Receptor-mediated phagocytosis elicits cross-presentation in nonprofessional antigen-presenting cells

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In cross-presentation by dendritic cells (DCs), internalized proteins are retrotranslocated into the cytosol, degraded by the proteasome, and the generated antigenic peptides bind to MHC class I molecules for presentation on the cell surface. Endoplasmic reticulum (ER) contribution to phagosomal membranes is thought to provide antigen access to the ER-associated degradation (ERAD) machinery, allowing cytosolic dislocation. Because the ERAD pathway is present in all cell types and exogenous antigens encounter an ER-containing compartment during phagocytosis, we postulated that forcing phagocytosis in cell types other than DCs would render them competent for cross-presentation. Indeed, FcRγIIA expression endowed 293T cells with the capacity for both phagocytosis and ERAD-mediated cross-presentation of an antigen provided as an immune complex. The acquisition of this ability by nonprofessional antigen-presenting cells suggests that a function potentially available in all cell types has been adapted by DCs for presentation of exogenous antigens by MHC class I molecules.

Cross-presentation refers to the binding of peptides derived from exogenous proteins by MHC class I molecules and their subsequent recognition by CD8$^+$ T cells (1). The process generally requires access of the exogenous antigen to the cytosol, and, like conventional MHC class I presentation, proteasomal activity (2). Resulting peptides are then translocated from the cytosol into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP) for subsequent binding to MHC class I molecules (3).

Dendritic cells (DCs) have been considered specialized antigen-presenting cells (APCs) for cross-presentation because they efficiently capture exogenous antigens and tightly regulate the pH and proteolytic activity of the endocytic pathway to minimize protein degradation (4–6). It has also been proposed that contribution of ER membrane to DC phagosomes allows exogenous antigens to access the ER-associated degradation (ERAD) machinery, normally used to dispose of misfolded proteins from the ER (7). Indeed, we showed that cross-presentation by DCs requires ERAD-mediated cytosolic translocation (8). Because all cell types are capable of ER quality control and use the ERAD pathway, we hypothesized that facilitating phagocytosis in nonprofessional APCs might promote ER recruitment to phagosomal membranes, rendering such cells competent for cross-presentation. Here, we show that expression of the Fc receptor FcγRIIA in the human 293T kidney cell line resulted in uptake of antibody-coated exogenous particles, ER contribution to phagosomes, and ERAD-mediated cross-presentation.

Results

293T Cells Expressing FcγRIIA Efficiently Internalize Antibody-Coated Particles. We generated 293T cells stably expressing an EGFP-tagged version of human FcγRIIA (293T FcR-EGFP) (Fig. 1*A*) (9). Efficient internalization of antibody-coated particles by FcγR-expressing cells that are normally nonphagocytic has been described (10, 11), and confocal microscopy clearly showed that 293T FcR-EGFP could phagocytose opsonized, fluorescent-labeled Zymosan

