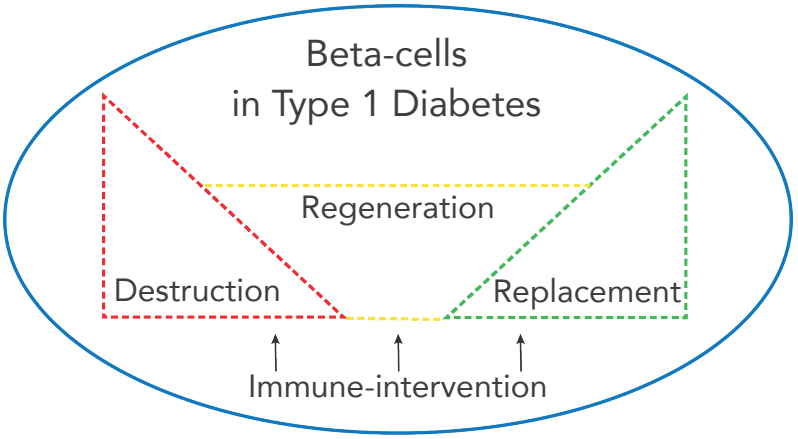
Destruction, Regeneration and Replacement of Beta-cells and Aspects of Immune-intervention in Type 1 Diabetes



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General introduction and outline of this thesis

Type 1 diabetes mellitus (T1DM) is a chronic disease caused by autoimmune mediated destruction of insulin producing beta-cells in the islets of Langerhans in the pancreas. It is the second most prevalent disease among children in the Western world. In the Netherlands, approximately 100,000-120,000 persons suffer from T1DM [1]. There has been a rise in childhood incidence worldwide during the second half of the 20th century attributed to changing environmental factors such as diet, increased hygiene and (entero)viral infections [2]. Currently there is no cure; treatment consists of frequent or continuous insulin administration to mimic beta-cell function. Despite intensive insulin regimes, T1DM still contributes to substantial morbidity and mortality. A significant fraction of patients with T1DM at some point during the course of their disease develop the life-threatening condition of either severe hypoglycemia or ketoacidosis, a state of uncontrolled catabolism due to insulin deficiency. Ketoacidosis can also be the first presentation of T1DM. In spite of insulin replacement therapy, the majority of T1DM patients will eventually develop chronic micro- and macrovascular complications such as nephropathy, retinopathy, peripheral neuropathy, myocardial infarction, stroke and peripheral vascular occlusions. T1DM therefore is associated with severe reduction of quality of life and significant health expenses.

Over the last decades, much progress has been made in our understanding of the pathophysiology and possible starting points of treatment of T1DM. But in spite of this progress, many questions remain unanswered. We know that T1DM is a T-cell mediated auto-immune disease, but the prerequisites for T-cell recruitment to the endogenous pancreatic islets are not fully understood. Specifically, it is unknown whether antigen presence is a prerequisite for T-cell recruitment. The first part of this thesis addresses beta-cell destruction. There is evidence that functional beta-cell mass can be restored after successful immune intervention in diabetes rodent models but we do not know the source of new insulin producing cells nor the role of ongoing autoimmunity in this process. The second part of this thesis addresses beta-cell regeneration. Clinical islet transplantation is an accepted treatment in a subset of T1DM patients. Yet, little is known about T-cell recruitment to islet transplants either. Furthermore there is a growing interest in the contribution of memory T-cells to recurrent autoimmunity in graft failure. T-cell antigen recognition and beta-cell destruction pathways have been largely unraveled, but it is unclear whether we can protect beta-cells by compromising these processes simultaneously, hence whether we can evade the immune system. The third part of this thesis addresses aspects of beta-cell replacement. Clinical trials have shown that anti-CD3 antibodies can temporarily preserve beta-cell function in recent onset T1DM patients but it is not known how safe this treatment is in terms recall immunity (the immune reaction towards pathogens to which patients have been exposed) and preservation of desired immunity against tumours. The fourth and final part of this thesis addresses aspects of immune intervention.

A major rodent model in T1DM research is the non-obese diabetes (NOD) mouse, originally developed in Japan during the selection of a cataract-prone strain. The NOD

mouse spontaneously develops an autoimmune form of diabetes sharing genetic and immuno-pathological features with human T1DM [3]. Yet, despite the similarities between mice and men in the development of autoimmune diabetes, there are many more differences, emphasizing prudence in translating results. Since accessibility to human pancreases during the course of the disease is limited and because reliable biomarkers of both disease activity and aspects of intervention such as efficacy and safety are not readily available, for many studies we currently have to rely on animal models.

Beta-cell destruction

T1DM results from a T-cell mediated autoimmune destruction of insulin-producing beta-cells in the islets of Langerhans in the pancreas, in genetically predisposed individuals [4]. Our immune system is programmed to react against non-self antigens: our protection to invaders from the outside world, such as viruses and bacteria. Through negative selection in the thymus, T-cells that react with *self* antigen are deleted, thereby preventing autoimmunity. In T1DM patients autoreactive T-cells escape this central tolerance for reasons not yet completely understood but among which post-translational modification is under a lot of attention [5]. Subsequent exposure of beta-cell proteins (e.g., proinsulin, IA-2, GAD65) to the immune system by for instance local stress or a viral attack, leads to priming and activating of naive autoreactive T-cells. These activated T-cells destruct more beta-cells, with additional exposure of beta-cell proteins to the immune system which continues to fuel beta-cell killing [4]. Why T1DM patients lose tolerance to islet antigens and why the immune system fails to suppress islet autoreactivity is not yet known. Furthermore, neither the nature and function of the different T-cells involved nor the prerequisites for T-cell recruitment are fully determined. In T1DM research, main focus has been on the role of CD4+ T-cells. Upon antigen encounter, such as beta-cell proteins presented by antigen presenting cells (APCs), CD4+T-cells can direct immune responses by differentiation into either effector or regulatory T-cells, depending on the context upon which these are primed [6]. It has become evident however, that CD8+ T-cells significantly contribute to the actual destruction of beta-cells. The pancreatic islets of recent onset T1DM patients show large CD8+ T-cell infiltrates and increased MHC-I expression, molecules necessary for antigen presentation to CD8+ T-cells [7,8]. More recently, specific beta-cell autoreactive CD8+ T-cells could be demonstrated within insulitic lesions of T1DM patients. This indicates that beta-cell destruction involves selective infiltration of autoreactive T-cells [9]. But how selective is this infiltration actually? Studies in a number of infection and autoimmune disease models have suggested that recruitment of T-cells into a site of extra lymphoid inflammation such as the pancreas does not require local expression of cognate peptide-MHC (pMHC) on tissue cells. Or put differently: the fact that T-cells are programmed to recognize a specific antigen does not necessarily imply that these cells need to recognize this antigen in order to infiltrate insulitic lesions. Alternatively, non-antigen specific cues such as cytokines and chemokines may recruit (bystander) T-cells [10,11].

A significant fraction of the islet associated CD8+ T-cells in NOD mice target the diabetogenic epitope *islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP)*$_{206\text{-}214}$ [12]. This IGRP epitope is shared between mice and men, and is a target of human diabetogenic T-cells [13,14]. In ***Chapter 2*** we monitored the recruitment of CD8+ T-cells specific for this epitope in gene targeted non-obese diabetic (NOD) mice expressing a T cell 'invisible' IGRP$_{206\text{-}214}$ epitope. The aim of our study was to ascertain whether local expression of cognate pMHC is a prerequisite for the recruitment to and/ or accumulation of CD8+ T-cells in the pancreas during the development of spontaneous autoimmune diabetes. As an alternative outcome, we assessed whether inflammatory cues themselves are sufficient to attract (bystander) T cells. Simplified: are specific T-cells, such as IGRP$_{206\text{-}214}$ -positive T-cells, still recruited to the pancreas when the antigen or epitope they are programmed for to recognize (IGRP$_{206\text{-}214}$), is invisible for them? Rationale behind this study was to understand the complex process of T-cell recruitment and/ or accumulation to endogenous islets during development of T1DM, which on the long run could bring us a step closer to intervening in this process.

Beta-cell regeneration

It is estimated that at diagnose of T1DM, 40-90% of the beta-cells have been destroyed. Among the number of issues that need to be addressed in T1DM research, finding a way to restore the severely compromised beta-cell mass/ function is one of them. There is evidence in both rodents and humans that beta-cells are able to adapt their cell mass to various physiological and pathological conditions [15,16,17,18,19,20,21]. The availability of therapies that decrease insulin requirements and temporarily normalize glycemia in T1DM patients [22,23,24] and that can fully reverse hyperglycemia in newly diagnosed diabetic NOD mice [25], raises the question of how functional beta-cell mass could be restored after successful immune intervention. In addition, the worldwide scarcity of pancreas/ islet allografts as an alternative for restoration of beta-cell mass/ function, fuels the exploration of the beta-cell regenerative capacity [26].

For over a century, both replication of pre-existing beta-cells and islet neogenesis, either via trans-differentiation of non-beta-cells or via differentiation of progenitor/ stem cells [27,28] have been proposed mechanisms of beta-cell regeneration. More recently, beta-cell recovery of exhausted beta-cells has been additionally suggested [29]. In the earliest beta-cell regeneration studies, islet cell regeneration was assessed from a morphological point of view. The development of techniques indicating islet cell proliferation, such as 3-H-thymidine incorporation and BrdU labelling, has been a step forward in addressing this issue in preclinical models [27]. However, the true origin of new insulin-producing cells remains difficult to prove. The technique of cell lineage tracing is based on the inheritable labelling of individual islet cells [30]. In T1DM research this means that the islet cell of interest, for instance the alpha-, beta- or duct cell can be marked in such a way that this mark will be transferred to its progeny. Ideally in cell lineage tracing, labelling occurs in a high percentage of the cells of interest, is specific, reliable, bright and without the need for signal enhancing at the same

time. Up until this thesis, beta-cell regeneration studies have been performed in *non*-autoimmune cell lineage tracing models [19,20,21]. Therefore not only the source of new insulin producing cells after successful immune intervention remains unclear, but also the role of ongoing autoimmunity in this process is unknown.

In ***Chapter 3*** we describe the development of lineage tracing models by generating (inducible) expression of red fluorescent protein (RFP) in pre-existing beta-cells and alpha-cells of gene targeted NOD mice. In NOD.RIP-tTA/tet07-Cre.ROSA-tdRFP mice, insulin producing beta-cells were targeted to express RFP in the absence of a Tetracycline antibiotic in food and drinking water (whereas presence of this antibiotic should prevent this). Beta-cells of NOD.RIPCreER.ROSA-tdRFP mice were targeted to express RFP upon injection with an Estrogen Receptor binder, such as Tamoxifen. Alternatively, glucagon producing alpha-cells were labelled with RFP in NOD.GluCre.ROSA-tdRFP mice. Reliable cell lineage tracing models in mice that spontaneously develop an autoimmune form of diabetes could add a novel tool to beta-cell regeneration studies in T1DM research. Because regenerative pathways and regenerative capacity might differ between species, prudence in translating results from animal models is required. However preclinical studies might give some clues and guidance in to what regenerative pathways may be considered.

Beta-cell replacement

Ever since the earliest rodent islet transplantation showed restoration of normoglycemia in diabetic recipients, attempts for clinical use of islet cell transplantation have been undertaken [31,32]. Currently islet transplantation is an accepted therapy for patients with complete insulin deficiency, unstable glycemic control and repeated severe hypoglycemia despite optimal diabetes management and compliance [33]. Two-third of T1DM patients that undergo islet cell transplantation show insulin independency 1 year after receiving their final islet infusion [34]. Unfortunately, graft function decreases over time and 5 years post transplantation 90% of the patients have returned to insulin dependency. The majority however continues to display some degree of islet allograft function with decreased insulin requirements, overall better metabolic control and most importantly less episodes of life-threatening hypoglycemia [35]. Challenges in clinical islet transplantation are manifold: there is a scarcity of donor material, significant islet cell loss occurs during the transplantation procedure and current immune suppressive regimens have significant side effects, including intrinsic beta-cell toxicity [36].

Allogeneic islet and pancreas transplants are derived from a donor with a different genetic make-up than the receiver and are thus subject to immune mediated rejection against which systemic immune suppression is required. The first evidence of ongoing autoimmunity as additional threat to graft survival came from a twin to twin (with identical genetic make-up) pancreas transplantation, which showed recurrence of diabetes after initial reversal of hyperglycemia [37]. Huurman *et al* showed in clinical islet transplantation studies that pre-transplantation autoimmune status is predictive of

clinical outcome [38,39]. Another piece of evidence came from Vendrame *et al*, who described that pancreas transplant derived autoreactive T-cells from T1DM patients that underwent simultaneous pancreas-kidney transplantation were able to destroy human islets transplanted into immune-deficient mice [40].

There is a growing interest in the role of recurrent or ongoing autoimmunity in the outcome of allograft islet transplantation. Yet, similar to T-cell recruitment to endogenous islets, little is known about T-cell recruitment to islet transplants. Specifically, there is an interest in the contribution of memory T-cells to recurrent autoimmunity in graft failure.

In ***Chapter 4*** we monitored the recruitment of the same epitope $IGRP_{206-214}$ CD8+ T-cells as previously described, into epitope competent- or epitope invisible grafts, which were transplanted either into diabetic wild type NOD mice (harboring both naive and memory epitope specific T-cells) or epitope invisible hosts (harboring only naive epitope specific T-cells). The aim of this study was two-fold: first, to ascertain whether local antigen expression is a prerequisite for recruitment of CD8+ T-cells in the setting of syngeneic islet transplantation or rephrased: is T-cell recruitment in an islet transplantation model comparable to T-cell recruitment into endogenous islets? And second, to specifically address the contribution of memory T-cells to islet graft failure due to recurrent or ongoing autoimmunity in this non-immune suppressive syngeneic islet transplantation model. Of note, since the transgenic NOD mice that were used are genetically identical, allo-immunity is not addressed here.

Islet cell transplantation is a promising therapy for type 1 diabetes mellitus but, among many other factors, compromised by recurrent islet autoimmunity. Transplantation of genetically immune protected islets to elude host immune responses could be one approach to improve clinical outcome. Direct cell-cell contact initiated by T-cell receptor recognition of antigenic peptide, presented by MHC class I at the surface of the target cells, appears to be critical for beta-cell destruction in T1DM [9,41,42,43]. Furthermore, perforin gene disruption has shown to delay onset of autoimmune diabetes in NOD mice, pointing to the perforin/ granzyme B pathway as the key effector in beta-cell destruction by cytotoxic T-cells [44,45]. In ***Chapter 5*** we tested whether down-regulation of MHC-I expression (a molecule necessary for antigen presentation to CD8+ T-cells), combined with inhibition of granzyme B activity (an enzyme involved in the actual beta-cell killing) via genetically engineered US2/ Serpin 9 expression, protects human beta-cells from acute recurrent islets autoimmunity both *in vitro* and *in vivo*. Or simplified: if compromising both immune recognition of the transplanted islets and the cytotoxic granzyme pathway, would prevent T-cell recruitment and (subsequent) beta-cell destruction.

In order to assess immune protection, reliable measurement of beta-cell toxicity is required. Beta-cell toxicity measurements however are influenced by the quality of the isolated islet fraction, as islet preparations contain a mixture of cell types including beta-cells, alpha-cells, delta-cells, duct cells, endothelial cells, stem cells and leukocytes. We therefore aimed to develop a new strategy to measure beta-cell toxicity and protection

from auto-reactive T-cell mediated killing. In ***Chapter 5*** we additionally tested whether a destabilized luciferase reporter gene, expressed under the human insulin promoter, allows for a specific beta-cell killing assay *in vitro*.

Aspects of immune-intervention

Since the 1980s several immunotherapeutic strategies in T1DM have been explored [46]. Targeted immune therapies, such as anti-CD3 therapy, have shown encouraging results in intervention treatment of T1DM, especially in subgroups. Phase II clinical trials with the anti-CD3 antibodies Otelixizumab en Teplizumab showed preserved beta-cell function for at least 18-24 months, decreased insulin requirements and improved levels of glycated haemoglobin in recent onset T1DM patients [21,22,23]. The mechanism of immune suppression mediated by anti-CD3 antibodies is complex, but is believed to depend largely on TCR internalization or unresponsiveness to antigen re-stimulation [47].

A major safety concern in the use of any immune modulating agent in type 1 diabetes mellitus is how well the immune reaction towards pathogens to which patients have been exposed (recall immunity) and desired tumour immunity are preserved. During the European phase II placebo-controlled Otelixizumab (humanized anti-CD3 antibody; ChAglyCD3) trial in recent onset T1DM patients, 75% of the treated patients showed transient Epstein Barr Virus (EBV) reactivation [48]. Although the number of EBV copies returned to normal levels within 1-3 weeks in all patients, comparable with that observed in individuals following infectious mononucleosis in general, this finding emphasizes the importance of addressing recall immunity. Other concerns of immune modulation in T1DM are recurrence or even fuelling of autoimmunity [49]. In ***Chapter 6*** we tested the proliferative T-cell responses towards common pathogens, towards the human tumour suppressor protein p53 and towards several auto-antigens *in vitro* in a sub cohort of the European phase II placebo-controlled Otelixizumab trial in recent onset T1DM patients. Up until now, no follow-up data about recall, tumour and autoimmunity after treatment with anti-CD3 has been available.

REFERENCES

1. Rijksinstituut voor Volksgezondheid en Milieu
2. Gillespie KM, Bain SC, Barnett AH et al. The rising incidence of childhood type 1 diabetes and reduced contribution of high-risk HLA haplotypes. Lancet 2004;364:1699-700
3. Anderson MS, Bluestone JA. The NOD mouse: a model of immune dysregulation. Annu Rev Immunol 2005;23:447-485
4. Roep BO. The role of T-cells in the pathogenesis of Type 1 diabetes: from cause to cure. Diabetologia 2003;46:305-321
5. Dunne JL, Overbergh L, Purcell AW et al. Posttranslational Modifications of Proteins in Type 1 Diabetes: The Next Step in Finding the Cure? Diabetes 2012; 61:1907-1914
6. FS Kleijwegt, BO Roep. Infectious tolerance as candidate therapy for Type 1 Diabetes: transfer of immunoregulatory properties from human regulatory T-cells to other T-cells and proinflammatory DCs. Critical Reviews in Immunology 2013;33(5)415-434]
7. Bottazzo GF, Dean BM, McNally JM et al. In situ characterization of autoimmune phenomena and expression of HLA molecules in the pancreas in diabetic insulitis. N Engl J Med 1985;313;353-360
8. Itoh N, Hanafusa T, Miyazaki A et al. Mononuclear cell infiltration and its relation to the expression of major histocompatibility complex antigens and adhesion molecules in pancreas biopsy specimens from newly diagnosed insulin-dependent diabetes mellitus patients. J Clin Invest 1993;92:2313-2322
9. Coppetiers M, Dotta F, Amirian N et al. Demonstration of islet-autoreactive CD8 T cells in insulitic lesions from recent onset and long-term type 1 diabetes patients. J Exp Med 2012;209:51-60
10. Chen AM, Khanna N, Stohlman SA et al. Virus-specific and bystander CD8 T cells recruited during virus-induced encephalomyelitis. J Virol 2005;79:4700-4708
11. Peterson JD, Berg R, Piganelli JD et al. Analysis of leukocytes recruited to the pancreas by diabetic T cell clones. Cell Imunol 1998;189:92-98
12. Lieberman SM, Evans AM, Han B et al. Identification of the beta cell antigen targeted by a prevalent population of pathogenic CD8+ T cells in autoimmune diabetes. Proc Natl Acad Sci U S A 2003;100:8384-8388
13. Unger WWJ, Pinkse GGM, Mulder-Van der Kracht S et al. Human clonal CD8 autoreactivity to an IGRP islet epitope shared between mice and men. Ann N Y Acad Sci 2007;1103:192-195
14. Unger WWJ, Pearson T, Abreu JRF et al. Islet-Specific CTL Cloned from a Type 1 Diabetes Patient Cause Beta-Cell Destruction after Engraftment into HLAA2 Transgenic NOD/SCID/IL2RG Null Mice. PLOS One 2008;7:e49213
15. Bouwens L, Rooman I. Regulation of pancreatic beta-cell mass. Physiol Rev 2005:85;1255-1270

16. Van Assche FA, Aerts L, De Prins F. A morphological study of the endocrine pancreas in human pregnancy. Br J Obstet Gynaecol 1978;85:818-820

17. Butler AE, Cao-Minh L, Galasso R et al. Adaptive changes in pancreatic beta cell fractional area and beta cell turnover in human pregnancy. Diabetologia 2010;53:2167-2176.

18. Sherry NA, Kushner JA, Glandt M et al. Effects of autoimmunity and immune therapy on beta-cell turnover in type 1 diabetes. Diabetes 2006;55:3238-3245

19. Nir T, Melton DA, Dor Y. Recovery from diabetes in mice by beta cell regeneration. J Clin Invest 2007;117:2553-2561

20. Xu X, D'Hoker J, Stangé G et al. Beta cells can be generated from endogenous progenitors in injured adult mouse pancreas. Cell 2008;132:197-207

21. Thorel F, Népote V, Avril I et al. Conversion of adult pancreatic alpha-cells to beta-cells after extreme beta-cell loss. Nature 2010;464:1149-1154

22. Keymeulen B, Vandemeulebroucke E, Ziegler AG et al. Insulin needs after CD3-antibody therapy in new-onset type 1 diabetes. N Engl J Med 2005; 352:2598-2608

23. Keymeulen B, Walter M, Mathieu C et al. Four-year metabolic outcome of a randomized controlled CD3-antibody trial in recent-onset type 1 diabetic patients depends on their age and baseline residual beta cell mass. Diabetologia 2010; 53:614-623

24. Herold KC, Hagopian W, Auger JA et al. Anti-CD3 monoclonal antibody in new-onset type 1 diabetes mellitus. N Engl J Med 2002;346:1692-1698

25. Chatenoud L, Thervet E, Primo J et al. Anti-CD3 antibody induces long-term remission of overt autoimmunity in nonobese diabetic mice. Proc Natl Acad Sci USA 1994;91:123-127

26. Harlan DM, Kenyon NS, Korsgren O et al. Immunology of Diabetes Society. Current advances and travails in islet transplantation. Diabetes 2009;58:2175-2184

27. Granger A, Kushner JA. Cellular origins of beta-cell regeneration: a legacy view of historical controversies. J Intern Med 2009;266:325-338

28. Bonner-Weir S, Li W, Ouziel-Yahalom L et al. Beta-cell growth and regeneration: replication is only part of the story. Diabetes 2010;59:2340-2348

29. Couri CE, Oliveira MC, Stracieri AB et al. C-peptide levels and insulin independence following autologous nonmyeloablative hematopoietic stem cell transplantation in newly diagnosed type 1 diabetes mellitus. JAMA 2009;301:1573-1579

30. Kretzschmar K, Fiona M. Watt FM. Lineage tracing. Cell 2012;148:33-45

31. Ballinger WF, Lacy PE. Transplantation of intact pancreatic islets in rats. Surgery 1972;72:175-186

32. Scharp DW, Lacy PE, Santiago JV et al. Insulin independence after islet transplantation into type 1 diabetic patient. Diabetes 1990;39:515-518

33. Ludwig B, Ludwig S, Steffen A et al. Islet versus pancreas transplantation in type 1 diabetes: Competitive or complementary? Curr Diab Rep 2010;10:506-511

34. Shapiro AM, Lakey JR, Ryan EA et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl Med 2000;343:230-238

35. Ryan EA, Paty BW, Senior PA et al. Five-year follow-up after clinical islet transplantation. Diabetes 2005;54:2060-2069

36. Robertson RP. Islet transplantation as a treatment for diabetes- a work in progress. N Engl J Med 2004;350:694-705

37. Sutherland DE, Sibley R, Xu XZ et al. Twin-to-twin pancreas transplantation: reversal and re-enactment of the pathogenesis of type 1 Diabetes. Trans Assoc Am Physicians 1984;97:80-87

38. Huurman VA, Hilbrands R, Pinkse GG et al. Cellular islet autoimmunity associates with clinical outcome of islet cell transplantation. PLoS One 2008;3: e2435

39. Hilbrands R, Huurman VA, Gillard P et al. Differences in baseline lymphocytes counts and autoreactivity are associated with differences in outcome of islet cell transplantation in type 1 diabetic patients. Diabetes 2009;58:2267-2276

40. Vendrame F, Pileggi A, Laughlin E et al. Recurrence of type 1 diabetes after simultaneous pancreas-kidney transplantation, despite immunosuppression, is associated with autoantibodies and pathogenic autoreactive CD4 T-cells. Diabetes 2010;59:947-957.

41. Katz J, Benoist C, Mathis D. Major histocompatibility complex class I molecules are required for the development of insulitis in non-obese diabetes mice. Eur J Immunol 1993;23:3358-3360

42. Serreze DC, Leiter EH, Christianson GJ et al. Major histocompatibility complex class I-deficient NOD-B2Mnull mice are diabetes and insulitis resistant. Diabetes 1994;43:505-509

43. Wicker LS, Leiter EH, Todd JA et al. Beta 2-microglobulin-deficient NOD mice do not develop insulitis or diabetes. Diabetes 1994;43:500-504

44. Kagi D, Odermatt B, Ohashi PS et al. Development of insulitis without diabetes in transgenic mice lacking perforin-dependent cytotoxicity. J Exp Med 1996;183:2143-2152

45. Thomas HE, Trapani JA, Kay TW. The role of perforin and granzymes in diabetes. Cell Death Differ 2010;17:577-585

46. Von Herrath M, Peakman M, Roep B. Progress in immune-based therapies for type 1 diabetes. Clin Exp Immunol 2013;172:186-202

47. Carpenter PA, Pavlovic S, Tso JY et al. Non-Fc receptor-binding humanized anti-CD3 antibodies induce apoptosis of activated human T cells. J Immunol 2000;165:6205-6213

48. Keymeulen B, Candon S, Fafi-Kremer S et al. Transient Epstein-Barr virus reactivation in CD3 monoclonal antibody-treated patients. Blood 2010;115:1145-1155

49. Monti P, Scirpoli M, Maffi P et al. Islet transplantation in patients with autoimmune diabetes induces homeostatic cytokines that expand autoreactive memory cells. J Clin Invest 2008;118:1806-1814

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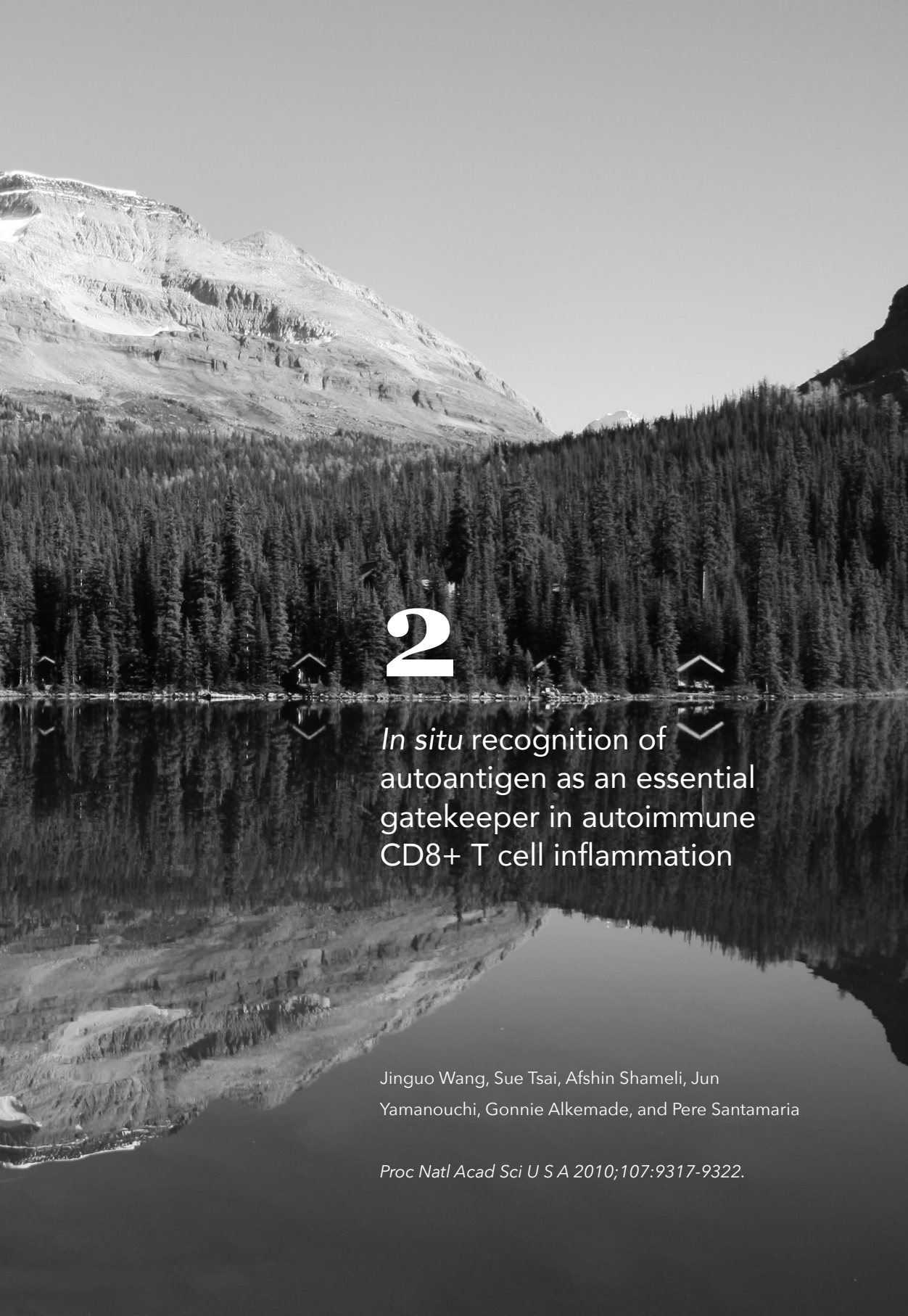

2

In situ recognition of autoantigen as an essential gatekeeper in autoimmune CD8+ T cell inflammation

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ABSTRACT

A current paradigm states that non-antigen-specific inflammatory cues attract non-cognate, bystander T-cell specificities to sites of infection and autoimmune inflammation. Here we show that cues emanating from a tissue undergoing spontaneous autoimmune inflammation cannot recruit naïve or activated bystander T-cell specificities in the absence of local expression of cognate antigen. We monitored the recruitment of CD8+ T-cells specific for the prevalent diabetogenic epitope $IGRP_{206-214}$ in gene-targeted nonobese diabetic (NOD) mice expressing a T-cell 'invisible' $IGRP_{206-214}$ sequence. These mice developed islet inflammation and diabetes with normal incidence and kinetics, but their inflammatory lesions could recruit neither naive (endogenous or exogenous) nor *ex-vivo*-activated $IGRP_{206-214}$-reactive CD8+ T-cells. Conversely, $IGRP_{206-214}$-reactive, but not non-autoreactive CD8+ T-cells rapidly homed to and accumulated in the inflamed islets of wild-type NOD mice. Our results indicate that CD8+ T-cell recruitment to a site of autoimmune inflammation results from an active process that is strictly dependent on local display of cognate pMHC, and suggest that CD8+ T-cells contained in extra-lymphoid autoimmune lesions are largely autoreactive.

