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Chaperone BAG6 is dispensable for MHC class I antigen processing and presentation

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A B S T R A C T

Antigen processing for direct presentation on MHC class I molecules is a multistep process requiring the concerted activity of several cellular complexes. The essential steps at the beginning of this pathway, namely protein synthesis at the ribosome and degradation via the proteasome, have been known for years. Nevertheless, there is a considerable lack of factors identified to function between protein synthesis and degradation during antigen processing. Here, we analyzed the impact of the chaperone BAG6 on MHC class I cell surface expression and presentation of virus-derived peptides. Although an essential role of BAG6 in antigen processing has been proposed previously, we found BAG6 to be dispensable in this pathway. Still, interaction of BAG6 and the model antigen tyrosinase was enhanced during proteasome inhibition pointing towards a role of BAG6 in antigen degradation. Redundant chaperone pathways potentially mask the contribution of BAG6 to antigen processing and presentation.

Keywords:
BAG6
Antigen processing
Antigen presentation
MHC class I

1. Introduction

Antigen processing and presentation in the MHC class I restricted pathway is a process comprising several subsequent steps of polypeptide processing and transportation. Generally, proteins are degraded to peptides by the proteasome, loaded onto MHC class I molecules, and presented as a complex to cytotoxic T lymphocytes (CTLs) at the cell surface (Rock and Goldberg, 1999). Based on the discovery of an intimate connection between antigen presentation and protein synthesis Yewdell and colleagues postulated the defective ribosomal product (DRiP) hypothesis (Yewdell et al., 1996). DRiPs are thought to be the main source of antigen and are defined as rapidly degraded nascent polypeptides due to their inability to achieve a functional state. Although the DRiP hypothesis was postulated almost two decades ago there are still considerable gaps in the knowledge about antigen processing upstream of proteasomal degradation. Interestingly, a report by Minami et al. (2010) found the chaperone BCL2-associated athanogene 6 (BAG6; also named Scythe or BAT3) to be essential for DRiP degradation and MHC class I cell surface expression (Minami et al., 2010). BAG6 is conserved in higher eukaryotes, ubiquitously expressed, and encoded in the MHC class III locus (Banerji et al., 1990; Ozaki et al., 1999; Wang and Liew, 1994). Even though BAG6 is classified as a chaperone, it has no apparent folding activity (Wang et al., 2011). Together with its binding partners TRC35 and UBL4A it associates with long hydrophobic patches in client proteins and prevents aggregation (Leznicki et al., 2013; Mariappan et al., 2010; Minami et al., 2010; Wang et al., 2011). Therefore, BAG6 has also been described as a "holdase" which keeps its substrate in a soluble state (Wang et al., 2011). Because of its binding properties, BAG6 is especially involved in the biogenesis and degradation of polypeptides carrying hydrophobic domains (Kawahara et al., 2013; Lee and Ye, 2013).

In this study, we revisited the role of BAG6 in MHC class I antigen processing proposed by Minami et al. (2010). However, we found no influence of BAG6 knockdown on MHC class I cell surface expression. Moreover, BAG6 was not transcriptionally regulated after stimulation with IFN-γ and presentation of virus-derived peptides was not altered in BAG6 knockdown cells. Together, these results suggest that BAG6 is dispensable or redundant for antigen processing and presentation on MHC class I.

2. Materials and methods

2.1. Mice, viruses, cell lines, and cytokines

C57BL/6 mice (H-2^b), BALB/c mice (H-2^d) and AAD mice (H-2^b, transgenic for an HLA-A*0201/H-$2D^d$ chimeric protein (Newberg et al., 1996)) were originally purchased from Charles River. Mice were kept in a specific pathogen-free facility and used at 8–12

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weeks of age. Animal experiments were approved by the review board of Regierungspräsidium Freiburg.

LCMV-WE was originally obtained from F. Lehmann-Grube (Hamburg, Germany) and propagated on the fibroblast line L929. Vaccinia virus Western Reserve strain (VV-WR) was originally obtained from H. Hengartner (University Hospital Zürich, Switzerland). Recombinant vaccinia virus (rVV) expressing tyrosinase (rVV-Tyr) was a kind gift from V. H. Engelhard (University of Virginia, VA, USA). Both vaccinia virus strains were propagated on BSC-40 cells.

B8-D^b (H-2^d) is a murine fibroblast cell line stably transfected with H-$2D^b$ (Basler et al., 2004). B8-D^b cells were cultured in IMDM. Primary murine embryonic fibroblasts from AAD mice, HeLa cells, HEK293 cells, and MelJuSo cells were maintained in DMEM. The human lung fibroblast cell line IMRS was maintained in MEM including 15% FCS. MelJuSo cells and IMRS cells were a kind gift from N. Koch (University of Bonn, Germany). All media were purchased from Invitrogen-Life Technologies and contained GlutaMAX, 10% FCS and 100 U/ml penicillin/streptomycin.

