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ARTICLE

Reg (regenerating) gene overexpression in islets from non-obese diabetic mice with accelerated diabetes: role of IFNβ

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Abstract
Aims/hypothesis The expression of IFNβ in beta cells results in accelerated type 1 diabetes. The REG family of beta cell proliferation factors have been described as autoantigens in autoimmune diabetes. The aim of this study was to determine the effect of IFNβ on *Reg* expression, and the implications of this in terms of autoimmunity.
Methods *Reg* gene expression was determined in islets from non-obese diabetic (NOD) RIP-HuIFNβ mice by cDNA microarray, quantitative real-time PCR and immunohistochemistry. The effect of IFNβ on *Reg1* and *Reg2* expression was assessed in the NOD insulinoma cell line NIT-1. IL-6, known to induce *Reg* expression, was measured in the insulitis microenvironment. Morphological studies were carried out to determine islet enlargement in this model.
Results *Reg2* was upregulated in islets from the NOD RIP-HuIFNβ mice at the onset of the autoimmune attack. IFNβ upregulates *Reg1* and *Reg2* genes in NIT-1 cells. The expression of *Il6* was increased in islets from transgenic mice and in NIT-1 cells exposed to HuIFNβ. Moreover, islets from transgenic mice were enlarged compared with those from wild-type mice.
Conclusions/interpretation *Reg* overexpression correlates well with the acceleration of diabetes in this model. The upregulation of *Reg* suggests that islets try to improve hyperglycaemia by regenerating the cells lost in the autoimmune attack. *Reg* expression is regulated by several factors such as inflammation. Therefore, the overexpression of an IFNβ-induced autoantigen (REG) in the islets during inflammation might contribute to the premature onset of diabetes.

Keywords Diabetes · IFNβ · Islets · NOD mice · REG · Regenerating gene

Abbreviations

H/E	haematoxylin and eosin
IIF	indirect immunofluorescence
NOD	non-obese diabetic
SCID	severe combined immune deficiency

Introduction

Type 1 diabetes results from a combination of genetic, immunological and environmental factors [1]. Although the aetiology of the disease is believed to have a major genetic component, environmental factors, such as viruses, may be determinants [2–4]. Type I interferons—antiviral cytokines—are mediators in type 1 diabetes [5–7]. The trans-

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genic expression of the human gene for IFNβ (HuIFNβ) in islet beta cells [8] accelerates autoimmune diabetes in non-obese diabetic (NOD) mice [9] and breaks the tolerance in non-diabetes prone strains. In this paper we report that the beta cell regeneration factor REG, also described as a diabetes autoantigen [10, 11] is overexpressed in islets from RIP-HuIFNβ transgenic mice.

Regenerating gene (*Reg*) was isolated from a cDNA library of rat regenerating islets [12]. REG family proteins, involved in liver, pancreatic, gastric and intestinal cell proliferation, belong to a conserved protein family sharing structural and functional properties. The increase of *Reg* expression in cancer [13, 14], inflammatory bowel disease [15] and autoimmune diabetes [10] could promote cell growth.

In mice, *Reg1* and *Reg2* genes encode proteins expressed in the pancreatic exocrine tissue and in regenerating islets [16, 17]. After partial pancreatectomy, experimental diabetes is improved by administering REG [18]. NOD mice expressing *Reg1* in beta cells show a delay in the onset of diabetes [19], whereas islets from *Reg* knockout mice showed a lower proliferative response [19]. Moreover, in NOD mice, diabetes improves following the administration of REG [20]. HIP/PAP, a REG family protein, is over-expressed in islets from patients with recent-onset type 1 diabetes and acts as T-cell autoantigen in NOD mice [10]. Type 1 and type 2 diabetes patients have autoantibodies to REG that delay beta cell proliferation [11]. Although *Reg* upregulation may reflect the effort of the islets in trying to repair the destruction of beta cells, REG proteins may act as autoantigens contributing to activate lymphocytes against REG, off-balancing the loss and generation of beta cells, and influencing diabetes onset.

Factors like nicotinamide, glucocorticoids, diet [21] and IL-6 [10], IL-8 [22] and IFNγ cytokines [14] regulate *Reg* expression. Our hypothesis is that an initial inflammation driven by IFNβ can upregulate *Reg* genes in an attempt to regenerate beta cells, possibly contributing to accelerate autoimmunity. In this study, we compared the expression of *Reg* genes in NOD mice and NOD RIP-HuIFNβ transgenic mice (a model of accelerated autoimmune diabetes) and correlated them with alterations in islet morphology.

Materials and methods

Cell lines

The NOD insulinoma cell line NIT-1 (CRL-2055; ATCC, Manassas, VA, USA) was cultured as described [23] and supplemented with HuIFNβ (50,000 U/ml; Betaferon; Schering, Madrid, Spain) for 72 h. To confirm the effect of HuIFNβ, MHC class I production was assessed by flow cytometry [24] with an anti-MHC class I antibody (H-2, M1/42.3.9.8.HKJ/TIB-126; ATCC) and an FITC-labelled goat anti-rat secondary antibody (SBA, Birmingham, AL, USA). Data were analysed with a FACScan Cell Analyzer (BD Biosciences, San Jose, CA, USA) and CellQuest software (BD Biosciences).

