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Primary Human and Rat β-Cells Release the Intracellular Autoantigens GAD65, IA-2, and Proinsulin in Exosomes Together With Cytokine-Induced Enhancers of Immunity

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The target autoantigens in several organ-specific autoimmune diseases, including type 1 diabetes (T1D), are intracellular membrane proteins, whose initial encounter with the immune system is poorly understood. Here we propose a new model for how these proteins can initiate autoimmunity. We found that rat and human pancreatic islets release the intracellular β-cell autoantigens in human T1D, GAD65, IA-2, and proinsulin in exosomes, which are taken up by and activate dendritic cells. Accordingly, the anchoring of GAD65 to exosome-mimetic liposomes strongly boosted antigen presentation and T-cell activation in the context of the human T1D susceptibility haplotype HLA-DR4. Cytokine-induced endoplasmic reticulum stress enhanced exosome secretion by β-cells; induced exosomal release of the immunostimulatory chaperones calreticulin, Gp96, and ORP150; and increased exosomal stimulation of antigen-presenting cells. We propose that stress-induced exosomal release of intracellular autoantigens and immunostimulatory chaperones may play a role in the initiation of autoimmune responses in T1D.

Type 1 diabetes (T1D) is an autoimmune disease characterized by circulating autoantibodies, lymphocytic infiltration of pancreatic islets of Langerhans, and cell-specific destruction of β-cells, leading to insulin deficiency (1). Prior to lymphocytic infiltration, physiological islet abnormalities have been described in the nonobese diabetic (NOD) mouse model of T1D, including upregulation of inflammatory cytokines (1) and increased endoplasmic reticulum (ER) stress in β-cells (2). Furthermore, the importance of ER stress and the beneficial effects of restoring organelle function have been reported (3).

Two main target autoantigens in T1D, GAD65 (4) and IA-2 (5), are rare intracellular membrane proteins in β-cells. Circulating autoantibodies to these proteins are an early marker of β-cell autoimmunity and can be used with HLA class II haplotyping to detect individuals who are at risk long before the clinical onset of disease (6). The mechanisms whereby these intracellular autoantigens are initially recognized by the immune system, taken up by antigen-presenting cells (APCs), and presented to self-reactive T cells have, however, not been clarified. A third autoantigen, insulin, constitutes ~50% of β-cell proteins and is released by regulated exocytosis of secretory granules (7). There is evidence that epitopes in proinsulin, the

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uncleaved prohormone form of insulin, escape immune tolerance in T1D (7,8).

MIN6 and INS-1 rat insulinoma cells, derived from a mouse and rat β-cell tumor, respectively, have been reported to release extracellular vesicles (EVs), including exosomes (9). Exosomes are small-diameter (30–150 nm) vesicles that are secreted in the extracellular space by most cell types after the formation of multivesicular bodies (MVBs) in the late endosomal pathway and their fusion with the plasma membrane (10).

Exosomes can display immunoregulatory functions (11). In the context of T1D, exosomes from MIN6 cells can stimulate autoreactive NOD T cells (9) and marginal zone-like B cells that accumulate in the pancreas of prediabetic NOD mice (12). In addition, islet-associated mesenchymal stem cell–like cells can produce immunostimulatory exosomes that activate autoreactive T and B cells in NOD mice (13). We and others (14–16) have been unable to detect the GAD65 protein in mouse islet cells and in insulinoma cells, and neither GAD65 nor IA-2 appear to be required for development of T1D in the NOD mouse (17,18).

Here we show that primary human and rat islet–derived exosomes contain the autoantigens GAD65, IA-2, and proinsulin/insulin. We further show that cytokine-induced ER stress results in the increased release of exosomes decorated with inflammatory chaperones that may promote β-cell autoimmunity.

RESEARCH DESIGN AND METHODS

Information on HLA-DR4$^{+/+}$ NOD mice, human islet donors, mass spectrometry, SDS-PAGE, and Western blotting (WB) are available in the Supplementary Data.

Islet and Cell Cultures

Islets isolated from P5 Sprague-Dawley rats (18), 5-week-old HLA-DR4$^{+/+}$ NOD mice (19), or human subjects (from European Consortium for Islet Transplantation Islets for Basic Research Program) were cultured as previously described (18). Monolayer cultures were derived from whole islets dispersed into single cells and seeded on Thermanox coverslips (Thermo Fisher Scientific) coated with HTB-9 cell–derived extracellular matrix (20). Bone marrow dendritic cells (BMDCs) were isolated from HLA-DR4$^{+/+}$ NOD mice (21) and cultured for 8 days with RPMI 1640 GlutaMAX medium with 11 mmol/L glucose, 10% FBS, 1% antibiotic-antimycotic, and granulocyte-macrophage colony-stimulating factor (200 μg/mL; PeproTech). INS-1E cells (22), MIN6 cells (23), and DR4 (DRA1*0101; DRB1*0401)–positive human Priess Epstein Barr virus–transformed B cells (24) were obtained and cultured as previously described (15,18).

Exosome Isolation

Rat islets, human islets, INS-1E cells, or Priess cells were seeded in exosome-depleted medium (25). Supernatant was collected every 2–3 days. For comparative analyses of exosomes released with and without cytokines, rat and human islets were divided into cell culture dishes (500 islets each) and rat interleukin (IL)-1β (10 units/mL) and interferon-γ (IFN-γ) (10 units/mL) (R&D Systems), or human IL-1β (20 units/mL) and IFN-γ (10 units/mL) (R&D Systems), were added to half of the plates. This concentration of cytokines results in mild ER stress in β-cells (18). Culture media were collected and replaced with fresh media with or without cytokines every 2 days for a total of 6 days. Islets were counted after each replacement of medium: the total loss of islets in medium with and without cytokines was ≤20% and ≤5%, respectively, for rat islets, and less for human islets. Trypan blue staining of dispersed islet cells revealed robust viability of cells, with or without cytokines, throughout the collection period. Exosomes from culture media were isolated by differential centrifugation (25). Briefly, media were centrifuged at 300*g* for 10 min to remove live cells, followed by 2,000*g* for 10 min to remove dead cells, and then by 10,000*g* for 35 min at 4°C to remove cell debris, large vesicles, and heavy membranes, including Golgi membranes, lysosomes, and apoptotic bodies. Supernatants were transferred to a new tube and centrifuged at 110,000*g* for 70 min at 4°C to isolate exosomes. The resulting pellet was resuspended, washed in 35 mL of PBS to eliminate serum and secreted proteins, and recentrifuged. The final pellet was resuspended in 50–100 μL of PBS and stored at −80°C.

Transmission Electron Microscopy

Exosomes or liposomes (15 μL, at different dilutions in PBS) were applied to carbon-coated 400-mesh grids (Canemco-Marivac) and negatively stained with 2% uranyl acetate, and images were obtained with a transmission electron microscope (Tecnai Spirit; FEI Company).

Nanoparticle Tracking Analysis

Exosomes and liposomes (1:1,000 in PBS) were analyzed using nanoparticle tracking analysis (NTA) instrument NS300 (NanoSight; Malvern Instruments Ltd.). Five measurements of 60 s were recorded consecutively for each sample, and a 532-nm laser diode source was used to analyze the fluorescence of boron-dipyrromethene (BODIPY)–labeled exosomes.

Proteomics

EV preparations (5 μg of protein) from untreated or cytokine-treated rat islets, untreated human islets, or INS-1E cells, were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS), as previously described (26).

Quantitation of Insulin in Exosomes by ELISA

EVs from human or rat islets were lysed in PBS with 1% Triton, and subjected to serial dilutions prior to quantitative analyses using ELISA kits for human or rat insulin and proinsulin (Mercodia).

Immunofluorescence

Fixing of monolayer cultures of islet cells, immunofluorescence staining, and confocal imaging were performed as previously described (18) using the following primary antibodies: mouse anti-GAD65 (15), 1:1,000; sheep anti-TGN38 (catalog #AHP499; Bio-Rad), 1:100; rabbit anti-flotillin1,

1:200; rabbit anti-CHOP (catalog #sc-575; Santa Cruz Biotechnology), 1:100; guinea pig anti-insulin (catalog #4011–01; Linco), 1:1,000; and rabbit anti-CD81 (catalog #SAB3500454; Sigma-Aldrich), 1:300.