INTRODUCTION

Recognition of cognate peptide–major histocompatibility complexes (pMHC) on the surface of dendritic cells (DC) by naive T-lymphocytes in lymph nodes draining a site of infection or autoimmune inflammation elicits the lymphocytes' activation, proliferation and differentiation into cytolytic effectors. Upon activation, lymphocytes also acquire the ability to survey non-lymphoid tissues for presence of their cognate target antigens, with a preference for inflamed tissues as well as tissues drained by the lymph nodes where activation took place (1-3). Studies in a number of infection and autoimmune disease models have suggested that recruitment of T-lymphocytes into a site of extra-lymphoid inflammation does not require local expression of cognate (foreign or self) pMHC on tissue cells or tissue-associated antigen-presenting cells (4-7). Accordingly, it is generally thought that non-antigen-specific inflammatory cues such as cytokines and chemokines emanating from the local microenvironment can recruit non-cognate (i.e. bystander) T-cells to a site of foreign or self antigen-triggered tissue inflammation (8-19). Notwithstanding that *in vitro*-activated bystander T-cell clones can transiently co-migrate with their cognate counterparts into non-inflamed tissue in adoptive T-cell transfer experiments; and that tissue-specific expression of cytokine and/or chemokine transgenes in normal tissues can trigger bystander T-cell inflammation, these models do not faithfully mimic the events that take place in spontaneous autoimmune inflammation. Specifically, it is unclear that bystander T-cell specificities can effectively compete with their cognate polyclonal counterparts, recognizing pMHC *in situ*, for occupation of the inflammatory space.

Type 1 diabetes (T1D) in both humans and NOD mice is a chronic autoimmune disease that results from inflammation of pancreatic islets and destruction of pancreatic β cells by T-cells targeting numerous β cell autoantigens (20, 21). A significant fraction of the islet-associated CD8+ cells in NOD mice recognize the mimotope NRP-V7 in the context of the MHC molecule K^d (22-25). These CD8+ T-cells are diabetogenic (22, 26), target a peptide from islet-specific glucose-6-phosphatase catalytic subunit-related protein ($IGRP_{206-214}$, similar to NRP-V7) (27), and circulate in the peripheral blood at a relative high frequency (>1/200 CD8+ T-cells), particularly as clinical disease nears (27, 28).

To ascertain whether local expression of cognate pMHC is a *sine-qua-non* condition for recruitment and/or accumulation of CD8+ T-cells to the pancreas during spontaneous autoimmune diabetes, we generated a gene-targeted NOD strain capable of developing islet inflammation but expressing a T-cell 'invisible' $IGRP_{206-214}$ epitope. We find that these mice develop insulitis and diabetes essentially like wild-type NOD mice but cannot recruit endogenous or exogenous $IGRP_{206-214}$-specific CD8+ T-cells, regardless of their activation state or degree of islet inflammation.

RESULTS AND DISCUSSION

Knock-in NOD mice expressing a T-cell 'invisible' IGRP$_{206-214}$ epitope

We generated a gene-targeted NOD strain expressing a mutant form of IGRP in which the two TCR-contact residues of IGRP$_{206-214}$ (25) are replaced with alanines (K209A and F213A) (Fig. 1A). The IGRP$_{K209A-F213A}$ peptide cannot trigger the activation or elicit the cytotoxicity of 8.3-CD8+ T-cells (25), which express a transgenic IGRP$_{206-214}$-reactive TCR (22), and does not impair, either *in vitro* or *in vivo*, their responsiveness to a subsequent challenge with IGRP$_{206-214}$ (Fig. S1). As expected, IGRP$_{K209A/F213A}$-homozygous knock-in NOD mice (NOD.IGRP$_{K209A/F213A}^{KI/KI}$) and wild-type NOD mice displayed indistinguishable thymic and splenic T-cell profiles (Fig. 1B) and exported similar numbers of IGRP$_{206-214}$-reactive CD8+ cells to the circulation (Fig. 1C). Analyses of pancreata from pre-diabetic animals indicated that both types of mice developed insulitis lesions of similar severity (Fig. 1D), similar CD4+ T-cell content and slightly different (but not statistically different) CD8+ T-cell content (Fig. 1E).

Notably, however, the islet-associated T-cells of pre-diabetic NOD.IGRP$_{K209A/F213A}^{KI/KI}$ mice did not contain IGRP$_{206-214}$-reactive CD8+ T-cells, as determined by NRP-V7/K^d tetramer staining (Fig. 1F), and did not produce IFNγ in response to NRP-V7 peptide-pulsed APCs (Fig. 1G). Impaired recruitment of IGRP$_{206-214}$-reactive CD8+ T-cells was associated with a significant increase in recruitment of other autoreactive T-cell specificities that are present at very low precursor frequencies in the islets of pre-diabetic mice (20, 21, 28), such as insulin-B$_{15-23}$-reactive CD8+ T-cells (29) (Fig. 1G). As a result, NOD and NOD.IGRP$_{K209A/F213A}^{KI/KI}$ mice developed T1D with virtually identical incidence curves (Fig. 1H). These data indicated that (i) IGRP$_{206-214}$-reactive CD8+ T-cells are completely excluded from insulitic lesions in the absence of local expression of IGRP$_{206-214}$; and that (ii) initiation and progression of spontaneous T1D in NOD mice does not require the accumulation of IGRP$_{206-214}$-reactive CD8+ T-cells into pancreatic islets.

Severely impaired recruitment of naïve IGRP$_{206-214}$-reactive CD8+ T-cells to the inflamed islets of NOD.IGRP$_{K209A/F213A}^{KI/KI}$ mice

To further investigate the role of local cognate pMHC vs. non-antigen-specific inflammatory cues in the recruitment of CD8+ T-cells to pancreatic islets, we ascertained whether naive and *in vitro*-pre-activated IGRP$_{206-214}$-reactive 8.3-CD8+ T-cells could undergo activation in the pancreatic lymph nodes (PLN) and/or home to the inflamed islets of pre-diabetic 10-12 wk-old NOD.IGRP$_{K209A/F213A}^{KI/KI}$ hosts (i.e. in response to pre-existing local inflammatory cues). Adoptively transferred naive CFSE-labeled 8.3-CD8+ T-cells (10^7) proliferated in the PLNs (and, to a much lesser extent, in the MLN and spleen) of insulitic NOD mice within a wk after adoptive transfer (Fig. 2A and B). Analysis of the islet-infiltrates of these insulitic NOD hosts 1, 2 and 3 wk after T-cell transfer revealed rapid recruitment (within 1 week) of actively proliferating 8.3-CD8+ T-cells (Figs. 2C-F). Notably, almost all the 8.3-CD8+ T-cells found within islets at this stage had undergone >2 cell divisions, and most of the cells that had only divided <3 times were found exclusively in the PLNs (Fig. 2C and Fig. S2), suggesting that recruitment of autoreactive

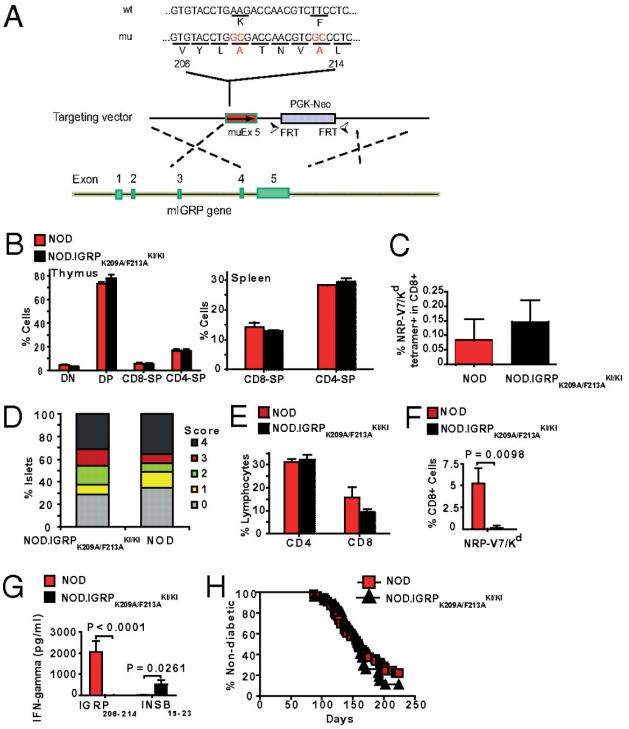


Figure 1 | NOD.IGRP$_{K209A/F213A}^{KI/KI}$ mice develop insulitis and diabetes without recruiting IGRP$_{206-214}$-reactive CD8+ T-cells into pancreatic islets.

(A) Targeting strategy. The FRT-flanked PGK-neo cassette was removed from targeted ES cells by transient transfection of Flp recombinase-encoding cDNA. **(B)** Distribution of lymphocyte subsets in thymi and spleens from NOD and NOD.IGRP$_{K209A/F213A}^{KI/KI}$ mice (n=3 and 5, respectively; 3 independent experiments): DN (double negative); DP (double positive); CD8-SP (CD8 single positive) and CD4-SP (CD4 single positive). **(C)** Frequency of NRP-V7 reactive CD8+ T-cells in peripheral blood. PBMC from 10 wk-old mice (NOD, n=7; NOD.IGRP$_{K209A/F213A}^{KI/KI}$, n=8) were stained with NRP-V7/K^d tetramers and anti-CD8 mAb. Data correspond to 4 independent experiments using 1-5 mice/experiment. **(D)** Insulitis scores. Pancreata from non-diabetic 32 week-old mice (NOD, n=3; NOD.IGRP$_{K209A/F213A}^{KI/KI}$, n=5) were examined for islet inflammation. Pancreata were from one cohort of NOD mice and two different cohorts of NOD.IGRP$_{K209A/F213A}^{KI/KI}$ mice. (E and F) CD4+ and CD8+ T-cell **(E)** and NRP-V7/K^d tetramer+ CD8+ T-cell content **(F)** in freshly isolated islets of NOD (n=6; 3 independent experiments) vs. NOD.IGRP$_{K209A/F213A}^{KI/KI}$ mice (n=7; 4 independent experiments). **(G)** Absence of IGRP$_{206-214}$-reactive CD8+ T-cells in the islet infiltrates of NOD.IGRP$_{K209A/F213A}^{KI/KI}$ (n=10; 10 independent experiments) vs. NOD mice (n=3; 3 independent experiments). Islet-associated CD8+ T-cells were cultured in IL-2 for 7 days and challenged with peptide-pulsed (10 mM) irradiated NOD splenocytes. The IFNγ content in the supernatants (at 48 h) was measured by ELISA. Data correspond to the means ± SEM. **(H)** Diabetes incidence in female NOD (n=56) and NOD.IGRP$_{K209A/F213A}^{KI/KI}$ (n=27) mice. The average blood glucose levels in newly diagnosed diabetic NOD and NOD.IGRP$_{K209A/F213A}^{KI/KI}$ mice are: 22.3 ± 2.5 vs. 23.7 ± 3.3 mM, respectively. In males, the incidence and average age at onset of disease were also similar in both strains (NOD: n=15; 40% diabetic at 133 ± 33 days; and NOD.IGRP$_{K209A/F213A}^{KI/KI}$: n=20; 50% diabetic at 149 ± 36 days). Data in B-F correspond to the means ± SEM. P values in F and G were obtained with Mann-Whitney U.

CD8+ T-cells into the pancreas is invariably preceded by antigen-induced activation in the PLNs. The islets (but not the PLNs) of hosts analyzed 2 weeks after T-cell transfer contained higher percentages of proliferating cells (Fig. 2C, E, and Fig. S2), and total 8.3-CD8+ T-cells (Fig. 2D, F). By the third week there was a further increase in the extent of cell division in islets (Fig. 2C and Fig. S2) in association with reductions in the percentages and total number of proliferated 8.3-CD8+ T-cells, presumably due to attrition by activation-induced cell death (i.e. in response to repetitive stimulation of differentiated CD8+ T-cells by cognate pMHC) (Fig. 2D-F). Thus, accumulation of autoreactive CD8+ T-cells in the inflamed islets of pre-diabetic NOD mice is associated with (i) T-cell activation and proliferation in the PLNs; (ii) recruitment of actively proliferating cells into pancreatic islets; and (iii) additional rounds of local (intra-islet) proliferation.

A remarkably different outcome was obtained when these experiments were done in age-matched, insulitic NOD.IGRP$_{K209A/F213A}^{KI/KI}$ hosts. Whereas the transfused 8.3-CD8+ T-cells readily homed to the spleen, PLNs and MLNs of insulitic NOD.IGRP$_{K209A/F213A}^{KI/KI}$ hosts (Fig. 2C,D), they did not proliferate in the PLNs (Figs. 2A-C and Fig. 2E, F, and Figs. S2 and S3), confirming that this event requires crosspresentation of b cell-derived IGRP$_{206-214}$. There was also a reduction in the proliferation of cognate 8.3-CD8+ T-cells in the MLNs and spleens of NOD.IGRP$_{K209A/F213A}^{KI/KI}$ vs. NOD mice (Fig. 2B, C), suggesting that some of the T-cells that are activated in the PLNs and/or islets (or the activating IGRP$_{206-214}$-loaded APCs) of wild-type NOD mice migrate to distant secondary lymphoid organs during disease progression. Most notably, the adoptively transferred cells failed to home to pancreatic islets of insulitic NOD.IGRP$_{K209A/F213A}^{KI/KI}$ hosts, where they could not be found throughout the 3 week study period (Fig. 2C-F), despite the presence of severe local inflammation (Fig. 1D). In fact, up to more than 6% (~10^4) of all the islet-associated lymphocytes of NOD hosts were donor-derived, compared to virtually none of those isolated from the NOD.IGRP$_{K209A/F213A}^{KI/KI}$ hosts (Fig. 2F). Thus, non-specific inflammatory cues emanating from insulitic lesions cannot single-handedly (in the absence of local cognate pMHC) recruit naive bystander IGRP$_{206-214}$-reactive CD8+ T-cells to the site.

T1D-irrelevant CD8+ T-cell specificities are not recruited to the inflamed pancreatic islets of wild-type NOD mice

To rule out the possibility that this outcome was a peculiarity of the IGRP$_{206-214}$-reactive CD8+ T-cell population, we tracked the recruitment of adoptively transferred naive Thy1.2+ LCMV gp33-specific CD8+ T-cells [a non-autoreactive, T1D-irrelevant T-cell population from LCMV gp33-TCR-transgenic (P14) NOD donor mice] in insulitic NOD.Thy1.1 hosts. This was done by co-transfusing these cells with an equal number of naive Thy1.2+ 8.3-CD8+ T-cells and by analyzing the hosts' lymphoid organs and pancreatic islets for presence of both T-cell pools 1, 7, 14 and 21 days after transfer. Whereas Thy1.2+ NRP-V7/K^d-tetramer+ and gp33/D^b-tetramer+ CD8+ T-cells were rapidly recruited to the spleen, PLNs and MLNs within 1 day of transfer, only the former homed to a significant degree to pancreatic islets (Fig. 3A,B). Accordingly, naive IGRP$_{206-214}$-reactive CD8+ T-cells need to engage cognate pMHC on local APCs and/or on b cells to access and/or accumulate (owing to retention and/or proliferation) in inflamed tissue.

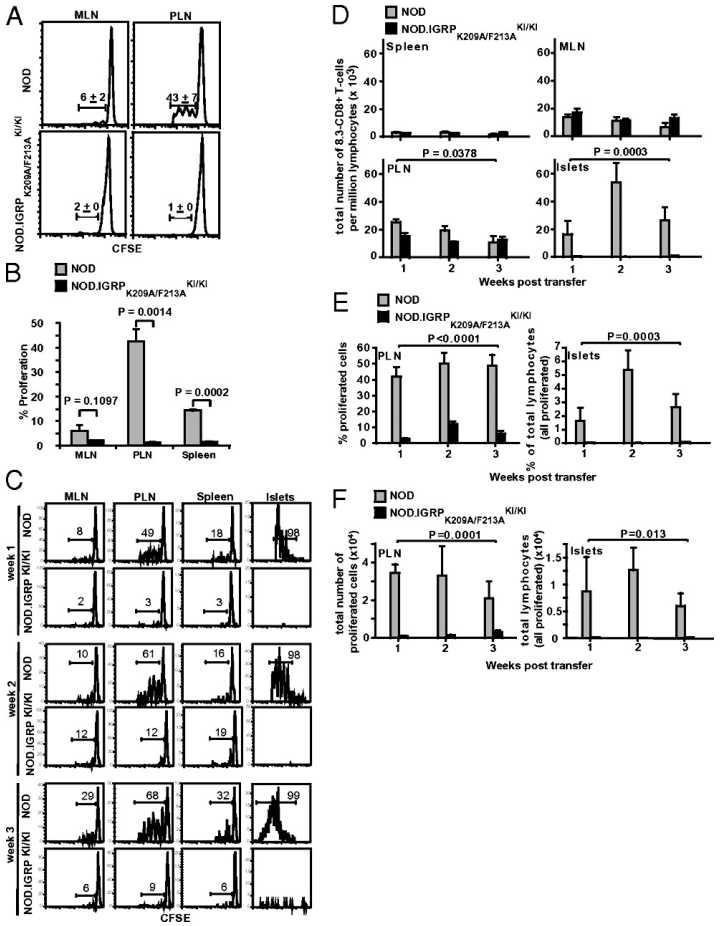


Figure 2 | Naive 8.3-CD8+ T-cells are not recruited into the inflamed pancreatic islets of NOD.IGRP$_{K209A/F213A}^{KI/KI}$ mice.

(A) Proliferation of naïve 8.3-CD8+ T-cells in the PLN. CFSE-labeled naïve 8.3-CD8+ T-cells were transfused into 10-12 wk-old NOD (n=3) or NOD.IGRP$_{K209A/F213A}^{KI/KI}$ (n=3) recipients (3 independent experiments, each using both host types). Dilution of CFSE was measured by flow cytometry 7 days post-transfer. Values correspond to the average percentage of proliferated CD8+ T-cells ± SEM. (B) Summary of data described in (A). P values were obtained with Mann-Whitney U. (C) Recruitment and proliferation of naïve 8.3-CD8+ T-cells from 8.3-NOD.*Thy1.1* donor mice in the lymphoid organs and islets of 10-12 wk-old insulitic NOD and NOD.IGRP$_{K209A/F213A}^{KI/KI}$ hosts 1, 2 and 3 wk after transfer. CFSE histograms correspond to Thy1.1+CD8+ cells. (D) Mean ± SEM of total numbers of 8.3-CD8+ T-cells per million lymphocytes. Data in (C) and (D) correspond to 3-6 experiments/time point and host type (1 mouse/time point/host in each experiment). (E) Mean ± SEM of percentages of proliferated cells (for PLNs) or total number of donor lymphocytes (for islet T-cell isolates, where all donor-derived T-cells were proliferating) (3-6 experiments/time point and host type; 1 mouse/time point/host in each experiment). (F) Absolute numbers of proliferated (for PLNs) or recruited (for islet T-cell isolates) 8.3-CD8+ T-cells (mean ± SEM) (3-6 experiments/time point and host type; 1 mouse/time point/host in each experiment). P values in D, E and F were obtained by two-way ANOVA.

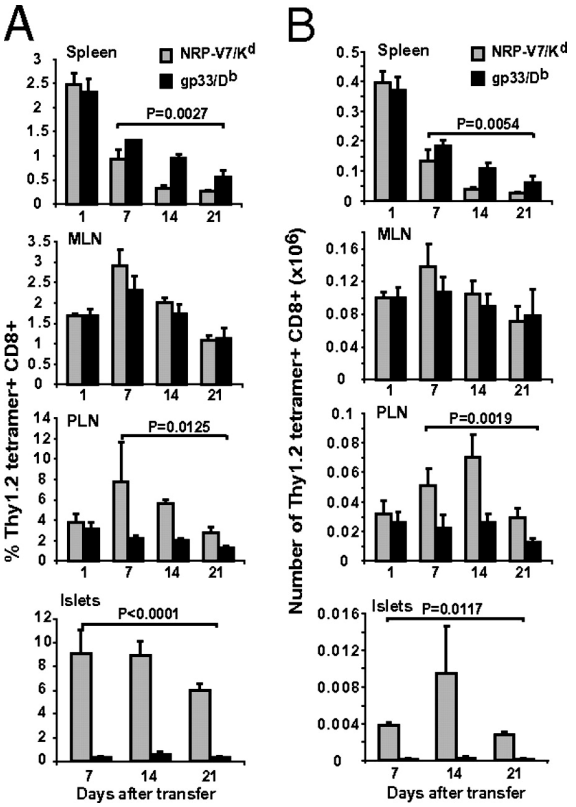


Figure 3 | Selective recruitment of cognate autoreactive T-cells into pancreatic islets of NOD mice. **(A)** Naïve 8.3-CD8+/Thy1.2+ and P14-CD8+/Thy1.2+ T-cells were mixed at 1:1 (8 x 10^6 cells each) and injected i.v. into 10-12 wk-old NOD.*Thy1.1* hosts. Hosts were sacrificed at various time points after transfer and their peripheral lymphoid organs and islet cell suspensions analyzed for presence of NRP-V7/K^d and GP33/D^b tetramer+ Thy1.2+ CD8+ T-cells. Data correspond to 3 mice/time point (n=12 mice) and 3 independent experiments (1 mouse/time point in each experiment) and are shown as mean ± SEM. **(B)** Data from (A) presented as absolute numbers of cells (mean ± SEM). P values were obtained with two-way ANOVA.

Pre-activated $IGRP_{206-214}$-specific Cytotoxic T lymphocytes also fail to home to the insulitic lesions of NOD.$IGRP_{K209A/F213A}^{KI/KI}$ hosts

To investigate the role of T-cell activation in the recruitment and/or accumulation of bystander T-cells to inflamed and non-inflamed islets, we transfused CFSE-labeled, *in vitro*-differentiated Thy1.1+ 8.3-CTLs (1.5 x 10^7) into non-insulitic (3 week-old) or insulitic (10-12 week-old) NOD and NOD.$IGRP_{K209A/F213A}^{KI/KI}$ hosts. Whereas CFSE+ 8.3-CTLs were rapidly recruited into non-insulitic NOD islets (Fig. 4A), leading to rapid loss of insulin-producing b cells and rampant development of diabetes in all hosts within 5 days (Fig. 4B), they were neither recruited to islets, nor caused any obvious b cell loss or diabetes in any non-insulitic NOD.$IGRP_{K209A/F213A}^{KI/KI}$ hosts for up to 6 weeks after transfer (Fig. 4A and B), suggesting that activated CD8+ T-cells cannot home to non-inflamed

tissue in the absence of local cognate pMHC. Similar results were obtained when 8.3-CTLs were transfused into insulitic hosts. 8.3-CTL were progressively recruited to, and accumulated in the PLNs and MLNs of both types of mice during the first 3 days after transfer (~2-3-fold on day 3 vs. day 1; Fig. 4C,D). In contrast, whereas CFSE+ 8.3-CTL accumulated in the islets of insulitic NOD mice (~7-9 fold on day 3 vs. day 1), they did not do so in the insulitic islets of NOD.IGRP$_{K209A/F213A}^{KI/KI}$ hosts (Fig. 4C,D), confirming a critical role for cognate pMHC on retention and accumulation of IGRP$_{206-214}$-specific CTL in non-lymphoid tissue. Taken together, these data suggest that T-cell occupation of the inflamed islet space in spontaneous autoimmune diabetes is not due to "diffusion" from the periphery in response to inflammatory and chemotactic cues, but rather to an active process that involves local recognition of cognate pMHC.

Our observations challenge the generally held assumption that T-cell infiltrates in inflamed extra-lymphoid tissues, such as pancreatic islets in diabetes, contain a mixture of both cognate and non-cognate (i.e. bystander) T-cell specificities. Our results demonstrate, in a model of highly polyclonal spontaneous autoimmunity, that bystander CD8+ T-cells, even after activation, are strongly selected against for retention and accumulation in the target organ. This contention does not argue against the idea that bystander T-cells can transiently migrate to a site of inflammation non-specifically, such as in response to cytokine-induced chemokine receptor ligands like CXCL9, CXCL10 and CXCL11 (30, 31). Rather, our data strongly argues that, in the absence of cognate pMHC, non-specifically recruited CD8+ T-cells cannot effectively compete with cognate T-cell specificities for occupation of space. Recent studies in dual TCR retrogenic, sublethally-irradiated bone marrow chimeras co-expressing T1D-relevant and irrelevant MHC class II-restricted autoreactive CD4+ T-cell specificities in NOD.*scid* hosts suggest that naive CD4+ T-cells may also require local engagement of cognate pMHC for recruitment to non-inflamed islets (32). However, whether bystander naïve and/or activated CD4+ T-cells can home to inflamed islets, particularly in a model of spontaneous polyclonal inflammation such as the one described herein, remains to be determined.

Whichever the case for CD4+ T-cells might be, and assuming that this paradigm can be generalized to other antigenic specificities, our data suggest that the majority of all CD8+ T-cells that are recruited to sites of autoimmune inflammation, such as pancreatic islets during diabetogenesis, are autoreactive. Autoreactive CD8+ T-cells need not have to engage cognate pMHC directly on the b-cell surface to be effectively retained at the target site; recognition of pMHC on vascular endothelial cells (2, 33) or on a tissue-resident professional APC population might be sufficient. This would explain why NOD mice expressing a RIP-driven adenoviral E19 transgene, whose beta cells express significantly reduced levels of pMHC class I (34), and NOD mice with a beta cell-specific disruption of beta-2 microglobulin, which cannot display pMHC class I complexes on the surface (35), recruit CD8+ T-cells to pancreatic islets.

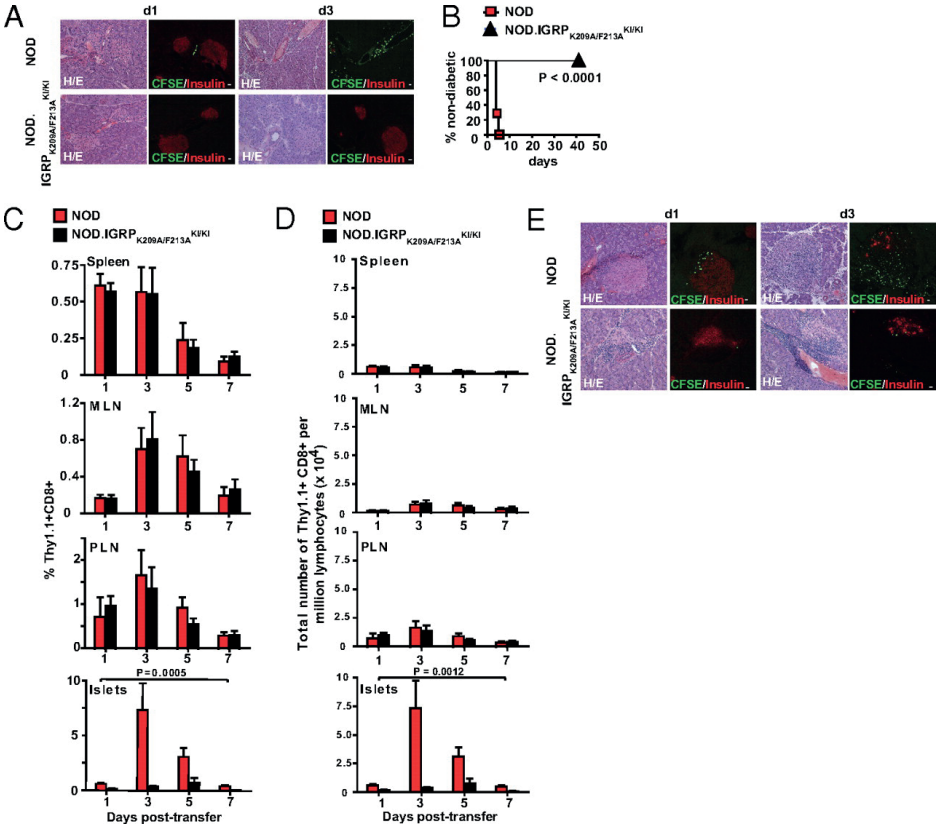


Figure 4 | The pancreatic islets of NOD.IGRP$_{K209A/F213A}^{KI/KI}$ mice also fail to recruit activated 8.3-CD8+ T-cells, even when inflamed.

(A) *In vitro*-activated, CFSE-labeled 8.3-CD8+ T-cells were injected i.v. into 3 wk-old NOD or NOD.IGRP$_{K209A/F213A}^{KI/KI}$ hosts. Pancreatic sections were stained with anti-insulin antibodies and examined for presence of CFSE+ T-cells by confocal microscopy on days 1 and 3 after transfer (3 mice/time point; 10-20 islets/mouse; 3 independent experiments). Panel shows representative images. An adjacent tissue section was stained with H&E (left). White bars represent 20 mm. On NOD hosts' day 1 samples, CFSE+ T-cells were predominantly found in the periinsular space. Note the near complete depletion of insulin+ cells in the CFSE+ T-cell-containing areas of NOD hosts' day 3 samples. **(B)** Incidence of diabetes in 3 wk-old NOD (n=7) or NOD.IGRP$_{K209A/F213A}^{KI/KI}$ recipients (n=6) of 8.3-CTL (2 independent experiments, each including 3-4 mice/strain type). P values were obtained with log rank test. **(C)** *In vitro*-activated, CFSE-labeled 8.3-CD8+ Thy1.1+ T-cells were injected i.v. into 10-12 wk-old NOD or NOD.IGRP$_{K209A/F213A}^{KI/KI}$ hosts. Hosts were analyzed for presence of Thy1.1+ CD8+ T-cells in different organs. Data correspond to 3-4 independent experiments/time point and strain (1 mouse/time point/strain in each experiment) and are shown as mean ± SEM. **(D)** Data from (C) presented as absolute numbers of cells per million lymphocytes (mean ± SEM). P values in C and D were obtained with two-way ANOVA. **(E)** Analysis of pancreas sections from 10-12 week-old mice transfused with CFSE-labeled *in vitro*-activated 8.3-CD8+ T-cells on days 1 and 3 after transfer, for presence of CFSE+ T-cells (3 independent experiments; 1 mouse/time point/strain in each experiment). Images of islets are representative of severe insulitis.

MATERIALS AND METHODS

Mice. 8.3-TCR-transgenic NOD mice (Thy1.2+) have been described (22). Thy1.1-congenic NOD mice (NOD.*Thy1.1*) and LCMV-Gp33-specific TCR-transgenic (P14) NOD mice were obtained from the T1D repository (Jackson Lab, Bar Harbor, ME). To generate $IGRP_{K209A/F213A}^{KI/KI}$ mice, we transfected an *igrp* targeting construct carrying a mutated exon 5 (encoding an $IGRP_{206-214}$ epitope in which the two TCR contact residues were replaced by Ala: (VYLATNVAL; K209A/F213A) into CK35 129/Sv-derived murine ES cells (Fig. 1). The FRT-flanked PGK-neo cassette was removed from targeted ES cells by transient transfection of Flp recombinase-encoding cDNA. Transfected ES clones were screened by Southern blot analysis using Avr II or Apa I-digested DNA and 5′ and 3′-specific probes differentiating wild-type (11.6 and 11.1 kb for 5′ and 3′ arms, respectively) versus targeted bands (7.7 and 6.7 kb, respectively). Type II recombinants arising from Flp-mediated deletion of the PGK-neo cassette were identified by Southern blotting using the 3′ probe described above. To produce NOD.$IGRP_{K209A/F213A}^{KI/WT}$ mice, we backcrossed the targeted $IGRP_{K209A/F213A}$ allele from germline-competent 129/Sv chimeras onto the NOD background for at least 6 generations. Genome-wide SNP analyses at the N6 backcross were done to confirm homozygosity for all known NOD diabetes-susceptibility alleles. Mice were intercrossed at the N6 or N7 generations to produce NOD.$IGRP_{K209A/F213A}^{KI/KI}$ homozygotes. These studies were approved by the Faculty of Medicine's Animal Care Committee and followed the guidelines of the Canadian Council of Animal Care.