Recombinant human and murine interferon-γ (IFN-γ) was purchased from Peprotech (Hamburg, Germany) and used at 200 U/ml for two days.

2.2. Transfections

Knockdown of BAG6 was performed through simultaneous transfection of four different siRNAs targeting BAG6. At 80% confluence, cells were transfected with mouse or human BAG6 siRNA (ON-TARGETplus SMART pool siRNA, Thermo Scientific) using DharmaFECT 1 transfection reagent (Thermo Scientific) according to the manufacturer's instruction. After 48 h, cells were transfected a second time to achieve maximum efficiency. Control cells were transfected with a mix of four non-targeting siRNAs (ON-TARGETplus Non-Targeting pool siRNA, Thermo Scientific).

For transient overexpression of BAG6, HeLa cells were transfected with a pCMV6-Entry expression plasmid encoding BAG6-Myc-DDK (OriGene Technologies). Control cells were transfected with empty vector. Transfection was performed at 80% confluence using FuGENE HD transfection reagent (Promega) according to the manufacturer's instructions. Cells were further analyzed 24 h post transfection. Transient overexpression of tyrosinase (Tyr) in HEK293 cells was achieved likewise using a pCMV6-Entry expression construct encoding tyrosinase-Myc-Flag and Trans-IT-LT1 transfection reagent (Mirus Bio LLC.) according to the manufacturer's instructions.

2.3. Flow cytometry

MHC class I surface expression was analyzed by flow cytometry using fluorescently-labeled antibodies. Cells were incubated together with antibodies diluted 1:150 in PBS, 2% (v/v) FCS, 2 mM EDTA for 20 min at 4 °C. Cells were washed and acquired with the use of the Accuri C6 flow cytometer system (BD Biosciences). Data were analyzed with FlowJo software (Tree Star). Anti-H-$2D^b$ FITC (clone KH95), anti-H-$2D^d$ PE (clone 43-2-12) and anti-HLA-ABC APC (clone G46-2.6) antibodies were obtained from BD Biosciences.

2.4. Real-time RT-PCR

Total RNA was extracted using NucleoSpin RNA II kit (Macherey-Nagel) according to the manufacturer's instructions. Extracted RNA was reverse-transcribed using the Reverse Transcription System (Promega) according to the manufacturer's instruction. Quantitative real-time RT-PCR was performed with the LightCycler Instrument II (Roche) and LightCycler Fast-Start DNA Master SYBR Green I kit (Roche). Gene expression was calculated relative to control cells and normalized to HPRT (hypoxanthine guanine phosphoribosyltransferase) expression using REST-384O software version 2 (Gene Quantification). Sense and antisense primers used for amplification were as follows: BAG6 (human) TACAATAACAATCACGAGGGCC, GGTGGTGTAGTGAGACATAGG; TRC53 (human) CGTGACCTTTGTGTCCAGAG, TTAAACAGAGAAACTGTAGCACGG; HPRT (human) TGGACAGGACTGAACGTCTTG, CCAGCAGGTCAGCAAAGAATTTA; LMP7 (human) AATGCAGGCTGTACTATCTGCG, TGCAGCAGGTCACTGACATCTG.

2.5. SDS PAGE and western blot

Cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris pH 8, 1% (v/v) Triton X-100, 0.5% (v/v) sodiumdesoxycholate, 0.1% (w/v) SDS) including protease inhibitors (cOmplete EDTA-free, Roche) for 30 min on ice. Lysates were centrifuged at 20,000 × *g* and 4 °C for 15 min. Supernatants were mixed with SDS sample buffer and boiled for 5 min at 95 °C. Proteins were separated by SDS-PAGE and blotted onto nitrocellulose membrane (Whatman). Membranes were blocked for 1 h in Roti-Block solution (Roth) followed by overnight incubation in primary antibodies at 4 °C. Membranes were washed and incubated for 2 h with appropriate peroxidase-conjugated secondary antibodies (Dako). Membranes were washed and proteins were visualized with enhanced chemiluminescence. Primary antibodies used: Rabbit anti-BAG6 (kind gift from R. S. Hegde, Cambridge, UK), mouse anti-α-tubulin (clone AA13, Sigma) and mouse anti-FLAG (clone M2, Sigma).

2.6. Generation of epitope-specific cytotoxic T lymphocyte (CTL) lines

LCMV-specific CTL lines were generated from female C57BL/6 or BALB/c mice infected i.v. with 200 PFU LCMV-WE. Four weeks post infection splenocytes from infected mice were cultured in IMDM 10% FCS, P/S, supplemented with 40 U/ml IL-2, 100 μM 2-mercaptoethanol and 10^{-6} M peptide. Cytokine-supplemented medium was added every other day for 8–14 days. Before CTLs were used in presentation assays, dead cells were removed by Ficoll density centrifugation.

