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Insulinoma-Released Exosomes or Microparticles Are Immunostimulatory and Can Activate Autoreactive T Cells Spontaneously Developed in Nonobese Diabetic Mice

Huiming Sheng,* Saleema Hassanali,* Courtney Nugent,† Li Wen,* Emma Hamilton-Williams,* Peter Dias,* and Yang D. Dai*

Exosomes (EXO) are secreted intracellular microparticles that can trigger inflammation and induce Ag-specific immune responses. To test possible roles of EXO in autoimmunity, we isolated small microparticles, mainly EXO, from mouse insulinoma and examined their activities to stimulate the autoimmune responses in NOD mice, a model for human type 1 diabetes. We demonstrate that the EXO contains strong innate stimului and expresses candidate diabetes autoantigens. They can induce secretion of inflammatory cytokines through a MyD88-dependent pathway, and activate purified APC and result in T cell proliferation. To address whether EXO or the secreted microparticles are possible autoimmune targets causing islet-specific inflammation, we monitored the T cell responses spontaneously developed in prediabetic NOD mice for their reactivity to the EXO, and compared this reactivity between diabetes-susceptible and -resistant congeneric mouse strains. We found that older NOD females, which have advanced islet destruction, accumulated more EXO-reactive, IFN-γ-producing lymphocytes than younger females or age-matched males, and that pancreatic lymph nodes from the prediabetic NOD, but not from the resistant mice, were also enriched with EXO-reactive Th1 cells. In vivo, immunization with the EXO accelerates insulitis development in nonobese diabetes-resistant mice. Thus, EXO or small microparticles can be recognized by the diabetes-associated autoreactive T cells, supporting that EXO might be a possible autoimmune target and/or insulitis trigger in NOD or congeneric mouse strains. The Journal of Immunology, 2011, 187: 1591–1600.
to study EXO-induced responses in an autoimmune scenario, at a stage of disease where pathogenic effectors, rather than regulatory T cells, may be preferentially activated in genetically susceptible individuals.

We have successfully isolated EXO from mouse insulinoma. We have shown that the EXO preparation has strong adjuvant effect to induce proinflammatory cytokines, including IL-6 and TNF-α. We have confirmed that MyD88 is required for this adjuvant effect (25), suggesting an involvement of TLR-mediated sensing of the innate stimuli enclosed in the insulinoma-derived EXO. We detected candidate islet Ags, particularly glutamic acid decarboxylase 65 kDa (GAD65), expressed in the EXO. Thus, EXO may act as both an endogenous adjuvant and a unique Ag carrier to trigger immune responses. Based on our observations that EXO can cause insulitis in resistant mice and EXO-reactive Th1 cells are enriched in prediabetic NOD mice, we propose that abnormal EXO or a dysregulated EXO-releasing pathway may trigger islet-specific autoimmunity in diabetes-susceptible individuals.

**Materials and Methods**

**Mice**

NOD/LtJ (NOD), NOD/RJlJ (nonobese diabetes resistant [NOR]), NOD.scid, and C57Bl/6 (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained as inbred strains at the animal facility of the Torrey Pines Institute for Molecular Studies. NOD/MkrTac strain, NOD.B10-H-2a (NOD-H-2a) (7), NOD.B10 Idsd congenic mice, line 6146 carrying B10-derived Idsd locus (26), were obtained from Taconic (Hudson, NY) and maintained as inbred strain at Torrey Pines Institute for Molecular Studies. NOD.Idds35 mice, line 1591, which carry both a B6-derived Idsd and a B10-derived Idsd T1D-protective loci (27), were housed at the animal facility of The Scripps Research Institute (San Diego, CA). Splenocytes from NOD.MyD88 knockout (MyD88.KO) mice were prepared by L. Wen (Yale University, New Haven, CT) and shipped to us overnight. Experimental protocols were conducted with approvals from the Ethical Review Committee of Torrey Pines Institute for Molecular Studies.