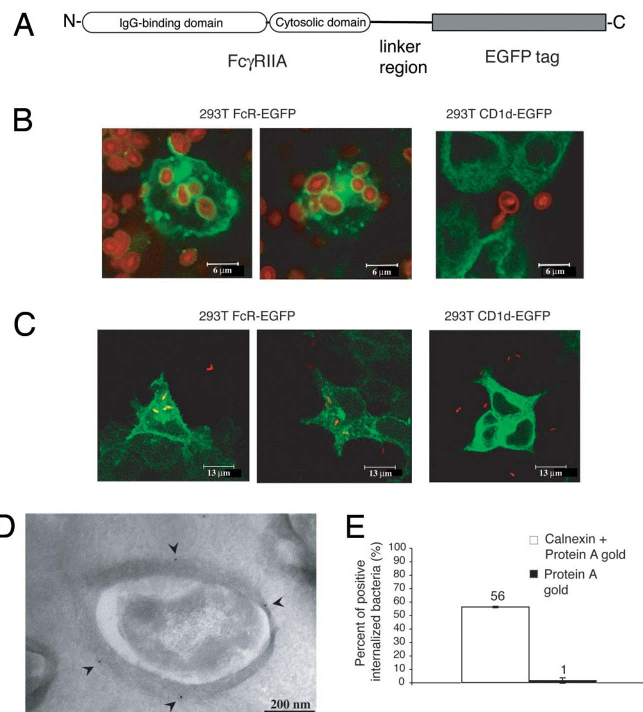

Fig. 1. FcγIIA expression in 293T cells elicits opsonized particle phagocytosis and ER recruitment to phagosomes. (*A*) Schematic representation of EGFP-tagged FcRγIIA. (*B* and *C*) Confocal microscopy analysis of opsonized Texas red-labeled Zymosan particles (*B*) or opsonized pDsRed *E. coli* (*C*) internalized by 293T cells expressing FcR-EGFP or EGFP-tagged CD1d cells as a control. (*D*) Calnexin localization in phagosomes by ImmunoGold labeling using anti-calnexin Ab followed by protein A-gold (black arrows). Phagosomes were formed in 293T FcR-EGFP.K^b cells by 20-min internalization of heat-killed *L. monocytogenes* opsonized with a goat anti-Listeria Ab. (*E*) Quantitation of ImmunoGold particle staining on phagosomes. Graph represents mean ± SD of 2 independent experiments; 100 *L. monocytogenes* containing phagosomes were counted for each experiment.

and bacteria (Fig. 1 *B* and *C*). No particles were phagocytosed by control 293T cells that expressed a recycling CD1d-EGFP construct (12). To establish a system competent for cross-presentation, the mouse MHC class I molecule H2-K^b was stably expressed in 293T

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FcR-EGFP cells. Flow cytometric analysis showed that FcR-EFGP-positive cells expressed H2-K^b on the cell surface (Fig. S1 *a* and *b*). H2-K^b trafficking in 293T FcR-EGFP.K^b cells, evaluated by [^{35}S]methionine pulse–chase labeling and immunoprecipitation, was similar to that in KG-1.K^b cells, a DC-like cell line (Fig. S1 *c* and *d*) (13).

ER Resident Calnexin Is Recruited to Phagosomes in 293T FcR-EGFP.K^b Cells. ER contribution to phagosomes has been reported in DCs and macrophages and correlated with the ability of DCs to use the ERAD pathway to transfer exogenous antigen into the cytosol (8, 14). To determine whether this phenomenon occurred in 293T FcR-EGFP.K^b cells, we performed immuno-electron microscopy after phagocytosis of opsonized heat-killed *Listeria monocytogenes*. Labeling cryoultrathin sections with a rabbit antibody against calnexin and protein-A gold clearly showed calnexin surrounding *L. monocytogenes*-containing phagosomes 20 min after internalization (Fig. 1 *D* and *E*). No significant peripheral labeling of phagosomes was observed upon incubation with protein-A gold alone (Fig. S2*a*). Within the same cells the anticalnexin antibody also labeled the ER (Fig. S2*b*), whereas a rabbit antisuperoxide dismutase antibody labeled mitochondria but not phagosomes (Fig. S2 *c* and *d*). Thus, ER recruitment to phagosomes occurs in 293T FcR-EGFP.K^b cells.

293T FcR-EGFP.K^b Cells Efficiently Internalize and Cross-Present Antigens in Immune Complexes (ICs). To evaluate the ability of 293T FcR-EGFP.K^b cells to internalize ICs, cells were incubated for 1 h with Alexa-Fluor 647-labeled soluble ICs (sICs) or precipitated ICs (pICs) containing ovalbumin (OVA) and chased for different times. Confocal microscopy clearly showed that both sICs and pICs were internalized (Fig. 2 *A* and *B*). To determine whether 293T FcR-EGFP.K^b cells, like DCs (15, 16) could cross-present ICs, they were incubated with sICs or pICs for 1 h and, after extensive washing and further incubation for 18 h to allow antigen processing, expression of MHC class I-peptide complexes was evaluated by using a mAb specific for the OVA-derived peptide SIINFEKL bound to the H2-K^b molecule [25D1.16 (17)]. Flow cytometric analysis showed specific staining of cells incubated with OVA ICs but not with control BSA ICs. Cross-presentation was FcR-dependent, because 293T.K^b cells expressing H2-K^b alone did not bind 25D1.16 (Fig. 2*C*). The results were confirmed by coculturing OVA IC-pulsed 293T FcR-EGFP.K^b cells with the T cell hybridoma B3Z, which secretes IL-2 in response to the SIINFEKL-K^b complex (Fig. 2*D*). Quantitative Western blot analysis revealed that the concentration of OVA antigen incubated with 293T FcR-EGFP.K^b cells was 70 μg/mL for pICs and 1.6 mg/mL for sICs (Fig. S3). Thus, 293T FcR-EGFP.K^b cells can cross-present, and pICs are presented more efficiently.

Roles of Proteasomal Activity, Phagosomal Acidification, and Lysosomal Proteolysis. To evaluate proteasome dependency we used the inhibitor lactacystin. IL-2 secretion by B3Z cells cocultured with 293T FcR-EGFP.K^b cells that had internalized sICs or pICs in the presence of lactacystin was reduced in a dose-dependent manner (Fig. 3*A*). Thus, proteasomal activity is required for cross-presentation by 293T FcR-EGFP.K^b cells. Because pICs were consistently cross-presented more efficiently than sICs despite their lower antigen content, we used pICs for subsequent studies.