Animals

Transgenic mice expressing the human gene for IFNβ under the control of the rat insulin I promoter (RIP-HuIFNβ) were generated by backcrossing the original C57BL6/SJL RIP-HuIFNβ transgenic mice with CD-1 mice [25]. NOD and NOD-severe combined immune deficiency (SCID) mice [26]—unable to produce mature T and B lymphocytes—were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Transgenic NOD mice were generated by backcrossing CD-1 RIP-HuIFNβ ten times to the NOD background. The acquisition of the genetic background was controlled by the analysis of microsatellites [8]. NOD RIP-HuIFNβ mice were crossed onto the NOD-SCID strain to generate NOD-SCID RIP-HuIFNβ. Non-transgenic littermates were used as controls for each strain of mice. Only female mice were analysed in this study. Mice were kept under specific pathogen-free conditions in a 12-h light–darkness cycle with free access to standard diet. For diabetes assessment, mice were monitored daily for glycosuria (Chroma 1 Glucose strips; Menarini, Barcelona, Spain). Principles of laboratory animal care (NIH publication) and the guidelines for the use and care of laboratory animals of the Generalitat de Catalunya (Spain) were followed.

Insulitis score

To determine the degree of insulitis, pancreases were snap frozen in an isopentane/cold acetone bath. Five μm cryostat sections performed at five non-overlapping levels were stained with haematoxylin and eosin (H/E). Groups of three to six mice were analysed at the age of 4 and 14 weeks, assessing a minimum of 20 islets per mouse. Insulitis was scored as described elsewhere [27]: 0, no insulitis; 1, peri-insular; 2, mild insulitis (<25% of the infiltrated islet); 3, 25–75% of the islet infiltrated; 4, total islet infiltration.

Islet isolation and purification

Pancreases were digested with collagenase (Worthington, Lakewood, NJ, USA) and islets were hand picked under a stereomicroscope as described [28]. To prepare cytosmears, freshly isolated islets were mechanically disrupted and centrifuged onto glass slides [29].

cDNA microarray

Total RNA was extracted from islets with TRIzol (Invitrogen, Life Technologies, Gaithersburg, MD, USA) and treated with DNAse (Ambion, Houston, TX, USA). RNA samples were pooled (three to five animals per condition) and purified with RNeasy Minelute Cleanup (Qiagen, Hilden, Germany). RNA integrity was assessed by electrophoresis in a denaturing 1% agarose gel. Fifteen micrograms of total RNA were reverse transcribed and directly labelled with Cy3- or Cy5-nucleotides with a CyScribe First Strand cDNA labelling kit (Amersham Biosciences, Freiburg, Germany). Labelled cDNA was purified in a CyScribe GFX column (Amersham Biosciences). *Escherichia coli* transcripts (Memorec; Miltenyi Biotec, Cologne, Germany) were included in the reverse transcription reaction as controls of the labelling process. Labelled cDNAs were hybridised in cDNA microarrays (Memorec) designed by our group with 200 diabetes and autoimmunity-related genes, spotted ×4. Hybridisations were performed at 62°C for 16 h following the manufacturer's instructions. After washing, microarrays were scanned (ScanArray 4000; GSI Lumonics, Boston, MA, USA) and normalised to *E. coli* control spots using QuantArray software (GSI Lumonics). Hybridisations were performed in triplicate, including a dye-swap. To test for statistical significance, data were expressed as the mean value of the $\log_2$ ratio between normalised intensities of samples from transgenic islets and from wild-type islets. Differential expression was considered significant when the p value for a one-sample t-test was <0.01, considering all spots (n=12 spots, three arrays with four internal replicate spots per array, passing normality test).

Laser-capture microdissection

A laser-capture microdissection technique was performed to obtain islets from diabetic mice which were very difficult to hand pick. Pancreatic cryostat sections (6 μm) were settled on P.A.L.M. slides (P.A.L.M. Microlaser Technologies, Bernried, Germany) and stained with H/E. Islets were microdissected (P.A.L.M. Microlaser Technologies) on a Zeiss microscope.

Real-time RT-PCR

RNA was extracted from islets (hand-picked or microdissected) with RNeasy Micro (Qiagen). RNA from NIT-1 cells was extracted with TRIzol reagent (Invitrogen) and treated with DNAse (Ambion). RNA was denatured and random hexamer was added. Then, RNA from hand-picked islets was reverse transcribed by using Moloney murine leukaemia virus (MMLV) reverse transcriptase (200 U/μl; Promega, Southampton, UK) in a reaction including MMLV 1×RT-buffer, 1 mmol/l dNTPs and RNAsin (40 U/μl) (Promega). RNA from microdissected islets was reverse transcribed by using SuperScript II (200 U/μl) (Invitrogen) in a reaction including 1×RT-buffer, 10 mmol/l dithiothreitol, 2 mmol/l dNTPs and RNAsin (40 U/μl) (Promega) at 42°C and 70°C for 60 and 15 min, respectively. Real-time PCR was performed using an ABI 7900 System (Applied Biosystems, Foster City, CA, USA) using Taqman primers and probes for *Reg1, Reg2, Il6, Ins2, Rn18s* and *Gapdh*. Relative values were determined by normalising the expression for each gene of interest to a housekeeping gene (*Rn18s* or *Gapdh*) and to a calibrator sample (mRNA from islets from NOD-SCID mice or untreated NIT-1 cells), as described in the $2^{-\Delta\Delta Ct}$ method [30].