Treatment and Transfections of Islet Monolayer Cultures

Rat islet cells were incubated with rat cytokines (IFN-γ and IL-1β, 10 units/mL each) for 4, 8, or 24 h.

Transfection with MAGT1-GFP (27) or GFP-Rab11-WT (28) plasmids was performed using Lipofectamine 2000 (Life Technologies). Cell labeling using BODIPY TR ceramide (Life Technologies) was performed according to the manufacturer protocol.

Uptake of Islet Exosomes by APCs

Rat islet exosomes (10 ng protein/mL) were labeled with 5 μmol/L of BODIPY TR ceramide (Life Technologies) in PBS for 30 min at room temperature (29), followed by two PBS washes and ultracentrifugations at 110,000*g* for 70 min at 4°C.

BODIPY-labeled exosomes (20–25 μg protein/mL) were incubated with 100,000 $DR4^{+/+}$ BMDCs or Priess cells labeled with 0.5 μmol/L carboxyfluorescein succinimidyl ester (Life Technologies). Uptake was analyzed on a Zeiss LSM700 confocal microscope with a stage-mounted incubation chamber at 37°C, 5% CO_2. Cells were then blocked with anti-mouse CD16/32 (catalog #101302; BioLegend), 1:200; stained with LIVE/DEAD Violet (Life Technologies) and anti-CD11c-APC antibody (catalog #17–0114–82; eBioscience), 1:800; and analyzed by a BD LSR II Flow Cytometer (BD Biosciences) and FlowJo software.

Immunostimulation of BMDCs by Islet-Derived Exosomes

Rat and human islet EVs (40 and 50 μg protein/mL) were incubated for 24 h with 50,000 $DR4^{+/+}$BMDCs. PBS and CpG-A (5 μg/mL; ODN1585; InvivoGen) were used as negative and positive controls, respectively. Concentration of TNF-α, IL-6, and IL-1β in the cell supernatant was measured by ELISA (eBioscience).

Preparation of GAD65 Liposomes Mimicking Exosomes

Liposomes were prepared according to the protocol by Masserini et al. (30). Ganglioside GM3, cholesterol and sphingomyelin (Avanti Polar Lipids Inc.) were dissolved in chloroform-methanol (2:1 v/v) at a molar ratio of 8:1:1. After rehydration of the lipid film, liposomes were size selected by extrusion through 100-nm Nuclepore track-etched membranes (Whatman) using a Lipex extruder (Northern Lipids Inc.). Endotoxin-free recombinant human GAD65 (1 mg/mL) produced in yeast (FIRS Laboratories RSR) was incubated with liposomes (1×10^{11} particles/mL), in a volume of 100 μL, at room temperature for 2 h, and purified by ultracentrifugation (100,000*g* for 70 min) to remove unbound protein. The anchoring of GAD65 to liposome membranes is achieved because of the presence of hydrophobic posttranslational modifications, including a double palmitoylation (31). The BCA Protein Assay Kit (Thermo Fisher Scientific) was used to analyze the concentration of GAD65 conjugated to liposomes.

Immunogenicity of GAD65 Liposomes

To test the antigenicity of free or liposome-conjugated GAD65, free recombinant human GAD65 protein, and GAD65 liposomes were incubated for 24 h with 50,000 $DR4^{+/+}$ NOD BMDCs. DR4 (DRA1*0101; DRB1*0401)–restricted T33.1 T cells were added, and incubation was continued overnight. IL-2 secretion by T cells was analyzed by ELISA.

Ethical Approval

Animals were used under École Polytechnique Fédérale de Lausanne animal regulation guidelines and an institutional animal care and use committee–approved protocol (#2919). Human islets were received from University Hospital of Geneva or San Raffaele Scientific Institute, Milan, through European Consortium for Islet Transplantation, and approved by the Institutional Review Board of Geneva University Hospital (CER no. 05–028) and the Ethics Committee of San Raffaele Institute (IPF002–2014).

Statistical Analyses

Mean values among three or more groups were compared by ANOVA using GraphPad Prism 6. If deemed significant, Tukey post hoc pairwise comparisons tests were performed. Mean values between two groups were compared using the Student *t* test. A confidence level of 95% was considered to be significant.

RESULTS

Rat and Human Pancreatic Islets Release the GAD65, IA-2, and Insulin/Proinsulin Autoantigens in EVs With Characteristics of Exosomes

EVs were isolated from primary rat and human pancreatic islet cells, and the rat insulinoma cell line INS-1E by differential centrifugation of culture supernatants (25). Transmission electron microscopy (TEM) analysis of EVs from rat and human islets demonstrated the presence of vesicles with sizes and shapes that were compatible with those of exosomes (32) (Fig. 1*A*). NTA indicated a uniform size distribution, with major peaks at 143 ± 5 and 139 ± 5 nm, respectively, for rat and human EVs (Fig. 1*B*), which are similar to the size of neural stem cell–derived exosomes (32).

The proteomes of EVs from rat or human islets, or INS-1E cells, were analyzed by LC-MS/MS and found to contain proteins previously identified in exosomes from other cell types (Supplementary Table 1). Those included flotillin1, which controls the sorting of specific proteins for exosomal secretion (33), the tetraspanins CD9 and CD81, which play a critical role in mediating protein loading and inclusion into endosomal membranes and exosomes (34,35); whereas, Alix and CD63 were not detected. Furthermore, Rab11, Rab27a, Rab27b, and Rab35, which are implicated in exosome biogenesis (36,37), and Rab1, which