Peptides, tetramers. The peptides $IGRP_{206-214}$ (VYLKTNVFL), $INSB_{15-23}$ (LYLVCGERG), NRP-V7 (KYNKANVFL), TUM (KYQAVTTTL), LCMV GP33 (KAVYNFATM) and the corresponding tetramers (PE-labeled) were prepared as described (27, 28). Briefly, the peptides were folded with recombinant human b2m and respective mouse heavy chains and subjected to gel filtration purification, biotinylation and ion exchange purification using an AKTA FPLC system (GE Healthcare). The final product was verified by both denaturing SDS-PAGE and native PAGE analysis.

Islet isolation. Pancreatic islets were isolated by hand-picking after collagenase P digestion of the pancreas, cultured overnight in IL-2-containing media (to avoid additional enzymatic digestion steps and thus enhance T-cell recovery and viability, as described (36)), disrupted into single cells, stained and analyzed by flow cytometry.

Flow cytometry. Peripheral blood and islet cell suspensions were stained with tetramers (5 mg/mL) in FACS buffer (0.1% sodium azide and 1% FBS in PBS) for 1 h at 4°C, washed, and incubated with FITC-conjugated anti-CD8a (5 mg/mL) for 30 min at 4°C. For other stains, thymuses, spleens and lymph node cell suspensions were analyzed by 3-color flow cytometry using anti–CD8-PerCP (53-6.7), anti–CD4-FITC (IM7) and tetramer-PE, or with anti–CD8-PerCP (53-6.7), tetramer-PE and FITC-conjugated anti-Thy1.2 mAb, or with anti–CD8-PerCP (53-6.7) and PE-conjugated anti-Thy1.1 mAb. Cells were washed, fixed in 1% PFA/PBS and analyzed by FACS. All mAbs were from BD Pharmingen. Data were analyzed by FlowJo (Tree Star, Inc.).

Specificity of islet-associated CD8+ T-cells. Islet-infiltrating cells from NOD or NOD.IGRP$_{K209A, F213A}^{KI/KI}$-mice mice were cultured for 7 days in the presence of 0.5 U/ml of rIL-2 to expand *in vivo*-activated islet-associated T-cells. Upon washing, CD8+ T-cells were cultured, in the absence of exogenous IL-2, with peptide-pulsed (10 mM) irradiated NOD splenocytes for 48h. The IFNγ content in the supernatants was measured by ELISA (Duoset, R&D systems, Minneapolis, MN). Values obtained with the negative control peptide TUM were subtracted.

Adoptive Transfer. Splenic CD8+ T-cells were purified using iMAG CD8 beads (BD Bioscience) following the manufacture's protocols, labeled with CFSE (2.5 mM), and injected i.v. (10^7 CD8+ T-cells). In co-transfer experiments employing P14 CD8+ T-cells, each mouse received a mixture of 8 x 10^6 8.3-CD8+ and 8 x 10^6 P14-CD8 T-cells. To generate *in vitro*-activated 8.3-CD8+ T-cells, splenocytes from 8.3-NOD or 8.3-NOD.*Thy1.1* donor mice were cultured in the presence of NRP-V7 peptide (1 mM) for 3 days in the absence of exogenous IL-2. These conditions generate highly diabetogenic CTL (Fig. 4B). The proliferating CD8+ T-cells (>95% of the cells) were then labeled with 2.5 mM CFSE and transfused i.v. (15 x 10^6) into unmanipulated 3 week or 10-12 week-old hosts. Mice were killed 1, 3, 5, 7, 14 or 21 days later and their spleens, PLN and MLN examined for presence of donor CD8+ T-cells (Tetramer+ and thy1.2+) or for dilution of CFSE in the CD8+ gate.

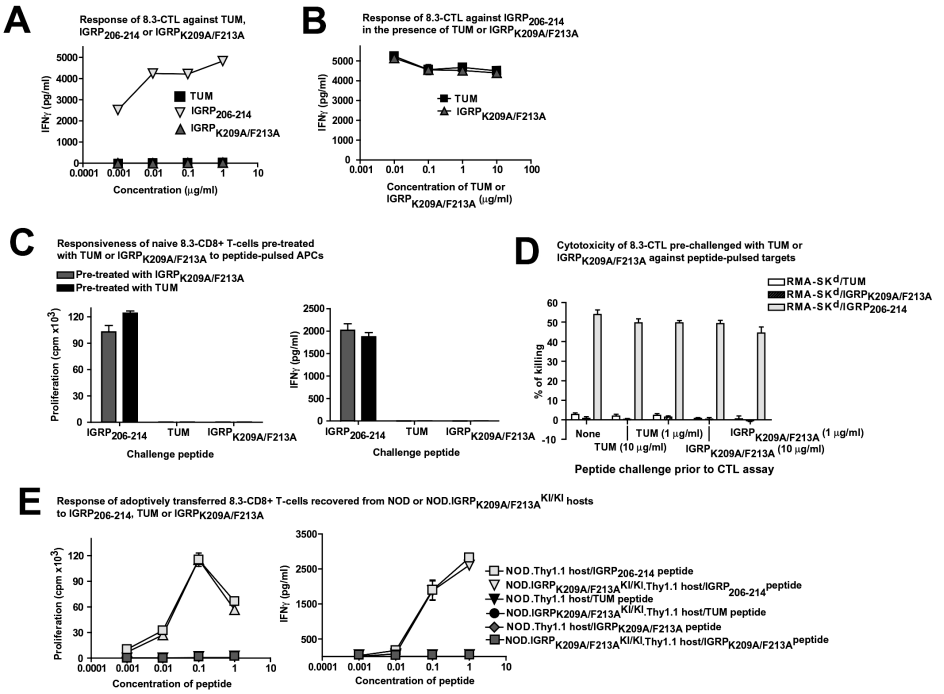
Immunopathology. Formalin-fixed, paraffin-embedded pancreas sections were stained with H&E and scored for insulitis (see below). To examine pancreatic islets for infiltration by transfused CFSE+ T-cells, pancreata were embedded in OCT medium and frozen in a dry ice/acetone bath. Cryosections (5 mm) were fixed with 3% paraformaldehyde, stained with guinea pig anti-insulin antibodies and Cy3-conjugated rabbit anti-guinea pig antibodies (Invitrogen, Carlsbad, CA). The sections were then mounted with prolong-Gold (Invitrogen) and analyzed with an Olympus FV1000 confocal microscopy system. Immediately adjacent sections were stained with H&E. We analyzed ~20 islets per mouse.

Insulitis scores. Scoring of insulitis lesions was performed as described (26). The degree of mononuclear cell infiltration was scored as: 0, none; 1, peri-insulitis; 2, infiltration covering <25% of the islet; 3, covering 25-50% of the islet; and 4, covering >50% of the islet.

Diabetes. Diabetes was monitored by measuring urine glucose levels twice weekly. Animals were considered diabetic after 2 consecutive readings greater than or equal to 3+. The average blood glucose levels in mice diagnosed as diabetic using this criteria are 21.96 $\pm$ 3.8 mM, and none of these mice have blood glucose levels below 16 mM.

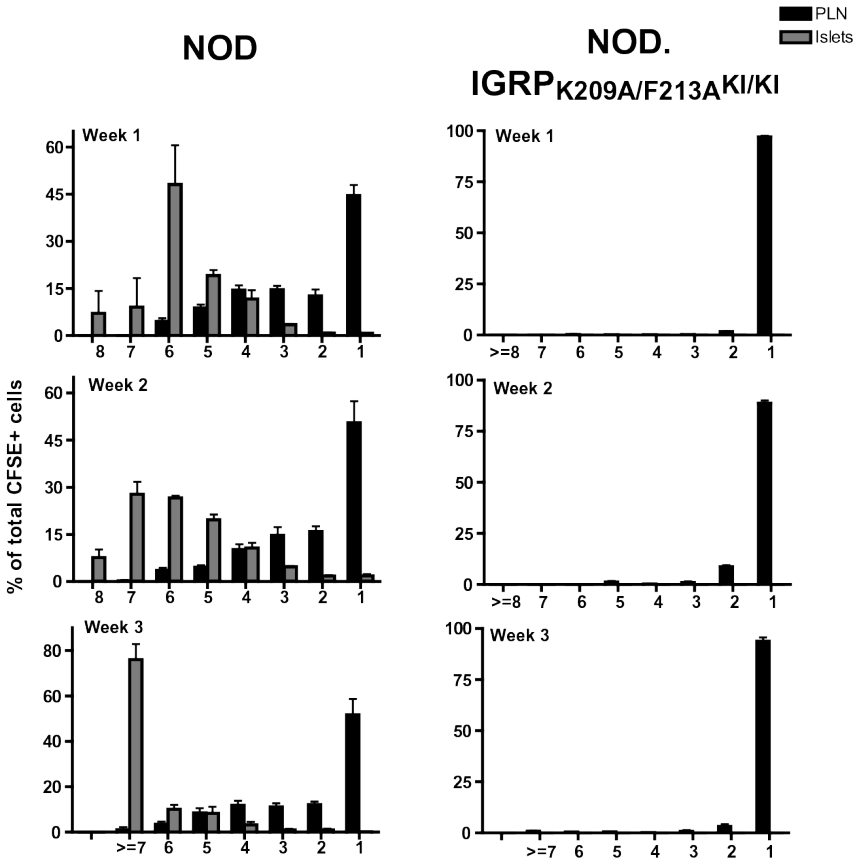
Statistical analyses. Data were compared by two-tailed Mann-Whitney U, Chi-Square, or two-way ANOVA tests. Statistical significance was assumed at $P < 0.05$.

Supplemental material. Fig. S1 shows that the IGRP$_{K209A/F213A}$ epitope is not recognized by, and does not alter the functional responsiveness of IGRP$_{206-214}$-reactive CD8+ T-cells either *in vitro* or *in vivo*. S2 shows that recruitment of naive 8.3-CD8+ T-cells into the pancreatic islets of NOD mice is preceded by antigen-induced proliferation in the PLN. Fig. S3 shows that naive 8.3-CD8+ T-cells do not proliferate in the PLN of NOD.*IGRP*$^{KI/KI}$ hosts.



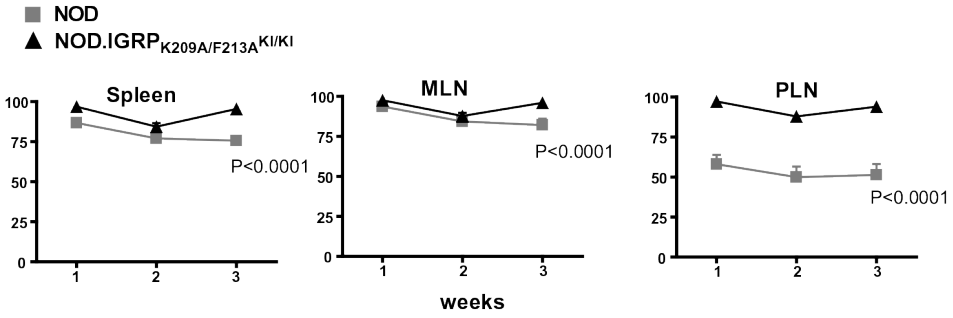
Supplementary figure 1 |

The $IGRP_{K209A/F213A}$ epitope is not recognized by, and does not alter the functional responsiveness of $IGRP_{206-214}$-reactive 8.3-CD8+ T cells either in vitro or in vivo. (A) Differentiated 8.3-CD8+ T cells secrete IFNγ in response to splenocytes pulsed with $IGRP_{206-214}$ but not $IGRP_{K209A/F213A}$. IFNγ content in the supernatants was measured at 24 h of culture. (B) $IGRP_{K209A/F213A}$ does not inhibit the responsiveness of differentiated 8.3-CD8+ T cells to $IGRP_{206-214}$. Differentiated 8.3-CD8+ T cells were cultured with $IGRP_{206-214}$-pulsed (0.1 μg/mL) splenocytes in the presence of various concentrations of TUM or $IGRP_{K209A/F213A}$ for 24 h and the supernatants collected to measure the IFNγ concentration. (C) Pretreatment of naive 8.3-CD8+ T cells with $IGRP_{K209A/F213A}$ (or TUM) peptide does not alter their subsequent responsiveness to $IGRP_{206-214}$. Naive splenic 8.3-CD8+ T cells were preincubated with 10 μg/mL $IGRP_{K209A/F213A}$ or TUM for 2 days. CD8+ T cells were then purified and tested for their ability to proliferate (Left) and secrete IFNγ (Right) in response to bone marrow-derived dendritic cells pulsed with 0.1 μg/mL $IGRP_{206-214}$, TUM, or $IGRP_{K209A/F213A}$. (D) Differentiated 8.3-CD8+ T cells cannot kill targets pulsed with $IGRP_{K209A/F213A}$, and preincubation of 8.3-CD8+ CTL with $IGRP_{K209A/F213A}$ does not inhibit their cytotoxic activity against RMA-SKd targets pulsed with $IGRP_{206-214}$. The 8.3-CD8+ CTLs were pre- incubated with bone marrow-derived dendritic cells pulsed with 1 or 10 μg/mL of either TUM or $IGRP_{K209A/F213A}$ for 24 h, purified away from DCs using mAb- coated magnetic beads, and used as effectors in a standard 51Cr-release assay using RMA-SKd cells pulsed with 10 μg/mL of $IGRP_{206-214}$, TUM or $IGRPK_{209A/F213A}$ at an 8:1 effector:target ratio in triplicate wells. Percentage of killing was calculated as (51Cr in test well − spontaneous release)/(maximum release − spontaneous release) × 100. (E) Encounter of $IGRP_{K209A/F213A}$ by 8.3-CD8+ T cells in vivo does not impair their functional responsiveness to $IGRP_{206-214}$ ex vivo. Ten million 8.3-CD8+ T cells (Thy1.2+) were adoptively transferred into NOD.Thy1.1 or NOD.$IGRP_{K209A/F213A}^{KI/KI}$.Thy1.1 hosts (8- to 12-weeks old). Seven days posttransfer, Thy1.2+ 8.3-CD8+ T cells were isolated from the spleen and lymph nodes of the hosts using antibody-coated magnetic beads and tested for proliferation and IFNγ against irradiated NOD splenocytes pulsed with TUM, $IGRP_{206-214}$, or $IGRP_{K209A/F213A}$. Data correspond to mean ± SE of triplicate cultures.



Supplementary figure 2 |

Recruitment of naive 8.3-CD8+ T cells into the pancreatic islets of NOD mice is preceded by antigen-induced proliferation in the PLN. Recruitment and proliferation of adoptively transferred naive 8.3-CD8+ T cells (107) from 8.3-NOD.Thy1.1 donor mice in the lymphoid organs and islets of insulitic NOD and NOD. IGRPK209A/F213AKI/KI hosts 1, 2, and 3 weeks after transfer. Histograms correspond to percentages of cells within each CFSE peak (i.e., from Fig. 2C) (mean ± SEM). Data correspond to three to six experiments for each time point and host type.



Supplementary figure 3 |
Naive 8.3-CD8+ T cells fail to proliferate in the PLN of NOD.IGRPKI/KI hosts. Proliferation of adoptively transferred naive 8.3-CD8+ T cells (107) from 8.3- NOD.Thy1.1 donor mice in the lymphoid organs of insulitic NOD and NOD.IGRPK209A/F213AKI/KI hosts 1, 2, and 3 weeks after transfer. Histograms correspond to percentages of nonproliferating CFSE+ cells (mean ± SEM). Data correspond to three to six experiments for each time point and host type. P values shown were obtained with two-way ANOVA. Values corresponding to the 1-week and 3-week time points in the spleen and MLNs were also statistically different as measured with Mann-Whitney U-test (spleen: P = 0.0011 and 0.0029, respectively; and MLN: P = 0.002 and 0.0343, respectively).

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AUTHOR CONTRIBUTIONS

J.W. and S.T. designed and executed experiments along with A.S., J.Y. and G.A. S.T. characterized the NOD.IGRP$_{K209A/F213A}^{KI/KI}$ strain. A.S. performed the experiments shown on Fig. 3. P.S. conceived the study, supervised the experiments, evaluated the data and wrote the manuscript along with J.W.

REFERENCES

1. von Andrian UH, Mackay CR.T-cell function and migration. N Engl J Med 2000; 343:1020-1034
2. Savinov AY, Wong FS, Stonebraker AC et al. Presentation of antigen by endothelial cells and chemoattraction are required for homing of insulin-specific CD8+ T cells. J Exp Med 2003;197:643-656
3. Mora JR, von Andrian UH. T-cell homing specificity and plasticity: new concepts and future challenges. Trends Immunol 2006;27:235-243
4. Stephens R, Randolph DA, Huang G et al. Antigen-nonspecific recruitment of Th2 cells to the lung as a mechanism for viral infection-induced allergic asthma. J Immunol 2002;169:5458-5467
5. Ostler T, Pircher H, Ehl S. 'Bystander' recruitment of systemic memory T cells delays the immune response to respiratory virus infection. Eur J Immunol 2003;33:1839-1848
6. Chen AM, Khanna N, Stohlman SA et al. Virus-specific and bystander CD8 T cells recruited during virus-induced encephalomyelitis. J Virol 2005;79:4700-4708
7. Galkina E, Thatte J, Dabak V et al. Preferential migration of effector CD8+ T cells into the interstitium of the normal lung. J Clin Invest 2005;115:3473-3483
8. Karin N, Szafer F, Mitchell D et al. Selective and nonselective stages in homing of T lymphocytes to the central nervous system during experimental allergic encephalomyelitis. J Immunol 1993;150:4116-4124
9. Picarella DE, Kratz A, Li CB et al. Transgenic tumor necrosis factor (TNF)-alpha production in pancreatic islets leads to insulitis, not diabetes. Distinct patterns of inflammation in TNF-alpha and TNF-beta transgenic mice. J Immunol 1993;150:4136-4150
10. Lee MS, von Herrath M, Reiser H et al. Sensitization to self (virus) antigen by in situ expression of murine interferon-gamma. J Clin Invest 1995;95:486-492
11. Faveeuw C, Gagnerault MC, Kraal G et al. Homing of lymphocytes into islets of Langerhans in prediabetic non-obese diabetic mice is not restricted to autoreactive T cells. Int Immunol 1995;7:1905-1913
12. Cantagrel A, Lambert N, Alam A.T cell receptor gene in synovial tissues of rheumatoid arthritis. Int Rev Immunol 1998;17:323-337
13. Peterson JD, Berg R, Piganelli JD et al. Analysis of leukocytes recruited to the pancreas by diabetogenic T cell clones. Cell Immunol 1998;189:92-98
14. Christen U, Benke D, Wolfe T et al. Cure of prediabetic mice by viral infections involves lymphocyte recruitment along an IP-10 gradient. J Clin Invest 2004;113:74-84
15. Carvalho-Pinto C, Garcia MI, Gomez L et al. Leukocyte attraction through the CCR5 receptor controls progress from insulitis to diabetes in non-obese diabetic mice. Eur J Immunol 2004;34:548-557
16. Rhode A, Pauza ME, Barral AM et al. Islet-specific expression of CXCL10 causes spontaneous islet infiltration and accelerates diabetes development. J Immunol 2005;175:3516-3524

17. Archambault AS, Sim J, Gimenez MA et al. Defining antigen-dependent stages of T cell migration from the blood to the central nervous system parenchyma. Eur J Immunol 2005;35:1076-1085

18. Chen J, Fujimoto C, Vistica BP et al. Active participation of antigen-nonspecific lymphoid cells in immune-mediated inflammation. J Immunol 2006;177:3362-3368

19. Smorodchenko A, Wuerfel J, Pohl EE et al. CNS-irrelevant T-cells enter the brain, cause blood-brain barrier disruption but no glial pathology. Eur J Neurosci 2007;26:1387-1398

20. Lieberman SM, DiLorenzo TP. A comprehensive guide to antibody and T-cell responses in type 1 diabetes. Tissue Antigens 2003;62:359-377

21. Tsai S, Shameli A, Santamaria P. CD8+ T cells in type 1 diabetes. Adv Immunol 2008;100:79-124

22. Verdaguer J, Schmidt D, Amrani et al. Spontaneous autoimmune diabetes in monoclonal T cell nonobese diabetic mice. J Exp Med 1997;186:1663-1676

23. Anderson B, Park B, Verdaguer J et al. Prevalent CD8+ T cell response against one peptide/MHC complex in autoimmune diabetes. Proc Natl Acad Sci USA 1999;96:9311-9316

24. Amrani A, Verdaguer J, Serra P et al. Progression of autoimmune diabetes driven by avidity maturation of a T-cell population. Nature 2000;406:739-742

25. Amrani A, Serra P, Yamanouchi J et al. Expansion of the antigenic repertoire of a single T cell receptor upon T cell activation. J Immunology 2001;167:655-666

26. Verdaguer J. Yoon JW, Anderson B et al. Acceleration of spontaneous diabetes in TCRbeta-transgenic nonobese diabetic mice by beta cell-cytotoxic CD8+ T-cells expressing identical endogenous TCRalpha chains. J Immunology 1996;157:4726-4735

27. Lieberman S, Evans AM, Hn B et al. Identification of the beta-cell antigen targeted by a prevalent population of pathogenic CD8+ T cells in autoimmune diabetes. Proc Natl Acad Sci USA 2003;100:8384-8388

28. Trudeau J, Kelly-Smith C, Verchere CB et al. Autoreactive T cells in peripheral blood predict development of type 1 diabetes. J Clin Invest 2003;111:217-223

29. Wong FS, Karttunen J, Dumont C et al. Identification of an MHC class I-restricted autoantigen in type 1 diabetes by screening an organ-specific cDNA library. Nat Med 1999;5**:**1026-1031

30. Loetscher M, Gerber B, Loetscher P et al. Chemokine receptor specific for IP10 and mig: structure, function, and expression in activated T-lymphocytes. J Exp Med 1996;184:963-969

31. Sallusto F, Lanzavecchia A, Mackay CR. Chemokines and chemokine receptors in T-cell priming and Th1/Th2-mediated responses. Immunol Today 1998;19:568-574

32. Lennon GP, Bettini M, Burton AR et al. T cell islet accumulation in type 1 diabetes is a tightly regulated, cell-autonomous event. Immunity 2009;31:643-653

33. Marelli-Berg FM, James MJ, Dangerfield et al. Cognate recognition of the endothelium induces HY-specific CD8+ T-lymphocyte transendothelial migration (diapedesis) in vivo. Blood 2004;103:3111-3116

34. Yamanouchi J, Verdaguer J, Han B et al. Cross-priming of diabetogenic T cells dissociated from CTL-induced shedding of beta cell autoantigens. J Immunol 2003;171:6900-6909

35. Hamilton-Williams EE, Palmer SE, Charlton B et al. Beta cell MHC class I is a late requirement for diabetes. Proc Natl Acad Sci USA 2003;100:6688-6693

36. Jarchum I, Takaki T, DiLorenzo TP. Efficient culture of CD8+ T cells from the islets of NOD mice and their use for the study of autoreactive specificities. J Immunol Methods 2008;339:66-73

3

Beta-cell regeneration: lineage tracing models

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ABSTRACT

Background

Several immunotherapies have been able to restore normoglycaemia in newly diabetic NOD mice. However, the mechanism behind restoration of the functional beta-cell mass, either through replication, functional recovery or neogenesis of beta cells, remains unclear. Furthermore, previous rodent studies have been conducted in non-autoimmune models: the role of the ongoing islet autoimmunity in this process is unknown. Developing cell lineage tracing models in mice that spontaneous develop an auto-immune form of diabetes could be of value in addressing the regenerative capacity of beta cells in type 1 diabetes.

Methods

We combined the rat insulin promoter with conditional Cre-mediated expression of red fluorescent protein (RFP) in mice of a Non Obese Diabetic (NOD) background. In NOD.RIP-tTA/tet07-Cre.ROSA-tdRFP mice, absence of Doxycyclin should result in beta cell RFP expression. Alternatively, in NOD.RIPCreER.ROSA-tdRFP mice, beta cells are programmed to express RFP upon tamoxifen treatment. Finally, we combined RFP reporter mice with the rat glucagon promoter for unconditional RFP- expression in alpha cells of NOD.GluCre.ROSA-tdRFP mice.

Results

The individual transgenes did not interfere with diabetes susceptibility. NOD.RIP-tTA/tet07-Cre.ROSA-tdRFP mice brightly and reliably express RFP in a high percentage (93.0% ± 1.3) of their beta cells in the total absence of Doxycyclin. Presence of Doxycyclin in food and drinking water from birth onwards, adequately suppressed RFP expression (94.3% ± 1.7). NOD.RIPCreER.ROSA-tdRFP beta cells express RFP in an equal high percentage (94.5% ± 1.3) upon tamoxifen treatment. NOD.GluCre.ROSA-tdRFP mice express RFP in a smaller but significant percentage of their alpha cells (58.0% ± 6.0).

Discussion

One of the key goals of future treatments for T1DM is abrogation of auto-immunity combined with restoration of beta cell mass. Our cell lineage tracing models add a novel tool to murine beta-cell regeneration research. These models could be used to address the origin of insulin producing cells after immune-intervention in preclinical studies. Furthermore these models could reveal the role of ongoing autoimmunity on beta-cell regeneration.

BACKGROUND

T1DM is a chronic disease caused by autoimmune mediated destruction of insulin producing beta-cells in the islets of Langerhans in the pancreas. This causes a decrease in beta-cell function and eventually leads to life-long insulin-dependency. Currently there is no cure; treatment consists of insulin administration to mimic beta-cell function. Despite intensive insulin regimes, T1DM still contributes to substantial morbidity and mortality. It is estimated that at diagnose of T1DM, 40-90% of the beta cells have been destroyed. Among the number of issues that need to be addressed in T1DM research, finding a way to put the immune attack to a halt and finding a way to restore beta cell mass/ function are key issues. One way of partially restoring beta cell mass is via replacement with allograft pancreatic tissue, either whole pancreas, or isolated islets of Langerhans. Currently different immune-suppressive strategies are being investigated for prolonged graft survival. However, the worldwide shortage of pancreas/ islet allografts combined with transplantation-related side effects, fuels the exploration of the beta-cell regenerative capacity [1].

There is evidence -both in rodents and humans- that beta cells are able to adapt their cell mass to various physiological and pathological conditions. In NOD mice, beta-cell proliferation initially increases during islet inflammation, even prior to significant changes in beta-cell area or glucose levels [2]. During pregnancy, beta-cell mass in rodents increases 2,5-5 times, resulting from both beta-cell hypertrophy and an increased number of beta-cells suggesting beta-cell replication [3,4]. In humans, older autopsy studies of pregnant women have revealed similar findings, with a significant increase in beta-cell mass (2.4x) with expansion of both islet size and number of beta-cells per islets [5]. However, a more recent study showed beta-cell mass does increase to a lesser extend (1.4x) with an increased number of small new cells, indicative of neogenesis rather than beta-cell replication. [6]. In experimental animal models, with partial or near-total destruction of the endocrine pancreas, regeneration of the beta-cells is seen as to different extends. Nir et al. show beta-cell replication as the main source of new beta-cells after 70-80% chemical beta-cell ablation by diphtheria toxin in a cell lineage tracing model [7]. Xu et al show that endogenous beta-cell progenitors can be activated in the mouse pancreas after partial duct ligation [8]. Near-total chemical ablation by diphtheria toxin in an alternative cell lineage tracing model revealed evidence for alpha-cell dedifferentiation [9]. Differences in outcome may be related to differences in experimental models, particularly concerning the percentage of beta-cell destruction as regenerative stimuli used in different models may be insufficient to trigger a neogenesis pathway. Of note, these studies have been performed on mice on a non-autoimmune background.

The availability of therapies that decrease insulin requirements and normalize glycated haemoglobin in T1DM patients [10,11,12] and that can fully reverse hyperglycemia in newly diagnosed diabetic NOD mice [13,14] raises the question of how functional beta-cell mass could be restored. For over a century, replication of pre-existing beta-cells and

islet neogenesis, either via transdifferentiation of non-beta cells, or via differentiation of progenitor/ stem cells have been proposed mechanisms of beta-cell regeneration [15,16]. More recently, beta-cell recovery of exhausted cells has been additionally suggested. A prospective phase 1-2 study in recent onset T1DM patients undergoing autologous non-myeloablative hematopoetic stem cell transplantation showed prolonged insulin independency compared to the natural course of the disease. [17]. The mechanism behind the increased beta-cell function allegedly does involve recovery of pre-existing beta-cells but this interpretation may not be sufficient to explain remission lasting more than seven years. A recent study in T2DM patients found double positive endocrine cells (insulin and glucagon/somatostatin) suggesting some degree of beta-cell neogenesis as a compensatory mechanism in newly diagnosed T2DM patients [18]. Although the classical pathophysiology of T2DM clearly differs from T1DM, some overlay in beta-cell inflammation with subsequent apoptosis of beta cell between the two conditions is being found. Spijker et al. demonstrated via cell lineage tracing studies in vitro, conversion of human beta-cells into glucagon producing alpha-cells, emphasizing human endocrine cell plasticity [19].

In the earliest beta-cell regeneration studies, islet cell regeneration was assessed from a morphological point of view. The development of techniques indicating islet cell proliferation, such as 3-H-thymidine incorporation and BrdU labelling, has been a step forward in addressing this issue in preclinical models [15]. However the true origin of new insulin-producing cells remains difficult to prove. The technique of cell lineage is based on the inheritable labelling of individual islet cells [20]. In T1DM research this means that the islet cell of interest, for instance the alpha-, beta- or duct cell can be marked in such a way that this mark will be transferred to its progeny.

Development of the Cre/loxP recombination system has had important implications for lineage tracing studies in mice. Cre recombinase is a small, bacteriophage P1-derived integrase that catalyses defined DNA recombination events between specific target sites, termed loxP (locus of crossover [x] in P1) [21]. The result of Cre-mediated recombination between two loxP sites depends on their specific orientation relative to another: a DNA sequence flanked by two directly repeated loxP elements is cut out as a circular molecule. In contrast, DNA flanked by two oppositely oriented sites will be inverted. By combining Cre/loxP with cell regulatory elements and a reliable and clear reporter, cells can be permanently and heritably marked. By using promoters responsive to the presence or absence of a certain drug, this labelling can be induced (conditional expression).

Cre/loxP systems have been combined with different reporters. Nir et al report approximately 20% beta-cell labeling with Human Placental Alkaline Phosphatase upon Tamoxifen treatment [7]. In a previous publication this group reports 30% labeling of beta cells [22]. Thorel et al report 95% Yellow Fluorescent Protein (YFP) labelling of beta-cells and a 90% labelling efficiency of alpha-cells, using the Rosa-26 YFP reporter [9].

Previous preclinical studies have been conducted in non-auto-immune models: the role

of the ongoing islet autoimmunity on beta-cell regeneration is unknown. Developing reliable cell lineage tracing models in mice that spontaneous develop an auto-immune form of diabetes [23] could be of value in addressing the regenerative capacity of beta-cells in type 1 diabetes, especially when combined with immune intervention studies.

MATERIALS AND METHODS

Mice.

