In vivo imaging of type 1 diabetes immunopathology using eye-transplanted islets in NOD mice

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Abstract

Aims/hypothesis Autoimmune attack against the insulin-producing beta cells in the pancreatic islets results in type 1 diabetes. However, despite considerable research, details of the type 1 diabetes immunopathology in situ are not fully understood mainly because of difficult access to the pancreatic islets in vivo.

Methods Here, we used direct non-invasive confocal imaging of islets transplanted in the anterior chamber of the eye (ACE) to investigate the anti-islet autoimmunity in NOD mice before, during and after diabetes onset. ACE-transplanted islets allowed longitudinal studies of the autoimmune attack against islets and revealed the infiltration kinetics and in situ motility dynamics of fluorescence-labelled autoreactive T cells during diabetes development. Ex vivo immunostaining was also used to compare immune cell infiltrations into islet grafts in the eye and kidney as well as in pancreatic islets of the same diabetic NOD mice.

Results We found similar immune infiltration in native pancreatic and ACE-transplanted islets, which established the ACE-transplanted islets as reliable reporters of the autoimmune response. Longitudinal studies in ACE-transplanted islets identified in vivo hallmarks of islet inflammation that concurred with early immune infiltration of the islets and preceded their collapse and hyperglycaemia onset. A model incorporating data on ACE-transplanted islet degranulation and swelling allowed early prediction of the autoimmune attack in the pancreas and prompted treatments to intercept type 1 diabetes.

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Research in context

What is already known about this subject?

- Autoimmune attack on the insulin-producing beta cells in the pancreatic islets causes type 1 diabetes
- The details of how type 1 diabetes pathogenesis progresses over time are still not fully known
- Recurrent diabetes following islet transplant limits the efficacy of islet transplant therapy in individuals with type 1 diabetes

What is the key question?

- Can reporter islets transplanted into the anterior chamber of the eye (ACE) inform early detection of the pathogenesis of type 1 diabetes in the pancreas and prompt timely intervention to halt or delay its development?

What are the new findings?

- ACE-transplanted islets in NOD mice are reliable reporters of the autoimmune attack on islets in the native pancreas
- Predicting the onset of hyperglycaemia in NOD mice is possible, based on early hallmarks of islet inflammation measured non-invasively and longitudinally in ACE-transplanted islets
- Delaying progression of type 1 diabetes or rejection of transplanted islets is possible through timely therapeutic intervention guided by ACE-transplanted islets

How might this impact on clinical practice in the foreseeable future?

- Considered alongside a clinical trial on islet transplantation in the eye, the current research highlights the potential of ACE-transplanted islets to guide and improve the development of new treatment modalities in type 1 diabetes prevention as well as in transplant applications

Conclusions/interpretation The current findings highlight the value of ACE-transplanted islets in studying early type 1 diabetes pathogenesis in vivo and underscore the need for timely intervention to halt disease progression.

Keywords Anterior chamber of the eye · Autoimmune diabetes · Diabetes recurrence · Diabetes transfer · Immune modulation · Intraocular transplantation · Islet degranulation · Islet inflammation · Islet swelling · Local intervention · NOD mice · Non-invasive longitudinal intravital imaging · Pancreatic islet transplant · Prediction of type 1 diabetes · Predictive mathematical model

Abbreviations

ACE	Anterior chamber of the eye
3D	Three-dimensional
IEQ	Islet equivalents
IVICL	In vivo immunocytolabelling
MFI	Median fluorescence intensity
MTH	Median time to hyperglycaemia
PET	Positron emission tomography

Introduction

Type 1 diabetes results from the autoimmune destruction of the insulin-producing beta cells, and currently there are no approved immunotherapies to prevent or treat it [1, 2]. Hence, the immune mechanisms that lead to beta cell destruction and the manifestation of diabetic symptoms are of major interest [3]; nevertheless, despite considerable progress in the past decades, they remain far from being fully

elucidated. Given the various risk and predisposition factors and heterogeneity of type 1 diabetes, it is likely that preventive and therapeutic approaches can be successful only if they are customised based on their mechanisms and initiated early enough to intercept irreversible damage to the functional islet mass (i.e. the point of no return). Therefore, early detection of the anti-islet autoimmunity and investigation of its in situ mechanisms could provide crucial insight into type 1 diabetes pathogenesis and new ways to prevent it.

Real-time in vivo evidence of type 1 diabetes immunopathogenesis is scarce due to difficulties in accessing the pancreatic islets in situ despite increased use of powerful non-invasive intravital imaging tools such as MRI, positron emission tomography (PET) and bioluminescence [4, 5]. For example, it is still unclear how effector immune cells engage their target beta cells, how immune damage is controlled, how beta cells and their microenvironment respond and/or contribute to their own demise, how all these are affected by different

immune therapies and what are the possible earliest indications of islet stress. Some information about the islet-directed autoimmunity has been obtained from confocal/two-photon microscopy studies showing how T cells regulate their motility within tissues [6–8] and how beta cells might respond to immune insults [7, 9]. Other studies have used highly invasive approaches to investigate directly islet grafts in the kidney or the pancreas [10–16]. However, there remains a significant gap in knowledge regarding the longitudinal changes occurring within pancreatic islets during progression of type 1 diabetes with or without immune therapy [12, 17].

We used an *in vivo* imaging platform with islets transplanted in the anterior chamber of the eye (ACE) that circumvents the above technical challenges and offers the following unique advantages: (1) immune cells can be imaged within target tissues with single-cell resolution non-invasively and longitudinally; (2) novel kinetic and dynamic profiles can be obtained based on quantitative variables derived from longitudinal real-time three-dimensional (3D) tracking of individual immune cells and (3) cellular phenotypes and tissue viability can be assessed *in situ* by *in vivo* immunocytolabelling (IVICL) [7, 8, 18, 19]. We capitalised on these technical advantages with the following aims: (1) to increase our understanding of the immunopathology of type 1 diabetes; (2) to demonstrate early prediction of imminent onset of hyperglycaemia resulting from the autoimmune attack on islets in the pancreas; (3) to initiate timely therapeutic intervention before the point of no return and (4) to evaluate the efficacy of local immune manipulation in preventing or slowing down the progression of anti-islet autoimmunity in the context of both type 1 diabetes and its recurrence in transplant applications.

