European Journal of Immunology

SHORT COMMUNICATION

Mature proteins derived from Epstein–Barr virus fail to feed into the MHC class I antigenic pool

Benjamin M. Fiebiger[1,2], Andreas Moosmann[3,4], Uta Behrends[1,2] and Josef Mautner[1,2]

[1] Clinical Cooperation Group Pediatric Tumor Immunology, Helmholtz-Zentrum München, München, Germany
[2] Children's Hospital, Technische Universität München, München, Germany
[3] Clinical Cooperation Group Immunooncology, Ludwig-Maximilians-Universität München, München, Germany
[4] Clinical Cooperation Group Immunooncology, Helmholtz Zentrum München, München, Germany

The immediate presentation of peptide epitopes on MHC class I (MHC I) after antigen expression has led to the concept that MHC I ligands are mostly derived from defective ribosomal products (DRiPs), a subset of newly synthesized proteins that are rapidly degraded by the proteasome. Whether and to what extent mature proteins contribute to the antigenic pool, however, has remained elusive. Here, we developed a conditional antigen expression system that allows studying antigen presentation from mature proteins by inducing their rapid proteasomal degradation in the absence of further antigen synthesis. Target cells in which expression of two Epstein–Barr virus (EBV) antigens was induced were rapidly recognized by antigen-specific $CD8^+$ T cells in a time- and dosage-dependent manner, demonstrating that antigen presentation was linked to antigen synthesis. By contrast, T cells failed to recognize target cells containing large amounts of mature protein even after induction of their rapid proteasomal degradation. Thus, the presentation of these antigens proved to be strictly dependent on protein synthesis whereas mature proteins failed to furnish the antigenic pool. These results have implications for the design of immunotherapeutic strategies that aim at targeting proteins with increased half-lives and are hence overexpressed in tumors.

Keywords: Antigen presentation · Epstein–Barr virus · MHC class I · T lymphocyte · Protein stability

Supporting Information available online

Introduction

Adaptive immunity relies on the presentation of peptides on major MHC molecules. The peptides presented on MHC class I (MHC I) molecules are predominantly derived from intracellular proteins that are degraded by the proteasome and transported by the transporter associated with antigen presentation into the lumen of the ER, where they are loaded onto newly synthesized MHC I molecules for recognition by $CD8^+$ T cells [1]. In order to accommodate the rapidity by which peptides derived from viral proteins are presented on MHC I after viral infection despite their relatively long half-lives [2], Yewdell and co-workers proposed that

Correspondence: Dr. Josef Mautner
e-mail: mautner@helmholtz-muenchen.de

antigenic peptides originate from defective ribosomal products (DRiPs), a subset of newly synthesized proteins that are defective in some manner and rapidly degraded [3, 4]. Evidence in support of the DRiP hypothesis has been provided by functional studies that linked peptide generation with protein translation [5–8], and by the finding that a significant proportion of all newly synthesized proteins is rapidly degraded [9]. Following these seminal observations, a number of ensuing studies performed with multiple antigens and in different cell types with different allomorphs pointed to DRiPs as the major, if not the sole source of antigenic peptides [4, 10, 11].

However, the contribution of DRiPs versus mature proteins to the antigenic peptide pool is typically assessed by abrogating protein synthesis with translation-inhibiting drugs such as cycloheximide (CHX), that might not only affect precursor pool size but also other features of the antigen presentation pathway, for example, protein degradation as well as synthesis, loading, and transport of MHC molecules [5, 12]. Also, the use of chimeric proteins as the source of antigen and viruses for antigen expression may have led to an overestimation of the contribution of DRiPs to the immunopeptidome; the fusion of genes may decrease the overall fidelity of protein synthesis or protein folding [13] and differences in viral translation mechanisms can greatly increase the fraction of DRiPs [14]. Likewise, studies performed with antigens with very long half-lives and hence, very slow turnover, might have led to an underestimation of the contribution of mature proteins to the antigenic pool [7, 10, 15]. Moreover, naturally processed peptides eluted from MHC I molecules were found to carry different post-translational modifications including glycosyl and phosphate side chains, suggesting that they derived from proteins that had reached a mature state [16].

Here, we assessed antigen presentation from mature versus newly synthesized proteins by using a novel expression system that facilitates to modulate both, antigen transcription and stability, without interfering with vital cellular processes.

