11-1-2010

Adjuvant immunotherapy increases β cell regenerative factor Reg2 in the pancreas of diabetic mice

Katrina Huszarik
Western University

Benjamin Wright
Western University

Christina Keller
Western University

Enayat Nikoopour
Western University

Olga Krougly
Western University

See next page for additional authors

The Journal of Immunology

Adjuvant Immunotherapy Increases β Cell Regenerative Factor *Reg2* in the Pancreas of Diabetic Mice

Katrina Huszarik, Benjamin Wright, Christina Keller, Enayat Nikoopour, Olga Krougly, Edwin Lee-Chan, Hui-Yu Qin, Mark J. Cameron, Werner K. Gurr, David J. Hill, Robert S. Sherwin, David J. Kelvin and Bhagirath Singh

J Immunol 2010; 185:5120-5129; Prepublished online 27 September 2010;
doi: 10.4049/jimmunol.1001596
http://www.jimmunol.org/content/185/9/5120

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Adjuvant Immunotherapy Increases β Cell Regenerative Factor *Reg2* in the Pancreas of Diabetic Mice

Katrina Huszarik,[*,†] Benjamin Wright,[*,†] Christina Keller,[*,†] Enayat Nikoopour,[*,†] Olga Krougly,[*,†] Edwin Lee-Chan,[*,†] Hui-Yu Qin,[*,†] Mark J. Cameron,[‡,§] Werner K. Gurr,[¶] David J. Hill,[‖,#,,††] Robert S. Sherwin,[¶] David J. Kelvin,[‡,§] and Bhagirath Singh[*,†]**

Insulin-producing β cells can partially regenerate in adult pancreatic tissues, both in human and animal models of type 1 diabetes (T1D). Previous studies have shown that treatment with mycobacterial adjuvants such as CFA and bacillus Calmette-Guérin prevents induction and recurrence of T1D in NOD mice with partial recovery of β cell mass. In this study, we investigated factors involved in the regeneration of β cells in the pancreas of NOD mice during diabetes development and after treatment with adjuvants. The Regeneration (*Reg*) gene family is known to be involved in regeneration of various tissues including β cells. *Reg2* expression was found to be upregulated in pancreatic islets both during diabetes development and as a result of adjuvant treatment in diabetic NOD mice and in C57BL/6 mice made diabetic by streptozotocin treatment. The upregulation of *Reg2* by adjuvant treatment was independent of signaling through MyD88 and IL-6 because it was not altered in MyD88 or IL-6 knockout mice. We also observed upregulation of *Reg2* in the pancreas of diabetic mice undergoing β cell regenerative therapy with exendin-4 or with islet neogenesis-associated protein. *Reg2* expression following adjuvant treatment correlated with a reduction in insulitis, an increase in insulin secretion, and an increase in the number of small islets in the pancreas of diabetic NOD mice and with improved glucose tolerance tests in streptozotocin-treated diabetic C57BL/6 mice. In conclusion, adjuvant immunotherapy regulates T1D in diabetic mice and induces *Reg2*-mediated regeneration of β cells. ***The Journal of Immunology*****, 2010, 185: 5120–5129.**

Type 1 diabetes (T1D) is an autoimmune disease characterized by destruction of the insulin-producing β cells in the pancreatic islets. Islet transplantation has been experimentally used for β cell replacement. An attractive alternative is exploring the endogenous production of β cells through stimulation of the regenerative capacities of the pancreas. Indeed, β cell regeneration has been shown to occur at a basal rate in normal adult tissues and to increase under conditions of metabolic stress such as pregnancy, obesity, and diabetes (1). In addition, injection of metabolically active peptides such as glucagon-like peptide-1 and islet neogenesis-associated protein (INGAP) peptide have been shown to increase β cell regeneration (2–4). The physiological mechanism of endogenous β cell regeneration is controversial and is thought to occur either by production of new β cells through the process of neogenesis (5) or by replication of existing β cells (6). A recent study suggests that β cells in the pancreas can regenerate by direct reprogramming of α cells (7).

In the NOD mouse model of T1D, insulitis develops at ~4 wk of age, and the onset of β cell destruction occurs shortly after. This destruction leads to a significant decrease in β cell mass by 8 wk of age, which is accompanied by an approximate 6-fold increase in β cell replication rates, suggesting that there is a compensatory regenerative effort in response to β cell destruction (8). Despite this, in T1D, β cell mass continues to be reduced because the endogenous regenerative response is overwhelmed by ongoing autoimmunity. Understanding the molecular mechanisms involved in this regenerative process is important for the development of novel β cell regenerative therapies.

The Regeneration (*Reg*) genes encode a family of conserved proteins that are members of the C-type lectin superfamily and are found in a number of animal species (9). They were originally identified in a cDNA library from rat regenerating islets (10) and are closely associated with regeneration in a number of tissues and in different disease models (11). There are seven types of *Reg* proteins in mouse and five types of *Reg* proteins in humans and rats (9), which have been categorized into four subfamilies. The *Reg2* subfamily has only been found to exist in mice and hamsters; however, it has now been categorized as being a member of the *Reg1* subfamily because of amino acid sequence homology (76% between mouse *Reg*1 and mouse *Reg2*) (9, 12). Of the *Reg* proteins, *Reg1* and INGAP have been definitively shown to play a role in β cell regeneration (13–15).

Pancreatic overexpression of the *Reg2* gene (and to a lesser degree of the *Reg1* gene) has been found in female NOD mice, which develop diabetes early in life, whereas NOD males, which are relatively protected, have a low mRNA level similar to the level found in a control mouse strain (16, 17). The specificity of

*Department of Microbiology and Immunology, Center for Human Immunology, [†]Robarts Research Institute, [‖]Lawson Health Research Institute, [#]Department of Medicine, **Department of Physiology and Pharmacology, and [††]Department of Pediatrics, University of Western Ontario, London; [‡]Division of Experimental Therapeutics, University Health Network and [§]Department of Immunology, University of Toronto, Toronto, Ontario, Canada; and [¶]Section of Endocrinology, Department of Internal Medicine, Yale University School of Medicine, New Haven, CT 06520

Received for publication May 13, 2010. Accepted for publication August 20, 2010.

This work was supported by grants from the Canadian Institutes of Health Research.

Address correspondence and reprint requests to Dr. Bhagirath Singh, Department of Microbiology and Immunology, University of Western Ontario, London, Ontario N6A 5C1, Canada. E-mail address: bsingh@uwo.ca

Abbreviations used in this paper: B6, C57BL/6; C, CFA treated; INGAP, islet neogenesis-associated protein; qRT-PCR, quantitative RT-PCR; *Reg*, Regeneration; S, saline treated; STZ, streptozotocin; T1D, type 1 diabetes; WT, wild-type.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1001596

the overexpression of *Reg2* in the diabetogenic process was reinforced by the fact that it was also observed in NOD male mice after treatment with cyclophosphamide, whereas this drug has no effect in male control mice. Other stimuli for *Reg*2 expression related to T1D are diet (18), type 1 IFN (19), and insulin-like growth factor-1 (20) among others.

Previously we showed that a single injection of the *Mycobacterium*-containing adjuvant CFA prevents the development of diabetes in NOD mice by downregulating autoimmunity (21, 22). Following studies by Faustman et al. (23, 24), several groups reported that CFA treatment restores normoglycemia in a small percentage of end-stage diabetic NOD mice (25–27). This was demonstrated by the reappearance of pancreatic β cells as observed by histological analysis of the islet tissue.

The precise role that CFA plays in the regeneration of β cell mass is unclear. Immunomodulation by itself is not enough to promote β cell regeneration in diabetic mice because immunotherapy using anti-CD3 mAb was not accompanied by regeneration of β cell mass (28, 29). Okamoto et al. (30) proposed that inflammatory stimuli cause upregulation of *Reg* gene family proteins within the islets. These proteins are secreted from the β cells and can act through their cognate receptors to stimulate β cell replication in an autocrine and/or paracrine manner. This led us to hypothesize that CFA treatment could stimulate β cell regeneration in diabetic NOD mice not only through prevention of autoimmunity but also by upregulating *Reg* genes involved in endogenous β cell regeneration, such as *Reg2* and *Reg3β*. In this study, we show that CFA treatment does indeed cause a significant upregulation of *Reg2* expression in both diabetic and nondiabetic animals. This effect is independent of signaling through the TLR adaptor molecule MyD88 or through IL-6, which has previously been linked to the regulation of *Reg* gene expression. We also show that *Reg*2 is upregulated following treatment with the established β cell regenerative agents INGAP peptide and the glucagon-like peptide-1 analog exendin-4. The increased *Reg*2 expression was accompanied by partial reversal of insulitis, an increased insulin production, and an increase in the number of islets in the pancreas of diabetic mice. Therefore, adjuvant immunotherapy regulates T1D in diabetic mice and induces *Reg2*-mediated regeneration of β cells.

Materials and Methods

Animals

Female NOD/Ltj and NOD.Scid mice were bred and maintained in the specific pathogen-free facility at the University of Western Ontario (London, Ontario, Canada). Female C57BL/6 (B6) and IL-6 knockout B6 (B6 IL-$6^{-/-}$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). MyD88 knockout B6 (B6.MyD$88^{-/-}$) mice were provided by Dr. R. Flavell from Yale University (New Haven, CT). Mice were maintained in the specific pathogen-free facility at the University of Western Ontario. Mice were used at 4–8 wk of age, unless otherwise stated. All experiments were performed in accordance with the Canadian Council for Animal Care guidelines.

