

Efficient Cross-presentation Depends on Autophagy in Tumor Cells

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Abstract

Cross-presentation of antigens is critical for the induction of adaptive immunity against tumor cells and infectious pathogens. Currently, it is not known how cross-presentation of tumor antigens is regulated by autophagy. Using both HEK 293T cells that expressed the model antigen OVA and melanoma cells as antigen donors, we show that macroautophagy in tumor cells is essential for cross-presentation by dendritic cells both *in vitro* and *in vivo*. Inhibition of autophagy abolished cross-presentation almost completely, whereas induction of autophagy dramatically enhanced the cross-presentation of tumor antigens. Moreover, purified autophagosomes were found to be efficient antigen carriers for cross-presentation. Our findings not only identified a novel role for autophagy as an active process in antigen sequestration and delivery to dendritic cells for cross-presentation, but also suggested, for the first time, that isolated autophagosomes may have potential as potent vaccines for immunotherapy against cancer and infectious diseases. [Cancer Res 2008;68(17):6889–95]

Introduction

Antigen cross-presentation is critical for the activation of T cells to viral and tumor antigens that are expressed by parenchymal cells and is necessary for the elimination of tumor cells and many pathogens. Cross-presentation is the process by which the antigens from “donor cells” are captured, processed, and then presented to antigen-specific T cells by host professional antigen-presenting cells (APC; ref. 1). Although the mechanisms of antigen capture, processing, and presentation by APCs have been well appreciated, the forms of the antigen that are actually transferred from the donor cells to the APC are not clear. Furthermore, how the donor cells process their antigens and supply them to dendritic cells (DC) for cross-presentation has not been well understood. The cellular proteins in the donor cell that are potentially available for cross-presentation are degraded either by the proteasome or in the lysosome after autophagy. It has been suggested that the substrates and/or products of either of these two pathways, e.g., stable intact proteins, proteasome-generated peptides, as well as peptides chaperoned by heat shock proteins, can serve as the relevant form of antigen for cross-presentation (2, 3). Defective ribosomal products (DRiP) and short-lived proteins are commonly degraded

by the proteasome pathway. It has been shown that an active proteasome in virus-infected cells may actually suppress the cross-presentation of short-lived proteins, including DRiPs and that proteasome activity is not required for the cross-priming of CD8 T cells against long-lived proteins (3). Macroautophagy (herein, autophagy) participates in the bulk degradation of long-lived proteins (4) and may play an important role in the digestion of short-lived proteins, such as misfolded proteins or DRiPs (5, 6). During autophagy, a double-membrane structure sequesters misfolded proteins and damaged organelles in the cytosol and forms autophagosomes. Fusion of autophagosomes with the MHC II-containing compartment, such as late endosomes and lysosomes, enhances MHC II presentation of cytosolic proteins and viral antigens (7, 8); however, the role of autophagy in cross-presentation, either *in vitro* or *in vivo*, has not been elucidated.

There are three major stages in the autophagy pathway (4). The initiation stage is the *de novo* formation of an isolation membrane (also called phagophore), which is regulated by the mammalian target of rapamycin (mTor), Beclin-1 (Atg6), and type III phosphatidylinositol-3-kinase (hVPS-34). The second stage is elongation during which the isolation membrane expands and damaged organelles or cytosolic materials are captured. A critical step in the second stage is the conjugation of Atg5 to the ubiquitin-like molecule, Atg12, and to membrane lipids by another ubiquitin-like molecule, Atg8. These two conjugation steps are critical for the expansion of the isolation membrane via tethering and hemifusion of the phagophore membranes (9). The third stage involves the formation of autophagosomes, which is followed by rapid transition into autolysosomes upon fusion with lysosomes, and targets the captured materials for degradation.

Because autophagy is another major cellular pathway which mediates protein degradation, we hypothesized that inhibition of autophagy, like inhibition of the proteasome-augmented cross-presentation of short-lived proteins (3), would enhance the cross-presentation of long-lived protein antigens. To make this determination, we measured the effects of inhibition or induction of autophagy on cross-presentation by mouse DCs to transgenic T cells using melanoma cells or OVA-expressing human HEK 293T cells as the antigen donor cells. Cross-presentation of antigens derived from whole tumor cells, in which autophagy was altered, was assessed by T-cell proliferation both *in vitro* and *in vivo*. In addition, autophagosome-enriched fractions and purified autophagosomes were used as the antigen source for the cross-presentation after being loaded onto DCs.

Materials and Methods

Mice. C57BL/6 mice were purchased from the Charles River Laboratories. TCR transgenic OT-I breeders were from the Jackson Laboratory. pmel-1 breeders transgenic for the TCR that recognizes mouse and human gp100_{25–33} were obtained from Dr. Nicholas P. Restifo (National Cancer Institute, NIH; ref. 10). All mice were maintained and used in accordance with the Earle A. Chiles Research Institute Animal Care and Use Committee.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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DNA construction and transfection. Plasmid DNA vector cloning: the Ub-X-GFP⁴-expressing plasmids were kindly provided by Dr. Maria G. Masucci (Microbiology and Tumor Biology Center, Karolinska Institutet, Stockholm, Sweden; ref. 11). The Ub-X-GFP-OVA and Ub-X-GFP-TFR-OVA fusion constructs were made by PCR with Vent polymerase and cloned into the lentiviral vector pWPT (Supplementary Fig. S1). The pWPT vector was kindly provided by Dr. D. Trono (Department of Microbiology, Geneva School of Medicine, Geneva, Switzerland) and modified to enable the convenient cloning method with the NEB USER Enzyme (New England Biolabs). LC3 fusion plasmids, pCMV-GFP-LC3, and pCMV-tdTomato-LC3 were kindly provided by Dr. T. Johansen (Biochemistry Department, Institute of Medical Biology, University of Tromsø, Tromsø, Norway) and subcloned into pWPT vector after PCR with Vent polymerase. CFP-LC3 and tdTomato-Ub fusion plasmids were constructed by PCR and cloned into pWPT vector. The second-generation lentiviral siRNA vector containing a new design of Mir30-modified short hairpin RNA (shRNAmir) against Beclin-1 (V2MM-9827) and nonspecific control shRNAmir plasmids were purchased from Open Biosystems. For the generation of recombinant lentiviruses, HEK 293 cells were transiently transfected with vector plasmid pWPT or pGIPz, virus packaging plasmid pPAX2, envelope plasmid VSV-G MD2, and helper plasmid pAdv. Viral supernatant was used to infect tumor cells and tumor cells were sorted based on GFP expression by flow cytometry (OVA fusion proteins) or selected with puromycin (Beclin-1).