**Preparation of EXO**

MIN6 insulinoma cell line was used for EXO preparation. This cell line was originally derived from a transgenic C57Bl/6 strain that overexpressed SV40 T Ag under an insulin promoter (28). A second insulinoma cell line, NIT-1, was also used for EXO preparation, which was, however, established from a SV40 T Ag transgenic NOD strain (29). To prepare EXO, insulinoma cells were cultured in 150-cm² culture flasks using high glucose DMEM, supplied with 10% FCS that was precentrifuged at 100,000 g for 90 min to remove serum EXO. Cultured supernatants were harvested by spinning in an ultracentrifuge (Sorvall Differential Ultracentrifuge, SSC-25, Stainless Steel) at 15,000 rpm for 20 min at 4°C. To perform SDS-PAGE, cell supernatants were centrifuged at 15,000 rpm for 1 h at 4°C. The supernatant (1 liter), EXO isolation was performed following a protocol provided by C. Thery (Curie Institute, Paris, France). Briefly, the culture supernatants were centrifuged at 3000 rpm for 15 min, followed by filtration using 200-nm pore-size membrane to remove cell debris and large particles. The filtered supernatants were concentrated in an Amicon Stirred Cells unit (Millipore, Billerica, MA) using an Ultrasel membrane disc of 300-kDa nominal m.w. limit (Millipore). EXO in the concentrated supernatant was harvested by spinning in an ultracentrifuge (Sorvall Discovery 90SE; Hitachi) at 100,000 × g for 90 min. The EXO pellets were then washed once with PBS and resuspended in PBS. Protein concentration was determined by Bradford protein assay (Bio-Rad, Hercules, CA). Normally, 1 liter MIN6 culture supernatant yields 0.5–1.0 mg EXO.

**Cytokine detection assay**

A cyometric bead array (CBA)-based flow cytometry method (BD Biosciences) was used to analyze for six different inflammatory cytokines, as follows: IL-6, IL-10, MCP-1, IFN-γ, TNF-α, and IL-12p70, according to the manufacturer’s protocol. Briefly, 50 μl culture supernatant of EXO-stimulated splenocytes that were collected from young (8- to 12-wk-old) NOD or congenic mouse strains was incubated with 50 μl mixture of six types of cytokine-capturing beads and 50 μl PE-detection reagent in a round-bottom 96-well plate at room temperature for 2 h, with protection from light. After two washes, the incubated beads were resuspended in 200 μl washing buffer and then subjected to FACS analysis. Because each type of the beads is coated with a different capture Ab specific for one of the six cytokines and has a distinct fluorescent intensity in the red channel when analyzed in a flow cytometer, the six individual cytokines can be simultaneously measured. The concentration of each cytokines was extrapolated from the standard curves by testing with the respective recombinant proteins of the cytokines.

**Proliferation assay**

To assess T cell activation by EXO, total splenocytes (5 × 10⁶ cells/200 μl well) were cultured with EXO, Con A, or medium only in 96-well flat-bottom plates for 72 h, followed by pulsing with 0.5 μCi/well ³H]thymidine (Amersham Biosciences, Pittsburgh, PA) for 18 h. Cells were harvested with a Micro Cell Harvester (Skatron Instruments, Sterling, VA), and incorporation of ³H]thymidine was measured on a Wallace MicroBeta Trilux counter (Perkin Elmer, Boston, MA). Stimulation indexes (SI) were calculated as cpm of EXO or Con A/cpm of medium control.

**ELISPOT**

The number of EXO-reactive, IFN-γ–secreting cells was evaluated using an ELISPOT assay. Briefly, ELISPOT plates (MAHA S4510; Millipore) were precoated overnight at 4°C with 5 μg/ml anti–IFN-γ Ab (BD Biosciences) and blocked with complete 10% FCS RPMI 1640 culture medium at room temperature for 2 h. Splenocytes were added at 10⁶/well in the presence of EXO or Con A, or medium. After culture for 48 h, the plates were washed, and IFN-γ–positive spots were detected using 2 μg/ml biotinylated anti–IFN-γ Ab (BD Biosciences), followed by addition of HRP-conjugated streptavidin (Sigma-Aldrich). The spots were developed using 3-amino-9-ethyl-carbazole substrate (BD Biosciences).

**IFN-γ secretion assay**

IFN-γ–secreting cells were identified and quantified by flow cytometry using a mouse cytokine secretion-capture assay kit (Miltenyi Biotec, Auburn, CA), which could capture the secreting cytokines and retain their surface. Briefly, splenocytes or lymph node cells (10⁶/sample/well) were first activated by EXO for 48 h, and washed twice with ice-cold buffer. To capture the secreted IFN-γ, the preactivated cells were resuspended in 40 μl ice-cold buffer and 10 μl IFN-γ capture reagent (provided in the kit), followed by incubation on ice for 5 min. After adding 1 ml/sample of 37°C warm medium, the cells were incubated again at 37°C for 45 min with slow rotation. The cells were then washed once and resuspended in 40 μl ice-cold buffer with 10 μl IFN-γ detection Ab (provided in the kit), together with additional fluorescent-labeled Abs to identify subsets of lymphocytes. After staining for another 15 min on ice, the cells were washed and resuspended in icro-cold buffer for flow cytometry analysis.

