



# Immunoregulated insulinitis and slow-progressing type 1 diabetes after duodenopancreatectomy

Pauline Faucher, Frédéric Beuvon, Daniela Fignani, Guido Sebastiani, Georgia Afonso, Zhicheng Zhou, Bertrand Dousset, Christian Boitard, Francesco Dotta, Roberto Mallone, et al.

## ► To cite this version:

Pauline Faucher, Frédéric Beuvon, Daniela Fignani, Guido Sebastiani, Georgia Afonso, et al.. Immunoregulated insulinitis and slow-progressing type 1 diabetes after duodenopancreatectomy. *Diabetologia*, 2021, Online ahead of print. 10.1007/s00125-021-05563-8 . inserm-03381517

**HAL Id: inserm-03381517**

**<https://inserm.hal.science/inserm-03381517>**

Submitted on 17 Oct 2021

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

## **Immuno-regulated insulinitis and slow-progressing type 1 diabetes after duodenopancreatectomy**

Pauline Faucher<sup>1,2</sup>, Frédéric Beuvon<sup>3</sup>, Daniela Fignani<sup>4,5</sup>, Guido Sebastiani<sup>4, 5</sup>, Georgia Afonso<sup>1</sup>, Zhicheng Zhou<sup>1</sup>, Bertrand Dousset<sup>6</sup>, Christian Boitard<sup>1,2</sup>, Francesco Dotta<sup>4,5</sup>, Roberto Mallone<sup>1,2</sup> and Etienne Larger<sup>1,2</sup>.

<sup>1</sup>Université de Paris, Institut Cochin, CNRS, INSERM, 75014 Paris, France.

<sup>2</sup>Assistance Publique Hôpitaux de Paris, Hôpitaux Universitaires de Paris Centre-Université de Paris, Cochin Hospital, Service de Diabétologie et Immunologie Clinique, 75014 Paris, France.

<sup>3</sup>Assistance Publique Hôpitaux de Paris, Hôpitaux Universitaires de Paris Centre-Université de Paris, Cochin Hospital, Service d'Anatomie Pathologique, 75014 Paris, France.

<sup>4</sup>University of Siena, Department of Medicine, Surgery and Neurosciences, Diabetes Unit, 53100 Siena, Italy.

<sup>5</sup>Fondazione Umberto Di Mario, c/o Toscana Life Sciences, 53100 Siena, Italy

<sup>6</sup>Assistance Publique Hôpitaux de Paris, Hôpitaux Universitaires de Paris Centre-Université de Paris, Cochin Hospital, Service de Chirurgie Digestive, 75014 Paris, France.

**ORCID identifiers:** 0000-0001-5472-3851 (P.F.), 0000-0003-0306-2402 (F.B.), 0000-0001-8803-5846 (D.F.), 0000-0003-4374-8564 (G.S.), 0000-0003-3759-7317 (G.A.), 0000-0001-9137-7951 (Z.Z.), 0000-0003-2526-7345 (B.D.), 0000-0003-3985-9405 (C.B.), 0000-0003-4947-7478 (F.D.), 0000-0002-9846-8861 (R.M.), 0000-0002-1017-1845 (E.L.).

**Corresponding author:** Etienne Larger, MD PhD; [etienne.larger@aphp.fr](mailto:etienne.larger@aphp.fr)

**Word count:** 3,209 words.

**Keywords:** type 1 diabetes, duodenopancreatectomy, autoimmunity, regulatory T cells, insulin secretion.

**Tweet:** A case report of slow-progressing type 1 diabetes: evidence of immuno-regulated insulinitis.

@Univ\_Paris, @APHP, @InstitutCochin, @Inserm, @Innodiagroup

## ABSTRACT

**Aims/hypothesis.** We report the case of a 56-year-old woman who underwent a partial pancreatectomy for a serous cystadenoma. She had been diagnosed with diabetes 6 years before and had Hashimoto's thyroiditis. Despite positive anti-GAD auto-antibodies (GADA) and previous surgery, she was transiently weaned off long-acting insulin. Glycaemia remained well controlled with low-dose long-acting insulin. Insulin needs eventually increased 8 years after surgery, in conjunction with anti-zinc transporter (ZnT)8 seroconversion and decreasing residual C-peptide. We hypothesised that the surgical pancreas specimens and blood autoimmune T-cell responses may provide correlates of this indolent clinical course.

**Methods.** Beta- and alpha-cell area and insulitis were quantified on pancreas head tissue sections obtained at surgery. Blood T-cell responses against beta-cell antigens were analysed by enzyme-linked immunospot.

**Results.** Pancreas sections displayed reduced beta-cell and normal alpha-cell area (0.27% and 0.85% of section areas, respectively). High-grade insulitis was observed, mostly in insulin-containing islets, with a peri-insulitis pattern enriched in regulatory FOXP3<sup>+</sup> T cells. *In-vitro* challenge with beta-cell antigens of circulating T cells collected 4 and 9 years after surgery displayed dominant and persistent interleukin-10 responses, but interferon- $\gamma$  responses increasing at 9 years, after anti-ZnT8 seroconversion was observed.

**Conclusions/interpretation.** Despite persistent GADA and the histopathological finding of insulitis and decreased beta-cell area 6 years after diabetes diagnosis, glycaemic control was maintained with low-dose insulin up to 8 years after surgery. Regulated T-cell responses towards beta-cell antigens and FOXP3<sup>+</sup> peri-insulitis suggest spontaneous long-term regulation of islet autoimmunity after substantial beta-cell loss, and eventual autoimmune progression upon anti-ZnT8 seroconversion.

## INTRODUCTION

Type 1 diabetes is the late consequence of an autoimmune process targeting insulin-producing pancreatic beta cells [1, 2]. The disease occurs on a susceptible genetic background following elusive environmental triggers [3] and is T-cell mediated, involving both CD4<sup>+</sup> and CD8<sup>+</sup> T cells that destroy beta cells. The presence of one or more auto-antibodies (aAbs) against GAD, islet antigen (IA)-2 and zinc transporter (ZnT)8 in adults is the hallmark of an ongoing autoimmune process. An improper balance between regulatory T cells (Tregs), which express the lineage marker forkhead box (FOX)P3, and effector T cells (Teffs) is central to the progression of this autoimmune process towards beta-cell destruction [4]. Up to the critical threshold that becomes insufficient to maintain euglycaemia, it is unknown whether the progression of beta-cell destruction is linear or follows a relapsing-remitting pattern, possibly reflecting cyclic disruption and restoration of the Treg/Teff balance [4].

We report the case of a patient positive for anti-GAD aAbs (GADA), whose diabetes remained well controlled with low-dose long-acting insulin for 8 years after partial pancreatectomy. Surgical pancreas specimens and blood samples were studied to search for histopathological and autoimmune T-cell correlates of this indolent clinical course.

## CASE REPORT

A 47-year-old woman was diagnosed with a serous cystadenoma of the uncinate process in 2002. The timeline of the clinical course is summarized in [Fig. 1](#) and detailed in electronic supplementary materials [ESM] Table 1. Diabetes was diagnosed 3 years later (2005), glycaemia was said to be “moderately elevated”, at the weight of 96 kg and BMI of 37 kg/m<sup>2</sup>. The patient had no family history of diabetes and no personal history of gestational diabetes at any of her 3 pregnancies. Metformin and gliclazide were started. She also had Hashimoto’s thyroiditis that required 112.5 µg/day L-thyroxin.

An uneventful duodenopancreatectomy (Whipple procedure with removal of the head of the pancreas) was performed 9 years later (February 2011), because of increasing tumour size (70 mm diameter) accompanied by abdominal pain, signs of vena cava compression with limb oedema and deteriorating diabetes (HbA<sub>1c</sub> 83 mmol/mol, 9.7%), with add-on insulin glargine started before surgery. Post-operative treatment included metformin, gliclazide, insulin glargine and pancreatic enzyme replacement therapy (lipase, Creon®, 150,000 U/d). Faecal elastase-1 concentration was 18 µg/g (normal values >200), confirming severe pancreatic exocrine failure and the need for replacement therapy.

She was referred to our Diabetology unit in July 2012, 17 months after surgery. She had lost 16 kg since surgery, and gliclazide was stopped due to hypoglycaemia. HLA typing was DQB1\*03:01/DRB1\*04:01 (neutral for type 1 diabetes susceptibility) and DQB1\*05:03/DRB1\*14:01, a rare haplotype with unknown association with disease susceptibility or protection. Anti-thyrepoxydase aAbs were positive (118 IU/mL; positive threshold >34). GADA were also positive at high titre, IA-2 and ZnT8 aAbs were negative.

Insulin glargine was stopped 3 months later (October 2012), and resumed after 2 months in light of positive GADA and intolerance to metformin doses higher than 1,500 mg/day. With this treatment, blood glucose levels remained at target, with HbA<sub>1c</sub> values between 43 and 55 mmol/mol (6.1-7.2%) for more than 6 years. In 2015, i.e. 3 years after surgery, fasting C-peptide concentration was 0.27 nmol/L (normal values 0.27-1.27 nmol/L) and raised to 0.35 nmol/L 6 min after a 1-mg intravenous glucagon challenge. Peripheral blood mononuclear cells (PBMCs) were collected for T-cell assays.