Fractionation and purification of autophagosomes. HEK 293T cells were transfected with CFP-LC3 and OVA antigen. After 18 h of treatment with bortezomib (100 nmol/L) and NH₄Cl (10 mmol/L), cells were disrupted by mild sonication at 115 V, 56 to 60 Hz using the G112SP1G Special Ultrasonic Cleaner (Laboratory Supplies, Co., Inc.). Here, bortezomib was used as an inhibitor of proteasome activity. NH₄Cl was used to block lysosomal fusion and degradation of autophagosomes. Cell lysates were precleared by centrifugation at 300 × g for 10 min, and then were separated into the crude autophagosome-containing large vesicles and the supernatant consisting of cytosolic components by a 15-min centrifugation at 10,000 × g. A portion of the crude large vesicle preparations was fractionated by self-generated Percoll gradient sedimentation. Vesicles from 5 million cells were resuspended in 20 mmol/L of HEPES (pH 7.0), with 0.3 mol/L of sucrose, 27% Percoll, and 1 mmol/L of EDTA. The gradient was centrifuged for 30 min at 36,000 × g in a NVT65 rotor in a Beckman Optima L-90K Ultracentrifuge (Beckman Coulter, Inc.). Twelve fractions were collected using Pasteur pipettes. For autophagosome purification, GFP-LC3-marked autophagosomes were pulled down using sheep anti-mouse IgG Dynabeads (DynaBiotec) in combination with mouse anti-GFP antibody (Stressgen Bioreagents) according to the manufacturer's recommendations. Fractions of the flow through, wash, and eluate were collected for further analysis. The distribution of autophagosomes within each fraction was determined by Western blot of the GFP-LC3 marker, and the ability of these fractions to activate OT-I CD8 T cells in the presence of DCs was analyzed by 6-carboxyfluorescein succinimidyl ester (CFSE) dilution.

Western blotting. HEK 293T cells (1 × 10⁶) expressing GFP-OVA fusion protein (treated or untreated) were lysed in 100 μL of radioimmunoprecipitation assay buffer. The lysates were mixed with 4× NuPAGE LDS sample buffer and samples (10 μL each) were resolved by 4% to 20% SDS-PAGE (Invitrogen). Proteins were transferred to a nitrocellulose membrane, incubated with primary antibodies diluted in blocking buffer (5% dry milk) overnight, and then exposed to horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h. Protein bands were revealed by using chemiluminescent reagents (Pierce). The primary antibodies included rabbit anti-actin (1:2,000; Sigma), biotin-conjugated anti-GFP (1:5,000; Rockland), anti-Beclin-1 (1:1,000; ProSci), and mouse anti-GFP (1:1,000; Stressgen). The secondary antibodies were avidin-HRP (1:10,000; eBioscience), goat anti-rabbit HRP (1:10,000; Jackson ImmunoResearch), and goat anti-mouse HRP (1:10,000; Jackson ImmunoResearch).

Fluorescent microscopy. Images of live cells were taken using a Leica inverted microscope capable of digital epifluorescence imaging. A GFP filter

(excited at 470/40, dichromatic mirror at 495, and long-pass emission filter at 500LP) and an Orange filter (excited at 525/50, dichromatic mirror at 555, and band-pass emission filter at 590/50) were used to capture fluorescent images.

Modulation of autophagy and proteasome activity. Autophagy of antigen donor cells was inhibited by the addition of 3-methyladenine (3-MA; 10 mmol/L; Sigma) or wortmannin (1.0 μmol/L; Calbiochem) for 12 to 18 h. To induce autophagy, donor cells were grown in the presence of rapamycin (20 nmol/L; Alexis), or HBSS (amino acid starvation), for 12 to 18 h. NH₄Cl (10 mmol/L; Sigma) or bortezomib (100 nmol/L; Millenium Pharmaceuticals) was used for 18 to 24 h to block lysosome or proteasome activity, respectively.

Atg12 or Beclin-1 knockdown by siRNA. Synthetic *Atg12* siRNAs and control siRNAs were purchased from Invitrogen. HEK 293T cells expressing GFP-OVA fusion protein were transfected with siRNAs together with INTERFERin (Polyplus Transfection) following the manufacturer's protocol. Knockdown of *Atg12* was verified by reverse transcription-PCR (RT-PCR) 24 h later. mRNA was isolated from fresh cells using the RNeasy Mini Kit (Qiagen) 24 to 40 h after siRNA transfection and reverse transcribed to first-strand cDNA using an oligo-dT primer (Promega). For knockdown of mouse *Beclin-1*, B16 F10 melanoma cells were infected with shRNAmir lentiviral vector (pGIPz; Open Biosystems) and selected with puromycin.

CFSE labeling and *in vitro/in vivo* CFSE dilution assay. Cross-presentation of antigens to OT-I or pmel-1 naïve T cells was assessed by measuring the dilution of CFSE by flow cytometry (9). DCs were isolated from the spleens of C57BL6 mice after sequential i.v. injection of plasmid DNA encoding murine Flt3 ligand and granulocyte macrophage colony-stimulating factor (12). For the *in vitro* CFSE assay, antigen donor cells (3 × 10⁵), with or without treatment, were irradiated at 10,000 cGy and incubated with 2 × 10⁶ DC for 6 h, the mixed cells were then washed thrice before the addition of 3 × 10⁶ CFSE-labeled OT-I or pmel-1 T-cells. Splenocytes from OT-I or pmel-1 mice were labeled with 5 μmol/L of CFSE according to the manufacturer's protocol (Invitrogen). T-cell proliferation was measured after 4 or 5 days of DC and T-cell coinoculation. For the *in vivo* CFSE assay, antigen donor cells (HEK 293T, FEMX, or B16 F10) treated with various chemicals or siRNAs were collected and washed extensively in PBS, and s.c. injected into both flanks of C57BL6 mice. The same day, 5 × 10⁶ Thy1.1⁺ OT-I or pmel-1 T-cells labeled with CFSE were adoptively transferred into these mice i.v. Lymph nodes were collected 5 days later and single-cell suspensions were prepared. Proliferation of CFSE-labeled T cells was analyzed and the percentage of adoptively transferred T cells to total CD8⁺ lymphocytes was determined by flow cytometry analysis.