Methods

Animals All studies were performed under approved protocols by the University of Miami's Institutional Animal Care and Use Committee (IACUC). Mouse strains purchased from the Jackson Laboratory (Bar Harbor, ME, USA) included NOD/ShiLtJ (stock number 001976; NOD), NOD.CB17-*Prkdc*^{scid}/J (stock number 001303; NOD.*scid*), B6.129S7-*Rag1*^{tm1Mom}/J (stock number 002216; B6/Rag1), NOD.129S7(B6)-*Rag1*^{tm1Mom}/J (stock number 003729; NOD.*Rag*^{-/-}) C57BL/6J (stock number 000664; B6) and DBA/2J (stock number 000671). NOD.*Gfp* mouse founders were kindly provided by R. M. Tisch (Department of Microbiology and Immunology, University of North Carolina, Chapel Hill, NC, USA) [20]. Mice were housed during the studies in micro-isolated cages in virus antibody-free rooms with free access to autoclaved/irradiated food and water under the supervision of the University of Miami's Department of Veterinary Resources (DVR).

Immunotherapy Anti-mouse CD3 ϵ monoclonal antibody (clone 145-2C11), purified low-endotoxin grade by Leinco Technologies (St Louis, MO, USA), was administered to NOD mice by *i.p.* injection at 50 μ g/day for 5 consecutive days [21, 22]. Prednisolone acetate (1%; Omnipred Alcon Laboratories, Fort Worth, TX, USA) and loteprednol etabonate (0.5%; Lotemax Bausch & Lomb, Tampa, FL, USA) ophthalmic solutions were applied topically on the cornea of transplanted mice at the frequencies indicated in the specific experiments.

Islet isolation and transplantation Pancreatic islets were obtained by enzymatic digestion of pancreases from donor male mice, followed by purification on density gradients using protocols standardised at the Diabetes Research Institute (DRI) Pre-Clinical Cell Processing and Translational Models Core. After overnight culture, isolated islets were implanted in fully anaesthetised mice in the ACE or under the kidney subcapsular space as previously described [8, 18, 19, 23]. Diabetic recipient mice were transplanted with 400–600 islet equivalents (IEQ; full mass) under the kidney capsule. Mice transplanted in the ACE for imaging studies received 20–40 IEQ/eye in one eye, or a full mass.

Adoptive transfer of autoimmune diabetes NOD.*scid* or NOD.*Rag*^{-/-} mice were used as recipients of syngeneic islets (obtained from NOD.*scid* or NOD.*Rag*^{-/-}, respectively) in the ACE and/or under the kidney capsule. After engraftment and full revascularisation (at least 20 days post-transplant), mice were injected with 5×10^6 or 20×10^6 (fresh or frozen) splenocytes obtained from NOD or NOD.*Gfp* mice with recent-onset diabetes, as previously described [24]. A lower number of splenocytes was used in some experiments to allow establishment of better-defined baselines via more prolonged measurements before initiating treatments.

Immune cell isolation and sorting Splenocytes were obtained from spontaneously diabetic NOD mice or NOD.*Gfp* mice. Red blood cells were lysed with ACK lysing buffer (Life Technologies, Carlsbad, CA, USA) and cells were counted for inoculum. For experiments with labelled CD4 and CD8 cells, single-cell suspensions were incubated with rat anti-CD16/32 (clone 24G2) to block non-specific antibody binding. Subsequently, splenic CD4⁺ or CD8⁺ cells were isolated by positive selection with either CD4 (LT34) or CD8a (Ly-2) magnetic microbeads using a MACS kit according to the manufacturer's instructions (MiltenyiBiotec.com). Positively selected cells were further enriched by cell sorting on a BD FACSaria version 1 instrument in the DRI Flow Cytometry Core to $\geq 95\%$ final purity, as previously described [25].

Diabetes monitoring Autoimmune diabetes onset was evaluated starting when female NOD mice were 7–8 weeks of age, and 1 week after cell reconstitution in NOD.*scid* mice

receiving autoreactive cells. Mice were monitored two-to-three times a week for blood glucose concentration (Diastix; Bayer.com) and positive glycosuria was confirmed by blood sugar levels and monitored thereafter in blood (tail vein pricking) using portable glucometers (OneTouchUltra2; Lifescan.com) [26]. Diabetes was defined as non-fasting blood glucose values ≥ 13.88 mmol/l in three consecutive readings. In diabetic transplanted mice, graft function was defined as non-fasting blood glucose < 11.11 mmol/l. Return to non-fasting hyperglycaemic state was considered to be a sign of diabetes recurrence.

In vivo imaging of ACE-transplanted islets Imaging of ACE-transplanted islets was performed as previously described in detail [8]. In brief, islets engrafted on top of the iris were mapped within the first week after transplantation in digital images of the eye and were revisited during the longitudinal imaging sessions. The islets were monitored by direct visualisation documented in high-resolution digital images and by confocal microscopy using a 633 nm laser backscatter (reflection). The islet-infiltrating immune cells were visualised based on the expression of fluorescence proteins or by direct IVICL in the ACE with fluorescence-labelled monoclonal antibodies obtained from BD (San Jose, CA, USA). 3D confocal micrographs using 10 $\times$ or 20 $\times$ water immersion objectives were acquired in Z-stacks spanning the full height of the ACE-transplanted islets during the experiments and quantitative analysis of the individual islet volume was performed based on the 3D images. As we previously discussed in detail [8], islets maintaining their volume above 70% relative to the baseline (typically acquired for individual islets during the first week after transplantation) were considered to be surviving. This was adopted in imaging studies as the operational definition of a minimal functional beta cell mass capable of producing enough insulin to maintain normal blood sugar levels. Immune cell infiltration into the islets was quantified based on the volume of the fluorescent cells within individual islets relative to the corresponding islet volume. Dynamic analysis of the islet-infiltrating immune cell in situ motility was performed in 3D within the islets in time-lapse recordings of confocal Z-stacks (i.e. four-dimensional imaging). Dynamic variables such as path length, displacement, velocity and meandering index were calculated as described previously in [8].