Another aAb measurement performed 6 years after the initial one (2019) documented increasing GADA titres and the appearance of high-titre anti-ZnT8, both persisting one year later. Anti-smooth muscle cell aAbs were positive on two occasions 1 year apart (1:2,000, then 1:50; positive threshold >1:20). One year later, fasting C-peptide values were lower than before (0.13 nmol/L), but still increased to 0.21 nmol/L 2 h after stimulation by a mixed meal test, documenting some residual insulin secretion. At the last follow-up in November 2020, fasting C-peptide was 0.10 nmol/L, and 0.15 nmol/L after a mixed meal test. A second PBMC sample was collected.

The patient gave written informed consent and the study was approved by the Cochin Hospital Ethics Committee.

## METHODS

### Immunohistochemistry

Beta-cell area, alpha-cell area and CD8<sup>+</sup>, CD4<sup>+</sup> and FOXP3<sup>+</sup> insulitis were assessed on 5 consecutive 4- $\mu$ m sections taken from 4 different distal regions of peri-tumoural pancreatic tissue (excluding the uncinate process). Immunohistochemical staining employed an avidin-biotin-peroxidase system. Briefly, sections were placed in a Bond III Automated Immunohistochemistry Vision Biosystem (Leica). Tissues were deparaffinised and pre-treated with EDTA buffer, pH 8.8 at 98°C for 20 min. After washing, peroxidase activity was blocked for 10 min using the Bond Polymer Refine Detection Kit DC9800 (Leica). Tissues were washed again and incubated with primary antibodies for 20 min: anti-insulin (RRID:AB\_10013624, 1/500), anti-glucagon (RRID:AB\_10013726, 1/1000), anti-CD8 (clone C8/144B, RRID:AB\_2075537; 1/100), anti-CD4 (clone 4B12, RRID:AB\_10554438; 1/200), anti-FOXP3 (clone 236A/E7, RRID:AB\_445284; 1/80), as previously described [5]. Tissues were subsequently coated with polymer for 10 min and developed with 3,3'-Diaminobenzidine for 8 min. Slices were washed with distilled water and counterstained with acid hemalum (Mayer). Quantifications were performed with a Hamamatsu Nanozoomer and the NDP.view software. Pancreatic and islet areas on insulin- or glucagon-stained slides were manually defined based on morphology, irrespective of islet size (possibly excluding clusters of few (<10) cells lacking an identifiable islet architecture). Insulin and glucagon area is expressed as the percentage of pancreatic area. Insulitis was scored as positive according to a threshold level of  $\geq 15$  CD8<sup>+</sup> cells/islet, a more restrictive definition than the consensus criterion of  $\geq 15$  CD45<sup>+</sup> cells/islet [6]. CD4<sup>+</sup> insulitis was assessed with the same criteria.

### Immunofluorescence

To validate FOXP3 staining, CD25<sup>hi</sup>CD127<sup>lo</sup> Tregs and CD25<sup>-</sup>CD127<sup>+</sup> Teffs were sorted and expanded as described [7], fixed and permeabilised for flow cytometry staining with anti-FOXP3 antibody (clone 259D/C7, RRID:AB\_11153143), or paraffin-embedded for immunofluorescence staining. Consecutive pancreatic sections were analysed by triple immunofluorescence to simultaneously evaluate the expression of CD8 or FOXP3 along with insulin and glucagon. After stepwise deparaffinisation and rehydration (xylene-I and xylene II, 20 min/each; ethanol 100%, 95%, 80%, 75%, 5 min/each), sections were subjected to heat-induced antigen retrieval using Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, 0.05% Tween-20, pH 9.0) for 20 min at 100°C. After cooling and incubation in 5% goat serum (Sigma) to reduce non-specific reactions, sections were stained in 5% goat serum overnight at 4°C with antibodies against CD8 (RRID:AB\_2075537; 1:50, final concentration 3.2  $\mu$ g/ml) or FOXP3 (clone 236A/E7, RRID:AB\_467556; 1:40, 12.5  $\mu$ g/ml). After 3 washes in PBS, sections were incubated with pre-diluted guinea pig anti-insulin (RRID:AB\_2800361) for 20 min, followed by rabbit anti-glucagon (RRID:AB\_10013726; 1:100, final concentration 352  $\mu$ g/ml) in 5% goat serum for 1 h. Secondary antibodies were subsequently added at 1:500 in PBS for 1 h: Alexa-Fluor594-labelled goat anti-guinea pig IgG (RRID:AB\_2535856), Alexa-Fluor647-labelled goat anti-rabbit IgG (RRID:AB\_2535813), Alexa-Fluor488-labelled goat anti-mouse IgG (RRID:AB\_2534088). Sections were counter-stained with DAPI (Sigma; 1:3,000) and mounted with Vectashield antifade (RRID:AB\_2336789) or

fluorescence mounting medium (Dako/Agilent) and analysed immediately or stored at 4°C prior to acquisition on a Leica TCS SP5 confocal laser scanning microscope with LAS software and manual counting of CD8<sup>+</sup> and FOXP3<sup>+</sup> cells. CD8<sup>+</sup> insulitis was defined based on  $\geq 15$  CD8<sup>+</sup> cells/islet. FOXP3<sup>+</sup> insulitis was defined based on  $\geq 1$  FOXP3<sup>+</sup> cells/islet.

### **T-cell assays**

Frozen-thawed PBMCs were processed as described [8, 9], plated at  $10^6$  cells/well in 96-well flat-bottom plates and stimulated for 48 h, as described [8], in the presence of 10 µg/ml of the following antigens: proinsulin (kindly provided by Lilly), insulin (Actrapid®, Novo Nordisk), C peptide (SynPeptide), preproinsulin signal peptide (GL Biochem), GAD65 (Diamyd), intracellular IA-2 (amino acids 214-591; kindly provided by J.F. Elliott, University of Alberta, Canada), adenoviral lysate (produced in-house) and phytohemagglutinin (PHA, 1 µg/ml; Sigma). All antigens were >95% pure and with an endotoxin concentration <5 EU/mg. At the end of the 48 h culture, non-adherent cells were washed and plated at  $2 \times 10^5$  cells/well in triplicate wells of 96-well PVDF enzyme-linked immunospot (ELISpot) plates coated with either an anti-interferon (IFN)- $\gamma$  or anti-interleukin (IL)-10 antibody (U-CyTech). After 6 h of incubation, plates were developed as described [8], counted on a BioSys 5000 Pro-SF Bioreader and results expressed as mean spot-forming cells/ $10^6$  PBMCs normalised across samples. T-cell responses were scored as positive for counts >2SD above spontaneous background responses in the absence of antigen, as determined by receiver-operator characteristic analysis.

## RESULTS

### Decreased beta-cell area and mixed CD8/FOXP3<sup>+</sup> insulitis at the time of surgery

Quantitative immunochemistry was performed on four blocks from the head of the pancreas, distant from the tumour and from areas of chronic pancreatitis (Fig. 2a). On slides stained for insulin (section area 56-250 mm<sup>2</sup>, total 513 mm<sup>2</sup>), a total of 119 islets were identified. On slides stained for glucagon (section area 44-219 mm<sup>2</sup>, total 416 mm<sup>2</sup>) a total of 320 islets were identified. Representative images are shown in Fig. 2b-c. The total fractional islet areas were 0.54% and 1.31% of the pancreas area on slides stained for insulin and glucagon respectively, ranging from 0.19% to 3.6% in different sections (Fig. 2d). When considering the 4 blocks altogether, the beta-cell/islet area and alpha-cell/islet area were 50% (range 0-97%) and 65% (range 18-100%), respectively (Fig. 2d). Sixty-five islets (55%) were insulin-positive (referred to as insulin-containing islets, ICIs, from hereon), with wide heterogeneity across blocks (range 0-95%); 263 islets (82%) were glucagon-positive (range 36-100%) (Fig. 2e).

Representative islets are shown that display peri-insulitis (Fig. 2f-h) and invasive (intra-islet) insulitis (Fig. 2i-k). Overall, CD8<sup>+</sup>, CD4<sup>+</sup> and regulatory (FOXP3<sup>+</sup>) peri-insulitis was observed in 28%, 16% and 8% of the islets, respectively. CD8<sup>+</sup>, CD4<sup>+</sup> and FOXP3<sup>+</sup> intra-islet insulitis was observed in 8%, 5% and 16% of the islets, respectively (Figure 2e).