Results and Discussion

The autophagy pathway in antigen donor cells affects cross-presentation. Because lysosomal degradation of proteins in the final stage of autophagy could decrease the supply of antigens for cross-presentation, we examined whether activation or inhibition of autophagy in donor cells would affect cross-presentation of tumor antigens. First, HEK 293T cells that expressed the V-GFP-TFR-OVA fusion protein were used as the antigen donor. The V-GFP-TFR-OVA fusion protein is a membrane-bound, long-lived protein (Supplementary Fig. S1). OVA-expressing HEK 293T cells were treated with rapamycin or subjected to starvation to induce autophagy, or they were treated with the phosphoinositide-3-kinase inhibitor, 3-MA, to prevent the initiation of autophagy. The treated HEK 293T cells were irradiated and incubated with DCs for 6 h and then CFSE-labeled naïve OT-I transgenic cells were added to the culture. T-cell proliferation was measured by flow cytometry analysis of the CFSE profile of gated CD8⁺ T cells. To our surprise, cross-presentation of OVA antigen was greatly increased after treatment with rapamycin (Fig. 1A) or starvation (data not shown). Conversely, inhibition of autophagy in antigen donor cells with 3-MA significantly decreased cross-presentation of OVA antigen to OT-I T-cells, close to the level of background T-cell proliferation

⁴ Ub, ubiquitin; X, M, R, or V; GFP, green fluorescent protein.