NOD.RIP-tTA/tet07-Cre.ROSA-tdRFP

NOD.RIP-tTA/tet07-Cre mice (from our lab, unpublished data) express a tetracycline-dependent transactivator under the control of a Rat Insulin Promoter. Absence of a Tetracycline (Doxycycline) leads to expression of Cre recombinase in beta-cells (Fig. 1A). This model is also known as 'Tet-off', as the presence of tetracycline turns Cre recombinase expression off.

C57Bl/6.ROSA-tdRFP mice are a Red Fluorescent Protein (RFP) reporter strain [24]. A tandem-dimer RFP (tdRFP) was inserted in an anti-sense orientation relative to the ubiquitously expressed ROSA26 locus to diminish leaky reporter expression (Fig. 1B). We backcrossed the targeted allele onto the NOD background for at least 10 generations and then introgressed this allele into NOD.RIP-tTA/tet07-Cre mice to produce NOD.RIP-tTA/tet07-Cre.ROSA-tdRFP mice. Presence of Cre recombinase under the Rat Insulin Promotor leads to expression of Red Fluorescent Protein in beta-cells.

NOD.RIPCreER.ROSA-tdRFP

NOD.RIPCreER mice (kind gift from D. Melton) express Cre recombinase fused to the Estrogen Receptor under the control of a Rat Insulin Promotor. Cre recombinase is initially kept in the cytosol of beta cells: nuclear translocation only occurs after (Estrogen-Receptor binding) Tamoxifen treatment (Fig. 1C). These mice were crossed with the RFP-reporter mice to produce NOD.RIPCreER.ROSA-tdRFP mice.

NOD.GluCre.ROSA-tdRFP

C57Bl/6.GluCre mice (kind gift from P. Herrera) were backcrossed with NOD mice for at least 10 generations. NOD.GluCre mice express Cre recombinase under the Rat Glucagon Promotor. Their alpha-cells constitutively express Cre recombinase. They were crossed with the RFP-reporter mice described above to produce NOD.GluCre.ROSA-tdRFP mice.

All mice were kept under specific pathogen free conditions. These studies were approved by the Faculty of Medicine's Animal Care Committee and followed the guidelines of the Canadian Council of Animal Care.

Doxycyclin.

NOD.RIP-tTA/tet07-Cre.ROSA-tdRFP mice were bred on irradiated Doxycyclin containing food (2.3 g/kg; Bio-Serv) and 1 mg/ml Doxycyclin containing drinking water as negative controls.

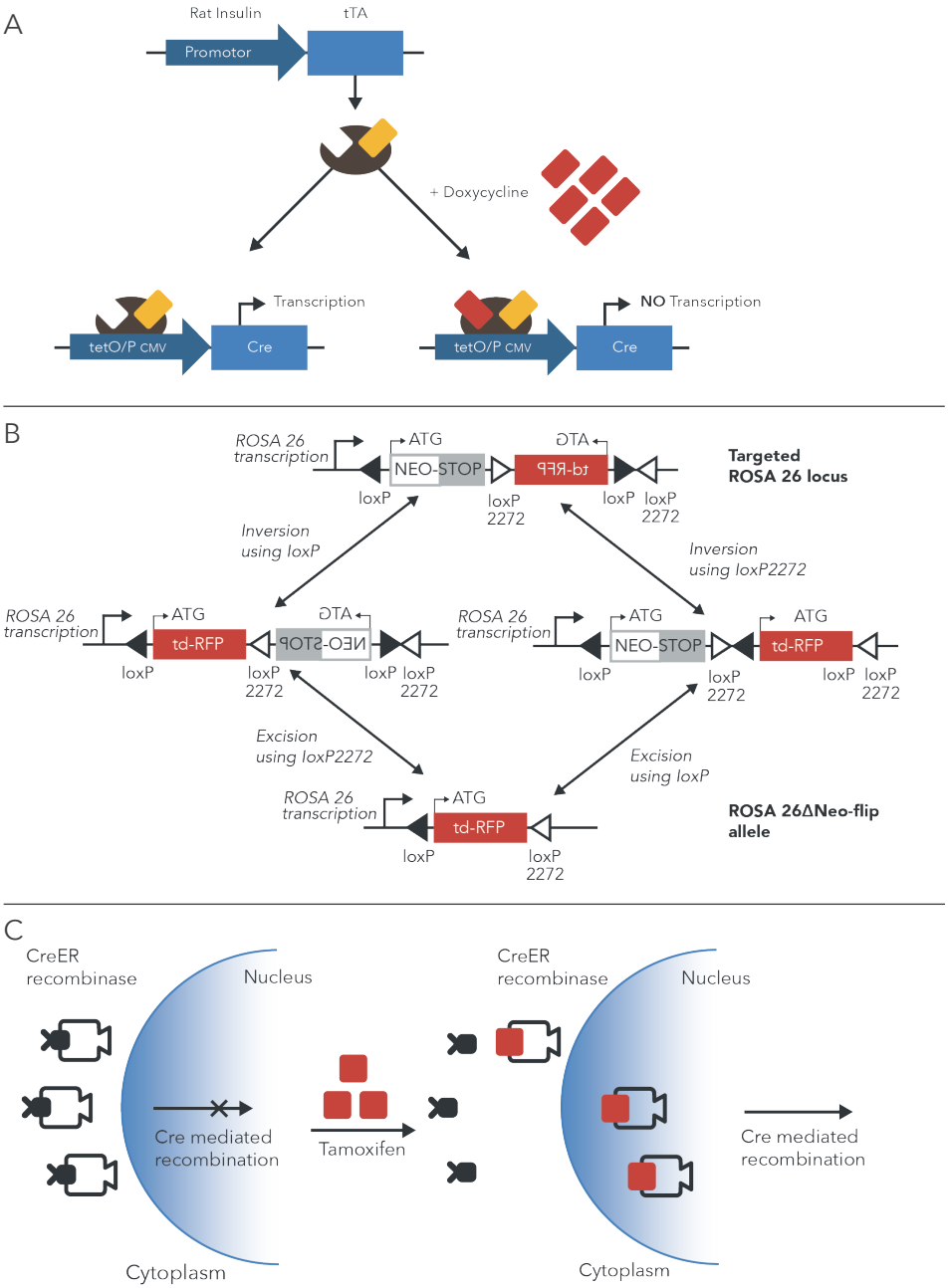

Fig 1 | A. Tetracycline transactivator (tTA) under a Rat Insulin Promoter (RIP). In the absence of Doxycycline, tTA binds to the tetO operator and drives expression of Cre recombinase in beta-cells. **B.** ROSA-tdRFP-reporter: tdRFP has been inversely inserted and is preceded by a stop-codon. Cre recombinase expression leads to removal of the stop-codon and correct orientation and expression of td-RFP [19]. **C.** Cre recombinase fused to the Estrogen Receptor (ER) under a Rat Insulin Promotor. Tamoxifen, which binds to ER, establishes nuclear translocation of Cre recombinase in beta-cells.

Tamoxifen.
Tamoxifen (Sigma) was dissolved in autoclaved corn oil 20 mg/ml containing 10% EtOH 100% at 37 °C using sonication. Tamoxifen was injected intraperitoneal (5 x 4 mg).

Immunopathology.
Pancreases were fixed in 3% paraformaldehyde for two hours before freezing. They were extensively washed in PBS overnight and dehydrated using rising concentrations of sucrose in PBS (5-10-20-30%). The tissue was subsequently frozen directly into Tissue TEC-OCT above ethanol/dry ice, and stored at -80 °C. Cryosections (5 µm) were fixed with 3% paraformaldehyde, stained with guinea pig anti-insulin antibodies and Alexa-488 goat-anti-guinea pig antibodies (Invitrogen) or goat-anti-glucagon antibodies (Santa Cruz Biotechnology) and Alexa 647 donkey-anti-goat antibodies (Invitrogen). The sections were mounted with Prolong gold (Invitrogen) and analyzed with an Olympus FV1000 confocal microscopy system.

Diabetes.
Diabetes was monitored by measuring urine glucose levels twice weekly. Animals were considered diabetic after two consecutive readings greater than or equal to 3+. The average blood glucose levels in mice diagnosed using these criteria are 22.0 ± 3.8 mmol/l, and none of these mice had blood glucose levels below 16 mmol/l.

Statistical Analysis.
Percentage of Cre expression in beta cells was estimated by dividing the amount of insulin+/RFP+ cells by the total amount of insulin+ cells per islet. Percentage of Cre expression in alpha cells was estimated by dividing the amount of glucagon+/ RFP+ cells by the total amount of glucagon+ cells per islet. Percentages are shown ± SEM. We analyzed ~15 islets per mouse and ~6 mice per strain. For NOD.GluCre.ROSA-tdRFP mice we analysed 4 islets per mouse and 4 mice in total.

RESULTS

The individual transgenes did not interfere with diabetes susceptibility.
The individual transgenes of both the conditional beta-cell tracing models and the unconditional alpha-cell tracing model, did not interfere with diabetes susceptibility. Diabetes incidence curves were comparable to wildtype NOD mice (Fig. 2).

NOD.RIP-tTA/tet07-Cre.ROSA-tdRFP and NOD.RIPCreER.ROSA-tdRFP mice specifically express RFP in their beta cells.
Transgenic NOD.RIP-tTA/tet07-Cre.ROSA-tdRFP mice reliably and brightly express RFP in 93.0% ± 1.3 of their beta-cells in the total absence of Doxycyclin, as shown by simultaneous insulin staining (Fig. 3A+B). Conversely, presence of Doxycyclin in food and drinking water of the mother from early pregnancy onwards, adequately suppressed RFP expression in litters (94.3% ± 1.7) (Fig. 3A+B).

In transgenic NOD.RIPCreER.ROSA-tdRFP mice we tested different Tamoxifen administration routes. Administration via gavage (20-20-10 mg) caused toxicity symptoms and was therefore discontinued. Tamoxifen (5 x 4 mg) administered intraperitoneal caused significant higher RFP expression than equal doses injected subcutaneously (results not shown). RFP expression in beta cells of NOD.RIPCreER.ROSA-tdRFP mice, as tested by simultaneous insulin staining, was comparable to our NOD.RIP-tTA/tet07-Cre.ROSA-tdRFP model with 94.5% ± 1.3 of the beta-cells expressing RFP upon Tamoxifen treatment (Fig. 3C).

Initially we experienced difficulties confirming RFP expression. Comparing different tissue handling regimes, we discovered that the intracellular RFP protein is only maintained when pancreases are fixed in 3% paraformaldehyde prior to freezing. Fresh-freezing methods turned out to interfere with preservation of RFP expression.

No RFP expression was found in pancreatic non-beta cells of either NOD.RIP-tTA/tet07-Cre.ROSA-tdRFP or NOD.RIPCreER.ROSA-tdRFP mice, as tested by simultaneous glucagon staining for alpha-cells (Fig. 3B). In addition, no RFP staining was found in non-endocrine tissue, as tested by confocal analysis of splenic and thymic tissue (data not shown).

NOD.RIPCreER.ROSA-tdRFP mice specifically express RFP in their alpha cells.
NOD.GluCre.ROSA-tdRFP mice unconditional express RFP in a smaller but significant percentage of their alpha-cells (58.0% ± 6.0 %) (Fig. 3D) as shown by simultaneous glucagon staining.

No RFP expression was found in pancreatic non-alpha cells of NOD.GluCre.ROSA-tdRFP mice, as tested by simultaneous insulin staining for beta-cells (Fig. 3D). In addition, no RFP staining was found in non-endocrine tissue, as tested by confocal analysis of splenic and thymic tissue (data not shown).

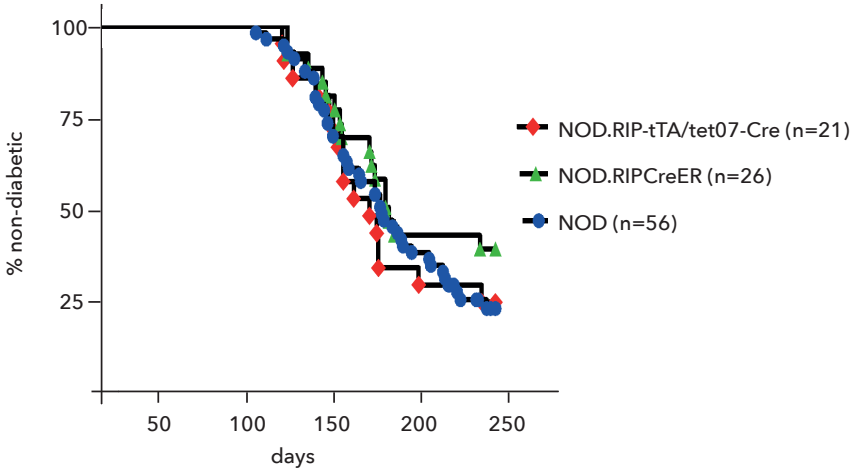

Fig 2 | T1DM incidence NOD.RIP-tTA/tet07-Cre.ROSA-tdRFP and NOD.RIPCreER.ROSA-tdRFP mice are comparable to wildtype NOD mice.

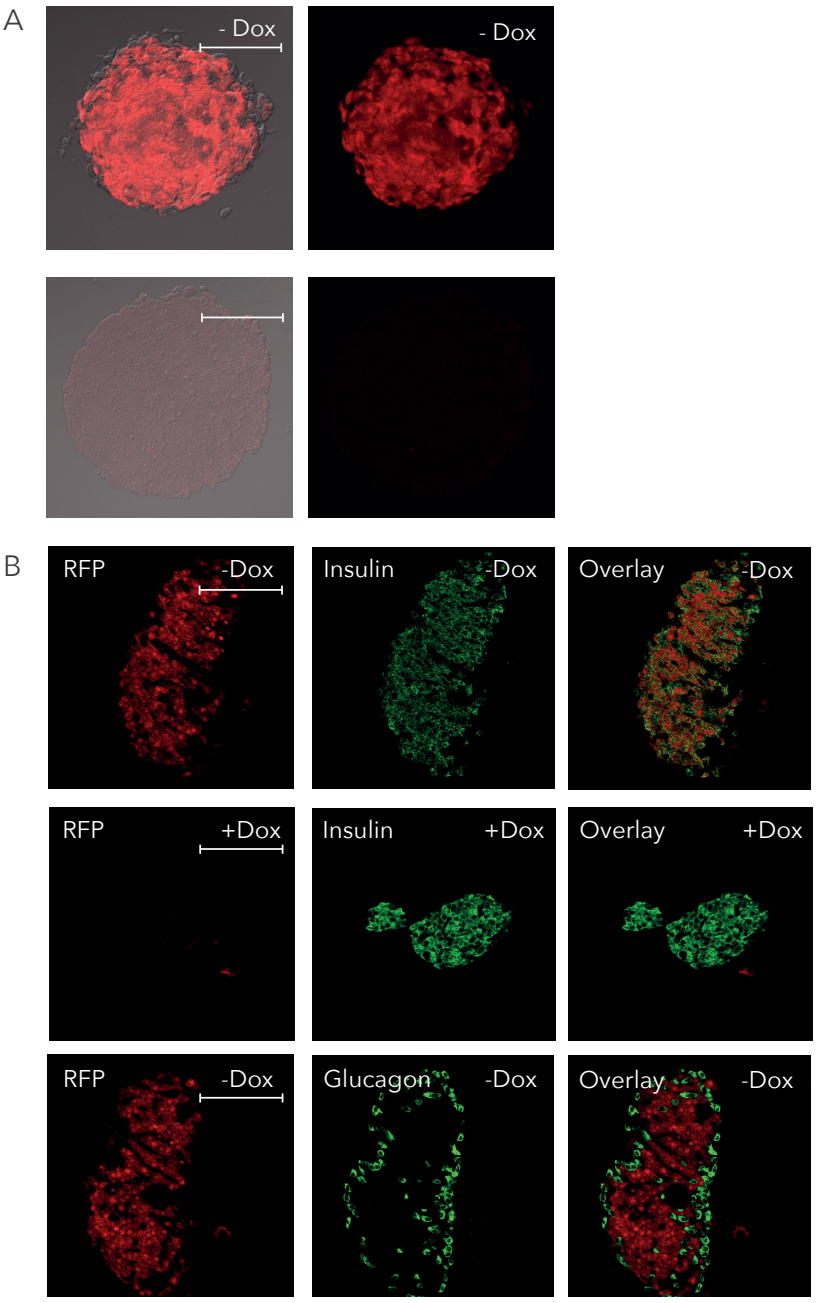

Fig 3 | A. Upper row: RFP expression in isolated islets of NOD.RIP-tTA/tet07-Cre.ROSA-tdRFP mice in the absence of Doxycyclin (left: brightfield + Cy3, right: Cy3). Lower row: isolated islets of NOD control **B.** RFP expression, insulin (upper two rows, in green)/ glucagon (lower row, in green) staining and overlay in islet cryosections of NOD.RIP-tTA/tet07-Cre.ROSA-tdRFP mice in the absence and presence of Doxycyclin.

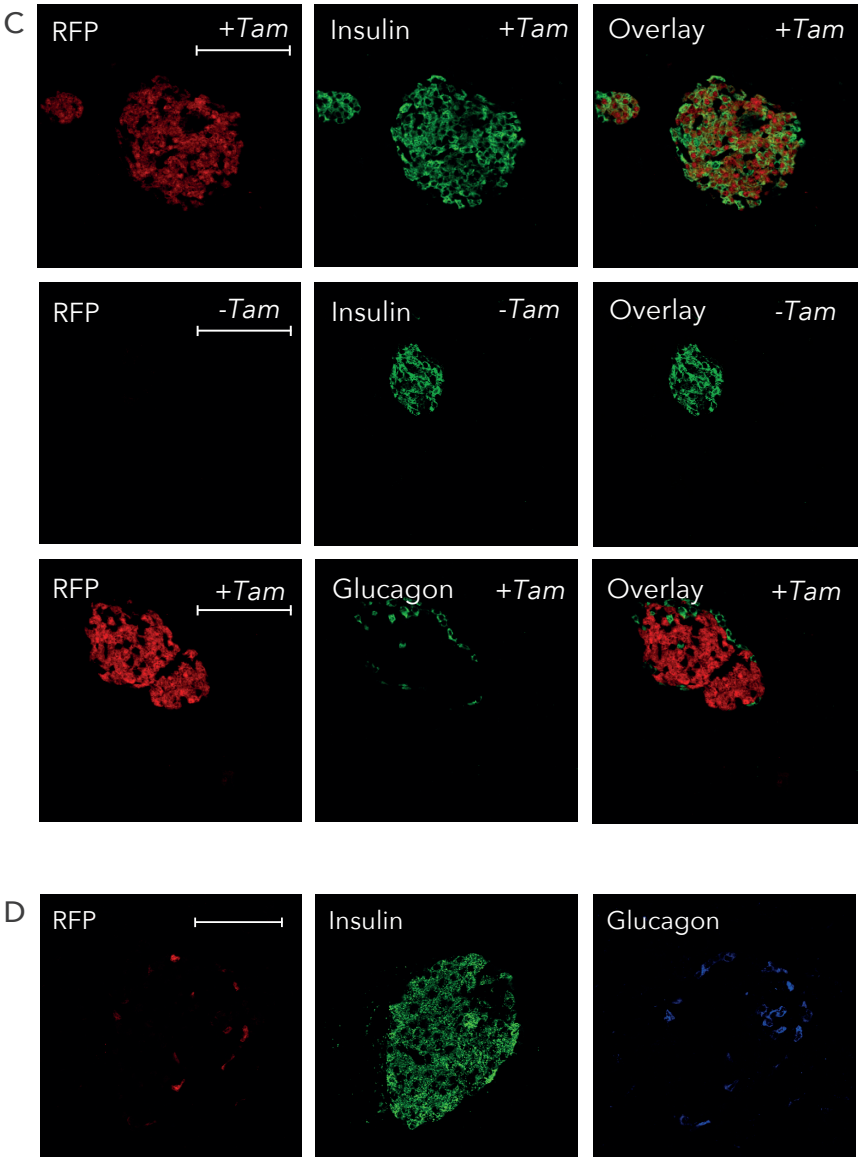

Fig 3 | C. RFP expression, insulin (upper two rows, in green)/ glucagon (lower row, in green) staining and overlay in islet cryosections of Tamoxifen treated NOD.RIPCreER.ROSA-tdRFP mice and untreated controls. **D**. RFP expression, insulin (green) and glucagon (blue) staining in islet cryosections of NOD. GluCre.ROSA-tdRFP mice.

DISCUSSION

One of the key goals of future treatments to cure T1DM is abrogation of auto-immunity combined with restoration of beta-cell mass. The availability of therapies that can restore beta-cell function/ mass initiated the search for the origin of these new insulin producing cells. In addition, the worldwide shortage of pancreas/ islet allografts combined with transplantation-related side effects [25], fuels the exploration of the beta-cell regenerative capacity.

Reliable cell lineage tracing models might help to distinguish between replication, functional recovery or neogenesis of beta-cells as predominant mechanism behind restoration of the functional beta-cell mass. Bright, inheritable labelling of pre-existing beta-cells (or pre-existing alpha-cells) in mice that spontaneously develop an auto-immune form of diabetes and are successfully treated with immune therapy, could help to unravel regeneration mechanisms, especially when combined with Bromodeoxyuridine (BrdU) labelling. This thymidine analogue can be incorporated in the newly-synthesized DNA of replicating cells. Upon successful immune-intervention in either NOD.RIP-tTA/ tet07-Cre.ROSA-tdRFP or Tamoxifen-treated NOD.RIPCreER.ROSA-tdRFP mice, finding RFP+/ insulin+/ BrdU+ cells would suggest replicating pre-existing beta cells as the predominant insulin producing source. RFP+/ insulin+/ BrdU- cells on the contrary would imply recovery of pre-existing beta cells whereas RFP-/ insulin+ cells would point in the direction of a non-beta cell source. In addition, finding RFP+/ insulin+ cells in NOD. GluCre.ROSA-tdRFP mice after successful immune intervention would suggest alpha-cells as the predominant source of new beta-cells, whereas RFP-/insulin+ cells would make this unlikely. Our current cell lineage tracing models only enable us to trace beta- and alpha-cells, if non-beta, non-alpha-cells are suggested as predominant source of new beta-cells, additional studies would be required. Due to neurotoxicity, continuous BrdU administration for longer periods of time is not considered safe. Pulse BrdU administration is a possible solution, with the downside of partial cell replication labelling.

The success of a murine cell lineage tracing model in T1DM research, depends on several aspects. First, none of the introduced transgenes should interfere with the T1DM incidence of the NOD mice. We have shown that Cre transgenes do not influence T1DM incidence. Second, Cre-mediated recombination should be cell type specific. Our immunohistochemistry studies indicate that RFP expression in our models is indeed limited to the cells that are driven by either an insulin or a glucagon promotor. And third, the efficiency of Cre-induced expression should be high. Compared to Cre-induced expression reported in the literature, both our NOD.RIP-tTA/tet07-Cre.ROSA-tdRFP and NOD.RIPCreER.ROSA-tdRFP models, and to a lesser extend our NOD.GluCre.ROSA-tdRFP mice efficiently express RFP in their respective beta- and alpha-cells. With higher expression rates, less statistical assumptions have to be made on the origin or fate of the targeted cells. This expression efficiency is directly linked to the brightness and reliability of the tdRFP reporter that is being used.

We successfully generated cell lineage tracing models with bright, inheritable labelling of pre-existing beta- or alpha-cells in mice of a NOD background. These mice spontaneous develop autoimmune diabetes and could therefore be used as a tool in T1DM research. Furthermore these models could be used to assess the role of the ongoing islet autoimmunity on beta cell regeneration. We predict that ongoing auto-immunity prevents any form of regeneration emphasizing the need for successful immune strategies.

Although there is evidence in both rodents and humans that beta-cells are able to adapt to various conditions, prudence in translating results from mouse models is required. After partial or near-total destruction of the mouse pancreas in a non-auto-immune environment, regeneration of beta-cells is seen as to different extends [7,8,9]. Interestingly, no evidence for beta-cell proliferation or regeneration was found in a study performed on human pancreatic tissue collected from 13 patients who underwent partial (50%) pancreactomy [26]. Differences in outcome may be related to differences in the percentage of beta cell destruction, which might be insufficient to trigger a neogenesis pathway. In addition, chronic pancreatic inflammation was the underlying cause in the majority of the patients, which might influence beta-cell regeneration capacity. Another explanation however could be that regeneration capacity and regeneration pathways might differ between mice and men, emphasizing precaution in translation of results. There are a number of reasons why animal studies currently are being conducted to address the questions raised above. First of all, there is limited accessibility of human pancreases during the course of the disease. Second, we can only measure beta-cell functional responses and not beta-cell mass in patients. Third, we do not have reliable biomarkers to assess the auto-immune process in humans [27]. And fourth, as opposed to mice, we have not been able to cure diabetes yet in humans and therefore do not know what happens to beta cells when the immune attack is terminated. Awareness of model limitations and prudence in translating preclinical studies are in order. However cell lineage tracing studies might give some clues and guidance as to what regenerative pathways could be pursued.

Remaining islet cells have been histologically demonstrated in individuals suffering from T1DM, even for as long as 50 years. This not only emphasizes the heterogeneous course of T1DM but could also be seen as an argument in favour of beta-cell recovery possibilities [28,29].

Identifying possible sources of insulin producing cells after future successful immune intervention could have significant clinical implications. If pre-existing beta-cells are the only source of restored beta-cell mass, the residual beta-cell mass at the time of intervention is expected to predict the outcome of any immune intervention. On the contrary, if other cells serve as beta-cell precursors, outcome should be independent residual beta cell mass [21]. Currently, different strategies are being undertaken to create insulin producing beta-cells from stem cells (either embryonic stem cells or induced pluripotent stem cells) and from endocrine progenitors [30]. In addition to

this, identifying regenerative pathways could eventually result in developing strategies capable of enhancing effectiveness of promising immune therapies.

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Conflicts of interest: none

REFERENCES

1. Harlan DM, Kenyon NS, Korsgren O et al. Immunology of Diabetes Society. Current advances and travails in islet transplantation. Diabetes 2009;58:2175-2184
2. Sherry NA, Kushner JA, Glandt M et al. Effects of autoimmunity and immune therapy on beta-cell turnover in type 1 diabetes. Diabetes 2006;55:3238-3245
3. Bouwens L, Rooman I. Regulation of pancreatic beta-cell mass. Physiol Rev 2005;85:1255-1270
4. Rieck S, Kaestner KH. Expansion of beta-cell mass in response to pregnancy. Trends Endocrinol Metab 2010;53:2167-2176
5. Van Assche FA, Aerts L, De Prins F. A morphological study of the endocrine pancreas in human pregnancy. Br J Obstet Gynaecol 1978;85:818-820
6. Butler AE, Cao-Minh L, Galasso R et al. Adaptive changes in pancreatic beta cell fractional area and beta cell turnover in human pregnancy. Diabetologia 2010;53:2167-2176
7. Nir T, Melton DA, Dor Y. Recovery from diabetes in mice by beta cell regeneration. J Clin Invest 2007;117:2553-2561
8. Xu X, D'Hoker J, Stangé G et al. Beta cells can be generated from endogenous progenitors in injured adult mouse pancreas. Cell 2008;132:197-207
9. Thorel F, Népote V, Avril I et al. Conversion of adult pancreatic alpha-cells to beta-cells after extreme beta-cell loss. Nature 2010;464:1149-1154
10. Herold KC, Hagopian W, Auger JA et al. Anti-CD3 monoclonal antibody in new-onset type 1 diabetes mellitus. N Engl J Med 2002;346:1692-1698
11. Keymeulen B, Vandemeulebroucke E, Ziegler AG et al. Insulin needs after CD3-antibody therapy in new-onset type 1 diabetes. N Engl J Med 2005;352:2598-2608
12. Keymeulen B, Walter M, Mathieu C et al. Four-year metabolic outcome of a randomized controlled CD3-antibody trial in recent-onset type 1 diabetic patients depends on their age and baseline residual beta cell mass. Diabetologia 2010;53:614-623
13. Chatenoud L, Thervet E, Primo J et al. Anti-CD3 antibody induces long-term remission of overt autoimmunity in nonobese diabetic mice. Proc Natl Acad Sci U S A. 1994;91:123-127
14. Tsai S, Shameli A, Yamanouchi J et al. Reversal of autoimmunity by boosting memory-like autoregulatory T cells. Immunity 2010;32:568-80
15. Granger A, Kushner JA. Cellular origins of beta-cell regeneration: a legacy view of historical controversies. J Int Med 2009;266:325-338
16. Bonner-Weir S, Li W, Ouziel-Yahalom L et al. Beta-cell growth and regeneration: replication is only part of the story. Diabetes 2010:59:2340-2348
17. Couri CE, Oliveira MC, Stracieri AB et al. C-peptide levels and insulin independence following autologous nonmyeloabaltive hematopoietic stem cell transplantation in newly diagnosed type 1 diabetes mellitus. JAMA 2009;301:1573-1579

18. Yoneda S, Uno S, Iwahashi H et al. Predominance of β-cell neogenesis rather than replication in humans with an impaired glucose tolerance and newly diagnosed diabetes. J Clin Endocrinol Metab 2013;98:2053-2061

19. Spijker HS, Ravelli RB, Mommaas-Kienhuis AM et al. Conversion of mature human β-cells into glucagon-producing α-cells. Diabetes 2013;62:2471-2480.

20. Kretzschmark K, Fiona M, Watt FM. Lineage tracing. Cell 2012;148:33-45

21. Hamilton DL, Abremski K. Site-specific recombination by the bacteriophage P1 lox-Cre system. Cre-mediated synapsis of two lox sites. J Mol Biol 1984;178:481-6

22. Dor Y, Brown J, Martinez OI et al. Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. Nature 2004;429:41-46

23. Yang Y, Santamaria P. Lessons on autoimmune diabetes from animal models. Clin Sci (Lond) 2006;110:627-639

24. Luche H, Weber O, Nageswara Rao T et al. Faithful activation of an extra-bright red fluorescent protein in "knock-in" Cre-reporter mice ideally suited for lineage tracing studies. Eur J Immunol 2007;37:43-53

25. Harlan DM, Kenyon NS, Korsgren O et al. Immunology of Diabetes Society. Current advances and travails in islet transplantation. Diabetes 2009;58:2175-2184.

26. Menge BA, Tannapfel A, Belyaev et al. Partial pancreactomy in adult cells does not provoke beta cell regeneration. Diabetes 2008;57:142-149

27. Ablamunits V, Sherry NA, Kushner JA et al. Autoimmunity and beta cell regeneration in mouse and human type 1 diabetes: the peace is not enough. Ann N Y Acad Sci 2007;1103:19-32.