In type 1 diabetes, insulitis is preferentially localized in ICIs as compared to insulin-deficient islets (IDIs, i.e. insulin-negative and glucagon-positive) [10]. We therefore analysed adjacent sections by triple immunofluorescence to verify whether this was the case also in our patient and to quantify the number of CD8<sup>+</sup> vs. FOXP3<sup>+</sup> cells. First, the specificity of FOXP3 staining was confirmed on tonsil tissue sections and on sorted, *in-vitro* expanded Tregs and Teffs (ESM Fig. 1). Representative images of CD8/insulin/glucagon and FOXP3/insulin/glucagon staining are shown in Fig. 3. Results were remarkably similar between sections stained for CD8 and FOXP3 (Fig. 4a-b). ICIs accounted for 58-63% of islets. CD8<sup>+</sup> and FOXP3<sup>+</sup> insulitis (defined as  $\geq 15$  CD8<sup>+</sup> cells/islet and  $\geq 1$  FOXP3<sup>+</sup> cells/islet, respectively) were present in 90% and 85% of ICIs vs. 32% and 35% of IDIs, respectively ( $p \leq 0.001$ ). Insulitis displayed a predominant peri-islet pattern for both CD8<sup>+</sup> and FOXP3<sup>+</sup> cells (85% and 73% in ICIs vs. 100% and 100% in IDIs, respectively). Also when analysing the number of CD8<sup>+</sup> cells and FOXP3<sup>+</sup> cells per islet (Fig. 4c-d), both were more abundant in ICIs than in IDIs. Overall, the number of CD8<sup>+</sup> cells was approximately 10-fold higher than for FOXP3<sup>+</sup> cells in peri-islet infiltrates, while it was similar in the less common intra-islet infiltrates. As reported [11], some infiltration was also noted in the exocrine tissue for CD8<sup>+</sup> cells; and, to a lesser extent, for FOXP3<sup>+</sup> cells.

Collectively, these findings document a heterogeneous pattern of beta-cell loss and ICI-polarised insulitis, as commonly observed in type 1 diabetic patients [12]. This insulitis displayed a preferential peri-islet pattern and an unusual FOXP3<sup>+</sup> infiltrate suggestive of an ongoing regulatory process.



### **Regulatory polarisation of circulating beta-cell-reactive T-cell responses waning over time**

Given the hallmarks of heterogeneous beta-cell loss and preservation along with the regulatory features of insulinitis observed in pancreatic sections, we analysed the responses against beta-cell antigens of circulating T cells obtained 4 and 9 years after surgery ([Fig. 5](#)). We employed an assay that stimulates unfractionated PBMCs with protein/polypeptide antigens to detect CD4<sup>+</sup> and, to a lesser extent, CD8<sup>+</sup> T-cell responses, without biasing towards IFN- $\gamma$  or IL-10 responses [8]. Weak IFN- $\gamma$  responses were initially present, mainly against proinsulin and IA-2, accompanied by a dominant IL-10 response against proinsulin and, to a lesser extent, C-peptide, GAD and insulin. In the follow-up sample taken 5 years later (i.e. after anti-ZnT8 seroconversion and the rise in insulin needs), IL-10 responses remained dominant but did not significantly increase. Conversely, IFN- $\gamma$  responses were more robust against all the beta-cell antigens tested, and significantly increased overall ( $p=0.03$ ). Both IFN- $\gamma$  and IL-10 responses remained stable against the adenoviral lysate and PHA positive controls. The dominant IFN- $\gamma$  responses detected in 2 age-matched type 1 diabetic patients are shown for comparison.

Collectively, these results suggest a pattern of T-cell autoimmunity with an initial regulatory polarisation that waned over time.

## DISCUSSION

Several lines of evidence support the diagnosis of type 1 diabetes in this patient: positive and increasing GADA titres; the late appearance of anti-ZnT8 aAbs; the associated thyroid autoimmunity and positivity for anti-smooth muscle cell aAbs; and the histopathological finding of insulitis and decreased beta-cell area 6 years after diabetes diagnosis. However, this patient maintained some endogenous insulin secretion for up to 8 years post-surgery, suggesting a slowly evolving form of type 1 diabetes. A plausible histopathological correlate of this slow clinical progression was an insulitis pattern enriched in FOXP3<sup>+</sup> T cells. Also in peripheral blood, T-cell responses against islet antigens were skewed towards secretion of the regulatory cytokine IL-10, while IFN- $\gamma$  is usually found in type 1 diabetic patients [13].

Given the age of this patient and the higher degree of beta-cell loss and insulitis observed in children [14-17], the most informative comparison is with published case series of adult recent-onset type 1 diabetic and at-risk single-aAb<sup>+</sup> cases. Moreover, quantification of insulitis in the pancreas of living type 1 diabetic patients is seldom available [18, 19], and the best-matched case series may be the one reported by the DiViD study on 6 living patients aged 24-35 years with recent-onset type 1 diabetes [19].

First, in the present case, 55% of the islets were insulin-positive, which is in the 18–66% range observed in the DiViD study. The proportion of ICIs and insulitis-positive islets was very variable across DiViD patients and, like in our case, from one section to another [19]. It should however be noted that the sections analysed herein were probably more distant between each other than the ones in the DiViD study. The beta-cell area in our patient was 0.27% of the total pancreas surface, which is even lower than the 0.44-1.2% observed in DiViD patients [19], and much lower than the average 0.8% observed in single-aAb<sup>+</sup> non-diabetic adult donors [20]. Moreover, the beta-cell/islet area ratio was lower than for alpha cells, whereas this ratio, although highly variable, is higher for beta cells in non-diabetic subjects [21, 22]. Thus, several lines of evidence suggest that significant beta-cell loss took place before surgery. Moreover, this beta-cell loss may have been underestimated because we only had access to tissue from the head of the pancreas, which is the less affected region in type 1 diabetic patients [23].

Second, CD8<sup>+</sup> lymphocytic infiltrates were detected in 36% of islets (28% peri-islet plus 8% intra-islet) as compared to 5-58% of CD3<sup>+</sup> ICIs in the DiViD study (i.e. 5%, 12%, 23%, 33%, 52% and 58% for the 6 DiViD patients) [19]. Also in other type 1 diabetes case series, the fraction of infiltrated islets is typically low (<10%) [10, 14, 15]. At earlier disease stages, little or no insulitis is observed (1-9% of islets) in single- and multiple-aAb<sup>+</sup> donors, even in those with predisposing HLA haplotypes [10, 15, 24, 25]. Thus, substantial insulitis lesions were present in this patient, more extensive than those observed in most type 1 diabetic cases.

Third, the striking histopathological hallmark of the present case was the abundance of infiltrating FOXP3<sup>+</sup> cells, which are virtually absent in the pancreas of type 1 diabetic donors, and even in non-diabetic cases [10]. In a series of 16 recent-onset type 1 diabetic cases, scattered FOXP3<sup>+</sup> cells

were detected in two islets of one single patient and not in non-diabetic controls, using the same antibody employed herein [26]. Similarly, FOXP3 gene expression in the DiViD series was modest [19]. Moreover, the insulinitis of type 1 diabetic patients mostly displays a peri-islet localization, with very little islet invasion [10, 25]. This was also the case of this patient, as all T cells were predominantly observed around the islets. However, FOXP3<sup>+</sup> cells were also found inside the islets.

This FOXP3-enriched insulinitis was mirrored in the blood by the presence of islet-reactive T cells that were skewed towards a regulatory, IL-10-secreting phenotype, as reported in slow-progressing type 1 diabetic and healthy adults [13, 27] and in ‘partially regulated’, IL-10<sup>+</sup>, pauci-aAb<sup>+</sup>, pauci-immune type 1 diabetic children and adolescents [16].

Despite significant beta-cell loss and islet infiltration, insulin requirements remained modest over time, suggesting a slowly evolving islet destruction. Although the weight loss after surgery likely contributed to these reduced insulin needs, this lack of progression is striking when considering the additional beta-cell deprivation imposed by duodenopancreatectomy. Collectively, these histopathological, immunological and clinical findings suggest that, after having destroyed a significant beta-cell fraction, the autoimmune reaction remained efficiently regulated over several years. We may speculate that the autoimmune process in the islets may have been hindered by exposure to the tumour microenvironment, which is often enriched in suppressive cytokines such as transforming growth factor (TGF)- $\beta$  that drive Treg differentiation [28]. Indeed, TGF- $\beta$  mRNA was reportedly expressed in two pancreatic serous cystadenomas [29]. The CD8<sup>+</sup> T-cell infiltration may have been another driver for FOXP3<sup>+</sup> T cells, as suggested in the NOD mouse [30].

Finally, the late raise of GADA titres, anti-ZnT8 seroconversion and increase in circulating IFN- $\gamma$  T-cell responses against islet antigens concomitant with increased insulin needs suggest that immune regulation was eventually lost. This progression may be in favour of a relapsing-remitting model of type 1 diabetes [4]. Several autoimmune diseases, e.g. vitiligo, multiple sclerosis and rheumatoid arthritis, follow a course marked by flares alternating with remission periods. In the present case, the early histopathological findings and late clinical and autoimmune progression are consistent with an initial autoimmune flare followed by several years of remission before eventual relapse. Nonetheless, some endogenous insulin secretion was maintained. Also in other autoimmune conditions, end-stage organ failure is not invariably attained even after significant tissue loss. For instance, subclinical hypothyroidism, i.e. positivity of anti-thyroperoxidase aAbs and increased thyroid-stimulating hormone levels with normal thyroxine levels, is reported to progress to overt hypothyroidism at a rate of only 4% per year [31]. Indeed, the prevalence of subclinical aAb<sup>+</sup> conditions is largely higher than that of overt functional failure for most organ-specific autoimmune diseases [32, 33]. In the case of type 1 diabetes, most slow-onset forms are associated with isolated GADA, as initially observed in this patient. While the majority eventually requires insulin treatment, one third remains insulin-free [34]. However, in the context of pancreas transplantation, anti-ZnT8 seroconversion has been shown to predict loss of graft function [35]. A similar phenomenon may have occurred in our patient.