**SDS-PAGE and Western blotting analysis**

MIN6 or NIT-1 cell lysates were prepared by resuspending cell pellets (5 × 10⁶ cells) in 200 μl cell-lysing buffer (containing 20 mM Tris·HCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM NaVO₄, 1% SDS, and 10 μg/ml aprotinin/leupeptin), and then incubated on ice for 15 min, followed by centrifugation at 15,000 rpm for 20 min at 4°C. To perform SDS-PAGE, cell lysates or EXO samples were denatured by incubation with sample buffer at 95°C for 10 min. SDS-PAGE-separated protein samples were electro-transferred to nitrocellulose membrane (Amersham, GE Healthcare Life Sciences, Piscataway, NJ) using a semidy transfer module (Bio-Rad Laboratories, Hercules, CA). The membrane was immunoblotted with 1 μg/ml primary Abs, followed by a respective secondary HRP-labeled anti-IgG (Amersham). The protein bands were visualized with an ECL detection system (Amersham). Clones 4-17 or 144 (provided by C. Hampe, University of Washington, Seattle, WA) were used for detecting GAD65. Antiheat shock protein cognate 70 (HSC70) was purchased from Stressgen (Stressgen Bioreagents, Victoria, BC, Canada). Anti-chromogranin A was purchased from Abcam (Cambridge, MA).

**Results**

Mouse MIN6 insulinoma can release EXO that expresses candidate islet Ags

EXO was isolated from the culture supernatant of MIN6 insuli-noma cells. As demonstrated by electron microscopy, the majority of the microparticles retain a round, vesicular shape, with a diameter <100 nm (Fig. 1A). Initial mass spectrometry analysis
using the total microparticle preparation identified several signature proteins of typical EXO (17), including tetraspanins, Alix, and TSG101, suggesting that the microparticle preparation contained EXO. To further analyze the protein content in the EXO, we separated the EXO on SDS-PAGE; after Coomassie staining, the gel was cut into four slices based on their m.w. (Fig. 1B), and each gel slice was subjected to mass spectrometry analysis. Approximately 200 protein molecules were identified (see Supplemental Table); other than molecules involved in intracellular vesicle sorting and protein degradation, some cytosolic and membrane proteins were also detected. Insulin was detected by mass spectrometry only once when total EXO preparation was analyzed. GAD65, one of the diabetes-associated islet Ags, did not appear in the protein list identified by mass spectrometry, although the GAD65 protein was expressed abundantly in MIN6 cells (Fig. 1C). A second insulinoma cell line, NIT-1, does not express GAD65 (Fig. 1C). The anti-GAD65 mAb (clone 144) binds to the N terminus of GAD65 protein. GAD65.KO brain tissue was used as a negative control. The band stained on the Western blot membrane was confirmed to be GAD65, not its glutamic acid decarboxylase 67 kDa isoform, by its m.w. Nevertheless, the GAD65 protein was detected in MIN6 EXO by Western blot, which might be more sensitive than mass spectrometry in analyzing a protein mixture. As shown in Fig. 1D, only EXO released by the MIN6, but not NIT-1 insulinoma, was stained positive by the GAD65-specific mAb, whereas both insulinomas-released EXOs contain HSC70 protein, which is commonly found in such microparticles (17). Thus, GAD65 is released by MIN6 cells, possibly via EXO secretion pathway, but at a low level under the given culture condition.

**FIGURE 1.** MIN6 insulinoma cells release EXO that expresses GAD65 protein. A, Images of EXO preparations under an electron microscope after negative staining. Arrow-pointed microparticles are the major structure observed. B, Preparing gel samples for identifying protein content of the EXO by mass spectrometry. MIN6 EXO (∼60 µg) was denatured in sample buffer and separated on a 10% SDS-PAGE gel. The gel was stained by Coomassie dye and cut into four slices, M1, 2, 3, and 4, as indicated, and submitted to the mass spectrometry facility in the Scripps Research Institute (San Diego, CA) for protein identification. C, Expression of GAD65 in insulinoma cell lines as detected in Western blot. Control brain samples were collected from GAD65.KO or wild-type NOD mice, and the blot was reprobed with anti-ERK to indicate similar amount of samples loaded. D, Detecting GAD65 and HSC70 protein in EXO preparations from MIN6 or NIT-1 insulinoma cell lines. The expression of GAD65 protein only in MIN6 EXO was confirmed in three separate experiments.