Dextran (sugars), which are safe for use in animals and humans [8, 27, 28], come in various molecular masses and can provide vital information on the vascular permeability based on their size cut-off. Vascular leakage within ACE-transplanted islet blood vessels was imaged based on the median fluorescence intensity (MFI) of the labelled dextrans (Alexa305-conjugated 10 kDa; FITC-conjugated 40 kDa and TRITC-conjugated 2000 kDa) that were injected (5 mg/ml), mixed, into the tail vein of the mice during live imaging. The

MFIs were acquired in line scans (15 pixels in width and 50 μ m in length) spanning the centre of islet blood vessels/capillaries and the islet parenchyma. Great care was taken to avoid intersecting other blood vessels along the line scans. Quantitative analysis of the MFIs was done semi-automatically with user feedback using Volocity software (Perkin Elmer, Akron, OH, USA) [8] and pseudo-ratiometric measurements of the changes in the MFI (i.e. vascular leakage) of the 10 kDa and 40 kDa dextrans were obtained relative to the reference (non-leaking) 2000 kDa.

Histopathology Tissues were collected in 10% buffered formalin solution or frozen at -80°C in OCT compound (Tissue-Tek; VWR.com). Sections were stained with H&E. For immunofluorescence microscopy, primary antibodies included polyclonal guinea pig anti-insulin (1:100; Dako, Carpinteria, CA, USA), rabbit anti-CD3 (1:50; CellMarque.com), rat anti-mouse/human B220 (1:50; eBioscience.com), rat anti-mouse-CD8 (1:25; BDBiosciences.com). Secondary antibodies (all obtained from LifeTechnologies.com and used at 1:200 dilution in universal FC block buffer) included goat anti-guinea pig AlexaFluor-488 and -647, goat anti-rat AlexaFluor-568 and -488 and goat anti-rabbit AlexaFluor-488 and -555. Images were obtained on an SP5 inverted confocal microscope (Leica.com). Analysis was performed using ImageJ Software (imagej.nih.gov/ij) [29].

Imaging-based model to predict the onset of type 1 diabetes in NOD mice using ACE reporter islets To obtain an estimate of the likelihood (probability) of developing hyperglycaemia (diabetes) within the near future, a logistic regression model was used with volume and backscatter data obtained in ACE reporter islets imaged non-invasively and longitudinally during type 1 diabetes progression in NOD.*scid* mice reconstituted by adoptive transfer of diabetogenic splenocytes derived from already diabetic female NODs. Measurements of islet volume (acquired with varying detection thresholds to capture the whole islet at the various time points) and backscatter (intensity [MFI] captured as volume with a fixed threshold in the backscatter channel) were obtained from several islets in different mice and normalised for each individual islet using the corresponding pre-onset value at baseline (i.e. measured ≥ 22 days prior to hyperglycaemia onset). These were then synchronised along the time axis by setting the onset of hyperglycaemia as day 0. Fitting these data independently with average trendlines of sigmoidal increase and/or decrease clearly showed that islet volume increased (swelling) and backscatter decreased (degranulation) in the days leading up to the onset of hyperglycaemia (diabetes) (ESM Fig. 1).

Next, binary data for impending onset of hyperglycaemia was obtained by considering all data points in the 5 days prior to actual onset (i.e. day -5 to day 0) as 1 and everything else as 0 (zero). Probabilities of onset binned by variable intervals

were then quantified for both the volume and backscatter of the individual islets and fit by a logistic regression model with the sigmoidal function (Fig. 5a)

$$p(x) = \frac{1}{1 + e^{-\alpha(x-\xi_0)}} \quad (1)$$

and by maximising the standard log likelihood function

$$\log_e L = \sum_{i=1}^n n_i [y_i \log_e p_i + (1 - y_i) \log_e (1 - p_i)] \quad (2)$$

Here, x is the variable (volume or backscatter), α and ξ_0 are the parameters of the model; n is the number of data points, p_i the predicted and y_i the observed probability of onset for interval i . A predicted probability of onset from each individual islet can then be obtained by combining the two probabilities from volume (p_V) and backscatter (p_B) data (with the basic assumption that they are independent predictors) as follows:

$$p = 1 - (1 - p_V)(1 - p_B) \quad (3)$$

For model validation, onset probabilities were calculated with the parameters derived here for a separate set of NOD mice (not used for fitting of the model) to verify the accuracy of the model in predicting hyperglycaemia onset based on volume and backscatter data obtained in a similar manner from ACE reporter islets (Fig. 1d–f). The probability of developing hyperglycaemia (diabetes) in each mouse was calculated according to the above model using the average probability based on all islets in each mouse (four or five islets).

Statistical analysis Data were plotted and analysed in GraphPad Prism version 6.07 (<https://www.graphpad.com/scientific-software/prism/>). Statistical analyses were done using parametric and non-parametric comparisons tests (Student's t test and one-way ANOVA followed by Tukey's multiple comparison test). No randomisation or blinding to group assignments were carried out in these studies. Islet allograft survival rate analysis was based on Kaplan–Meier survival rate curves, and comparison of the median survival times was done by the logrank (Mantel–Cox) test. Significant difference was considered as $p < 0.05$.