Morphological analysis

The islet area from CD-1, NOD and NOD-SCID mice (4 weeks old, n=12 transgenic mice and n=10 controls) was assessed by the point-count method as reported elsewhere [31] and analysed in an image analyser (OpenLab 2.0; Improvision, Coventry, UK). Pancreatic sections (5 μm) were stained with H/E. Islet area was determined (OpenLab 2.0) only taking into account the endocrine tissue. A minimum of 20 islets per animal was analysed. The size of beta cells was determined by measuring the area after specific staining with anti-glucagon, somatostatin and pancreatic polypeptide (Dako, Carpinteria, CA, USA) as described [32] in a minimum of 20 islets per condition.

Immunohistochemistry

Consecutive pancreatic cryostat sections (5 μm) were sequentially stained by indirect immunofluorescence (IIF) with: (1) antibodies to REG1 (mouse monoclonal IgG2a [16] and REG2 (rabbit polyclonal IgG to REG2); (2) labelled goat anti-mouse Alexa 488 IgG2a (ICN, Aurora, OH, USA) or FITC-goat anti-rabbit (SBA); (3) guinea-pig anti-insulin (ICN) and (4) tetramethyl rhodamine isothiocyanate-labelled goat anti-guinea pig (ICN). The sections were observed in a fluorescence UV microscope and an image analyser (OpenLab 2.0). On cytosmears stained as described above, 100–200 beta cells—positive for insulin staining—were evaluated. To ensure specific staining of REG, control slides were stained without the primary antibody, which revealed no specific staining. Each IIF staining was performed in triplicate.

Statistical analysis

Data are expressed as means±SE. Statistical analyses to compare independent groups were performed using a t-test when groups passed normality and showed equal variance

tests. Otherwise, a Mann–Whitney *U*-test was performed. Differences were considered significant when a value of <0.05 was reached (p<0.01 for microarray experiments).

Results

Reg2 mRNA is upregulated in NOD RIP-HuIFNβ islets

To determine the effect of IFNβ in the islets, we compared gene expression in islets from NOD RIP-HuIFNβ mice with those from NOD mice at the age of 4 weeks, at the onset of the autoimmune attack. The cDNA microarray analysis showed that *Reg2* expression was higher in healthy NOD RIP-HuIFNβ when compared with non-transgenic littermates (p<0.0001). As expected, *B2m* expression was also increased (p<0.01) (data from three independent experiments) (Table 1). However, other genes of autoantigens (*Gad1, Gad2 and Ins1*) were not upregulated in transgenic animals (Table 1). Moreover, the gene expression of other islet molecules was not increased (glucagon, somatostatin and pancreatic polypeptide; data not shown). *Reg2* upregulation was confirmed by quantitative RT-PCR (Fig. 1): *Reg2* was 3.5-fold upregulated (p<0.001) in NOD RIP-HuIFNβ when compared with NOD wild-type at the age of 4 weeks. *Reg2* upregulation was not detected in 14-week-old NOD mice when compared with 4-week-old healthy NOD mice (data not shown). *Reg1* was not altered (results from three different experiments).

Reg2 overexpression in diabetic RIP-HuIFNβ mice

Islets from diabetic mice were obtained by laser-capture microdissection. RNA from microdissected islets was obtained for real-time RT-PCR. *Reg2* was overexpressed in islets from early-onset diabetic RIP-HuIFNβ transgenic mice when compared with diabetic NOD mice (14.1-fold change, p<0.05) (Fig. 2). *Reg1* mRNA increase (3.4-fold) was not statistically significant. Since REG induces beta cell proliferation, *Ins2* expression was also determined. *Ins2* expression was higher in the islets from diabetic NOD transgenic mice than in the islets from diabetic NOD wild-type (10.9-fold change, p<0.05), correlating with *Reg2* overexpression.

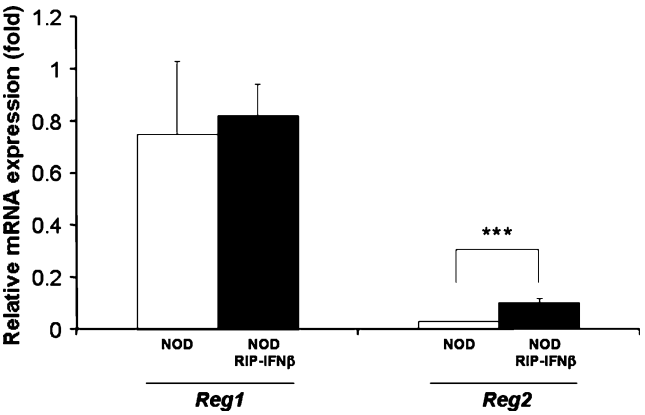

Fig. 1 Histogram of quantitative RT-PCR for *Reg1 and Reg2* expression in islets from NOD (*white bars*) and NOD RIP-HuIFNβ (*black bars*) non-diabetic mice (4 week old). Results from three experiments expressed as the relative mRNA expression of *Reg* to *Gapdh* compared with a calibrator sample (NOD-SCID islets). The $2^{-\Delta\Delta Ct}$ method was followed. ***p<0.001