The pH of phagosomes decreases during maturation because of the activity of the vacuolar V-ATPase, which pumps H^+ ions into the phagosomal lumen (18). Phagosomal acidification facilitates IC cross-presentation by DCs, likely because it enhances antigen–antibody dissociation and facilitates cytosolic delivery (16). Phagosomal acidification was also required for cross-presentation by 293T FcR-EGFP.K^b cells; treatment of 293T FcR-EGFP.K^b cells that had internalized OVA pICs with concanamycin B (ConB), a V-ATPase inhibitor, inhibited stimulation of the B3Z hybridoma in a dose-dependent manner (Fig. 3*B*).

Fig. 2. 293T FcR-EGFP.K^b cells internalize and cross-present soluble and precipitated OVA ICs. (*A* and *B*) Confocal microscopy analysis of Alexa-Fluor 647-labeled OVA precipitated (*A*) or soluble (*B*) ICs internalized by 293T FcR-EGFP.K^b cells. Cells were incubated for 1 h with ICs, washed, and incubated for the indicated times. In the untreated sample (UT), no ICs were added. (*C*) (*Upper*) Cross-presentation of pICs or sICs by 293T FcR-EGFP.K^b cells assessed by flow cytometry with Alexa-Fluor 647-conjugated 25D1.16 mAb. (*Lower*) 293T.K^b cells, soluble OVA (sOVA), soluble BSA (sBSA), BSA pICs, and sICs were used as controls. Exogenous SIINFEKL peptide was added as a positive control. (*D*) pIC and sIC OVA cross-presentation by 293T FcR-EGFP.K^b cells was measured by IL-2 ELISA on cell supernatants after coculture with the B3Z hybridoma. BSA pICs and ICs, soluble OVA (sOVA), and soluble BSA (sBSA) were used as controls. Graph represents mean ± SD from triplicate samples. All results are representative of at least 4 independent experiments.

Lysosomal proteolysis has also been shown to be important for cross-presentation by DCs (19). To address this issue in 293T FcR-EGFP.K^b cells, we used leupeptin, an inhibitor of cathepsins B and L. IL-2 secretion by B3Z cells was not reduced when 293T FcR-EGFP.K^b cells were incubated with OVA pICs and leupeptin. In fact, it was slightly increased, suggesting that cathepsin B and L activities are dispensable for cross-presentation (Fig. 3*C*). Levels of cathepsin B in 293T-FcR-EGFP.K^b cells were similar to those in DCs, as detemined by Western blot analysis. Lactacystin, ConB, and leupeptin treatment did not affect presentation of exogenously added SIINFEKL peptide (Fig. 3 *D*–*F*).