Tyr-specific CTL-lines were generated from male AAD mice infected i.p. with 2×10^6 PFU rVV-Tyr. Splenocytes from memory mice were cultured for three weeks as described above. To increase percentage of specific T cells, CTLs were restimulated twice with peptide-loaded, irradiated (20 Gy) AAD splenocytes on day 8 and 16.

2.7. Synthetic peptides

The synthetic peptides GP276-286 (SGVENPGGYCL), NP118-126 (RPQASGYVM) and NP396-404 (FQPQNGQFI) were obtained from P. Henklein (Charité, Berlin, Germany). The synthetic peptide Tyr369-377 (YMDGTMSQV) was obtained from Sigma.

2.8. Antigen presentation assay

B8-D^b cells or AAD mouse embryonic fibroblasts (MEFs) were transfected with BAG6 siRNA as described above. Transfected cells were harvested and infected with LCMV or rVV with a multiplicity of infection (MOI) of 10 for 2 h, 4 h, or 5 h. Serial dilution of infected cells was performed to achieve different effector to stimulator ratios (E/S). Epitope-specific CTL lines were added to infected cells and incubated in presence of 10 μg/ml brefeldin A for 3 h at 37 °C. Activation of CTLs was determined by intracellular IFN-γ staining and flow cytometry. All samples were prepared as duplicates.

2.9. Intracellular IFN-γ staining

Intracellular IFN-γ staining was performed as described previously (Basler et al., 2004) except that anti-CD8a APC (clone 53-6.7, eBioscience) (1:150) was used to stain cytotoxic T cells.

2.10. Immunoprecipitation

Cells transiently transfected with an expression construct for tyrosinase-Myc-Flag or mock transfected cells were incubated with 10 μM MG132 (Sigma) or DMSO (1:1000) for 5 h at 37 °C. Cells were then harvested and lysed in lysis buffer (20 mM Tris pH 7.8, 50 mM NaCl, 10 mM $MgCl_2$, 1% (v/v) NP40) including protease inhibitors (cOmplete EDTA-free, Roche) for 30 min on ice. Lysates were centrifuged at 20,000 × *g* and 4 °C for 15 min. Supernatants were loaded onto protein G affinity gel (Sigma) together with anti-Myc antibody (clone 9E10, Sigma) and incubated overnight at 4 °C on a rotator. The affinity gel was washed three times in high salt buffer (50 mM Tris pH 8, 650 mM NaCl, 5 mM EDTA, 0.5% (v/v) Triton X-100) and three times in low salt buffer (50 mM Tris pH 8, 150 mM NaCl, 5 mM EDTA, 0.5% (v/v) Triton X-100). Then, precipitated proteins were heated in SDS sample buffer for 5 min at 95 °C and subsequently analyzed by SDS-PAGE and western blot as described above.

2.11. Statistical analysis

The unpaired two-tailed Student's *t* test was applied for statistical analysis using GraphPad Prism software.

3. Results

3.1. MHC class I surface expression is independent of BAG6

If DRiPs result from incomplete capture of nascent polypeptides by chaperones, deletion of chaperones should increase DRiP rates. A higher proportion of DRiPs should in turn increase MHC class I surface expression due to increased peptide supply. On the other hand, deletion of chaperones could lead to inefficient shuttling and degradation of antigen. To analyze the influence of BAG6 on MHC class I surface expression, the murine fibroblast cell line B8-D^b (H-2^d + H-$2D^b$) and HeLa cells were subjected to BAG6 knockdown. Subsequently, surface expression of MHC class I molecules was