Results and discussion

A conditional cell system to modulate antigen expression and stability

To investigate whether mature proteins feed into the MHC I antigen pool, we fused a destabilizing domain (DD) to the Epstein–Barr virus (EBV) antigens BRLF1 and EBNA1, as well as EBNA1ΔGA, an EBNA1 deletion mutant lacking the glycine-alanine repeat [17] to generate DD-antigen-GFP as well as DD-antigen-IRES-GFP fusion constructs (IRES, internal ribosomal entry site) (Fig. 1A).DD derives from a mutated version of FKBP12 and has been shown to confer its instability to fusion partners, thereby causing their rapid proteasomal degradation [18]. Importantly, DD can be stabilized by FK-506 and other cell-permeable derivatives such as Shield1, which bind to DD and rescue the protein from degradation [18] (Fig. 1B).

To allow for conditional expression, these fusion genes were cloned under the control of a tetracycline-regulatable promoter in the pRTS1 vector [19] and lymphoblastoid B-cell line (LCL) stably transfected with these episomally replicating plasmids. Following incubation of the transfectants with 1 μg/mL of the tetracycline derivative doxycycline (Dox) and 0.5 μM Shield1 for 24 h, Dox was washed out to terminate gene transcription and the cells cultivated for additional 24 h in presence or absence of 0.5 μM Shield1. Subsequently, protein levels were analyzed by flow cytometry and western blotting. As compared with cells treated continuously with Shield1, BRLF1, and EBNA1ΔGA protein levels were greatly decreased when the cells were cultured in the absence of the stabilizing compound, thereby qualifying them as potentially suitable model antigens for studying antigen presentation of mature proteins (Fig. 1C–E and Supporting Information Fig. 1). Interestingly, Shield1 had no effect on DD-EBNA1-GFP protein levels, demonstrating that the glycine-alanine repeat is able to counteract the destabilizing effect of DD. The C-terminal fusion of GFP to the antigen had no effect on protein stability, because identical results were obtained in western blot analyses with DD-antigen-IRES-GFP constructs, in which GFP is expressed as separate protein (data not shown).

Since antigen might still be synthesized from residual mRNA after Dox removal and furnish the antigenic pool, we investigated the stability of antigen transcripts by qPCR. mRNA levels of all fusion genes sharply dropped within 4 h and were barely detectable after 24 h (Fig. 1F), suggesting that fusion protein synthesis ceased shortly after Dox removal.

Recognition of the model antigens by $CD8^+$ T cells

To test whether cells expressing the model antigens were recognized by antigen-specific $CD8^+$ T cells, the BRLF1 and the EBNA1ΔGA transfectants were treated with increasing concentrations of Dox for 36 h and then probed with the BRLF1 and EBNA1-specific $CD8^+$ T cells. Specific T-cell recognition was already detected with the lowest concentration of Dox used (30 ng/mL) and further increased with higher doses (Fig. 2A). This increase in T-cell recognition was paralleled by an increase in fusion protein levels (Fig. 2B). To investigate, when after induction antigen presentation became detectable, the cell lines were treated with 1 μg/mL Dox for different periods of time and then probed with the T cells. Again, T-cell recognition already started at the earliest time point tested (6 h) and further increased following prolonged incubations (Fig. 2C).

Induced degradation of mature protein does not lead to T-cell recognition

To test whether mature proteins contributed to the antigenic pool, the cells were treated with 1 μg/mL Dox and 0.5 μM Shield1 for 36 h and then cultivated for additional 4 h in the absence of Dox