REG1 and REG2: immunohistology of the islets

We detected a clear increase of REG1 and REG2 expression in the islets from healthy NOD RIP-HuIFNβ compared with healthy NOD mice at the age of 4 weeks (Fig. 3a). REG expression was markedly increased in diabetic transgenic mice compared with NOD diabetic mice. A weak increase in REG expression was also observed in the exocrine tissue from NOD RIP-HuIFNβ in close contact with the islets. As positive control of the effect of HuIFNβ, we determined the MHC class I hyperexpression in the islets from NOD RIP-HuIFNβ as described elsewhere [8]. In order to correlate REG overexpression with the insulitis score, we compared the staining pattern in healthy NOD animals with advanced insulitis (14 weeks of age) with that of NOD mice with early insulitis (4 weeks of age), but no differences were found; this was in agreement with our microarray results (data not shown). Moreover, REG2 expression was slightly

Table 1 Microarray experiments: gene expression in islets from NOD RIP-HuIFNβ compared with NOD

UniGene code	Gene symbol	$\log_2$ ratio (mean±SE)	p value	Function
Mm. 46360	*Reg2*	3.48±0.14	<0.0001	Beta cell proliferation factor. Autoantigen in type 1 diabetes
Mm. 163	*B2m*	1.43±0.33	0.0034	Antigenic presentation restricted to MHC I (β2-microglobulin)
Mm. 16620	*Gad1*	0.17±0.22	NS	GAD. Autoantigen in type 1 diabetes
Mm. 4784	*Gad2*	−0.31±0.24	0.23	GAD. Autoantigen in type 1 diabetes
Mm. 46269	*Ins1*	−0.47±0.07	<0.0001	Regulator of glucose metabolism. Autoantigen in type 1 diabetes
Mm. 5289	*Gapdh*	−0.26±0.10	NS	Housekeeping gene

All subjects were female, non-diabetic 4-week-old mice. Data are expressed as the mean of the $\log_2$ ratio between both samples. Differential expression was considered when the p value for a one-sample t-test was <0.01 (n=12 spots, four from each of three different experiments)

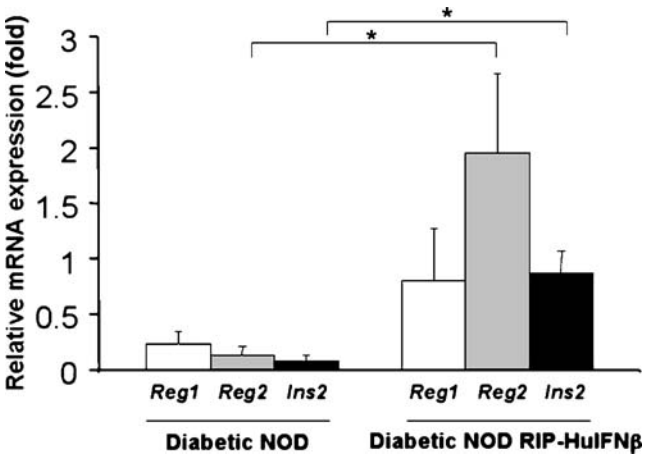

Fig. 2 Histogram of quantitative RT-PCR for *Reg1 and Reg2* expression in microdissected islets from recent-onset diabetic mice (NOD and NOD RIP-HuIFNβ). *Reg1* (*white bars*), *Reg2* (*grey bars*) and *Ins2* (*black bars*) expression was normalised to that of *Rn18s* RNA, and NOD-SCID islets were used as a calibrator. Results from three independent experiments. The $2^{-\Delta\Delta Ct}$ method was followed. *$p<0.05$

increased in the islets from NOD-SCID RIP-HuIFNβ mice compared with wild-type NOD-SCID mice (Fig. 3a). These mice have neither insulitis nor diabetes, due to their lack of functional lymphocytes, but hyperexpress MHC class I in the islets due to the effect of IFNβ. In general, the staining pattern of REG agrees with that of insulin. To confirm co-localisation of REG and insulin, single islet cells were stained in cytosmears. REG2 and REG1 expression clearly co-localised to insulin staining (Fig. 3b), as described [16].

Insulitis is not the only cause of *Reg2* hyperexpression

Since inflammation upregulates *Reg* expression, we assessed insulitis score. Insulitis score was statistically significantly higher in 4-week-old healthy NOD RIP-HuIFNβ when compared with 4-week-old NOD mice ($p<0.001$), and was well correlated with *Reg2* induction (Fig. 4). However, insulitis was higher in 14-week-old NOD mice when compared with 4-week-old NOD mice ($p<0.05$), but *Reg2* upregulation was not found when both groups of animals were compared.

Effect of IFNβ per se on *Reg* expression

To confirm the effect of IFNβ on *Reg* expression, the NOD insulinoma cell line NIT-1 was cultured with HuIFNβ. The effect of the cytokine on NIT-1 cells was confirmed by