Glucose monitoring

Mice were monitored for diabetes development and reversal by urine glucose with Diastix strips (Bayer, Elkhart, IN) and by blood glucose analysis using a glucose meter (Bayer). Mice were recorded as diabetic based on two consecutive positive urine glucose (>11.5 mmol/l) or blood glucose (>16 mmol/l) results.

Treatments

Streptozotocin (STZ) (Sigma-Aldrich, St. Louis, MO) was reconstituted in PBS and injected i.p. at a concentration of 200 mg/kg. Mice were fasted for 6–8 h prior to STZ treatment. Mice were injected i.p. or in hind footpad with 50 or 100 μl CFA or IFA (Sigma-Aldrich) emulsified in saline (1:1) or with saline alone. Recombinant exendin-4 (Cedarlane Laboratories, Hornby, Ontario, Canada) was reconstituted in saline (8 μg/ml), and 100 μl was injected i.p. daily for 3 consecutive days.

Islet isolation and culture

Islets from NOD mice were isolated and handpicked as before (31). Islets were incubated with INGAP peptide, IGLHDPSHGTLPNGS (5 μg/ml) in DMEM, or in DMEM only (Invitrogen Life Technologies, Carlsbad, CA). The DMEM was supplemented with 5 μg/ml penicillin, 100 U/ml streptomycin (Invitrogen Life Technologies), and 10% (v/v) FCS at 37°C. Peptides were synthesized and purified in our laboratory as described previously (32).

RNA extraction

For whole pancreatic RNA, 50 mg tissue from NOD mice was homogenized in buffer RLT (Qiagen, Mississauga, Ontario, Canada) containing 2-ME (Sigma-Aldrich) using PowerGen 700 homogenizer (Fisher Scientific, Pittsburgh, PA). For RNA from islets, homogenization through a Qiashredder column (Qiagen) was performed. Total RNA was extracted from homogenates using RNeasy Midi kit for whole tissue or RNeasy Mini kit for islets (Qiagen), and contaminating DNA was removed using the DNase 1 treatment kit from Ambion (Austin, TX). The RNA was quantified by measuring absorbance at 260 nm using a Nanodrop 1000 spectrophotometer (NanoDrop Products, Wilmington, DE).

End-point RT-PCR

Five micrograms of total RNA from individual samples was used to synthesize cDNA using oligo $dT_{12–18}$ primer and SuperScript II reverse transcriptase (Invitrogen Life Technologies) according to the protocol. Complimentary DNA was amplified by PCR using the SuperScript first-strand synthesis system with gene-specific primers (Sigma-Aldrich) as listed in Table I. Amplification was performed in the GeneAmp PCR System 2400 thermocycler (Applied Biosystems, Foster City, CA). PCR products were separated on a 1.5% ethidium bromide containing agarose gel by electrophoresis and were visualized under UV light using a gel documentation system (Bio-Rad, Hercules, CA). Band intensities were quantified using SigmaGelTM^{gel} analysis software (Jandel Scientific, San Rafael, CA).

Real-time quantitative RT-PCR

One to 2 μg total RNA from each sample was reverse transcribed into cDNA using oligo $dT_{12–18}$ primers from Superscript III first-strand Synthesis SuperMix for quantitative RT-PCR (qRT-PCR) (Invitrogen Life Technologies). Resultant cDNA was diluted to 225 ng/μl in diethyl pyrocarbonate water and PCR amplified using Quantifast SYBR Green PCR Kit (Qiagen). Gene-specific primers (95–100% efficient; Sigma-Aldrich) (Table I) were used at a concentration of 1.25 μM. Amplification was performed in a Corbett Rotor-Gene 6000 thermocycler (Corbett Life Sciences, San Francisco, CA). A two-step melting/annealing program was used with 40 cycles of amplification. The efficiency of each set of primers was determined by the standard curve method, and the Pfaffl method (33) was subsequently used for quantification of cycle threshold values using β-actin as the housekeeping gene.

Western blot analysis

Ten milligrams of whole pancreatic tissue was snap frozen and homogenized in lysis buffer (50 mM Tris-HCl [pH 7.4], 300 mM NaCl, 5 nM EDTA, 0.02% [w/v] sodium azide, 1% Triton X-100, and complete protease inhibitors [Roche Applied Science, Laval, Quebec, Canada]) using PowerGen 700 homogenizer. Total protein (10–50 μg) from each sample was separated by nonreducing SDS-PAGE and transferred onto a nitrocellulose membrane using a TransBlot semidry Western blotting apparatus (Bio-Rad). Membranes were blocked with skim milk and incubated overnight at 4°C with monoclonal rat anti-mouse *Reg2* (R&D Systems, Minneapolis, MN) and polyclonal goat anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA) at 1 μg/ml. The secondary Abs HRP-labeled goat anti-rat IgG and HRP-labeled donkey anti-goat IgG (R&D Systems) were used at 1/1000 dilution. Blots were developed using chemiluminescent reagent (GE Healthcare, Buckinghamshire, U.K.) and a transillumination apparatus (Alpha Innotech, Johannesburg, South Africa).

Histology and immunohistochemistry

Whole pancreatic tissue was fixed for 24 h in 10% neutral-buffered formalin (EMD, Darmstadt, Germany), embedded in paraffin, cut (5–6 μm), and mounted on microscope slides. Tissue was stained with H&E, and insulitis was scored as described previously (31).

For immunohistochemical staining, Ag retrieval was performed using sodium citrate buffer, endogenous peroxidase activity was quenched by H_2O_2, and nonspecific binding was blocked with 10% goat normal serum (Invitrogen Life Technologies). After staining, islets were photographed using a QImaging camera (QImaging, Surrey, British Columbia, Canada),

an Axioskop 2 microscope (Carl Zeiss, Thornwood, NY), and Northern Eclipse imaging software.

For light microscopy imaging of insulin, sections were incubated with chicken polyclonal anti-insulin primary Ab (1/200 dilution; Abcam, Cambridge, MA), followed by biotin-labeled goat anti-chicken IgG secondary Ab (1/500 dilution; Molecular Probes, Eugene, OR) and the Vectastain elite avidin/biotin complex immunoperoxidase system (Vector Laboratories, Burlingame, CA). Diaminobenzidine substrate was used for visualizing Abs.

For fluorescent microscopy imaging of insulin and *Reg2*, sections were incubated with rabbit polyclonal anti-mouse *Reg2* Ab (1/40 dilution) (34) or chicken polyclonal anti-insulin Ab (1/200 dilution). The sections were fluorescently labeled with Alexa Fluor 594 (red)-conjugated goat anti-rabbit IgG Ab or Alexa Fluor 488 (green)-conjugated goat anti-chicken IgG (Molecular Probes) at 1/500 dilution. Nuclei were stained using blue fluorescent Hoechst Stain (Molecular Probes).

For the analysis of pancreatic tissue from diabetic NOD mice that reverted from diabetes following CFA treatment, the number of islets per tissue section from reverted mice was compared with the number of islets per section from untreated diabetic mice. The individual islets were sorted into groups based on diameter, and the average numbers of islets per tissue section were scored.

Intraperitoneal glucose tolerance test

Mice were fasted for 8–10 h, fasting blood glucose levels were measured, and mice were injected i.p. with 10 μl/g body weight of 100 mg/ml D-glucose (BDH, Toronto, Ontario, Canada). Blood glucose was measured at 15, 30, 60, and 120 min after injections.

Statistical analysis

All data p values were determined using the unpaired Student t test with Microsoft Excel software, and the values of $p < 0.05$ were considered significant.

Results

Reg *family genes are upregulated in NOD mice during diabetes development*

An increase in the replication rate of β cells during diabetes development in NOD mice has been reported previously (8). Generally, NOD mice do not begin to develop insulitis and subsequently diabetes until after 6–8 wk of age. Therefore, expression of regenerative genes in 4-wk-old mice was defined as the baseline level to compare with expression in 8- and 12-wk-old mice because there is known to be a significant reduction in β cell mass and increase in β cell replication in NOD mice at these ages (8). As shown in Fig. 1, we found the expression of two members of the *Reg* gene family, *Reg2* and *Reg3β*, was significantly upregulated in female NOD mice at 8 and/or 12 wk of age. The semiquantitative RT-PCR data in Fig. 1 confirmed an age-related increase in *Reg2* and *Reg3β* gene expression in NOD mice. See Table I for a list of primer sequences used for PCR.

Reg2 *expression is upregulated in NOD mice following CFA treatment*

It has been shown that CFA injection in diabetic NOD mice results in the regeneration of β cell mass (25–27, 35). We hypothesized that CFA upregulates the transcription of genes involved in endogenous β cell regeneration. To test this hypothesis, NOD mice were injected with CFA, and expression of genes associated with embryonic β cell development including *pax*4, *pax*6, and *pdx*1 as well as several members of the *Reg* gene family including *Reg1* and *Reg2* was monitored in an age-dependent manner. Only the expression of *Reg2* was elevated in the CFA-treated group at all time points investigated (Fig. 2*A*). We also examined the expression of regenerative and developmental genes in the pancreas of diabetic mice and again found that *Reg2* was the only gene that was upregulated after a single injection of CFA (Fig. 2*B*).

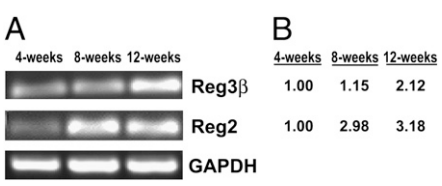

FIGURE 1. Upregulation of *Reg* genes in pancreas of NOD mice with age. RT-PCR was performed on total RNA extracted from the pancreas of 4-, 8-, and 12-wk-old female NOD mice. Expression of GAPDH housekeeping gene is shown as a loading control. *A*, PCR products were separated on a 1.5% agarose gel containing ethidium bromide for band visualization. *B*, Quantification of band intensities adjacent on the left, using SigmaGelTM[gel] analysis. Values indicate fold change compared with the 4-wk-old sample.

