Lymphocyte-Derived Exosomal MicroRNAs Promote Pancreatic β Cell Death and May Contribute to Type 1 Diabetes Development

Highlights

- T lymphocytes release exosomes containing specific microRNAs
- T lymphocyte exosomes can transfer microRNAs to rodent and human pancreatic β cells
- The transferred microRNAs trigger chemokine expression and apoptosis of β cells
- Blockade of microRNAs transferred in β cells decreases diabetes incidence in NOD mice

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In Brief

Guay et al. show that T cells release exosomes containing specific microRNAs that trigger chemokine expression and apoptosis in recipient pancreatic β cells in type 1 diabetes. Inactivation of miR-142-3p/-5p and miR-155 in β cells results in higher insulin levels, lower insulitis scores, and reduced inflammation and protects NOD mice from diabetes development.
Lymphocyte-Derived Exosomal MicroRNAs Promote Pancreatic β Cell Death and May Contribute to Type 1 Diabetes Development

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SUMMARY

Type 1 diabetes is an autoimmune disease initiated by the invasion of pancreatic islets by immune cells that selectively kill the β cells. We found that rodent and human T lymphocytes release exosomes containing the microRNAs (miRNAs) miR-142-3p, miR-142-5p, and miR-155, which can be transferred in active form to β cells favoring apoptosis. Inactivation of these miRNAs in recipient β cells prevents exosome-mediated apoptosis and protects non-obese diabetic (NOD) mice from diabetes development. Islets from protected NOD mice display higher insulin levels, lower insulitis scores, and reduced inflammation. Looking at the mechanisms underlying exosome action, we found that T lymphocyte exosomes trigger apoptosis and the expression of genes involved in chemokine signaling, including Ccl2, Ccl7, and Cxcl10, exclusively in β cells. The induction of these genes may promote the recruitment of immune cells and exacerbate β cell death during the autoimmune attack. Our data point to exosomal-miRNA transfer as a communication mode between immune and insulin-secreting cells.

INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disorder characterized by infiltration of the islets of Langerhans by immune cells and by selective elimination of the insulin-secreting β cells (Eizirik et al., 2009). The infiltration of immune cells has been observed in non-obese diabetic (NOD) mice, a well-known model of T1D (Anderson and Bluestone, 2005; Atkinson and Letter, 1999), but also in the islets of human diabetic donors (Atkinson et al., 2014; Krogvold et al., 2016; Lundberg et al., 2017). Patients suffering from T1D require lifelong insulin administration to regulate whole body glucose metabolism and homeostasis. Understanding the dialogue between pancreatic β cells and the infiltrating immune cells is crucial to promote the design of novel strategies to prevent the development of this disease and/or to find a cure.

Part of the detrimental effects of the immune cells on β cell survival is known to be exerted through the release of pro-inflammatory cytokines (Eizirik et al., 2009). However, lymphocytes were recently found to release another class of immune mediators: the exosomes (Thery et al., 2009). Exosomes are small extracellular vesicles of 50–150 nm in diameter that originate from the late endosomal pathway and are secreted in the extracellular space upon fusion of multivesicular bodies with the plasma membrane (Thery et al., 2009). Exosomes are released by numerous cell types, including lymphocytes and pancreatic islets (Guay and Regazzi, 2015; Robbins et al., 2016), and are present in the blood and other body fluids (Weber et al., 2010). These extracellular vesicles transport proteins, mRNAs, and non-coding RNAs that can be transferred in active form to recipient cells, suggesting an important role in cell-to-cell communication (Kosaka et al., 2010; Thomou et al., 2017; Valadi et al., 2007; Zhang et al., 2010). Until recently, most of the studies on exosomes were performed in \textit{in vitro} or \textit{ex vivo} cell models, but in vivo studies are now emerging. For example, the use in mice of the Cre-loxP system or of a human-specific microRNA (miRNA) elegantly demonstrated the transfer of the exosome...
cargo between distally located cells (Ridder et al., 2014; Thomou et al., 2017; Zomer et al., 2015). Of interest, the transfer of nucleic acids by exosomes was found to be promoted by inflammatory conditions (Ridder et al., 2014).

miRNAs constitute one of the main cargoes transported by exosomes. These small non-coding RNAs are key post-transcriptional modulators of gene expression and can be transferred in active form via exosomes to acceptor cells (Kosaka et al., 2010; Montecalvo et al., 2012; Thomou et al., 2017; Valadi et al., 2007; Zhang et al., 2015). miRNAs play an important role in the control of β cell activities. Indeed, they are involved in the differentiation and functional maturation of β cells and regulate insulin secretion and cell survival (Dumortier et al., 2013; Eliasson and Esguerra, 2014; Guay et al., 2011; Jacovetti et al., 2015; Lynn et al., 2007). Deregression of miRNA expression has been associated with the development of T1D and T2D and with age-associated decline in β cell proliferation (Filios and Shalev, 2015; Guay and Regazzi, 2016; Nesca et al., 2013; Tugay et al., 2016). Moreover, some miRNAs were found to induce β cell dysfunction and death under inflammatory conditions in both rodent and human islets (Grieco et al., 2017; Roggli et al., 2012).

In the present study, we investigated the involvement of miRNAs in the initial phases of T1D development in NOD mice. We found that islet insulitis is associated with an exosome-mediated transfer of a particular group of miRNAs, namely miR-142-3p, miR-142-5p, and miR-155, from lymphocytes to β cells, resulting in the selective death of the insulin-secreting cells. In vitro, inactivation of these miRNAs inside the β cells protected them against apoptosis induced by T lymphocyte exosomes and, in vivo, prevented T1D development in NOD mice. Taken together, our results suggest that the transfer of miRNAs mediated by exosomes released by lymphocytes contributes to the destruction of the β cells and could be part of the mechanisms leading to T1D development.

RESULTS

Increased Abundance of miR-142-3p, miR-142-5p, and miR-155 in β Cells of Pre-diabetic NOD Mice

In a previous study, we performed a global miRNA expression profiling of pancreatic islets of 4- and 8-week-old female NOD mice (which correspond to control and pre-diabetic state, respectively) to understand the role of miRNAs in β cell death associated with T1D development (Roggli et al., 2012). At 8 weeks of age, NOD mice are still normoglycemic but T lymphocytes are starting to infiltrate the islets (Roggli et al., 2012). The miRNAs displaying the most important deregulation in the islets of 8-week-old animals were miR-142-3p/-5p, miR-150, and miR-155, which are known to control the activity of immune cells (Dudda et al., 2013; Kroesen et al., 2015; Wu et al., 2007), but are normally expressed at very low levels in insulin-secreting cells. Indeed, we found that these miRNAs are much more abundant in lymphocytes compared with islet cells (Figure S1A). The expression of these miRNAs is modified to a much lesser extent in the islets of age-matched diabetes-free NOD-SCID mice (Figure 1A), indicating that the changes observed in pre-diabetic mice are most likely linked to the development of the disease. The increase in the levels of miR-142-3p/-5p and miR-155 were detected also in fluorescence-activated cell sorting (FACS)-sorted β cells of pre-diabetic NOD mice (Figure 1B), confirming that the rise of these miRNAs occurs in insulin-secreting cells. In agreement with previous findings in rat islets (Bravo-Egana et al., 2012), the expression of these miRNAs was not affected by incubation of control C57Bl/6N mouse islets with the pro-inflammatory cytokines interferon γ (IFN-γ), tumor necrosis factor alpha (TNF-α), and interleukin-1β (IL-1β) (Figure 1C). Therefore, the increase of miR-142-3p/-5p and miR-155 in β cells in 8-week-old NOD mice is unlikely to be caused by cytokines secreted by immune cells infiltrating the islets.