28. Foulis AK, Liddle CN, Farquharson MA et al. The histopathology of the pancreas in type 1 (insulin-dependent) diabetes mellitus: a 25-year review of deaths in patients under 20 years of age in the United Kingdom. Diabetologia 1986;29:267-274

29. Coppieters KT, Dotta F, Amirian N et al. Demonstration of islet-autoreactive CD8 T cells in insulitic lesions from recent onset and long-term type 1 diabetes patients. J Exp Med 2012;209:51-60

30. Borowisk M, Melton DA. How to make beta cells. Curr Opinion Cell Biology 2009;21:727-732

4

Local autoantigen expression as essential gatekeeper of memory T-cell recruitment to islet grafts in diabetic hosts

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ABSTRACT

It is generally believed that inflammatory cues can attract non-cognate, 'bystander' T-cell specificities to sites of inflammation. We have shown that recruitment of naïve and *in vitro*-activated autoreactive CD8+ T-cells into endogenous islets requires local autoantigen expression. Here we demonstrate that absence of an autoantigen in syngeneic extra-pancreatic islet grafts in diabetic hosts renders the grafts 'invisible' to cognate memory (and naïve) T-cells. We monitored the recruitment of $IGRP_{206-214}$-reactive CD8+ T-cells into $IGRP_{206-214}$-competent and $IGRP_{206-214}$-deficient islet grafts in diabetic wild type or $IGRP_{206-214}^{-/-}$ nonobese diabetic (NOD) hosts (harboring either naïve and memory, or only naive $IGRP_{206-214}$-specific T-cells, respectively). All four host-donor combinations developed recurrent diabetes within two weeks. Wild type hosts recruited $IGRP_{206-214}$-specific T-cells into $IGRP_{206-214}^{+/+}$ but not $IGRP_{206-214}^{-/-}$ grafts. In $IGRP_{206-214}^{-/-}$ hosts, there was no recruitment of $IGRP_{206-214}$-specific T-cells, regardless of donor type. Graft-derived $IGRP_{206-214}$ activated naïve $IGRP_{206-214}$-specific T-cells, but graft destruction invariably predated their recruitment. These results indicate that recurrent diabetes is driven exclusively by autoreactive T-cells primed during the primary autoimmune response, and demonstrate that local antigen expression is a *sine-qua-non* requirement for accumulation of memory T-cells into islet grafts. These findings underscore the importance of tackling autoreactive T-cell memory after beta-cell replacement therapy.

INTRODUCTION

Nonobese diabetic (NOD) mice develop a form of T1D that results from destruction of β-cells by $CD4^+$ and $CD8^+$ T-cells recognizing many autoantigenic peptides (1). A significant fraction of islet-associated $CD8^+$ cells recognize the mimotope NRP-V7 in the context of the MHC molecule K^d (2). These cells are a significant component of the earliest NOD islet $CD8^+$ infiltrates (2; 3), are diabetogenic (4; 5) and target residues 206-214 of islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) (6). The peripheral $IGRP_{206-214}$-reactive $CD8^+$ T-cell pool is sizeable (7) and, upon recruitment into islets, undergoes a local avidity maturation process that contributes to disease progression (8).

Studies in infection and autoimmune disease models have suggested that recruitment of T-cells into sites of extra-lymphoid inflammation does not require local expression of cognate peptide–MHC (pMHC) (9-11). We have recently shown, however, that cues emanating from pancreatic islets undergoing spontaneous autoimmune inflammation in NOD mice cannot recruit naïve or newly activated bystander T-cell specificities. This was established by monitoring the recruitment of naïve or *in vitro*-activated $IGRP_{206-214}$-specific $CD8^+$ T-cells in gene-targeted NOD mice expressing a T-cell 'invisible' $IGRP_{206-214}$ sequence. These mice developed diabetes with normal incidence, but their insulitic lesions could not recruit either cell type. These results indicated that recruitment of naïve T-cells or effector CTL to a site of autoimmune inflammation results from an active process that is strictly dependent on local display of cognate pMHC (12).

Here, we asked whether this revised paradigm also applies to: (i) recruitment of memory (autoantigen-experienced) autoreactive T-cells; and/or (ii) recruitment of naïve and memory T-cells to syngeneic islet grafts. We reasoned that the 'non-physiological' lymphatic and vascular anatomy of islets grafts transplanted under the kidney capsule (13-15), coupled to a high rate of graft cell death (16), should allow recruitment of 'graft-irrelevant' (i.e. non-autoreactive) memory T-cells to the site in response to local inflammatory cues, including those caused by grafting. We demonstrate that recruitment of $CD8^+$ T-cells to islet grafts during disease recurrence *exclusively* involves autoantigen-specific T-cells from the memory pool, excluding a role for bystander T-cell specificities or graft antigen-activated autoreactive T-cells.

RESEARCH DESIGN AND METHODS

Mice. NOD.IGRP$_{K209A/F213A}$ $^{KI/KI}$ mice, encoding an immunologiclaly silent IGRP$_{206-214}$ epitope have been described (12). These studies were approved by the local Animal Care Committee.

Diabetes. Diabetes was monitored twice a week by measuring urine glucose levels and confirmed by tail vein blood glucose measurements. All recipient mice had at least two successive blood glucose measurements >22.2 mmol/l and were transplanted within 1-2 weeks of diabetes onset.

Peptides and Tetramers. The peptides IGRP$_{206-214}$, NRP-V7, TUM and the corresponding tetramers (PE-labeled) were prepared as described (17).

Flow Cytometry. Cell suspensions were stained with pMHC tetramers and FITC- or PerCP-conjugated anti-CD8α and anti-CD4 mAbs (BD Pharmingen) for 60 min at 4 °C, fixed in 1% Paraformaldehyde/PBS, and analyzed by Fluorescence-Activated Cell Sorting (FACS).

Islet Isolation. Pancreatic islets were isolated by hand-picking after collagenase P digestion of the pancreas and cultured overnight at 37°C, 5% CO_2.

Islet transplantation and graft harvest. Five hundred islets were transplanted under the left kidney capsule. Successful engraftment was defined as restoration of glycemic control for >48h. Graft failure was defined as non-fasting blood glucose >15 mmol/l.

Specificity of islet-associated CD8$^+$ T-cells. The grafts of recurrent-diabetic hosts were cut into ~2 mm^3 fragments and cultured for 1 week in 0.5 units/ml rIL-2. T-cells were analyzed by FACS as described. Measurements of IFNγ secretion by graft-associated T-cells (2 x 10^4/well) in response to peptide-puled irradiated NOD splenocytes (10^5/well) were determined by ELISA (R&D Systems) and normalized to values obtained with TUM.

Adoptive Transfer. Purified splenic CD8+ T-cells were labeled with CFSE (2.5 mM), and injected i.v. (5x10^6) 24h after transplantation. Mice were killed 7days later and the grafted and non-grafted kidney-draining Lymph Nodes (LNs), spleens, Pancreatic Lymph Nodes (PLNs) and Mesenteric Lymph Nodes (MLNs) examined for dilution of CFSE in the CFSE+CD8+ gate.

Statistical Analyses. Data were compared by Mann-Whitney *U* or χ^2 Logrank tests. Statistical significance was assumed at $P<0.05$.

Supplementary Materials. Provides supporting data on the recruitment of IGRP$_{206-214}$- or Insuli-B$_{15-23}$ reactive CD8+ T-cells to islet grafts, as well as representative FACS profiles.

RESULTS

NOD.IGRP$_{K209A/F213A}^{KI/KI}$ mice (referred to as IGRP$_{206-214}^{-/-}$ or 'epitope-deficient' mice) develop diabetes with the same incidence and kinetics as wild-type NOD ('epitope-competent') mice, but cannot trigger the activation or recruitment of naive or *in vitro*-activated IGRP$_{206-214}$-reactive CD8$^+$ T-cells (12). Here, we investigate if the naïve IGRP$_{206-214}$-reactive CD8$^+$ T-cells of epitope-deficient hosts and/or their memory counterparts arising in epitope-expressing hosts are recruited into epitope-competent or epitope-deficient islet grafts (from NOD.*scid* and NOD.*rag2*$^{-/-}$.IGRP$_{K209A/F213A}^{KI/KI}$ donors, respectively).

We first tracked the recruitment of IGRP$_{206-214}$-reactive CD8$^+$ T-cells from diabetic IGRP$_{206-214}^+$ hosts (harboring both naïve and memory IGRP$_{206-214}$-reactive CD8$^+$ T-cells) into IGRP$_{206-214}^+$ or IGRP$_{206-214}^{-/-}$ grafts. The presence of IGRP$_{206-214}$-reactive CD8$^+$ T-cells recruited into the graft was analyzed by flow cytometry, using pMHC tetramers. We also measured the amount of IFNγ that graft-infiltrating T-cells secreted in response to peptide-pulsed irradiated splenocytes, as an additional read-out of T-cell recruitment.

Diabetic IGRP$_{206-214}^+$ hosts receiving IGRP$_{206-214}^{-/-}$ islets developed recurrence of disease, but did so a few days later than those receiving IGRP$_{206-214}^+$ islets (12.7±3.5 vs. 5.9±0.7 days; Figs. 1A top, 1B and 1C left). This indicated that recruitment of naïve and/or pre-activated IGRP$_{206-214}$-reactive CD8$^+$ T-cells contributes to, but is dispensable for graft destruction in diabetic IGRP$_{206-214}^+$ hosts. Importantly, however, whereas IGRP$_{206-214}$-reactive T-cells accounted for a significant fraction of IGRP$_{206-214}^+$ graft-associated CD8+ T-cells (18.2±4.7%), they were undetectable in IGRP$_{206-214}^{-/-}$grafts (Fig. 2A-2C and Supplementary Fig. 1A). Furthermore, the lymph nodes draining the grafted (left) kidney in mice receiving IGRP-$_{206-214}$-expressing islet grafts harbored more IGRP$_{206-214}$-reactive CD8+ T-cells than those draining the contralateral (non-grafted) kidney, and this was not seen in diabetic hosts grafted with IGRP$_{206-214}^{-/-}$ islets (Fig. 3A). In addition, the pancreatic lymph nodes (PLNs) and the spleen, and to a lesser extent the mesenteric lymph nodes (MLNs), of mice grafted with IGRP$_{206-214}^+$ islets contained more IGRP$_{206-214}$-reactive CD8+ T-cells than those from mice grafted with IGRP$_{206-214}^{-/-}$ islets (Figs. 3B-3C). This suggests that graft-derived IGRP$_{206-214}$ induces the activation and retention of host naïve and/or pre-activated IGRP$_{206-214}$-reactive CD8+ T-cells in graft-proximal lymphoid organs.

We next investigated whether the IGRP$_{206-214}$-reactive CD8$^+$ T-cells recruited to the epitope-expressing grafts include naïve T-cells primed by graft-derived IGRP$_{206-214}$. We followed the fate of IGRP$_{206-214}^+$ and IGRP$_{206-214}^{-/-}$ islet grafts and IGRP$_{206-214}$-reactive CD8$^+$ T-cells in diabetic IGRP$_{206-214}^{-/-}$ hosts, which are unable to generate antigen-experienced IGRP$_{206-214}$-reactive CD8$^+$ T-cells from an otherwise normal pool of naïve T-cell precursors. IGRP$_{206-214}^{-/-}$ hosts rejected IGRP$_{206-214}^+$ and IGRP$_{206-214}^{-/-}$ islets with kinetics similar to those seen in NOD mice (Figs. 1A-1C). Yet, IGRP$_{206-214}$-reactive CD8$^+$ T-cells were barely detectable in IGRP$_{206-214}^+$ and IGRP$_{206-214}^{-/-}$ grafts implanted into IGRP$_{206-214}^{-/-}$ hosts (Figs. 2A and 2B), indicating that the grafts do not recruit newly primed IGRP$_{206-214}$-reactive CD8$^+$ T-cells, at least within the first two weeks after transplantation. Interestingly, IGRP$_{206-214}^+$ grafts in

$IGRP_{206\text{-}214}^{-/-}$ hosts recruited slightly more $InsB_{15\text{-}23}$-reactive CD8+ T-cells than in $IGRP_{206\text{-}214}^{+}$ hosts (Supplementary Figs. 1B and 1C). Although these differences were not statistically significant, they suggest that in these mice the $IGRP_{206\text{-}214}$-reactive CD8+ T-cell niche is occupied by other memory T-cell specificities. In addition, the islet graft-associated CD8+ T-cells express markers of memory (i.e. are $CD44^{high}$, $CD62L^{-}$ and $CD127^{+}$; data not shown).

In agreement with the above data, the proximal lymphoid organs (graft-draining LN, PLN and spleen) and blood of $IGRP_{206\text{-}214}^{-/-}$ hosts transplanted with antigen-expressing islets contained fewer $IGRP_{206\text{-}214}$-reactive $CD8^{+}$ T-cells than their $IGRP_{206\text{-}214}^{+}$ host counterparts, suggesting that graft-derived antigen does not induce a detectable peripheral expansion of *naïve* autoreactive T-cells (Fig. 3D). In addition, since the percentages of $IGRP_{206\text{-}214}$-reactive $CD8^{+}$ T-cells in the graft-draining LNs, PLNs and spleen of $IGRP_{206\text{-}214}^{+}$ hosts grafted with $IGRP_{206\text{-}214}$-deficient islets were also low (Fig. 3D, right panels), we conclude that the peripheral expansion of $IGRP_{206\text{-}214}$-reactive $CD8^{+}$ T-cells seen in $IGRP_{206\text{-}214}^{+}$ islet-grafted $IGRP_{206\text{-}214}^{+}$ hosts (Figs. 3A-C) largely, if not exclusively involves antigen-experienced T-cells. Interestingly, NOD hosts grafted with $IGRP_{206\text{-}214}^{-/-}$ islets accumulated $IGRP_{206\text{-}214}$-reactive $CD8^{+}$ T-cells in the bloodstream (Fig. 3D) suggesting that, in the absence of antigen in the graft and graft-draining lymphoid organs, pre-activated $IGRP_{206\text{-}214}$-reactive $CD8^{+}$ T-cells are 'trapped' in the bloodstream.

Finally, we asked whether absence of graft-antigen-primed naïve $IGRP_{206\text{-}214}$-reactive $CD8^{+}$ T-cells in the epitope-expressing grafts of $IGRP_{206\text{-}214}^{-/-}$ hosts was caused by inability of graft-derived $IGRP_{206\text{-}214}$ to activate cognate naïve CD8+ T-cells, or to protracted recruitment and/or accumulation of these T-cells into the graft. This was done by tracking the proliferation of naïve splenic CFSE-labeled $IGRP_{206\text{-}214}$-reactive $CD8^{+}$ T-cells from 8.3-TCR-transgenic mice in NOD hosts grafted with $IGRP_{206\text{-}214}^{+}$ or $IGRP_{206\text{-}214}^{-/-}$ islets the previous day. Various host lymphoid organs were examined for dilution of CFSE in the CFSE+CD8+ gate 7 days after T-cell transfer. Naive 8.3-CD8+ T-cells proliferated vigorously in the LNs draining $IGRP_{206\text{-}214}$+ (but not $IGRP_{206\text{-}214}^{-/-}$) grafts and, to a lesser extent, the PLN and spleen, where some of the proliferation appears to be induced by host-derived (residual) $IGRP_{206\text{-}214}$ (Fig. 4A and B). There were very few donor 8.3-CD8+ T-cells in $IGRP_{206\text{-}214}^{+}$ or $IGRP_{206\text{-}214}^{-/-}$ grafts (0.06 + 0.03% vs. 0.06 + 0.016% of CD8+ cells, respectively) (Fig. 4C). These observations indicate that destruction of $IGRP_{206\text{-}214}^{+}$ and $IGRP_{206\text{-}214}^{-/-}$ grafts in $IGRP_{206\text{-}214}^{+}$ hosts (Fig. 1A) predates recruitment of newly activated T-cells.

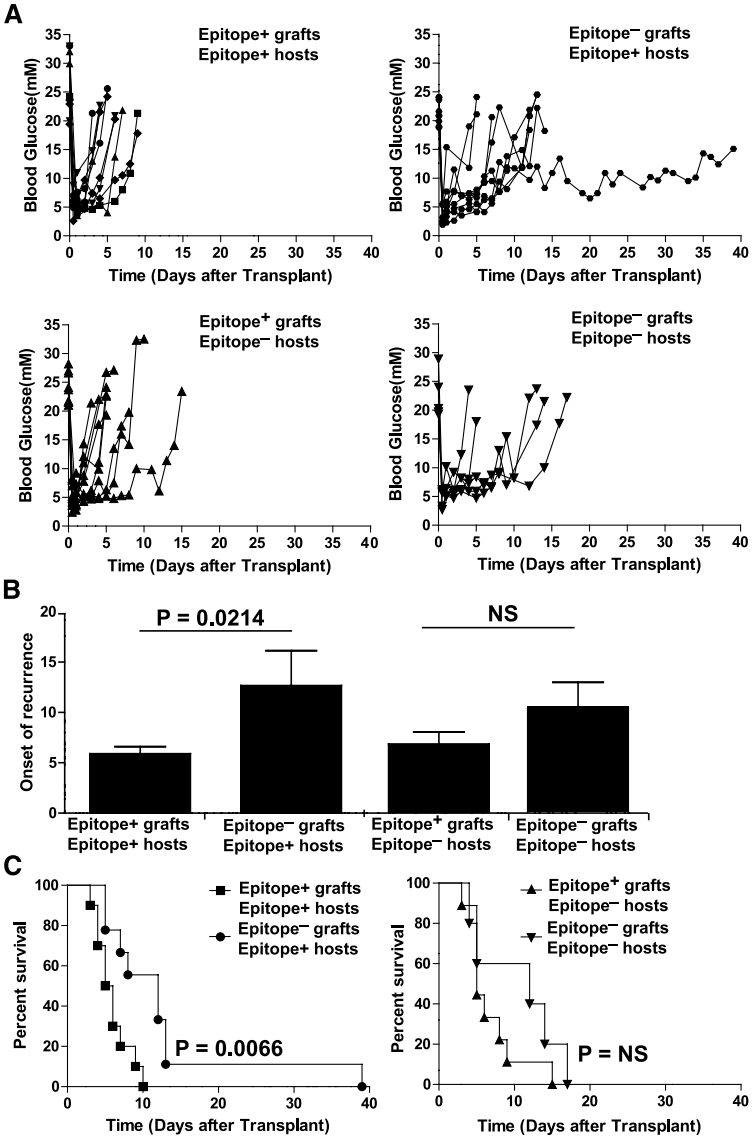


Figure 1 | Survival of islet grafts from IGRP$_{206\text{-}214}$-competent or -deficient donors in spontaneously diabetic IGRP$_{206\text{-}214}$-competent or -deficient NOD hosts.

(A) Individual blood glucose curves of diabetic NOD hosts receiving NOD.*scid* (n=10) or NOD.*rag2*$^{-/-}$.IGRP$_{K209A/F213A}^{KI/KI}$ islets (n=9), and diabetic NOD.IGRP$_{K209A/F213A}^{KI/KI}$ hosts receiving NOD.*scid* (n=9) or NOD.*rag2*$^{-/-}$.IGRP$_{K209A/F213A}^{KI/KI}$ islets (n=5). **(B)** Average onset of disease recurrence after transplantation (in days) in the four different donor/host type combinations. P values were obtained by Mann-Whitney U. **(C)** Survival curves of grafts in diabetic NOD (left) or NOD.IGRP$_{K209A/F213A}^{KI/KI}$ hosts (right). P values were calculated via Log Rank test. For **B** and **C**, differences between epitope+ and epitope– grafts in epitope+ hosts remained statistically significant upon exclusion of the epitope– graft that survived out to 40 days (P=0.0395 in **B**; and P=0.0353 in **C**).

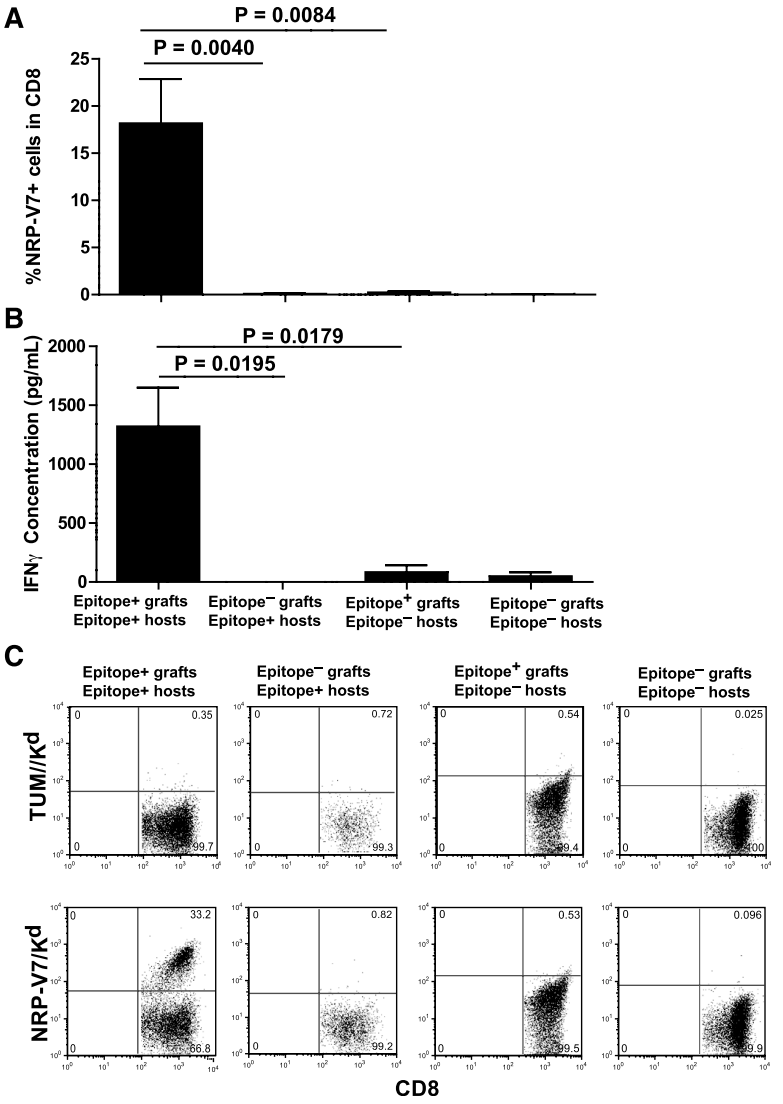


Figure 2 | Recruitment of IGRP$_{206-214}$-reactive CD8+ T-cells from diabetic IGRP$_{206-214}$-competent or -deficient NOD hosts into islet grafts from IGRP$_{206-214}$-competent or -deficient donors or NOD.rag2$^{-/-}$.IGRP$_{K209A/F213A}^{KI/KI}$ donors.

(A) Percentages of NRP-V7/K^d tetramer+ in islet-graft-associated CD8+ T-cells. Data (average + SEM) correspond, from left to right, to 8, 4, 4 and 3 grafts per group, respectively. **(B)** IFNγ secretion by islet graft-associated CD8+ T-cells in response to NRP-V7 peptide-pulsed NOD DCs. Data correspond, from left to right, to 5, 4, 4 and 3 grafts per group, respectively. **(C)** Representative FACS staining profiles of CD8+ T-cells isolated from islet grafts in the four different donor/host type combinations. TUM/K^d was used as a negative control tetramer. P values in A and B were obtained with Mann-Whitney U. Grafts were harvested immediately after the last blood glucose measurement in Fig. 1.

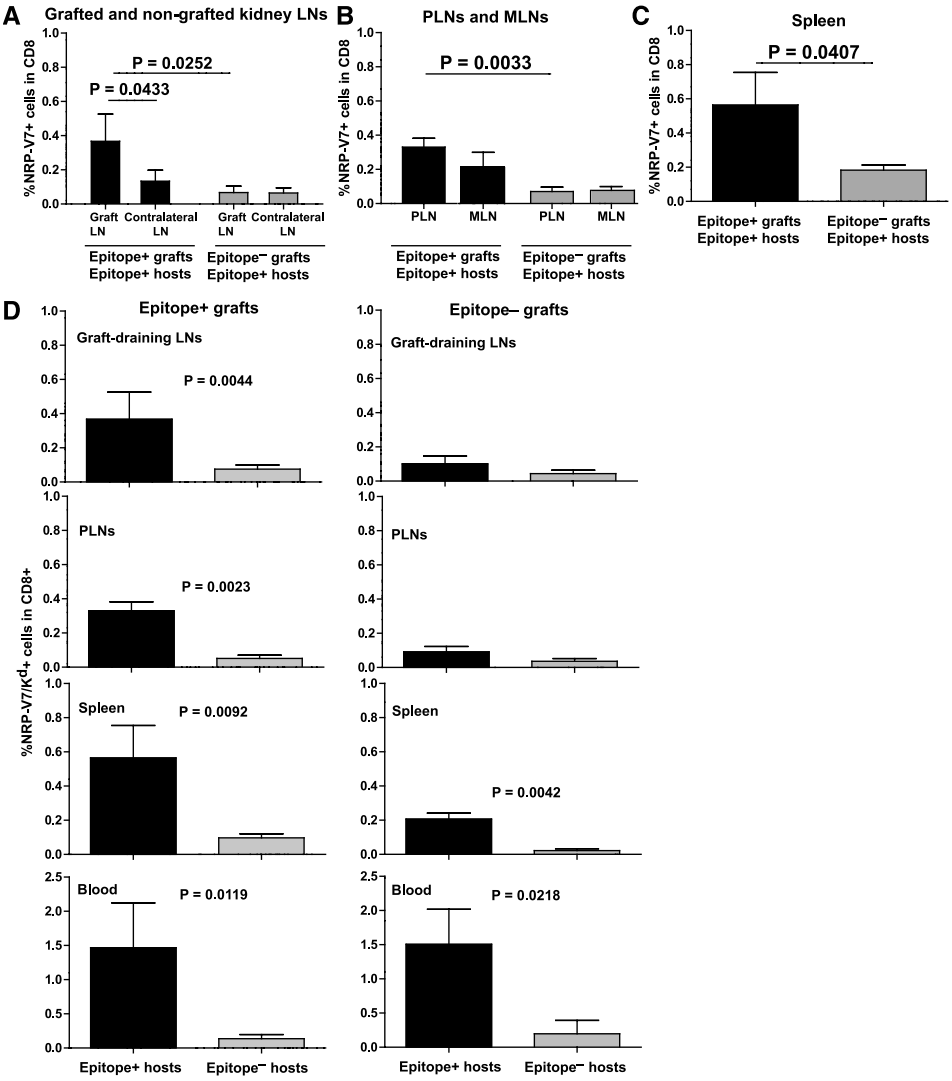


Figure 3 | Frequencies of IGRP$_{206\text{-}214}$-reactive CD8+ T-cells in lymphoid organs and blood of diabetic IGRP$_{206\text{-}214}$-competent or -deficient hosts grafted with IGRP$_{206\text{-}214}$–competent or -deficient islets.
(A-C) Percentages of NRP-V7/K^d tetramer+ cells in CD8+ T-cells from lymph nodes draining the grafted vs. contralateral kidneys **(A)**, the PLN vs. MLNs **(B)** and the spleen **(C)**. Data (average + SEM) correspond to 6 and 8 mice, respectively. **(D)** Frequencies of IGRP$_{206\text{-}214}$-reactive CD8+ T-cells in lymphoid organs and blood of IGRP$_{206\text{-}214}$-competent vs. IGRP$_{206\text{-}214}$-deficient hosts grafted with IGRP$_{206\text{-}214}$-competent (left) vs. IGRP$_{206\text{-}214}$-deficient islets (right). Data (average + SEM) correspond to 6, 6, 8 and 5 mice per group, respectively. Background staining with the negative control tetramer TUM/K^d was subtracted. P values were obtained with Mann-Whitney U.

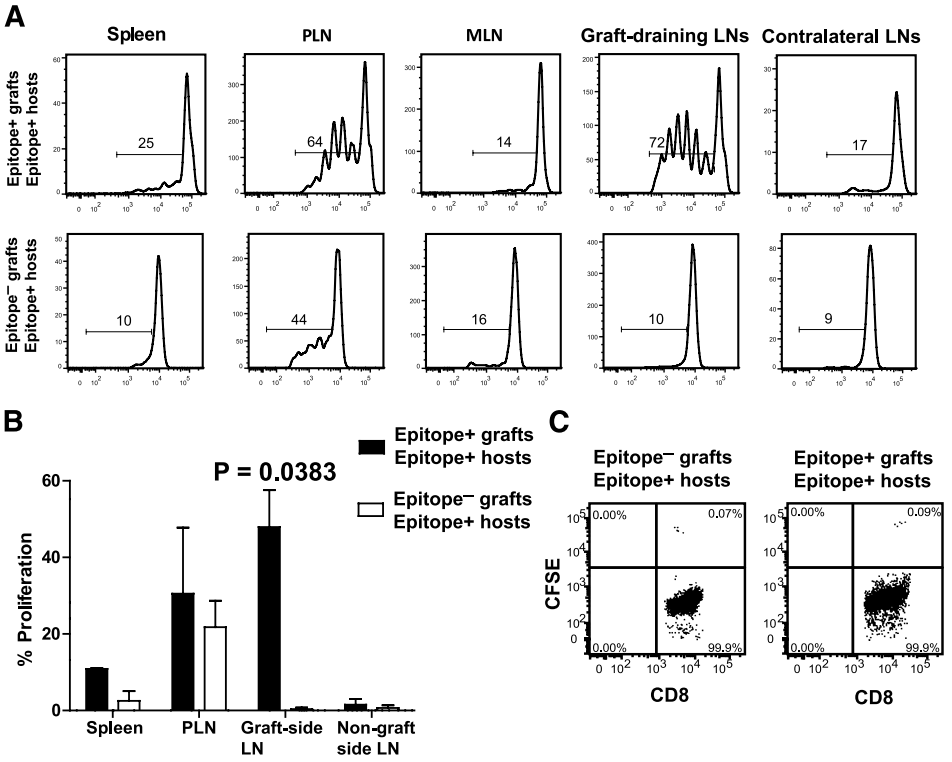
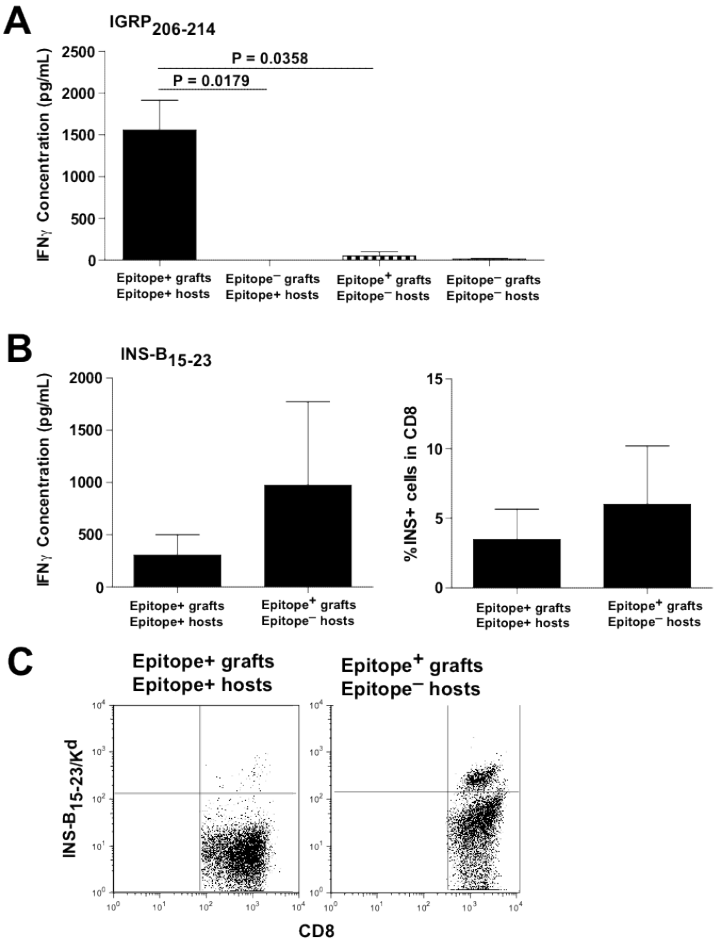


Figure 4 | Proliferation of naive CFSE-labelled 8.3-CD8+ T-cells in lymphoid organs of IGRP$_{206\text{-}214}$-competent hosts grafted with IGRP$_{206\text{-}214}$-competent vs. IGRP$_{206\text{-}214}$-deficient islets.