Quantitative real-time PCR analysis of *Reg* gene expression in the pancreas of NOD mice following CFA treatment was performed to quantify changes in regenerative gene expression. *Reg2* expression was increased ~100-fold in saline-treated diabetic mice compared with saline-treated 4-wk-old mice, confirming that there is an age-related increase in *Reg2* expression in NOD mice (Fig. 3*A*). A small increase in expression of the *Reg1* and *Reg3δ* (INGAP) genes with age was also observed (Fig. 3*A–C*). *Reg2* expression was significantly upregulated following CFA treatment in young nondiabetic (4 wk old), prediabetic (8 wk old), and diabetic NOD mice (Fig. 3*A*). The change in expression compared with controls was ~30-fold in young mice compared with only ~1.5-fold in diabetic mice, which is most likely because of the increase in basal *Reg2* expression that occurs as mice age.

Reg2 gene expression was the highest in the CFA-treated diabetic group being ~4-fold greater than the CFA-treated 4-wk-old group (Fig. 3*A*). An increase in *Reg2* gene expression occurred as early as 2 d following CFA treatment in diabetic mice, and expression decreased with time postinjection but remained greater than the level of expression in the control mice at 1 mo post-treatment (Fig. 3*D*).

Similar to the pattern of gene expression, *Reg2* protein expression was present in the pancreas of 4-wk-old mice 24 h after CFA treatment, whereas expression was absent in 4-wk-old saline-treated mice (Fig. 3*E*). *Reg2* protein was also expressed in the pancreas of diabetic mice 24 h after CFA treatment, whereas there was only slight expression of the protein in saline-treated diabetic mice (Fig. 3*F*).

Reg2 *expression is localized to the islets following CFA treatment*

To determine whether Reg2 upregulation following CFA treatment occurred specifically in pancreatic tissue, Reg2 expression in the adjacent splenic tissue was analyzed by end-point PCR. No Reg2 expression could be detected in the spleen of CFA- or saline-treated mice (data not shown). Previous studies have shown that Reg2 expression is localized to islet β cells in NOD mice during diabetes development (34). We found that there was an approximate 8-fold increase in Reg2 expression specifically within the islet tissue following CFA treatment in 4-wk-old mice (Fig. 4*A*). We also observed Reg2 protein expression within the islets of immunostained tissue sections from CFA-treated NOD mice at different times postinjection (representative image shown in Fig. 4*B*). Reg2 expression was localized to the cytoplasm and occurred mainly within the β cell area as seen by merged images of sections that were double immunostained for insulin and Reg2 (Fig. 4*B*).

Reg2 *expression is upregulated in B6 mice following STZ and/or CFA treatment*

To better clarify the role of *Reg2* upregulation in islet regeneration and to confirm results found in the NOD mouse, *Reg2* expression was analyzed in an STZ-induced model of diabetes. In this model, a single high-dose injection (200 mg/kg) of STZ was administered to B6 mice to induce diabetes. At this high dose of STZ, diabetes develops mainly by direct toxic effects on β cells (36, 37).

Table I. *List of genes with primer sequences used in RT-PCR and qRT-PCR*

	Gene Symbol	Forward Primer (5′→3′)	Reverse Primer (5′→3′)	Product Size (bp)
RT-PCR	*Pdx1*	cagtgaggagcagtactac	gatgtgtctctcggtaagttc	569
	Pax4	gctctccgttttcagtttgc	ctttagctgggcaattcgag	613
	Pax6	tctaatcgaagggccaaatg	cataactccgcccattcact	343
	Reg1	catctgccaggatcagttgc	aggtaccataggacag	549
	Reg2	gatcagcatggctcagaaca	gtgccaacgacggttacttt	364
	Reg3β	cagacctggtttgatgcaag	aattcgggatgtttgctgtc	382
	β-Actin	atccgtaaagacctctatgc	aacgcagctcagtaacagtc	286
	GAPDH	atcactgccacccagaagac	ccctgttgctgtagccgtat	430
qRT-PCR	*Reg1*	gatcagttgcccagaaggtt	ctgacaccaggtagcctgaa	124
	Reg2	cactgccaaccgtggttat	gacaaaggagtactgtgcctca	75
	Reg3δ (INGAP)	ccatggtgtctcacaagacc	tgatgcgtggagaagacagt	117
	IL-6	gaggataccactcccaacaga	aagtgcatcatcgttgttcat	140
	β-Actin	gcccagagcaagagaggtat	cacacgcagctcattgtaga	116

We found that treatment with high-dose STZ resulted in a significant increase in pancreatic *Reg2* expression within 24 h following injection ($p < 0.01$) (Fig. 5). Injection of CFA 1 d after STZ treatment significantly increased *Reg2* expression in high-dose STZ-treated groups ($p < 0.02$) (Fig. 5). The highest expression of *Reg2* occurred in the high-dose STZ plus CFA treatment group, which showed a 23-fold greater expression of *Reg2* than the PBS plus CFA treatment group (Fig. 5).

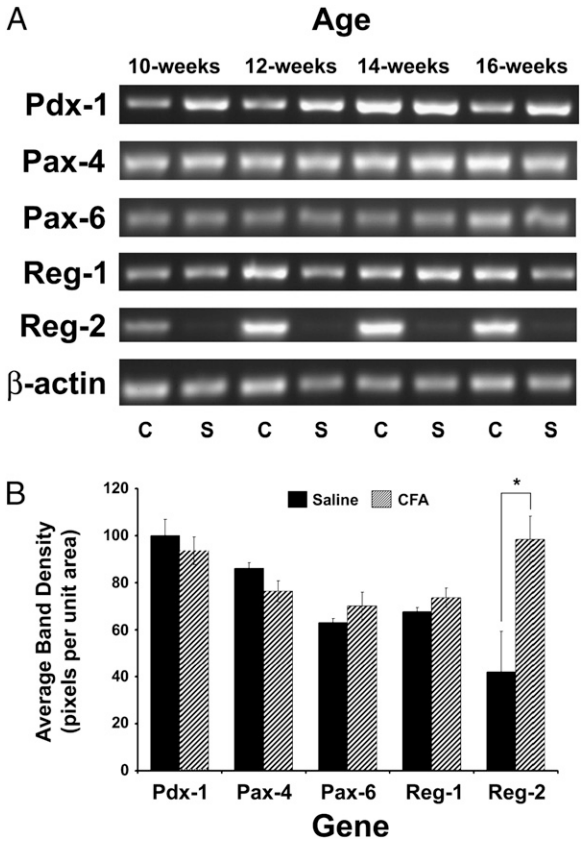

FIGURE 2. Expression of β cell developmental and regenerative genes in the pancreas of prediabetic and diabetic NOD mice following CFA treatment. RT-PCR was performed on RNA from whole pancreatic tissue from female NOD mice that were injected i.p. with 100 μl CFA emulsified in saline or saline alone. *A*, Eight-week-old female mice were injected with CFA or saline, and RT-PCR was performed on pooled RNA from two mice per group every 2 wk after injection. *B*, Gene expression in diabetic mice was analyzed 1 wk after CFA or saline injection. Quantification of band intensities was performed using SigmaGelTM[gel] analysis software and normalized to β-actin expression. Results are shown as the average band density ± SEM ($n = 5$) per group. $*p < 0.05$. Note that the differences in expression between groups cannot be compared directly because the relationship between band density and gene expression is not linear. C, CFA treated; S, saline treated.

CFA upregulation of Reg2 *expression is not MyD88 dependent*

Mycobacterial adjuvants such as CFA are known to stimulate innate immune responses through activation of a number of cell surface receptors including members of the TLR family. MyD88 is an adaptor molecule that is involved in downstream signaling through all TLRs, except for TLR3 (38). To determine whether innate immune responses that signal through MyD88 are required for *Reg2* upregulation after CFA treatment, *Reg2* expression was analyzed in B6 knocked out MyD88 mice (MyD88$^{-/-}$). In MyD88$^{-/-}$ mice, *Reg2* expression was significantly upregulated after CFA treatment compared with mice that were treated with saline, and the magnitude of the increase was similar to that seen in wild-type mice. This suggests that there is no defect in *Reg2* upregulation after CFA treatment in the MyD88$^{-/-}$ mice (Fig. 6) and that other significant pathways may be involved in the upregulation of *Reg*2 by CFA.

CFA upregulation of Reg2 *expression is not IL-6 dependent*

Adjuvants including CFA have been shown previously to upregulate inflammatory cytokines such as IL-6 in the spleen and other lymphoid tissues (39). A similar upregulation of IL-6 gene expression within the pancreas of young NOD mice was found in pancreatic tissue from CFA versus saline groups 2 d after treatment (Fig. 7*A*). The *Reg* genes contain an upstream response element for the inflammatory cytokine IL-6 and are responsive to IL-6 in vitro. We found that in islets from NOD mice incubated with IL-6 in vitro, the expression of *Reg2* was upregulated ~3.5-fold compared with media (Fig. 7*B*). In comparison, there was a 1.5 fold upregulation of *Reg1* expression in islets after treatment with IL-6 (Fig. 7*B*). This led us to hypothesize that IL-6 induced by adjuvant therapy causes upregulation of *Reg2* expression in the pancreas in vivo by binding its cognate response element upstream of the *Reg2* gene. To test this hypothesis, we immunized IL-6 knockout mice with CFA and analyzed their pancreatic tissue for *Reg2* expression. However, we found that *Reg2* expression was significantly upregulated even in IL-6 knockout mice, and this upregulation was similar to that seen for wild-type mice (Fig. 7*C*). This indicates that *Reg2* upregulation is independent of IL-6.