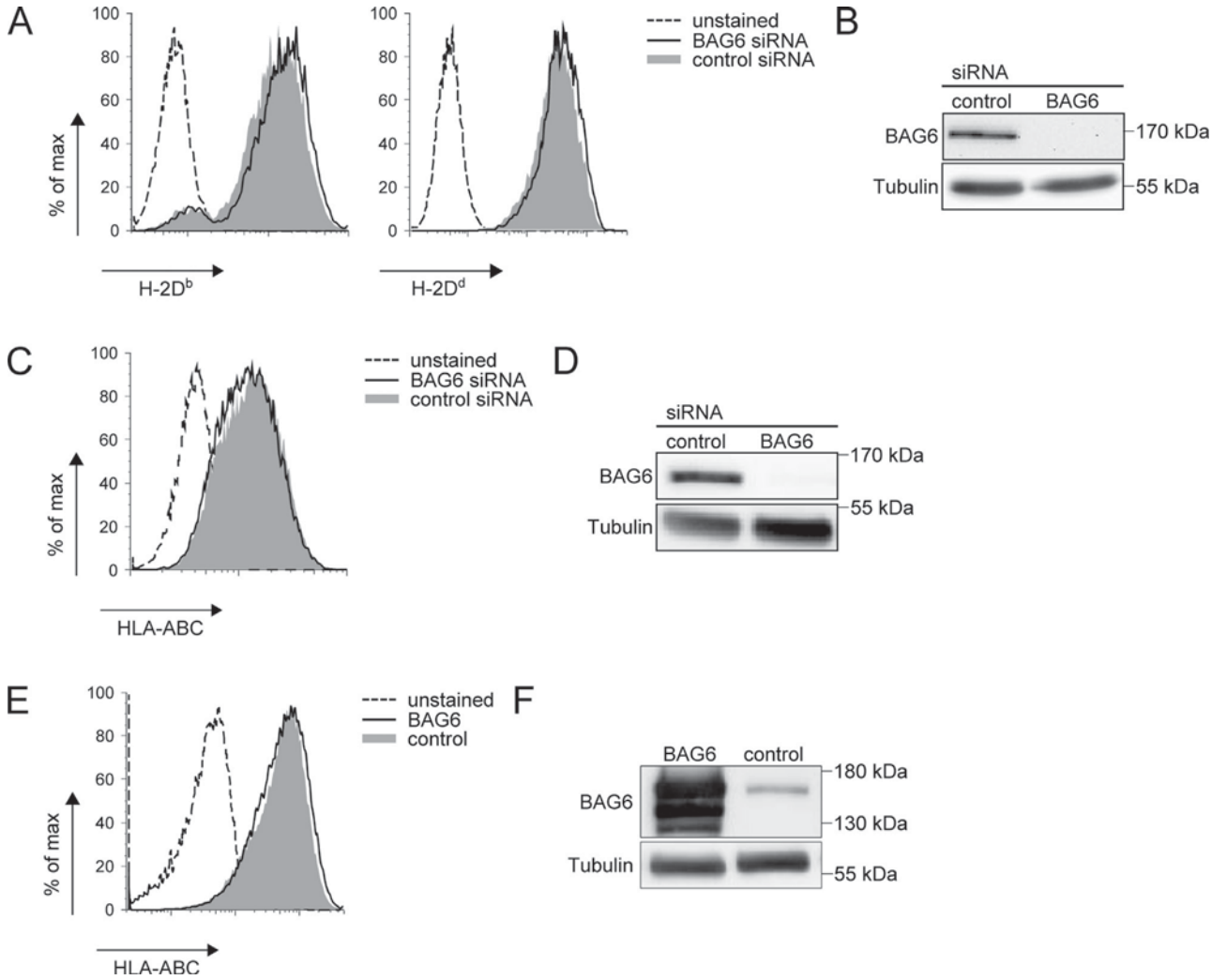

Fig. 1. Knockdown or overexpression of BAG6 has no influence on MHC class I surface expression. (A and C) B8-D^b cells (A) and HeLa cells (C) were transfected with BAG6 siRNA or control siRNA and surface expression of MHC class I molecules H-$2D^b$ and H-$2D^d$ (A) or HLA-A,B,C (C) was analyzed by flow cytometry. (B and D) Western blot analysis of whole cell lysates prepared from B8-D^b cells (B) or HeLa cells (D) transfected with BAG6 siRNA or control siRNA. (E) HeLa cells were transfected with a BAG6 expression construct or an empty plasmid (control) and surface expression of MHC class I molecules HLA-A,B,C was analyzed by flow cytometry. (F) Western blot analysis of whole cell lysates prepared from HeLa cells transfected with a BAG6 expression construct or an empty control plasmid. Tubulin was used as loading control. For all experiments one out of two independent experiments is shown.