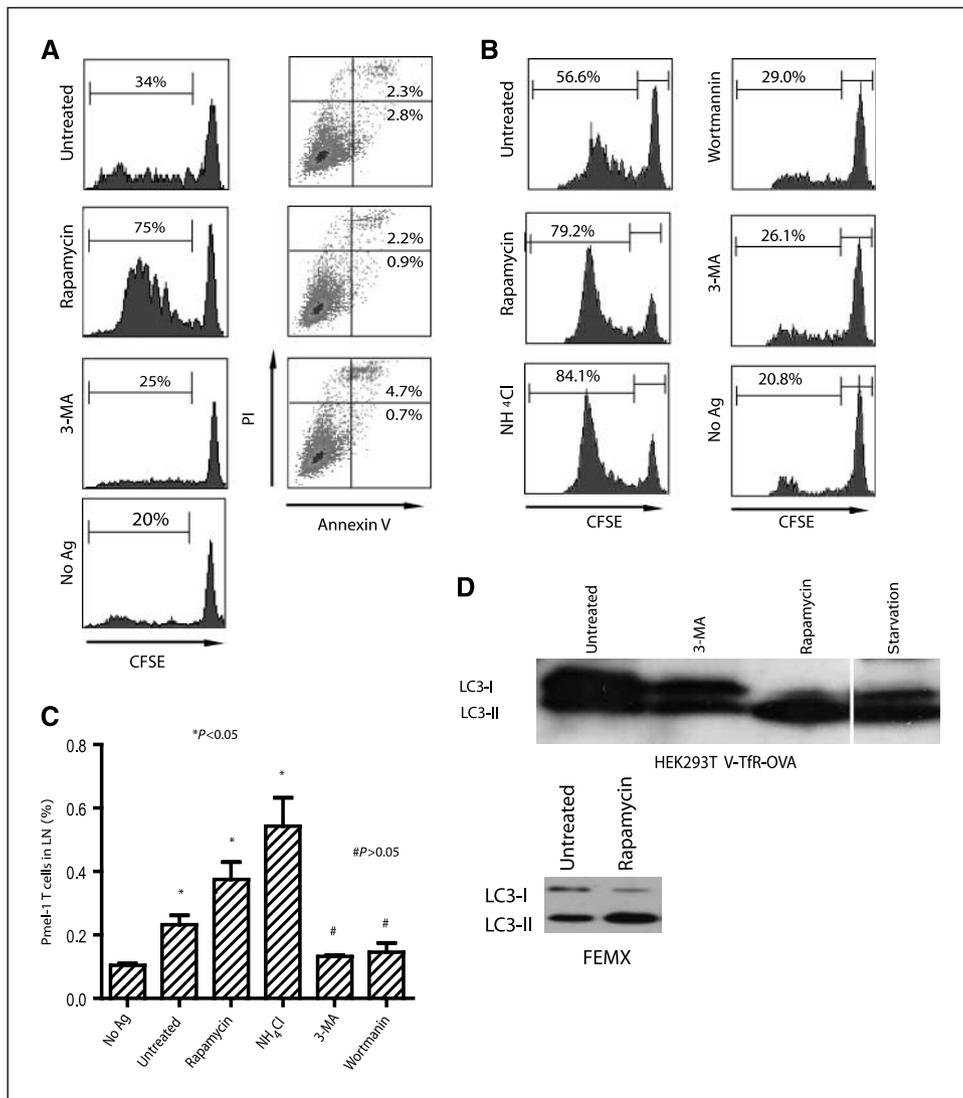


Figure 1. Cross-presentation of OVA and gp100 to transgenic T cells was greatly affected by the inhibition or induction of autophagy in the antigen donor cells. **A**, modulation of cross-presentation of the OVA model antigen *in vitro*. HEK 293T cells expressing the GFP-OVA fusion protein were treated with the autophagy-inhibitors 3-MA or wortmannin or the autophagy-inducer rapamycin for 18 h, or subjected to starvation by culturing donor cells in HBSS for 18 h. Propidium iodide and FITC-labeled Annexin V were used to stain treated cells to measure the levels of apoptosis or necrosis, respectively. To assess cross-presentation, treated tumor cells were irradiated and incubated with DCs for 6 h. CFSE-labeled naive T cells from OT-I TCR transgenic mice were added to the mixture of DC and 293T donor cells and cultured for 4 to 5 d. The dilution of CFSE label in OT-I T cells was determined by flow cytometry. The results are expressed as the percentage of OT-I T cells that had undergone at least one division 4 d after coculture with DC and donor cells. The data represent typical results of three to five independent experiments. **B** and **C**, modulation of cross-presentation of the melanoma gp100 antigen *in vivo*. Cells from the human melanoma cell line FEMX were treated with the indicated agents as described above and then injected into the flanks of naive C57BL/6 mice. CFSE-labeled naive spleen cells from pmel-1 transgenic mice were adoptively transferred into tumor-bearing mice, and lymph nodes draining the tumors were collected at day 6. Both the CFSE profile of pmel-1 CD8 T cells (**B**) and the percentage of pmel-1 T cells among the lymph node lymphocytes (**C**) were determined by flow cytometry. Mice that received no tumor or untreated FEMX cells were included as the controls. Each group consisted of four mice and the experiment was repeated once with similar results. The difference between treated and untreated groups was significant (*, $P < 0.05$); however, the difference between the no Ag and 3-MA or the wortmannin-treated group was not significant (#, $P > 0.05$). **D**, HEK 293T cells expressing OVA antigen or FEMX melanoma cells were treated with 20 nmol/L of rapamycin or 10 mmol/L of 3-MA for 18 h. Lysates were prepared from both untreated and treated cells. Lysate from starved HEK 293T cells was included as a positive control. Ten micrograms of total proteins were loaded and subjected to SDS-PAGE and Western blot analysis with rabbit anti-LC3 antibody.

without antigen (Fig. 1A). Moreover, percentage of cell death, which was judged by staining treated HEK 293T cells with annexin and propidium iodide or 7-AAD, did not seem to be related to the alteration in cross-presentation due to autophagy modulation. No significant apoptosis nor necrosis of tumor cells was identified 18 hours after any of the treatments, indicating that cell viability was not affected by either the induction or inhibition of autophagy before irradiation (Fig. 1A). However, cells underwent progressive death after irradiation and cocultivation with DCs. Induction of autophagy prior to irradiation hindered cell death, whereas

inhibition of autophagy accelerated cell death (Supplementary Fig. S2). To ensure that these observations were not an artifact of the OVA model, we also examined cross-presentation of an endogenous tumor antigen, gp100, expressed by the human melanoma cell line, FEMX, after inhibiting and inducing autophagy. By assessing proliferation of adoptively transferred gp100-specific pmel-1 transgenic T cells (10), we found that *in vivo* cross-presentation of gp100 was also regulated by autophagy. Treatment of FEMX tumor cells with rapamycin before injection into mice significantly increased cross-presentation of gp100

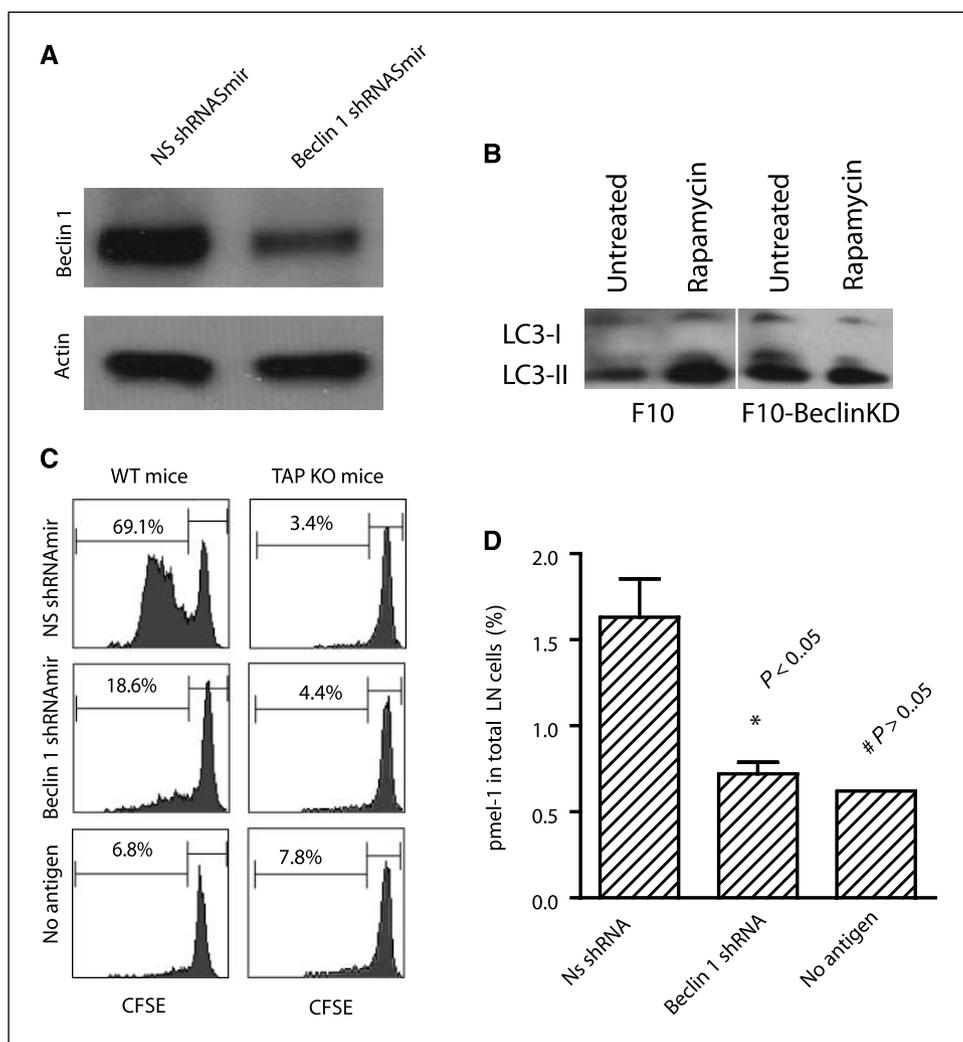


Figure 2. Knockdown of Beclin-1 in antigen donor cells decreased cross-presentation of tumor antigens by DC. *A*, B16 F10 melanoma cells were transduced with lentiviral vectors encoding a shRNA specific for mouse *Beclin-1* or a nonspecific control shRNA. The specific knockdown of *Beclin-1* was confirmed by Western blot analysis with anti-Beclin-1 antibody. Anti- β -actin antibody was included as the control. *B*, F10 melanoma cells expressing either control or *Beclin-1* shRNAs were treated with 20 nmol/L of rapamycin for 18 h. Lysates were prepared from both untreated and treated cells. Ten micrograms of total proteins were loaded and subjected to SDS-PAGE and Western blot analysis with rabbit anti-LC3 antibody. *C*, B16 F10 melanoma cells that were transduced with *Beclin-1* or control shRNA were injected into the flanks of both C57BL/6 wild-type or TAP-1 knockout mice ($n = 4$) that received 5×10^6 Thy1.1⁺ pmel-1 transgenic T cells. Six days after tumor injection, the CFSE profile of transferred pmel-1 T cells from the draining lymph nodes was determined. *D*, the percentage of pmel-1 T cells found among the lymphocytes in the draining lymph nodes of each mouse was determined by flow cytometry. Columns, mean; bars, SD ($n = 4$). The difference between control and *Beclin-1* knockdown was significant; however, the difference between *Beclin-1* knockdown and no antigen was not significant. The data represent the results from three independent experiments.