Reg2 *expression is upregulated following treatment with INGAP peptide*

INGAP peptide induces upregulation of a number of cell cycle-associated genes in neonatal rat islets when cultured in vitro (40), and it has been previously used to stimulate β cell regeneration

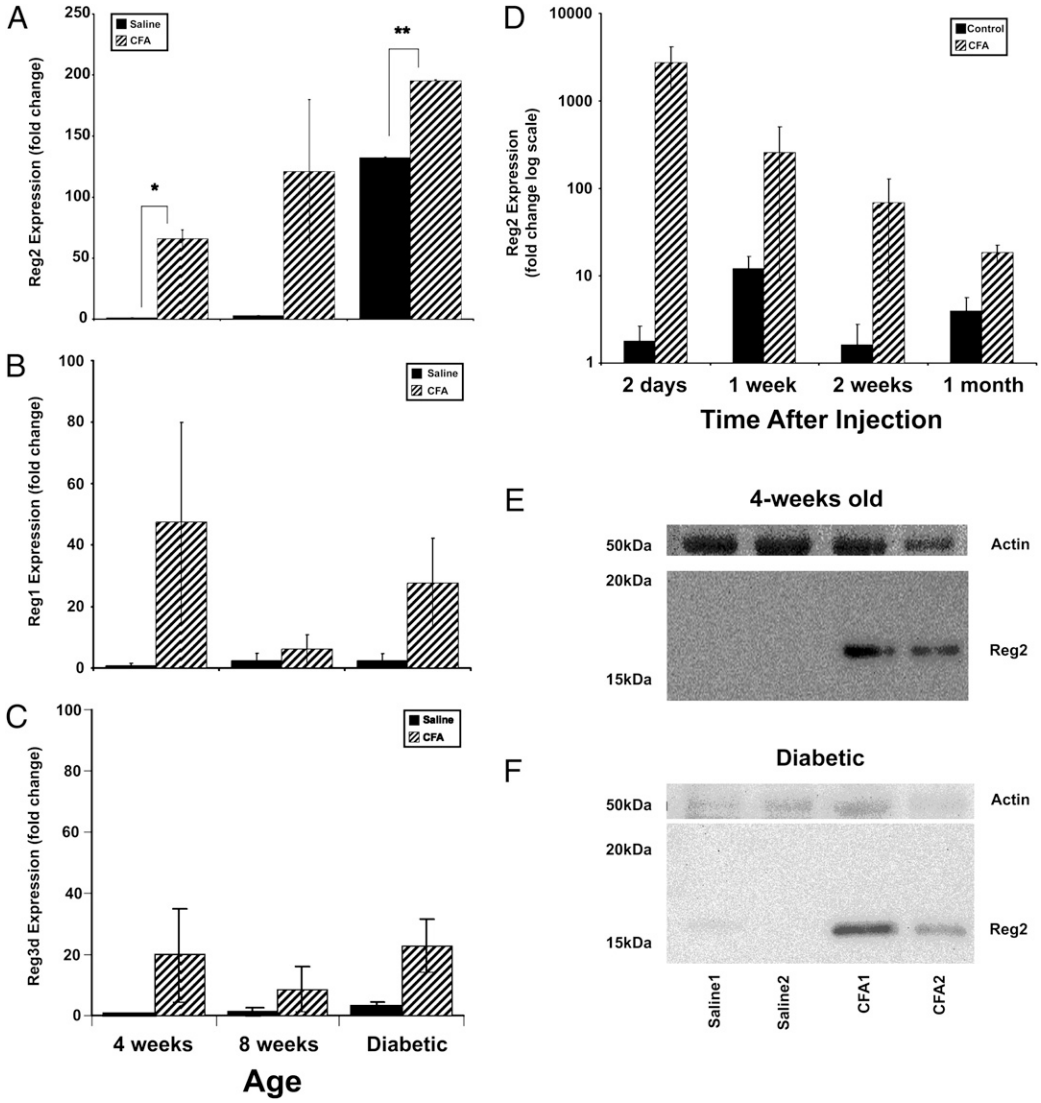

FIGURE 3. Reg2 gene and protein expression in the pancreas of prediabetic and diabetic NOD mice following CFA treatment. Female NOD mice were injected i.p. with 100 μl CFA emulsified in saline or saline alone. *A–D*, Reverse transcription quantitative real-time PCR analysis was performed on RNA from whole pancreatic tissue using gene-specific primers. Results are shown as the average fold change in expression ± SEM as compared with the 4-wk-old saline-treated group (*A–C*) or the 2-d saline-treated group (*D*). *A–C*, Mice were treated with CFA at various ages, and expression of Reg genes from two to three pooled samples of RNA per group was measured 1 wk after injection (three to five mice per pooled sample). $*p < 0.01$; $**p < 0.0005$. *D*, Diabetic mice were treated with CFA, and Reg2 expression from unpooled samples of RNA from two to three mice per group was measured at various times after injection. Western blots showing Reg2 protein expression in whole pancreatic lysates from two groups of 4-wk-old (*E*) and diabetic NOD mice (*F*) 1 d after CFA or saline injection. Actin expression is shown as a loading control. Bands were visualized using chemiluminescent staining.

and diabetes reversal in several animal models (41) and is currently in clinical trials for treating human T1D (42). To further substantiate a relationship between *Reg*2 expression and β cell regeneration, expression of *Reg2* in this established β cell regeneration model was investigated.

We used the protocol previously developed by Barbosa et al. (40) to explore the effect of INGAP peptide on *Reg2* expression in islets from NOD mice. As shown in Fig. 8, *Reg2* was significantly upregulated in islets treated with INGAP peptide for 24 h compared with those that were treated with media only. *Reg2* expression was ~3.5-fold greater in the INGAP-treated islets compared with the islets treated with media alone.

Reg2 expression is upregulated by exendin-4 treatment

Multiple daily injections of exendin-4 were previously found to increase β cell regeneration and β cell mass in NOD mice (43–45). We treated 4-wk-old NOD mice with three daily injections of exendin-4 or with saline as a control. We found that there was a significant increase in *Reg2* expression in whole pancreatic tissue from the exendin-4 treatment group compared with the saline control group (Fig. 9). The magnitude of the difference in expression between the two groups was ~25-fold (Fig. 9).

CFA treatment influences autoimmunity and islet mass in diabetic NOD mice

A role for several of the *Reg* genes in stimulating β cell regeneration and ultimately diabetes reversal has been shown previously (15, 41); however, no role for *Reg2* in this process has been defined clearly. To correlate upregulation of *Reg2* expression with diabetes reversal, blood glucose levels were monitored in diabetic NOD mice that were treated with CFA. A single injection of CFA has been shown previously to reverse diabetes in a small percentage of NOD mice (23, 35). We did not observe a reduction in the blood glucose levels of diabetic mice within 1 mo following

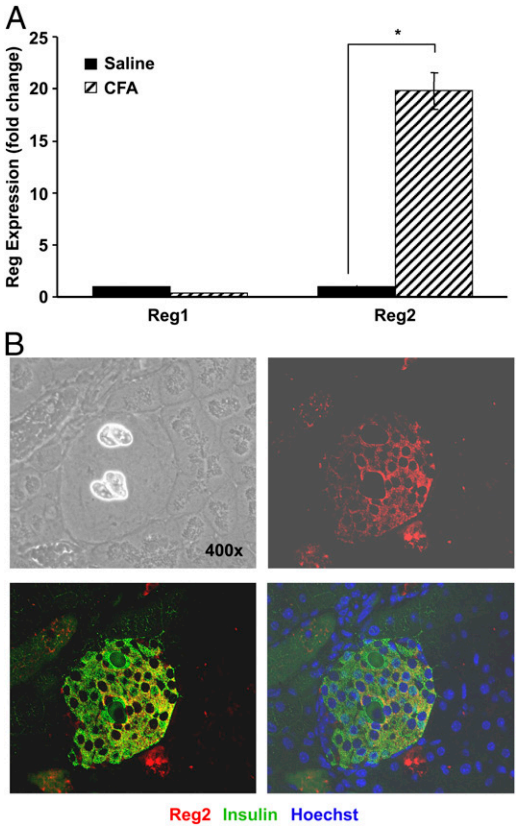

FIGURE 4. Localization of *Reg2* expression in islets following CFA treatment. Female NOD mice were injected i.p. with 100 μl CFA emulsified in saline or saline alone. *A*, One week after CFA injection, islets from 4-wk-old mice were extracted from the pancreatic tissue by collagenase digestion, and islets from eight mice per group were pooled. Reverse transcription quantitative real-time PCR analysis was performed on RNA from pooled islets using *Reg2*-specific primers, and data were quantified by the Pfaffl method (33) using β-actin as a housekeeping gene. Results are shown as the average fold change in expression ± SD of triplicate ($n = 3$) PCRs as compared with the saline control group. $*p < 0.005$. *B*, Representative differential contrast microscopy (*top left panel*) and fluorescent microscopy (*top right*, *bottom left*, and *bottom right panels*) images of an islet from a 16-wk-old mouse that was treated with CFA 4 wk earlier. Five-micrometer-thick formalin-fixed and paraffin-embedded tissue sections were double immunochemically stained using polyclonal primary Abs reactive against *Reg2* (red) and insulin (green) as well as Hoechst nuclear stain (blue). Shown are fluorescent images of *Reg2* only (*top right*), *Reg2* and insulin merged (*bottom left*), and *Reg2*, insulin, and Hoechst merged (*bottom right*). Original magnification ×400.