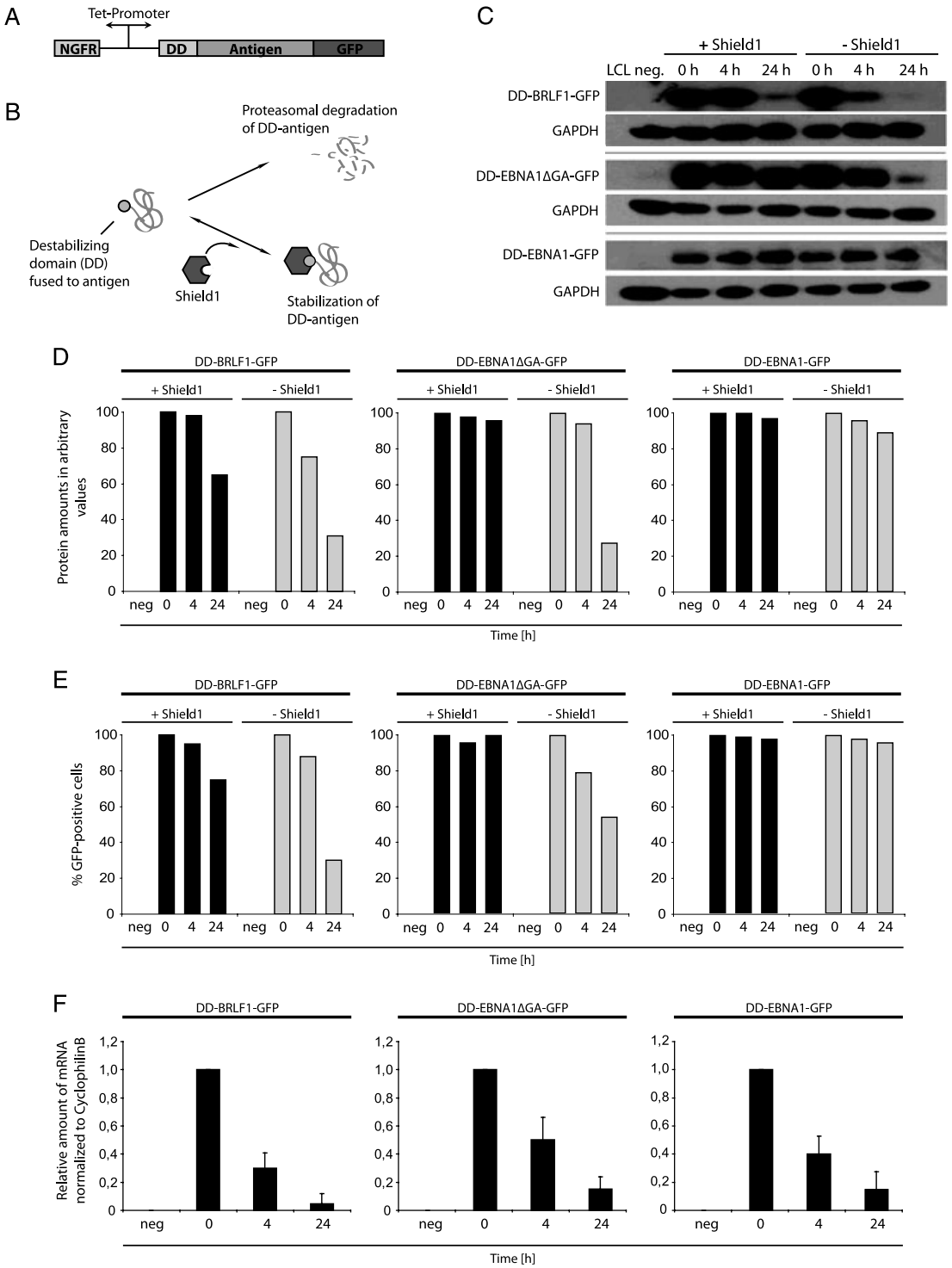

Figure 1. A conditional cell system to modulate antigen transcription and stability. (A, B) Schematic depiction of the expression vector. (A) The EBV proteins BRLF1, EBNA1, and EBNA1ΔGA were N-terminally fused to a destabilizing domain (DD) and at the C-terminus to GFP or an IRES-GFP cassette. (B) DD confers instability to the fusion proteins, causing their rapid proteasomal degradation. Shield1 reversibly binds to and stabilizes DD, and thereby rescues the fusion proteins from degradation. The fusion genes were expressed from a tetracycline-regulatable bidirectional promoter in the pRTS1 vector that also expressed a truncated version of the nerve growth factor receptor (NGFR) for enriching transfected cells by MACS. (C–E) Stability of the fusion proteins. Gene expression was induced by treating the cells with 1 μg/mL Dox and 0.5 μM Shield1 for 24 h. Cells were then washed and cultivated for the indicated time periods in the presence or absence of Shield1. Fusion protein levels were analyzed by (C) western blot, (D) quantified with an Odyssey infrared imager, and (E) by measuring GFP fluorescence by flow cytometry. (F) Measurement of mRNA stability by quantitative RT-PCR. Expression of the fusion genes was induced by treating the cells with 1 μg/mL Dox for 24 h. Subsequently, the cells were washed to remove Dox and cultivated for additional 4 or 24 h before measuring fusion gene mRNA transcripts by qPCR. Data are shown as mean + SD of $n = 3$ and are representative of four independent experiments.