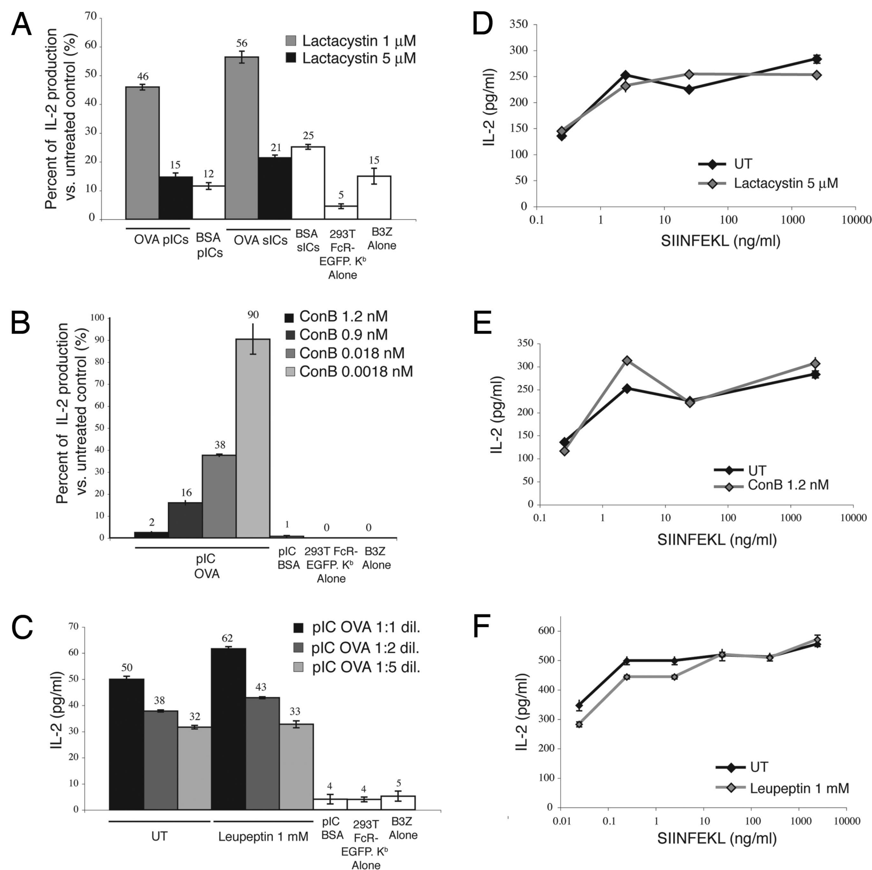

Fig. 3. Cross-presentation by 293T FcR-EGFP.K^b cells depends on the proteasome and phagosomal acidification and is slightly enhanced by leupeptin treatment. (*A*–*C*) 293T FcR-EGFP.K^b cells were treated with lactacystin (1 μM, gray bars, or 5 μM, black bars in *A*), ConB (*B*), or leupeptin (*C*) for 1 h before OVA IC uptake. After overnight incubation in the presence of inhibitors, cells were cocultured with the B3Z hybridoma. BSA pICs and sICs were used as controls. In *A* and *B* graphs represent mean ± SD from triplicate samples expressed as percentage of the maximum signal seen in untreated cells. In *C* mean ± SD from triplicate samples are shown. (*D*–*F*) As a control, 293T FcR-EGFP.K^b cells were incubated with SIINFEKL peptide and 5 μM lactacystin (gray lines in *D*), 1.2 nM ConB (gray lines in *E*), 1 mM leupeptin (gray lines in *F*) or left untreated (UT, black lines). Graphs represent mean ± SD from triplicate samples. All results are representative of at least 3 independent experiments.

Phagosomal pH and Lysosomal Degradation in 293T-FcR Cells, Human DCs, and Macrophages. DCs tightly regulate their phagosomal pH to maintain neutrality over a prolonged period, resulting in decreased lysosomal protease activity and MHC class I epitope preservation for efficient cross-presentation (20, 21). We compared phagosomal pH changes over time in 293T cells, human macrophages, and DCs by using pH-sensitive fluorescent probes internalized by FcR-mediated phagocytosis. To avoid problems associated with EGFP fluorescence, we used 293T cells expressing an untagged FcγRIIA construct. Fig. 4*A* shows that phagosomes acidified more rapidly in 293T-FcR than in DCs, but more slowly than in macrophages. To evaluate their relative lysosomal proteolytic activities, 293T, 293T-FcR cells, human DCs, and macrophages were lysed at pH 4.5, which is optimal for lysosomal proteases, and degradation was measured as described (22). Macrophage extracts were much more proteolytic than those from DCs, as expected (6), but lysosomal proteolytic activity in 293T-FcR cells was not significantly different from DCs (Fig. 4*B*).

Mechanisms of Cross-Presentation by 293T FcR-EGFP.K^b Cells. To investigate whether cross-presentation by 293T FcR-EGFP.K^b cells required the same molecular components used by DCs, we used exotoxin A (ExoA), a *Pseudomonas aeruginosa*-derived toxin that inhibits retrotranslocation of ERAD substrates into the cytosol. This process is believed to be mediated by the Sec61 channel (8, 23, 24). Unlike our earlier results with DCs and the DC-like cell line KG-1, ExoA (WT ExoA) significantly inhibited protein synthesis in 293T FcR-EGFP.K^b cells (Fig. S4*a*), causing decreased H2-K^b surface expression and decreased FcR-EGFP expression (Fig. S4 *b* and *c*). We therefore used a nontoxic (nt) variant, nt ExoA, in which the deletion of a single amino acid (Glu-553) abolishes its ADP-ribosylating activity (25, 26). Incubation of 293T FcR-EGFP.K^b cells with nt ExoA did not inhibit protein synthesis, H2-K^b surface expression, or FcR-EGFP expression levels (Fig. S4 *b* and *c*). To test whether nt ExoA affected cross-presentation 293T FcR-EGFP.K^b cells were incubated with OVA pICs together with nt ExoA or equal concentrations of BSA as a control. Stimulation of B3Z cells was reduced in a dose-dependent manner by nt Exo treatment, whereas recognition of exogenously added SIINFEKL peptide was unaffected (Fig. 5 *A* and *C*). We also found that nt ExoA could block the ability of the WT toxin to inhibit protein synthesis, measured by reduction of H2-K^b surface expression in 293T FcR-EGFP.K^b cells (Fig. S4*d*). Inhibition was not caused by competition for binding to a putative cell surface receptor (Fig. S5). Because ExoA can block peptide retrotranslocation from microsomes (23), the most likely explanation is competition of nt ExoA and WT ExoA for retrotranslocation. This idea is consistent with the hypothesis that nt ExoA inhibits dislocation of antigen into the cytosol.