CFA injection. There was, however, a reduction in the degree of insulitis in islets at 1-wk post-CFA injection (Fig. 10*A*) that correlated with an increased expression of *Reg2* (Fig. 3*A*). Qualitative analysis showed that the insulin-positive area within individual islets was inversely correlated with the degree of insulitis (Fig. 10*B*), and therefore, there appeared to be more insulin staining within tissue sections from CFA-treated mice compared with controls. To confirm the reversal of diabetes by CFA treatment, we treated 26 confirmed diabetic NOD mice with a single injection of CFA (50μl in each hind footpad). Mice were maintained by daily injection of 1 U insulin and were monitored for glycemia. Between 40 and 60 d after adjuvant therapy, insulin treatment was discontinued, and we found at least five mice (19.2%) maintained normoglycemia. In addition to recovery from disease, these mice also showed evidence of β cell regeneration as indicated by an increase in the number of pancreatic islets compared with untreated diabetic mice (Fig. 10*C*). This increase occurred for small-

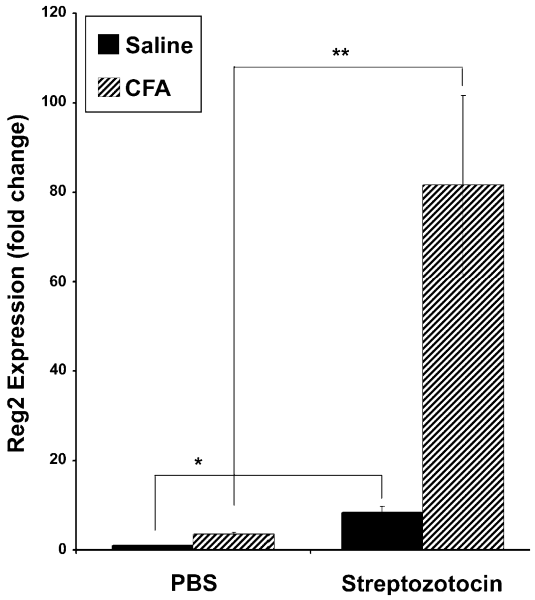

FIGURE 5. Pancreatic *Reg2* expression following CFA treatment in STZ-induced diabetic B6 mice. Four-week-old female B6 mice were given a single i.p. injection of 200 mg/kg STZ dissolved in PBS or with PBS only as a control. One day later, the mice were given a single i.p. injection of 100 μl CFA emulsified in saline or saline alone. *Reg2* expression was then measured 1 d after the CFA treatment by performing qRT-PCR on RNA from whole pancreatic tissue using gene-specific primers. PCR data were quantified by the Pfaffl method (33). Results are shown as the average fold change in *Reg2* expression ± SEM ($n = 3$) per group as compared with saline only control groups. $*p < 0.01$; $**p < 0.02$.

and medium-sized islets but not large-sized islets, which suggests that the increase in islet numbers is due to the formation of new islet cells through islet neogenesis. These results confirm previous studies that treatment with CFA alone can reverse diabetes (41). We are currently exploring ways to improve the rate of diabetes reversal in diabetic NOD mice by mycobacterial adjuvant treatment.

CFA treatment influences diabetes in STZ-treated B6 mice

The effect of CFA treatment on diabetes in the high-dose STZ treatment model has not been investigated previously. We explored the effect of CFA treatment on β cell regeneration in this model. Approximately 60% of mice that were treated with high-dose STZ became diabetic within 3 wk postinjection. We injected the

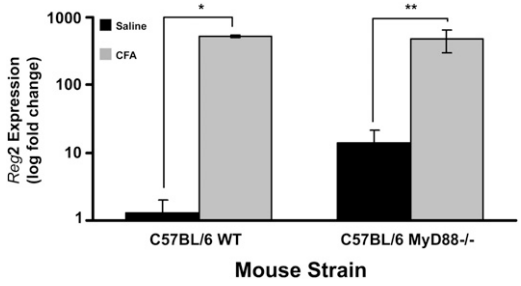

FIGURE 6. *Reg2* expression after CFA treatment in B6 MyD88$^{-/-}$ mice. Four-week-old B6 wild-type (WT) or B6 MyD88$^{-/-}$ mice were injected i.p. with CFA emulsified in saline or saline only. Seven days later, RNA was extracted from whole pancreatic tissue, and qRT-PCR was performed using gene-specific primers. Gene expression was analyzed by the Pfaffl method (33) using β-actin as a housekeeping gene. Results are shown as the average fold change in *Reg2* expression from three mice per group ± SEM as compared with the saline control group. $*p < 0.0001$; $**p < 0.005$.

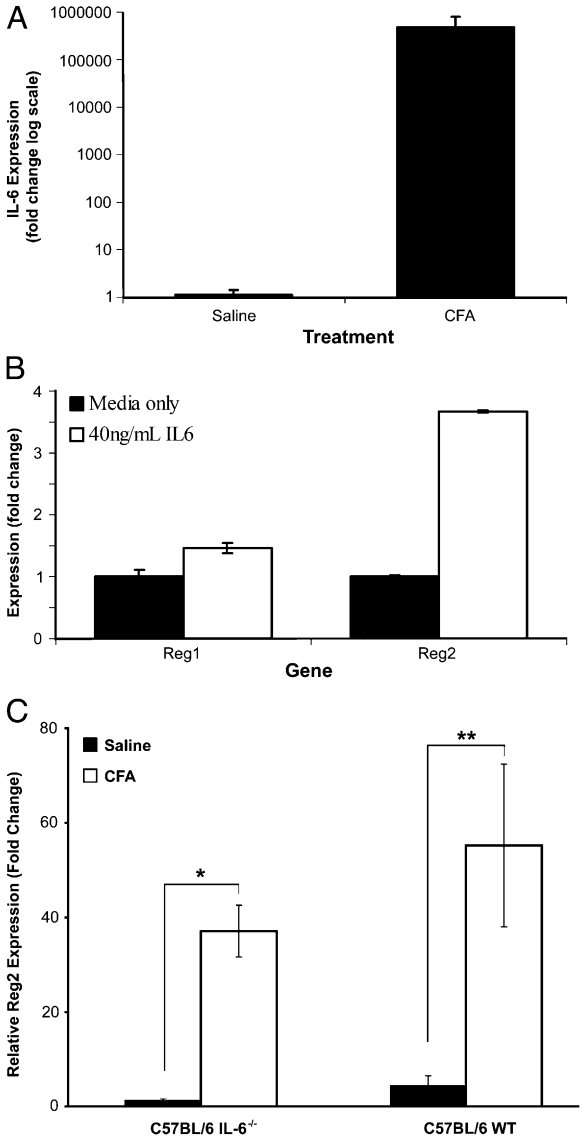

FIGURE 7. Role of IL-6 in *Reg2* upregulation after CFA treatment. *A*, Four- to 6-wk-old female NOD mice were injected i.p. with CFA emulsified in saline or saline only. RNA was extracted from whole pancreatic tissue, and qRT-PCR was performed using gene-specific primers. Gene expression was analyzed by the Pfaffl method (33) using β actin as a housekeeping gene. Results are shown as the average fold change in *Reg2* expression per group ± SEM ($n = 3$) as compared with saline control group. *B*, Islets from 4-wk-old female NOD mice were extracted from pancreatic tissue by collagenase digestion. Pooled islets from 12 mice were divided into two different wells in a 6-well culture dish and treated with 40 ng IL-6 in DMEM or with media only as a control, which was followed by an overnight incubation at 37°C. After incubation, RNA was extracted for qRT-PCR analysis of gene expression, which was quantified by the Pfaffl method (33) using β actin as a housekeeping gene. Results are shown as the average fold change in *Reg1* or *Reg2* expression ± SD of triplicate ($n = 3$) PCR tubes as compared with the media control group. *C*, Four-week-old female IL-6 knockout (IL-$6^{-/-}$) and wild-type (WT) B6 mice were injected i.p. with CFA emulsified in saline or saline only. One day later, RNA was extracted from whole pancreatic tissue, and qRT-PCR was performed using gene-specific primers. Gene expression was analyzed by the Pfaffl method using β-actin as a housekeeping gene. Results are shown as the average fold change in *Reg2* expression per group ± SEM ($n = 4$) as compared with the B6 IL-$6^{-/-}$ saline control group. $*p < 0.001$; $**p < 0.03$.

diabetic mice with CFA or saline as a control and monitored nonfasted blood glucose levels for 20 d. We found an initial increase in glucose levels, but there was no difference in the average

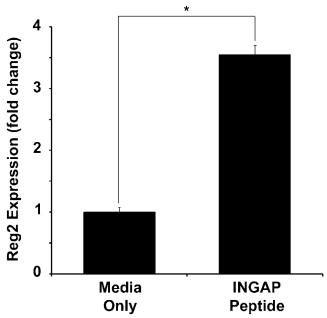

FIGURE 8. *Reg2* expression in the pancreas of NOD mice after in vitro treatment with INGAP peptide. Pooled islets from 14 4-wk-old female NOD mice were divided into three different wells in a 6-well culture dish and treated with either 25 μg INGAP peptide or DMEM alone. Islets were kept at 37°C for 24 h after treatment, and then, RNA was extracted for qRT-PCR to quantify *Reg2* expression. Results are shown as the average fold change in *Reg2* expression ± SD of triplicate ($n = 3$) PCR tubes as compared with the media control group. $*p < 0.0001$.

nonfasted blood glucose levels between the two groups (Fig. 11*A*). There was, however, a significant increase in the overall ability of the CFA-treated mice to regulate blood glucose as indicated by lower average blood glucose levels in the glucose tolerance test compared with the saline-treated group (Fig. 11*B*). It is likely that a longer time frame is needed to restore β cell mass to reverse diabetes. A recent study suggests that it could take 5–10 mo for islets to regenerate β cells to restore normoglycemia in diabetic mice (7).