Results

ACE-transplanted islets are reliable reporters on the immunopathology of type 1 diabetes in NOD mice We used the ACE platform in different experimental models of recurrent type 1 diabetes. Implanting islets from NOD.*scid* mice into the ACE of spontaneously diabetic NOD mice resulted in swift destruction of the islets (Fig. 1a), and diabetes recurred within 2 weeks. Median time to hyperglycaemia (MTH) was 10 days (range 5–13 days) and was not significantly different from that

observed with islets implanted under the kidney capsule (MTH 8 days; range 5–12 days) (Fig. 1b). Similarly, NOD.*scid* mice transplanted with syngeneic ACE-NOD.*scid* islets developed diabetes after reconstitution with diabetogenic splenocytes [24]. Time to diabetes onset was comparable between mice receiving islets in the ACE (MTH 33 days; range 25–54 days) and non-transplanted counterparts (MTH 35 days; range 25–40 days) (Fig. 1c).

Next, we monitored ACE-transplanted islets in reconstituted NOD.*scid* recipients and performed high-resolution imaging of individual islets longitudinally (Fig. 1d) to assess their survival rate based on qualitative and quantitative analyses of their granularity and volume before, during and after onset of hyperglycaemia. These studies revealed significant degranulation (Fig. 1e) and swelling (Fig. 1f) of ACE-transplanted islets starting approximately 1 week before the onset of hyperglycaemia, followed by their relatively quick deterioration and subsequent collapse in the post-onset period. In another set of studies, young female NOD.*scid* mice received NOD.*scid* islets in the ACE and under the kidney capsule simultaneously. Following adoptive transfer, the mice were euthanised at diabetes onset and pancreases and islet graft-bearing eyes and kidneys were obtained and immunostained for CD3-, CD4-, CD8- and CD220-expressing immune cells. Results showed no significant difference in the immune cell infiltrates (Fig. 1g–i) and similar CD4:CD8 cell ratios (Fig. 1j) in all three compartments.

Kinetics and in situ dynamics of islet-infiltrating immune cells and islet-cell apoptosis in vivo

In the same adoptive transfer model, we performed longitudinal IVICL of islet-infiltrating CD4⁺ and CD8⁺ cells in the same ACE-transplanted NOD.*scid* islets before and at diabetes onset [8]. Quantitative 3D image analysis showed significantly increased islet infiltration by CD4⁺ and CD8⁺ cells (Fig. 2a, b) and increased CD4:CD8 ratios (Fig. 2c) at diabetes onset compared with pre-diabetes onset. Further quantification of islet-cell death in association with immune cell infiltration by IVICL using DAPI and Annexin-V before and after diabetes onset in the same mice, but on days different from antibody injections, revealed a several-fold increase in DAPI- and Annexin-V-positive islet cells at onset compared with pre-diabetes (Fig. 2d). Furthermore, quantitative analysis of the in situ cellular motility within the islet grafts showed significant differences in the dynamics of the CD4⁺ and CD8⁺ cells before, during and after diabetes onset (Fig. 2e–h). Before onset, CD8⁺ cells had significantly higher velocity than CD4⁺ cells (Fig. 2g). They also exhibited higher overall motility (path length and displacement) and this became even more pronounced at diabetes onset (Fig. 2e, f). At onset, both CD4⁺ and CD8⁺ cells displayed significantly increased velocities compared with pre-diabetes, and their overall motility was significantly reduced post-onset (Fig. 2g). Both CD4⁺

and CD8⁺ cells had relatively low meandering index, indicative of confined and less directional overall motility (Fig. 2h).

To further elucidate the kinetics of islet infiltration, we designed experiments where immune cells from recently diabetic wild-type NOD mice and NOD.*Gfp* mice [20] were FACS sorted and adoptively transferred into NOD.*scid* mice bearing NOD.*scid* ACE-transplanted islets. Diabetogenic CD4⁺ or CD8⁺ T cells (expressing GFP or not) were separated using magnetic beads, further purified (>90%) by cell sorting and were used fresh or frozen. Flow-through-remaining splenic cells were also used. At the time of reconstitution, viable cells were counted and mixed in the appropriate proportions to recapitulate physiological proportions in the spleen from NOD mice (e.g. 10% CD8⁺ cells, 20% CD4⁺ cells and 70% remaining-flow-through cells). When preparing the cell

mixtures for inoculum, only one of the populations (either CD4⁺ or CD8⁺ T cells) expressed GFP, while the rest of the cells were unlabelled. We chose this procedure to have all the critical cell types required for diabetes transfer while having only one subset GFP labelled to allow its non-invasive tracking throughout the progression of type 1 diabetes (Fig. 2i). Both CD4⁺ and CD8⁺ cells appeared in ACE-transplanted islets with similar kinetics that paralleled morphological changes in the islets and their eventual collapse (Fig. 2j).

Permeability changes in the islet microvasculature during progression of type 1 diabetes Increased vascular permeability is a hallmark of inflammation and results in increased immune cell infiltration and accumulation of extracellular fluids leading to tissue swelling (i.e. oedema) [30]. Therefore, we investigated