antigen as indicated by both the CFSE profile and the number of pmel-1 T cells in the draining lymph nodes (Fig. 1*B* and *C*). Most importantly, both autophagy inhibitors, 3-MA and wortmannin, almost completely abolished the cross-presentation of gp100 to gp100-specific transgenic T cells. As expected, treatment of either OVA-expressing HEK 293T or FEMX cells with rapamycin led to the conversion of autophagy protein Atg8, from an LC3-I form to an LC3-II form (Fig. 1*D*). These results indicate that cross-presentation of tumor antigens depends on the early phase of autophagy (i.e., formation of early autophagosomes). Interestingly, when NH_4Cl was included to prevent lysosome acidification and fusion, and the subsequent degradation of proteins, cross-presentation was augmented. These data suggested that although the early phases of autophagy (initiation and formation of autophagosomes) are required for efficient cross-presentation, the late phase of autophagy during which the encapsulated proteins are degraded in lysosomes hinders cross-presentation. Apoptosis and necrosis of tumor cells have been reported to affect antigen cross-presentation (13, 14); here, we showed that autophagy in antigen donor cells also has a dramatic effect on the efficiency of cross-presentation for both model and endogenous tumor antigens.

Knockdown of the autophagy initiation gene, *Beclin-1*, in antigen donor tumor cells greatly reduced cross-presentation. The abovementioned inhibitors of autophagy are not specific and

may affect other cellular processes. Therefore, we sought to confirm the role of autophagy in cross-presentation using RNA interference to knock down the essential autophagy initiation gene, *Atg6/Beclin-1*, in B16 F10 murine melanoma cells. *Atg6/Beclin-1* regulates autophagy by forming a complex with class III phosphatidylinositol-3-kinase, a critical lipid kinase involved in membrane trafficking and fusion, and modulating its activity (15). To knock down *Beclin-1*, a stable B16 F10 cell line that expressed shRNA was generated using a lentiviral vector. The *Beclin-1* protein level in transduced B16 F10 was reduced to approximately one-half that found in B16 F10 melanoma cells that expressed the control nonspecific shRNA (Fig. 2*A*). To show that F10 melanoma cells expressing shRNA against *Beclin-1* were in fact defective in autophagy induction, the rapamycin-induced conversion of LC3 was examined in F10 cells expressing either control shRNA or *Beclin-1*-specific shRNA. As expected, rapamycin increased LC3-II found in F10 cells expressing control shRNA but not F10 cells expressing shRNA specific to *Beclin-1* (Fig. 2*B*). To examine the effects of Beclin-1 knockdown on cross-presentation, each melanoma cell line was injected into mice infused with CFSE-labeled gp100-specific pmel-1 T-cells. Melanoma cells with a knockdown of *Beclin-1* were far less able to cross-present gp100 as evidenced by significantly less T-cell proliferation compared with T cells that were exposed to tumor cells treated with control siRNA (Fig. 2*C*). Knocked-down

Beclin-1 reduced the percentage of pmel-1 T-cells in the draining lymph nodes to the background level of control mice without tumor injection (Fig. 2D). Because *Beclin-1* knockdown could have affected direct presentation by B16 F10 cells, we did an additional experiment in TAP-1-deficient mice; endogenous DCs from these mice will not be able to cross-present peptides to T cells. The near-complete absence of pmel-1 proliferation in TAP-1-deficient mice injected with tumors treated with control or *Beclin-1* siRNA essentially ruled out direct presentation. Thus, cross-presentation was significantly impaired in *Beclin-1* knockdown mice. This also confirmed an earlier study which showed that cross-presentation was the major pathway by which T cells were activated and induced to proliferate in mice inoculated with B16 melanoma cells (16).

Knockdown of *Atg12*, a critical gene for the formation of autophagosomes in antigen donor cells, significantly reduced cross-presentation. In order to determine what portion

of the autophagy pathway was important for cross-presentation, we investigated whether the formation of autophagosomes (the second stage of autophagy) was required for efficient cross-presentation. The elongation phase of autophagy, which leads to the formation of autophagosomes that capture damaged organelles or cytosolic material, requires the initial conjugation of Atg12-Atg5 and the subsequent conjugation with Atg8 (LC3) and membrane phospholipids (4). This aspect of the pathway was inhibited by specific knockdown of *Atg12* by siRNA in OVA-expressing HEK 293T cells. Decreased *Atg12* expression was documented by RT-PCR (Fig. 3A). The lack of *Atg12* protein would prevent incorporation of Atg8 into the lipid membrane and interfere with the formation of the autophagosome. Thus, we would expect fewer LC3-positive autophagosomes in *Atg12* knockdown cells. This can be readily seen in Fig. 3B, in which there were far fewer LC3-positive punctates in donor cells transfected with *Atg12* siRNA