(A) Representative CFSE dilution profiles. **(B)** Average + SEM of the percentage of proliferated cells. **(C)** Representative flow profiles of graft-associated CD8+ T-cells of these mice. Data in A-C correspond to 3 mice per host/donor combination. P values were obtained with Mann-Whitney U. (LN: Lymph node; MLN: mesenteric lymph node; PLN: pancreatic lymph node).



Supplementary Figure 1 | Recruitment of $IGRP_{206-214}$- and Insulin B_{15-23}-reactive CD8+ T-cells from diabetic $IGRP_{206-214}$-competent or -deficient NOD hosts into islet grafts from $IGRP_{206-214}$-competent or -deficient donors or NOD.rag2$^{-/-}$.$IGRP_{K209A/F213A}^{KI/KI}$ donors.

(A) IFNγ secretion by islet graft-associated CD8+ T-cells in response to $IGRP_{206-214}$ peptide-pulsed NOD DCs. Data correspond, from left to right, to 5, 4, 3 and 3 grafts per group, respectively. **(B)** IFNγ secretion by islet graft-associated CD8+ T-cells in response to $InsB_{15-23}$ peptide-pulsed NOD DCs. Data correspond, from left to right, to 5, 3, 4 and 3 grafts per group, respectively. **(C)** Examples of FACS staining profiles of graft-associated CD8+ T-cells containing low versus high percentages of $InsB_{15-23}/K^d$ tetramer-reactive cells.

DISCUSSION

The data presented herein challenge a current paradigm stating that non-antigen-specific inflammatory cues can attract and retain non-cognate, 'bystander' T-cell specificities to sites of inflammation, including syngeneic islet transplants in diabetic mice. We demonstrate that absence of the cognate autoantigen in a syngeneic extra-pancreatic islet graft in a diabetic host renders the graft 'invisible' to cognate memory (and naïve) T-cells. Local antigen expression (in addition to MHC class I expression (18)) is thus a *sine-qua-non* requirement for accumulation of autoreactive CD8+ T-cells into islet grafts.

The absolute need for local autoantigen expression is highlighted by two important considerations. First, $IGRP_{206\text{-}214}/K^d$ (NRP-V7)-reactive $CD8^+$ T-cells are among the most prevalent in NOD islet infiltration (8). Second, the vascular beds irrigating islet grafts, including the subcapsular kidney space have a porous, fenestrated architecture (13-15) that could conceivably render them permeable to bystander T-cells. It is therefore remarkable that autoantigen-experienced (i.e. memory) $IGRP_{206\text{-}214}$-reactive T-cells, despite their prevalence in the periphery, do not accumulate into $IGRP_{206\text{-}214}$-deficient grafts. Recruitment of memory $CD8^+$ T-cells to islet grafts thus follows the same rules that we have described for the recruitment of naïve and *in vitro*-activated CD8+ T-cells into endogenous islets (i.e. requiring a cognate pMHC interaction *in situ*). A recent report demonstrates a striking similarity in human insulitis: all the $CD8^+$ T-cells found in the inflamed islets of type 1 diabetic patients bound self-pMHC complexes (19).

Our findings further imply that individual autoantigenic specificities, even when prevalent, play relatively minor roles in the anamnestic autoimmune response contributing to graft destruction in autoimmune disease-affected hosts. Our results also clearly indicate that destruction of syngeneic islet grafts in diabetic NOD mice is largely, if not exclusively effected by autoantigen-experienced T-cells primed during the primary autoimmune response. Although graft antigen-loaded APCs residing in the graft-draining lymph nodes can readily induce the activation of naive autoreactive CD8+ T-cells, graft destruction precedes recruitment of these T-cells into the graft. The high physical and functional pMHC-binding avidities of antigen-experienced T-cells coupled to their ability to mount rapid recall responses to limiting amounts of antigen (20) likely affords them a competitive advantage, particularly during the first two weeks after transplantation. Differences in the bio-distribution of memory vs. naïve T-cells may be another contributing factor. These considerations, however, do not exclude the likely involvement of graft antigen-primed naïve autoreactive T-cells in chronic loss of graft function, such as for example in the context of partially matched islet allografts.

Recurrent autoimmunity in allogeneic islet cell transplantation has become a topic of growing interest. For example, the pre-transplant peripheral frequencies of autoreactive T-cells in diabetic recipients are predictive of islet allograft fate, and post-transplant increases are associated with loss of graft function (21-24), suggesting that recurrent autoimmunity may contribute to allograft destruction. Although clinical islet

transplantation is a more complex situation, our model has allowed us to dissect the specific roles of bystander immunity vs. anamnestic and naive autoimmunity to islet graft rejection. Our observations emphasize the importance of developing therapies capable of preventing: (i) priming of naïve alloreactive T-cells causing allograft rejection; (ii) recruitment of memory autoreactive T-cells causing anamnestic autoimmunity in the immediate post-transplant period; and (iii) and the priming of naïve autoreactive T-cells causing chronic loss of graft function, paying special attention to the pre-transplant autoreactivity status of the diabetic host.

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G.A., X.C-C, S.T. and J.W. researched data and contributed to the discussion and editing of the manuscript. J.Y. and B.X. assisted with experiments. J.W. and B.R. contributed to the discussion and reviewed and edited the manuscript. P.S. designed and supervised the study. P.S. is the guarantor of this work, had full access to all the data, and takes full responsibility for the integrity of the data and the accuracy of data analysis. The authors have no conflicts of interest to declare.

REFERENCES

1. Babad J, Geliebter A, DiLorenzo TP. T-cell autoantigens in the non-obese diabetic mouse model of autoimmune diabetes. Immunology 2010;131:459-465
2. Anderson B, Park B, Verdaguer J et al. Prevalent CD8+ T cell response against one peptide/MHC complex in autoimmune diabetes. Proc Natl Acad Sci USA 1999;96:9311-9316
3. DiLorenzo T, Graser T, Ono Tet al. MHC class I-restricted T-cells are required for all but end stages of diabetes development and utilize a prevalent T cell receptor a chain gene rearrangement. Proc Natl Acad Sci USA 1998;95:12538-12543
4. Verdaguer J, Yoon J, Anderson B et al. Acceleration of spontaneous diabetes in TCRb-transgenic nonobese diabetic mice by beta cell-cytotoxic CD8+ T-cells expressing identical endogenous TCRa chains. J Immunology 1996;157:4726-4735
5. Verdaguer J, Schmidt D, Amrani A et al. Spontaneous autoimmune diabetes in monoclonal T cell nonobese diabetic mice. J Exp Med 1997;186:1663-1676
6. Lieberman S, Evans A, Han B et al. Identification of the b-cell antigen targeted by a prevalent population of pathogenic CD8+ T cells in autoimmune diabetes. Proc Natl Acad Sci USA 2003;100:8384-8388
7. Trudeau J, Kelly-Smith C, Verchere B et al. Autoreactive T cells in peripheral blood predict development of type 1 diabetes. J Clin Invest 2003;111:217-223
8. Amrani A, Verdaguer J, Serra P, et al. Progression of autoimmune diabetes driven by avidity maturation of a T-cell population. Nature 2000;406:739-742
9. Stephens R, Randolph DA, Huang G et al. Antigen-nonspecific recruitment of Th2 cells to the lung as a mechanism for viral infection-induced allergic asthma. J Immunol 2002;169:5458-5467
10. Ostler T, Pircher H, Ehl S. 'Bystander' recruitment of systemic memory T cells delays the immune response to respiratory virus infection. Eur J Immunol 2003;33:1839-1848
11. Chen AM, Khanna N, Stohlman SA et al. Virus-specific and bystander CD8 T cells recruited during virus-induced encephalomyelitis. J Virol 2005;79:4700-4708
12. Wang J, Tsai S, Shameli A et al. In situ recognition of autoantigen as an essential gatekeeper in autoimmune CD8+ T cell inflammation. Proc Natl Acad Sci U S A 2010;107:9317-9322
13. Lukinius A, Jansson L, Korsgren O. Ultrastructural evidence for blood microvessels devoid of an endothelial cell lining in transplanted pancreatic islets. The American journal of pathology 1995;146:429-435
14. Vajkoczy P, Menger MD, Simpson E et al. Angiogenesis and vascularization of murine pancreatic islet isografts. Transplantation 1995;60:123-127
15. Morini S, Brown ML, Cicalese L et al. Revascularization and remodelling of pancreatic islets grafted under the kidney capsule. Journal of anatomy 2007;210:565-577
16. Davalli AM, Scaglia L, Zangen DH et al. Vulnerability of islets in the immediate posttransplantation period. Dynamic changes in structure and function. Diabetes 1996;45:1161-1167

17. Tsai S, Shameli A, Yamanouchi J et al. Reversal of autoimmunity by boosting memory-like autoregulatory T cells. Immunity 2010;32:568-580

18. Serreze DV, Chapman HD, Varnum DS et al. Initiation of autoimmune diabetes in NOD/Lt mice is MHC class I-dependent. J Immunol 1997;158:3978-3986

19. Coppieters KT, Dotta F, Amirian N et al. Demonstration of islet-autoreactive CD8 T cells in insulitic lesions from recent onset and long-term type 1 diabetes patients. The Journal of experimental medicine 2012;209:51-60

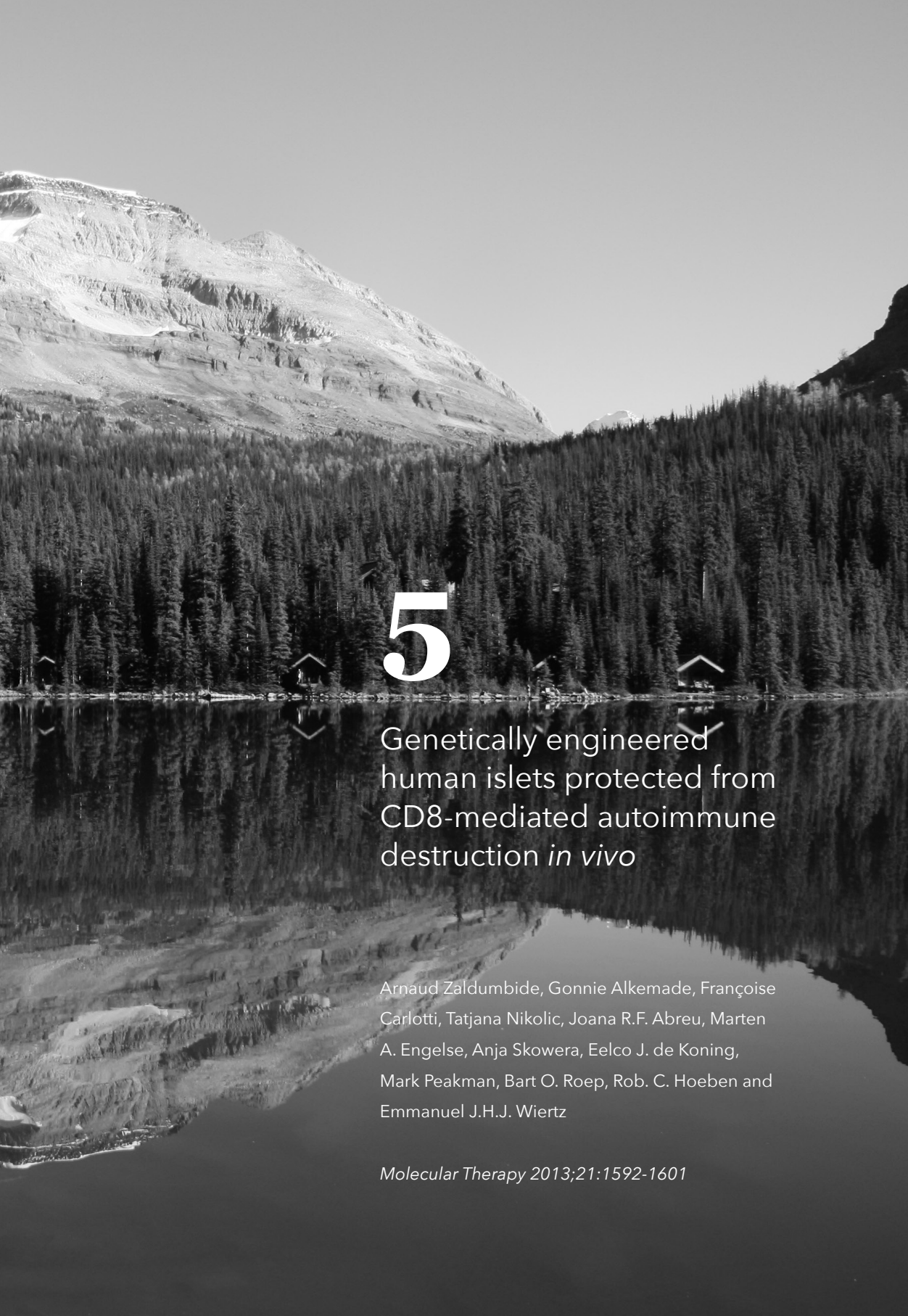
20. Tailor P, Tsai S, Shameli A et al. The proline-rich sequence of CD3epsilon as an amplifier of low-avidity TCR signaling. Journal of immunology 2008;181:243-255

21. Roep BO, Stobbe I, Duinkerken G et al. Auto- and alloimmune reactivity to human islet allografts transplanted into type 1 diabetic patients. Diabetes 1999;48:484-490

22. Huurman VA, Hilbrands R, Pinkse GG et al. Cellular islet autoimmunity associates with clinical outcome of islet cell transplantation. PloS one 2008;3:e2435

23. Hilbrands R, Huurman VA, Gillard P et al. Differences in baseline lymphocyte counts and autoreactivity are associated with differences in outcome of islet cell transplantation in type 1 diabetic patients. Diabetes 2009;58:2267-2276

24. Pinkse GG, Tysma OH, Bergen CA et al. Autoreactive CD8 T cells associated with beta cell destruction in type 1 diabetes. Proceedings of the National Academy of Sciences of the United States of America 2005;102:18425-18430



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Genetically engineered human islets protected from CD8-mediated autoimmune destruction *in vivo*

Arnaud Zaldumbide, Gonnie Alkemade, Françoise Carlotti, Tatjana Nikolic, Joana R.F. Abreu, Marten A. Engelse, Anja Skowera, Eelco J. de Koning, Mark Peakman, Bart O. Roep, Rob. C. Hoeben and Emmanuel J.H.J. Wiertz

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ABSTRACT

Islet transplantation is a promising therapy for type 1 diabetes, but graft function and survival are compromised by recurrent islet autoimmunity. Immunoprotection of islets will be required to improve clinical outcome. We engineered human beta-cells to express herpesvirus-encoded immune evasion proteins, 'immunevasins'. The capacity of immunevasins to protect beta-cells from autoreactive T-cell killing was evaluated *in vitro* and *in vivo* in humanized mice. Lentiviral vectors were used for efficient genetic modification of primary human beta-cells without impairing their function. Using a novel beta-cell specific reporter gene assay we show that autoreactive cytotoxic CD8+ T-cell clones isolated from patients with recent-onset diabetes selectively destroyed human beta-cells, and that co-expression of the human cytomegalovirus-encoded US2 protein and serine proteinase inhibitor 9 offers highly efficient protection *in vitro*. Moreover, co-implantation of these genetically modified pseudoislets with beta-cell specific cytotoxic T-cells into immunodeficient mice achieves preserved human insulin production and c-peptide secretion. Collectively, our data provide proof of concept that human beta-cells can be efficiently genetically modified to provide protection from killing mediated by autoreactive T-cells and retain their function *in vitro* and *in vivo*.

INTRODUCTION

Type 1 diabetes results from selective and progressive destruction of insulin-producing cells by autoreactive $CD8^+$ T-cells. [1,2] The direct cell-cell contact initiated by T-cell receptors recognizing a beta-cell specific antigenic peptide presented by the MHC class-I (MHC-I) at the surface of the target cell appears to be critical for beta-cell destruction in type 1 diabetes. In humans, during acute insulitis, beta-cells show hyper-expression of MHC-I and selective infiltration by islet-specific autoreactive $CD8^+$ T-cells. [3] Also mice lacking MHC-I expression do not develop diabetes, [4-6] whereas adoptive spleen cell transfer from NOD mice to recipients selectively expressing MHC-I on beta-cells leads to beta-cell destruction. [7,8] In addition, the Fas signaling pathway is not essential in the destruction of beta-cells, [9,10] while disruption of the perforin gene delays the onset of autoimmune diabetes in NOD mice, pointing to the perforin/granzyme pathway as a key effector in beta-cell destruction by cytotoxic T-cells. [11,12]

To date, immunotherapies to cure, prevent, or delay disease onset in humans have been inefficient. [13,14] Options for type 1 diabetes patients to restore normoglycemia are limited to daily insulin injection, combined kidney-pancreas or experimental islet transplantation. However, numerous factors have been reported to potentially affect islet allograft function and survival. [15] Importantly, the close correlation between loss of islet-graft function and frequencies of circulating autoreactive islet-specific T cells observed in transplanted patients, in particular when the donor and the recipient share the same HLA class I haplotypes, points to the importance of recurrent autoimmunity in islet graft failure. [16-19] Along the same line, it appears that, in case of autotransplantation, islet grafts are much less affected, suggesting that persistent attacks from autoimmunity, alloimmunity and possibly the toxicity of immunosuppressive treatments have a major responsibility in graft loss. [20]

An alternative could be the transplantation of genetically immunoprotected beta-cells. We have previously explored the capacity of herpesvirus-derived immunevasins to elude the host immune responses (reviewed in [21-23]). In this study we aim to combine the US2 protein of human cytomegalovirus, known to interfere with antigenic peptide presentation by inducing proteasomal degradation of MHC-I molecules [24], and Serpin-9, a serine protease inhibitor specific for granzyme B activity. [25]

Furthermore, the identification of specific human beta-cell protectants is hindered by the difficulty of studying the dialogue between human insulin-producing cells and the immune system. Even *in vitro*, the cellular heterogeneity of dissociated islet preparations makes the standard release-based assays imperfect to specifically assess beta-cell cytotoxicity. Islet preparations contain a mixture of cell types, including β-cells, α-cells, δ-cells, ε-cells, duct cells, endothelial cells, stem cells, and leukocytes, that all may interfere with the assays to selective assess beta-cell cytotoxicity. [26]

Here we describe the development of a new strategy to measure beta-cell cytotoxicity and protection from autoreactive T-cell-mediated killing. We report that lentivirus

vectors can be used to genetically modify human islet cells. Moreover, using this powerful technology, we demonstrate that downregulation of MHC-I combined with the inhibition of granzyme B activity protects human beta-cells from acute recurrent islet autoimmunity both *in vitro* and *in vivo*.

RESULTS

Primary human islet cells can be transduced efficiently by lentiviral vectors.
Lentiviral vectors are the gene transfer system of choice for stable genetic modification of primary cells. To evaluate their capacity to genetically modify freshly isolated human islets, a vector carrying an enhanced GFP reporter gene under the control of the human cytomegalovirus immediate early promoter was used (LV-CMV-GFP). Seventy-two hours post transduction, islets were inspected for reporter gene expression (Fig. 1a). A MOI of 5 was well tolerated without any apparent signs of virus-induced toxicity. In addition, exposure to the virus did not significantly affect islet function, as exemplified by their capacity to secrete insulin upon glucose stimulation (Fig. 1b). Yet, GFP expression within the islet structures was heterogeneous. Following insulin/DNA staining, whole mount confocal microscopy confirmed that in most islets only cells at the rim of the islets were efficiently transduced, demonstrating that in intact human islets only a limited number of cells are permissive to lentiviral vector transduction (Fig. 1c). To increase the transduction efficiency, human islets were dispersed, and the resulting single-cell suspension was exposed to the lentivirus vector LV-CMV-GFP. Subsequently, pseudoislets were formed by self-aggregation of the transduced cells. Seven days post re-aggregation, the 3D structures were macroscopically similar to islets. The majority of the cells expressed GFP as determined by flow cytometry (Fig. 1d). To evaluate the functionality of genetically modified pseudoislet structures, a static glucose-stimulated insulin secretion test was performed and glucose responsiveness was compared to non-transduced and intact islets. Neither dispersion nor transduction hampered islet functionality, since no differences in insulin release were detected after glucose stimulation (Fig. 1e).

Genetically modified pseudoislets are functional *in vivo*.
To assess the fate of these pseudoislets *in vivo*, non-transduced and LV-CMV-GFP transduced human pseudoislets were transplanted under the kidney capsule of NSG mice. The release of human insulin after intra-peritoneal glucose administration was measured. Assuming that one islet would contain approximately 1000 to 1500 cells, [27] ~3000 islet equivalents (IEQ) were transplanted and compared to $5x10^6$ dispersed/re-aggregated cells. Pseudoislets efficiently released insulin upon glucose challenge, since no significant differences were observed when compared to intact islets (Fig. 2a). In order to define the limitation of the insulin assay used, we transplanted NSG mice with $2.5x10^6$ cells (~1500 pseudoislets) or $5x10^6$ cells (~3000 pseudoislets) and monitored human insulin release. As anticipated, the amount of insulin secreted correlated with the number of transplanted cells (Fig. 2b). Similar results were obtained with a human c-peptide assay (data not shown). To confirm the survival of the insulin-producing cells,

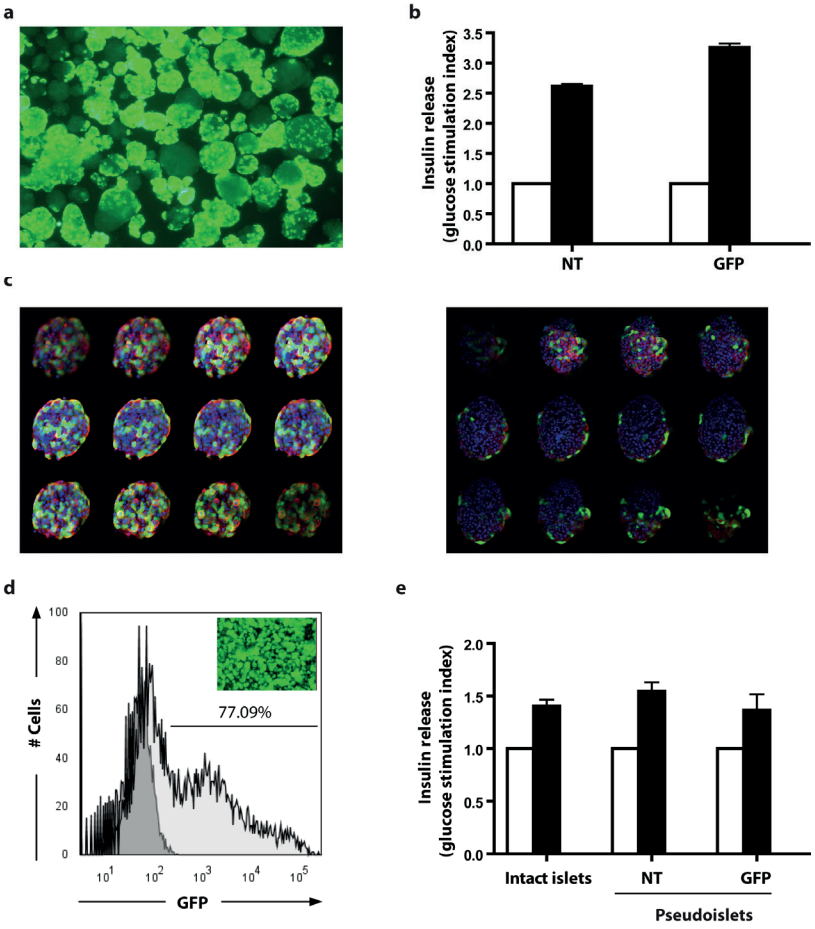


Figure 1 | Genetic modification of human islets *in vitro*.

a) Fluorescent microscopy pictures of freshly isolated human islets transduced with LV-CMV-GFP (MOI=5). GFP expression was assessed by fluorescent microscopy (200x magnification). **b)** Comparative glucose responsiveness of non-transduced islets (NT) and GFP-modified islets (GFP) determined by glucose-stimulated insulin-secretion test. Insulin release data are shown as glucose stimulating index (i.e. insulin-release at low glucose - white bars - is used as reference and set to 1 and used as reference for high glucose (black bars) induction. **c)** Series of optical sections of two representative islets (2μm) taken by confocal microscopy. Insulin is depicted in red, GFP in green and nuclei are visualized by DAPI (blue) staining. While some GFP expression could be observed in the central core of the islet (left panel), in most islets GFP expression was limited to the outer rim of the islet (right panel). **d)** Fluorescent microscopy picture of GFP-modified pseudoislets (MOI=2) and quantification of the GFP positive cells by FACS. Light grey histogram shows GFP-transduced cells and non-transduced dispersed cells are shown in dark grey histogram. Experiments are performed 6-days post-transduction. **e)** Glucose responsiveness of pseudoislets or genetically modified pseudoislets compared to intact islets from the same donor. Similar to panel b, insulin release data are shown as glucose stimulating index. Low glucose (white bars) concentration is set to 1 and used as reference for high glucose (black bars) induction (GFP: green fluorescent protein; MOI: multiplicity of infection; NT: non-transduced).

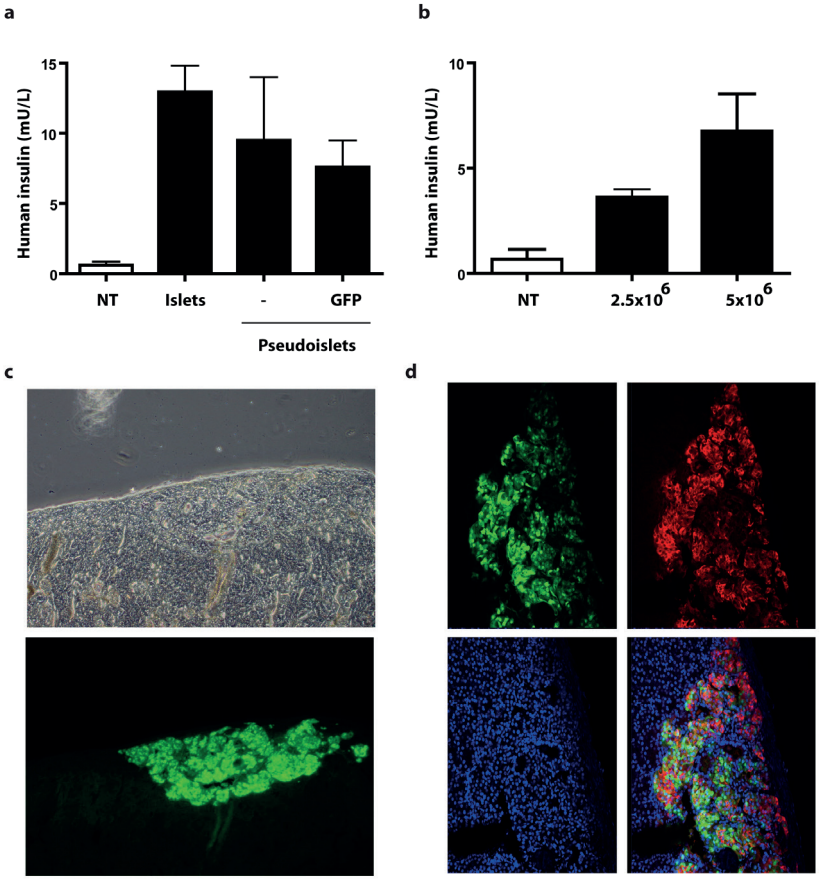


Figure 2 | Function of genetically modified pseudoislets in vivo.
a) Intraperitoneal glucose-tolerance test performed on mice transplanted with 3000 intact islets (n=1) or 5.10^6 GFP modified pseudoislets (n=4) or non-modified islets (n=2). N represents the number of transplanted mice. Results are represented as average of 3 different time points at 4, 11 and 19 days after transplantation. **b)** Similar experiment performed with GFP-modified pseudoislets formed with $2.5.10^6$ cells or 5.10^6 cells (n=2). Non-transplanted mice were used as negative control. **c)** Fluorescent microscopy of the kidney performed after nephrectomy 19 days post transplantation of pseudoislets containg 5.10^6 cells. **d)** Immunostaining of the graft. Insulin is shown in red, GFP in green and nuclei are stained by DAPI in blue. Sections were analyzed by confocal microscopy.

the graft was removed 19-days post-transplantation and analyzed by confocal imaging for GFP fluorescence and, after immunostaining, for insulin reactivity. Macroscopically, a strong and sustained GFP expression was detected at the site of re-implantation (Fig. 2c). Insulin immunostaining demonstrated a large proportion of GFP positive beta-cells (Fig. 2d). From these data we conclude that lentivirus-mediated gene transfer into cells from freshly isolated human islets is feasible, and that the cells can be re-aggregated to form functional pseudoislets.

The human insulin promoter drives beta-cell specific expression in human islet cells.
Next, in order to acquire specific expression of the gene of choice in beta-cells, the CMV promoter was replaced by the human insulin promoter (HIP) (Fig. 3a). To assess HIP promoter specificity, we first compared CMV-GFP lentivirus transduction efficiency in human embryonic kidney cells (HEK) or rat insulinoma cell lines (INS-1E) and confirmed that both cell types can be efficiently modified by lentiviruses (Fig. 3b, upper panel). Secondly, we performed similar experiments using the HIP-GFP lentivirus and detected only few GFP positive HEK cells whereas 25% of the INS-1E expressed GFP (Fig. 3b, lower panel). Finally, we verified HIP specificity and efficiency in human primary cells. One week post transduction, HIP-GFP human pseudoislets were analyzed for GFP expression using confocal microscopy (Fig. 3c). Altogether, these data demonstrate that the HIP promoter facilitates efficient transgene expression and limits this expression to beta-cells.

Autoreactive HLA-A2-restricted PPI-directed CTL clones kill HLA-A2 human islet cells *in vitro*.
To quantify beta-cell death, the GFP cassette was replaced by a destabilized luciferase reporter gene, allowing short half-life luciferase expression specifically in beta-cells (Fig. 3a). To validate this method, we designed an *in vitro* killing assay by incubating HIP-Luc2CP modified human islet cells with autoreactive $CD8^+$ T-cells isolated from a recent onset T1D patient and directed against an epitope located in the signal peptide of the preproinsulin molecule (PPI). [28] CTL killing capacity was validated in a standard chromium release assay using K562 surrogate beta-cells (Figure S1). Using fractions of different purities from the same donor, killing assays were performed with different target/effector ratios (corrected for purity of the fraction). These experiments demonstrate that the luciferase assay is not affected by the quality of the isolated islet fraction (Fig. 4a). Similarly, killing assays performed in parallel with HLA-A2-restricted PPI-directed CTL, incubated with HIP-Luc2CP islet cells from HLA-A3 and HLA-A2 donors, demonstrated that PPI-directed CTL were able to specifically kill HLA-A2 beta-cells, as seen by a massive drop in luciferase activity. When HLA-A3 donor cells were used as targets, no significant decrease in light emission was observed (Fig. 4b). Moreover, when using HLA-A2 restricted pp65CMV-specific CTL, the viability of the HLA-A2 positive beta-cells was not affected (Fig. 4c), which is consistent with the absence of pp65CMV target epitope on human beta cells. This demonstrates that beta-cell death is dependent on the presence of the PPI-specific CTL.