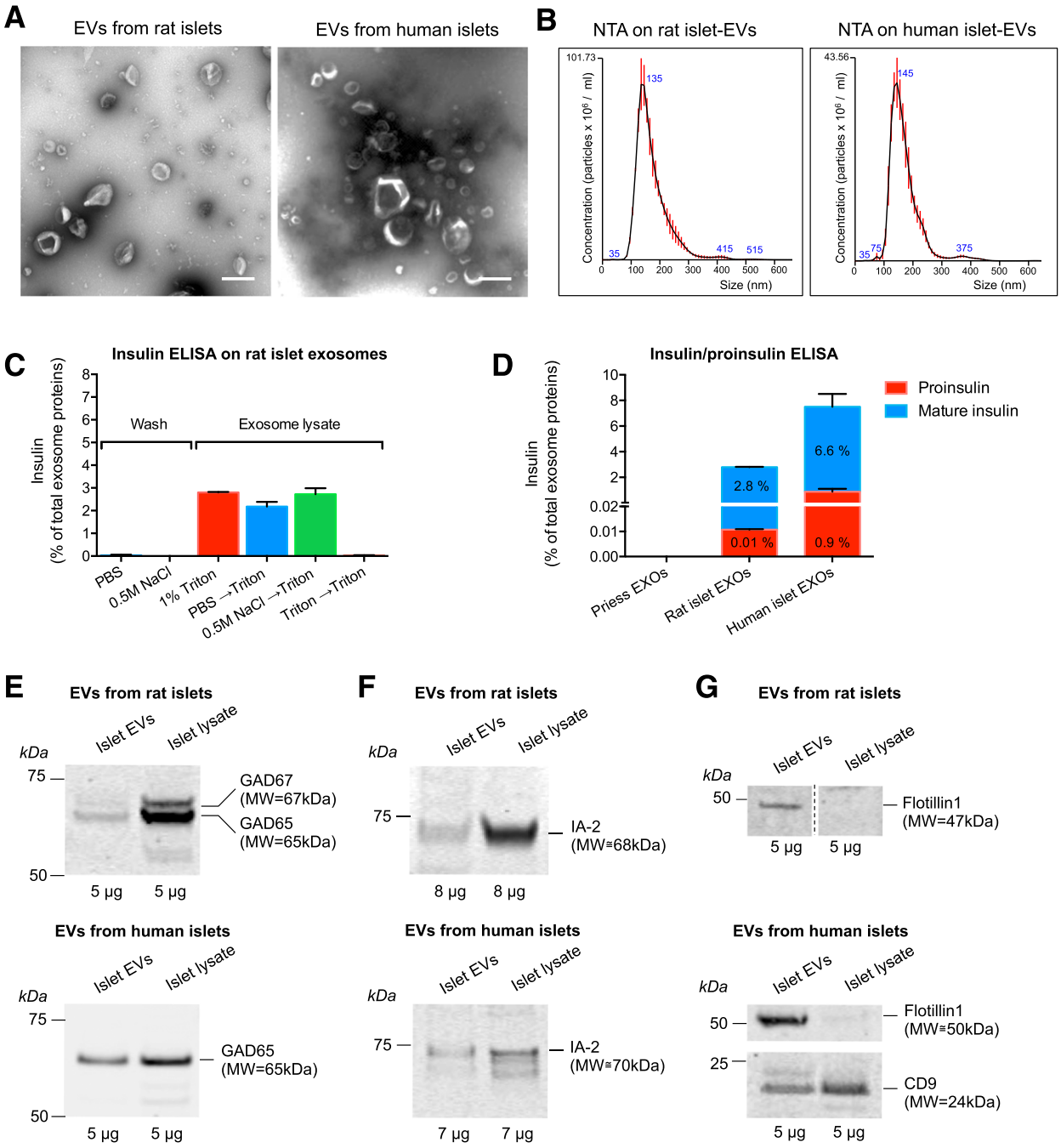

Figure 1—Human and rat islets release EVs, mainly exosomes (EXOs), containing the exosomal markers flotillin1 and CD9, and the β-cell autoantigens GAD65, IA-2, and proinsulin/insulin. *A*: TEM analysis of rat and human islet EVs with "cup-shaped" morphology and 30–200 nm in diameter, with an average size of 120 nm, in both rat and human preparations. Vesicle sizes were estimated by measuring the diameters of 20 exosomes in five different fields. Although some vesicles are larger (150–200 nm), the main constituents of the EV preparations are exosomes (30–150 nm). Scale bars: 200 nm. *B*: Particle size distribution of EVs from rat and human islets using NTA. Data are representative of three independent experiments. *C*: Aliquots of purified rat islet exosomes were resuspended in PBS, PBS supplemented with NaCl to a final concentration of 0.5 mol/L, or PBS containing 1% Triton X-100, and incubated for 1 h followed by sedimentation at 110,000*g* for 1 h. Pellets were extracted in PBS/1% Triton X-100, and supernatants were subjected to serial dilutions and analyzed for insulin/proinsulin by ELISA. Insulin is released only from exosomes by detergent, suggesting that is an integral part of exosomes rather than a contaminant from islet cell culture media. Analyses were performed in technical triplicates. Results are reported as the mean ± SD. *D*: Insulin/proinsulin content of human and rat islet exosomes measured by ELISA after extraction in PBS containing 1% Triton X-100. Data for human insulin/proinsulin are shown as the mean ± SD of three independent experiments. Data for rat insulin/proinsulin are reported as the mean ± SD for the triplicate rat islet EV lysates shown in *C*. Data are representative of three independent experiments. WB analyses of GAD65 (*E*), IA-2 (*F*), and CD9 and flotillin1 (*G*) expression in total lysates or EV preparations of rat and human islets. Human islets only express the GAD65 isoform of GAD, while rat islets express both GAD65 and the nonautoantigenic isoform GAD67. The results shown are representative of two to five independent experiments.

is involved in vesicle trafficking from ER to Golgi intermediate membranes (38), were detected in islet EVs together with the autoantigen proinsulin/insulin (Supplementary Table 1). As shown in Fig. 1*C*, insulin was an integral part of islet exosomes, rather than a contamination from secreted insulin, and accounted for 7–8% of human and 2–3% of rat islet exosome proteins, as measured by ELISA (Fig. 1*D*). Proinsulin constituted ~12% and ~0.5% of total insulin in human and rat islet exosomes, respectively (Fig. 1*D*). The rare β-cell autoantigens GAD65 and IA-2 were not detected in islet EVs, likely due to their hydrophobicity and poor peptide ionization. However, both proteins were detected in human islet exosomes after prepurification by SDS-PAGE.

We next analyzed the expression of GAD65 and IA-2, as well as the exosomal markers flotillin1 and CD9, by WB of islet EVs and total islet lysates. These analyses unequivocally identified the autoantigens GAD65 and IA-2 in both human and rat EVs (Fig. 1*E* and *F* and Supplementary Fig. 1*A* and *B*). Human islets only express the GAD65 isoform, whereas rat islets primarily express the GAD65 isoform but also the nonautoantigenic isoform GAD67, which can form heterodimers with GAD65 and become membrane bound in rat islet β-cells (15). Both isoforms were detected in rat islet exosomes (Fig. 1*E* and Supplementary Fig. 1*A*). In contrast to a previous report (9), GAD65 was not detected in lysates of the mouse insulinoma cell line MIN6. Furthermore, as reported previously (14,15), GAD67, but not GAD65, was detected in mouse islet and rat INS-1E cells (Supplementary Fig. 1*A*). The ratio of GAD65 in exosomes and total lysates was ~1:2 in human islets and 1:5 in rat islets (Fig. 1*E*). Since approximately half of total β-cell GAD65 is firmly membrane anchored (39), the concentration ratio of GAD65 in EVs to total β-cell membranes was ~1:1 for human and ~2:5 for rat. The transmembrane IA-2 autoantigen of 68–70 kDa (40) was detected in human and rat islet exosomes (Fig. 1*F* and Supplementary Fig. 1*B*); the concentration ratio between exosomes and total islet lysate was ~1:2 for IA-2 in human and ~1:6 in rat islets (Fig. 1*F*).

WB analyses revealed a 25-fold enrichment of the exosomal marker flotillin1 in EVs from both human and rat pancreatic islets compared with islet lysates (Fig. 1*G*). CD9 was detected in human islet EVs but was not enriched over lysates (Fig. 1*G*). The exosome marker Alix was detected in islet lysates and EVs from INS-1E cells, but was below the detection limit in islet EVs (Supplementary Fig. 1*C*), which is consistent with the notion that, in islet cells, flotillin1-, CD9- and CD81-positive exosomes represent a population of exosomes distinct from and more abundant than Alix-, syndecan/syntenin- and CD63-positive exosomes (41).

These results suggest that human and rat pancreatic islets release EVs presenting characteristics of flotillin1-, CD9-, and CD81-positive exosomes. Importantly, islet exosomes contain the GAD65, IA-2 and insulin/proinsulin autoantigens associated with the development of T1D in humans.

Trafficking of the Autoantigen GAD65 to EVs May Involve a Pathway From the Golgi Compartment to MVB via the Perinuclear Recycling Endosome Compartment

We hypothesized that the specific intracellular trafficking of GAD65 facilitates its loading into EVs/exosomes. Two pathways have been proposed for sorting membrane proteins to MVBs and exosomes. The first involves endocytosis of plasma membrane proteins and transport through early endosomes to late endosomes and MVBs (10). The second involves transport from the *trans* Golgi network (TGN) to recycling endosomes and late endosomes/MVBs (29,36). Membrane-anchored GAD65 is primarily localized to the TGN and peripheral vesicles in β-cells but is not detected at the plasma membrane (31,42). Furthermore, the transmembrane protein IA-2 and proinsulin/insulin are processed in the ER and Golgi prior to their respective targeting to the membrane and lumen of insulin secretory granules, which is where proinsulin undergoes the final processing to insulin prior to regulated secretion (7).

We explored the possibility that islet β-cells form MVBs and exosomes using the TGN pathway, and asked whether GAD65 can be detected along this route. The small GTP-binding protein Rab11 is localized in the perinuclear endosomal recycling compartment (ERC) and controls trafficking to and from this compartment (43). Moreover, Rab11 is implicated in the exosomal biogenesis pathway originating at the TGN (36) and appears to promote the fusion of MVBs with the plasma membrane to release exosomes (44). We analyzed the localization of Rab11, GAD65, and the TGN marker TGN38 in rat islet cells expressing GFP-Rab11a. As reported previously (42), GAD65 colocalized with TGN38 in the TGN (Fig. 2*A*, arrowheads). Furthermore, GAD65 and GFP-Rab11a colocalized in the perinuclear ERC, as well as in a few smaller vesicular structures close to the surface of β-cells (Fig. 2*A*, arrows). Colocalization between GAD65 and flotillin1 was detected both in vesicle-like compartments in proximity to the Golgi compartment as well as in the periphery of the β-cells (Fig. 2*B*, arrows). Similarly, CD81 and insulin/proinsulin colocalized extensively in vesicles budding off from the TGN (Fig. 2*C*, arrows) or localized in the periphery (Fig. 2*C*, arrowheads).