p97 is a AAA-ATPase (ATPase associated with different cellular activities) essential for retrotranslocation of ERAD substrates into the cytosol (27). Point mutations in both of its ATPase domains render p97 incapable of releasing substrates associated with the retrotranslocation channel, trapping them within the ER lumen (27). To test the role of p97 in cross-presentation by 293T FcR-

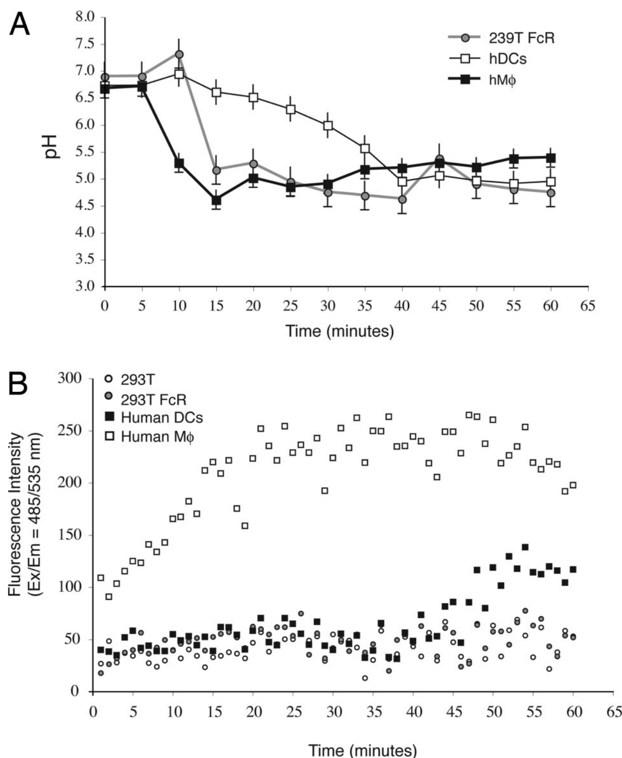

Fig. 4. 293T-FcR cells rapidly acidify their phagosomes but have low levels of acidic pH-optimal protease activity. (*A*) For phagosomal pH measurements 293T FcR cells, human DCs, or human Mϕ were incubated for 5 min on ice with microspheres covalently coupled to carboxyfluorescein-SE and coated with human IgG. Fluorescent emissions were collected at the indicated time points for 1 h. Graph represents mean ± SEM of 6 independent experiments. (*B*) To estimate lysosomal proteolytic activity 293T cells, 293T FcR cells, human DCs, or human Mϕ were lysed with detergent at pH 4.5. Fluorescent emissions in lysates incubated with BODIPY FL casein at 37 °C were monitored every 2 min for 1 h. Results are representative of at least 3 independent experiments.

EGFP.K^b cells, they were transiently transfected with bicistronic constructs encoding WT or double mutant [dominant negative (DN) mutant] p97 and the surface marker Thy 1.1. These contructs allowed determination of transfection efficiency by flow cytometric analysis of Thy 1.1 surface expression, which ranged between 65% and 85%. IL-2 secretion by B3Z cells cocultured with OVA pIC-exposed 293T FcR-EGFP.K^b cells was reduced by ≈40% by DN p97 expression and increased by ≈25% by WT p97 expression, compared with Thy 1.1 vector control cells (Fig. 5*B*). Neither WT nor DN p97 expression affected presentation of SIINFEKL peptide (Fig. 5*D*). These results indicate that p97 ATPase activity, critical for ERAD retrotranslocation, is also important for cross-presentation by 293T FcR-EGFP.K^b cells.

Discussion

Although still subject to debate, considerable evidence suggests that ER membranes are recruited to phagosomes in DCs and macrophages (28–30). Recently, it was reported that the ER-resident SNARE syntaxin-18 localizes to phagosomes in 293T FcγRIIA-expressing cells, suggesting that ER recruitment occurs in cells that are not generally considered APCs (31). We and others have shown that the ERAD retrotranslocation machinery is required for cross-presentation by DCs (8, 32). Because ERAD is a function essential to all cell types, we hypothesized that FcR-mediated phagocytosis and subsequent ER contribution to phagosomes might allow cross-presentation by cells normally incapable of it. The data show that efficient phagocytosis is the only strict requirement for retrotranslocation of exogenous proteins into the cytosol, which then leads to proteasomal degradation and peptide association with MHC class I molecules in the ER.