**FIGURE 2.** EXO collected from MIN6 culture supernatant is proinflammatory. A, Splenocytes (10^6/200 µl/well) from 8-wk-old NOD female mice were cultured with 5 µg/ml MIN6 EXO for 48 h in a flat-bottom 96-well plate. A CBA assay was performed to monitor the levels of six different inflammatory cytokines in the culture supernatants or the non-stimulated control. Similar results were observed in three separated experiments. B, Experiment was similarly performed as in A, except that an IL-12-blocking Ab (clone C17.8) or a rat Ig isotype control was added at a final concentration of 20 µg/ml during the culture with EXO.
tation might be too low to be detected using this CBA assay. The cytokine levels produced by the EXO-stimulated splenocytes were dose and time dependent (Table I): even after just 6 h of incubation with a low dose of EXO (1 μg/ml), TNF-α was elevated 8-fold above background, indicating that a rapid innate signal was triggered by EXO. This instant TNF-α release was due to EXO-induced activation of splenocytes because the cytokine was not detected by culturing EXO or splenocytes separately. At 20 μg/ml of 24-h incubation with EXO, IL-6 increased 32-fold from 15.6 to 497.3 pg/ml, IFN-γ increased 22-fold from 2.7 to 59.9 pg/ml, and TNF-α increased 54-fold from 9.0 to 482.7 pg/ml (Table I). In contrast, an EXO preparation from cultured NOD splenocytes was nonstimulatory, whereas the same volume of supernatant from MIN6 culture yielded sufficient EXO to induce the innate response. Therefore, the EXO secreted by the MIN6 cells contain some endogenous, adjuvant-like components, capable of inducing the inflammatory cytokines.

**The innate response induced by EXO requires MyD88-mediated TLR-signaling pathway**

To examine whether TLR-mediated innate signaling pathway is required for the EXO-induced cytokine response, we tested total splenocytes from a MyD88.KO mouse. Fig. 3A shows that the cytokine response to EXO stimulation (12 h) was almost completely impaired in the MyD88.KO splenocytes as compared with wild-type NOD cells; a similar lack of response in the MyD88.KO cells was observed after a longer (24-h) incubation time, although IFN-γ was slightly increased, ∼2-fold above the nonstimulated background. In contrast, in the wild-type NOD female splenocytes, IFN-γ increased at least 20-fold at 12 h, and reached >100-fold at 24 h after stimulation with 10 μg/ml MIN6 EXO (data not shown). In a 72-h lymphocyte proliferation assay, the wild-type NOD splenocytes responded to the EXO (10 μg/ml) with a SI >50. In contrast, the MyD88.KO splenocytes were completely unable to proliferate at the same dose ranges (SI < 3), but responded strongly to Con A (Fig. 3B). These results suggest that the possible innate stimuli enclosed in the EXO may activate TLR signals via MyD88-dependent pathways.

**APC and purified subsets are activated by the EXO to upregulate class II MHC and costimulatory molecules**

To examine whether EXO could activate APC, we monitored the expression of CD86 (B7.2) on class II MHC-positive splenocytes. Fig. 4A demonstrates that EXO stimulation increased the percentage of CD86 and I-A<sup>β</sup> MHC double-positive population from 2.6 to 11.5% in total splenocytes. Fig. 4B shows the expression of class II MHC, CD80, CD86, or ICAM-1 on the splenic B220<sup>+</sup>, CD11c<sup>+</sup>, and CD11b<sup>+</sup> APC subpopulations after EXO stimulation. Clearly, professional APC were activated effectively by EXO as detected by their upregulated expression of MHC II or costimulatory molecules. It is noticed that nonstimulated splenic CD11c<sup>+</sup> and CD11b<sup>+</sup> APC expressed higher level of class II MHC than B220<sup>+</sup> APC and EXO stimulation only upregulated CD86 or CD80, but not class II MHC on these two populations (Fig. 4B). Because EXO are small particles under 100 nm diameter, it is possible that certain APC subpopulation(s), such as dendritic cells, may be more efficient than others, such as macrophages or B cells, in picking up this size range of microparticles. We isolated CD11c<sup>+</sup>, CD11b<sup>+</sup>, or B220<sup>+</sup> APC populations from NOD splenocytes by a positive selection protocol using MACS microbeads coated with the respective Abs, and examined their responses to EXO. At 5 μg/ml EXO concentration and 12-h incubation time, both purified CD11c<sup>+</sup> and CD11b<sup>+</sup> APC populations responded rapidly (also observed at an 8-h time point) to EXO stimulation by releasing mainly IL-6 and TNF-α (Fig. 4C); the response by the purified B220<sup>+</sup> APC (∼90% purity) was much lower, with a marginal (∼2-fold) increase of TNF-α, and IL-6 increased insignificantly.