Discussion

There is considerable evidence that a low level of β cell turnover normally exists within the adult pancreas, and the generation of new β cells is accelerated by physiological stress or damage, such as occurs during T1D development in NOD mice (8). However, this attempted regeneration is not enough to prevent disease, most likely because regenerated β cells are destroyed by ongoing autoimmunity. Several recent studies have shown that regeneration of β cell mass and reversal of diabetes in the NOD mouse is possible following blocking of autoimmunity by CFA treatment (23–27, 35). The objective of this study was to determine the

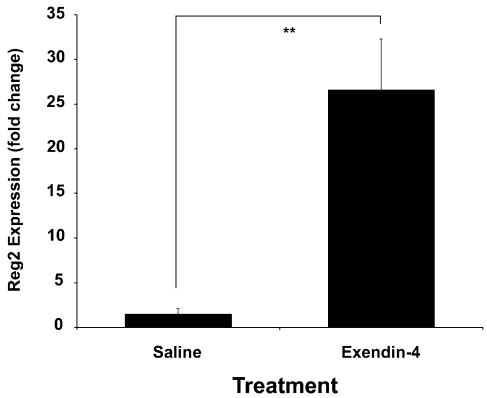

FIGURE 9. *Reg2* expression in pancreas of NOD mice following in vivo treatment with exendin-4. Four-week-old female NOD mice were injected i.p. daily for 3 d with 0.8 μg exendin-4 in 100 μl saline or with 100 μl saline alone. RNA from whole pancreatic tissue was extracted for qRT-PCR to quantify *Reg2* expression. Results are shown as the average fold change in *Reg2* expression ± SEM ($n = 5$) per group as compared with the saline control group. $**p < 0.05$.

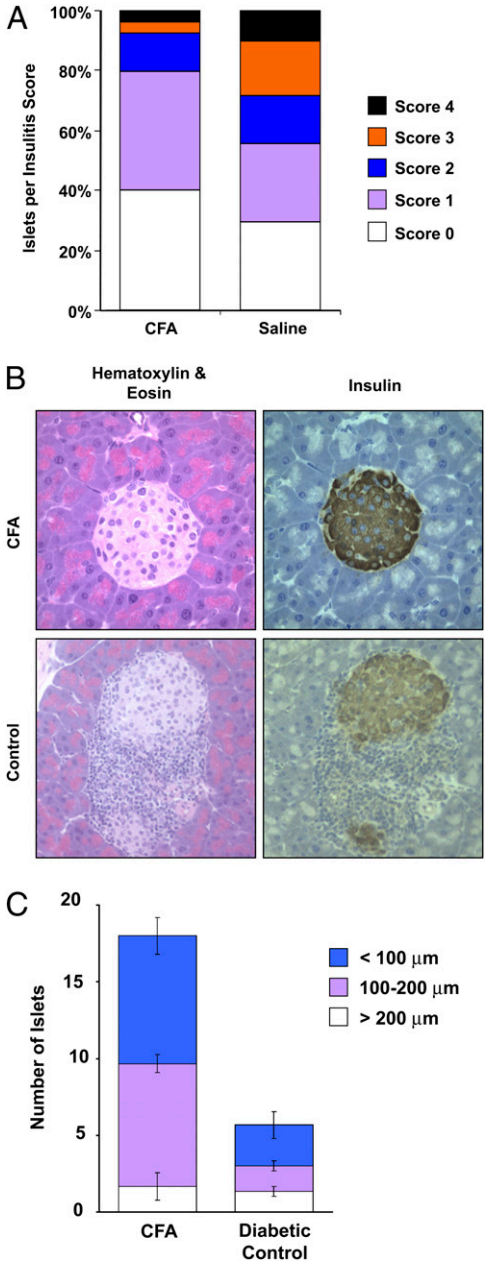

FIGURE 10. Improved insulitis scores, insulin expression, and islet mass in the pancreas of diabetic NOD mice following CFA treatment. Diabetic female NOD mice were injected i.p. with 100 μl CFA emulsified in saline or saline alone (*A*, *B*) or in the footpad with 50 μl CFA (*C*), and pancreatic tissue was extracted 1 mo (*A*, *B*) or 5 mo (*C*) later. Five-micrometer-thick formalin-fixed and paraffin-embedded tissue sections were stained with H&E (*A–C*) or Abs specific for insulin (*B*). *A*, Multiple tissue sections from five mice per group were analyzed, and the degree of insulitis was graded in ≥50 islets from each group. The individual islets were graded as follows: 0, no infiltration; 1, <25% infiltration (peri-insulitis); 2, 25–50% infiltration (mild insulitis); 3, >50% infiltration; and 4, islet destruction (severe insulitis). *B*, Representative microscopy images showing cell infiltration (*left panels*) and insulin expression (*right panels*) in a healthy islet from a CFA-treated mouse at ×1000 magnification (*top panels*) and in a damaged islet from a saline-treated mouse at ×400 magnification (*bottom panels*). *C*, Pancreatic tissue sections from three mice that recovered from diabetes following CFA treatment were analyzed for the number of islets per tissue section and compared with the number of islets per section from untreated diabetic mice. The individual islets were sorted into groups based on diameter, and the average number of islets per tissue section is shown. The results are significantly different ($p < 0.05$) between the two groups of mice for total number of islets, number of medium sized islets (100–200 μm), and number of small-sized islets (<100 μm).

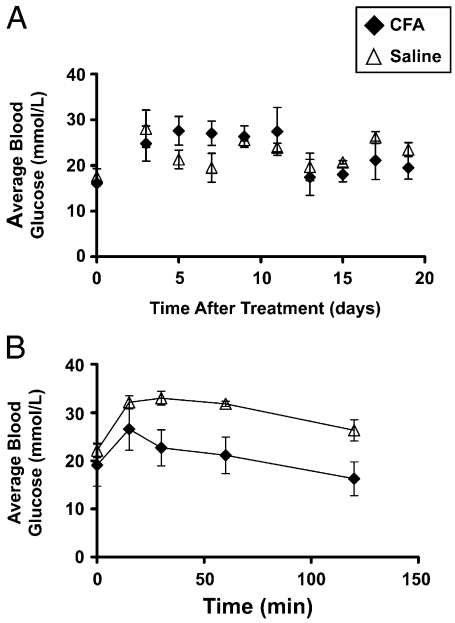

FIGURE 11. Partial disease reversal following CFA treatment in STZ-induced diabetic B6 mice. Four-week-old B6 mice were given a single i.p. injection of STZ (200 mg/kg body weight) and were then monitored for diabetes development by measuring blood glucose levels via the tail vein. Once all mice were diabetic, they were injected i.p. on day 0 with 100 μl CFA emulsified in saline or saline alone. *A*, Nonfasted blood glucose levels were measured every other day. *B*, A glucose tolerance test was performed on day 20 by injecting mice i.p. with glucose (1 mg/g) and measuring blood glucose via the tail vein at 15, 30, 60, and 120 min after injection. The average blood glucose ± SEM ($n = 4$) per group is shown in *A* and *B*.

molecular mechanisms involved in β cell regeneration during T1D development and following CFA-mediated reversal of diabetes.

We found that several members of the *Reg* gene family were upregulated with age in NOD mice and that *Reg2* was further upregulated in diabetic NOD mice following CFA treatment. An increased pancreatic *Reg2* expression was also found in the STZ model of diabetes both during development of disease and following CFA treatment. Previous studies have shown that *Reg* genes, including *Reg2*, are upregulated in the pancreas in several diabetes models including the NOD mouse (16, 18–20, 46) and have identified *Reg2* as a potential factor involved in endogenous β cell regeneration during diabetes development. Our results support these findings and suggest that *Reg2* is also involved in β cell regeneration following adjuvant immunotherapy in NOD mice. This upregulation of *Reg*2 expression following CFA treatment may be occurring via an inflammatory signaling pathway involving IL-6 in the pancreas (19, 39). We found CFA treatment caused in vivo upregulation of IL-6 within the pancreas, whereas IL-6 treatment was shown to induce in vitro upregulation of *Reg*2 in islets. However, we found that the upregulation of *Reg2* expression following CFA treatment in IL-6 knockout mice was not diminished; therefore, indicating that IL-6 does not play a role in the signaling pathway for *Reg2* upregulation. We also found that *Reg2* was upregulated following CFA treatment in MyD88 knockout mice, indicating that CFA signaling through MyD88-dependent TLRs is not required for *Reg2* induction. This follows logically from our result for IL-6 because expression of inflammatory cytokines in response to mycobacterial adjuvants occurs at least partially by signaling through MyD88-dependent TLR pathways. Previous studies have shown that expression of *Reg* genes is increased in response to inflammatory molecules

other than IL-6 such as IFN-β and TNF-α (9, 19, 47). It is possible that *Reg2* upregulation following CFA treatment involves one of these inflammatory mediators through a MyD88-independent pathway or through a cytokine-independent signaling pathway.