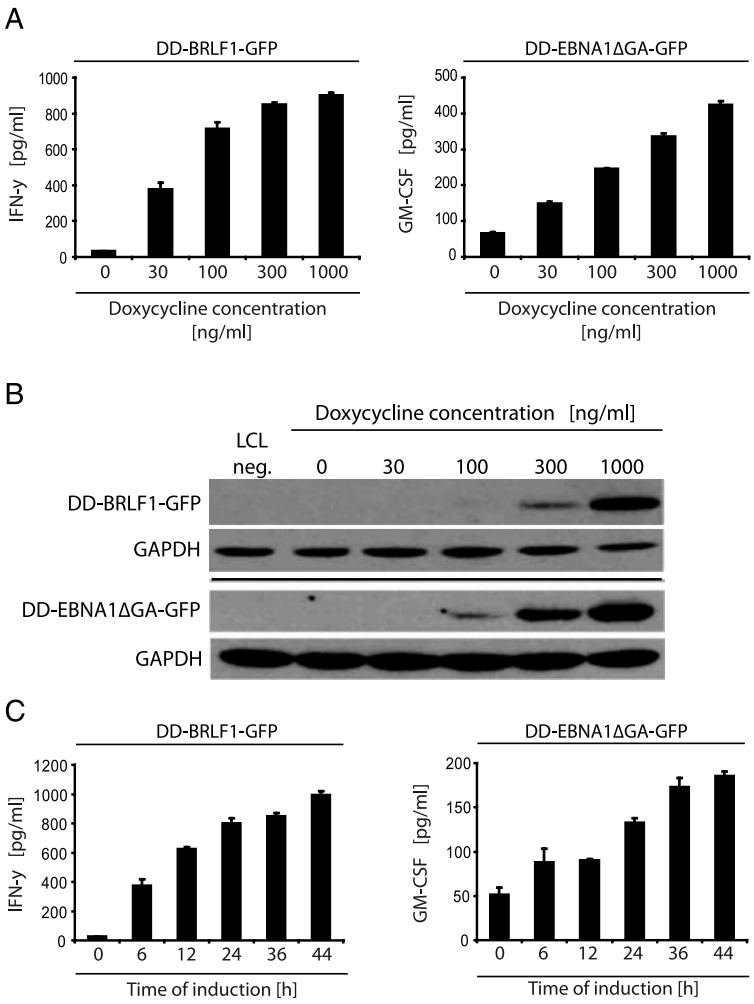

Figure 2. Time- and doxycycline-dependent recognition of transfected cells by antigen-specific T cells. (A) Stably transfected cells were treated with increasing amounts of Dox for 36 h and then probed with antigen-specific $CD8^+$ T cells. Cytokine secretion by the T cells was measured by ELISA. (B) Expression of the fusion proteins in these cells was analyzed by western blot. GAPDH was used as gel loading control. (C) To assess the kinetics of T cell recognition, the transfected cells were treated with 1 μg/mL Dox for the indicated time periods and then probed with the antigen-specific $CD8^+$ T cells. Data are shown as mean + SD of $n = 3$ and are representative of three independent experiments.

to allow for the degradation of residual fusion gene mRNAs. At this time point, de novo synthesis had waned while substantial amounts of mature protein had accumulated in the cells, which allowed assessing their contribution to antigen presentation. The cells were then treated with an acid stripping buffer to remove peptides from cell surface MHC I molecules [20]. While untreated cells were strongly recognized, T-cell recognition of acid stripped cells was almost completely abrogated (Fig. 3A). T-cell recognition was restored when stripped cells were re-induced for 6 h with Dox, demonstrating that the stripping procedure did not affect cell viability. However, cotreatment of the cells with the protein translation inhibitor CHX, or the proteasome inhibitor MG132, prevented T-cell recognition of reinduced cells, indicating that antigen presentation involved protein synthesis and required proteasomal degradation. To test whether mature proteins fed into the antigenic pool, the stripped cells were cultured in the presence or absence of Shield1 for additional 6 and 24 h before addition of the T cells. Despite the rapid and almost complete degradation of mature protein in the absence of Shield1 (Fig. 3B), the cells were not recognized by the T cells. These results demonstrate that the model antigens failed to feed into the MHC I antigen presentation pathway once they had reached a mature state.