**T cells contribute to EXO-induced lymphocyte proliferation**

Because NOD total splenocytes proliferated strongly after EXO stimulation (Fig. 5A), we asked whether T cells are the proliferating population by testing the proliferation response after depleting CD4<sup>+</sup> and CD8<sup>+</sup> T cells from the splenocytes. As a result, the proliferation capability was impaired in the absence of T cells. In one experiment, total NOD splenocytes reached a SI as high as 65 at the dose of 10 μg/ml EXO, whereas the T cell-depleted splenocytes responded 3-fold less to the same dose of EXO (Fig. 5A) despite that only ∼50–60% depletion of T cells was achieved in this experiment. T cell proliferation was also demonstrated by a 2-fold increase of the percentage of total CD4<sup>+</sup> T cells in the EXO-stimulated splenocytes (Fig. 5B). Strikingly, no reactivity was observed when NOD.scid splenocytes were used in the proliferation assay (Fig. 5A), suggesting that T and/or B lymphocytes were the only populations that proliferated in response to EXO. The lack of proliferation of NOD.scid splenocytes to the EXO was not due to defects in their innate responses because the NOD.scid splenocytes also produced high levels of inflammatory cytokines when stimulated by EXO, although the cytokine release pattern was different (Fig. 5C).

**Prediabetic animals accumulate EXO-reactive, IFN-γ-secreting T cells**

To examine whether EXO stimulation could activate diabetes-associated T cell responses in NOD mice, three different methods were used to monitor the IFN-γ response to the EXO: 1) total EXO-responder, IFN-γ-secreting cells in splenocytes were detected by ELISPOT assay; 2) IFN-γ and other inflammatory cytokines in culture supernatants were examined by a flow cytometry-based CBA assay; and 3) single IFN-γ-secreting Th1 cells were identified by an IFN-γ capture assay (see Materials and Methods). Fig. 6A shows that the levels of EXO-induced IFN-γ response correlated well with the developmental stages of diabetes in the ELISPOT assay, with the oldest NOD females exhibiting the highest number of IFN-γ<sup>+</sup> spots, and the male NODs the lowest. In addition, CBA assay was performed to compare NOD female and male mice for their responses to EXO stimulation in vitro. Among the six inflammatory cytokines, IFN-γ was found different in that female splenocytes produced 10-fold higher IFN-γ on average than male splenocytes after stimulated with EXO (Fig. 6B). Three

| Table I. Exosomes stimulate splenocytes to release inflammatory cytokines |
|-----------------------------|-----------------------------|
| **Stimulation Times** | **Cytokines (pg/ml)** | **EXO Doses (μg/ml)** |
|-----------------------------|-----------------------------|
| 6 h  | IL-6 | 15.6 | 20.3 | 28.5 | 35.5 |
|     | IFN-γ | 2.7 | 2.4 | 5.5 | 3.5 |
|     | TNF-α | 9.0 | 77.6 | 121.8 | 193.5 |
| 12 h | IL-6 | ND | 81.3 | 116.7 | 161.3 |
|     | IFN-γ | ND | 3.4 | 4.8 | 10.0 |
|     | TNF-α | ND | 124.9 | 172.5 | 266.9 |
| 24 h | IL-6 | 17.4 | 334.3 | 333.5 | 497.3 |
|     | IFN-γ | 8.9 | 362.4 | 31.4 | 59.9 |
|     | TNF-α | 11.8 | 319.0 | 366.3 | 482.7 |

*Table 1. Exosomes stimulate splenocytes to release inflammatory cytokines*

| Splenocytes (10^7/200 μl/well) from one male NOD were stimulated with different doses of EXO for various periods. Cytokine levels in the culture supernatants were monitored by the CBA assay. |

ND, not done.
of four 9- to 12-wk-old females produced large amount of IFN-γ (632, 688, and 1044 pg/ml) after 48-h stimulation, whereas all three age-matched males responded below 100 pg/ml (35, 72, and 87 pg/ml). Thus, the EXO-responding, IFN-γ–producing cells are accumulated in prediabetic NOD female mice. This correlation was further confirmed by testing NOD congenic strains with different susceptibility to T1D. As shown in Fig. 6C, NOD, the most susceptible strain, produced the highest number of IFN-γ spots (∼100 spots/10⁶ splenocytes), whereas only <10 spots were identified when NOR splenocytes were stimulated by the EXO and a partially resistant strain, NOD.B10 Idd5.2, exhibited an intermediate response. Similarly, IFN-γ, but not the other five inflammatory cytokines, was also found different between NOD and the resistant strain. Fig. 6D shows one example that splenocytes from a NOD female produced 3- to 5-fold higher amount of IFN-γ than an age- and gender-matched NOD.B10 Idd5.2 mouse.