Similar to the results for gene expression, we found there to be a greater amount of *Reg2* protein in the pancreas following CFA treatment in both 4-wk-old and diabetic NOD mice. This was likely due to an increase in production of the protein, given similar changes in mRNA; however, it is also possible that CFA acted to stabilize *Reg2* protein from degradation. The fact that CFA treatment caused an increase in both *Reg2* gene and protein expression over and above the already endogenously upregulated levels in diabetic mice strongly supports a role for *Reg2* in mediating β cell regeneration following CFA treatment.

CFA treatment in diabetic mice was previously thought to reverse disease by downregulating autoimmunity to allow regeneration of β cell mass to occur through endogenous mechanisms (23–27, 35). In this study, CFA treatment caused upregulation of *Reg2* gene and protein expression in nondiabetic mice and influenced disease in a nonautoimmune STZ-induced model of diabetes, suggesting that CFA might act to stimulate regeneration directly and independently from its ability to downregulate autoimmunity. As *Reg*2 is being considered an autoantigen, CFA might modulate autoimmune responses toward *Reg* proteins to facilitate regeneration of β cells without being vulnerable to destruction by immune cells. The mechanism by which *Reg2* acts to induce β cell regeneration is not known. A CFA-stimulated increase in *Reg2* gene expression occurred specifically in the islets, and *Reg2* protein expression was localized to the β cell area, which indicates that *Reg2* may be acting to induce β cell replication through an autocrine and/or paracrine mechanism similar to *Reg*1.

Previous studies have shown that exendin-4 increases β cell regeneration and β cell mass through both neogenesis and β cell replication (45). *Reg2* expression was significantly upregulated after only 3 d of exendin-4 treatment in nondiabetic NOD mice. In addition, *Reg2* was shown to be increased in isolated islets from NOD mice following treatment with INGAP peptide in vitro, which has been shown previously to induce β cell neogenesis in isolated rat islets (40). The upregulation of *Reg2* expression in these two well established models of β cell regeneration provides further evidence that *Reg2* is directly involved in the process of β cell regeneration.

The regeneration of pancreatic islet β cells is important for the prevention and cure of diabetes mellitus. We have demonstrated that different members of the mitogenic *Reg* gene family, *Reg2* and *Reg 3β*, are upregulated during the course of diabetes development in the pancreas of NOD mice and *Reg*2 was also upregulated following CFA treatment. We also demonstrate that *Reg2* gene is localized to the islets following CFA treatment. Controversy exists as to the cellular source of the *Reg2* (gene and protein) expression in the pancreas. Watanabe et al. (48) demonstrated that the *Reg* gene is normally expressed in the pancreatic acinar cells, and the protein encoded by this gene is found in a fairly high amount in pancreatic secretion in humans (49). Also, Sanchez et al. (17) demonstrated that *Reg* mRNA and protein expression remained restricted to exocrine tissue, both in cyclophosphamide-treated mice, which represent a prediabetic stage, and in overtly diabetic NOD females. Finally, we also demonstrate that INGAP peptide and exendin-4 can upregulate *Reg*2 gene expression in the pancreas of 4-wk-old prediabetic NOD mice. De Leon et al. (50) showed that a common subset of genes are regulated by exendin-4 after partial pancreatectomy including members of the *Reg* gene family *Reg2*, *Reg3γ*, and *Reg3δ*. These and other studies linking treatment with CFA, INGAP peptide, and exendin-4 to *Reg2* upregulation in pancreas suggest a role for *Reg* genes in pancreatic growth and function.

In conclusion, we suggest that *Reg2*, a member of the *Reg* gene family, is upregulated during diabetes development and could stimulate β cell regeneration. However, this regenerative response is not enough to compensate for β cell loss as a result of autoimmune destruction, and diabetes eventually ensues. The injection of mycobacterial adjuvants into diabetic NOD mice further upregulates *Reg2* expression, which could potentially induce regeneration of β cell mass. We have confirmed previous studies that diabetes reversal following a single CFA treatment can be achieved in ∼20% of new-onset diabetic NOD mice and may take up to 40 d post-injection to occur (26, 27, 35). These mice showed evidence of β cell regeneration as indicated by an increase in the number of pancreatic islets compared with untreated diabetic mice, (Fig. 10*C*). This increase occurred for small- and medium-sized islets but not large-sized islets, suggesting that the increase in islet numbers is due to the formation of new islet cells through islet neogenesis. This is interesting as the recent studies by Thorel et al. (7) observed that in fully diabetic mice, α cells can reprogram to become β cells, but the process takes 2–4 wk to start, and complete recovery of insulin production takes between 5 and 10 mo. Our studies suggest that *Reg* gene family members such as *Reg2* may be useful targets for β cell regenerative therapies (42, 51) to reverse T1D.

Acknowledgments

We thank Shani Mintzberg for technical assistance.

Disclosures

The authors have no financial conflicts of interest.

References

1. Butler, A. E., J. Janson, S. Bonner-Weir, R. Ritzel, R. A. Rizza, and P. C. Butler. 2003. β-Cell deficit and increased β-cell apoptosis in humans with type 2 diabetes. *Diabetes* 52: 102–110.
2. Bhushan, R., K. E. Elkind-Hirsch, M. Bhushan, W. J. Butler, K. Duncan, and O. Marrioneaux. 2009. Improved glycemic control and reduction of cardiometabolic risk factors in subjects with type 2 diabetes and metabolic syndrome treated with exenatide in a clinical practice setting. *Diabetes Technol. Ther.* 11: 353–359.
3. Perfetti, R., J. Zhou, M. E. Doyle, and J. M. Egan. 2000. Glucagon-like peptide-1 induces cell proliferation and pancreatic-duodenum homeobox-1 expression and increases endocrine cell mass in the pancreas of old, glucose-intolerant rats. *Endocrinology* 141: 4600–4605.
4. Rafaeloff, R., S. W. Barlow, L. Rosenberg, and A. I. Vinik. 1995. Expression of *Reg* gene in the Syrian golden hamster pancreatic islet regeneration model. *Diabetologia* 38: 906–913.
5. Seaberg, R. M., S. R. Smukler, T. J. Kieffer, G. Enikolopov, Z. Asghar, M. B. Wheeler, G. Korbutt, and D. van der Kooy. 2004. Clonal identification of multipotent precursors from adult mouse pancreas that generate neural and pancreatic lineages. *Nat. Biotechnol.* 22: 1115–1124.
6. Dor, Y., J. Brown, O. I. Martinez, and D. A. Melton. 2004. Adult pancreatic β-cells are formed by self-duplication rather than stem-cell differentiation. *Nature* 429: 41–46.
7. Thorel, F., V. Népote, I. Avril, K. Kohno, R. Desgraz, S. Chera, and P. L. Herrera. 2010. Conversion of adult pancreatic α-cells to β-cells after extreme β-cell loss. *Nature* 464: 1149–1154.
8. Sreenan, S., A. J. Pick, M. Levisetti, A. C. Baldwin, W. Pugh, and K. S. Polonsky. 1999. Increased β-cell proliferation and reduced mass before diabetes onset in the nonobese diabetic mouse. *Diabetes* 48: 989–996.
9. Liu, J. L., W. Cui, B. Li, and Y. Lu. 2008. Possible roles of reg family proteins in pancreatic islet cell growth. *Endocr. Metab. Immune Disord. Drug Targets* 8: 1–10.
10. Terazono, K., H. Yamamoto, S. Takasawa, K. Shiga, Y. Yonemura, Y. Tochino, and H. Okamoto. 1988. A novel gene activated in regenerating islets. *J. Biol. Chem.* 263: 2111–2114.
11. Zhang, Y. W., L. S. Ding, and M. D. Lai. 2003. Reg gene family and human diseases. *World J. Gastroenterol.* 9: 2635–2641.
12. Castellarin, M. L., M. Petropavlovskaia, M. A. Lipsett, and L. Rosenberg. 2007. The identification and sequence analysis of a new Reg3γ and Reg2 in the Syrian golden hamster. *Biochim. Biophys. Acta* 1769: 579–585.