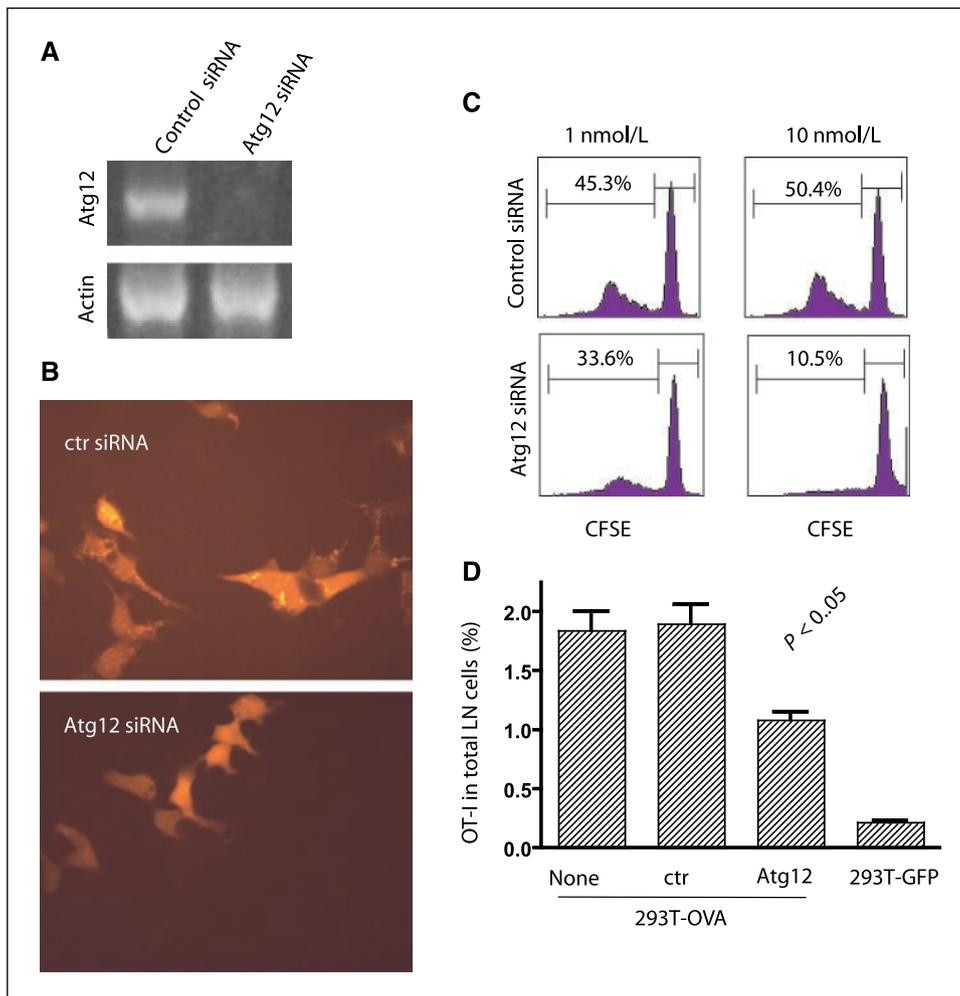


Figure 3. Cross-presentation was reduced when autophagy was inhibited by knockdown of *Atg12*. *A*, RT-PCR for *Atg12* mRNA in HEK 293T cells expressing the GFP-OVA fusion protein after transient transfection with *Atg12* or control siRNA. Actin mRNA was included as the control. *B*, diminished formation of tdTomato-LC3 punctates after *Atg12* knockdown. HEK 293T cells stably expressing tdTomato-LC3 fusion protein following infection with a viral vector were transfected with *Atg12* siRNA or control siRNA. Forty-eight hours later, transfected cells were treated with rapamycin and NH_4Cl for 6 h to induce the formation of punctates. *C*, reduced cross-presentation of OVA from donor cells after transfection with *Atg12* siRNA *in vitro*. HEK 293T cells that expressed GFP-OVA fusion protein were transfected with *Atg12* or luciferase siRNA 24 h before they were loaded onto DC. CFSE-labeled OT-I naïve T cells were added after 6 h of DC and 293T coculture and the dilution of CFSE-label in OT-I T cells at day 4 was determined by flow cytometry. The results are typical of five independent experiments. The numbers indicate the percentage of OT-I T cells that had undergone at least one cell division. *D*, reduced cross-presentation of OVA from donor cells after transfection with *Atg12* siRNA *in vivo*. HEK 293T cells expressing GFP-OVA fusion protein were transfected with either control siRNA or *Atg12* siRNA. Two days later, control or *Atg12* siRNA-treated cells were injected into both flanks of mice that also received 5×10^6 Thy1.1+ OT-I transgenic T cells. HEK 293T cells expressing GFP protein were used as the negative control. The percentage of OT-I T cells found in the draining lymph nodes of each mouse was determined individually by flow cytometry 5 d after injection. Columns, mean; bars, SD ($n = 4$). Typical results from three independent experiments.

compared with cells transfected with control siRNA. This confirmed that autophagy was reduced and fewer autophagosomes had been produced. The donor cells' ability to cross-present antigen *in vitro* following knockdown of *Atg12* was notably reduced (Fig. 3C). *In vivo* cross-presentation was also decreased when antigen donor cells were transfected with *Atg12* siRNA before inoculation into mice (Fig. 3D). Four days after tumor inoculation, the percentage of OT-I T-cells in the draining lymph nodes of mice immunized with donor tumor cells transfected with *Atg12* siRNA was significantly reduced compared with the percentage of T cells in mice immunized with untransfected or control siRNA-transfected donor cells. These results further support an important role for autophagy in the efficient cross-presentation of protein antigens from tumor cells.

Autophagosomes are efficient carriers of protein antigens from donor tumor cells. Having showed both that NH_4Cl

blockade of the fusion between autophagosomes and lysosomes enhanced cross-presentation, and that autophagosome formation was required for cross-presentation, our results strongly suggested that autophagosomes could be the antigen carriers for cross-presentation. Using fluorescent microscopy, we visualized the localization of the protein antigen Ub-V-GFP-OVA in the LC3-positive autophagosome punctates in HEK 293T cells treated with NH_4Cl and bortezomib (Supplementary Fig. S3A). Bortezomib, a known inhibitor of proteasome activity, was also found to induce autophagy in our experiments (Supplementary Fig. S3B). To determine whether autophagosomes served as an antigen carrier for efficient cross-presentation, we lysed tumor cells by sonication and separated the whole-cell lysate into supernatant and pellet by high-speed centrifugation ($10,000 \times g$ for 15 minutes). Cytosolic proteins and microsomes remained in the supernatant whereas large vesicles, which included mitochondria, lysosomes, and autophagosomes were found in the pellet. Although the supernatant contained more GFP-OVA antigen than the pellet (data not shown), the pelleted vesicles were significantly better in stimulating the proliferation of OT-I T-cells when used to pulse DCs (Fig. 4A). Using a Percoll gradient and high-speed centrifugation, we further fractionated the vesicles from the pellet according to their density. Autophagosomes are light vesicles and band at lower density than mitochondria and lysosomes (15). Individual fractions were recovered and incubated with DCs and then used to stimulate CFSE-labeled naïve OT-I T-cells. The ability of each individual fraction to cross-present OVA correlated directly with the amount of LC3, a specific marker of autophagosomes, in that fraction (Fig. 4B). Although LC3, and therefore, presumably, autophagosomes were found in many fractions, the three fractions in which LC3 could not be detected were devoid of cross-presentation ability. Using melanoma cells, we also found that fractions enriched