T Lymphocytes Release Exosomes Containing miR-142-3p, miR-142-5p, and miR-155

Since immune cells release large amounts of exosomes, we hypothesized that the rise of miR-142-3p/-5p, and miR-155 in islet cells of pre-diabetic animals may result from an exosome-mediated transfer favored by the proximity of T lymphocytes and β cells during the initial phases of the disease. To explore this hypothesis, we isolated by ultracentrifugation the vesicles released by human Jurkat T cells (exot) and by CD4+CD25+ T lymphocytes of NOD mice (exoNOD). Analysis of these preparations by electron microscopy (Figures 2A and 2B) and NanoSight technology (Figures 2D and 2E) revealed the presence of round-shaped small extracellular vesicles surrounded by a lipid bilayer membrane with a diameter of 50–200 nm. These small extracellular vesicles carried the characteristic exosomal markers CD81, Alix, and Tsg101 (Thery et al., 2009) (Figure 2C). We next investigated if these small extracellular vesicles can be efficiently internalized by insulin-secreting cells. After an overnight incubation, DiD-labelled exoT were found to be taken up by most cells of the β cell line MIN6B1 (Figure 2F).

We next examined the miRNA content of T cell exosomes. All three miRNAs, miR-142-3p/-5p, and miR-155, were found to be present in the extracellular vesicles released by T lymphocytes (Figures S1B and S1C). To ensure that these miRNAs are confined inside exosomes, the samples were treated with RNase. In contrast to a synthetic oligonucleotide (miR-29a mimic) spiked into the samples, the level of the endogenous miRNAs was not affected by the RNase treatment and were degraded only when the exosome membrane integrity was altered with the detergent Triton X-100 (Figure 2G).

Exosomal miRNAs from T lymphocytes Are Transferred to β Cells

To assess whether the miRNAs present in the exosomes of T lymphocytes can be transferred to β cells, dispersed mouse islet cells were incubated with exoNOD for 72 hr (Figure 3A). This treatment led to a significant increase in the abundance of miR-142-3p/-5p and miR-155 in islet cells (Figure 3B). Similar results were obtained when MIN6B1 or rodent islet cells were exposed to exoT (Figures 3C and S2B). The sequence of miR-142-3p and miR-142-5p is identical in humans and rodents and, therefore, it is not possible to distinguish the miRNAs delivered via the exosomes from those endogenously produced in mouse or rat β cells. In contrast, human miR-155 (hsa-miR-155) differs by one nucleotide from its rodent counterparts (Figure S2A) and can be readily distinguished by qPCR using species-specific primers (Table S1). This strategy enabled us to show that the rise of miR-155 observed in the receiving rodent
islet cells is entirely due to an exosome-mediated transfer of hsa-miR-155 from the human Jurkat T cell line and not from an increased synthesis of endogenous mmu-miR-155 (Figures 3C, 3D, and S2C). This finding was further confirmed using exosomes purified from CD4+/CD25⁻/CD4⁺ T cells of miR-155 knockout mice (Figures S2D and S2E). Indeed, while the other miRNAs were still increased in recipient MIN6B1 cells, the level of miR-155 remained unchanged upon incubation with exosomes isolated from miR-155 knockout mice (Figures S2F–S2H). To demonstrate that hsa-miR-155 is transferred in active form inside the β cells, the receiving cells were transfected with a luciferase sensor. As expected the incubation of MIN6B1 cells with exoT for 48 or 72 hr led to a decrease in the luciferase activity of the hsa-miR-155 sensor (Figure 3E), confirming that the miRNA is transferred in active form in the receiving cells. Taken together, these results provide evidence for an exosome-mediated transfer of miRNAs from immune cells to β cells analogous to that observed in other biological processes (Ridder et al., 2014; Thomou et al., 2017; Zhang et al., 2015).

T Lymphocyte Exosomes Promote β Cell Apoptosis and Induce the Upregulation of Genes Involved in Cytokine and Chemokine Signaling

We next investigated the impact of T lymphocyte exosomes on β cell function and survival. Incubation of β cells in the presence of exoT did not affect insulin secretion or content (Figures S3A–S3D) but led to a significant increase in cell apoptosis, which was prevented by the pan-caspase inhibitor zVAD (Figures 3F, 3G, S3E, and S3F). The cytotoxic action of T cell exosomes was reproduced in dispersed mouse islet cells incubated with exosomes from T cells of NOD mice (Figure 3H). The apoptotic effect of exoT cannot be attributed to the presence of residual traces of cytokines released by lymphocytes. In fact, incubation of β cells in the medium surrounding the purified exosomes did not modify the level of the miRNAs and did not affect the survival of the cells (Figures S3G and S3H). To determine if this process occurs also in humans, human islet cells were incubated with exosomes (exo-hT) of CD4⁺ T cells isolated from human blood donors. Similar to the findings obtained in rodents, exposure of human islet cells to exo-hT resulted in the transfer of miR-142-3p, miR-142-5p, and miR-155 (Figure 3I) and in an increase in cell death measured by Hoechst or by TUNEL staining (Figures 3J and 3K).

Interestingly, the cytotoxic effect of the exosomes was restricted to β cells. Indeed, the increase in apoptosis was reproduced in FACS-sorted rat β cells (98% ± 2% purity) but not in an α cell-enriched fraction (91% ± 4% α cells) (Figures 4A–4D).
and in non-fluorescent islet cells (Figure 4C). Consistent with an increase in the level of the eosinophil-associated ribonuclease exoT can be equally detected in auto-fluorescent b et al., 2012). ImageStream flow cytometry, revealed that DiD-la-

To elucidate the mechanisms through which T cell exosomes exert their effect on b cells, we analyzed by microarray the changes in the mRNA profile elicited in mouse islet cells by exoNOD. Upon exposure to exoNOD, we detected about 90 upregulated and 20 downregulated genes (nominal p value < 0.05 and fold change >2) (Table S2). Pathway analysis revealed a significant enrichment for genes involved in cytokine and chemokine signaling, including Ccl2, Ccl7, and IFNg (Figure S4A). Several of these changes were confirmed by qPCR (Figures 5A and 5B). Using this technique, we also obtained evidence for an increase in Cxcl10 (p = 0.05), a chemokine known to be involved in T1D development (Frigerio et al., 2002; Roep et al., 2010) (Figure 5A). The array data revealed also a strong increase in the mRNA profile elicited in mouse islet cells by treatment with exoNOD (Figure 5C), indicating that at least part of the effect of the exosomes is mediated by the transfer of these miRNAs. Exposure of human islets to the exosomes released by activated human CD4+ T cells (exo-hT) led to a similar increase in CCL2, CCL7, and CXCL10 (Figure 5D), indicating that this phenomenon is not restricted to rodents. To better understand the cell-specific apoptotic effect of exosomes, we incubated FACS-sorted α and β cells with exoNOD for 72 hr. We found that miRNAs are transferred to both cell types (see Figures 4D and 4E), but the rise of Ccl2 and Cxcl10 was only observed in β cells (Figure 5E). A similar trend was observed for Ccl7. These results suggest that the selective effect of the exosomes is likely to be caused by the activation of cell death signaling pathways operating predominantly in β cells.