Concluding remarks

Although compelling evidence suggests that the ligands for MHC I molecules derive from DRiPs, the detection of naturally processed peptides carrying post-translational modifications implied that mature proteins also feed into this presentation pathway. In our study, both EBV antigens obviously failed to generate antigenic peptides from mature proteins. Although we cannot formally exclude that some degradation products fed into the presentation pathway, the discrepancy between T-cell recognition of cells expressing barely detectably amounts of protein and the failure to recognize cells in which large amounts of mature protein were degraded, suggests that the contribution of mature protein to the antigenic pool is immunologically insignificant. Using a similar approach, Dolan and co-workers recently showed that retirees can feed into the antigenic pool [15, 21]. Although in both studies DD-fusion proteins were used, the stability of the chimeric model

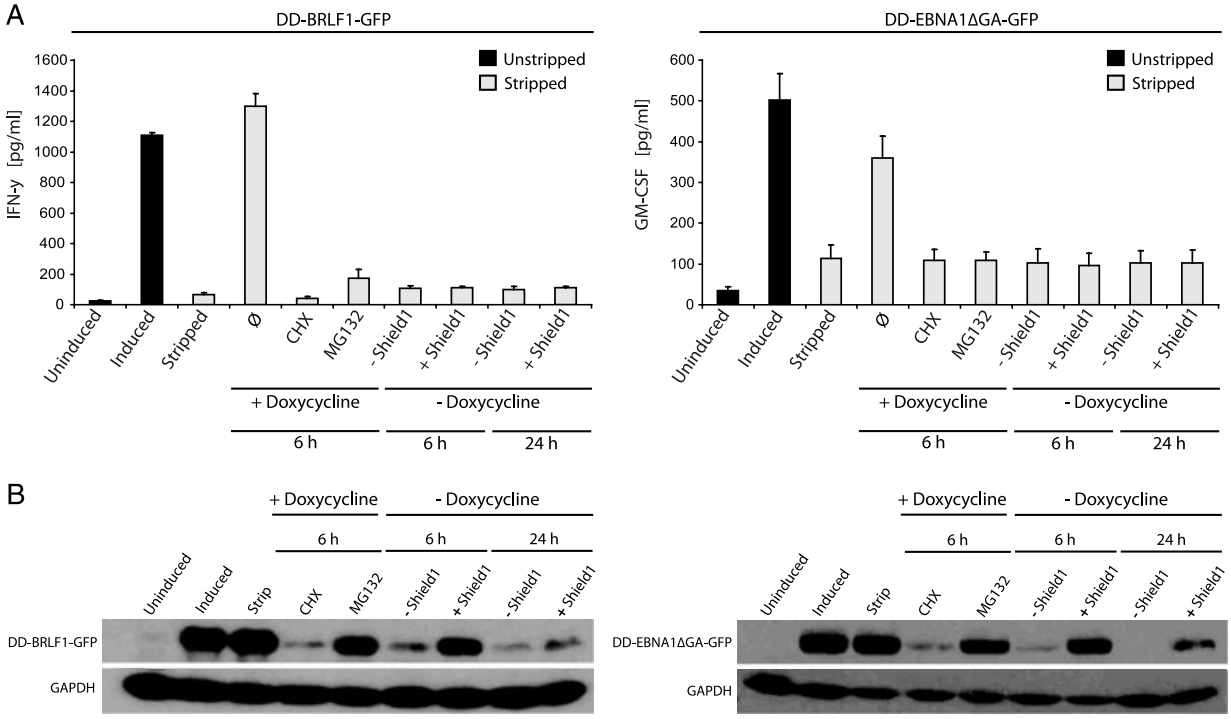

Figure 3. Induced degradation of mature protein fails to restore MHC I antigen presentation. (A) Antigen expression in transfected cells was induced with 1 μg/mL Dox for 36 h. Subsequently, the cells were cultivated in the absence of Dox for additional 4 h to allow for degradation of antigen mRNA and then tested with the T cells. Induced cells were treated with an acid stripping buffer to remove peptides from MHC I. Then they were probed with the T cells either directly, or after treating the cells again with 1 μg/mL Dox for 6 h in the presence or absence of the translation inhibitor cycloheximide (CHX) or the proteasome inhibitor MG132. Furthermore, stripped cells were cultivated in the presence or absence of Shield1 for 6 and 24 h and then tested with the T cells. (B) Western blot analysis of antigen levels in the cells described in (A). Although cultivation of induced and stripped cells in the absence of Shield1 led to a rapid degradation of antigen, these cells were not recognized by the T cells, demonstrating that mature fusion proteins did not feed into the MHC I presentation pathway. (A, B) Data are shown as mean + SD of $n = 3$ and are representative of three independent experiments.