Laulagnier et al. (29) found that a population of exosomes released by RBL-2H3 cells displayed lipid composition similar to that of Golgi membranes and preferably absorbed a fluorescent BODIPY ceramide dye used for the labeling of Golgi membranes. We confirmed that BODIPY ceramide specifically labels the Golgi compartment in rat islet cells (Fig. 2*D*), and analyzed the specificity of BODIPY ceramide labeling of rat islet exosomes using NTA. No significant differences were detected in the vesicle size distribution between the light scattering and the fluorescence acquisition settings (Fig. 2*E*), suggesting that the

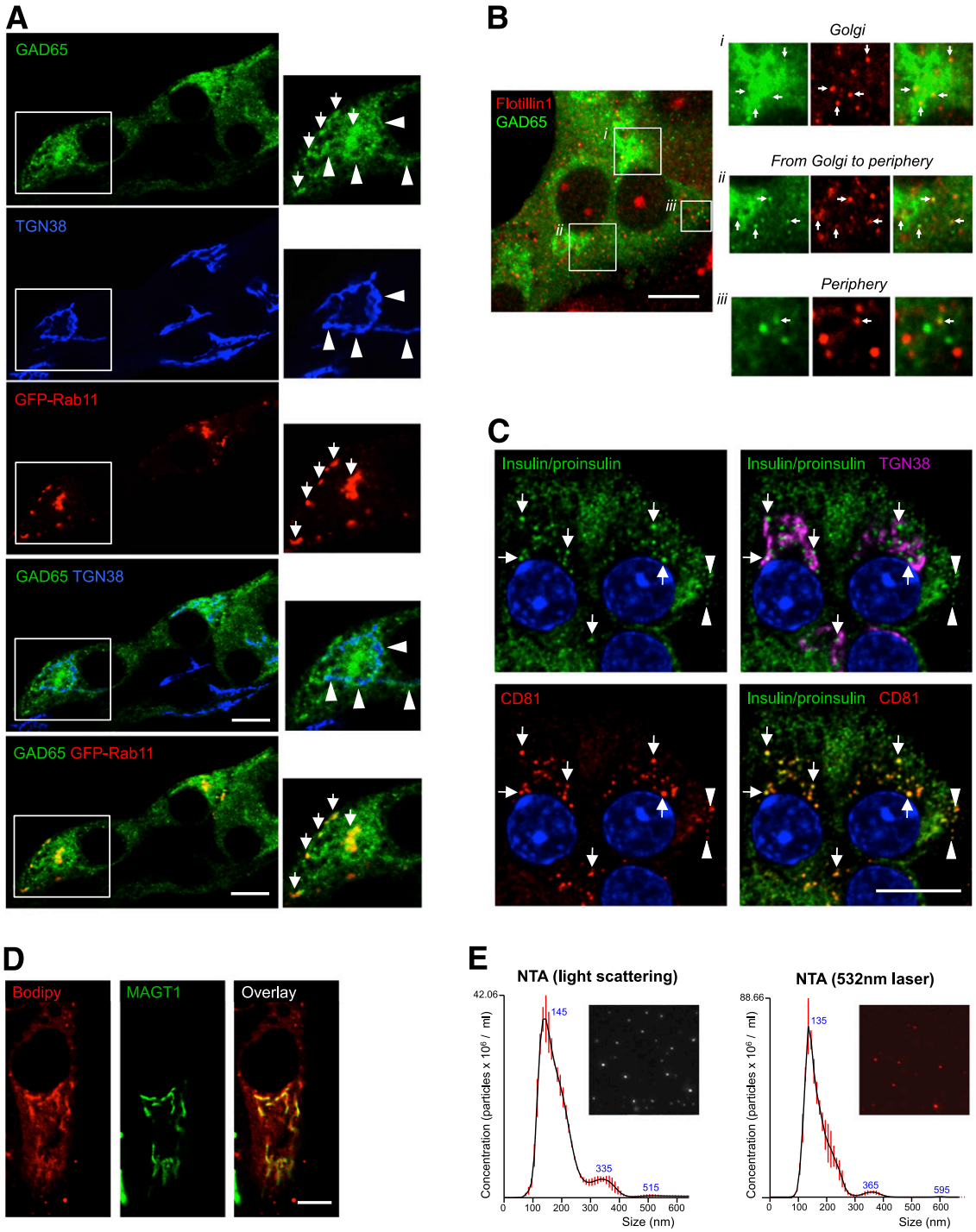

Figure 2—Analyses of a putative pathway for biogenesis of β-cell EVs from the TGN. *A*: Confocal analyses of primary rat islet cells transfected with GFP-Rab11 and immunostained for GAD65 (green) and the *trans* Golgi marker TGN38 (blue), revealing colocalization of GAD65 and TGN38 (arrowheads), minimal colocalization of TGN38 and GFP-Rab11, and colocalization of GAD65 and GFP-Rab11 (arrows) in the perinuclear ERC close to the Golgi region and in vesicular structures in the cell periphery. *B*: Confocal analyses of immunostaining of primary human islet cells for GAD65 (green) and the exosomal marker flotillin1 (red) reveals colocalization (arrows) in vesicular structures in proximity to the Golgi compartment (*Bi*), between Golgi and peripheral structures (*Bii*), and in the periphery close to the plasma membrane (*Biii*). *C*: Confocal analyses of immunostaining of primary rat islet cells for insulin/proinsulin (green), the exosomal marker CD81 (red), and TGN38 (magenta) reveal colocalization of CD81 and insulin/proinsulin in vesicles budding off the Golgi compartment (arrows) and localized in the periphery (arrowheads). Nuclei are stained in blue with DAPI. *D*: Primary rat islet cells transfected with a plasmid encoding the Golgi protein MAGT1-GFP and incubated with BODIPY ceramide for 30 min reveal specific staining of lipids in Golgi membranes by the BODIPY ceramide dye. *E*: NTA of BODIPY ceramide–labeled rat islet exosomes reveal a uniform size distribution with a major peak at 137 ± 5 nm similar to that determined with no fluorescence filter, suggesting that the labeling procedure is effective and exosome specific. All scale bars: 10 μm.

labeling procedure indeed targets exosomes. Thus, the lipid composition of islet exosomes resembles that of the Golgi, suggesting that, in primary islet cells, a sizable fraction of MVBs may indeed originate from Golgi membranes.

Taken together, these results support an exosomal biogenesis pathway for β-cell autoantigens that originates in the TGN. For GAD65, the pathway appears to pass through the ERC and involve Rab11.

Uptake of Islet EVs Results in Stimulation of HLA-DR4$^{+/+}$ Dendritic Cells

We next assessed the ability of professional APCs to take up islet EVs. Incubation of BODIPY-labeled islet exosomes with BMDCs from HLA-DR4$^{+/+}$ transgenic mice (Fig. 3*A* and *B* and Supplementary Fig. 2*A* and *C*) and DR4$^{+/+}$ human Priess B cells (Supplementary Fig. 2*B* and *D*) revealed an efficient and rapid uptake within 2–3 h, as shown by fluorescent microscopy and flow cytometry analyses. To test the potential ability of EVs to activate APCs, rat islet EVs were incubated with DR4$^{+/+}$ BMDCs. During 24 h of incubation, secretion of the proinflammatory molecules TNF-α, IL-6, and IL-1β was significantly increased in the presence of islet EVs compared with PBS (Fig. 3*C*). These results suggest that EVs from primary islets are efficiently taken up by APCs, resulting in their stimulation.