In conclusion, we here describe the case of a type 1 diabetic patient who, despite positivity for GADA and other aAbs, significant insulinitis and beta-cell loss, and partial pancreatectomy, remained well-controlled with low-dose insulin till anti-ZnT8 seroconversion 14 years after diabetes onset. Both islet infiltrates and circulating islet-reactive T cells were skewed towards a regulatory phenotype. These observations suggest an islet autoimmune process that remained spontaneously regulated for several years after substantial beta-cell destruction. The definition of the mechanisms behind these indolent forms of more or less “benign” autoimmunity may lead to better understanding of type 1 diabetes physiopathology and to the definition of novel therapeutic targets [36, 37].

**Acknowledgments.** We thank Dr. Raphaël Scharfmann (INSERM U1016 Cochin Institute, Paris) for reviewing the manuscript.

**Data availability.** Data are available from the authors on request.

**Funding.** This work was performed within the *Département Hospitalo-Universitaire* (DHU) AutHorS and supported by grants from the JDRF (2-SRA-2016-164-Q-R), the *Fondation pour la Recherche Médicale* (EQU20193007831), the *Agence Nationale de la Recherche* (ANR-19-CE15-0014-01) and the Innovative Medicines Initiative 2 Joint Undertaking under grant agreements 115797 and 945268 (INNODIA and INNODIA HARVEST), which receive support from the EU Horizon 2020 program, the European Federation of Pharmaceutical Industries and Associations, JDRF, and The Leona M. & Harry B. Helmsley Charitable Trust. ZZ is supported by JDRF Postdoctoral Fellowship 3-PDF-2020-942-A-N. The funding sources had no involvement in study design; in the collection, analysis, and interpretation of data; in the writing of the report; and in the decision to submit the manuscript.

**Authors’ relationships and activities.** The authors declare that there are no relationships or activities that might bias, or be perceived to bias, their work.

**Contribution statement.** PF performed histology quantifications; FB, DF, GS performed histopathology studies; GA, ZZ performed T-cell assays; BD performed surgery; CB, FD provided intellectual input; RM, EL designed the study, interpreted the data and wrote the manuscript; EL is the diabetologist in charge of the patient. All authors approved the version to be published. The corresponding author confirms that he had full access to all the data in the study and had final responsibility for the decision to submit for publication.

## Figure legends

**Figure 1. Schematic timeline of the evolution of aAbs, clinical parameters, treatments and related pancreas histopathology and blood T-cell analyses.** The patient's clinical history before and after surgery (2011) is depicted. The time of sampling for pancreas tissue (2011) and PBMCs (2015, 2020) is indicated.

**Figure 2. Tissue macroscopy, immunohistochemistry and quantification of endocrine areas and insulinitis at surgery.** **a.** Macroscopy of the tumour and surrounding pancreas. The tumour (red arrow), the duct of Wirsung (white arrow) and the head of the pancreas where tissue sections were taken (black arrow) are indicated. Scale bar, 10 mm. **b-c.** Representative tissue sections stained for insulin (INS, b) and glucagon (GCG, c). **d.** Percent tissue section area for islets, beta ( $\beta$ ) cells and alpha ( $\alpha$ ) cells (d, left); and percent islet tissue area for beta and alpha cells (d, right). **e.** Percent positive islets for the indicated markers. Each symbol represents one of the 4 tissue blocks analysed; black symbols display values for the 4 blocks considered altogether. **f-h.** Representative peri-insulitis stained for CD8 (f), CD4 (g) and FOXP3 (h). **i-k.** Representative invasive insulinitis (intra-islet) stained for CD8 (i), CD4 (j) and FOXP3 (k). Scale bar, 100  $\mu$ m.

**Figure 3. Immunofluorescence images of pancreas sections at surgery.** **a-c.** Representative staining for CD8 (green), insulin (INS, red) and glucagon (GCG, blue) of one ICI (a), one IDI (b) and of exocrine tissue (c). **d-f.** Representative staining for FOXP3 (green), INS (red) and GCG (blue) of one ICI (d), one IDI (e) and of exocrine tissue (f). Scale bars, 50  $\mu$ m.

**Figure 4. Immunofluorescence quantification.** **a-b.** Fraction of ICIs and IDIs (top rows), insulinitis-positive ICIs and IDIs (middle rows) and of peri-islet insulitis in insulitis-positive ICIs and IDIs in CD8-stained sections (a) and FOXP3-stained sections (b). CD8<sup>+</sup> and FOXP3<sup>+</sup> insulitis was scored as positive in islets with  $\geq 15$  CD8<sup>+</sup> cells and  $\geq 1$  FOXP3<sup>+</sup> cells, respectively.  $p \leq 0.001$  for the comparison between insulitis-positive ICIs and IDIs by Fisher exact test (for both CD8 and FOXP3 staining). **c-d.** Number of CD8<sup>+</sup> cells (c) and FOXP3<sup>+</sup> cells (d) in ICIs (red symbols), IDIs (blue symbols) and exocrine tissue (gray symbols). For ICIs and IDIs, cell numbers are displayed for total islets (circles) and for those displaying a peri-islet infiltrate (squares) and an intra-islet infiltrate (diamonds). \*\*\* $p \leq 0.0004$ , \* $p = 0.02$  by Mann-Whitney test.

**Figure 5. IFN- $\gamma$  and IL-10 T-cell responses against beta-cell autoantigens 4 and 9 years after surgery (2015, 2019).** Frozen-thawed PBMCs were analysed by ELISpot following a 48 h culture in the presence of the indicated beta-cell antigens, an adenoviral (AdV) lysate and PHA positive controls or no antigen. Each symbol represents the mean number of spot-forming cells/ $10^6$  PBMCs from triplicate wells. The dotted line indicates the positive cut-off, corresponding to 2SD above mean values from wells stimulated in the absence of antigen. The responses detected in 2 age-matched type 1 diabetic (T1D) patients are shown for comparison. \* $p = 0.03$  by Wilcoxon signed rank test.

## References

- [1] Atkinson MA, Eisenbarth GS, Michels AW (2014) Type 1 diabetes. *Lancet* 383: 69-82. [https://doi.org/10.1016/S0140-6736\(13\)60591-7](https://doi.org/10.1016/S0140-6736(13)60591-7)
- [2] Katsarou A, Gudbjornsdottir S, Rawshani A et al (2017) Type 1 diabetes mellitus. *Nat Rev Dis Primers* 3: 17016. <https://doi.org/10.1038/nrdp.2017.16>
- [3] Craig ME, Kim KW, Isaacs SR et al (2019) Early-life factors contributing to type 1 diabetes. *Diabetologia* 62: 1823-1834. <https://doi.org/10.1007/s00125-019-4942-x>
- [4] von Herrath M, Sanda S, Herold K (2007) Type 1 diabetes as a relapsing-remitting disease? *Nat Rev Immunol* 7: 988-994. <https://doi.org/10.1038/nri2192>
- [5] Le Goux C, Vacher S, Pignot G et al (2017) mRNA Expression levels of genes involved in antitumor immunity: Identification of a 3-gene signature associated with prognosis of muscle-invasive bladder cancer. *Oncoimmunology* 6: e1358330. <https://doi.org/10.1080/2162402X.2017.1358330>
- [6] Campbell-Thompson ML, Atkinson MA, Butler AE et al (2013) The diagnosis of insulinitis in human type 1 diabetes. *Diabetologia* 56: 2541-2543. <https://doi.org/10.1007/s00125-013-3043-5>
- [7] Putnam AL, Brusko TM, Lee MR et al (2009) Expansion of human regulatory T-cells from patients with type 1 diabetes. *Diabetes* 58: 652-662.
- [8] Martinuzzi E, Afonso G, Gagnerault MC et al (2011) acDCs enhance human antigen-specific T-cell responses. *Blood* 118: 2128-2137. <https://doi.org/10.1182/blood-2010-12-326231>
- [9] Mallone R, Mannering SI, Brooks-Worrell BM et al (2011) Isolation and preservation of peripheral blood mononuclear cells for analysis of islet antigen-reactive T cell responses: position statement of the T-Cell Workshop Committee of the Immunology of Diabetes Society. *Clin Exp Immunol* 163: 33-49. <https://doi.org/10.1111/j.1365-2249.2010.04272.x>
- [10] Carré A, Richardson SJ, Larger E, Mallone R (2021) Presumption of guilt for T cells in type 1 diabetes: lead culprits or partners in crime depending on age of onset? *Diabetologia* 64: 15-25. <https://doi.org/doi.org/10.1007/s00125-020-05298-y>
- [11] Rodriguez-Calvo T, Ekwall O, Amirian N, Zapardiel-Gonzalo J, von Herrath MG (2014) Increased immune cell infiltration of the exocrine pancreas: a possible contribution to the pathogenesis of type 1 diabetes. *Diabetes* 63: 3880-3890. <https://doi.org/10.2337/db14-0549>
- [12] Coppieters KT, Dotta F, Amirian N et al (2012) Demonstration of islet-autoreactive CD8 T cells in insulinitic lesions from recent onset and long-term type 1 diabetes patients. *J Exp Med* 209: 51-60. <https://doi.org/10.1084/jem.20111187>
- [13] Arif S, Tree TI, Astill TP et al (2004) Autoreactive T cell responses show proinflammatory polarization in diabetes but a regulatory phenotype in health. *J Clin Invest* 113: 451-463. <https://doi.org/10.1172/JCI19585>
- [14] In't Veld P (2011) Insulinitis in human type 1 diabetes: The quest for an elusive lesion. *Islets* 3: 131-138. <https://doi.org/10.4161/isl.3.4.15728>
- [15] Campbell-Thompson M, Fu A, Kaddis JS et al (2016) Insulinitis and beta-Cell Mass in the Natural History of Type 1 Diabetes. *Diabetes* 65: 719-731. <https://doi.org/10.2337/db15-0779>
- [16] Arif S, Leete P, Nguyen V et al (2014) Blood and islet phenotypes indicate immunological heterogeneity in type 1 diabetes. *Diabetes* 63: 3835-3845. <https://doi.org/10.2337/db14-0365>
- [17] Leete P, Mallone R, Richardson SJ, Sosenko JM, Redondo MJ, Evans-Molina C (2018) The Effect of Age on the Progression and Severity of Type 1 Diabetes: Potential Effects on Disease Mechanisms. *Curr Diab Rep* 18: 115. <https://doi.org/10.1007/s11892-018-1083-4>