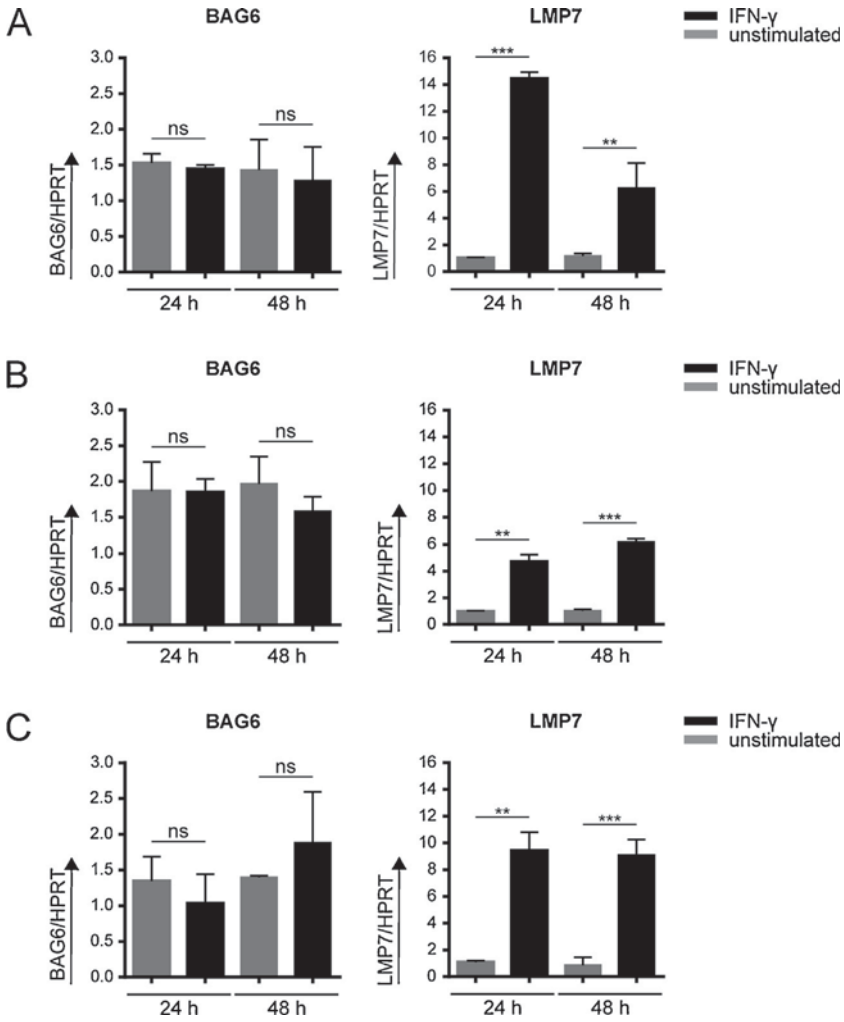

Fig. 2. BAG6 is not transcriptionally regulated after IFN-γ stimulation. HeLa cells (A), IMRS cells (B) or MelJuSo cells (C) were stimulated with IFN-γ for 24 h or 48 h or were left unstimulated and mRNA levels of BAG6 and LMP7 were determined by real-time RT-PCR. Expression levels were calculated relative to unstimulated cells and normalized to hypoxanthine guanine phosphoribosyl transferase (HPRT) expression. Bar graphs show means ± SD from three independent experiments. ns (not significant) $P > 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (unpaired two-tailed Student's *t* test).

determined by flow cytometry. No difference in surface expression of the transfected MHC class I molecule H-2D^b and the endogenous molecule H-2D^d could be observed between B8-D^b cells transfected with BAG6 siRNA and cells transfected with control siRNA (Fig. 1A). Successful knockdown leading to ablation of the BAG6 protein below the detection limit of western analysis was confirmed by SDS-PAGE and immunoblotting (Fig. 1B). The same result was obtained with HeLa cells in which total MHC class I surface expression (HLA-A,B,C) did not differ between BAG6 knockdown and control cells (Fig. 1C). Very successful knockdown in HeLa cells was again confirmed by SDS-PAGE and immunoblotting (Fig. 1D).

To rule out limiting effects of BAG6 on MHC class I surface expression, overexpression was performed in HeLa cells. Cells were transiently transfected with an expression construct encoding for BAG6 and subjected to flow cytometric analysis 24 h post transfection (Fig. 1E). No influence on HLA-A,B,C surface expression could be detected although lysates of transfected cells subjected to SDS-PAGE and immunoblotting revealed very high expression levels of BAG6 compared to control cells (Fig. 1F).

Taken together, we could not observe an influence of BAG6 on MHC class I surface expression. Loss of BAG6 might be effectively compensated by other cytosolic chaperones in the setting applied here. On the other hand, BAG6 is not a limiting factor in antigen processing since its overexpression had no effect on MHC class I surface expression.

3.2. *BAG6 is not transcriptionally regulated after interferon gamma (IFN-γ) stimulation*

Stimulation of cells with the cytokine IFN-γ induces or enhances transcription of several genes whose products delay viral replication and enhance peptide presentation on MHC class I (Platanias, 2005; Sen and Lengyel, 1992). Like many of these genes, BAG6 is encoded in the MHC locus and cytokine regulated expression control could be possible (Banerji et al., 1990). Yet, stimulation of HeLa cells with IFN-γ for 24 h or 48 h did not induce upregulation of BAG6 mRNA (Fig. 2A, left side). In contrast, expression of the immunoproteasome subunit LMP7, a well-known IFN-regulated gene, was strongly upregulated after stimulation (Fig. 2A, right side). Although Kamper et al. found expression of the *bag6* gene to be upregulated after stimulation of the melanoma cell line MelJuSo and the lung fibroblast cell line IMRS with IFN-γ we could neither confirm this finding for HeLa cells, nor for the other two cell lines (Fig. 2B and C) (Kamper et al., 2012). Overall, our results do not point towards a central role of BAG6 in a type II interferon regulated antiviral immune response.