Combined US2/Serpin9 expression protects surrogate beta-cells and human primary beta-cells from PPI-directed CTL killing *in vitro*.
To explore the protective effect of combined MHC-I downregulation and granzyme B inactivation, we generated a bicistronic lentivirus vector encoding the immune-evasion gene US2 and the serine protease inhibitor specific for granzyme B activity (Fig 5a). First, the protective effect was evaluated on surrogate beta-cells generated by overexpression of preproinsulin in K562-A2 cells and then transpose to primary human islets. Insulin mRNA level in surrogate beta-cells was verified by RT-PCR and qPCR (Fig. 5b). Transduction of K562-A2 with a US2-containing lentivirus vector led to decreased HLA class I expression as quantified by flow cytometry (Fig. 5c, lower panel). After transduction of HEK 293T

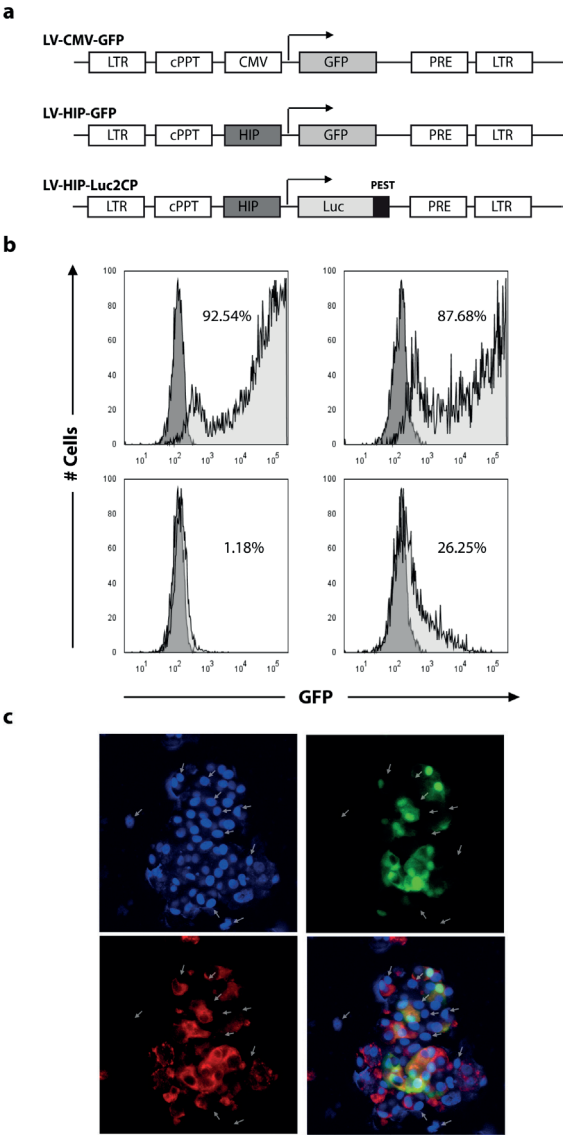


Figure 3 | Human insulin promoter specificity.

a) Schematic representation of the lentivirus constructs used: LV-CMV-GFP; LV-HIP-GFP; LV-HIP-Luc2CP. (LTR: Long terminal Repeat; cPPT: central polypurine tract; PRE: Posttranscriptional Regulatory E (HEK: human embryonic kidney; HIP: human insulin promotor). The arrow indicates the transcription initiation) **b)** Comparative GFP-expression as determined by FACS in HEK 293T cells (left column) and INS1E cells (right column) after transduction with LV-CMV-GFP (MOI=1) (upper panel) or LV-HIP-GFP (MOI=1) (lower panel). Non-transduced cells were used as negative control and shown in dark grey histogram. **c)** Whole mount immunostaining using anti-insulin antibody (red) performed on HIP-GFP transduced pseudoislets. Nuclei were stained by DAPI in blue. White arrows indicate the insulin negative cells.

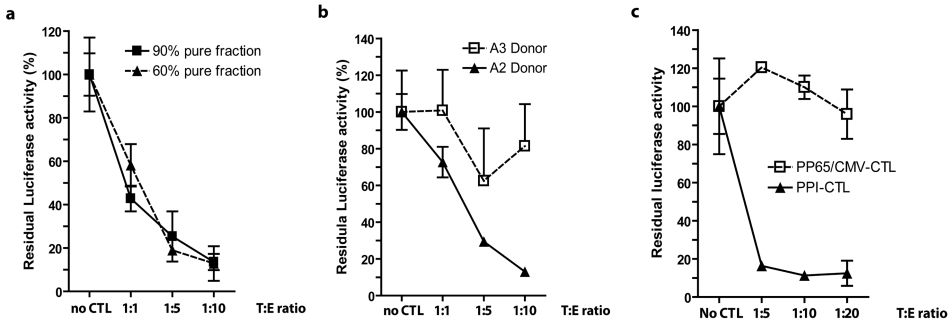
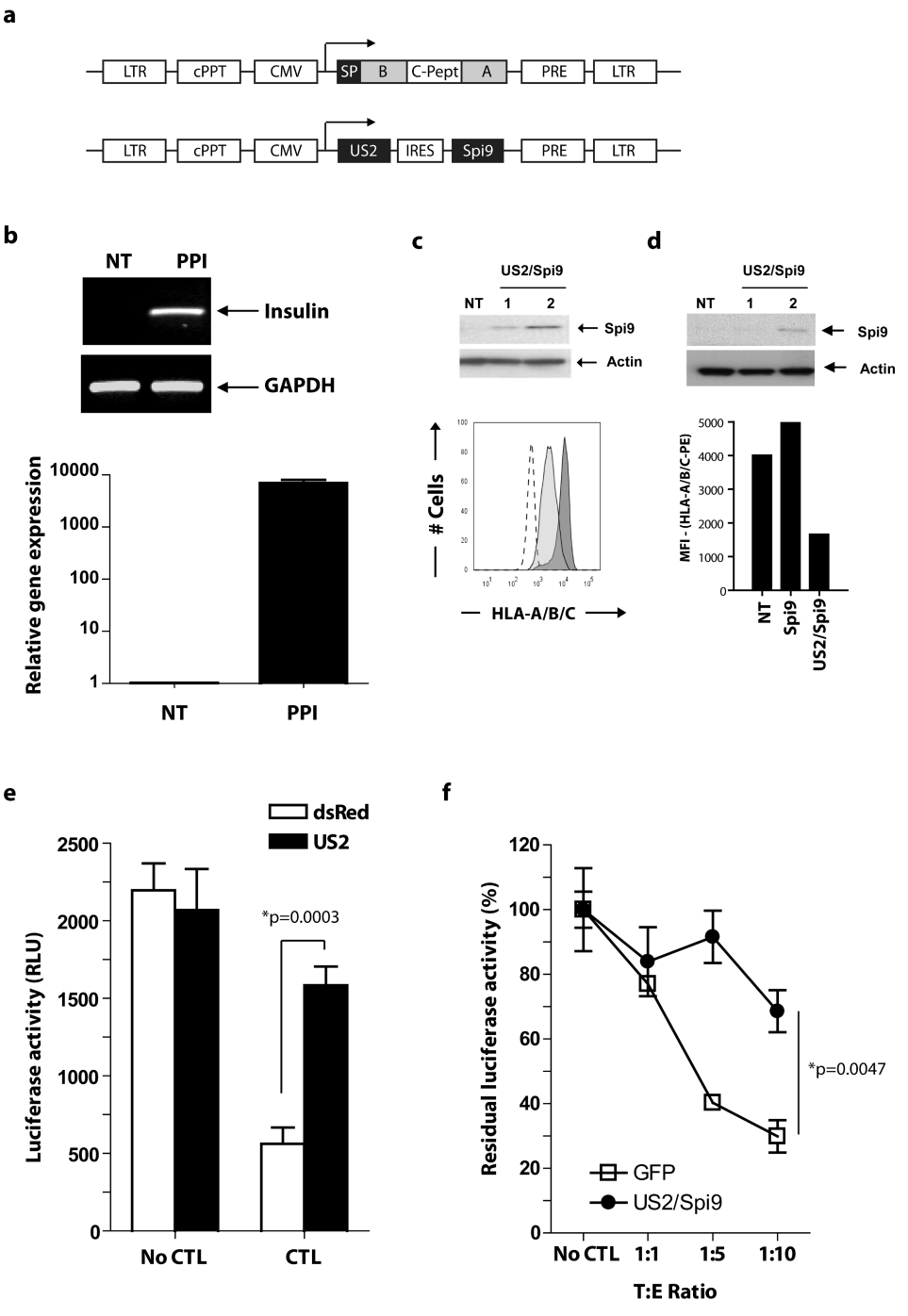


Figure 4 | Autoreactive HLA-A2-specific PPI-directed CTL clones kill HLA-A2 human islet cells *in vitro*.
a) Luciferase killing assay performed on 60% purity (triangle) or 90% pure beta-cell preparations (square) HIP-Luc2CP HLA-A2 human islet cells using PPI-directed CTLs with increasing Target:Effector ratios. Results are shown as residual luciferase activity 48h post coculture. **b)** Luciferase killing assay performed on HLA mismatched (HLA-A3; open square) or matched (HLA-A2; triangle) LV-HIP-Luc2CP-transduced human islet cells using PPI-directed CTLs (with increasing Target:Effector ratios). **c)** Luciferase killing assay performed on HLA-A2 LV-HIP-Luc2CP transduced human islet cells using increasing Target:Effector ratios of pp65-CMV directed CTLs (open squares) or PPI-directed CTLs (triangles). Experiments have been performed in triplicates, normalized to luciferase activity without CTL (set to 100%) and were shown as means of triplicate measurements (± standard deviations). Matched killing assay have been performed and confirmed on HLA-A2 islet cells from 6 different donors (CTL: cytotoxic lymphocyte; PPI: peptire of preproinsulin).

cells at an MOI of 1 or 2, the expression of Serpin-9 was verified by Western blotting (Fig. 5c,upper panel). Similar results were obtained in primary human islets (Fig. 5d). Following co-transduction of surrogate beta-cells with CMV-Luc2CP and CMV-DsRed or CMV-US2 lentiviruses, a killing assay was performed with CTLs against PPI. After 48h of co-culture, massive cell death of DsRed-expressing cells was observed. HLA-A/B/C downregulation mediated by US2 led to protection of the surrogate beta-cells from PPI-specific CTL killing (Fig. 5e). Importantly, US2/Serpin-9 co-expression in primary human islets had no effect on insulin release upon glucose stimulation (supplementary figure S2a) and protected human beta-cells from autoreactive $CD8^+$ T-cells as seen by residual luciferase activity after co-culture when compared to GFP-modified islets (Fig. 5f).

Primary human beta-cells can be efficiently immuno-protected from PPI-directed CTL killing *in vivo*.

As an *in vivo* proof of concept, equal amounts of GFP or US2/Serpin-9 modified pseudoislets (~3000) and PPI-directed CTLs (E:T ratio 1:100) were transplanted under the kidney capsule of NSG mice and human insulin and c-peptide were monitored following intraperitoneal glucose tolerance tests (Fig. 6a,b). In agreement with the *in vitro* results, human insulin or C-peptide secretion by GFP pseudoislets was low following co-transplantation with autoreactive CTL. US2/Spi9 expression had no effect on islets functionality (Supplementary figure S2b) and US2/Serpin-9 expressing cells maintained both insulin (and c-peptide) secretion, to a level similar to the one measured in absence of autoreactive T-cells (Figure 2a and 2b), indicating that US2/Serpin-9 expression does not impact on islet viability *in vivo* and protects beta-cells from autoimmune CTL attack.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*p=0.0003
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Residual luciferase activity (%)
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80
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40
20
0
*p=0.0047
GFP
US2/Spi9
No CTL
1:1
1:5
1:10
T:E Ratio

< Figure 5 | Combined US2/Serpin9 expression protects surrogate beta-cells from PPI-directed CTL killing.
a) Schematic representation of the lentivirus constructs used: LV-CMV-PPI and LV-CMV-US2-bc-Spi9. **b)** Reverse-transcription PCR (upper panel) and reverse-transcription qPCR (lower panel) validating insulin-gene expression in LV-CMV-PPI transduced A2/K562 cells. The GAPDH housekeeping gene was used as internal control. **c)** Serpin-9 expression assessed by western blot using Spi9 antibody following LV-CMV-US2-bc-Spi9 transduction (MOI=1 or MOI=2; non transduced cells –NT- were used as control) of HEK 293T cells (upper panel) and HLA-ABC expression in US2/Spi9 A2/K562 cells assessed by FACS analysis (lower panel). Unstained A2/K562 cells (dashed-line) and HLA-A/B/C stained K562 (dark grey area) were used as controls, HLA-A/B/C surface expression of US2/Spi9 modified A2/K562 cells is shown in light grey area. **d)** Serpin-9 expression and US2 inhibitory effect on HLA-ABC level in human pseudoislets. Following LV-CMV-US2-bc-Spi9 transduction of primary human islet cells (MOI=1 or MOI=2; non transduced cells –NT- were used as control), Serpin-9 expression was assessed by western blot (upper panel). Effect of US2 on HLA-ABC expression in islet cells was determined, following US2/Spi9 transduction MOI=2, by median fluorescent intensity of the HLA-ABC-PE antibody used and compared to non transduced islets (NT) cells or serpin-9 transduced islets (Spi-9) (lower panel). **e)** Luciferase killing assay performed on DsRed LV-CMV-Luc2CP A2/K562 (white bars) or US2/Spi9 LV-CMV-luc2CP-transduced A2/K562 (black bars) using PPI-directed CTLs (Target:Effector 1:5). **f)** Luciferase killing assay performed on LV-HIP-Luc2CP transduced HLA-A2 human islet cells modified by DsRed (opened square) or US2/Spi9 (closed circle) using PPI-directed CTLs. Experiments have been performed in triplicates, normalized to luciferase activity without CTL (set to 100%). Data were shown as means of triplicate measurements (± standard deviations). P value has been calculated by Unpaired Student's t test relative to non protected control.

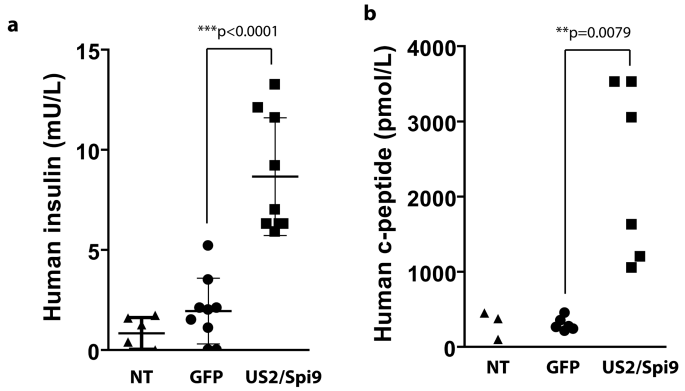

Figure 6 | Immune protection of human islet cells.
a, b) Intraperitoneal glucose-tolerance test performed on mice co-transplanted with 5.10^6 LV-GFP modified pseudoislets (n=3) or LV-US2/Spi9 modified islets (n=3) and PPI-directed CTLs (Target:Effector 100:1). n represents the number of transplanted mice each performed with a different donor. **(a)** Human insulin release values and **(b)** human c-peptide concentration after IPGTT. Three tolerance tests have been performed per mice in the first 3 weeks post transplantation (4, 11 and 19 days). Data are represented as scatter plot showing every glucose tolerance test results (a total of 9 values from bleeding at day 4, 11 and 19 for insulin and a total of 6 values from bleeding at day 11 and 19 for C-peptide). Non-transplanted mice (NT) were used as negative control (n=2). ** is considered to be statistically significant, *** is considered to be extremely statistically significant.

DISCUSSION

Islet transplantation offers a promising approach for restoring endogenous insulin secretion in diabetic patients. However, recurrent islet autoimmunity has been shown to be a major hurdle thwarting the clinical efficacy of beta-cell replacement therapy. Here we propose an alternative with transplantation of genetically immunoprotected pseudo-islets. We demonstrate that third generation lentiviral vectors can be used as efficient gene carriers for protecting primary human beta-cells without affecting their function, thereby confirming earlier studies in intact human islets. [29] As described earlier for rodents, [30] the self-aggregation phenomenon of islet cells offers an attractive opportunity to protect these endocrine "micro-organs" to a large extent prior to transplantation. Although we did not study the precise composition of these pseudoislets in detail, but focused on beta-cells only, our data show that both treatments (viz. dispersion and transduction) are not accompanied by a significant loss in insulin production rate in response to glucose. These data are in line with a recent study aiming at the evaluation of immunoisolation method and showing that pseudo-islets of rat islet cells are superior to primary intact islets in term of survival and function. [31]

The luciferase-based survival assay presented here is an alternative to the currently used traditional tracer-release assays, which have their limitations for monitoring cell cultures (reviewed in [32]). This assay requires a limited number of cells (from 5.000 to 10.000 cells per sample) and the results obtained appeared to be relatively insensitive to variations in the purity of islet preparations. The use of other reporters and substrates (e.g. an amino luciferin derivative specific for caspases 3, 7, 8 or 9) are currently under investigation and may provide new insights into the molecular mechanisms involved in the killing process. [33,34] The assay relies on the specificity of the promoter: the use of a ubiquitously active promoter to drive luciferase-gene expression in assays to measure beta-cell survival was confounded by the increase in luciferase activity due to the expansion of other cell types (data not shown). [26]

Besides systemic immunosuppressive injection or immunomodulatory therapy using educated regulatory T-cells, [35] other strategies to protect or improve insulin secretion of the transplanted beta-cells, for example using encapsulated islets, are currently under investigation. However, the perfect material allowing selective permeability and reduced bioreactivity is not available yet (reviewed in [36]). Similarly to our approach, the direct genetic modification of islets prior to transplantation has also been explored in studies aiming at improving islet function by expression of heme oxygenase (HO-1), downregulation of protein tyrosine phosphatase using siRNA, or prevention of beta-cell death by overexpression of viral proteins (Epstein-Barr virus vIL10, baculovirus p35, and the adenovirus gp19 and RIDa/b). [37-40] However, studies illustrating the feasibility in human islets are rare but efficient thereby lentivirus-mediated expression of cFLIP in as little as 10% of the human islet cells was sufficient to protect these cells from IL-1β, TNF-α, and IFN-γ proinflammatory cytokines treatment. [41]

Herpesviruses have the capacity to establish a lifelong infection of their host, a phenomenon associated with the expression of proteins with potent immunosuppressive properties, eluding $CD8^+$ and $CD4^+$ T-cells, NK-cells and innate immunity. [42,43] Among the different immunevasins, US2 is extremely effective in targeting MHC-I molecules for destruction. Shortly after their synthesis, most of the newly generated MHC class I heavy chains are degraded. [24,44] US2 has been suggested to down-regulate all HLA-A and -G and most HLA-B alleles, whereas several HLA-B alleles and all HLA-C and -E alleles are likely to be insensitive to US2-mediated degradation. Importantly, the fact that US2 does not down-regulate HLA-E may be useful in the context of immune protection, as HLA-E serves as an inhibitor of NK-cells.

We confirmed that US2 expression induces downregulation of class-I in surrogate beta-cells and that its co-expression with serine protease inhibitor 9 protects human islet cells against autoreactive T-cells. The possible risk of increased susceptibility to natural killer (NK) cells associated with MHC-I downregulation could be counteracted with Serpin-9 co-expression by blocking granzyme B activity. [45] The significance of Serpin-9 in protecting beta-cells against PPI autoreactive T-cells has been recently reinforced by a study showing that cytotoxic degranulation is the predominant *modus operandi* of PPI-CD8+ mediated killing. [46]

Environmental factors including proinflammatory cytokines might influence CTL killing capacity and beta-cell survival during transplantation. The clinical transplantation setting where islets are directly infused via the portal vein might complicate the problem. [15] Participation of proinflammatory cytokines, instant blood mediated immune response (IBMIR) and activated complement or beta-cell stress on autoreactive CTL killing capacity and on beta-cell antigenic peptide generation remains to be investigated.

The use of NSG mice allowed us to monitor the human islet survival in mild hyperglycemic environment (basal glucose level is about 1mM higher in mouse than human) to specifically evaluate autoreactive CTL activity without interference from innate immunity and NK activity. [47] The killing assay using human pseudoislets and a human autoreactive T-cell clone isolated from recent onset T1D patient, although not relevant for a long term study of the graft, provides a suitable model to determine the fate of human islet cells in the context of human CD8 autoreactivity. The results prove that these cellular aggregates remained functional and glucose-responsive, indicating that they were capable of resisting autoimmune T-cells.

The lentiviral system used to deliver the protective transgenes is believed to provide a long term expression of US2/Spi9. However cessation of transgene expression, due to promoter silencing, is still possible, and could lead to eradication of the modified cells by immune system. The cytomegalovirus promoter, used here, is very active in finally differentiated cells, like beta-cells but as many viral promoters its expression could be silenced. [48] When translated to the clinic, other promoter, in particular cellular promoters, should be envisaged (Ubiquitin C promoter for example) and included in a specific comparative study.

To summarize, the novelty of our report is three-fold. First we engineered a novel assay to assess specific, autoimmune-mediated destruction of primary human beta-cells, allowing for the definition of selective beta-cell loss *in vitro*. This approach facilitates the creation of a screening platform for identification of new compounds that inhibit the interplay between beta-cells and autoreactive T-cells. Secondly, we designed a preclinical humanized mouse model to allow assessment of the fate of primary human beta-cells in an autoimmune environment. Finally, we showed that lentiviral vectors represent an efficient system for gene transfer into human diploid islet cells that can be subsequently reaggregated into functional pseudoislets. This offers new possibilities for genetic modification as a means of protecting human islet cells against the effect of autoreactive and possibly alloreactive T cells. By targeting two molecular pathways (viz. MHC class I synthesis and the perforin cell death pathway), we could increase the resilience of human beta-cells to acute, antigen-specific autoimmune attack. This study constitutes a proof of concept in mild hyperglycemic condition. The questions of the protective effect in extreme hyperglycemic environment where the increase in insulin production (and by definition the increase of antigenic leader peptide epitope) could exacerbate CTL killing, the scaling up of gene transfer techniques under clinically applicable procedures, the stability of transgene expression, the efficacy of downregulation of different HLA haplotypes and the risk of tumor development remain to be addressed before any translational research. Yet, we can speculate that this type of approach could be successfully extended to protect against any auto- and alloreactivity. A combine approach using other highly potent viral immune evasion proteins could be envisaged to specifically target the HLA type or to increase the level of protection. [21] The next Holy Grail will be the translation of these evasion strategies without the use of viral carrier nor prior disruption of the islet integrity.

MATERIALS AND METHODS

Cells

HEK293T cells were maintained in DMEM + 10% FCS supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml); K562-A2 cells were maintained in IMDM + 10% FCS supplemented with 0,7 mg/ml geneticin (Gibco BRL, Gaithersburg, MD), penicillin (100 units/ml), streptomycin (100 µg/ml). PPI-specific CD8 T cells were cloned previously from a HLA-A201 Type 1 diabetic patient as described previously. [28] For maintenance and expansion, PPI-specific and pp65CMV-directed CTLs were cultured for two weeks with irradiated allogeneic PBMCs, irradiated PPI_{15-24} or CMV_{pp65} peptide-pulsed HLA-A2-expressing EBV-LCL in IMDM supplemented with 10% human serum, 0.5% LeucoA, 0.1 ng/ml recombinant human (rh)-IL12, 10 ng/ml rh-IL7, 25 U/ml rh-IL2 and 5 ng/ml rh-IL15. Cells were frozen in a solution of 20% human pooled serum and 10% DMSO and kept in liquid nitrogen until use. Upon thawing, cells were allowed to rest in IMDM supplemented with 10% human serum, IL-2 (50U/ml) and IL-15 (0.1ng/ml).

DNA constructs

pLV-CMV-US2-bc-Spi9 has been generated from 2 intermediate cloning vectors pLV-CMV-US2-bc-GFP and pLV-CMV-Spi9-bc-GFP. pLV-CMV-US2-bc-GFP has been generated by cloning the US2-bc-GFP cassette from the LZRS-US2-bc-GFP described previously [49] into the pLV-CMV vector. A pJET-Spi9 vector has been generated by cloning a Spi9 PCR fragment into pJET2.1/blunt vector (Fermentas) using the following primers Spi9 Fw5'-TACATAAGGTTACACTAT-3' and Spi9 Rv 5'-AACCCTTGTGTTAAGTAA-3'on human placental cDNA (Agilent technologies). pLV-CMV-Spi9 has been generated by introducing a NotI/XbaI Spi9 containing fragment from pJET-Spi9 into pLV-CMV-bc-GFP. Next, the GFP fragment from pLV-CMV-US2-bc-GFP was removed after NsiI/MscI digestion and blunting reaction, and was replaced by a SmaI/SmaI Spi9 containing fragment from pLV-CMV-Spi9-bc-GFP to generate the final pLV-CMV-US2-bc-Spi9 vector.

The HIP derived constructs have been generated by cloning the human insulin promoter -326/+30 by PCR from human genomic DNA using the following primers FW: 5'-GCGCTCGAGTCTCCTGGTCTAATGTGGAA-3' and Rv: 5'-GCGAAGCTTCTCTTCTGATGCAGCCTGTC-3'. The PCR product containing the XhoI/HindIII linker was then used to replace the CMV promoter in pLV-CMV-GFP to create pLV-HIP-GFP. pLV-HIP-Luc2CP was generated by cloning the HIP-Luc2CP containing fragment from pGL4-HIP-Luc2CP into lentivirus backbone.

Lentiviruses and cell transduction

All vectors are derived from pRRL-cPPT-CMV-IRES-GFP-PRE. Third generation self-inactivating lentivirus vectors were produced as described previously [50]. Lentivirus vectors are quantified by antigen capture ELISA measuring HIV p24 levels (ZeptoMetrix Corporation, New-York, USA). The infectious titer is derived from the p24 concentration by the conversion that 1ng p24 corresponds to 2500 infectious particles. Whole islets or freshly dispersed islets were seeded in ultralow attachment 6-well plates and maintained in DMEM + 10% FCS + Pen/Strep. Viral supernatants were added to fresh medium supplemented with 8μg/mL polybrene (Sigma), and the cells were incubated overnight. Transduction efficiency was determined by FACS analysis after washing in PBS containing 0.1% BSA and analyzed on a FACS LSRII (BD Pharmingen Inc, San Diego, CA, USA).

Luciferase killing assay

Transduced dispersed islet cells were seeded in U-shape 96-well plates and incubated for 48h with HLA-A2 specific CTLs (directed against pp65CMV- or PPI) in IMDM + 10% human serum supplemented with IL-2 (50U/ml) and IL-15 (0.1ng/ml). After 2 days of co-culture, cells were lysed in Luciferase Lysis buffer (25 mM Tris–HCl, pH 7.8, 2 mM CDTA, 2 mM DTT, 10% glycerol, 1% Triton X-100). Luciferase activity was determined by luminometry using the Promega Luciferase Assay Reagent. Experiments were performed in triplicate for every target:effector ratio used. Results shown are represented as residual luciferase activity with the luciferase activity of transduced islets incubated without CTL set at 100%.

Western blot

Cells were treated with Luciferase Lysis buffer supplemented with a cocktail of protease inhibitors (Roche). Samples for Western-blot analyses were prepared by boiling protein extracts with Sample buffer (10% glycerol, 2% SDS, 60 mM Tris-Cl (pH 6.7), 2.5% -mercaptoethanol, and 2.5% bromophenol blue from a 1:20-diluted saturated solution) for 5 minutes at 100°C, and analyzed on SDS-PAGE. Proteins were transferred to Immobilon-P (Immobilon-P transfer membrane (polyvinylidene difluoride); Millipore, Etten-Leur, The Netherlands) and visualized by standard protocols with anti-insulin (1:1000, SantaCruz sc14664), and anti-Actin (1:1000 clone C4; ICN Biomedicals, Inc., Zoetermeer, The Netherlands).

Reverse-transcription PCR and real-time PCR

Total cellular RNA was extracted from K562 cells using Trizol. 500 ng RNA was reverse transcribed using Superscript RT II kit (Invitrogen, Karlsruhe, Germany). Expression of the genes of interest was detected using the following primers: Insulin Fw 5′-GCAGCCTTTGTGAACCAACA-3′, Insulin Rv 5′-CGGGTCTTGGGTGTGTAGAAG-3′; GAPDH Fw 5′-ACAGTCAGCCGCATCTTCTT-3′, GAPDH Rv 5′-AATGAAGGGGTCATTGATGG-3′. Real-time PCR was performed in triplicate using the SybrGreen master mix kit (Applied Biosystems) and an Applied Biosystems Step One Plus machine. GAPDH mRNA was used as reference.

Human islet isolation

Human pancreata were harvested from brain-dead organ donors after informed consent was obtained in writing from family members. Leiden University Medical Centre had permission to isolate islets and to use them for scientific research if they are insufficient for clinical islet transplantation, in accordance with national laws and institutional ethical requirements. Human islet isolations were performed in the GMP-facility of the Leiden University Medical Center. Purity of the final islet preparation was assessed by 1 mM Dithizone (Sigma-Aldrich) staining and ranged from 75 and 95%. The purified islets were cultured in CMRL-1066 supplemented with 10% human serum, ciprofloxacin 20 μg/mL, gentamycine 50 μg/mL, L-glutamine 2 nM, fungizone 250 ng/mL, HEPES 10 mM, and nicotinamide 10 mM, and cultured at 37°C in a humidified atmosphere of 5% CO2 for 1–7 days prior to use.

Pseudoislets formation

Dissociation of primary human islets was performed by 5-10 minutes 0.05% trypsin treatment at 37°C. After washing step in DMEM 10% FCS (Invitrogen, Karlsruhe, Germany) containing media, dissociated cells were passed though a 40μm filter and seeded in ultra-low attachment 6-well plates at ~200.000 cells/ml (Corning, NY 14831). Spontaneous re-aggregation of islet cells started to be observed 24h post dissociation. Pseudoislets were maintained 6 days in culture prior reimplantation and medium was refreshed every 2 days.

Mice

6-12 week old male NOD SCID IL-2R-/- mice (NSG) were obtained from the Jackson Laboratory, Europe, and bred in the animal facilities of the LUMC. Animals were

housed under specific pathogen-free conditions. All studies were approved by the local Animal Care Committee.

Human islets/pseudoislets transplantation

3000 islet equivalents and 5 x 10^6 dispersed and reaggregated islet cells, either non-transduced or GFP–transduced, were transplanted under the left kidney capsule, using a polyethylene catheter and precision syringe (Hamilton, Reno, NV). Grafts were removed 19 days post transplantation. Formalin- fixed paraffin-embedded sections were stained for insulin and DNA content. Insulin and DNA staining, and GFP epifluorescence were assessed by confocal microscopy. Evaluation of PPI-specific CTL killing *in vivo* was performed by co-transplantation of 5 x 10^6 reaggregated cells, either GFP or US2/Spi9 transduced, with HLA-A2 PPI-directed cytotoxic T-cells at a 100:1 target:effector ratio. Islets isolated from 3 different donors have been genetically modified by GFP or US2/Spi9 lentiviral vectors and transplanted under the kidney capsule of 3 different NSG mice. Islet functionality was evaluated after IPGTT by human insulin and c-peptide measurement.

Immunostaining and confocal microscopy

Intact islets or pseudoislets were fixed in PBS containing 4% paraformaldehyde. Permeabilization was performed using PBS/1% Triton. Blocking was done with PBS/BSA 3%, and first and secondary antibodies were diluted in PBS/BSA 3%. Anti-Insulin (H86 - Santa Cruz Biotechnology, Santa Cruz, Calif., USA) was used at 1:200 and secondary antibody coupled to Alexa 568 was used at a dilution of 1:500. Nuclei were stained with 4'-6-diamidino-2-phenylindole (DAPI). Samples were subjected to optical sectioning using a laser scanning confocal microscope (Zeiss).