To quantify EXO-responding, IFN-γ–secreting Th cells, we performed IFN-γ–secreting/capturing assay after stimulating pancreatic lymph node cells (Pan-LN) or inguinal lymph node cells (Ing-LN) with EXO in culture. Fig. 7A shows a >10-fold increase of the Th1 cells observed in the Pan-LN of NOD female mice after EXO stimulation, from 0.17% increased to 1.8% of total Pan-LN, whereas no increase was observed for a resistant NOD congenic strain, NOD.Idd3/5. It is noticed that many Pan-LN of NOD increased their IFN-γ secretion after EXO stimulation, but at a low level, as indicated in a histogram plot in Fig. 7B (top panel), rather than a polarized, fully activated, Th1 phenotype. Control Ing-LN of the NOD mice showed less of an increase (from 0.25 to 0.48%) after EXO stimulation (Fig. 7B, bottom panel), further confirming that EXO-reactive Th1 cells preferentially accumulate in the Pan-LN of prediabetic animals.

**EXO immunization accelerates insulitis in diabetes-resistant NOR mice**

To examine whether EXO can trigger or accelerate islet inflammation or insulitis in diabetes-resistant mice, we directly injected EXO i.v., without addition of other adjuvant, into three diabetes-resistant mouse strains, NOR, NOD.H-2b, and C57BL/6 mice. After 3 or 7 d, their pancreatic glands were collected for histological examination. It has been reported that both NOD.H-2b and...
The severity of insulitis or lymphocyte infiltration is significantly increased in the group treated with EXO for 7 d (p = 0.01), but the difference between the untreated and 3-d–treated groups was insignificant.

**Discussion**

Exosomes derived from tumor cells are naturally occurring small nanoparticles that not only contain potent adjuvant-like substances, but also carry tumor-specific Ags (19, 20). It has also been reported that EXO can activate myeloid-derived suppressor cells (30) and regulatory T cells (24, 31), which may explain the difficulties in designing EXO vaccine to treat cancer patients (32). Because these physiologically important microparticles contain mostly self Ags, to which immune tolerance is well established, except for those rare individuals who are highly susceptible to a particular autoimmune disease, the chance of successfully inducing effective antitumor immunity or breaking self tolerance using EXO as an immunogen is low. Thus, the consequence of EXO-induced immune responses might be heavily dependent on the types/contents of EXO as well as the genetic background of the hosts. To our knowledge, this is the first study characterizing immune responses to EXO in an autoimmune scenario, the NOD mouse model of T1D, in which immunopathology rather than immunosuppression is the outcome of an organ-specific inflammatory response. We found that EXO can induce innate immunity via a MyD88–mediated TLR-signaling pathway, and that EXO-reactive Th1 cells accumulated in prediabetic NOD female mice, but not in NOD males or resistant congenic strains. These findings emphasize the importance of genetic background and memory precursors in controlling adaptive immune responses to EXO. It remains unknown whether the accumulated EXO-reactive Th1 cells in the prediabetic NOD mice were primed directly by the endogenous EXO secreted by the pancreatic islet cells, or via cross-reaction to other Ags. Nevertheless, EXO or microparticles secreted by islet cells should be useful tools for studying tissue-specific autoantigens and their cognate autoreactive T cells.