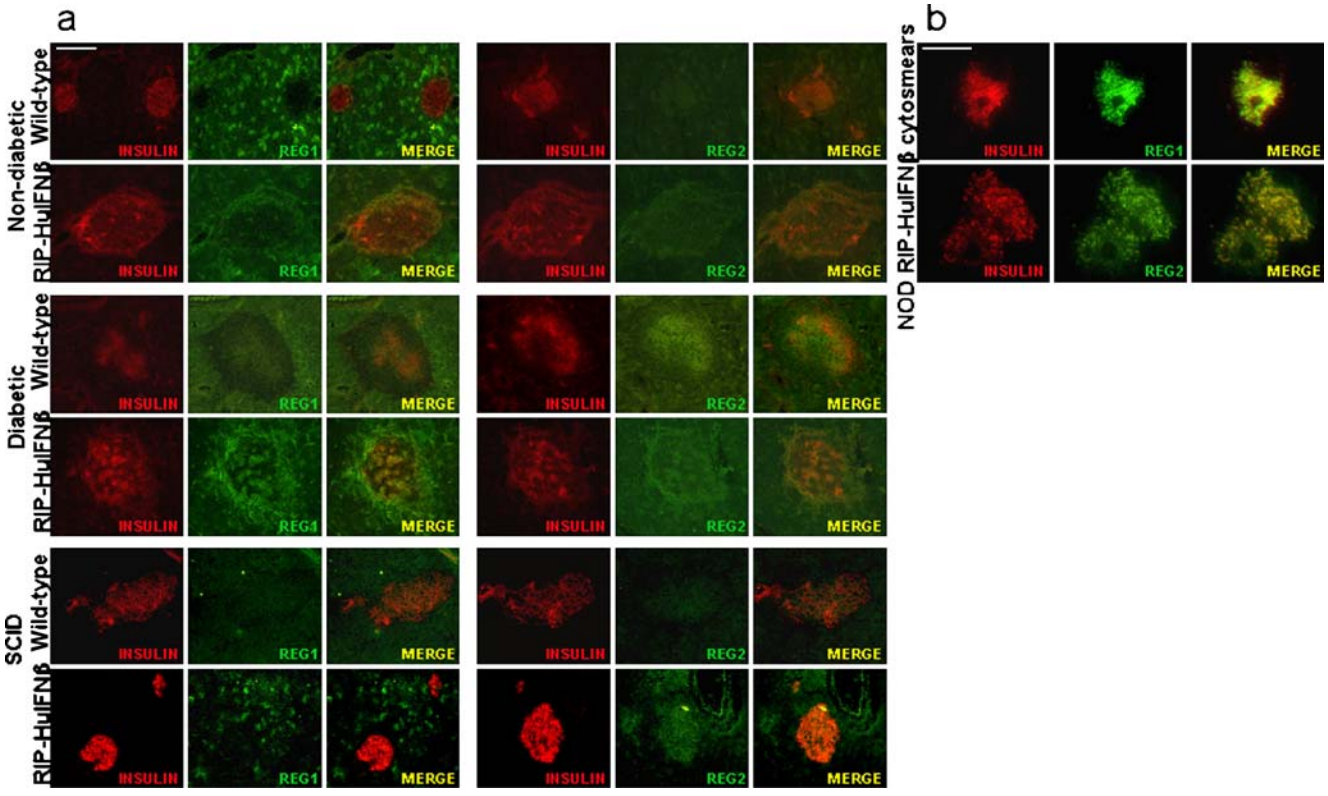

Fig. 3 REG1 and REG2 levels in the pancreatic islets. **a** Double IIF staining for REG (*green*) and insulin (*red*). REG1 (*left panels*) and REG2 (*right panels*) production in islets from NOD RIP-HuIFNβ mice, as compared with those from NOD and NOD-SCID mice. Magnification: ×200; the *bar* represents 100 μm. **b** Double IIF staining in isolated beta cells from non-hyperglycaemic NOD RIP-HuIFNβ mice. REG1 and REG2 levels (*green*) in insulin-positive cells (*red*). Magnification: ×1,000; the bar represents 25 μm

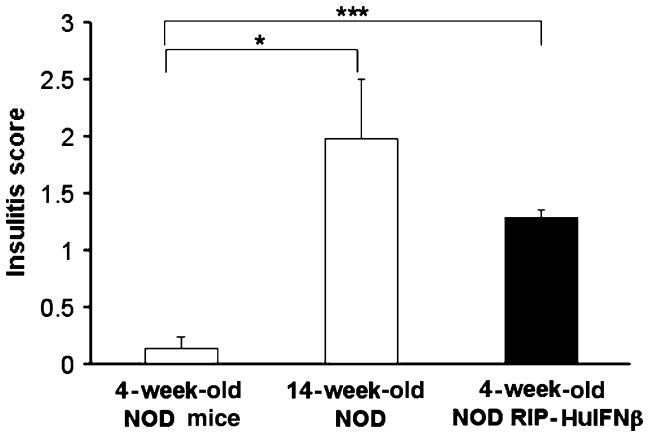

Fig. 4 Insulitis score in NOD (*white bars*) and NOD RIP-HuIFNβ (*black bars*) mice at the points studied by cDNA microarray analysis (3–6 animals per condition and 20 islets per animal). Islets from non-hyperglycaemic 14-week-old NOD females showed a significantly higher insulitis score (*p<0.05) than at 4 weeks. There was also a significant difference (***p<0.001) between islet infiltration from NOD RIP-HuIFNβ mice and wild-type NOD mice at the age of 4 weeks

assessing MHC class I overexpression in the membrane of NIT-1 cells (100.2±19.6), which increased significantly after culturing with HuIFNβ (439.4±43) (p<0.001). Quantitative RT-PCR showed that HuIFNβ upregulates the expression of *Reg1* (3.1-fold change, p<0.05) and *Reg2* (5.5-fold change, p<0.001) (Fig. 5) in the NIT-1 cell line (data from four independent experiments). Moreover, we compared *Reg* expression in the islets from NOD-SCID RIP-HuIFNβ and NOD-SCID. Islets from NOD-SCID RIP-HuIFNβ mice showed a slight *Reg2* upregulation (Fig. 5) compared with NOD-SCID, although differences were not statistically significant (data from three experiments).