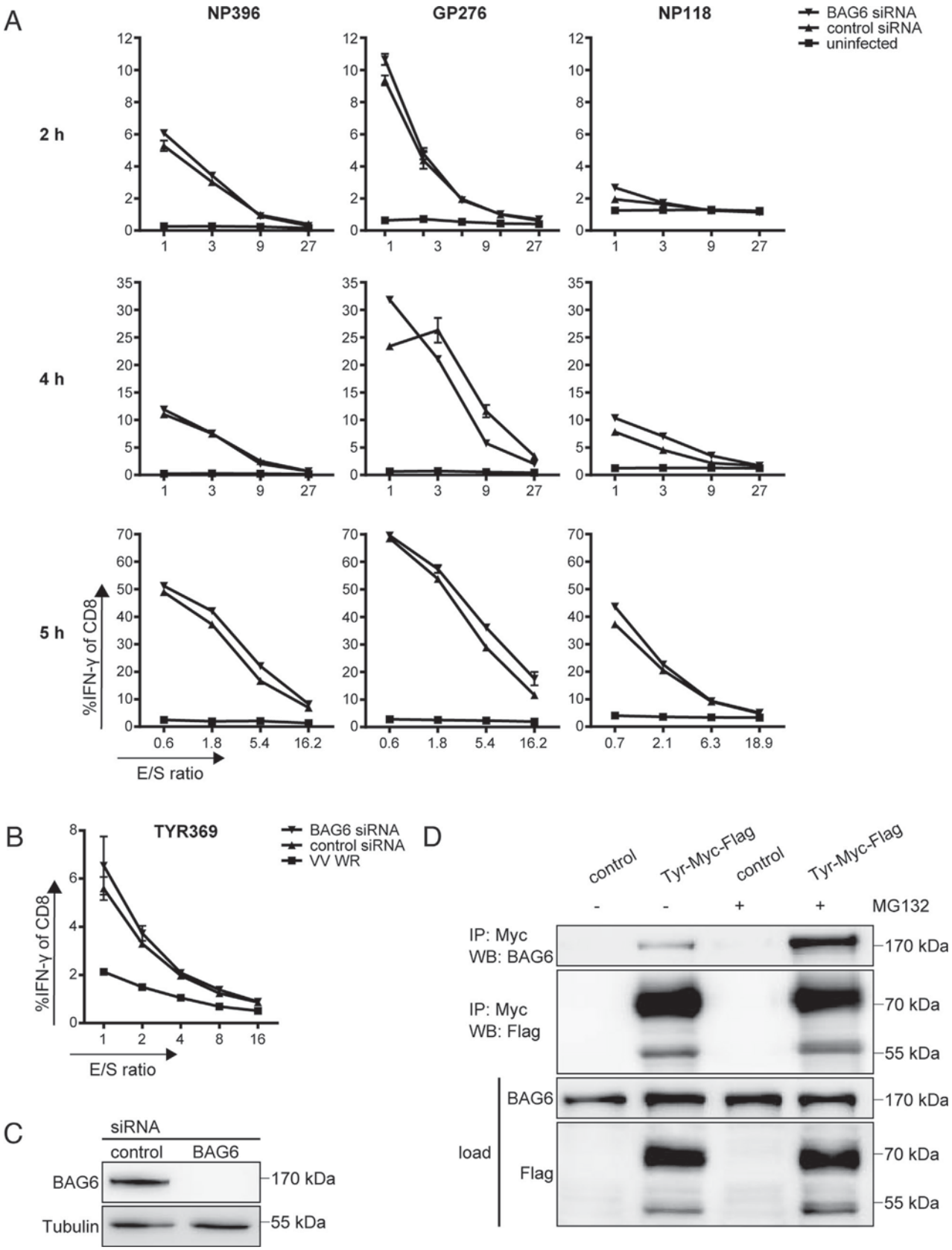

Fig. 3. BAG6 is dispensable for presentation of virus-derived epitopes. (A and B) Cells were transfected with BAG6 siRNA or control siRNA and in (A) infected with lymphocytic choriomeningitis virus (LCMV), (MOI = 10). Epitope-specific CTLs were added to infected cells in different effector to stimulator ratios (E/S) for 3 h and the percentage of IFN-γ^+ of $CD8^+$ cells was determined by flow cytometry as a measure for epitope presentation. Values represent mean ± SD of duplicate determinations. (A) B8-D^b cells infected with LCMV for 2, 4 or 5 h were analyzed for presentation of NP396-404, GP276-286 or NP118-126. (B) Transfected MEFs from AAD mice were stimulated with IFN-γ for 2 days, infected for 5 h with rVV-Tyr or VV-WR and then analyzed for the presentation of Tyr369-377(D). (C) Western blot analysis of whole cell lysates prepared from AAD MEFs transfected with BAG6 siRNA or control siRNA and stimulated with IFN-γ for 2 days. Tubulin was used as loading control. (D) HEK293 cells were transiently transfected