antigens as well as their expression levels might have differed. Besides, differences in the detection method or the experimental approach involving, for example, CHX treatment [15] may have led to these discrepant results. Studying the presentation of natural, unmodified antigens in their physiological context may lead to a more thorough understanding of this matter.

While these findings may also imply that different pathways exist for different antigens, they also raise concerns about targeting proteins with $CD8^+$ T cells that are overexpressed in tumors. If overexpression is caused by an increased half-life of the protein, as for example in the case of p53 [22], higher levels of mature protein may not necessarily lead to an increased immunological visibility of the tumor cells, even if the degradation of mature protein is enhanced by drugs or viral gene products [23].

Materials and methods

Antibodies and reagents

Monoclonal antibodies against BRLF1 (Argene), EBNA1 (clone 1H4, provided by Dr. E. Kremmer; Helmholtz Zentrum München), and GAPDH (Millipore) were used. HRP-conjugated antimouse (GE Healthcare) and anti-rat (Jackson ImmunoResearch) secondary antibodies as well as anti-rat DyLight 800-conjugated and anti-mouse DyLight 800-conjugated secondary antibodies (both from Rockland) were used at 1:10,000 dilution.

Unless otherwise indicated, CHX (Sigma-Aldrich) was used at a final concentration of 20 μg/mL, Dox (Sigma-Aldrich) at 1 μg/mL, MG132 (Santa Cruz) at 10 μM, and Shield1 (Clontech) at 0.5 μM.

Cell culture

LCLs were generated by the infection of peripheral blood B cells with EBV. LCLs were grown as suspension cultures in RPMI-1640 medium supplemented with 10% fetal calf serum, 1% nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 1.25 μg/mL fungizone, and 50 μg/mL gentamycin.

The $CD8^+$ T cells JM-BRLF1, recognizing the BRLF1 peptide YVLDHLIVV on HLA-A*0201, and SJ-EBNA1 specific for the EBNA1-derived peptide HPVGEADYFEY presented on HLA-B*3501, were cultured in T-cell media consisting of AIM-V (Invitrogen) medium supplemented with 10% heat-inactivated human serum, 2 mM L-glutamine, 1.25 μg/mL fungizone, and 10 mM Hepes.

For T-cell stimulation, autologous LCLs were loaded with peptide (1 μg/mL) for 2 h and then irradiated with 80 Gy. Following wash out of unbound peptides, 5×10^5 LCL were mixed with 1×10^6 of $CD8^+$ T cells and then cultured in T-cell media and in 24-well plates as described in [24]. Every third day, 10 U/mL IL-2 was added to the medium. The cells were restimulated in the same way after 2 weeks. Cytokine secretion by the T cells was measured in triplicates by ELISA after incubating 1×10^5 target cells with 5×10^4 T cells in a final volume of 200 μL as described in [24]. Because SJ-EBNA1 T cells produce more GM-CSF than IFN-γ, this cytokine was used as readout for antigen recognition of these T cells. Plotted data represent the mean + SD. Unless otherwise indicated, all experiments in this article were performed at least thrice with similar results.

Construction of expression vectors

To generate the vectors DD-BRLF1-GFP, DD-BRLF1-IRES-GFP, DD-EBNA1-GFP, DD-EBNA1ΔGA-GFP, and DD-EBNA1ΔGA-IRES-GFP, the respective open reading frames of the antigens were inserted downstream of DD in the pPTuner plasmid (Clontech). Subsequently, the gene coding for GFP was inserted in frame downstream of the fusion gene or an IRES-GFP cassette added. Identity and integrity of the created plasmids were verified by restriction enzyme digestion and sequence analysis of the modified regions.