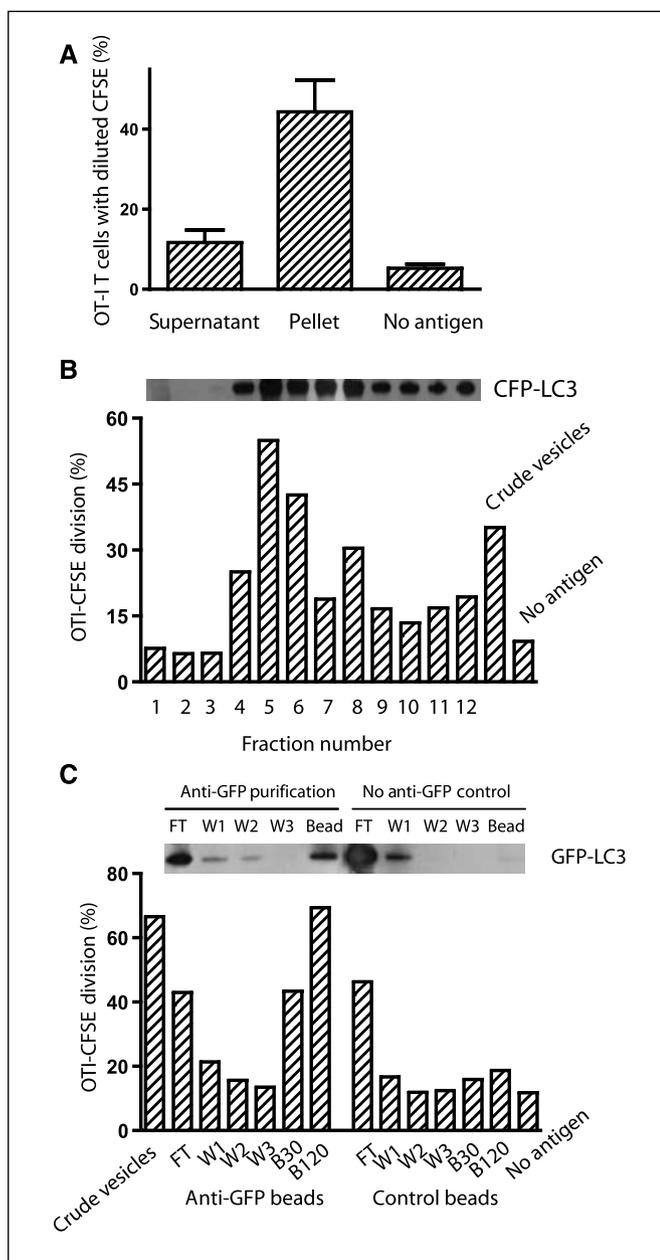


Figure 4. Autophagosomes are the source of antigen for cross-presentation. **A**, cross-presentation activity was found primarily in the large vesicles obtained from centrifugation of cell lysates. HEK 293T cells expressing OVA and CFP-LC3 were treated with bortezomib and NH_4Cl for 24 h and then lysed by mild sonication. The lysate was fractionated into the supernatant and a pellet containing large vesicles by high-speed centrifugation ($10,000 \times g$ for 15 min). The pellet was resuspended in the original volume. DCs were incubated with an equal volume of supernatant or resuspended pellet and used to stimulate CFSE-labeled OT-I T cells *in vitro*. The dilution of the CFSE label in OT-I T cells at day 4 was determined by flow cytometry. *Columns*, mean of four independent experiments; *bars*, SD. **B**, the highest cross-presentation activity and the greatest amount of LC3 was found in the lighter density fraction of large vesicles. The pellet was resuspended in 27% Percoll solution and subjected to ultra-speed centrifugation ($36,000 \times g$ for 30 min). One-milliliter fractions were collected from the top to the bottom of the gradient. The distribution of CFP-LC3 in the gradients was analyzed by Western blot with anti-GFP antibody (*above the bar graph*). DCs were loaded with each fraction and used to stimulate CFSE-labeled OT-I T cells *in vitro*. The dilution of CFSE-label in OT-I T cells at day 4 was determined by flow cytometry (*bar graph*). Data represent a typical result from three independent experiments. **C**, purified autophagosomes delivered OVA very efficiently for cross-presentation. HEK 293T cells were transiently transfected with plasmids encoding GFP-LC3 and OVA. Forty-eight hours after transfection, cells were treated and lysed as above, and the large vesicles were prepared from lysates. The resuspended pellet was purified using a mouse anti-GFP antibody and magnetic beads conjugated with anti-mouse IgG. The control was done in the absence of the mouse anti-GFP antibody. Different fractions, including the crude vesicles before purification, flow through (FT), three washing steps (W1, W2, and W3), and the bead fractions (B30 and B120) were obtained and kept in their original volume. Thirty microliters of each fraction were loaded onto DC, which were used to stimulate OT-I T cells as above (FT, W1, W2, W3, and B30). An additional tube with 120 μL of the bead fraction was included (B120). *Top*, Western blot analysis of each fraction with anti-GFP antibody. Data represent a typical result from two independent experiments.