3.3. Presentation of LCMV-derived epitopes is independent of BAG6

To address whether generation of virus-derived peptide epitopes is dependent on BAG6, we investigated antigen presentation in lymphocytic choriomeningitis virus (LCMV)-infected cells after BAG6 knockdown. B8-D^b cells were transfected with BAG6 siRNA and subsequently infected with LCMV-WE for 2, 4 or 5 h. Peptides presented on MHC class I were detected with peptide-specific CTL lines specific for the epitopes nucleoprotein (NP) 118–126 (H-$2L^d$), NP396-404 (H-$2D^b$) and glycoprotein (GP) 276–286 (H-$2D^b$) by staining for the CTL surface marker CD8 and intracellular IFN-γ. CTLs produce IFN-γ upon stimulation of their receptor with the appropriate peptide-MHC complex. Thus, the proportion of IFN-γ producing CTLs of all CD8 bearing CTLs reflects the amount of peptides presented by infected cells. GP276-286 represents an epitope derived from the secretory pathway, whereas NP118-126 and NP396-404 are derived from a cytosolic protein. Compared to control cells, BAG6 knockdown cells are not confined in their ability to present LCMV-derived epitopes (Fig. 3A).

3.4. Presentation of retrotranslocated tyrosinase-derived epitope is not affected by BAG6 knockdown

BAG6 maintains retrotranslocated ER-associated protein degradation (ERAD) substrates in a soluble state and thereby allows proteasomal degradation of these substrates (Claessen and Ploegh, 2011; Wang et al., 2011). To study the presentation of an epitope derived from a retrotranslocated protein we chose the tyrosinase epitope Tyr369-377(D). Tyrosinase is a transmembrane glycoprotein produced in melanocytes and melanoma cells that is targeted to the ER (Jimbow et al., 2000; Petrescu et al., 2000). Misfolded tyrosinase is exported back to the cytosol where it is deglycosylated leading to the conversion of N371 to D371 through deamination (Skipper et al., 1996). The HLA-A*0201-restricted epitope Tyr369-377(D) spans across this modified amino acid and allows analysis of an epitope derived exclusively from a retrotranslocated antigen. To study presentation of Tyr369-377(D), we made use of the transgenic mouse strain AAD (H-2^b, HLA-A2.1/H2-D^d) expressing a hybrid MHC class I molecule consisting of the alpha-1 and alpha-2 domain of human HLA-A*0201 and the alpha-3 transmembrane and cytoplasmic domain of mouse H-$2D^d$ (Newberg et al., 1996). AAD mouse embryonic fibroblasts (MEFs) were transfected with BAG6 siRNA and stimulated with IFN-γ to induce MHC class I expression. Cells were then infected with recombinant vaccinia virus expressing tyrosinase (rVV-Tyr) for 5 h. Tyr369-377(D)-MHC complexes were detected with specific CTL lines generated from AAD mice and analyzed by staining for CD8 and intracellular IFN-γ. BAG6 knockdown did not alter Tyr369-377(D) presentation (Fig. 3B) although no detectable BAG6 protein remained after knockdown (Fig. 3C). While BAG6 seemed to be dispensable for Tyr369-377(D) presentation, endogenous BAG6 could be pulled down with transiently expressed tyrosinase in HEK293 cells (Fig. 3D). This interaction was even enhanced when proteasomal degradation was blocked by MG132 treatment indicating a role of BAG6 in tyrosinase degradation.

Overall, we found no evidence for an essential role of BAG6 in generating LCMV- or tyrosinase-derived ligands for the presentation on MHC class I. However, we cannot formally exclude the possibility that other epitopes might depend on BAG6 for effective processing and presentation.

4. Discussion

Antigen processing and presentation have crucial impacts on the efficiency and outcome of an immune response. A possibility to rapidly detect translation of foreign proteins within a cell is the use of DRiPs as substrates for antigen processing. As a consequence, the "immunopeptidome" surveyed by CTLs mirrors translation rates rather than protein concentrations (Caron et al., 2011; Fortier et al., 2008; Khan et al., 2001). Bourdetsky and colleagues could indeed show that peptides presented on MHC class I shifted faster from light to heavy isotope forms than their source proteins when cells were switched to media containing amino acids with heavy isotopes (Bourdetsky et al., 2014). This raises the question whether DRiPs and DRiP-derived peptides are simple by-products of protein synthesis or if cells have evolved mechanisms regulating DRiP formation and/or processing. Yewdell and Nicchitta (2006) suggested the existence of "immunoribosomes" specialized on the production of substrates for the MHC class I restricted antigen processing pathway (Yewdell and Nicchitta, 2006). Such immunoribosomes may have a different subunit composition or localize in a different intracellular compartment than conventional ribosomes. Although intellectually attractive, this hypothesis has not yet been confirmed experimentally. Instead, ribosomes are more likely to function as a platform for associated factors acting upon the nascent chain and influencing its fate (Kramer et al., 2009). While more and more factors are being identified to mediate co-translational and post-translational protein quality control little is known about the contribution of these factors to antigen processing. Specifically designed to recognize non-native polypeptides chaperones are amongst the candidates ideally suited for DRiP processing. Chaperones mediate an important layer of quality control and can facilitate degradation of non-native polypeptides through interaction with factors of the ubiquitin-proteasome system (Hohfeld et al., 2001; Rodrigo-Brenni and Hegde, 2012).