- [18] Imagawa A, Hanafusa T, Tamura S et al (2001) Pancreatic biopsy as a procedure for detecting in situ autoimmune phenomena in type 1 diabetes: close correlation between serological markers and histological evidence of cellular autoimmunity. *Diabetes* 50: 1269-1273.
- [19] Krogvold L, Wiberg A, Edwin B et al (2016) Insulitis and characterisation of infiltrating T cells in surgical pancreatic tail resections from patients at onset of type 1 diabetes. *Diabetologia* 59: 492-501. <https://doi.org/10.1007/s00125-015-3820-4>
- [20] Diedisheim M, Mallone R, Boitard C, Larger E (2016) beta-cell Mass in Nondiabetic Autoantibody-Positive Subjects: An Analysis Based on the Network for Pancreatic Organ Donors Database. *J Clin Endocrinol Metab* 101: 1390-1397. <https://doi.org/10.1210/jc.2015-3756>
- [21] Cabrera O, Berman DM, Kenyon NS, Ricordi C, Berggren PO, Caicedo A (2006) The unique cytoarchitecture of human pancreatic islets has implications for islet cell function. *Proc Natl Acad Sci USA* 103: 2334-2339. <https://doi.org/10.1073/pnas.0510790103>
- [22] Brissova M, Fowler MJ, Nicholson WE et al (2005) Assessment of human pancreatic islet architecture and composition by laser scanning confocal microscopy. *J Histochem Cytochem* 53: 1087-1097. <https://doi.org/10.1369/jhc.5C6684.2005>
- [23] Poudel A, Savari O, Striegel DA et al (2015) Beta-cell destruction and preservation in childhood and adult onset type 1 diabetes. *Endocrine* 49: 693-702. <https://doi.org/10.1007/s12020-015-0534-9>
- [24] In't Veld P, Lievens D, De Grijse J et al (2007) Screening for insulitis in adult autoantibody-positive organ donors. *Diabetes* 56: 2400-2404. <https://doi.org/10.2337/db07-0416>
- [25] Rodriguez-Calvo T, Richardson SJ, Pugliese A (2018) Pancreas Pathology During the Natural History of Type 1 Diabetes. *Curr Diab Rep* 18: 124. <https://doi.org/10.1007/s11892-018-1084-3>
- [26] Willcox A, Richardson SJ, Bone AJ, Foulis AK, Morgan NG (2009) Analysis of islet inflammation in human type 1 diabetes. *Clin Exp Immunol* 155: 173-181. <https://doi.org/10.1111/j.1365-2249.2008.03860.x>
- [27] Tree TI, Lawson J, Edwards H et al (2010) Naturally arising human CD4 T cells that recognize islet autoantigens and secrete IL-10 regulate pro-inflammatory T cell responses via linked suppression. *Diabetes* 59: 1451-1460. <https://doi.org/10.2337/db09-0503>
- [28] Oleinika K, Nibbs RJ, Graham GJ, Fraser AR (2013) Suppression, subversion and escape: the role of regulatory T cells in cancer progression. *Clin Exp Immunol* 171: 36-45. <https://doi.org/10.1111/j.1365-2249.2012.04657.x>
- [29] Van Laethem JL, Resibois A, Rickaert F et al (1997) Different expression of transforming growth factor beta 1 in pancreatic ductal adenocarcinoma and cystic neoplasms. *Pancreas* 15: 41-47. <https://doi.org/10.1097/00006676-199707000-00006>
- [30] Grinberg-Bleyer Y, Saadoun D, Baeyens A et al (2010) Pathogenic T cells have a paradoxical protective effect in murine autoimmune diabetes by boosting Tregs. *J Clin Invest* 120: 4558-4568. <https://doi.org/10.1172/JCI42945>
- [31] Biondi B, Cooper DS (2018) Subclinical Hyperthyroidism. *N Engl J Med* 378: 2411-2419. <https://doi.org/10.1056/NEJMc1709318>
- [32] Van den Driessche A, Eenkhoorn V, Van Gaal L, De Block C (2009) Type 1 diabetes and autoimmune polyglandular syndrome: a clinical review. *Neth J Med* 67: 376-387.
- [33] Merbl Y, Zucker-Toledano M, Quintana FJ, Cohen IR (2007) Newborn humans manifest autoantibodies to defined self molecules detected by antigen microarray informatics. *J Clin Invest* 117: 712-718. <https://doi.org/10.1172/JCI29943>
- [34] Sorgjerd EP, Skorpen F, Kvaloy K, Midthjell K, Grill V (2012) Time dynamics of autoantibodies are coupled to phenotypes and add to the heterogeneity of autoimmune diabetes in

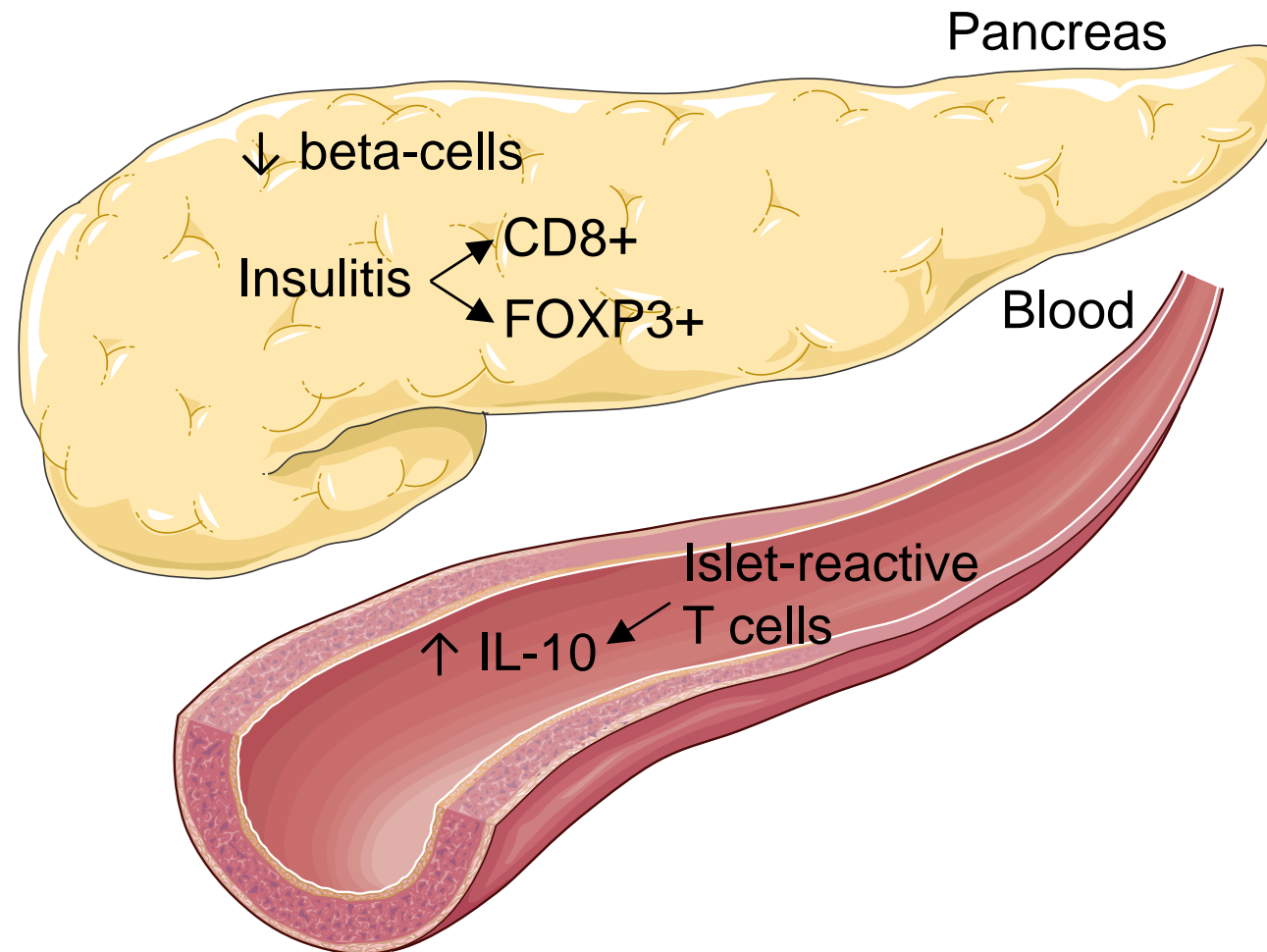
adults: the HUNT study, Norway. *Diabetologia* 55: 1310-1318. <https://doi.org/10.1007/s00125-012-2463-y>