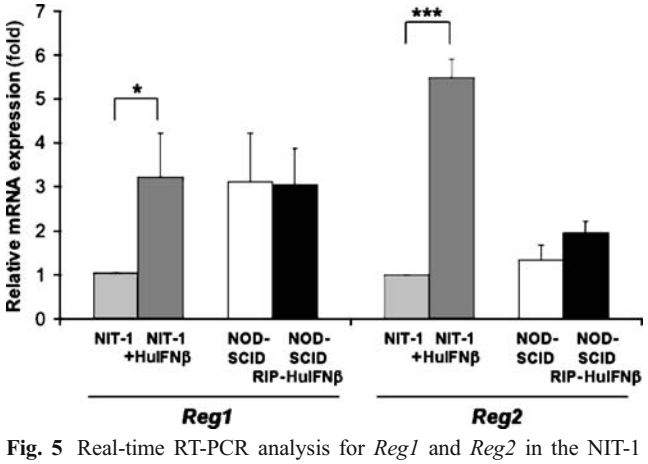

Fig. 5 Real-time RT-PCR analysis for *Reg1* and *Reg2* in the NIT-1 cell line and in NOD-SCID islets. Cells exposed to HuIFNβ for 72 h showed a 3.9- (*p<0.05) and 5.48- (***p<0.001) fold increase in *Reg1* and *Reg2* mRNA, respectively, when compared with basal cultured NIT-1 cells (data from four independent experiments). NOD-SCID RIP-HuIFNβ mice showed higher *Reg2* expression than NOD-SCID mice, but differences were not statistically significant. Results were normalised to *Gapdh* expression and compared with a calibrator (untreated NIT-1 cells and NOD-SCID islets, respectively). *Light grey bars*, NIT-1 cells (basal); *dark grey bars*, NIT-1 cells with HuIFNβ; *white bars*, NOD-SCID mice; *black bars*, islets from NOD-SCID RIP-HuIFNβ transgenic mice

Upregulation of *Il6*, a described enhancer of *Reg* promoter, by IFNβ

As IFNβ increases IL-6 production [33], IL-6 acts as an enhancer in the upstream region of *Reg* genes [17], and inflammatory cytokines are produced in the insulitis, we determined *Il6* islet expression. NOD RIP-HuIFNβ transgenic mice overexpressed *Il6* in the islets (12.1±1.8) when compared with islets from NOD wild-type mice (2.8±0.8) (4.3-fold change, p<0.001, data from three independent experiments) (Fig. 6). To evaluate the effect of HuIFNβ while excluding that of insulitis, we measured *Il6* expression in the islets from NOD-SCID RIP-HuIFNβ and NOD-SCID mice. Islets from NOD-SCID RIP-HuIFNβ mice showed a slightly increased *Il6* expression (Fig. 6) compared with NOD-SCID, although differences were not statistically significant (data from three experiments). The effect of HuIFNβ on *Il6* expression was determined in NIT-1 cells: the expression of *Il6* increased in NIT-1 cells exposed to HuIFNβ (2.5-fold change, p<0.05, data from four experiments). By using protein low-density arrays (a cytokine antibody array, a system to assess the expression profiles of multiple cytokines in pancreatic lysates; Raybiotech, Atlanta, GA, USA) we confirmed that the amounts of IL-6 in the pancreases from healthy and diabetic NOD RIP-HuIFNβ

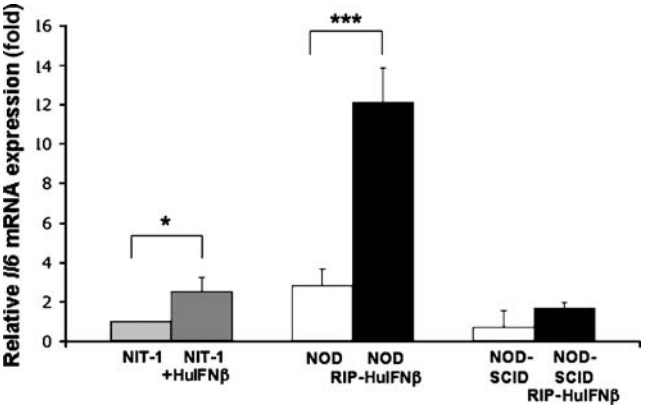

Fig. 6 Effect of IFNβ on *Il6* mRNA expression. Real-time RT-PCR analysis for *Il6* mRNA in NIT-1 cells (basal or cultured with HuIFNβ), and in NOD and NOD-SCID mice (transgenic and wild-type). *Il6* mRNA was significantly increased by IFNβ in NIT-1 cells (*p<0.05) and in NOD islets (***p<0.001) (data from four and three independent experiments, respectively). Results were normalised to *Gapdh* expression and compared with a calibrator (untreated NIT-1 cells and NOD-SCID islets, respectively). *Light grey bars*, NIT-1 (basal); *dark grey bars*, NIT-1 with HuIFNβ; *white bars*, NOD and NOD-SCID mice; *black bars*, NOD RIP-HuIFNβ and NOD-SCID RIP-HuIFNβ transgenic mice

mice were higher that in wild-type mice by 1.9- and 2.1-fold, respectively, (data not shown).