GAD65 Exosome Mimetics Activate GAD65-Specific T Cells

In order to investigate the potential autoantigenicity of GAD65 in islet exosomes, we used the GAD65-specific T-cell hybridoma, T33.1, which recognizes the GAD65$_{274–286}$ peptide in the context of HLA-DR4 (24,45). Since the quantity of EVs that can be isolated from primary islets is limited (~2–3 μg/day from 10,000 islets) and the concentration of GAD65 in islet EVs is low (~0.05% of total exosomal protein), we sought to engineer a surrogate for natural islet exosomes. Purified, endotoxin-free human recombinant GAD65 was inserted into liposomes that approximated the size and lipid composition of exosomes (10), as follows: unilamellar vesicles, 100 nm in diameter, containing cholesterol, sphingomyelin, and ganglioside GM3 (Fig. 4*A*). Different concentrations of either free or liposome-bound GAD65 were incubated for 24 h with DR4$^{+/+}$ BMDCs as APCs, and then T33.1 hybridoma cells (GAD65-specific T cells) were added and incubated for 24 h. This concentration range of GAD65 induces the

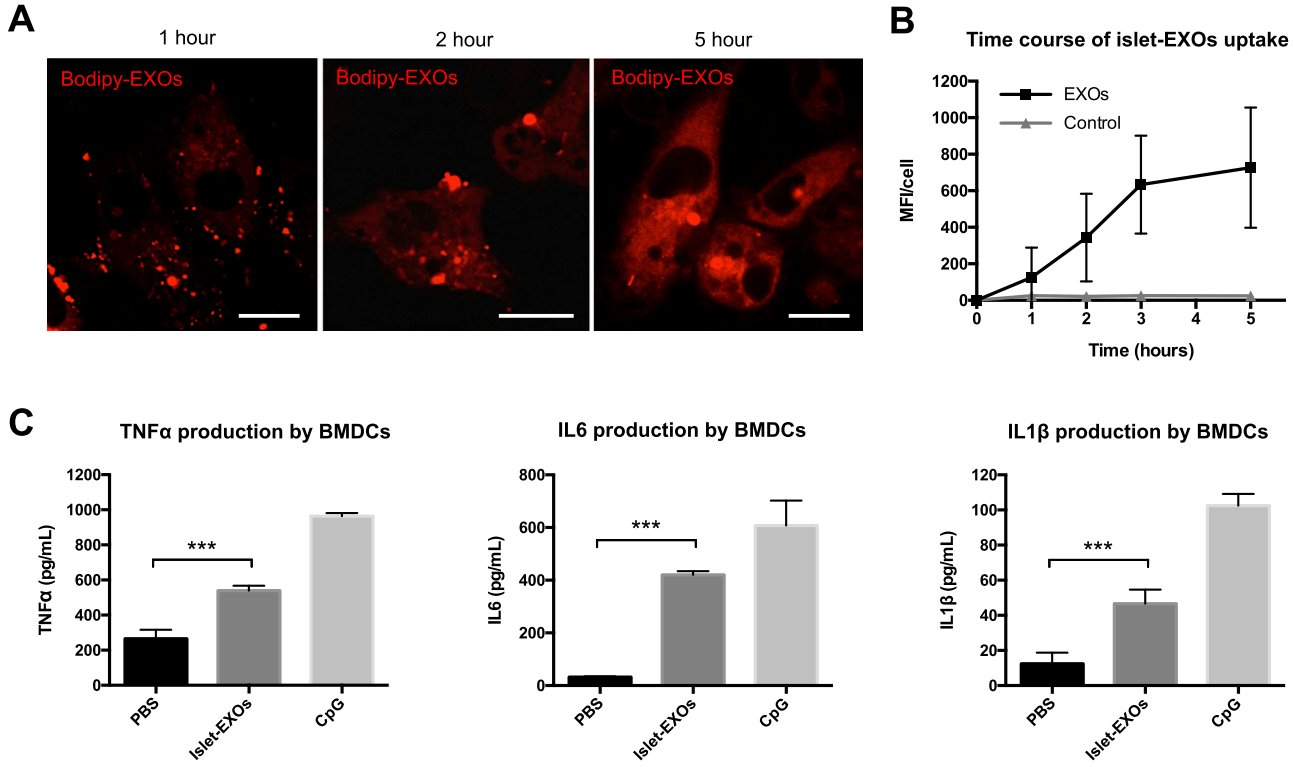

Figure 3—Activation of BMDCs upon uptake of islet exosomes (EXOs). *A*: Uptake of BODIPY ceramide–labeled rat islet exosomes by DR4$^{+/+}$ BMDCs analyzed by confocal live-cell image acquisitions. At 1 h, BODIPY exosomes were detected at the surface of cells. Exosome internalization was observed ~2 h after incubation, whereas a more diffuse red staining was detected at 5 h, indicating diffusion of the dye within the cells. Scale bars: 10 μm. *B*: Uptake of BODIPY exosomes by BMDCs. Free BODIPY dye processed the same way as the BODIPY-labeled islet exosomes was used as a control. The results are shown as mean fluorescence intensity (MFI) ± SD for a total of 20 cells sampled from three different fields. *C*: Secretion of the proinflammatory cytokines TNF-α, IL-6, and IL-1β by DR4$^{+/+}$ BMDCs after stimulation by rat islet exosomes or PBS and CpG as negative and positive controls, respectively. Analyses were performed in technical triplicate. The results are reported as the mean ± SD and are representative of two independent experiments. Statistical analyses were performed using a one-way ANOVA analysis (***$P < 0.001$).

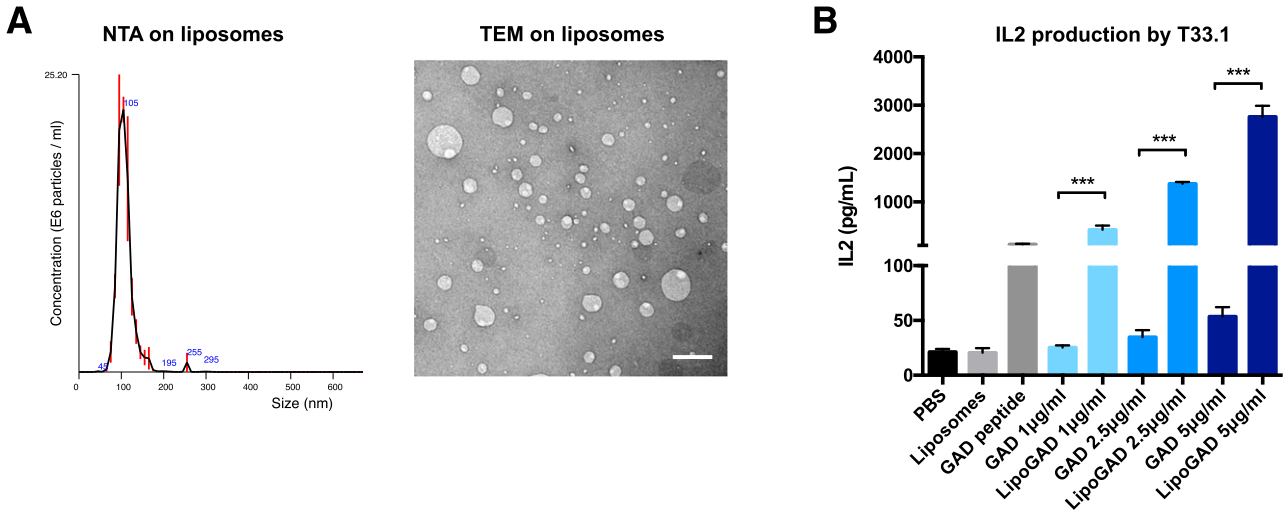

Figure 4—GAD65 exosome mimetics induce strong GAD65-specific T-cell activation. *A*: NTA and TEM analyses of exosome mimetic liposomes reveal an average size of ~100 nm. Scale bar: 200 nm. *B*: Activation of GAD65-specific DR4-restricted T33.1 hybridoma cells by $DR4^{+/+}$ BMDCs that had been incubated with either free GAD65 protein or GAD65 anchored to liposomes (LipoGAD). PBS or empty liposomes (in equivalent amount to the highest concentration of GAD65 liposomes used in the assay) are negative controls and 0.5 μg/mL $GAD_{274–286}$ peptide (Genscript) is a positive control. Analyses were performed in technical triplicate. Statistical analyses were performed using a one-way ANOVA. The results are representative of two independent experiments. ***$P < 0.001$.

activation of T33.1 cells and the release of IL-2 in a dose-dependent manner (24,45). $GAD65_{274–286}$ was used as a positive control at a concentration that consistently induces IL-2 release at ~150–200 μg/mL from T33.1 cells. A 50-fold increase in IL-2 was detected in the supernatant of T33.1 cells after stimulation with GAD65 liposomes compared with free antigen (Fig. 4*B*). Of note, we recently showed (18) that, compared with free GAD65, palmitoylated GAD65, is more efficiently internalized by APCs and enhances the stimulation of T cells. Together, these findings suggest that the presentation of GAD65 in the context of exosome-like particles significantly enhances T-cell stimulation.