[35] Occhipinti M, Lampasona V, Vistoli F et al (2011) Zinc transporter 8 autoantibodies increase the predictive value of islet autoantibodies for function loss of technically successful solitary pancreas transplant. *Transplantation* 92: 674-677. <https://doi.org/10.1097/TP.0b013e31822ae65f>

[36] Ehlers MR (2018) Who let the dogs out? The ever-present threat of autoreactive T cells. *Sci Immunol* 3. <https://doi.org/10.1126/sciimmunol.aar6602>

[37] Mallone R, Eizirik DL (2020) Presumption of innocence for beta cells: why are they vulnerable autoimmune targets in type 1 diabetes? *Diabetologia* 63: 1999-2006. <https://doi.org/10.1007/s00125-020-05176-7>





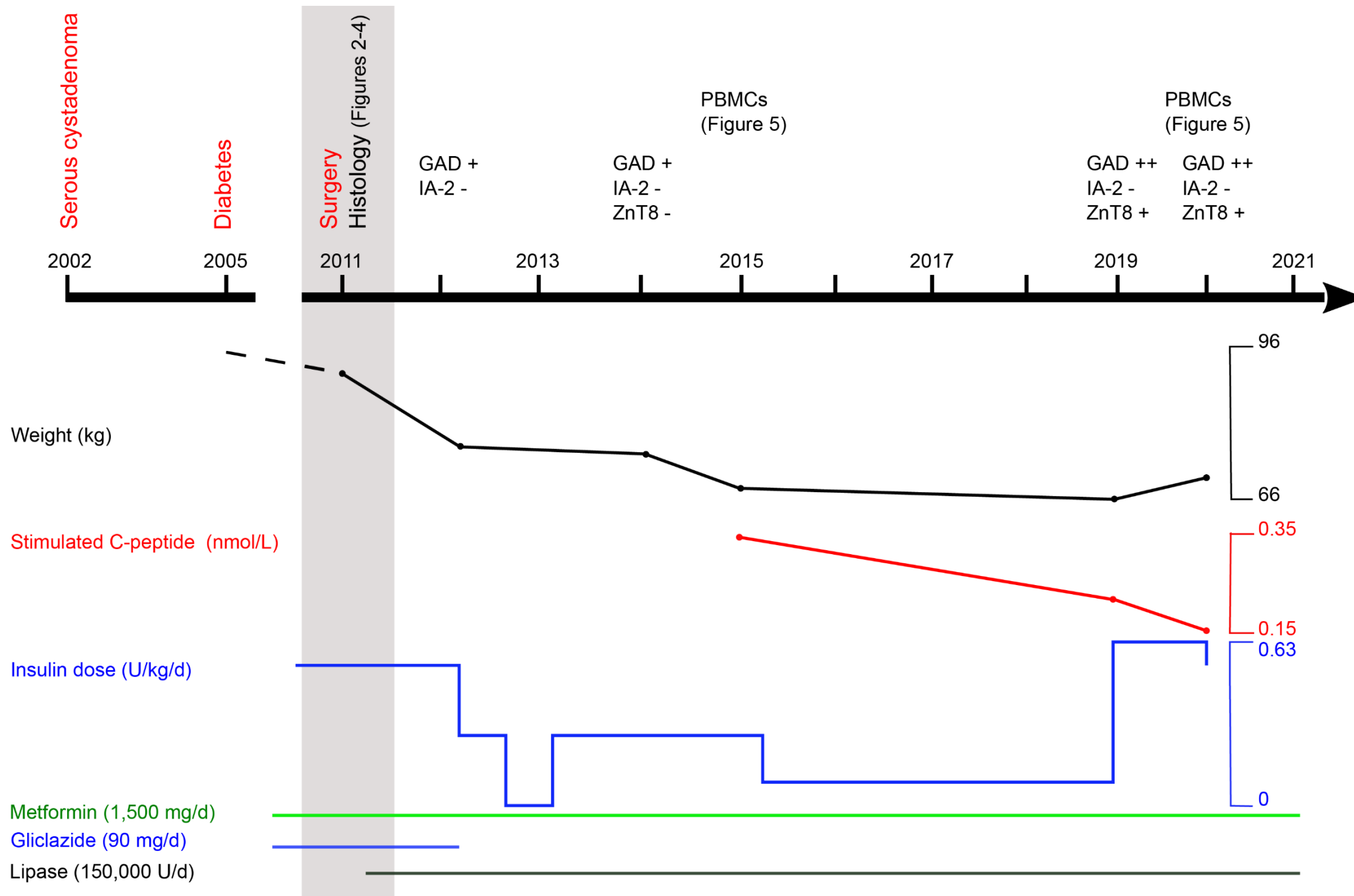
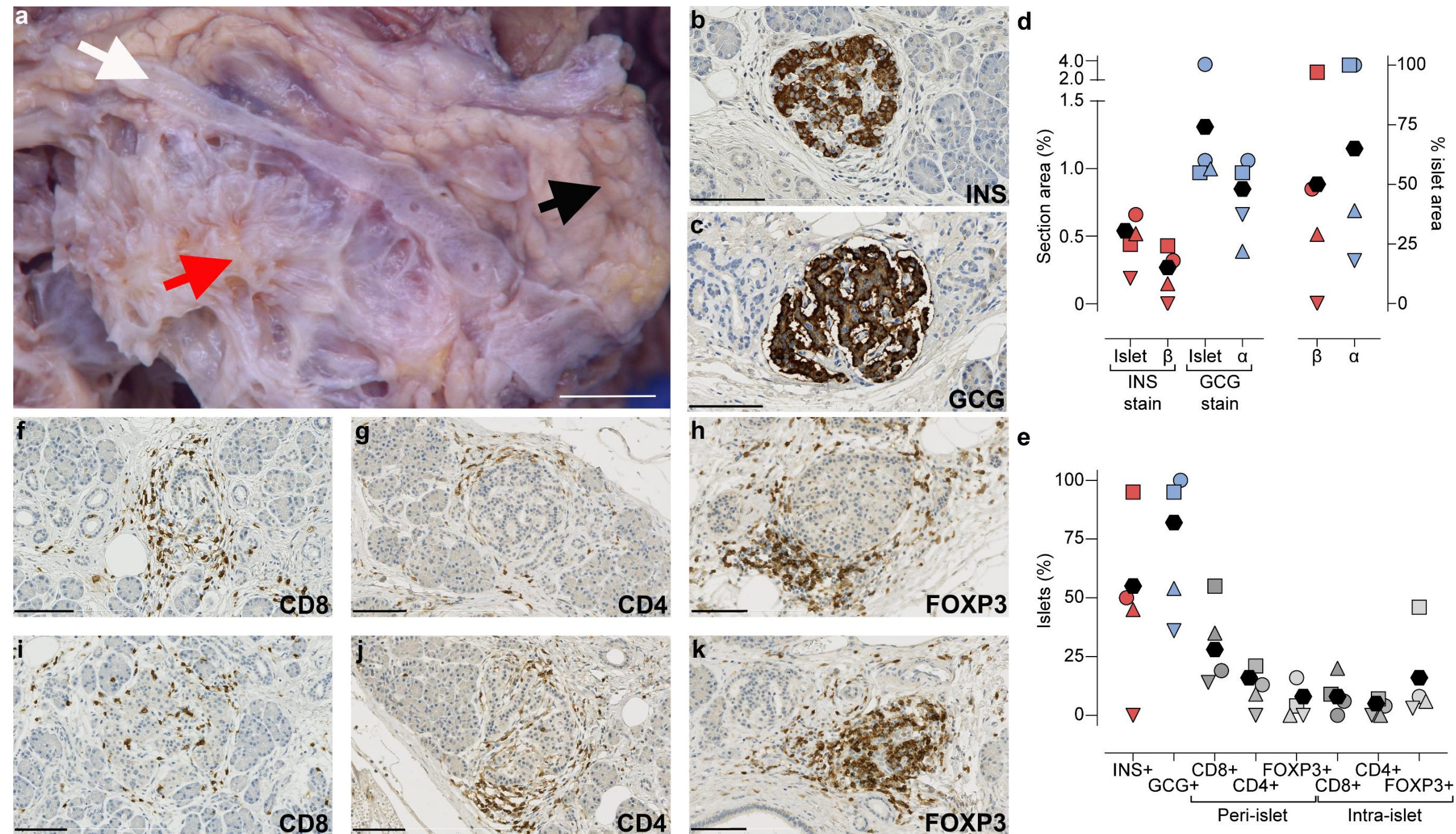