ER recruitment to phagosomes in the 293T FcR system was confirmed by the detection of the ER-resident protein calnexin on phagosomal membranes surrounding opsonized heat-inactivated *L. monocytogenes* (Fig. 1*D*). Acquisition of ERAD components by phagosomes can thus explain cross-presentation in these cells, which is supported by the experiments with nt ExoA and DN p97 (Fig. 5). Together with ricin, gelonin, and cholera toxin, ExoA is a member of the ribosome-inactivating protein family that exploits the cellular ERAD machinery for its cytosolic delivery (33). The ability of nt ExoA to block protein synthesis inhibition by the native toxin argues that it functions upstream of the ribosomal inactivating activity (Fig. S4*a*). The lack of competition for cell surface binding (Fig. S4*b*) indicates that inhibition is occurring at an intracellular step, likely by blocking the retrotranslocation channel, as suggested by the work of Koopmann *et al.* (23). Although ExoA and other ERAD substrates have been shown to interact with Sec61 (23, 34, 35), the precise identity of the retrotranslocation channel is still unclear. Derlin-1 has been implicated as has Sec61, and p97 can interact with substrates potentially associated with both during retrotranslocation (8, 36, 37). Inhibition of cross-presentation by the p97 DN mutant and its enhancement by overexpression of WT p97 further supports the involvement of ERAD in cross-presentation by 293T-FcR cells (Fig. 5*B*). Whether other phagocytic receptors found on DCs [such as $\alpha_v\beta_5$ and CD36, allowing cross-presentation of antigenic components from apoptotic bodies (38)], can transfer this ability to conventional, non-APCs remains to be determined.

Despite having ≈20-fold lower OVA content, pICs were more efficient than sICs in promoting cross-presentation (Figs. 2*C* and 3*A*). This difference might be explained by better recruitment of ER components to the phagosomal membrane or by the delivery of more OVA antigen per cell by pICs compared with their soluble counterpart, suggested by the relative fluorescent intensities upon phagocytosis (Fig. 2 *A* and *B*). FcγRIIA might also be differently engaged by insoluble versus sICs. Although signaling is essential for phagocytosis itself (39), it may also play a role in subsequent events required for efficient cross-presentation, as recently reported for B cells (40). The IgG abundance in pICs (Fig. S3*b*) and the consequent engagement of multiple FcRs could promote stronger downstream signaling and more efficient cross-presentation.

Cross-presentation usually involves proteasomal degradation, but in some cases lysosomal proteolysis can generate peptides that bind to MHC class I molecules. Depending on the antigen, one or both pathways can contribute to cross-presentation in vivo (41). In DCs, and in the experiments involving 293T FcR-EGFP.K^b cells reported here, cross-presentation was inhibited by lactacystin (Fig. 3*A*), indicating the involvement of proteasomal activity and implying antigen transfer into the cytosol (15, 16). Nevertheless, inhibiting acidification by ConB also inhibited cross-presentation (Fig. 3*B*). It has been suggested that cross-presentation of ICs requires phagosomal acidification to dissociate antigen from the antibody (16). Acidification of phagosomes in the 293T-FcR system is more rapid than in DCs, consistent with data reporting a rapid pH drop in other cell lines expressing FcγRIIA (11), but slower than in macrophages (Fig. 4*A*). Proteolysis within the phagosome may not be involved, judging by both the generally low lysosomal proteolytic activity of 293T-FcR cells (Fig. 4*B*) and the lack of an inhibitory effect of leupeptin on cross-presentation (Fig. 3*C*). In fact, leupeptin treatment reproducibly enhanced cross-presentation in this system. The data argue that cross-presentation of IC antigens by 293T-FcR cells does not involve phagosomal proteolysis and endocytic loading of MHC class I molecules.