Several of the differentially expressed genes are elicited in response to the activation of nuclear factor-kB (NF-κB), a signaling pathway known to trigger β cells apoptosis (Melloul, 2008). Consistent with this observation, exposure of MIN6B1 cells to ExoT was associated with a nuclear translocation of NF-κB (Figures 5F and S4). The fraction of cells in which NF-κB localized in the nucleus after ExoT treatment was very similar and 4B). To assess whether this cell-specific effect is due to a selective uptake of the exosomes, we took advantage of the auto-fluorescence properties of the rodent β cells (Kohler et al., 2012). ImageStream flow cytometry, revealed that DiD-labelled exoT can be equally detected in auto-fluorescent β cells and in non-fluorescent islet cells (Figure 4C). Consistent with these findings, upon incubation with exoNOD miR-142-3p, miR-142-5p, and miR-155 were transferred to both FACS-sorted α and β cells (Figures 4D and 4E, respectively).

The role of these RNAs in β cells has so far not been explored but, in other cell types, Ear proteins have cyto-toxic and chemomtrattractant effects (Gupta et al., 2013; Rosenberg, 2015). Interestingly, overexpression of miR-142-3p/5p family members, Ear1, Ear2, and Ear12 (Table S2). The role of these RNases in β cells has so far not been explored but, in other cell types, Ear proteins have cytotoxic and chemotactant effects (Gupta et al., 2013; Rosenberg, 2015). Interestingly, overexpression of miR-142-3p/5p and miR-155 in mouse islet cells led to an increase in the level of Ccl2, Ccl7, and Cxcl10 similar to that observed upon incubation with exoNOD (Figure 5C), indicating that at least part of the effect of the exosomes is mediated by the transfer of these miRNAs. Exposure of human islets to the exosomes released by activated human CD4+ T cells (exo-hT) led to a similar increase in CCL2, CCL7, and CXCL10 (Figure 5D), indicating that this phenomenon is not restricted to rodents.

Figure 2. Characterization of the Exosomes Released by T lymphocytes
Exosomes were isolated from the culture media of Jurkat cells (exoT) or NOD T lymphocytes (exoNOD) by sequential centrifugation.

(A and B) Visualization of the extracellular vesicles (arrows) released from (A) Jurkat cells or (B) NOD T lymphocytes by electron microscopy. Scale bars, 100 nm.

(C) Analysis of the presence of the exosomal markers Alix, CD81, and Tsg101 by western blotting in our extracellular vesicle preparations. The supernatant surrounding the exosome pellet after ultracentrifugation was used as negative control (Neg. Ctl).

(D and E) Analysis of the size distribution of (D) ExoT and (E) ExoNOD vesicles using the NanoSight technology.

(F) ImageStream flow cytometry analysis of MIN6B1 cells incubated with DiD-labelled ExoT (4 representative pictures out of 20,171 cells analyzed). Scale bars, 7 μm.

(G) miRNA levels in exoT. A miR-29a mimic, used as positive control for RNase digestion, was spiked in the ExoT preparation. The samples were then treated or not (NT) with Triton X-100 (T) and incubated with RNases (+R). miRNA levels were measured by qPCR and are expressed as percentage of NT (n = 3 independent experiments). Mean ± SD.
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to that observed upon exposure to pro-inflammatory cytokines (Figure 5F). Interestingly, the translocation of the transcription factor could be reproduced by directly overexpressing miR-142-3p, miR-142-5p, or miR-155 (Figure 5F), confirming a causal link with the transfer of these miRNAs.

Inactivation of miR-142-3p, miR-142-5p, and miR-155 Prevents Exosome-Induced Cell Death

To determine whether the transferred miRNAs contribute to the deleterious effect of T cell exosomes on β cell activities, we directly increased their level using miRNA mimics (Figure S5A and SSC). While none of the tested miRNAs affected insulin secretion or content (Figures 6A and SSB), overexpression of miR-142-3p, miR-142-5p, or miR-155 increased apoptosis both in MIN6B1 (Figure S5D) and in rat islet cells (Figure 6B). If the transfer of specific miRNAs from T cells to β cells contributes to apoptosis induced by exosomes, then inactivation of these miRNAs in receiving cells should prevent cell death. We first verified this hypothesis in vitro using anti-miRs that specifically block the action of the transferred miRNAs. Anti-miRs will not avoid the transfer of the miRNAs but will sequester them upon delivery inside the recipient β cells, blocking the interaction with their targets. Transfection of islet cells with anti-miR-142-3p, anti-miR-142-5p, or anti-hsa-miR-155 attenuated the rise of these non-coding RNAs (Figures S6A–S6C) and prevented apoptosis induced by exoT (Figure 6C). This effect was specific to exosome-induced apoptosis since these anti-miRs failed to protect β cells exposed to pro-inflammatory cytokines (Figures S6E–S6G). While decreasing the level of endogenous miR-155, transfection of rat islet cells with an anti-miR against murine miR-155 did not prevent cell death (Figures 6C and S5D). Taken together, these results suggest that miR-142-3p, miR-142-5p, and miR-155 transferred by exosomes from T lymphocytes to β cells induce recipient cell death.