Fig. 1





**Fig. 2**



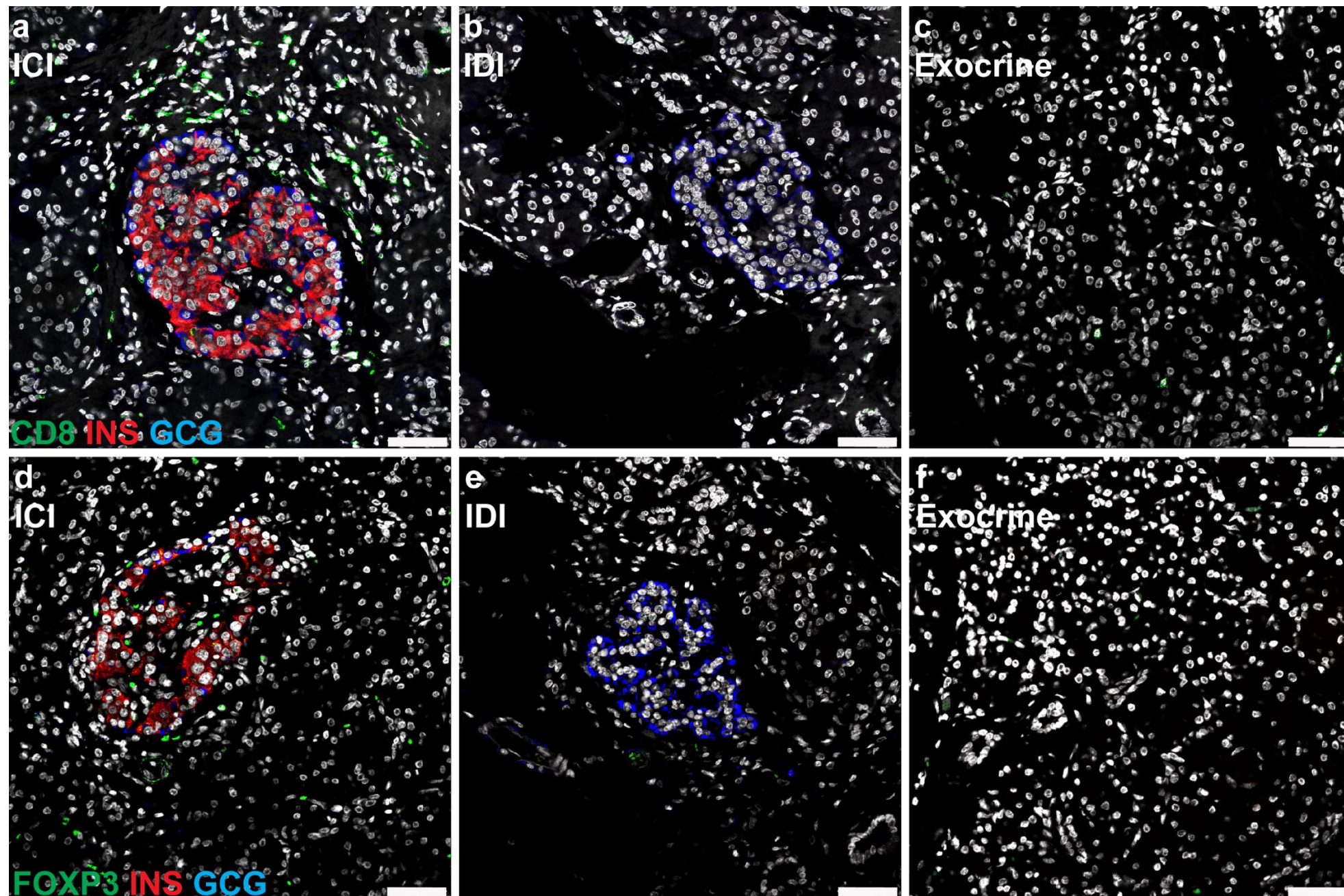


Fig. 3

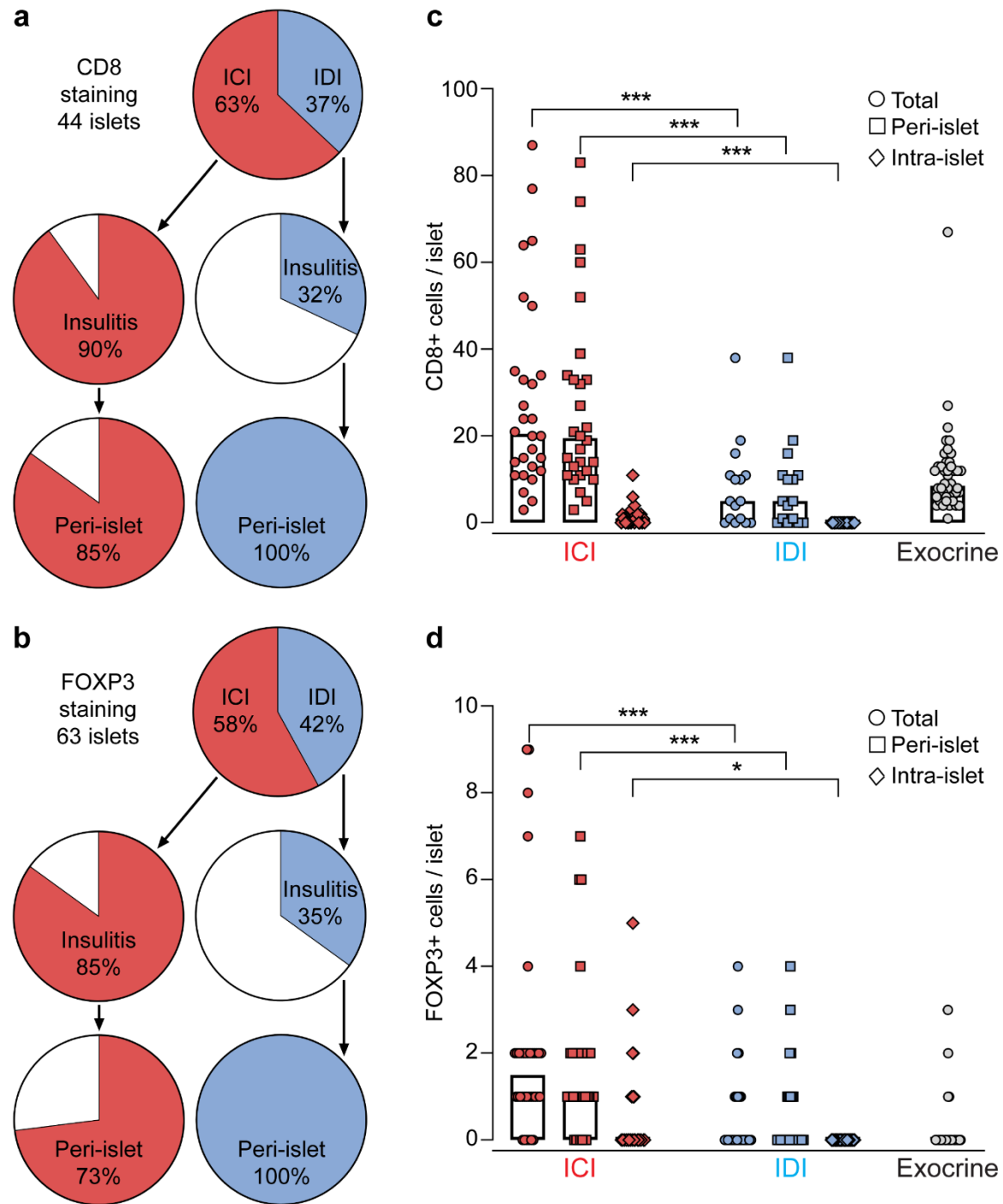


Fig. 4

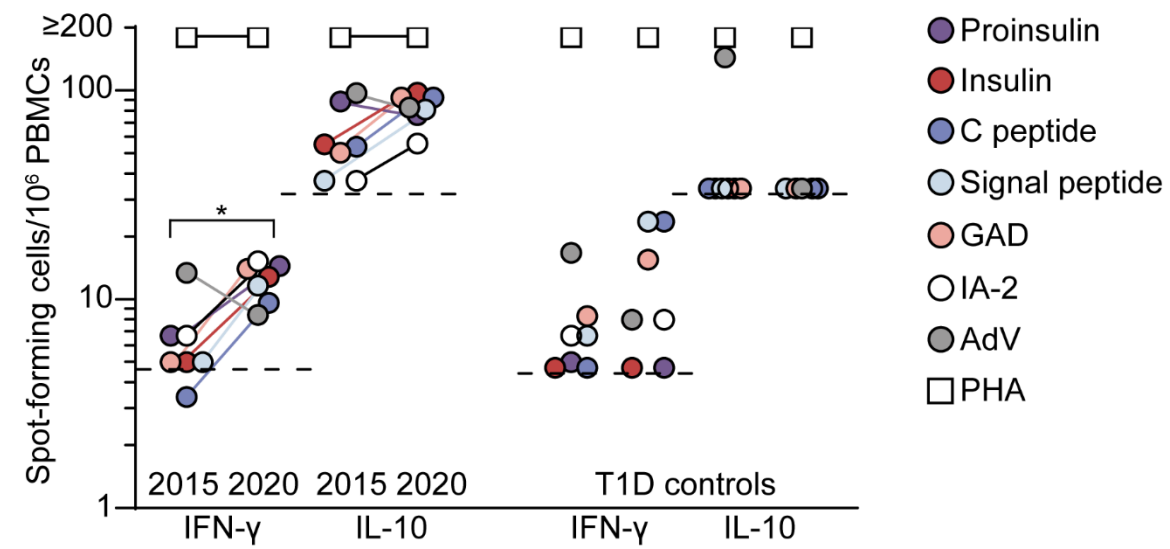


Fig. 5

## ELECTRONIC SUPPLEMENTARY MATERIALS (ESM)

### Immuno-regulated insulinitis and slow-progressing type 1 diabetes after duodenopancreatectomy

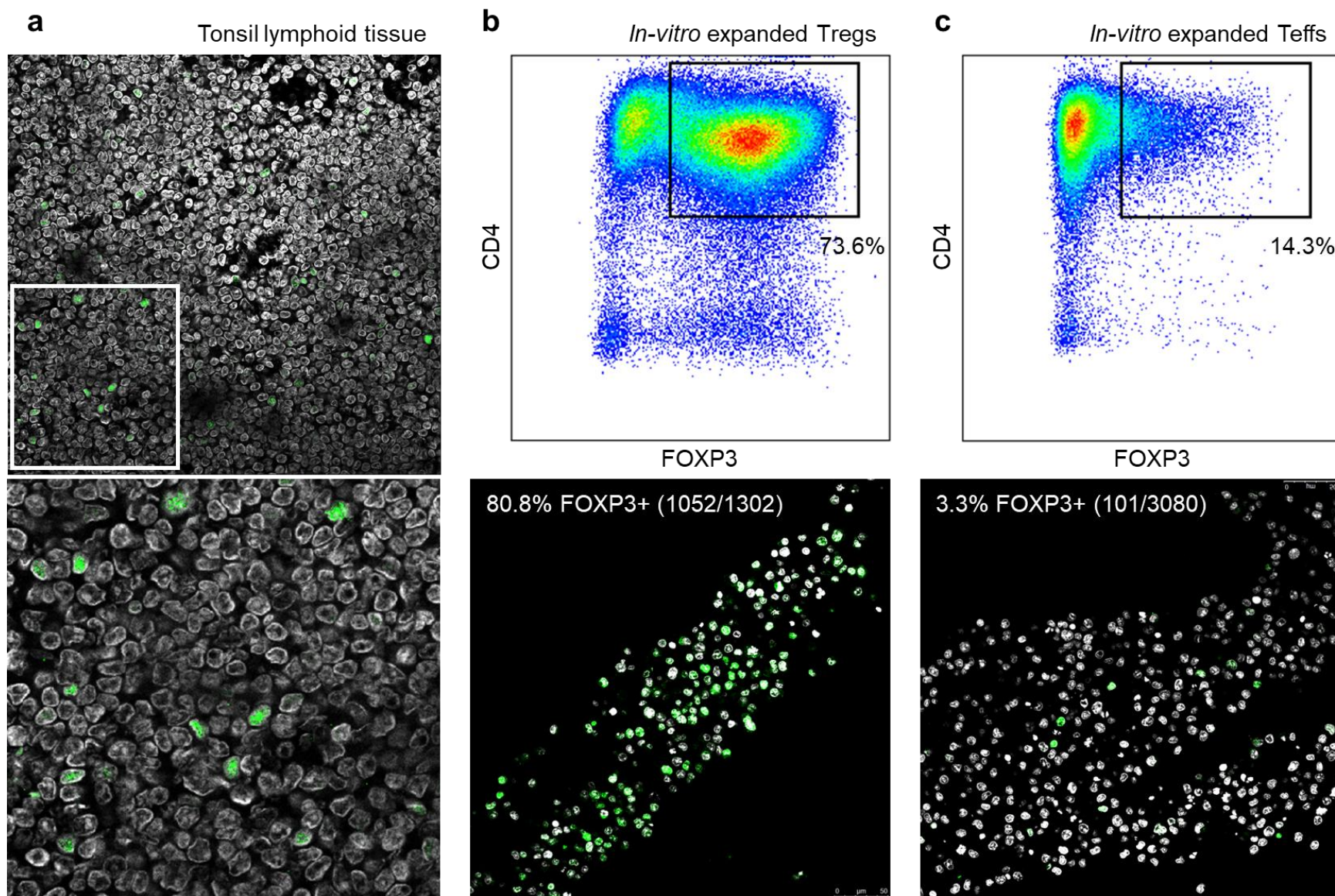
Pauline Faucher, Frédéric Beuvon, Daniela Fignani, Guido Sebastiani, Georgia Afonso, Zhicheng Zhou, Bertrand Dousset, Christian Boitard, Francesco Dotta, Roberto Mallone and Etienne Larger.

**ESM Table 1. Evolution of autoantibodies, clinical parameters and treatments.**

| Date    | Years before/after surgery | GADA (U/mL)      | IA-2A (U/mL) | ZnT8A (U/mL) | Weight (kg) | Stimulated C-peptide (nM) | HbA1c (mmol/mol) (%) | Insulin dose (UI/kg/d) | Metformin (mg/d) | Gliclazide (mg/d) | Lipase (kUI/d) |
|---------|----------------------------|------------------|--------------|--------------|-------------|---------------------------|----------------------|------------------------|------------------|-------------------|----------------|
| 2002    | -9 → serous cystadenoma    |                  |              |              |             |                           |                      |                        |                  |                   |                |
| 2005    | -6 → diabetes              |                  |              |              | 96          |                           |                      |                        | 1,500            | 90                |                |
| 02/2011 | 0 → surgery/histology      |                  |              |              | 92          |                           | 83 (9.7%)            | 0.54 glargine          | 1,500            | 90                |                |
| 07/2012 | 1.4                        |                  |              |              | 76          |                           | 39 (5.7%)            | 0.28 glargine          | 1,500            | —                 | 150            |