Fig. 1 ACE-transplanted islets give an early indication of the status of autoimmunity against pancreatic islets and are attacked with similar kinetics as in the kidney and pancreas. **(a)** Longitudinal quantitative analysis of individual ACE-transplanted islet volume in female NOD mice with established diabetes ($n = 24$ islets; expressed as percentage of volume at pre-diabetes for each islet and shown as means $\pm$ SEM). The ACE-transplanted islets were considered as having lost their beta cells and insulin-producing ability when $\geq 30\%$ reduction in the individual islet volume was measured compared with baseline, based on our prior analysis [8]. **(b)** Kaplan–Meier survival rate curves of NOD.*scid* islet grafts in spontaneously diabetic female NOD mice following transplantation in either the ACE ($n = 6$) or under the kidney capsule (KC, $n = 10$). A total of 400–600 IEQs were transplanted either in both eyes (ACE) or in one kidney. Graft loss was defined as return to hyperglycaemia (non-fasting blood glucose values >13.88 mmol/l after at least five consecutive days of normoglycaemia) in the originally diabetic NOD recipients. **(c)** Survival rate curves showing the time to diabetes recurrence after adoptive transfer of $\sim 20 \times 10^6$ splenocytes obtained from spontaneously diabetic NOD mice. NOD.*scid* mice ($n = 15$) received ~ 20 islets from NOD.*scid* donors in the ACE. Islets were allowed to engraft for ≥ 20 days before inoculation with immune cells. Non-transplanted NOD.*scid* mice ($n = 5$) receiving autoreactive splenocytes were used as a control group. Comparisons of islet survival rate was by logrank (Mantel–Cox) test; $p > 0.05$ in **(b)** and **(c)**. **(d)** Serial images of a NOD.*scid* mouse eye with transplanted islets before (at baseline) and after adoptive transfer of diabetogenic splenocytes 5 days pre-diabetes onset, at onset and 15 days post-onset of diabetes (hyperglycaemia). Representative longitudinal Z-stack confocal micrographs of the boxed islet pair (shown as maximum projections; 2D view) or in 3D (magnified) illustrate the changes in the islets before, during and after onset of overt hyperglycaemia (scale bars, 100 μ m). **(e)** Islet granularity index at baseline ($n = 9$ islets), before (pre-onset; $n = 8$ islets) and at onset of diabetes/hyperglycaemia ($n = 14$ islets). The index is the ratio of the MFI in the backscatter channel within the islet at pre-diabetes and at diabetes onset relative to baseline (prior to adoptive transfer). The imaging acquisition parameters were fixed throughout the studies. Data were pooled from 6 mice and shown as means $\pm$ SEM (** $p < 0.01$ and *** $p < 0.001$ by Student's *t* test). **(f)** Longitudinal quantitative analysis of individual islet volume in the same mice (expressed as percentage of volume at pre-diabetes for each islet and shown as means $\pm$ SEM) before, during and after onset of hyperglycaemia (grey area); $n = 24$ islets from 6 mice. **(g–j)** Relative ratios of B220- **(g)**, CD4- **(h)** and CD8- **(i)** to CD3-positive cells (shown as medians with interquartile ranges in red) and corresponding CD4:CD8 ratio **(j)** (shown as means $\pm$ SEM) in tissue sections of the native pancreas (Panc), the kidney subcapsular space (KC) and eye (ACE) of the same NOD.*scid* mice ($n = 5$) at onset of diabetes/hyperglycaemia following adoptive transfer of diabetogenic splenocytes. Each data point in **(g–i)** represents an immunostained tissue section ($n = 46$, $n = 14$ and $n = 12$ sections in Panc, KC and ACE, respectively)

whether the swelling of the ACE reporter islets measured in the days preceding onset of diabetes is associated with increased permeability/leakage of islet blood vessels. In the setting of an adoptive transfer model, we injected intravenously a mixture of fluorescently labelled dextrans (10, 40 and 2000 kDa; see ESM Fig. 2) and measured the MFI at the time of injection and at 40 min thereafter. Pseudo-ratiometric measurements were used to evaluate vascular leakage (see Methods for further details). These in vivo measurements were repeated in the same islets of the same mice after allowing engraftment for ≥ 20 days (baseline), then ~ 10 days after adoptive transfer (pre-diabetes) and at

diabetes onset. Results showed high and unchanged vascular permeability for the 10 kDa dextran before or after transfer (Fig. 3a–g), whereas leakage of the 40 kDa dextran increased progressively after adoptive transfer compared with baseline (Fig. 3a–f, h). Accordingly, the vascular ‘leakage index’ was increased considerably after adoptive transfer (Fig. 3i).

ACE reporter islets predict type 1 diabetes early and enable timely intervention to intercept hyperglycaemia We investigated whether initiating clinically relevant immunotherapy [31] guided by early in vivo signs of inflammation in ACE reporter islets could interrupt beta cell destruction and prevent progression to hyperglycaemia in NOD mice. Prediabetic female NODs transplanted in the ACE with NOD.*scid* islets were imaged longitudinally and their blood glucose was monitored regularly. At the first signs of inflammation in the ACE reporter islets (e.g. degranulation and swelling), systemic treatment was initiated with anti-mouse CD3 ϵ monoclonal antibody or PBS [21, 22]. Results showed significantly lower mean blood glucose in anti-CD3-treated mice during the 5 day treatment course (coinciding with ~ 16 weeks of age) and in the first 30 days following treatment (~ 20 weeks of age) compared with PBS-treated control mice (Fig. 4a). All anti-CD3-treated mice remained normoglycaemic during this period (100%), whereas $\sim 67\%$ of the PBS-treated control mice had progressed to hyperglycaemia (Fig. 4b). Median time to onset of diabetes was 78 and 18 days in the anti-CD3- and PBS-treated mice, respectively.

Well-timed local treatment can modulate the autoimmune destruction of islet beta cells We investigated whether manipulating the immune system locally by application of eye-drops has an impact on recurrent autoimmunity against islets transplanted in diabetic recipient mice. Diabetic female NOD mice bearing ACE-transplanted islets from NOD.*Rag*^{−/−} mice were treated with prednisolone acetate (1%) eye-drops for 21 days starting at transplantation. This caused significant prolongation in the survival rate of ACE-transplanted islets ($p = 0.0044$, prednisolone treatment vs PBS or sham treatment) (Fig. 4c). Median survival times were 23 and 17 days in the PBS and sham controls, respectively, vs 76 days in prednisolone-treated recipients.