Injection of a miRNA Sponge in 4-Week-Old NOD Mice Prevents T1D Development

Our findings suggest that a transfer of a subset of miRNAs from immune cells to β cells taking place during the initial phases of T1D could potentially contribute to the development of the disease. To assess the pathophysiological relevance of this exosome-mediated cell-to-cell signaling in vivo, we engineered an Adeno-associated virus (AAV) producing a GFP transcript containing multiple binding sites for miR-142-3p/-5p, miR-150, and miR-155. This “miRNA sponge” is driven by the insulin promoter and specifically sequesters these miRNAs once delivered inside the β cells (Figure 7A) (Ebert et al., 2007). As at the beginning of our study it was not clear whether miR-150 would be involved in diabetes development, we decided to also include in the construct the binding site of this miRNA. The miRNA sponge or a control AAV lacking the miRNA binding sites were intraperitoneally injected in 4-week-old female NOD mice. As expected, GFP mRNA and protein expression was detected in the islet of 8-week-old mice injected with the sponge or the control AAV (Figures S7A and S7B). Importantly, GFP was not detected in the exocrine pancreas and in immune cells invading the islets, confirming the specificity of the insulin promoter (Figure S7C). At 8 weeks of age, both control and sponge-treated NOD mice displayed similar body weights, plasma glucose levels and glucose tolerance, and showed low levels of lymphocyte infiltration (Figures S7D–S7F, S8A, and S8B). However, while at 32 weeks of age 80% of the control mice became diabetic, only 40% of the animals receiving the miRNA sponge developed the disease (Figure 7B). Histological analysis of the pancreases at the time of sacrifice revealed that these disease-free mice retain a higher insulin volume compared with the animals developing diabetes (Figure 7C). Moreover, their islets more often displayed low insulin levels (Figure 7D). This was not associated with an increase in FoxP3-positive Treg lymphocytes suggesting a decrease in inflammation rather than changes in the cell populations of the immune infiltrate (Figure S8C). Consistent with this hypothesis, we found reduced levels of CXCL10 in islet cells (Figure 7E). In line with our in vitro observations, CXCL10 was more frequently found in insulin-positive cells compared with glucagon-positive cells, and the expression of this chemokine in β cells was significantly reduced in sponge-protected mice (Figure 7E). CXCL10 levels were already lower in sponge-treated 8-week-old mice compared with control animals suggesting a diminished chemotactic ability of the β cells expressing the miRNA sponge (Figure 7F).

Taken together, these in vivo data support the relevance of the exosome-mediated transfer of genetic material in the autoimmune reaction and indicate that the inactivation of miR-142-3p/-5p, miR-150, and miR-155 in β cells is able to protect NOD mice against T1D development.

DISCUSSION

The autoimmune attack of β cells is a hallmark of T1D, but the events culminating in the specific loss of the insulin-secreting cells remain poorly understood. In the present study, we provide evidence for the involvement of an exosome-mediated transfer of miRNAs from T lymphocytes to β cells in the initial phases of T1D development. Exosomes have been shown to participate to the etiology of other autoimmune disorders (Robbins et al., 2016; Turpin et al., 2016). Indeed, these small extracellular...
vesicles can activate specific signal transduction pathways, participate in the formation of immune complexes and synapses, and contribute to the maintenance of the inflammatory status. We observed that exposure of \( \beta \)-cells to exosomes released from T lymphocytes does not alter their secretory capacity but increases their apoptotic rate. An important feature of the autoimmune attack of pancreatic islets is the selective killing of \( \beta \)-cells. Interestingly, the exosomes released by T cells reproduce this \( \beta \)-cell-specific event and have no deleterious impact on \( \alpha \)-cells. This cell selectivity cannot be explained by a specific targeting of the exosomes to \( \beta \)-cells, since these small extracellular vesicles are internalized by other islet cell types. \( \beta \)-cell-specific apoptosis is most likely the result of the activation by exosomes of cell death pathways operating only in insulin-secreting cells. Human and rodent \( \alpha \)-cells have been reported to be resistant to metabolic and immune stresses that induce \( \beta \)-cell death (Marroqui et al., 2015; Takeda et al., 2012; Vasu et al., 2015). In agreement with this hypothesis, Ccl2, Ccl7, and Cxcl10 were solely increased in \( \beta \)-cells and not in \( \alpha \)-cells upon exosome treatment. Moreover, in NOD mice developing diabetes CXCL10 was more frequently detected in insulin-positive cells than in glucagon-positive cells.

The exosome cargo includes proteins and nucleic acids, in particular miRNAs. We previously identified a subset of miRNAs that are highly abundant in the islets of pre-diabetic NOD mice (Roggli et al., 2012). We show here that this increase cannot be explained by the exposure of \( \beta \)-cells to pro-inflammatory cytokines but is rather the result of an exosome-mediated shuttling of miR-142-3p/-5p and miR-155 from T lymphocytes to \( \beta \)-cells. Our data provide strong evidence indicating that the transfer of these miRNAs is instrumental to induce \( \beta \)-cell death. Indeed, overexpression of these miRNAs is sufficient to induce \( \beta \)-cell apoptosis, and blockade of these miRNAs inside the \( \beta \)-cells prevents apoptosis induced by T cell exosomes but not by pro-inflammatory cytokines. Individually, none of the predicted targets of the miRNAs is able to explain the activation of NF-\( \kappa \)B or the observed increase in apoptosis. This is not surprising because miRNAs are known to function by inducing relatively small changes in the expression of a large number of genes rather than by causing major changes in the level of a single transcript. Computational analysis suggests that miR-142-3p, miR-142-5p, and miR-155 share some common targets (Table S3), some of which are known to participate in the regulation of cytokine and/or NF-\( \kappa \)B signaling. Thus, the signaling cascades activated in response to an increase of these miRNAs may potentially converge. Indeed, in agreement with reports in other...
Figure 5. Regulation of β Cell Gene Expression by T Cell Exosomes

(A and B) Confirmation by qPCR of gene expression changes observed by global microarray profiling of mouse islet cells incubated with exoNOD. The results are expressed (A) as fold changes or (B) as RFU, Ifng being undetected under control conditions (n = 4 independent experiments).
cell types (Sharma et al., 2012; Yin et al., 2017), miR-142-3p, miR-142-5p, and miR-155 were all found to modulate NF-κB activity, pointing to a common mode of action. Thus, although the overexpression of each individual miRNA can potentially lead to cell death, the effect of the T cell exosomes is likely to result from the synergistic action of the three miRNAs. In fact, blockade of any of the three transferred miRNAs was sufficient to prevent exosome-induced apoptosis.

We were able to prove the relevance of this novel exosome-mediated cell-to-cell communication mode for T1D development by taking advantage of an AAV construct containing multiple binding sites for miR-142-3p/-5p, miR-150, and miR-155. Indeed, NOD mice injected with this miRNA sponge showed a 50% reduction in the incidence of the disease compared with the control group. As expected, this protection against T1D development was accompanied with a higher islet insulin volume and lower insulitis scores. Whether the protective effect of the miRNA sponge is limited to β cell death or also prevents the initial steps of the disease remains to be defined. Since an efficient transfer of miRNAs from lymphocytes to β cells involves the close proximity of the two cells, this event is very unlikely to constitute the initial trigger of the disease. However, once the autoimmune reaction has been initiated by viral antigens, auto-antigens, or other inflammatory mediators, and immune cells are recruited to the islets, β cell chemokine production resulting from the transfer of the miRNAs may contribute to maintain and further exacerbate the insulitis. This scenario is consistent with the reduced immune infiltration of the islets observed in mice treated with the miRNA sponge. To precisely define the contribution of exosome-mediated miRNA transfer to the different stages of the diseases, large cross-sectional studies monitoring the impact of the miRNA sponge on insulitis progression and β cell loss during the natural course of diabetes will be needed.

Interestingly, Ying et al. (2017) recently demonstrated the detrimental effect of miR-155 released in exosomes from macrophages residing in adipose tissue of obese mice on systemic insulin sensitivity and glucose tolerance. These metabolic markers were improved in miR-155 knockout animals. Therefore, the release by immune cells of miR-155 in exosomes may possibly be implicated in the etiology of both T1D and T2D.