Cytokine-Induced ER Stress Increases EV Release by Pancreatic Islets and Induces the Expression of Immune Stimulatory Chaperones

ER stress and the resulting β-cell dysfunction are implicated in the pathogenesis of T1D (3,46). We addressed the question of whether the induction of ER stress in primary β-cells by T-helper type 1 (Th1) cytokines affects the quantity and/or composition of released EVs. Rat islets were incubated with or without low-concentration IL-1β and IFN-γ. We confirmed the induction of ER stress in cytokine-treated primary β-cells by detecting upregulation and nuclear translocation of the transcription factor CHOP (2) (Fig. 5*A*).

No differences in the morphology and size of vesicles from cytokine-treated and untreated islets were observed by TEM or NTA (Fig. 5*B* and *C*). However, the analysis of total protein content by BCA Protein Assay Kit, or by counting the total number of EVs by NTA, revealed a twofold to threefold increase in EVs released from cytokine-treated islets versus nontreated islets (Fig. 6*A* and *B*).

LC-MS/MS analysis of the protein profile of EVs isolated from cytokine-treated islets revealed the upregulation of immunomodulatory proteins involved in the ER stress response. These included the chaperones Gp96, calreticulin, and the hypoxia-upregulated protein ORP150, which were increased 2-, 3-, and 3.5-fold, respectively, in EVs from cytokine-treated compared with untreated islets (Fig. 6*C*). These proteins are highly immunogenic when they leave the ER and appear on the surface of cells or outside cells (47). In contrast, other proteins were found at similar concentrations in cytokine-treated versus untreated preparations, including exosomal markers (CD9, CD81, and flotillin1), GAPDH, and proteins involved in the cytosolic stress response, such as Hsp90 (Fig. 6*C*). WB analysis confirmed the induction of the ER chaperones calreticulin and Gp96 in exosomes from cytokine-treated islets, whereas exosomes from untreated cells were negative. In contrast, the ER chaperone protein disulfide isomerase, which can also be detected on the surface of cells and secreted from cells (48), was detected at similar levels in exosomes from treated and untreated islets, suggesting that the induction of calreticulin, Gp96, and ORP150 in islet exosomes by cytokines is a selective process. The concentrations of GAD65, IA-2, insulin, and flotillin1, however, were similar in EVs from cytokine-treated and untreated islets, although their total amount was increased commensurate with the increase in released EVs (Fig. 6*D*). The data presented here suggest that the induction of ER stress in β-cells results in the targeting of selected immunostimulatory ER chaperones to exosomes. Furthermore, exosomes isolated from human islets treated with IL-1β and IFN-γ induced a stronger activation of $DR4^{+/+}$ BMDCs compared with exosomes from untreated

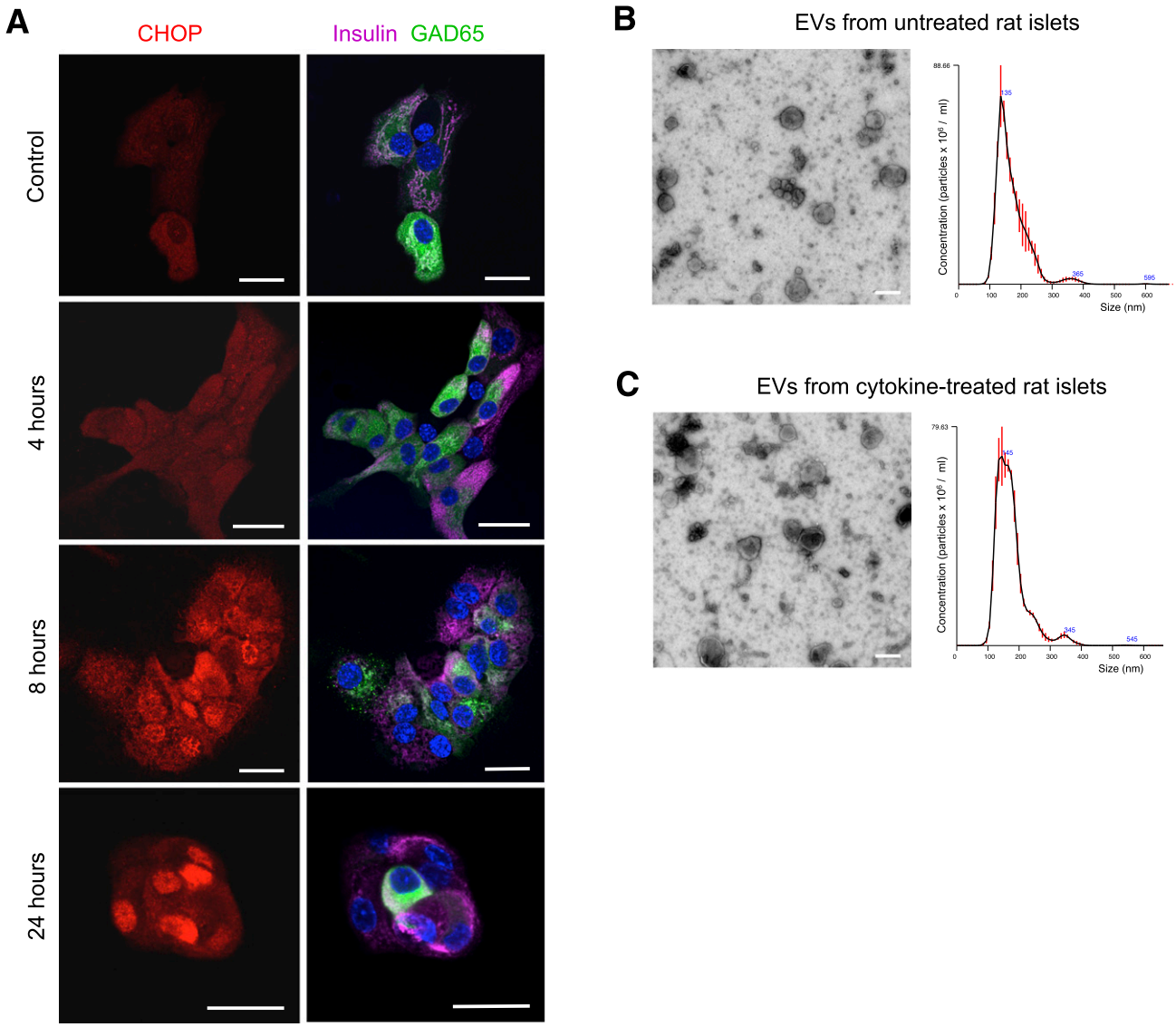

Figure 5—Cytokine-induced ER stress does not affect the size and physical properties of exosomes released by rat pancreatic islets. *A*: Primary pancreatic islet cells immunostained for the stress marker CHOP (left panels) and insulin and GAD65 (right panels) upon cytokine exposure for the indicated number of hours. Nuclei are stained in blue with DAPI. The progressive upregulation and nuclear translocation of the transcription factor CHOP indicates the induction of ER stress in treated cells. Scale bar: 30 μm. TEM and NTA on EVs isolated from untreated islets (*B*) and cytokine-treated islets (*C*). NTA indicates the presence of a uniform size distribution of EVs, with a similar major peak for untreated islets (141 ± 4 nm) and cytokine-treated islets (144 ± 10 nm). Scale bars: 200 nm.

islets, as shown by secretion of the immunostimulatory cytokines IL-6 and TNF-α (Fig. 6*E* and *F*).