It has been shown previously that tumor cell-derived EXO requires MyD88–dependent TLR-signaling pathway to induce IL-6 and TNF-α secretion from myeloid cells (25). EXO derived from different tissues or cell lines may vary in their proinflammatory activity, and the characteristics of the possible innate stimuli within MIN6 insulinoma remain unknown, but we confirmed that the innate response also required MyD88. Several heat shock proteins were found in our EXO preparations. Chalmers et al. (30) demonstrated that heat shock protein 72 expressed on tumor cell-derived EXO could activate myeloid-derived suppressor cells in a TLR2/MyD88-dependent manner, whereas Zhang and colleagues (25) demonstrated that neither TLR2 nor TLR4 was involved in their system testing EXO-induced activation of the myeloid-derived suppressor cells. EXO released from Leishmania could modulate monocytes’ cytokine response by promoting IL-10, but inhibiting TNF-α (33). Interestingly, human primary astrocytes can release exosomes carrying mitochondrial DNA (34), which were shown to have innate stimulatory activity, possibly via the TLR9-signaling pathway (35). It was noteworthy that our EXO preparation contains endogenous gag and env proteins (Supplemental Table); whether endogenous retroviruses, which have been implicated as etiological agents of T1D (36, 37), contribute to the immune responses to EXO is an interesting possibility. Whether EXO produced by different types of tumor cells or primary tissues or organisms vary in their usages of different innate pathways or TLRs remains to be studied.
Without the MyD88 gene, both female and male NOD mice were almost completely resistant to diabetes development at 30 wk of age (38), indicating an essential contribution of the innate TLR-signaling pathway to this T cell-mediated autoimmunity. Wen et al. (38) reported that neither TLR3 or TLR4 deficiencies in NOD mice could mediate protection from diabetes, whereas TLR9-deficient NOD mice were partially protected (39); Kim et al. (40) showed that their TLR2.KO NOD mice were partially protected; Dutz and colleagues (41) reported that altering either the TLR2 or TLR9 pathways had some moderate effects on T1D development in NOD mice. Interestingly, MyD88.KO NOD mice gained their diabetes susceptibility when housed under germ-free environment (38). Thus, gut flora must affect disease development in NOD mice, possibly by shaping the immune repertoire or promoting immune regulation. The requirements for sensing inflammatory stimuli in the pancreas may be different from that of responding to the microflora in the gut. Because MyD88 is required for the EXO-induced innate response, determining which

FIGURE 6. EXO can activate diabetes-associated, IFN-γ-secreting cells. A, Total splenocytes (10^6/200 μl/well) from NOD mice of different ages or genders (wk, weeks old; F, females; M, males) were tested for their reactivity to MIN6.EXO (1 μg/ml) in an IFN-γ ELISPOT assay. All mice were not diabetic at the time of testing, as indicated by urine glucose. The results were reproducible in a separate experiment, with similar observation. B, Splenocytes from four female and three male NOD mice (9–12 wk old) were stimulated with EXO for 48 h, and CBA assay was performed to measure cytokines in the culture supernatants. The average cytokine concentrations for each gender are shown. C, EXO-induced IFN-γ responses among different NOD congenic strains (8- to 10-wk-old female mice) were compared by ELISPOT method. Data represent one of two experiments with similar observation. D, IFN-γ levels in the culture supernatants of EXO-stimulated splenocytes were compared between two age-matched NOD and NOD.B10 Idd5.2 (Idd5.2) female mice in a CBA cytokine assay.

FIGURE 7. Accumulation of EXO-responding, IFN-γ-secreting Th cells in pancreatic lymph nodes of NOD mice. A, Pan-LN (5 × 10^5/well) from age-matched NOD and NOD Idd3/5 (Idd3/5) female mice (three mice/group) were mixed with irradiated syngenic splenic APC (1:1) and then cultured with medium or 5 μg/ml EXO for 48 h. IFN-γ-secreting CD4^+ T cells were identified in an IFN-γ-secreting/capturing assay (see Materials and Methods). B, Histogram plot of EXO-stimulated Pan-LN is presented by gating on CD4^+ T cells (top panel, filled, medium control; empty, cultured with EXO for 48 h). Ing-LN from the NOD and Idd3/5 mice were used as controls to culture with (empty) or without EXO (filled), and the results are shown in histogram plot (bottom panel).
TLR(s) may be involved in responding to EXO will help further clarify the endogenous innate stimulatory materials enclosed in the EXO.

Among the six inflammatory cytokines we examined, IL-6 and TNF-α were the major cytokines produced by purified splenic APC populations (CD11c+ DC, CD11b+ macrophages, and B220+ cells), although IL-10 was also detected after EXO stimulation. MCP-1 was produced only by CD11b+ macrophages, and IFN-γ was undetectable for all three purified APC populations. When total splenocytes were cultured with EXO, IFN-γ level increased after 24-h culture, indicating an activation of T cells at this time point. However, some CD4+CD8+ splenocytes were also found to produce IFN-γ when cultured with EXO. This is particularly interesting because it has been shown that NK cells may be one of the early islet infiltrators triggering the inflammatory response (42), and an abnormal EXO release from the islets may attribute to this early NK infiltration.