Enlarged islets in RIP-HuIFNβ mice

Since REG causes beta cell proliferation, we studied the endocrine area of the islets hyperexpressing REG. The area of the islets from NOD RIP-HuIFNβ mice, with mild insulitis, was 18,356±1,755 μm^2, 33.8% larger than those of NOD wild-type mice (13,717±1,532 μm^2) (p<0.05). Moreover, the area of the islets from CD-1 RIP-HuIFNβ, with peri-insulitis, was 17,231±770 μm^2, 25% increased when compared with CD-1 wild-type mice (12,686±543 μm^2) (p<0.01). Islets enlarged in 21% of the area (17,315± 1,354 μm^2) were also found in NOD-SCID RIP-HuIFNβ mice (insulitis-free) when compared with NOD-SCID mice (14,304±1,139 μm^2) (p<0.01) (Fig. 7a). Hence, we conclude that the increase of the islet area observed in transgenic mice did not depend on the degree of insulitis but on the presence of IFNβ. Transgenic mice, on the three genetic backgrounds studied, showed a lower percentage of small islets (<10,000 μm^2) and a higher percentage of large islets (>20,000 μm^2) (Fig. 7b). Moreover, the distribution of the islets in categories was similar in NOD, NOD-SCID and CD-1 mice. Thus, the expression of IFNβ in beta cells correlates with a higher percentage of large islets. The increase of the endocrine area may be caused by an increase in the beta cell size or in the islet cell number. No significant differences were found in the size of beta cells when comparing CD-1 transgenic mice with control mice (431± 16.1 vs 407±15.7 μm^2). Moreover, beta cell density in the islets was the same in CD-1 RIP-HuIFNβ (988.4 cells/mm^2) as in wild-type mice (988.3 cells/mm^2). These data support the hypothesis that islet hyperplasia in transgenic mice is caused by an increase in the number of cells per islet. However, this islet hyperplasia did not cause any metabolic dysfunction; body weight, pancreatic weight, insulinaemia, pancreatic insulin contents and i.p. glucose tolerance tests were not altered in non-diabetic transgenic mice [8].

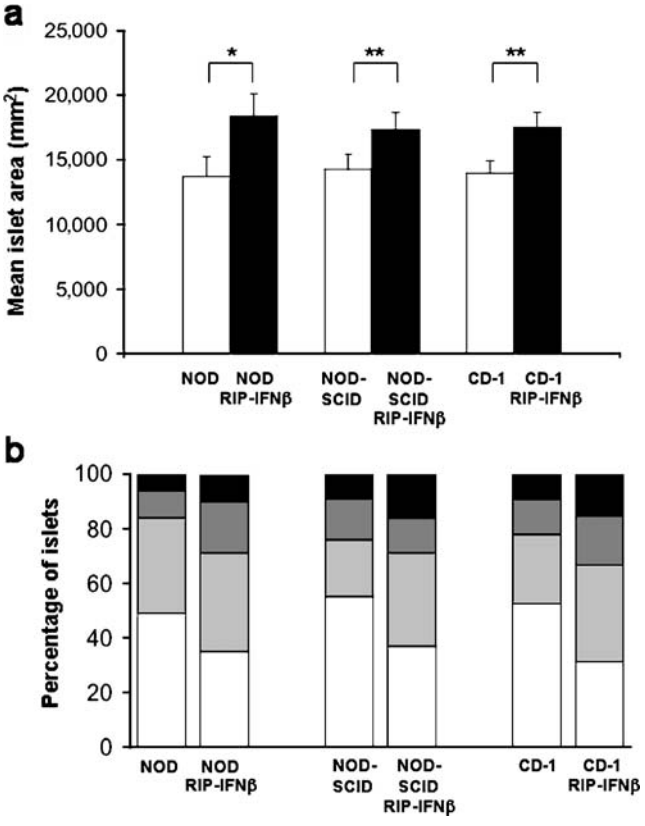

Fig. 7 Morphological studies. **a** Histogram of the mean of islet area (μm^2) in transgenic mice (*black bars*) and wild-type (*white bars*) NOD, NOD-SCID and CD-1 mice at the age of 4 weeks. A minimum of 20 islets/mouse was evaluated (three animals/condition). RIP-HuIFNβ transgenic mice showed an increased islet area when compared with control (*p<0.05 and **p<0.001). **b** Percentage of islets classified in each of the four area (μm^2) categories. A minimum of 20 islets/mouse was evaluated (three animals/condition) in NOD, NOD-SCID and CD-1 mice (RIP-HuIFNβ transgenic and control mice). *White*, small islets (0–10,000 μm^2); *light grey*, medium islets (10,000–20,000 μm^2); *dark grey* big islets (20,000–30,000 μm^2); *black*, mega islets (>30,000 μm^2)