Next, we investigated whether modifying the localised therapy based on in vivo signs of islet inflammation could improve the therapeutic outcome of the ACE-transplanted islets compared with those in the native pancreas. NOD.*Rag*^{−/−} mice, transplanted with NOD.*Rag*^{−/−} islets in the ACE in both eyes and reconstituted with diabetogenic GFP-expressing splenocytes [20, 32], were treated (starting 2 weeks after adoptive transfer) with loteprednol etabonate (0.5%) eye-drops in the right eye and, as a control, PBS in the left eye. Loteprednol etabonate is known to be rapidly deactivated systemically, so that its effects should be mainly limited to the treated eye

Fig. 2 Longitudinal quantification of the infiltration kinetics and in situ motility dynamics of immune cells in ACE-transplanted islets in vivo. **(a)** Fluorescence confocal micrographs (Z-stacks shown as maximum projections) of a representative ACE-transplanted NOD.*scid* islet following adoptive transfer of diabetogenic splenocytes and prior to diabetes/hyperglycaemia onset. Infiltrating CD4⁺ (green) and CD8⁺ (red) cells were labelled by IVICL using Alexa488 and PE-labelled monoclonal antibodies, respectively. The islet and iris (grey) were visualised by backscatter (reflection) of a 633 nm laser. Scale bar, 100 μm. **(b)** CD4⁺ and CD8⁺ cell density within ACE-transplanted islets measured pre-diabetes ($n = 12$ islets from 3 mice) and at onset of diabetes ($n = 14$ islet from 5 mice). **(c)** CD4:CD8 ratio pre-diabetes and at diabetes onset; $n = 8$ islets from 3 mice for pre-diabetes and $n = 8$ islet from 5 mice for diabetes onset. **(d)** DAPI-positive and Annexin-V-positive islet cells at diabetes onset expressed as percentage of pre-diabetes ($n = 9$ islets from 3 mice). **(e–h)** Dynamic motility variables of autoreactive CD4⁺ and CD8⁺ cells. Path length **(e)** is the total travelled distance. Displacement **(f)** is the straight line between beginning and end of travel. Velocity **(g)** is the average speed of translocation along the path length. Meandering index **(h)** is a measure of the movement directionality vs randomness during travel. Analysis was done within islets in 3D based on 20 min time-lapse recordings (4D imaging; xyz with 2 min intervals) acquired in ACE-transplanted islets at pre-diabetes (>14 days before onset; $n = 98$ islet-

infiltrating cells), at diabetes onset (± 1 day; $n = 602$ cells) and post-onset of diabetes/hyperglycaemia (>7 days; $n = 148$ cells). Data pooled from 5 mice and medians and interquartile ranges are shown in red (see also ESM Fig. 1). **(i)** Fluorescence confocal micrographs (Z-stacks shown as maximum projections) of representative ACE-transplanted NOD.*scid* islets before and 1 week after adoptive transfer, and 2, 1 and 0 weeks from onset of hyperglycaemia (diabetes). The inoculum consisted of 20×10^6 diabetogenic splenocytes from recent-onset diabetic NOD mice containing either purified CD4⁺ or CD8⁺ T cells from spontaneously diabetic NOD.*Gfp* mice in the appropriate proportions to recapitulate physiological rates in spleens from NOD mice (e.g. 10% CD8⁺ cells, 20% CD4⁺ cells and 70% remaining-flow-through cells). Mice ($n = 5$ in each group) were monitored longitudinally by confocal microscopy for intraocular islet morphological changes and GFP-positive immune cell infiltration, as well as metabolic function (glycosuria and non-fasting blood glucose) to stage the diabetes progression (i.e. pre, onset, post). **(j)** Quantitative analysis showing progressive islet infiltration by GFP-expressing CD4⁺ or CD8⁺ cells (expressed as cell density based on volume ratio of GFP to corresponding islet; shown on the left y-axis) and corresponding changes in islet volume relative to baseline (pre-transfer; shown on the right y-axis). Data throughout this figure are shown as means $\pm$ SEM. * $p < 0.05$ and *** $p < 0.001$ (Student t test in **b**, ANOVA in **d**)

Fig. 3 Longitudinal measurement of vascular permeability (leakage) within ACE-transplanted islets during type 1 diabetes progression. NOD.*scid* mice received NOD.*scid* islets in the ACE that were allowed to engraft for at least 20 days before adoptive transfer of 20×10^6 splenocytes obtained from spontaneously diabetic NOD mice. Mice were monitored longitudinally by confocal microscopy for intraocular islet morphological changes and vascular leakage, as well as metabolic function (glycosuria and non-fasting blood glucose) to stage the diabetes progression (i.e. pre, onset, post). (a–f) Representative vascular permeability measurements at the time of injection (t0; a, c, e) and 40 min (t40; b, d, f) after injection acquired before (baseline; a, b) and after adoptive transfer of diabetogenic splenocytes, ~10 days before diabetes onset (pre-

onset; c, d) and at onset of diabetes/hyperglycaemia (± 1 day; e, f). Vascular permeability was measured as the MFI for Alexa305-conjugated 10 kDa (blue) and FITC-conjugated 40 kDa (green) dextrans (corrected to the reference non-leaking 2000 kDa dextran labelled with TRITC and expressed as a percentage of the average MFI within the blood vessel; see also ESM Fig. 2). (g, h) AUC measurements of vascular permeability for 10 kDa (g) and 40 kDa dextrans (h) at t0 and t40 (see also ESM Fig. 2). * $p < 0.05$ by Student's *t* test; † $p < 0.05$ by ANOVA). (i) Fold increase (relative to baseline) in the leakage index for 10 kDa and 40 kDa dextrans pre-diabetes and at diabetes onset after adoptive transfer. Data are shown as means $\pm$ SEM ($n = 4$ mice)

[33–35]. Results showed evident differences in the infiltration and destruction kinetics of the ACE-transplanted islets in association with loteprednol etabonate vs PBS treatments and treatment frequency. Islet infiltration in the PBS-treated eye increased progressively and islet swelling occurred earlier than in the contralateral loteprednol etabonate-treated eye (Fig. 4d; see also ESM Fig. 3 and ESM Video 1). By contrast, islets in the loteprednol etabonate-treated eye showed swelling only several days after switching to the once-a-day regimen (Fig. 4d). Islet infiltration proceeded unhampered in the PBS-treated eye and islets collapsed about 10 days after exhibiting early signs of swelling. Notably, their collapse coincided with onset of hyperglycaemia. Islet infiltration was blunted by the resumption of three-times-a-day loteprednol etabonate treatment, which also reduced islet swelling despite diabetes onset. Switching back to once-a-day application was paralleled by

increased infiltration, eventually resulting in the collapse of these islets. Survival rate analyses showed significantly prolonged survival of ACE-transplanted islets in association with loteprednol etabonate treatment compared with those in the pancreas, despite changing the treatment frequency (ESM Fig. 3d).