| 10/2012 | 1.7                        | <b>132</b>       | 1.0          |              | 80          |                           | 45 (6.3%)            | 0                      |                  |                   |                |
| 04/2014 | 3                          | <b>89.5</b>      | 1.0          | 7.0          | 77          |                           | 43 (6.1%)            | 0.33 glargine          |                  |                   |                |
| 01/2015 | 4 → PBMCs #1               |                  |              |              | 68          | 0.35                      | 55 (7.2%)            | 0.15 glargine          |                  |                   |                |
| 03/2019 | 8                          | <b>&gt;2,000</b> | 1.0          | <b>204</b>   | 66          | 0.21                      | 62 (7.8%)            | 0.63 glargine, aspart  |                  |                   |                |
| 11/2020 | 9.5 → PBMCs #2             | <b>&gt;2,000</b> | 1.0          | <b>90</b>    | 69          | 0.15                      | 61 (7.7%)            | 0.56 glargine, aspart  |                  |                   |                |

IA-2A, IA-2 autoantibodies; ZnT8A, ZnT8 autoantibodies. Positive autoantibody titres are marked in bold. Thresholds for autoantibody positivity: GADA 4 U/ml, IA-2A 1 U/ml, ZnT8A 20 U/ml.





**ESM Figure 1. Validation of the anti-FOXP3 antibody (clone 236A/E7, RRID:AB\_467556) used for immunohistochemistry and immunofluorescence analyses. a.** Representative staining of tonsil lymphoid tissue (top), and higher magnification of the indicated area (bottom). **b-c.** Flow cytometry dot plots (top; gated on viable CD3<sup>+</sup> cells) and representative immunofluorescence fields of paraffin-fixed *in-vitro* expanded Tregs (b) and Teffs (c).