The precise mechanism by which ER membrane is delivered to phagosomes is not understood. Syntaxin-18 localization on 293T-FcR phagosomes (31) suggests that vesicular fusion or tubular extension may render the ERAD machinery available for retro-

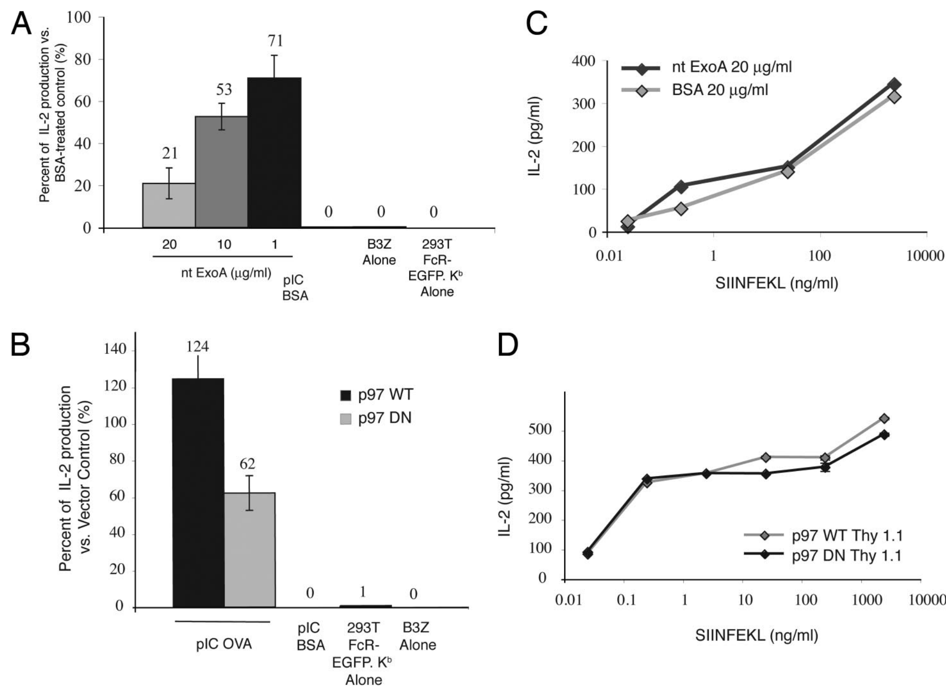

Fig. 5. nt ExoA and DN p97 inhibit cross-presentation of OVA pIC by 293T FcR-EGFP.K^b cells. (*A*) 293T FcR-EGFP.K^b cells were treated with nt ExoA at the indicated concentrations for 1 h before OVA pIC uptake. After overnight incubation in the presence of nt ExoA, cells were cocultured with the B3Z hybridoma. BSA pICs and sICs were used as controls. Graph represents mean ± SD from 3 independent experiments expressed as percentage of the maximum signal seen in BSA-treated cells used as control. (*B*) OVA pIC cross-presentation efficiency was decreased in 293T FcR-EGFP.K^b cells transfected with DN p97 AAA-ATPase and enhanced in WT p97 transfectants. BSA pICs were used as controls. Graph represents mean ± SD from 3 independent experiments. Values are expressed as percentage of the maximum signal seen in vector transfected control cells. (*C*) Presentation of exogenous SIINFEKL peptide by 293T FcR-EGFP.K^b cells treated with 20 μg/mL of BSA (gray lines) or nt ExoA (black lines) was measured by IL-2 ELISA release. (*D*) 293T FcR-EGFP.K^b cells transfected with WT p97 or DN p97 present the SIINFEKL peptide similarly to B3Z cells. In *C* and *D* graphs represent mean ± SD from triplicate samples and are representative of at least 3 independent experiments.

translocation and recruiting the MHC class I loading complex to phagosomes as described for DCs (14, 28, 42). This process could also create a transient continuity between these compartments, allowing exogenous antigens to directly access the ER for ERAD-mediated cytosolic translocation (43).

Cross-presentation by nonmyeloid cells, such as melanoma cells (44) and endothelial cells (45, 46), has been reported. These and our results suggest reevaluating the concept that DCs are highly specialized cross-presenting machines and indicate a potential role for nonprofessional APCs in autoimmunity and immune responses to tumors. DCs do have unique properties that facilitate the stimulation of $CD8^+$ T cell responses in vivo, such as the capacity to migrate from peripheral tissues to lymph nodes and the surface expression of costimulatory molecules required for the activation of naïve T cells (47). However, like DCs, 293T-FcR-expressing cells recruit ER to phagosomes, allow antigens to access the cytosol, and cross-present ICs effectively. Their phagosomes acquire the LAMP-1 lysosomal marker ≈2 h after initiation of phagocytosis (31), which is similar to the rate of phagosome–lysosome fusion in DCs (28). Internalized proteins remain intact in DCs, B cells, and fibroblasts much longer than in macrophages (22), and our studies suggest that this is likely to be true in 293T cells. Thus, rapid lysosomal proteolysis and high expression levels of lysosomal proteases by macrophages is the exception rather than the rule and is not optimal for cross-presentation because of epitope destruction (48). The endocytic properties characteristic of DCs that facilitate cross-presentation appear to be a combination of the high phagocytic activity of a macrophage and the low proteolytic activity found in nonphagocytic cells. 293T-FcR cells can be transfected with high efficiency, suggesting that they could be used for siRNA screens to identify trafficking pathways responsible for the acquisition of ER components by phagosomes.