The chaperone BAG6 is involved in post-translational insertion of tail-anchored proteins into the ER membrane (Leznicki et al., 2010; Mariappan et al., 2010). While assisting in protein biosynthesis on the one hand, BAG6 also mediates degradation of mislocalized nascent chains (Hessa et al., 2011) and ERAD substrates (Claessen and Ploegh, 2011; Claessen et al., 2014; Payapilly and High, 2014; Wang et al., 2011). Moreover, Rodrigo-Brenni et al. (2014) recently described RNF126 as an E3 ligase interacting with BAG6 and specifically ubiquitylating chaperone bound client proteins (Rodrigo-Brenni et al., 2014). The fact that BAG6 has been described as a "holdase" conferring no evident folding activity onto its substrates makes this chaperone even more interesting with regard to protein quality control and degradation (Wang et al., 2011).

Here, we addressed whether BAG6 can influence antigen processing and presentation in the MHC class I restricted pathway. Knockdown of BAG6 had no influence on MHC class I cell surface expression indicating that loss of BAG6 can be effectively counterbalanced by other cytosolic chaperones under physiological conditions. This result is in contrast to a previous report by Minami et al. (2010) who found reduced MHC class I cell surface expression in BAG6 knockdown cells (Minami et al., 2010) but in agreement with a more recent study by Koch and colleagues who did not find such an effect (Kamper et al., 2012). We have currently no explanation for this discrepancy, especially, since we could not detect any residual BAG6 expression after transfection with BAG6 siRNA. Presentation of three LCMV-derived epitopes was entirely unaffected

with an expression construct for tyrosinase (Tyr)-Myc-Flag or empty plasmid (control) and treated with 10 μM MG132 or solvent DMSO for 5 h. Cell lysates were subjected to immunoprecipitation (IP) with anti-Myc antibody followed by western blot (WB) analysis with BAG6 or Flag specific antibodies. Load indicates protein expression levels in total protein lysates. For all experiments one out of two independent experiments is shown.

although one of the epitopes analyzed (GP276-286) derives from an ER-targeted glycoprotein. Hence, degradation of the GP276-286 precursor could be handled by the ERAD pathway or the mislocalized protein disposal pathway in both of which BAG6 is involved. Strikingly, the tyrosinase-derived epitope Tyr369-377(D), which strictly requires retrotranslocation from the ER in order to be presented, was also not affected by BAG6 knockdown. Together, these results indicate that BAG6 is dispensable for antigen processing and presentation on MHC class I. Enhanced interaction of BAG6 and tyrosinase under proteasome inhibition on the other hand still indicates that BAG6 could be a potential player in antigen processing. The redundancy in cellular chaperone networks could, however, mask the contribution of BAG6 to this pathway. BAG6 together with its E3 ligase RNF126 indeed resembles the HSP70/HSP90 chaperone system, which cooperates with the E3 ligase CHIP to promote substrate degradation (Connell et al., 2001; Rodrigo-Brenni et al., 2014). The finding that BAG6 substrates could still be degraded in the absence of BAG6 and/or RNF126, albeit more slowly and less efficiently, further indicates the existence of one or several redundant pathways (Rodrigo-Brenni et al., 2014). Moreover, BAG6 and HSP70 seem to compete for substrate binding as BAG6 could prevent HSP70-mediated refolding of denatured luciferase in an in vitro assay (Wang et al., 2011). Although BAG6 preferably binds to longer hydrophobic patches (Mariappan et al., 2010) and HSP70/HSP90 to shorter hydrophobic patches (Jackson, 2013; Rudiger et al., 1997) considerable overlap in both systems is likely. Definition of the potential proportion of antigen that is handled by BAG6 would therefore be possible if BAG6-mediated degradation could be inhibited after substrate binding to the chaperone. Thereby, antigen could be trapped on BAG6 and degradation via a different route would be blocked.

Compartmentalized antigen processing was first suggested by Lev et al. (2010) (Lev et al., 2010) and could be another explanation for our findings. In this case, BAG6 might be involved in degradation of bulk proteins while being dispensable for epitope generation. Taken together, we propose that BAG6 is dispensable for antigen processing and presentation on MHC class I but it might become essential under conditions of limited chaperone availability.

Conflict of interest

The authors declare no conflicts of interest.

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