The precise mechanisms through which the exosomes of T lymphocytes trigger β cell death remain to be fully established. In vitro, activation of NF-κB signaling as well as the induction of several members of the Ear family that are cytotoxic in other cell types (Gupta et al., 2013; Rosenberg, 2015) may contribute to the increase in the apoptotic rate observed upon exposure to exosomes. In vivo, T lymphocyte exosomes may be part of the mechanisms favoring islet inflammation, insulitis progression, and T1D development. Under inflammatory conditions, β cells are known to produce chemokines such as CCL2 and CXCL10, which are believed to favor insulitis and diabetes development (Frigerio et al., 2002; Martin et al., 2008). The expression of these inflammatory mediators was also observed in pancreatic sections or in islet samples of human T1D donors (Eizirik et al., 2012; Roep et al., 2010). In our study, global expression profiling of mouse islets incubated with exosomes from NOD T effector cells revealed the upregulation of several genes linked

(C–E) Gene expression levels were measured by qPCR in (C) dispersed mouse islet cells transfected with miR-142-3p, miR-142-5p, and miR-155 mimics. (D) human islet cells incubated with exo-hT, and (E) FACS-sorted rat islet fractions enriched for α or β cells incubated with exoNOD. The results are expressed as fold changes (n = 4 independent experiments).

(F) Nuclear translocation of NF-κB in MIN6B1 cells incubated with exoT or pro-inflammatory cytokines (IFN-γ, TNF-α, and IL-1β), or transfected with miR-142-3p, miR-142-5p, and miR-155 mimics (n = 3–4 independent experiments).

Mean ± SD; *p < 0.05, **p < 0.01 by Student’s t test. See also Figures S3 and S4.
Please cite this article in press as: Guay et al., Lymphocyte-Derived Exosomal MicroRNAs Promote Pancreatic β Cell Death and May Contribute to Type 1 Diabetes Development, Cell Metabolism (2018), https://doi.org/10.1016/j.cmet.2018.09.011
to cytokine and chemokine signaling, including Ccl2, Ccl7, Cxcl10, and Ifng. Interestingly, Ccl2 was found to modulate NF-κB signaling in MIN6 cells (Cai et al., 2011) and other cell types (Viedt et al., 2002; Viedt and Orth, 2003). At least part of these gene expression changes may be driven by the transfer of miRNAs from immune cells to β cells. Indeed, overexpression of miR-142-3p, miR-142-5p, and miR-155 in β cells led to an increase in Ccl2, Ccl7, and Cxcl10 expression and favored nuclear translocation of NF-κB. CXCL10 levels are reduced in β cells of NOD mice injected with the AAV-sponge and protected from diabetes development. Therefore, in vivo, the exposure to exosomes may exacerbate the release of cytokines and chemokines from β cells elicited in response to inflammatory mediators produced by T lymphocytes, favoring insulitis progression and T1D development.

The exosome-mediated dialogue between lymphocytes and β cells may operate in both directions. Indeed, these vesicles were reported in other immune diseases to activate T lymphocytes either directly or indirectly via the antigen-presenting cells (Turpin et al., 2016). The release of exosomes from human and rodent islet cells or by insulinoma cell lines has been reported by several laboratories (Bashratyan et al., 2013; Cianciaruso et al., 2017; Guay et al., 2015; Sheng et al., 2011). Sheng et al. (2011) observed that injection of exosomes produced by MIN6 cells in NOR mice favors lymphocyte islet infiltration and increases insulitis. In vitro, exosomes from MIN6 cells were found to trigger both innate and antigen-specific signals by inducing lymphocyte proliferation and by activating autoreactive T helper 1 cells and marginal zone-like B cells (Bashratyan et al., 2013; Sheng et al., 2011). The precise mechanisms through which β cell exosomes trigger the immune response remain to be unveiled. Exosomes released by rat and human islet cells contain three autoantigens linked to T1D, namely GAD65, IA-2, and proinsulin, and are taken up by dendritic cells causing their activation (Cianciaruso et al., 2017). The presence of these autoantigens in exosomes released by β cells could explain how these proteins become the target of the immune system even if they are localized mainly inside the cells. Indeed, exosome-mimetic liposomes transporting GAD65 could favor antigen presentation and T cell activation. Finally, other components of β cell exosomes were also involved in stimulation of antigen-presenting cells (Cianciaruso et al., 2017).

In the present study, we focused on miRNAs transferred from CD4+ T effector cells to β cells. However, other immune cells infiltrating the islets release exosomes with a similar miRNA content. For example, miR-150 and miR-155 are released in exosomes originating from monocytes and macrophages, respectively, and have functional impacts on recipient cells (Ying et al., 2017; Zhang et al., 2010). The composition of the immune infiltrate and the origin of the exosomes may differ with the progression of the disease. Our data do not exclude the contribution of exosomes produced by other immune cells infiltrating the islets in β cell dysfunction and loss. We believe that the transfer of miRNAs to β cells during T1D development is mainly promoted by the close proximity of the activated immune cells that invade the islets. However, the amount and the composition of the exosome cargo varies depending on the activation state of the immune cells (Gutierrez-Vazquez et al., 2013; Okoye et al., 2014; van der Vlist et al., 2012). Indeed, the exosomes released by active T cells were reported to contain higher levels of miR-142-3p (Sukma Dewi et al., 2017). In vivo, it is possible that the presence of autoreactive T cell receptors or of other specific components at the exosome surface of infiltrating immune cells may facilitate the fusion with the β cells and the delivery of the miRNAs. Moreover, the exosomes released by immune cells may carry additional molecules potentially affecting the activity of the target cells, including other RNAs, proteins, and lipids. A better understanding of the mechanisms governing the targeting of the exosomes and of the cargo they deliver will not only help elucidating the mechanisms triggering T1D but may also shed new light on the etiology of other autoimmune diseases.

Our findings have relevant implications for the design of better approaches to prevent T1D development. Strategies to avert the loss of β cells will need to take into account the existence of this newly discovered cell-to-cell signaling mechanism. The exosome-mediated transfer of miRNAs with harmful effects on β cells is probably exacerbating the deleterious impact of pro-inflammatory cytokines released by the invading immune cells, and is likely to synergize with other well-described events involved in the autoimmune reaction. Thus, therapeutic interventions tackling several aspects of the immune attack would probably be more effective in protecting the β cells.

**Limitations of the Study**

In this study, we showed that a viral construct blocking the activity of a group of miRNAs transferred from lymphocytes to β cells reduces the incidence of T1D in NOD mice. Although these mice constitute a widely used model of T1D and reproduce many of the features of the disease, the use of a viral construct to block miRNA activity may alter cellular signaling pathways in a unphysiological manner, and the results may not be directly applicable to human disease. Further studies are needed to determine whether the results obtained with the viral construct can be replicated using other approaches, and to investigate the potential of targeting miRNAs transferred from immune cells to β cells as a therapeutic strategy for the prevention of T1D.
the features of the disease in humans (Pearson et al., 2016), additional investigations will be needed to assess the translational potential of our findings. In fact, human T1D is characterized by less-pronounced intra-islet infiltration, and several immunotherapies preventing the disease in NOD mice were found to be inefficient in humans (Kolb and von Herrath, 2017). Future experiments will also have to investigate the efficacy of the treatment at different stages of the disease. Indeed, in this study the miRNA sponge was injected prior to the initiation of the autoimmune reaction and we ignored whether the treatment would be equally efficient after intra-islet infiltration has occurred. This information will be essential to evaluate the therapeutic potential for human T1D of strategies targeting the exosome-mediated process unveiled in this study.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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**SUPPLEMENTAL INFORMATION**

Supplemental information includes eight figures and six tables and can be found with this article online at https://doi.org/10.1016/j.cmet.2018.09.011.