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for autophagosomes mediated the cross-presentation of gp100 antigen to naïve pmel-1 T-cells.⁵

To confirm that autophagosomes labeled with LC3 were the carriers of antigen for efficient cross-presentation, autophagosomes were purified from lysates of HEK 293T cells that expressed both GFP-LC3 and OVA proteins by using a mouse anti-GFP antibody and anti-mouse IgG-conjugated magnetic beads (Fig. 4C). Mock purification in the absence of anti-GFP antibody was also done to ensure that purification was specific to GFP-LC3. Western blot analysis confirmed the specificity of purification. GFP-LC3-positive materials were obtained only when the anti-GFP antibody was used. After purification, each fraction was loaded onto DCs which were used to stimulate naïve OT-I T-cells as described above. The bead fraction collected in the presence of antibody to GFP conferred strong cross-presentation ability to DCs in a dose-dependent fashion; DCs pulsed with the same fraction isolated in the absence of GFP antibody failed to stimulate OT-I T-cells even if more material was used for loading.

Cross-presentation of tumor antigens has been shown to involve both intact whole proteins and chaperoned protein products that were generated either during protein synthesis or degradation (16, 17). Additional factors that may influence cross-presentation include the role of particulate versus soluble antigens and the role of apoptotic versus necrotic cells as antigen donors for professional APCs. The half-life of proteins and the quantity available are also important factors in cross-presentation. Our results identified another novel, critical regulatory element of cross-presentation and strongly suggest that autophagy in the antigen donor cells is a central process by which antigens are sequestered, accumulated, and degraded prior to cross-presentation. Proteins targeted to the proteasome for degradation (short-lived proteins and DRiPs) are not efficiently cross-presented; however, proteins that accumulate in autophagosomes, e.g., long-lived proteins, or accumulated DRiPs and short-lived proteins after inhibition of proteasome function,

could subsequently be delivered to DCs for cross-presentation if fusion with lysosomes was blocked.

Autophagy is a highly conserved, global cellular process in many cell lineages and tumor cell lines. A number of pathogens, including bacteria, viruses, and parasites, induce autophagy in infected cells (18). Our findings also suggest that autophagy represents a physiologically relevant mechanism for cross-presentation of antigens derived from either tumor cells or pathogens. A deficiency of autophagy has been associated with tumorigenesis and persistence of viral infection (4, 19). Our results imply that defective autophagy could promote tumorigenesis or chronic infections by diminishing intrinsic cellular immunity (autophagy-mediated degradation of intracellular pathogens), reducing innate immunity (reduced activation of innate immune cells), and by inhibiting adaptive immunity (less efficient cross-presentation). Our results suggest a new strategy by which the efficacy of vaccines that depend on cross-presentation may be increased, i.e., by induction of autophagy *in vivo* or using isolated autophagosomes as the antigen vehicles to cross-prime antitumor immune response. Our preliminary data (Supplementary Fig. S4) from both mouse melanoma and lung cancer models supports the latter possibility, and we are planning new clinical trials using autophagosomes isolated from tumor cells as the novel cancer vaccine.

Disclosure of Potential Conflicts of Interest

H-M. Hu has ownership interest in Ubivac, LLC. The other authors disclosed no potential conflicts of interest.

Acknowledgments

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⁵ Unpublished data.

References

1. Heath WR, Carbone FR. Cross-presentation, dendritic cells, tolerance and immunity. *Annu Rev Immunol* 2001; 19:47-64.
2. Serna A, Ramirez MC, Soukhanova A, Sigal LJ. Cutting edge: efficient MHC class I cross-presentation during early vaccinia infection requires the transfer of proteasomal intermediates between antigen donor and presenting cells. *J Immunol* 2003;171: 5668-72.
3. Norbury CC, Basta S, Donohue KB, et al. CD8+ T cell cross-priming via transfer of proteasome substrates. *Science* 2004;304:1318-21.
4. Klionsky DJ, Emr SD. Autophagy as a regulated pathway of cellular degradation. *Science* 2000;290: 1717-21.
5. Komatsu M, Waguri S, Chiba T, et al. Loss of autophagy in the central nervous system causes neurodegeneration in mice. *Nature* 2006;441:880-4.
6. Hara T, Nakamura K, Matsui M, et al. Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. *Nature* 2006;441:885-9.
7. Paludan C, Schmid D, Landthaler M, et al. Endogenous MHC class II processing of a viral nuclear antigen after autophagy. *Science* 2005;307:593-6.
8. Schmid D, Pypaert M, Munz C. Antigen-loading compartments for major histocompatibility complex class II molecules continuously receive input from autophagosomes. *Immunity* 2007;26:79-92.
9. Nakatogawa H, Ichimura Y, Ohsumi Y. Atg8, a ubiquitin-like protein required for autophagosome formation, mediates membrane tethering and hemifusion. *Cell* 2007;130:165-78.
10. Overwijk WW, Theoret MR, Finkelstein SE, et al. Tumor regression and autoimmunity after reversal of a functionally tolerant state of self-reactive CD8+ T cells. *J Exp Med* 2003;198:569-80.
11. Dantuma NP, Lindsten K, Glas R, Jellne M, Masucci MG. Short-lived green fluorescent proteins for quantifying ubiquitin/proteasome-dependent proteolysis in living cells. *Nat Biotechnol* 2000;18:538-43.
12. He Y, Pimenov AA, Nayak JV, Plowey J, Faló LDJ, Huang L. Intravenous injection of naked DNA encoding secreted fli3 ligand dramatically increases the number of dendritic cells and natural killer cells *in vivo*. *Hum Gene Ther* 2000;11:547-54.
13. Albert ML, Sauter B, Bhardwaj N. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature* 1998;392:86-9.
14. Sauter B, Albert ML, Francisco L, Larsson M, Somersan S, Bhardwaj N. Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. *J Exp Med* 2000;191: 423-34.
15. Stromhaug PE, Berg TO, Fengsrud M, Seglen PO. Purification and characterization of autophagosomes from rat hepatocytes. *Biochem J* 1998;335:217-24.
16. Shen L, Rock KL. Cellular protein is the source of cross-priming antigen *in vivo*. *Proc Natl Acad Sci U S A* 2004;101:3035-40.
17. Binder RJ, Srivastava PK. Peptides chaperoned by heat-shock proteins are a necessary and sufficient source of antigen in the cross-priming of CD8+ T cells. *Nat Immunol* 2005;6:593-9.
18. Schmid D, Munz C. Innate and adaptive immunity through autophagy. *Immunity* 2007;27:11-21.
19. Orvedahl A, Alexander D, Tallozy Z, et al. HSV-1 ICP34.5 confers neurovirulence by targeting the Beclin 1 autophagy protein. *Cell Host Microbe* 2007; 1:23-35.