T1D is a Th1-mediated autoimmune disease, and activation of tissue-specific, IFN-γ-secreting Th cells is a crucial checkpoint controlling disease progression (43). However, the endogenous pathway inducing these pathogenic effector cells is not fully understood. Because I-A^d class II MHC is required for the insulitis and diabetes in NOD mice and T cells are among the earliest islet-infiltrating lymphocytes (44), we proposed that altered presentation of islet Ags may contribute to specific activation of high-affinity Th cells (45, 46). We found that EXO were effective in activating IFN-γ-secreting Th1 cells in NOD mice. Presumably, these Th1 cells represent the islet-Ag–specific memory cells that are primed endogenously in the pancreatic islets or the draining lymph nodes. We noticed that despite a significant increase of EXO-reactive Th1 cells in old NOD female mice, the number of highly polarized Th1 cells was very low, as indicated in the ELISPOT assay. Also, although many EXO-activated Th cells in the Pan-LN of NOD mice can produce IFN-γ, the amount of IFN-γ they produced was small, as measured in the IFN-γ-secreting/capturing assay. One possibility is due to a low expression of candidate autoantigens within the EXO. It might be interesting to test whether increased expression of the autoantigens could enhance IFN-γ production. It is also possible that other islet Ag sources such as apoptotic bodies released following β cell death (47) that may express large amount of candidate Ags can stimulate T cells to produce more IFN-γ. Alternatively, polyclonal activation of many Th cell clones that are not highly polarized, but biased to produce some IFN-γ, might be sufficient to cause damage of the islets. In fact, it has been shown that even at very early stage of prediabetic NOD mice, TCR repertoire in pancreatic lymph nodes, although restricted, is highly diverse and dynamic (48), and we had similar observation in studying the TCR repertoire of islet-infiltrating T cells (I. Marrero, A. Vong, Y. Dai, and J. Davies, submitted for publication).

It will be helpful to test whether insulinoma-derived EXO can activate some highly diabetogenic T cell clones (49, 50), providing that EXO and its Ags may be processed and presented via some unique pathway(s). Our study is inconclusive about whether the EXO-induced IFN-γ-secreting cells could be due to partial cross-reactivity (51) of the disease-related memory precursors to some unknown exosomal Ags or even due to bystander activation (52). Study of a particular Ag and its cognate T cells using EXO as an Ag carrier may help address further whether EXO could be responsible for inducing a tissue-specific autoimmunity. It is noteworthy that of the five different inflammatory cytokines we examined, only IFN-γ was found to differ between diabetes-susceptible and -resistant strains, or between NOD male and female mice when total splenocytes were cultured with EXO. Such difference cannot be identified after stimulation with LPS. This indicates that EXO may be a possible endogenous target that can preferentially activate a small percentage of memory autoreactive T cells in vitro.

We have found that candidate diabetes-associated autoantigens, particularly GAD65, are expressed in the EXO produced by MIN6 insulinoma cells. GAD65-specific autoantibodies and T cell responses are detected very early in prediabetic patients (53). The GAD65 protein in the pancreatic islets of NOD mice (54) and the NIT-1 insulinoma cells is barely detectible, but its expression in the MIN6 cells and their secreted EXO is effective (Fig. 1C). We have also detected another candidate diabetic autoantigen, chromogranin A (55), in MIN6 cells, as well as in their secreted EXO (data not shown). We have learned that most of candidate diabetes Ags such as GAD65 and IA-2, except for insulin, are not a necessity causing diabetes in NOD mice (56–58); it might be an interesting question whether a disease-triggering event, maybe abnormal EXO secretion, could cause autoimmune targeting at several islet Ags simultaneously. The hypothesis that insulin acts as the primary islet Ag (58) is intriguing because islet β cells

**FIGURE 8.** Induction of insulitis in NOR mice by EXO immunization. The 4- to 5-mo-old NOR female mice were injected (i.v.) once with 15 μg EXO in PBS. After 3 or 7 d, pancreases were collected and submitted for H&E stain. Levels of islet infiltration were scored in blind, and the percentages of islets within each level of lymphocyte infiltration are shown for individual mice. Nine age/gender-matched NOR mice purchased from The Jackson Laboratory were used as untreated controls. The mean of infiltration for each mouse was calculated as follows: the sum of percentages of infiltration for each islet/number of total islets examined for each mouse.
apparently are not the only cell populations attacked in the islets and might be secondary to the peri-insulitis targeting at the islet glial cells (13), which we found also express GAD65 protein and release innate stimuli (H. Sheng and Y. Dai, unpublished data). This leads us to wondering whether the GAD65-specific T cell responses developed early in NOD mice (59, 60) may be triggered by the glial cells. Interestingly, not all types of EXO are proinflammatory; for example, oligodendrocyte-released EXO can carry large amount of myelin autoantigens, but are incapable of inducing a concomitant inflammatory response (61). Whether NOD mouse strain and/or the islet glial cells are producing abnormal EXO that are more inflammatory than diabetes-resistant strains remains to be studied.

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Disclosures
The authors have no financial conflicts of interest.

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