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**AUTHOR CONTRIBUTIONS**

R.R. conceived the study. C.G., J.K.K., and R.R. designed the experiments. C.G., S.R., V.M., A.D., B.J.G., C.J., K.B., and M.P. carried out in vitro experiments and collected the data. J.K.K., N.L.M., and A.J. conducted the in vivo experiments on NOD mice and collected the data. F.M. and G.S. performed histological analysis. C.G., J.K.K., S.R., A.D., F.M., G.S., C.B., P.R., F.D., and R.R. contributed to the analysis and interpretation of the data. C.G. and R.R. wrote the manuscript. All authors critically reviewed the manuscript and approved its final version.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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**REFERENCES**


# STAR METHODS

## KEY RESOURCES TABLE

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CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Dr. Romano Regazzi (romano.regazzi@unil.ch).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Rodent

All animal procedures were performed in accordance with the NIH guidelines and protocols were approved by the Swiss Research Councils and Veterinary Offices or the Ethical Committee for Animal Experiments of the University of Groningen. Male Wistar rats (200-250g) and 4-8 weeks old female NOD/ShiLtJ mice were obtained from Charles River Laboratories (L’arbresle, France). Male C57Bl/6N mice (12-15 week-old) and 4 and 8 weeks-old female NOD scid mice (NOD.CB17-Prkdcscid/Rj) were from Janvier Labs (Le Genest St-Isle, France). miR-155 knockout mice (miR-155-/-) were from Jackson Laboratories (Jax 007745).

Human Islets

Human islet preparation were obtained from Tetu-Bio (Lot HP-18157-01, Germany) or from the Centre d’Etude Europeen pour le Diabète in Strasbourg (isolation protocol authorization for scientific research #PFS12-0013). Dissociated islet cells were cultured in CMRL medium (Invitrogen) supplemented with 10% fetal calf serum (Gibco), 10 mM HEPES, pH 7.4, 100 μg/mL streptomycin and 100 IU/mL penicillin and 2 mmol/l glutamine. Detailed information about human islet donors are provided in Table S4 and in Key Resources Table.

Cell Lines

Jurkat cells (J77 clone 20 from O. Acuto) (Rebeaud et al., 2008) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (previously depleted from serum-exosomes), 50 μg/mL streptomycin and 50 IU/mL penicillin at densities comprised between 0.2 and 1 x 10^6 cells/mL. Jurkat culture media was collected each two to three days for exosome isolation. MIN6B1 cells, a murine insulin-secreting cell line (Lilla et al., 2003), were cultured at a density of 1.5 x 10^5 cells/cm^2 in DMEM-GlutaMAX medium (Invitrogen) containing 25 mM glucose and 4 mM L-glutamine, and supplemented with 15% fetal calf serum, 70 μM β-mercaptoethanol, 50 μg/mL streptomycin and 50 IU/mL penicillin. All cell lines were cultured at 37°C in a humidified atmosphere (5% CO2, 95% air) and tested negative for mycoplasma contamination.

METHOD DETAILS

Isolation of Rodent Pancreatic Islet Cells

Rodent islets were isolated by collagenase digestion of the pancreas (Gotoh et al., 1987) followed by Histopaque density gradient to separate them from digested exocrine tissue. At the end of the isolation, islets were hand-picked and incubated overnight at 37°C in RPMI 1640- GlutaMAX medium (Invitrogen) containing 11.1 mM glucose and 2.06 mM L-glutamine and supplemented with 10% fetal calf serum (Gibco), 10 mM HEPES, pH 7.4, 1 mM sodium pyruvate, 100 μg/mL streptomycin and 100 IU/mL penicillin. Dissociated islet cells were obtained by incubating the islets in Ca^2+/Mg^2+ free phosphate buffered saline, 3 mM EGTA and 0.002% trypsin for 5 min at 37°C. For some experiments, rat islet cells were separated by Fluorescence-Activated Cell Sorting (FACS) based on β cell autofluorescence, as previously described (Jacovetti et al., 2015; Kohler et al., 2012). Sorted islet cells were seeded on plastic dishes coated with extracellular matrix secreted by 804 G rat bladder cancer cells (804 G ECM), as described elsewhere (Parnaud...
et al., 2008). Enrichment of α and β cells was evaluated by double immunofluorescence staining using polyclonal guinea pig anti-insulin (dilution 1:100, A0564, Dako) and polyclonal mouse anti-glucagon (dilution 1:1000, Abcam Ab10988) primary antibodies and goat anti-guinea pig Alexa-Fluor-488 and goat anti-mouse Alexa-Fluor-555 (diluted 1:500, Molecular Probes, Thermofisher A11073 and A21422, respectively) secondary antibodies. On average, β cell fractions contained 98.1% ± 2.2% insulin-positive cells and 1.4% ± 1.5% glucagon-positive cells and α cell-enriched fractions contained 5.2% ± 3.5% insulin-positive cells and 91.2% ± 4.3% glucagon-positive cells.

**T Lymphocyte Isolation and Culture**

Mouse T effector cells were purified from lymphoid tissues of 8 weeks-old NOD/ShiLtJ mice, or miR-155KO mice (or wild type control littermates) using anti-CD4 and anti-CD25 coated beads (Miltenyi Biotec). Human CD4+ T cells were obtained from 3 anonymous donors that gave informed consent in accordance with the US National Institutes of Health guidelines. Briefly, buffy coats were purchased from the Blood Transfusion Center in Lausanne, Switzerland. Peripheral blood mononuclear cells were obtained by density centrifugation over a Ficoll-Hypaque gradient (Amersham Bioscience) and washed three times. CD4+ T cells were enriched from purified mononuclear cells by positive selection using anti-CD4 magnetic microbeads (Miltenyi Biotec). After isolation, murine CD4+/CD25+ T cells and human CD4+ T cells were incubated in RPMI 1640-medium supplemented with 10% fetal calf serum (previously depleted from serum-exosomes), 100 μg/mL streptomycin and 100 IU/mL penicillin and were stimulated for 72 hr with 20 ng/mL IL-12 and 200 UI/mL IL-2 (R&D system Europe) and Dynabeads T-activator CD3/CD28 (bead-to-cell ratio of 1) (Invitrogen). After 3 days, media and cells were collected for exosome isolation and/or RNA extraction.