DISCUSSION

It is unclear why the GAD65, IA-2, and insulin/proinsulin autoantigens become targets of autoimmunity in human T1D, or how they trigger the attention of the immune system. Notably, GAD65 and IA-2 are scarce intracellular β-cell membrane proteins, which are processed in the ER and Golgi compartments before targeting secretory pathways. IA-2 is a transmembrane protein localized in the limiting membrane of large dense-core insulin secretory vesicles, whereas GAD65 is a peripheral membrane protein found in the membranes of distinct smaller microvesicles (15). While mature insulin is present in sufficient amounts in the circulation to induce and maintain immunological tolerance, proinsulin is an intracellular β-cell protein targeted by autoimmunity in NOD mice and men (7,9). The data presented here reveal that primary islets release immunogenic exosomes containing the β-cell intracellular autoantigens GAD65 and IA-2, and proinsulin. Hybrid peptides between proinsulin and other secretory granule proteins are targets of $CD4^+$ T cells in T1D and are implicated in the loss of self-tolerance to insulin in human T1D (8).

The biogenesis pathway for CD9- and CD81-expressing exosomes may originate in the TGN and involve Rab11-controlled transport through recycling endosomes (10). The data presented here suggest that the trafficking

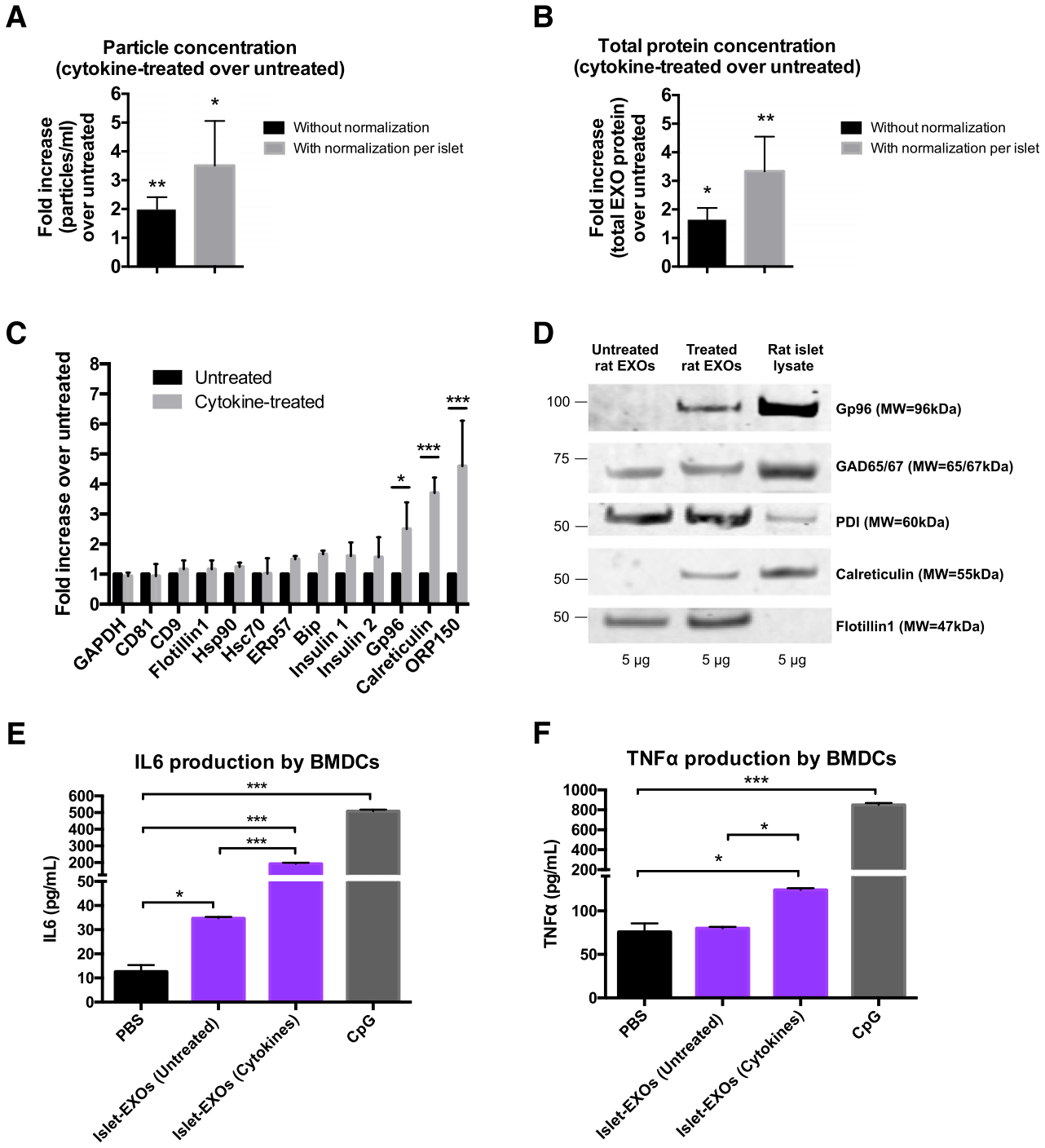

Figure 6—Th1 cytokine treatment results in increased exosome (EXO) release and corelease of immunogenic chaperones by islets cells. Release, over a total period of 6 days, of exosomes from cytokine-treated (10 units/mL IFN-γ and 10 units/mL IL-1β) vs. untreated rat islets shown as particle concentration by NTA (*A*) and total protein content by use of the BCA Protein Assay Kit (*B*). Bars represent the quantitation of exosome secretion without (black bars) and with (gray bars) correction for the number of islets. Data are expressed as the mean ± SD of four independent experiments. Statistical analyses were performed using a paired *t* test (**P* < 0.05, ***P* < 0.01). *C*: Ratio of LC-MS/MS analyses of EV proteins from cytokine-treated and untreated rat islet cells showing cytokine-induced upregulation of proteins involved in the ER stress response. Data are expressed as the mean ± SD of three independent experiments. Statistical analyses were performed using a one-way ANOVA (**P* < 0.05, ****P* < 0.001). *D*: WB analyses of EVs isolated from cytokine-treated and untreated rat islet cells showing specific induction of the ER chaperones Gp96, calreticulin, and ORP150 by cytokine treatment. Data are representative of three independent experiments. *E* and *F*: Exosomes isolated from untreated and cytokine-treated human islets were incubated (50 µg/mL) with DR4$^{+/+}$ BMDCs for 24 h. CpG (5 µg/mL) was used as positive control. Activation of the BMDCs was assessed by the secretion of the proinflammatory cytokines IL-6 (*E*) and TNF-α (*F*). Data are expressed as the mean ± SEM (n = 3) and are representative of two independent experiments. Statistical analyses were performed using a one-way ANOVA: **P* < 0.05, ****P* < 0.001.

of GAD65 from TGN to exosomes may follow this pathway.

β-Cells have an extensive and highly active ER, reflecting their role in synthesizing and secreting large amounts of insulin. The enormous protein synthesis capacity of β-cells, however, makes them markedly susceptible to ER stress. Th1 cytokines secreted by inflammatory cells induce ER stress in β-cells (46). Disturbances in the normal function of the ER to modify and fold newly synthesized proteins result in the induction of the unfolded protein response, which is aimed at restoring cell ER homeostasis but can eventually trigger apoptosis if an imbalance in the folding capacity persists. In this study, we show that the induction of ER stress by proinflammatory cytokines results in a twofold to threefold increase in exosome secretion by islet cells and, consequently, exosomal proteins.

Different scenarios may explain the increased release of EVs from β-cells during ER stress. First, exosomes play roles in intercellular communication (10), and increased secretion may serve to signal ER stress conditions to neighboring cells. Second, exosomes may be a vehicle for disposing of cell material that is not needed or desirable for optimal responses to ER stress in cells seeking to regain homeostasis.