Materials and Methods

Peptides and Antibodies. The H2-K^b-binding SIINFEKL peptide from $OVA_{257–264}$ was synthesized by the Keck Facility at Yale University. Rabbit anti-OVA IgG was from Polysciences. Rabbit anti-BSA IgG was purified from rabbit anti-BSA serum (Serotec) by protein A-Sepharose binding, acid elution, and overnight PBS dialysis. Alexa Fluor 647-conjugated 25D1.16 mAb recognizing H2-K^b-SIINFEKL complexes has been described (17). Phycoerythrin-conjugated anti-H2-K^b mAb was from GeneTex.

Reagents and Inhibitors. The plasmid encoding His-tagged nt *P. aeruginosa* ExoA (nt ExoA) (from L. Silbart, University of Connecticut, Storrs) was used to produce nt ExoA (26). ConB was from Axxora. Leupeptin and puromycin were from Sigma. Lactacystin was from Biomol.

Plasmids. Human FcRγIIA cDNA (from J. Ravetch, The Rockefeller University, New York) was cloned into pEGFP-N1 (Clontech) and subcloned into pLPCX retroviral vector (Clontech). FcRγIIA and EGFP coding sequences were separated by the linker region as described (9). For untagged FcR, the cDNA was directly cloned into pLPCX. H2-K^b coding DNA was cloned into pBMN-neo retroviral vector. cDNAs encoding WT and DN p97 were cloned into the pMSCV Thy 1.1 bicistronic plasmid.

Cells and Cultures. Retrovirus production and transduction was performed as described (49). The T cell hybridoma B3Z specific for SIINFEKL-H2-K^b has been described (50). Primary human DCs and macrophages were differentiated from human mononuclear cells for 5 days in RPMI medium 1640 supplemented with 20% FBS and hIL-4 (800 units/mL) and hGM-CSF (1,000 units/mL) for DCs or hM-CSF (1,000 units/mL) for macrophages.

Flow Cytometry. Cells were stained with fluorochrome-conjugated antibodies in 5% bovine calf serum and 0.05% sodium azide in PBS on ice for 20 min, washed, and analyzed by flow cytometry using a FACSCalibur (BD Biosciences) and FlowJo software.

Cross-Presentation in 293T FcR-EGFP.K^b Cells. Five hundred μL of pICs or sICs (see *SI Text*) were added to cells in 1.5 mL of DMEM/1% FBS in 6-well plates. After 1-h incubation, the cells were extensively washed, incubated overnight, fixed with 0.2% paraformaldheyde, and stained with 25D1.16 mAb (17) or counted, fixed, and cocultured with B3Z T cell hybridoma at a 1:1 ratio. IL-2 was quantitated by ELISA (BD Biosciences). Where indicated, the cells were pretreated for 1 h with inhibitors before IC uptake and during overnight incubation before cell fixation. For p97 experiments, 293T FcR-EGFP.K^b cells transiently transfected with Thy 1.1-p97 constructs or Thy 1.1 vector control by Lipofectamine 2000 (Invitrogen) were assayed for cross-presentation 24 h posttransfection.

Fluorescence and Confocal Microscopy. Texas red-conjugated Zymosan A BioParticles were incubated with opsonizing reagent (Invitrogen) according to the manufacturer's protocol. *Escherichia coli* expressing pDsRed-NI (Clontech) was opsonized with anti-LPS pAb (Serotec). Opsonized material or fluorescent ICs (see *SI Text*) were added to cells plated on poly-L-lysine-treated coverslips (Sigma). After 1-h incubation, the cells were washed, incubated for the indicated times, fixed, and examined with a Leica TCS SP2 confocal microscope.

Immuno-Electron Microscopy. *L. monocytogenes*, heat-killed for 1 h at 80 °C, were opsonized with goat anti-*Listeria* antibody (Meridian Life Science) and incubated with 293T FcR-EGFP.K^b cells for 20 min at 37 °C and processed for immunoelectron microscopy as described in *SI Text*.

Phagosomal pH and Protease Activity Assays. Phagosomal pH (51) and protease activity (22) in 293T-FcR, human DCs, and macrophages was measured as described.

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