**Exosome Isolation and Quantification**

Exosomes were isolated by ultra-centrifugation as described previously (Lasser et al., 2012). Briefly, the culture media (depleted from serum-exosomes) of Jurkat cells or primary T cells were collected and centrifuged first at 300 x g for 3 min to pellet cells and then at 2,000 x g for 10 min to discard dead cells. Supernatants were centrifuged at 10,000 x g for 30 min to remove cell debris. Exosomes were then isolated from the supernatant by ultracentrifugation at 100,000 x g for 2 hr. The pellet containing the exosomes was washed with PBS and re-centrifuged at 100,000 x g for 2 hr. Exosomes were collected in a minimal volume of PBS. The same volume of PBS (without exosomes) was added to control experimental conditions. Protein concentration was determined by Bradford assay (BioRad). Recipient MIN6B1 or islet cells were incubated with 50 μg/mL of exosome preparation previously passed on a 0.45 μm filter (Figuillini et al., 2014; Guay et al., 2015). The amount of vesicles added to the recipient cells was estimated using the FluoroCet exosome quantification kit (System Biosciences), which is based on the measurement of the esterase activity associated with exosomes. According to these measurements, the recipient cells were exposed to exosome concentrations of about 0.8x10^{11}/mL for exoT, 0.7x10^{11}/mL for exoNOD and 2 x10^{11}/mL for exo-hT. These concentrations are within the same range as those previously reported in murine and human plasma samples (Matsumoto et al., 2016; Thomou et al., 2017).

**Electron Microscopy**

Suspensions were adsorbed on 200 Mesh Cupper grids coated with formvar-C for 2 min at RT. The grids were then stained using 2% phosphotungstic acid for 2 min and observed on a transmission electron microscope (Jeol 1400 JEM, Tokyo, Japan) equipped with a Gatan camera (Orius 600) and Digital Micrograph Software.

**Nanosight Analysis**

Exosome size distribution was measured by Nanoparticle Tracking Analysis using the NanoSight system (NanoSight, UK). The device measures the Brownian motion of particles whose speed of motion, or diffusion coefficient (D), is related to particle size through the Stokes-Einstein equation (Soo et al., 2012).

**Western Blot**

Proteins from exosome samples or from cellular lysates were migrated on 10% SDS-PAGE gels. Following electrophoresis, proteins were transferred onto PVDF membranes and blocked at room temperature in Tris-buffered saline/0.3% Tween20 containing 4% of BSA. The membranes were then incubated overnight at 4°C with CD81 (sc-166028), Alix (sc-49268) or TSG101 (sc-6037) antibodies (Santa Cruz Biotechnology) diluted 1/1000 in 1% BSA. The bands were detected by chemiluminescence (Pierce) after incubation with a horseradish peroxidase-conjugated secondary antibody (Bio-Rad).

**Image Stream Analysis**

Exosomes from Jurkat cells were labelled with 1μM of the lipophilic dye DiD (1,1’-dioctadecyl-3,3’,3’-tetramethylindocarbocyanine, 4-chlorobenzenesulfonate salt, Molecular Probes) during the last ultracentrifugation (2 hr at 100,000 x g). The labelled-exosome pellet was washed twice in PBS before another round of ultracentrifugation (2 hr at 100,000 g). MIN6B1 cells or dissociated rat islet cells were incubated overnight in the presence of DiD-labelled exoT, before being analyzed on Amnis ImageStream™ Mark II (Merk Millipore).
RNase Treatment
Exosome preparations were re-suspended in 300 µL PBS and spiked with 20 µmol of a synthetic oligonucleotide corresponding to the mature sequence of miR-29a (Exiqon). Samples were then divided into four aliquots and treated or not with 1% Triton X-100 (Sigma) and incubated with or without RNase A (0.5 U) and RNase T1 (15 U) (Ambion) for 30 min at 37°C before RNA extraction.

RNA Assays
RNA was extracted from exosomes, Jurkat, MIN6B1, or islet cells using the miRNeasy micro kit (Qiagen). Mature miRNA levels were measured using the miRCURY LNA Universal RT microRNA PCR kit (Exiqon) starting from 200 ng of RNA. Cellular miRNA levels were normalized to miR-7 levels, a miRNA highly expressed in β cells and not modified under the studied conditions. For miRNA measurement, RNA samples were treated with DNase prior to analysis. Profiling of miRNA was performed by Arraystar using Agilent Technologies and Agilent GeneSpring GX v12.1 software for data analysis. Gene expression levels were measured by qPCR using miScript II RT and SYBR Green PCR kits (Qiagen) and results were normalized to 18s rRNA and Hprt. Finally, GFP levels were assessed using SYBR Green PCR Master Mix (Life Technologies) with primers specific for GFP and normalized to Gapdh levels. Primer sequences are provided in Table S5. All qPCR measurements were performed in triplicates and expression levels of miRNAs and mRNAs were analyzed using the 2^(-ΔΔCt) method.

Transfection of Insulin-Secreting Cells
MIN6B1 or dispersed rat islet cells were transfected using Lipofectamine 2000 (Invitrogen) with RNA oligonucleotide duplexes (Exiqon) corresponding to the mature miRNA sequence (overexpression) or with single-stranded miRCURY LNA inhibitors (Exiqon) that specifically block the miRNAs (down-regulation). A control miRNA mimic or the control miRCURY LNA inhibitor (Exiqon) were used as negative controls for miRNA overexpression and down-regulation, respectively.

Insulin Secretion and Content
After incubation for 72 hr in the presence of exosomes (or control PBS), MIN6B1 or dissociated rat islet cells were pre-incubated for 30 min at 37°C in Krebs-Ringer bicarbonate buffer (KRBH) containing 25 mM HEPES, pH 7.4, 0.1% BSA (Sigma-Aldrich) and 2 mM glucose. The cells were then incubated for 45 min in KRBH containing 0.5% BSA and 2 or 20 mM glucose. At the end of the incubation period, media were collected and total cellular insulin contents recovered in acid-ethanol (0.2 mM HCl in 75% ethanol). Insulin levels were measured using an insulin enzyme immunoassay kit (SPI-Bio). All experiments were performed in triplicates.

Cell Death Assays
MIN6B1 and dissociated islet cells were incubated for 72 hr in presence of T cell exosomes with or without 0.1 mM of the pan caspase inhibitor Z-VAD-FMK (R&D systems). At the end of the incubation, 1 µg/mL Hoechst 33342 (Invitrogen) was added for 1-2 min. Cells displaying pycnotic nuclei were scored under fluorescence microscopy (AxioCam MRc5, Zeiss). A minimum of thousand cells were counted per condition. TUNEL staining on human β cells was performed 48 hr after exposure to exo-hT using the In Situ Cell Death Detection Kit (Roche) combined to FLEX polyclonal guinea pig anti-insulin (dilution 1:10, IR00261-2, Dako) followed by incubation with fluorescently labeled goat anti-guinea-pig AlexaFluor 555 antibody (dilution 1:400, A21435, Thermofisher). NFκB translocation was assessed in MIN6B1 cells after exposure for 48 hr to exoT or for 24 hr to pro-inflammatory cytokines (30 ng/mL INFγ, 10 ng/mL TNFα and 0.1 ng/mL IL-1β). Briefly, MIN6B1 cells cultured on coverslips were fixed, permeabilized, and incubated with a mouse anti-NFκB p65 antibody (dilution 1:100, sc-372, Santa Cruz Biotechnology) followed by goat anti-mouse Alexa Fluor 488 antibody (A-11029, Invitrogen). Cell nuclei were stained with Hoechst 33342 (1 µg/mL, Invitrogen). Coverslips were mounted on microscope glass slides with Fluor-Save mounting medium (VWR International SA) and were visualized with a Zeiss Axiocvision fluorescence microscope. Experiments were performed in single replicates.