Cytokine treatment also resulted in a change in the composition of EVs. Whereas the composition and relative quantity of autoantigens and exosome markers were similar with or without cytokine treatment, the latter resulted in a corelease of ER chaperone proteins known to act as strong proinflammatory molecules (49,50). These proteins, calreticulin, Gp96, and ORP150, can be found outside the ER, on the cell surface, and released extracellularly (47). They can trigger immune responses by promoting phagocytosis and/or by directly exerting adjuvant capacity (47,50). Some of these ER proteins, or antibodies directed to them, have been implicated in autoimmunity (51). It is possible that islet exosomes carrying a combination of autoantigens and proinflammatory signals may be particularly immunogenic.

We considered the possibility that the ER chaperones in exosome preparations from stressed β-cells originated from ER microsomes—generated during cytokine-induced islet cell death—that coincidentally copurified with exosomes. While we cannot exclude this possibility, several factors argue against a significant contribution of ER-derived microsomes to our EV preparations. First, the relative amount of exosomal markers and autoantigens in EVs was similar with and without cytokine treatment. Thus, a dilution effect of contaminating microsomes was not observed. Second, in contrast with the immunogenic ER chaperones mentioned above, the relative amount of protein disulfide isomerase, an ER chaperone not associated with the induction of immunogenicity (52), was not increased in EVs by cytokine treatment, suggesting that only specific ER proteins were enhanced. Third, the morphology and size distribution of exosomes were homogeneous and not altered by cytokine treatment, which excludes differential abundance and/or the presence of ER microsomes.

The pathway involved in the transport of ER proteins into exosomes under stress conditions has not been elucidated. During immunogenic cell death, calreticulin undergoes an ER-to-Golgi anterograde transport (53). An anterograde transport of the ER proteins in conditions of ER stress would be consistent with a Golgi origin of some islet exosomes and a trafficking pathway where autoantigens, exosome markers, and heat shock proteins assemble in exosomes. Alternatively, as several studies have identified contact sites and interaction between ER membranes and endosomal compartments (54), including MVBs (55), it is possible that the ER chaperones are transported directly from the ER to endosomal compartments. ER stress-inducing cytokine treatment of mouse islets increases the detection of another ER chaperone, glucose-regulated protein 78, in the plasma membrane of primary islet cells and INS-1E cells, as well as in the medium of INS-1E cells (56). These findings support the notion that inflammatory conditions induced by IL-1β and IFN-γ may result in aberrant translocation and secretion of some ER chaperones. Cytokine treatment also resulted in citrullination of glucose-regulated protein 78, and targeting of a citrullinated peptide by both B cells and T cells was observed in NOD mice (56). Further studies that identify potential posttranslational modifications of ER chaperones and autoantigens associated with islet exosomes may clarify their contribution to the induction of autoimmunity.

Islet-derived EVs are rapidly and efficiently taken up by APCs expressing the T1D susceptibility haplotype DRB1*0401, resulting in their activation. Activation is increased using EVs isolated from cytokine-treated islets. To investigate the potential autoantigenicity of islet exosomes, we adopted an established GAD65-specific T-cell assay using DR4$^{+/+}$APCs and a DR4-restricted GAD65-specific T-cell hybridoma (24). The limited amounts of exosomes that can be obtained from primary islets did not allow for obtaining sufficient amounts of GAD65 for the antigen-specific stimulation of T33.1 cells. To overcome this limitation, we developed a platform whereby purified GAD65 was bound to liposomes approximating the size and lipid composition of islet exosomes. APCs loaded with GAD65 liposomes were one to two orders of magnitude more efficient in inducing the activation of autoreactive T cells compared with the free antigen. Furthermore, preliminary experiments revealed that, compared with ovalbumin, calreticulin enhances the stimulatory effect of GAD65 liposomes on BMDCs and activation of GAD65-specific T33.1 cells.

The engineered platform based on exosome mimetics presents an initial attempt to assess the immunogenic potential of GAD65 in lipid membranes with characteristics of exosomes. It should be stressed, however, that the liposomes carry GAD65 on the surface, whereas exosomes likely present GAD65 on the luminal face. Furthermore, the immunogenicity of natural exosomes likely results from

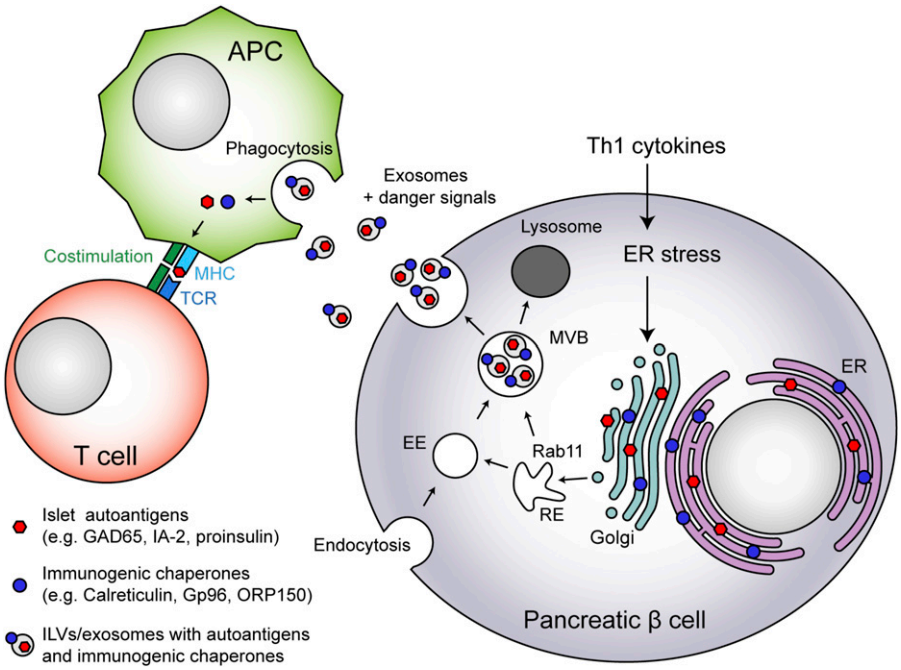

Figure 7—Proposed schematic model of the combined release of autoantigens and proinflammatory ER proteins in β-cell exosomes under cytokine-induced ER stress conditions. ER stress can be induced in pancreatic β-cells by Th1 cytokines and other inflammatory factors, leading to the upregulation of ER proteins involved in the unfolded protein response and possibly their translocation to the Golgi. Once in the Golgi, these ER proteins can be transferred, loaded into the ILVs of MVBs, and released in exosomes. A localization in common between the islet autoantigens during their biosynthesis pathway is the Golgi compartment; thus, their loading into exosomes, together with ER proteins, is a possibility. In addition, ER stress can induce an increase in the number of exosomes released by pancreatic β-cells. ER proteins outside the cell may be recognized as danger signals by APC, leading to cell activation and phagocytosis of autoantigen-containing exosomes. This may result in autoantigens being processed and presented to self-reactive T cells, triggering autoimmunity against pancreatic β-cells. EE, early endosome; ILV, intraluminal vesicle; RE, recycling endosome; TCR, T-cell receptor.

their specific composition of proteins and lipids, which may control exosomal uptake and processing by APCs, among other functions. However, our reductionist approach can be explored to investigate the role of individual components in APC activation and antigen presentation. With further development, exosome mimetics may become a useful therapeutic strategy to tune tolerance versus immunity to GAD65 and other autoantigens, when combined with molecules with known anti-inflammatory properties.

In conclusion, we propose that the release in exosomes of the autoantigens GAD65, IA-2, and insulin/proinsulin by pancreatic β-cells may play a role in triggering autoimmunity, especially under proinflammatory conditions eliciting ER stress and dysfunction, with consequent corelease of immunostimulatory ER chaperones (Fig. 7).

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Author Contributions. C.C. conceived the study, designed the study, analyzed the data, performed the experiments, and wrote the paper with input from the other authors. E.A.P. designed the study, analyzed the data, and performed the experiments. M.P. performed the experiments. R.H., D.D., M.A.A., L.P., S.H., M.A.S., M.D.P., and J.A.H. contributed reagents, human islets, and/or analyses tools. S.B. designed the study, analyzed the data, and wrote the paper with input from the other authors. S.B. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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