For conditional expression, the DD-antigen-GFP as well as DD-antigen-IRES-GFP genes were cloned into the pRTS-1 vector [19] and the resulting plasmids stably transfected into LCL1.11 and LCL-MSC by electroporation. The pRTS-1 vector also encodes for a C-terminally truncated nerve growth factor receptor that permitted to enrich transfected cells by MACS [17].

Western blot analysis

Whole cell extracts were prepared from 3×10^6 cells using RIPA (radio-immunoprecipitation assay) cell lysis buffer containing Protease Inhibitor Cocktail (Roche). After adding SDS-loading buffer, the extracts were heated for 5 min at 95°C and the samples then separated on a 10% SDS-PAGE gel. After blotting, the membranes were incubated with specific antibodies and bound proteins visualized by chemiluminescence using the ECL plus detection kit (GE Healthcare). To determine relative protein concentrations, the membranes were hybridized with DyLight 800-conjugated secondary antibodies and then analyzed with the Odyssey infrared imager (LI-COR).

RNA isolation and quantitative RT-PCR

Cells were harvested and total cellular RNA was extracted using the RNeasy Kit (Qiagen). Reverse transcription was performed with 1 μg RNA using the SuperScript II reverse transcriptase (Invitrogen) and oligo-dT primer (Roche). Quantitative RT-PCR analysis was performed with the Lightcycler 2.0 (Roche). To quantify mRNA amounts, the LC FastStart Reaction Mix SYBR Green I (Roche) and specific primer pairs were used. To detect mRNA of the fusion genes, GFP-specific primers were used (5′-GGCAACTACAAGACCCGC-3′ (forward) and 5′-AAGTCGATGCCCTTCAGCT-3′ (reverse)). CyclophilinB mRNA served as internal control for normalization (5′-CTGCGGCCGATGAGAAGAAGAAGGG-3′ (forward) and 5′-GGGAAGCGCTCACCGTAGATGCTC-3′ (reverse)).

Acknowledgements: This study was supported by the Deutsche Forschungsgemeinschaft SFB455 (JM).

Conflict of interest: The authors declare no financial or commercial conflict of interest.

References

1 **Kloetzel, P. M.**, Generation of major histocompatibility complex class i antigens: functional interplay between proteasomes and tppii. *Nat. Immunol.* 2004. **5**: 661–669.

2 **Esquivel, F., Yewdell, J. and Bennink, J.**, Rma/s cells present endogenously synthesized cytosolic proteins to class i-restricted cytotoxic t lymphocytes. *J. Exp. Med.* 1992. **175**: 163–168.

3 **Yewdell, J. W., Anton, L.C. and Bennink, J. R.**, Defective ribosomal products (drips): a major source of antigenic peptides for mhc class i molecules? *J. Immunol.* 1996. **157**: 1823–1826.

4 **Yewdell, J. W.**, Drips solidify: progress in understanding endogenous mhc class i antigen processing. *Trends Immunol.* 2011. **32**: 548–558.

5 **Qian, S. B., Reits, E., Neefjes, J., Deslich, J. M., Bennink, J. R. and Yewdell, J. W.**, Tight linkage between translation and mhc class i peptide ligand generation implies specialized antigen processing for defective ribosomal products. *J. Immunol.* 2006. **177**: 227–233.

6 **Reits, E. A., Vos, J. C., Gromme, M. and Neefjes, J.**, The major substrates for tap in vivo are derived from newly synthesized proteins. *Nature* 2000. **404**: 774–778.

7 **Khan, S., de Giuli, R., Schmidtke, G., Bruns, M., Buchmeier, M., van den Broek, M. and Groettrup, M.**, Cutting edge: neosynthesis is required for the presentation of a T-cell epitope from a long-lived viral protein. *J. Immunol.* 2001. **167**: 4801–4804.

8 **Cardinaud, S., Starck, S. R., Chandra, P. and Shastri, N.**, The synthesis of truncated polypeptides for immune surveillance and viral evasion. *PLoS ONE* 2010. **5**: e8692.

9 **Schubert, U., Anton, L. C., Gibbs, J., Norbury, C. C., Yewdell, J. W. and Bennink, J. R.**, Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. *Nature* 2000. **404**: 770–774.

10 **Dolan, B. P., Bennink, J. R. and Yewdell, J. W.**, Translating drips: progress in understanding viral and cellular sources of mhc class i peptide ligands. *Cell. Mol. Life Sci.* 2011. **68**: 1481–1489.