Luciferase
MIN6B1 cells were transfected using Lipofectamine 2000 (Invitrogen) with a pGL3 plasmid containing the hsa-miR-155 binding sequence in the 3’ UTR (Skalsky et al., 2007) and the psicheck1 vector (Promega). After 7 hr, culture media of transfected MIN6B1 was changed and exoT was added for 48 hr or 72 hr. Luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity to minimize experimental variabilities. Experiments were performed in triplicates.

Generation of Viral Vectors
The dsAAV-RIP-GFP (AAV-control) was constructed on the backbone of the dsAAV8 plasmid (Nathwani et al., 2006) by replacing the original LP1 promoter and FIX coding sequences with two multiple cloning sites and inserting the coding sequences for the Rat Insulin promotor (RIP) (Addgene plasmid 15029) and green fluorescent protein (GFP) into the multiple cloning site, using the PstI-AccIII and EcoRI-Notl restriction sites. For the construction of the dsAAV-RIP-GFP-sponge (AAV-sponge), the interrupted palindromic SanDI (Fermentas) restriction site was used to insert miRNA binding sites for miR-142-3p/-5p, miR-150 and miR-155 (Table S6) (Kluiver et al., 2012). Viral particles were generated by a triple transfection of HEK293 cells using the 25 kDa linear polyethyleneimine (Polysciences Inc) transfection method (Reed et al., 2006). AAV particles were purified by iodixanol gradient centrifugation as previously described (Zolotukhin et al., 1999). Viral titers were determined by qPCR with specific primers for the RIP and GFP.
In Vivo Experiments
Female NOD/ShiLtJ mice were injected intraperitoneally (i.p.) with 150 μL PBS containing 1 x 10^{12} viral genome particles at the age of 4 weeks. Blood glucose levels were measured once per week on restrained, un-anesthetized animals through tail vein bleeds with a glucometer and test strips (Life Scan). NOD mice were diagnosed as diabetic if blood glucose levels above 14 mM on 2 consecutive days. Glucose tolerance test was performed by injecting i.p. 1.5 g glucose per kg of body weight on 4 hr fasted NOD mice of 10 weeks of age, from the diabetes incidence cohort. Experiments were not performed blinded. One mouse from the AAV-ctl group was excluded and euthanized at 13 days after injection as it met ethical endpoints (loss of more than 15% of body weight after injection and showed signs of discomfort).

Histology and Immunohistochemistry
Formalin-fixed pancreatic tissues were embedded in paraffin using standard techniques. 4-μm sections were deparaffinized, rehydrated, and incubated with blocking solution. Sections were incubated overnight at 4°C with antibodies against GFP (Life Technologies), followed by signal amplification using the TSA Fluorescein system (Perkin Elmer) or with CD3 antibody (dilution 1:500, A0452, Dako) followed by the fluorescein-labeled secondary antibody goat anti-rabbit AlexaFluor-555 (dilution 1:200, A21428, Molecular Probes, Thermofisher).

Insulitis score was evaluated by Hematoxylin-Eosin staining of formalin-fixed paraffin embedded pancreata sections. A score ranging from 0 to 3 was assigned to each identified islet by manual bright field microscope visualization (0: intact islet; 1: peri-insulitic islet; 2: <50% infiltrated islet area; 3: >50% infiltrated islet area) (Figure S8).

Immunofluorescence analysis was performed to quantify insulin, glucagon and Cxcl10 positive volume. 5 μm formalin-fixed paraffin embedded pancreata sections were subjected to double or triple immunofluorescence staining using respectively polyclonal guinea pig anti-insulin (dilution 1:500, A0564, Dako), monoclonal mouse anti-glucagon (dilution 1:300, MAB1249, R&D Systems) and polyclonal rabbit anti-murine CXCL10 (dilution 1:25, 500-P129, Peprotech); fluorescently labeled secondary antibodies were goat anti-guinea pig Alexa-Fluor 488 (dilution 1:500, A11073, Molecular Probes, Thermofisher), goat anti-mouse 647 (dilution 1:500, A21236, Molecular Probes, Thermofisher) or goat anti-rabbit AlexaFluor 594 conjugated (dilution 1:500, A11037, Molecular Probes, Thermofisher). Stained sections were imaged and analyzed using Leica Sp5 confocal microscopy. Five different focal planes for each identified islet were scanned at 40X magnification. The analysis of positive insulin and CXCL10 volume (μm^{3}) and CXCL10-insulin and CXCL10-glucagon colocalization rate (%) was quantified using Velocity 6.3 software (Perkin Elmer) by measuring the quantity of insulin and CXCL10 positive fluorescent voxels signal in five different focal planes of each scanned islet and by measuring the ratio between double positive voxels CXCL10-insulin and CXCL10-glucagon on total insulin and glucagon positive voxels for each scanned islet.

Immunofluorescence analysis to quantify insulin, glucagon and FoxP3+ cells, was performed by triple immunofluorescence staining with polyclonal guinea pig anti-insulin (dilution 1:500, A0564, Dako), monoclonal mouse anti-glucagon (dilution 1:300, MAB1249, R&D Systems) and rat monoclonal anti-murine FoxP3, FITC conjugated (dilution 1:100, 11-5773-82, eBioscience); fluorescent labeled secondary antibodies goat anti-guinea pig Alexa Fluor 405 conjugated (dilution 1:500, Ab175678, Abcam), goat anti-mouse 594 conjugated (dilution 1:500, A11032, Molecular Probes, Thermofisher), Rabbit anti-fluorescein 488 conjugated (dilution 1:80, A11090, Molecular Probes, Thermofisher) and tertiary antibody goat anti-rabbit 488 conjugated (dilution 1:200, A11034, Molecular Probes, Thermofisher), were used. Stained sections were imaged and analyzed using Leica Sp5 confocal microscopy as described above and FoxP3+ cells were quantified through manual cell count.

QUANTIFICATION AND STATISTICAL ANALYSIS
Data are expressed as mean ± SD. Statistical significance was determined using parametric unpaired two-tailed Student’s t test or, for multiple comparisons, with one-way analysis of variance (ANOVA) of the means, followed by post-hoc Dunnett or Tukey test, or unparametric Mann-Whitney U test (Graph Pad Prism6). The percentage of nondiabetic mice was calculated by Kaplan–Meier survival analysis and were compared using the Log rank (Mantel-Cox) test. p values less than 0.05 (p < 0.05) were considered statistically significant.

DATA AND SOFTWARE AVAILABILITY
The accession number for the genomic data reported in this paper is GEO: GSE92439.