Predicting the onset of autoimmune diabetes in NOD mice based on ACE reporter islets The above results demonstrated measurable changes in the volume and backscatter of ACE reporter islets before the onset of overt hyperglycaemia resulting from the autoimmune destruction of a substantial proportion of the insulin-producing beta cell mass in the pancreas of NOD.*scid* mice receiving diabetogenic splenocytes. To obtain a predicted probability of developing hyperglycaemia prior to its onset in this setting, we used a logistic regression model with imaging-

Fig. 4 Effect of early initiated systemic and localised immune interventions on diabetes onset and ACE reporter islets. **(a)** Longitudinal non-fasting blood glucose levels in female NOD mice (starting at 14–15 weeks of age) transplanted with ACE reporter NOD.*scid* islets and treated with systemic anti-CD3 monoclonal antibody (50 μ g/day i.p. for 5 consecutive days) or PBS ($n = 6$ and $n = 3$ mice, respectively). Data were pooled before, during and after the treatment period (grey area). * $p < 0.05$ by Student t test. **(b)** Kaplan–Meier survival rate curves showing per cent of normoglycaemic (diabetes-free) mice before, during and after treatment (grey area) with either anti-CD3 monoclonal antibody or PBS. **(c)** Survival rate of islets from NOD.*Rag*^{−/−} mice transplanted into the ACE of female NOD mice with established diabetes. The recipients were treated locally with prednisolone acetate eye-drops (Pred; $n = 4$ mice) or PBS ($n = 3$ mice) or were left untreated (Sham; $n = 6$ mice). Eye-drops were applied for 90 s to the eye bearing the islet grafts three times a day during the first week, twice daily during the second week and once a day during the third week (grey area). Treatment was stopped 21 days after

transplantation. **(d)** Longitudinal analysis of islet volume (black symbols/lines) and infiltration kinetics (green symbols/lines) in GFP-splenocyte-reconstituted NOD.*Rag*^{−/−} recipients of ACE-transplanted islets in both eyes. The mice were reconstituted with 5×10^6 diabetogenic splenocytes to allow sufficient time for establishing baseline measurements during the longer engraftment of the adoptively transferred splenocytes. The mice were treated locally with loteprednol etabonate (0.5%) eye-drops (LE; in one eye; circles/solid lines) and with PBS in the other eye (squares/dotted lines) with alternating frequencies (three times a day and once a day), as indicated. Treatment was initiated three times a day, starting on the day of adoptive transfer, and was reduced to once a day 24 days later. Another 10 days later, the three-times-a-day treatment regimen was resumed and maintained for 10 days before switching back to once a day treatment. Data are based on $n = 15$ islets (LE) and $n = 10$ islets (PBS) pooled from two mice and shown as means $\pm$ SEM. See also ESM Fig. 3 and ESM Video 1

based data only as input. Volume and backscatter data, obtained at various time points from several ACE-transplanted islets in different mice during their progression to diabetes, were normalised to baseline (measured ≥ 22 days prior to diabetes onset) and synchronised to diabetes onset (day 0; also see ESM Fig. 4a–c). Binned frequencies of ‘impending onset’ (onset of overt hyperglycaemia within 5 days) for volume and backscatter data were fit with sigmoidal functions (Eq. 1) for separate logistic regressions (Fig. 5a; see Methods for details). The model was validated on another set of mice (reconstituted using the same procedure but not used for model fitting and development) to obtain an estimate of the predicted probability at various time points prior to hyperglycaemia onset. In this dataset, model-predicted probabilities for imminent onset based on the volume and backscatter of individual ACE-transplanted islets in each mouse were combined into an overall predicted probability (Eq. 3) and plotted as a function of time. Results showed that

the predicted average probability of impending diabetes onset for each mouse increased steadily as they approached the actual onset of hyperglycaemia, with estimates ranging from ~60% around day −10 to >90% within the last 5 days prior to actual onset (Fig. 5b; see also ESM Fig. 4d).

Discussion

We have previously demonstrated the versatility of the ACE platform in studying various aspects of the pancreatic islet-cell immunobiology in vivo [7, 8, 36–46]. We and others have consistently shown that, despite immune privilege, both allo- and autoimmune responses against ACE-transplanted islets recapitulate those against islets at other ectopic sites (e.g. kidney capsule and ear) and in the native pancreas [9, 17, 47–49]. It is likely that initial inflammation and later graft

Fig. 5 Predictive model for type 1 diabetes onset in NOD mice based on ACE-transplanted reporter islets. **(a)** Observed probabilities of diabetes onset for binned islet volume (normalised values; circles) and backscatter (squares) measured based on data from all islets used for fitting of the logistic regression model (see Methods). The islet volume and backscatter data were derived from ACE-transplanted reporter islets in NOD.*scid* mice reconstituted with diabetogenic splenocytes ($n = 27$ islets from 7 mice; see also ESM Fig. 4a–c). **(b)** Model-predicted estimates of the probability of diabetes onset in non-diabetic NOD.*scid* mice ($n = 4$)