11 **Apcher, S., Manoury, B. and Fahraeus, R.**, The role of mrna translation in direct mhc class i antigen presentation. *Curr.Opin. Immunol.* 2012. **24**: 71–76.

12 **Lev, A., Takeda, K., Zanker, D., Maynard, J. C., Dimberu, P., Waffarn, E., Gibbs, J. et al.**, The exception that reinforces the rule: crosspriming by cytosolic peptides that escape degradation. *Immunity* 2008. **28**: 787–798.

13 **Yen, H. C., Xu, Q., Chou, D. M., Zhao, Z. and Elledge, S. J.**, Global protein stability profiling in mammalian cells. *Science* 2008. **322**: 918–923.

14 **Berglund, P., Finzi, D., Bennink, J. R. and Yewdell, J. W.**, Viral alteration of cellular translational machinery increases defective ribosomal products. *J. Virol.* 2007. **81**: 7220–7229.

15 **Dolan, B. P., Li, L., Veltri, C. A., Ireland, C. M., Bennink, J. R. and Yewdell, J. W.**, Distinct pathways generate peptides from defective ribosomal products for $CD8^+$ T-cell immunosurveillance. *J. Immunol.* 2011. **186**: 2065–2072.

16 **Engelhard, V. H., Altrich-Vanlith, M., Ostankovitch, M. and Zarling, A. L.**, Post-translational modifications of naturally processed mhc-binding epitopes. *Curr. Opin. Immunol.* 2006. **18**: 92–97.

17 **Mautner, J., Pich, D., Nimmerjahn, F., Milosevic, S., Adhikary, D., Christoph, H., Witter, K. et al.**, Epstein-barr virus nuclear antigen 1 evades direct immune recognition by $CD4^+$ T helper cells. *Eur. J. Immunol.* 2004. **34**: 2500–2509.

18 **Banaszynski, L. A., Chen, L. C., Maynard-Smith, L. A., Ooi, A. G. and Wandless, T. J.**, A rapid, reversible, and tunable method to regulate protein function in living cells using synthetic small molecules. *Cell* 2006. **126**: 995–1004.

19 **Bornkamm, G. W., Berens, C., Kuklik-Roos, C., Bechet, J. M., Laux, G., Bachl, J., Korndoerfer, M. et al.**, Stringent doxycycline-dependent control of gene activities using an episomal one-vector system. *Nucleic Acids Res.* 2005. **33**: e137.

20 **Sugawara, S., Abo, T. and Kumagai, K.**, A simple method to eliminate the antigenicity of surface class i mhc molecules from the membrane of viable cells by acid treatment at ph 3. *J. Immunol. Methods* 1987. **100**: 83–90.

21 **Dolan, B. P., Sharma, A. A., Gibbs, J. S., Cunningham, T. J., Bennink, J. R. and Yewdell, J. W.**, Mhc class i antigen processing distinguishes endogenous antigens based on their translation from cellular vs. viral mrna. *Proc. Natl. Acad. Sci. USA* 2012. **109**: 7025–7030.

22 **Vermeij, R., Leffers, N., van der Burg, S. H., Melief, C. J., Daemen, T. and Nijman, H. W.**, Immunological and clinical effects of vaccines targeting p53-overexpressing malignancies. *J. Biomed. Biotechnol.* 2011. **2011**: 702146.

23 **Brosh, R. and Rotter, V.**, When mutants gain new powers: news from the mutant p53 field. *Nat. Rev. Cancer* 2009. **9**: 701–713.

24 **Nimmerjahn, F., Milosevic, S., Behrends, U., Jaffee, E. M., Pardoll, D. M., Bornkamm, G. W. and Mautner, J.**, Major histocompatibility complex class ii-restricted presentation of a cytosolic antigen by autophagy. *Eur. J. Immunol.* 2003. **33**: 1250–1259.

Abbreviations: **CHX**: cycloheximide · **Dox**: doxycycline · **DRiP**: defective ribosomal product · **LCL**: lymphoblastoid B cell line

Full correspondence: Dr. Josef Mautner, Children's Hospital, Technische Universität München, Kölner Platz 1, D-80804 Munich, Germany
Fax: +49-89-7099500
e-mail: mautner@helmholtz-muenchen.de

Received: 19/4/2012
Revised: 27/7/2012
Accepted: 30/8/2012
Accepted article online: 4/9/2012