Discussion

The main finding of this study is *Reg2* hyperexpression in the infiltrated islets from NOD RIP-HuIFNβ transgenic mice, correlating with islet enlargement and premature onset of diabetes: islets overexpress *Reg2* both before and at the onset of the disease, but *Reg1* expression did not increase significantly. Insulitis was not the only cause of this overexpression, because severely infiltrated islets from NOD mice did not hyperexpress *Reg*. The direct effect of IFNβ upregulating *Reg1* and *Reg2* was demonstrated in the NOD insulinoma cell line NIT-1. *Reg2* hyperexpression associated with IFNβ always correlated with a relevant increase in IL-6, both in the islets from RIP-HuIFNβ transgenic mice and in NIT-1 cell line, consistent with the described regulation of REG by IL-6. Moreover, mice with accelerated IFNβ-driven diabetes showed higher levels of insulin than diabetic NOD mice, as expected in islets hyperexpressing *Reg2*. However, the increase in *Reg1* and *Reg2* observed in the islets from NOD-SCID RIP-HuIFNβ mice compared with NOD-SCID was not statistically significant. Interestingly, the islets from NOD RIP-HuIFNβ and NOD-SCID RIP-HuIFNβ transgenic mice displayed an increased area compared with wild-type mice. This islet hyperplasia did not cause a metabolic dysfunction of the beta cells.

The role of type 1 IFNs in autoimmunity has been discussed elsewhere [34]. This is the first report assessing the role of type 1 IFN in increasing the expression of REG, a diabetes autoantigen. The upregulation of *Reg2* in NOD

RIP-HuIFNβ transgenic mice could reflect the regeneration of beta cells during the autoimmune attack accelerated by the antiviral cytokine. Our results showed that IFNβ also increases IL-6 in the beta cells. The correlation of IL-6 and *Reg2* hyperexpression observed in our model agrees with the previously reported IL-6 response elements in murine *Reg* genes [10, 14] and with the islet inflammation and hyperplasia detected in transgenic mice expressing IL-6 in the islet beta cells [35]. Since inflammation upregulates *Reg* expression, we assessed insulitis score. We conclude that insulitis is not the only cause of *Reg2* hyperexpression, because old NOD mice did not hyperexpress *Reg* although they exhibited strong insulitis. Moreover, the islets from NOD-SCID RIP-HuIFNβ transgenic mice (insulitis-free) produced REG2 in the islets. Our results suggest that beta cell damage or stress is required for REG2 hyperexpression to promote cell growth or repair.

During a viral infection, IFNβ modulates inflammatory and immune responses by upregulating *MHC I* and *Il6* genes, among others [36]. The link between infections and type 1 diabetes has not been demonstrated, but infected islets themselves may produce inflammatory cytokines [37], affecting the clinical outcome of beta cell autoimmunity [38]. However, virally induced cytokines and chemokines can accelerate or prevent autoimmunity depending on the host strain, age, sex, immunological competence, time, location and level of expression. For example, another viral stimulus, the synthetic double-stranded RNA poly (I:C), inducer of IFNα and other cytokines, can either prevent or exacerbate the pathogenic effects of diabetogenic viruses, depending on the genetic background and the timing of administration [39, 40]. In our model, IFNβ expression in NOD islets results in an early onset of autoimmune diabetes. Since many virus-infected or stressed cells produce IFNβ, our data suggest that *Reg* upregulation may regenerate beta cells that might accelerate the autoimmune process towards beta cells, contributing to an early onset of type 1 diabetes.

Since REG causes beta cell proliferation, we determined possible changes in islet morphology in transgenic mice. Enlarged islets were found in RIP-HuIFNβ transgenic mice. Mega islets have been associated with dendritic cells and macrophages and with beta cell activity [41]. Different factors may contribute to mega islet formation in RIP-HuIFNβ mice: innate immunity, insulitis and beta cell damage/activity.

A transient attempt at beta cell regeneration was observed at the early stages of insulitis in NOD mice [41]. It has been reported that leucocytes influence endocrine cell growth [42]. The expression of *Reg* in the islets and ductal cells of prediabetic NOD mice [10], suggests that the early insulitis or the associated cytokines (IL-6, type I IFNs) may induce regeneration, also described in diabetic transgenic mice expressing IFNγ in the islets [43] and in a cyclophosphamide-induced type 1 diabetes model [44]. New beta cell formation and destruction has been found in long-standing type 1 diabetes patients, thus suggesting regeneration in chronic autoimmunity [45].

A REG family member has been described recently as an autoantigen in diabetes, but unlike other autoantigens, it seems to be upregulated during the prediabetic period [10]. Preliminary results from our group suggest that the response of islet-infiltrating T cells from transgenic (RIP-HuIFNβ) and wild-type (NOD) mice to REG2, is higher than the response to GAD (data not shown), thus supporting the role of REG as an autoantigen. The expression of the most well-known type 1 diabetes autoantigens is not upregulated at the beginning of the autoimmune process. Therefore, the autoimmune attack could be accelerated by the upregulation of *Reg* in the islets. After the clinical onset of type 1 diabetes, most patients enter a period of remission (honeymoon) as a consequence of diminished autoimmunity and/or of beta cell regeneration. Autoantibodies to REG have been found in diabetic patients and these autoantibodies may neutralise the proliferative effect of REG [11]. Whether *Reg* upregulation described in our model plays a key role both in regeneration and in diabetes onset remains to be determined.

In summary, the autoantigen REG overexpressed in islets from NOD RIP-HuIFNβ transgenic mice correlates with enlarged islets in non-diabetic animals and with an acceleration of diabetes onset. REG could provide novel molecular targets for the prognosis and progression of type 1 diabetes, for the recurrence of autoimmunity after pancreas or islet transplantation, and for the design of immunotherapy.

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