revascularisation contribute to compromising immune privilege afforded by the ACE. In addition, while the ACE might not fully recapitulate all anatomical features of the native pancreatic microenvironment [12, 50], our studies in two experimental murine models of autoimmune diabetes further demonstrated similar features and kinetics of the autoimmune response against islets in the ACE, kidney and pancreas (Figs 1 and 2). Therefore, ACE-transplanted islets appear to be reliable reporters of immune responses against islets elsewhere in the body. Notably, these islets exhibited early signs of inflammation and this enabled reliable prediction of the autoimmune destruction of pancreatic islets prior to diabetes onset (Fig. 5 and ESM Fig. 4); these included islet degranulation and swelling, increased peri- and intra-islet insulinitis and increased permeability of islet microvasculature. Diabetes symptoms, such as glucose intolerance, hyperglycaemia and glycosuria, manifest only when a critical functional beta cell mass has been disrupted. Hence, the ACE platform could guide the timing of mechanistic investigations of beta cell disruption during early type 1 diabetes pathogenesis. It could also facilitate the development of new therapeutic strategies before manifestation of symptoms. Furthermore, the morphological features of ACE-transplanted islet inflammation, namely degranulation and swelling, could be assessed without the need for high-powered imaging. This could enable early detection in transplantation and timely immune intervention to protect islet grafts from allorejection and recurrent autoimmune diabetes in potential clinical application, where the eye could serve as a primary site for islet transplant in diabetic individuals or in the reporter concept to monitor the status of islets transplanted elsewhere in the body [38, 42, 51].

It is well-established that peri-insulitis is an early indicator of islet inflammation in NOD mice. However, monitoring pancreatic insulitis non-invasively and longitudinally is not feasible in vivo. Our studies using ACE-transplanted islets overcame

reconstituted with diabetogenic splenocytes (by adoptive transfer) acquired at various time points during type 1 diabetes development and prior to onset of hyperglycaemia. Mice shown in (b) were not used for model development. Estimates for each mouse were obtained based on the averaged probabilities calculated at each time point using the volume and backscatter data from the ACE-transplanted reporter islets, as described in the Methods (see ESM Fig. 4d). Data are shown as means $\pm$ SEM ($n = 4$ islets in mouse 1 and 2, $n = 5$ islets in mouse 3 and 4)

this limitation and revealed simultaneous accumulation of both CD4⁺ and CD8⁺ cells within islets and longitudinal changes in their motility patterns during diabetes progression (Fig. 2). These findings suggested greater involvement of CD8⁺ cells in the autoimmune response against islets than previously appreciated. Notably, despite their overall increased cellular motility at onset, both CD4⁺ and CD8⁺ cells had slower dynamics when compared with a purely allogeneic setting, where CD8⁺ cells were primarily involved in the rejection of islet allografts (ESM Fig. 1; also see [8]). This could be due in part to the lower antigen spectrum of the autoimmune response against beta cells [52] vs the potentially broader antigenic profile in the allogeneic setting [8]. Another hallmark of tissue inflammation is increased permeability of blood vessels. Vascular permeability depends on many factors including endothelial pathologies, inflammation and the shape/size of molecules [53, 54]. Consistent with indirect evidence from MRI studies showing accumulation of contrast agents in the pancreas prior to and at diabetes onset in NOD mice and individuals with type 1 diabetes [55–58], our findings showed increased vascular permeability within the islet microvasculature in the days preceding hyperglycaemia onset (Fig. 3 and ESM Fig. 2). Thus, vascular leakage within ACE-transplanted islets could also serve as an early in vivo indicator of inflammation to prompt timely therapeutic interventions.

Despite immunosuppression, autoimmune diabetes can reoccur in islet and pancreas transplants thereby limiting their long-term therapeutic benefits in diabetic recipients [59, 60]. We investigated whether initiating clinically relevant systemic immunotherapy at the first signs of inflammation, as detected by ACE-transplanted islets, could protect against diabetes. We found that anti-CD3 antibody treatment improved blood glucose control and delayed diabetes onset in the stringent NOD model (Fig. 4a, b). We also explored localised immunomodulation at the graft site, as local treatment can provide a safer alternative to

systemic treatment with its associated off-target effects [5, 35, 61] and because addition of locally administered agents could be considered for specific modulation of the auto-/allo-immune response in islet transplants [5]. Our studies in which a corticosteroid was applied to the eye showed significant prolongation in the survival of ACE-transplanted islets in diabetic recipient mice (Fig. 4 and ESM Fig. 3). These findings demonstrated the potential for providing additional protection for transplanted islets from the autoimmune response via local/topical interventions. Considered alongside a Phase 1/2 clinical trial on intraocular islet transplant in type 1 diabetes patients (ClinicalTrials.gov registration no. NCT02846571), these findings could impact islet transplant therapy in the near future [51].

In summary, our findings revealed early in vivo hallmarks of inflammation in ACE-transplanted islets that enabled early prediction of type 1 diabetes and allowed timely interventions to intercept its progression. Therefore, the ACE platform can be instrumental in guiding and improving the development of new treatment modalities in type 1 diabetes and transplant applications.

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Data availability Data supporting the results reported in this article are available on request from the authors.

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Duality of interest P-OB is cofounder and CEO of Biocrine, an unlisted biotech company that is using the ACE technique as a research tool. MHA is consultant for the same company. All other authors declare that there is no duality of interest associated with their contribution to this manuscript.

Contribution statement MHA, RDM and AP conceived and designed the study, conducted experiments, analysed and interpreted data and wrote the manuscript. GF, ASB and CF planned experiments, analysed and interpreted data and edited the manuscript. MLC, UU, CF, AS, LFH, AT, VA and AT-G, conducted experiments, collected data and proof-read the manuscript. PB planned experiments, analysed and interpreted data, developed the mathematical model and wrote the manuscript. CR contributed to discussion and advice on experimental design and reviewed the manuscript. AC and P-OB conceived the study, designed experiments, interpreted data and edited the manuscript. All authors approved the version of the manuscript to be published. MHA, RDM, PB, AP and P-OB are the guarantors of this work. The contents of this manuscript are solely the responsibility of the authors and do not necessarily represent the official views of the funding agencies. This article was prepared while AP was employed at the University of Miami. He is currently employed at NIH/Center for Scientific Review. The opinions expressed in this article are the authors' own and do not reflect the view of the NIH, the Department of Health and Human Services or the United States government.

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