13. Taylor-Fishwick, D. A., S. Rittman, H. Kendall, L. Roy, W. Shi, Y. Cao, G. L. Pittenger, and A. I. Vinik. 2003. Cloning genomic INGAP: a Reg-related family member with distinct transcriptional regulation sites. *Biochim. Biophys. Acta* 1638: 83–89.
14. Unno, M., K. Nata, N. Noguchi, Y. Narushima, T. Akiyama, T. Ikeda, K. Nakagawa, S. Takasawa, and H. Okamoto. 2002. Production and characterization of Reg knockout mice: reduced proliferation of pancreatic β-cells in Reg knockout mice. *Diabetes* 51(Suppl. 3): S478–S483.
15. Watanabe, T., Y. Yonemura, H. Yonekura, Y. Suzuki, H. Miyashita, K. Sugiyama, S. Moriizumi, M. Unno, O. Tanaka, H. Kondo, et al. 1994. Pancreatic β-cell replication and amelioration of surgical diabetes by Reg protein. *Proc. Natl. Acad. Sci. USA* 91: 3589–3592.
16. Baeza, N., D. Sanchez, B. Vialettes, and C. Figarella. 1997. Specific reg II gene overexpression in the non-obese diabetic mouse pancreas during active diabetogenesis. *FEBS Lett.* 416: 364–368.
17. Sanchez, D., N. Baeza, R. Blouin, C. Devaux, G. Grondin, K. Mabrouk, O. Guy-Crotte, and C. Figarella. 2000. Overexpression of the reg gene in non-obese diabetic mouse pancreas during active diabetogenesis is restricted to exocrine tissue. *J. Histochem. Cytochem.* 48: 1401–1410.
18. Qiu, L., E. O. List, and J. J. Kopchick. 2005. Differentially expressed proteins in the pancreas of diet-induced diabetic mice. *Mol. Cell. Proteomics* 4: 1311–1318.
19. Planas, R., A. Alba, J. Carrillo, M. C. Puertas, R. Ampudia, X. Pastor, H. Okamoto, S. Takasawa, W. Gurr, R. Pujol-Borrell, et al. 2006. Reg (regenerating) gene overexpression in islets from non-obese diabetic mice with accelerated diabetes: role of IFNβ. *Diabetologia* 49: 2379–2387.
20. Lu, Y., A. Ponton, H. Okamoto, S. Takasawa, P. L. Herrera, and J. L. Liu. 2006. Activation of the Reg family genes by pancreatic-specific IGF-I gene deficiency and after streptozotocin-induced diabetes in mouse pancreas. *Am. J. Physiol. Endocrinol. Metab.* 291: E50–E58.
21. Qin, H. Y., M. W. Sadelain, C. Hitchon, J. Lauzon, and B. Singh. 1993. Complete Freund's adjuvant-induced T cells prevent the development and adoptive transfer of diabetes in nonobese diabetic mice. *J. Immunol.* 150: 2072–2080.
22. Sadelain, M. W., H. Y. Qin, J. Lauzon, and B. Singh. 1990. Prevention of type I diabetes in NOD mice by adjuvant immunotherapy. *Diabetes* 39: 583–589.
23. Faustman, D. L., S. D. Tran, S. Kodama, B. M. Lodde, I. Szalayova, S. Key, Z. E. Toth, and E. Mezey. 2006. Comment on papers by Chong et al., Nishio et al., and Suri et al. on diabetes reversal in NOD mice. *Science* 314: 1243, author reply 1243.
24. Kodama, S., W. Kühtreiber, S. Fujimura, E. A. Dale, and D. L. Faustman. 2003. Islet regeneration during the reversal of autoimmune diabetes in NOD mice. *Science* 302: 1223–1227.
25. Chong, A. S., J. Shen, J. Tao, D. Yin, A. Kuznetsov, M. Hara, and L. H. Philipson. 2006. Reversal of diabetes in non-obese diabetic mice without spleen cell-derived β cell regeneration. *Science* 311: 1774–1775.
26. Nishio, J., J. L. Gaglia, S. E. Turvey, C. Campbell, C. Benoist, and D. Mathis. 2006. Islet recovery and reversal of murine type 1 diabetes in the absence of any infused spleen cell contribution. *Science* 311: 1775–1778.
27. Suri, A., B. Calderon, T. J. Esparza, K. Frederick, P. Bittner, and E. R. Unanue. 2006. Immunological reversal of autoimmune diabetes without hematopoietic replacement of β cells. *Science* 311: 1778–1780.
28. Phillips, J. M., L. O'Reilly, C. Bland, A. K. Foulis, and A. Cooke. 2007. Patients with chronic pancreatitis have islet progenitor cells in their ducts, but reversal of overt diabetes in NOD mice by anti-CD3 shows no evidence for islet regeneration. *Diabetes* 56: 634–640.
29. Ablamunits, V., N. A. Sherry, J. A. Kushner, and K. C. Herold. 2007. Autoimmunity and β cell regeneration in mouse and human type 1 diabetes: the peace is not enough. *Ann. N. Y. Acad. Sci.* 1103: 19–32.
30. Okamoto, H., and S. Takasawa. 2002. Recent advances in the Okamoto model: the CD38-cyclic ADP-ribose signal system and the regenerating gene protein (Reg)-Reg receptor system in β-cells. *Diabetes* 51(Suppl. 3): S462–S473.
31. Young, H. Y., P. Zucker, R. A. Flavell, A. M. Jevnikar, and B. Singh. 2004. Characterization of the role of major histocompatibility complex in type 1 diabetes recurrence after islet transplantation. *Transplantation* 78: 509–515.
32. Stephens, T. A., E. Nikoopour, B. J. Rider, M. Leon-Ponte, T. A. Chau, S. Mikolajczak, P. Chaturvedi, E. Lee-Chan, R. A. Flavell, S. M. Haeryfar, et al. 2008. Dendritic cell differentiation induced by a self-peptide derived from apolipoprotein E. *J. Immunol.* 181: 6859–6871.
33. Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29: e45.
34. Gurr, W., M. Shaw, Y. Li, and R. Sherwin. 2007. RegII is a β-cell protein and autoantigen in diabetes of NOD mice. *Diabetes* 56: 34–40.
35. Tian, B., J. Hao, Y. Zhang, L. Tian, H. Yi, T. D. O'Brien, D. E. Sutherland, B. J. Hering, and Z. Guo. 2009. Upregulating $CD4^+CD25^+FOXP3^+$ regulatory T cells in pancreatic lymph nodes in diabetic NOD mice by adjuvant immunotherapy. *Transplantation* 87: 198–206.
36. Lenzen, S. 2008. The mechanisms of alloxan- and streptozotocin-induced diabetes. *Diabetologia* 51: 216–226.
37. Like, A. A., and A. A. Rossini. 1976. Streptozotocin-induced pancreatic insulitis: new model of diabetes mellitus. *Science* 193: 415–417.
38. Werling, D., and T. W. Jungi. 2003. TOLL-like receptors linking innate and adaptive immune response. *Vet. Immunol. Immunopathol.* 91: 1–12.
39. Billiau, A., and P. Matthys. 2001. Modes of action of Freund's adjuvants in experimental models of autoimmune diseases. *J. Leukoc. Biol.* 70: 849–860.
40. Barbosa, H., S. Bordin, L. Stoppiglia, K. Silva, M. Borelli, H. Del Zotto, J. Gagliardino, and A. Boschero. 2006. Islet neogenesis associated protein (INGAP) modulates gene expression in cultured neonatal rat islets. *Regul. Pept.* 136: 78–84.
41. Rosenberg, L., M. Lipsett, J. W. Yoon, M. Prentki, R. Wang, H. S. Jun, G. L. Pittenger, D. Taylor-Fishwick, and A. I. Vinik. 2004. A pentadecapeptide fragment of islet neogenesis-associated protein increases β-cell mass and reverses diabetes in C57BL/6J mice. *Ann. Surg.* 240: 875–884.
42. Dungan, K. M., J. B. Buse, and R. E. Ratner. 2009. Effects of therapy in type 1 and type 2 diabetes mellitus with a peptide derived from islet neogenesis associated protein (INGAP). *Diabetes Metab. Res. Rev.* 25: 558–565.
43. Ogawa, N., J. F. List, J. F. Habener, and T. Maki. 2004. Cure of overt diabetes in NOD mice by transient treatment with anti-lymphocyte serum and exendin-4. *Diabetes* 53: 1700–1705.
44. Sherry, N. A., W. Chen, J. A. Kushner, M. Glandt, Q. Tang, S. Tsai, P. Santamaria, J. A. Bluestone, A. M. Brillantes, and K. C. Herold. 2007. Exendin-4 improves reversal of diabetes in NOD mice treated with anti-CD3 monoclonal antibody by enhancing recovery of β-cells. *Endocrinology* 148: 5136–5144.
45. Xu, G., D. A. Stoffers, J. F. Habener, and S. Bonner-Weir. 1999. Exendin-4 stimulates both β-cell replication and neogenesis, resulting in increased β-cell mass and improved glucose tolerance in diabetic rats. *Diabetes* 48: 2270–2276.
46. Baeza, N. J., C. I. Moriscot, W. P. Renaud, H. Okamoto, C. G. Figarella, and B. H. Vialettes. 1996. Pancreatic regenerating gene overexpression in the non-obese diabetic mouse during active diabetogenesis. *Diabetes* 45: 67–70.
47. Dusetti, N. J., G. V. Mallo, E. M. Ortiz, V. Keim, J. C. Dagorn, and J. L. Iovanna. 1996. Induction of lithostathine/reg mRNA expression by serum from rats with acute pancreatitis and cytokines in pancreatic acinar AR-42J cells. *Arch. Biochem. Biophys.* 330: 129–132.
48. Watanabe, T., H. Yonekura, K. Terazono, H. Yamamoto, and H. Okamoto. 1990. Complete nucleotide sequence of human reg gene and its expression in normal and tumoral tissues: the reg protein, pancreatic stone protein, and pancreatic thread protein are one and the same product of the gene. *J. Biol. Chem.* 265: 7432–7439.
49. Carrère, J., O. Guy-Crotte, E. Gaia, and C. Figarella. 1999. Immunoreactive pancreatic Reg protein in sera from cystic fibrosis patients with and without pancreatic insufficiency. *Gut* 44: 545–551.
50. De León, D. D., C. Farzad, M. F. Crutchlow, J. Brestelli, J. Tobias, K. H. Kaestner, and D. A. Stoffers. 2006. Identification of transcriptional targets during pancreatic growth after partial pancreatectomy and exendin-4 treatment. *Physiol. Genomics* 24: 133–143.
51. Gross, D. J., L. Weiss, I. Reibstein, J. van den Brand, H. Okamoto, A. Clark, and S. Slavin. 1998. Amelioration of diabetes in nonobese diabetic mice with advanced disease by linomide-induced immunoregulation combined with Reg protein treatment. *Endocrinology* 139: 2369–2374.