Glucose-induced insulin-secretion test

Intact islets or pseudoislets were washed in PBS and transferred to a 8µm pore size transwell plate (Corning® HTS Transwell®-96 well, Sigma-Aldrich) and pre-incubated in a modified Krebs-Ringer Bicarbonate buffer (KRBH) containing 115mM NaCl, 5mM KCl, 24mM NaHCO3, 2.2mM CaCl2, 1mM MgCl2, 20mM HEPES, 2 g/l human serum albumin (Cealb, Sanquin, The Netherlands), pH 7.4 for 2 hours. The transwell plate was then successively transferred for 1 hour to KRBH with 2 mM and 20 mM glucose at 37°C. Insulin concentrations were determined in the supernatants by ELISA (Mercodia, Uppsala, Sweden).

Intraperitoneal glucose tolerance test

For the Intraperitoneal Glucose Tolerance Tests mice were fasted overnight and injected with 2 g/kg Glucose 1 M i.p. Blood was drawn from the tail vein at t=0, t=30 and t=45 min. IPGTT was validated by determination of blood glucose concentrations measured using a glucose meter (Accu Chek, Roche, Basel, Switzerland). Human insulin content and human C-peptide concentrations were determined by ELISA according to the manufacturer's instructions. (Human insulin ELISA 10-113-10 and human C-peptide ELISA 10-1136-01, Mercodia, Uppsala, Sweden).

Statistical analysis

All data were presented as mean ± standard deviation (SD). For Human Insulin and human C-peptide values presented in Figure 6, data were subjected to nonparametric statistical analysis using two-way ANOVA test with Bonferroni correction using GraphPad Prism software. A value of $p < 0.05$ was considered statistically significant.

Acknowledgements

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No potential conflicts of interest relevant to this article were reported.

SUPPLEMENTARY MATERIAL

CTL killing assay

Cytotoxicity was analyzed by a classical chromium release assay. Briefly, following incubation with 100 µl Na-chromate (^{51}Cr, 3.7 MBq) for 1 h, K562-PPI-A2, K562-A2 cell lines were seeded in triplicate at various effector-to-target (E:T) together with CTLs. After 4 h incubation at 37°C in 5% CO_2, supernatants were collected, and the release of ^{51}Cr was measured with a gamma-counter (Wallac/PerkinElmer, Waltham, MA, USA). Spontaneous and maximum releases were obtained by incubation with medium and 1% triton in PBS, respectively. The specific lysis was calculated by the following formula: percentage of specific lysis = 100 × (experimental release − spontaneous release) / (maximum release − spontaneous release).

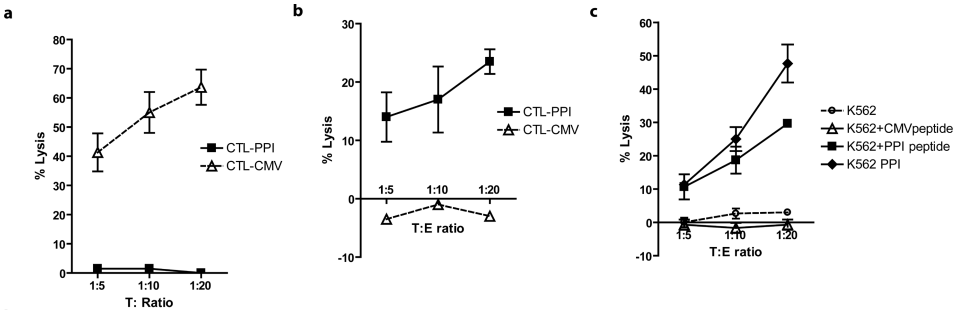

Figure S1 | CTLs killing capacity.

a) Chromium-release assay performed on A2/K562 cells preincubated with 10ng CMV peptide and incubated with CMV-directed CTLs or PPI-directed CTLs. **b)** Similar experiment as seen in a) performed on A2/K562 preincubated with 10 ng PPI purified peptide. **c)** Chromium-release assay performed using PPI directed CTL on A2/K562-A2 or A2/K562 with 10ng PPI peptide or on PPI overexpressing A2/K562 cells.

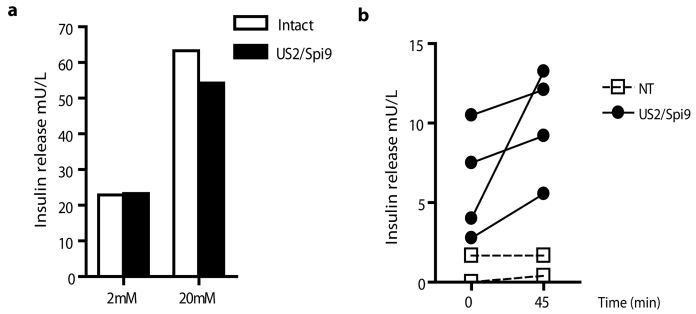

Figure S2 | Effect of US2/Spi9 on islets functionality.

a) Comparative insulin release of intact islets (white bars) and US2/Spi9-modified islets (black bars) determined by glucose-stimulated insulin-secretion test following incubation in 2mM or 20mM glucose. **b)** IPGTT performed on mice co-transplanted with 5.10^6 LV-GFP (n=1) or LV-US2/Spi9 modified pseudoislets (n=2). Non transplanted mice (NT) are used as control (n=1). Blood samples were taken at 4, and 11days post transplantation before the intraperitoneal glucose ionjection (t=0) and 45min after glucose injection (t=45min)

REFERENCES

1. In't Veld P, Lievens D, De Grijse J et al. Screening for insulitis in adult autoantibody-positive organ donors. Diabetes 2007;56:2400-2404.
2. Cnop M, Welsh N, Jonas JC et al. Mechanisms of pancreatic beta-cell death in type 1 and type 2 diabetes: many differences, few similarities. Diabetes 2005;54 Suppl 2:S97-107
3. Coppieters KT, Dotta F, Amirian N et al. Demonstration of islet-autoreactive CD8 T cells in insulitic lesions from recent onset and long-term type 1 diabetes patients. J Exp Med 2012;209:51-60
4. Katz J, Benoist C, Mathis D. Major histocompatibility complex class I molecules are required for the development of insulitis in non-obese diabetic mice. Eur J Immunol 1993;23:3358-3360
5. Serreze DV, Leiter EH, Christianson GJ et al. Major histocompatibility complex class I-deficient NOD-B2null mice are diabetes and insulitis resistant. Diabetes 1994;43:505-509
6. Wicker LS, Leiter EH, Todd JA et al. Beta 2-microglobulin-deficient NOD mice do not develop insulitis or diabetes. Diabetes 1994;43:500-504
7. Hamilton-Williams EE, Palmer SE, Charlton B et al. Beta cell MHC class I is a late requirement for diabetes. Proc Natl Acad Sci U S A 2003;100:6688-6693
8. Kay TW, Parker JL, Stephens LA et al. RIP-beta 2-microglobulin transgene expression restores insulitis, but not diabetes, in beta 2-microglobulin null nonobese diabetic mice. J Immunol 1992;157:3688-369.
9. Chervonsky AV, Wang Y, Wong FS et al. The role of Fas in autoimmune diabetes. Cell 1997;89:17-24
10. Apostolou I, Hao Z, Rajewsky K et al. Effective destruction of Fas-deficient insulin-producing beta cells in type 1 diabetes. J Exp Med 2003;198:1103-1106
11. Kagi D, Odermatt B, Ohashi PS et al. Development of insulitis without diabetes in transgenic mice lacking perforin-dependent cytotoxicity. J Exp Med 1996;183:2143-2152
12. Thomas HE, Trapani JA, Kay TW. The role of perforin and granzymes in diabetes. Cell Death Differ 2010;17:577-585
13. Roep BO, Atkinson M. Animal models have little to teach us about type 1 diabetes: 1. In support of this proposal. Diabetologia 2004;47:1650-1656
14. Roep BO, Atkinson M, Von Herrath M. Satisfaction (not) guaranteed: re-evaluating the use of animal models of type 1 diabetes. Nat Rev Immunol 2004;4:989-997
15. Harlan DM, Kenyon NS, Korsgren O et al. Current advances and travails in islet transplantation. Diabetes 2009;58:2175-2184
16. Pinkse GG, Tysma OH, Bergen CA, et al. Autoreactive CD8 T cells associated with beta cell destruction in type 1 diabetes. Proc Natl Acad Sci U S A 2005;102:18425-18430

17. Huurman VA, Hilbrands R, Pinkse GG, et al. Cellular islet autoimmunity associates with clinical outcome of islet cell transplantation. PLoS One 2008;3:e2435

18. Huurman VA, Velthuis JH, Hilbrands R, et al. Allograft-specific cytokine profiles associate with clinical outcome after islet cell transplantation. Am J Transplant 2009;9: 382-388

19. Hilbrands R, Huurman VA, Gillard P et al. Differences in baseline lymphocyte counts and autoreactivity are associated with differences in outcome of islet cell transplantation in type 1 diabetic patients. Diabetes 2009;58:2267-2276

20. Blondet JJ, Carlson AM, Kobayashi T et al. The role of total pancreatectomy and islet autotransplantation for chronic pancreatitis. Surg Clin North Am 2007;87:1477-1501

21. Griffin BD, Verweij MC Wiertz EJ. Herpesviruses and immunity: the art of evasion. Vet Microbiol 2010;143:89-100

22. Horst D, Verweij MC, Davison AJ et al. Viral evasion of T cell immunity: ancient mechanisms offering new applications. Curr Opin Immunol 2011;23:96-103

23. Zaldumbide A, Hoeben RC. How not to be seen: immune-evasion strategies in gene therapy. Gene Ther 2008;15:239-246

24. Wiertz EJ, Tortorella D, Bogyo M et al. Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. Nature 1996;384:432-438

25. Medema JP, De Jong J, Peltenburg LT et al. Blockade of the granzyme B/perforin pathway through overexpression of the serine protease inhibitor PI-9/SPI-6 constitutes a mechanism for immune escape by tumors. Proc Natl Acad Sci U S A 2001;9811515-11520

26. Carlotti F, Zaldumbide,A, Loomans CJ, et al. Isolated human islets contain a distinct population of mesenchymal stem cells. Islets 2010;2:164-173

27. Bishop AE, Polak JM. The Anatomy, Organisation and Ultrastructure of the Islets of Langerhans. (Blackwell Science,Oxford,UK, 1997)

28. Skowera A, Ellis RJ, Varela-Calvino R, et al. CTLs are targeted to kill beta cells in patients with type 1 diabetes through recognition of a glucose-regulated preproinsulin epitope. J Clin Invest 2008;118: 3390-3402

29. Kobinger GP, Deng S, Louboutin JP et al. Transduction of human islets with pseudotyped lentiviral vectors. Hum Gene Ther 2004;15: 211-219

30. Callewaert H, Gysemans C, Cardozo AK et al. Cell loss during pseudoislet formation hampers profound improvements in islet lentiviral transduction efficacy for transplantation purposes. Cell Transplant 2007;16: 527-537

31. O'Sullivan ES, Johnson AS, Omer A et al. Rat islet cell aggregates are superior to islets for transplantation in microcapsules. Diabetologia 2010;53: 937-945

32. Jerome KR, Sloan DD, Aubert, M. Measurement of CTL-induced cytotoxicity: the caspase 3 assay. Apoptosis 2003:8:563-571

33. O'Brien MA, Daily WJ, Hesselberth PE, et al. Homogeneous, bioluminescent protease assays: caspase-3 as a model. J Biomol Screen 2005;10:137-148

34. Kanno A, Umezawa Y, Ozawa, T. Detection of apoptosis using cyclic luciferase in living mammals. Methods Mol Biol 2009;574:105-114

35. Kleijwegt FS, Laban S, Duinkerken G et al. Transfer of regulatory properties from tolerogenic to proinflammatory dendritic cells via induced autoreactive regulatory T cells. J Immunol 2011;187:6357-6364

36. O'Sullivan ES, Vegas A, Anderson DG et al. Islets transplanted in immunoisolation devices: a review of the progress and the challenges that remain. Endocr Rev 2011;32: 827-844.

37. Carter JD, Ellett JD, Chen M et al. Viral IL-10-mediated immune regulation in pancreatic islet transplantation. Mol Ther 2005;12:360-368

38. Hollander K, Bar-Chen M, Efrat S. Baculovirus p35 increases pancreatic beta-cell resistance to apoptosis. Biochem Biophys Res Commun 2005;332:550-556

39. Kojaoghlanian T, Joseph A, Follenzi A et al. Lentivectors encoding immunosuppressive proteins genetically engineer pancreatic beta-cells to correct diabetes in allogeneic mice. Gene Ther 2009;16:340-348

40. Lu B, Wu H, Gu P, et al. Improved glucose-stimulated insulin secretion by intra-islet inhibition of protein-tyrosine phosphatase 1B expression in rats fed a high-fat diet. J Endocrinol Invest 2012;35:63-70

41. Fenjves ES, Ochoa MS, Cechin S et al. Protection of human pancreatic islets using a lentiviral vector expressing two genes: cFLIP and GFP. Cell Transplant 2008;17:793-802

42. Wiertz E, Hill A, Tortorella D et al. Cytomegaloviruses use multiple mechanisms to elude the host immune response. Immunol Lett 1997;57:213-216

43. Wiertz EJ, Mukherjee S, Ploegh HL. Viruses use stealth technology to escape from the host immune system. Mol Med Today 1997;3:116-123

44. Barel MT, Pizzato N, Le Bouteiller P et al. Subtle sequence variation among MHC class I locus products greatly influences sensitivity to HCMV US2- and US11-mediated degradation. Int Immunol 2006;18:173-182

45. Falk CS, Mach M, Schendel DJ et al. NK cell activity during human cytomegalovirus infection is dominated by US2-11-mediated HLA class I down-regulation. J Immunol 2002;169:3257-3266

46. Knight RR, Kronenberg D, Zhao M et al. Human beta-Cell Killing by Autoreactive Preproinsulin-Specific CD8 T Cells Is Predominantly Granule-Mediated With the Potency Dependent Upon T-Cell Receptor Avidity. Diabetes 2012;62:205-213

47. Shultz LD, Ishikawa F, Greiner DL. Humanized mice in translational biomedical research. Nat Rev Immunol 20007;7:118-130

48. Brooks AR, Harkins RN, Wang P, et al. Transcriptional silencing is associated with extensive methylation of the CMV promoter following adenoviral gene delivery to muscle. J Gene Med 2004;6:395-404

49. De La Garza-Rodea AS, Verweij MC, Boersma H et al. Exploitation of herpesvirus immune evasion strategies to modify the immunogenicity of human mesenchymal stem cell transplants. PLoS One 2011;6:e14493

50. Carlotti F, Bazuine M, Kekarainen T et al. Lentiviral vectors efficiently transduce quiescent mature 3T3-L1 adipocytes. Mol Ther 2004;9:209-217

6

Preservation of recall immunity in anti-CD3 treated recent onset type 1 diabetes patients.

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ABSTRACT

Background
The safety of any immune modulating agent in type 1 Diabetes Mellitus (T1DM) involves its selectivity on auto-immunity and its preservation of recall and tumour immunity.

Methods
We performed lymphocyte proliferation tests on seven newly diabetic patients treated with anti-CD3 (Otelixizumab; ChAglyCD3) and five newly diabetic patients treated with placebo, on average two years after therapy.

Results
Proliferative responses towards common viral, bacterial and yeast antigens upon *in vitro* stimulation with a range of recall antigens in anti-CD3 treated T1DM patients were highly similar to those in placebo-treated T1DM patients. Likewise, T-cell responses towards auto-antigens were equally low between the two groups, several years after diagnosis of T1DM. The proliferative response upon stimulation with the human suppressor protein p53 was invariably high in both anti-CD3 and placebo treated patients, implying preserved anti-tumour immunity in anti-CD3 treatment.

Conclusions
As long-term focus on side-effects is key, we demonstrate in this sub-cohort of recent onset T1DM patients treated with Otelixizumab that recall immunity is preserved in spite of high-dose anti-CD3 treatment, adding to the safety of anti-CD3 treatment as an immune-modulatory agent in the treatment of T1DM.

INTRODUCTION

Type 1 Diabetes Mellitus is a chronic and progressive autoimmune disease in which auto-reactive CD4+ and CD8+ T-cells attack insulin-producing beta-cells in the pancreatic islets. Targeted immune therapies have shown encouraging results in the treatment of T1DM. Preclinical studies in non obese diabetic (NOD) mice have demonstrated reversal of hyperglycaemia upon anti-CD3 treatment [1]. In clinical studies initial toxicity problems with anti-CD3 treatment, due to non-specific FcR binding, were overcome by creating humanized non-FcR binding anti-CD3 antibodies. Phase 2 clinical trials with Otelixizumab [2,3] and Teplizumab [4] have shown to preserve beta cell function for at least 18-24 months, decreasing insulin requirements and normalizing glycated haemoglobin levels in patients with recent onset T1DM.

In the successful Phase II Otelixizumab trial achieving long term preserved beta-cell function, the antibody dosage was considerably higher then that elected for the Phase III DEFEND1 study, which did not reach its primary endpoint of preserved beta-cell function. One of the reasons for dose reduction was transient Epstein Barr Virus (EBV) reactivation in the high Otelixizumab study, seen in 75% of the treated patients [2,5]. EBV copies peaked between 3 and 4 weeks after treatment with Otelixizumab and returned to pretreatement levels within a few weeks as an efficient humoral and cellular immune response specific to EBV had developed, comparable with the response observed in otherwise healthy individuals following EBV infection [2,5]. Over the 48 month follow-up period, no biological or clinical signs of EBV reactivation or EBV-related disease were observed [3]; there was no higher incidence of infections, and no lymphoma or other types of cancer. No differences in CD3+ lymphocyte counts were found between months 6 and 48.

Another safety concern in the use of immune modulating agents is how well recall immunity (the immune reaction towards pathogens patients have prior been exposed to) to other pathogens than EBV is preserved. No follow-up data on recall immunity after treatment with anti-CD3 has been available.

Previously, we reported auto- and recall immunity in patients who successfully underwent kidney-pancreas transplantation and were induced with either anti-thymocyte globulin (ATG) or Daclizumab, a monoclonal antibody against the interleukin-2 receptor (CD25) [6]. T cell autoimmunity towards islet antigens was low in both groups. Yet, immune memory responses towards bacterial, viral and tumour antigens were significantly lower in the ATG -treated group when compared to Daclizumab-treated patients, implying Daclizumab has a selective effect on auto-immunity but preserves desired recall responses.

An additional concern in the treatment of T1DM is the recurrence of autoimmunity. Monti *et al.* [7] proposed homeostatic expansion of autoreactive T-cells in T1DM patients receiving islet allografts under anti-IL-2 receptor mAb induction therapy, followed by low dose tacrolimus and rapamycin maintenance therapy. Homeostatic expansion of

autoreactive T-cells could lead to exacerbation of auto-immunity and precipitation of disease. The mechanism behind the success of anti-CD3 treatment in recent onset T1DM patients is largely unknown. Short-lived antigenic modulation, apoptosis and anergy are found immediately after treatment [9]. There is compelling evidence that the lasting effect is achieved through the induction of regulatory CD4+ and CD8+ T cells [8,9]. In vitro studies on prediabetic glutamic acid decarboxylase (GAD) 65-antigen specific autoreactive T-cells revealed that both Daclizumab and Otelixizumab operate in a non-depleting fashion, by proliferation inhibition and antigen modulation, hence not by creating a niche for autoreactive T-cells [10].

MATERIALS AND METHODS

We performed lymphocyte proliferation tests on average two years after therapy on seven newly diabetic patients treated with anti-CD3] (Otelixizumab; ChAglyCD3) and five newly diabetic patients treated with placebo, as previously described [2,6]. These patients represent a sub-cohort of the European phase II placebo-controlled trial, in which a cumulative dose of 48 mg Otelixizumab was administered intravenously over 6 days [2].

In short, peripheral blood mononuclear cells (PBMC) were isolated from freshly drawn heparinized blood. 150.000 PBMC were cultured in tissue-coated, round bottomed 96-well plates (Costar, Cambridge, MA, USA) in Iscove's modified Dulbecco's medium with 2 mmol/l glutamine (GIBCO, Paisley, Scotland, UK) supplemented with 10% human type AB pool serum in the presence of antigen, recombinant IL-2 or medium alone in 150 ul at 37°C, 5% CO2. After 5 days, RPMI-1640 (Dutch Modification; GIBCO) containing 0.5 uCi [^{3}H]-thymidine per well was added, and incubation was continued for 16 hours. Cultures were harvested on glass-fibre filters and [^{3}H]-thymidine incorporation was measured by liquid scintillation counting. The stimuli used were insulin (Sigma), recombinant human proinsulin and islet autoantigen IA-$2_{602\text{-}979}$, GAD65 (Diamyd Medicals, Stockholm, Sweden), tumour suppressor protein p53, recombinant IL-2, tetanus toxoid (1.5 Limes flocculationes/ml or 12.0 international units/ml) (National Institute of Public Health and Environmental Protection, the Netherlands), Candida, purified protein derivate (PPD) (tuberculin) and *Haemophilus influenzae* matrix protein M1. The results are expressed as stimulation indexes (SI); CPM in the presence of stimulus divided by CPM with medium alone.

RESULTS

Upon *in vitro* stimulation with different recall antigens, anti-CD3 treated T1DM patients had proliferative responses towards common viral, bacterial and yeast antigens that were highly comparable to those in placebo-treated T1DM patients (Fig.1). In addition, several years after diagnose, T-cell responses towards auto-antigens did not differ significantly between these two groups.

The proliferative response upon stimulation with the human suppressor protein p53 was invariably high in both the anti-CD3 and the placebo treated group. These responses underline preserved anti-tumour immunity in anti-CD3 treatment. This observation is in line with the 48 month clinical follow-up where no lymphoma or other types of cancer were observed [3].

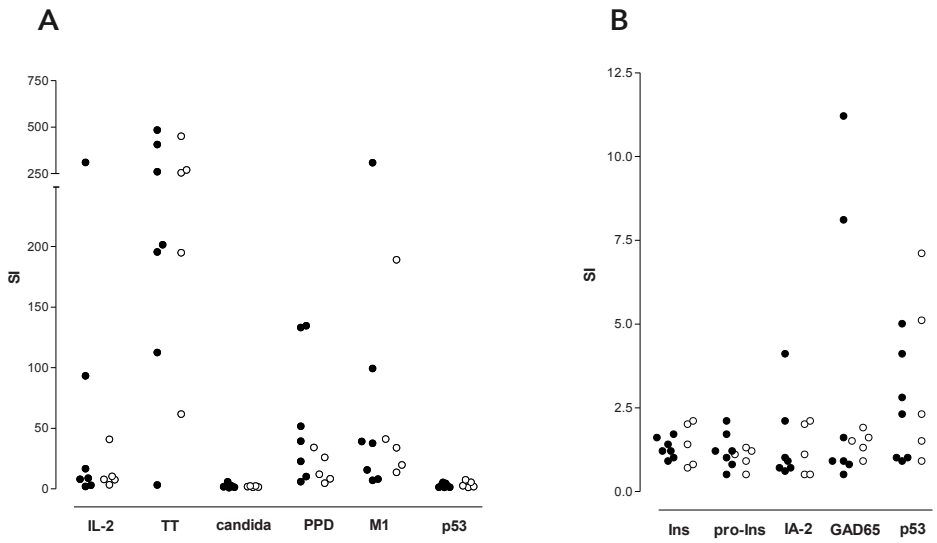

Figure 1 | Memory responses towards recall antigens (A) and auto-antigens (B).
Open circles for placebo (n=5), closed circles for anti-CD3 treatment (n=7). Lymphocyte proliferation tests, expressed as stimulation indices of median responses in triplicate experiments. Average background for placebo treated and anti-CD3 treated patients was 333±80 and 450±194 cpm, respectively.

DISCUSSION

Over the last decade the use of anti-CD3 in the treatment of T1DM has moved from bench to bedside. Two separate phase II trials with anti-CD3 mono-therapy have shown prolonged preservation of beta cell function with decreased insulin requirements. While we continue to focus on long-term side-effects, we demonstrate in this sub-cohort of recent onset T1DM patients treated with Otelixizumab that in spite of high-dose anti-CD3 treatment recall immunity is preserved. This follow-up data adds to the safety of anti-CD3 treatment as an immune-modulatory agent in the treatment of T1DM.

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DUALITY OF INTEREST

Drs. Keymeulen and Mathieu participated in a GSK funded phase 1 study with subcutaneous Otelixizumab and a phase 3 study with intravenous Otelixizumab (Defend 2). Dr. Roep was member of the scientific advisory board of TolerX from October 2010 till April 2011. Dr. Waldmann is co-founder of TolerX.

REFERENCES

1. Chatenoud L, Thervet E, Primo J et al. Anti-CD3 antibody induces long-term remission of overt autoimmunity in nonobese diabetic mice. Proc Natl Acad Sci USA 1994; 91:123-127
2. Keymeulen B, Vandemeulebroucke E, Ziegler AG et al. Insulin needs after CD3-antibody therapy in new-onset type 1 diabetes. N Engl J Med 2005; 352:2598-2608
3. Keymeulen B, Walter M, Mathieu C et al. Four-year metabolic outcome of a randomized controlled CD3-antibody trial in recent-onset type 1 diabetic patients depends on their age and baseline residual beta cell mass. Diabetologia 2010; 53:614-623
4. Herold KC, Hagopian W, Auger JA et al. Anti-CD3 monoclonal antibody in new-onset type 1 diabetes mellitus. N Engl J Med 2002; 346:1692-1698
5. Keymeulen B, Candon S, Fafi-Kremer S et al. Transient Epstein-Barr virus reactivation in CD3 monoclonal antibody-treated patients. Blood 2010; 115: 1145-1155
6. Van de Linde P, Van der Boog PJM, Tysma OMH et al. Selective unresponsiveness to beta cell autoantigens after induction immunosuppression in pancreas transplantation with anti-interleukin-2 receptor antibody versus anti-thymocyte globulin. Clin Exp Immunol 2007; 149:56-62
7. Monti P, Scirpoli M, Maffi P et al. Islet transplantation in patients with autoimmune diabetes induces homeostatic cytokines that expand autoreactive memory T cells. J Clin Invest 2008; 118:1806-1814
8. Chatenoud L, Bluestone JA.CD3-specific antibodies: a portal to the treatment of autoimmunity. Nat Rev Immunol 2007; 7:622-632
9. Bisikirska B, Colgan J, Luban J et al. TCR stimulation with modified anti-CD3 mAb expands CD8+ T cell population and induces CD8+CD25+ Tregs. J Clin Invest 2005; 115:2904-2913
10. Van de Linde P, Tysma OMH, Medema JP et al. Mechanisms of antibody immunotherapy on clonal islet reactive T cells. Human Immunol 2006; 67:264-273

7

General discussion and conclusions

Type 1 diabetes mellitus results from a T-cell mediated autoimmune destruction of insulin-producing beta cells in genetically predisposed individuals [1]. Currently there is no cure; treatment consists of frequent or continuous insulin administration to mimic beta-cell function. Despite intensive insulin regimes, T1DM still contributes to substantial morbidity and mortality.

Beta-cell destruction

Clinical autopsy studies of recent onset T1DM patients compellingly indicate that beta-cell destruction involves antigen-specific infiltration of autoreactive CD8+ T-cells into insulitic pancreas lesions [2,3,4]. However, studies in a number of infection and autoimmune disease models have suggested additional (bystander) T-cell recruitment in a *non*-antigen specific manner, for instance via cytokines and chemokines [5,6,7]. Presence of bystander T-cells in inflamed tissue could theoretically influence the course of the disease as cytokine production or T-cell receptor engagement as a result of cross-reactivity may provide additional activation signals [5]. And even if these bystander T-cells do not influence the immune pathogenesis of T1DM, the question whether or not T-cell recruitment is solely antigen-specific or antigen-driven stands out, since such knowledge could have possible implications for future therapeutic strategies. Despite the fact that in vitro-activated bystander T-cell clones can transiently co-migrate with their antigen-specific counterparts and that tissue specific expression of cytokine and/or chemokine transgenes in tissue van trigger bystander T-cell inflammation, at least in animal models where the islet infiltrate is often enormous, previous models do not faithfully mimic the events that take place in spontaneous autoimmune inflammation [8]. Specifically it is unclear whether bystander T-cells can effectively compete with their antigen-specific counterparts in occupying inflammatory space.

In ***Chapter 2*** we describe the generation of a genetically engineered NOD strain expressing a T-cell invisible $IGRP_{206-214}$ epitope in beta-cells. These mice developed insulitis and autoimmune diabetes with the same incidence and kinetics as wildtype NOD mice and displayed indistinguishable thymic and splenic profiles. As opposed to wildtype NOD mice however, the islet-associated T-cells of these pre-diabetic mice did not contain $IGRP_{206-214}$-reactive CD8+ cell, as determined by NRP-V7/K^d tetramer staining, nor did these T-cells produce IFNγ in response to NRP-V7 peptide-pulsed APCs. We did find a significant increase in recruitment of other autoreactive T cell specificities, such as insulin-B_{15-23}-reactive CD8+ cells, that were present at very low precursor frequencies in their wildtype counterparts. Additional adoptive transfer experiments revealed that activation and proliferation of naive $IGRP_{206-214}$-reactive CD8+ cell in pancreatic lymph nodes as well as recruitment to the inflamed islets of pre-diabetic NOD mice expressing the T-cell invisible $IGRP_{206-214}$ epitope was severely impaired. Furthermore, pre-activated $IGRP_{206-214}$ specific cytotoxic T-lymphocytes failed to home to the insulitic lesions of these gene-targeted NOD mice.

These data indicate that $IGRP_{206-214}$-reactive CD8+ T-cells are excluded from insulitic

lesions in the absence of local expression of $IGRP_{206-214,}$ suggesting that T-cell occupation to the inflamed islet space in spontaneous autoimmune diabetes is not due to 'diffusion' from the periphery in response to inflammatory and chemotactic cues, but rather to an active process that involves local recognition of cognate pMHC. Furthermore, these data indicate that initiation and progression of spontaneous T1DM in NOD mice does not require the accumulation of $IGRP_{206-214}$-reactive CD8+ T-cells into pancreatic islets. Our observations challenge the generally held assumption that T cell infiltrates in inflamed extra lymphoid tissues, such as pancreatic islets in diabetes, contain a mixture of both cognate and non-cognate (bystander) T cells. Our findings do not argue against the idea that bystander T-cells can transiently migrate to a site of inflammation non-specifically, but rather strongly argue that in the absence of non-cognate pMHC, non-specifically recruited T-cells cannot effectively compete with cognate T cell specificities for occupation of space.

How does this translate clinically? We demonstrate that antigen presence is important in cytotoxic T-cell recruitment and this finding could be translated into strategies that interfere with T-cell recruitment. Our model shows that removing a major diabetogenic epitope in NOD mice, such as IGRP, will not suffice in preventing diabetes nor will it influence the course of the disease. In our T-cell invisible $IGRP_{206-214}$ epitope model, this was illustrated by the significant increase in recruitment of other autoreactive T-cell specificities such as insulin-B_{15-23} reactive CD8+ T-cells. However, inducing tolerance to antigens via the induction of regulatory T-cells may be a possibility to interfere with T-cell recruitment [9]. Furthermore the importance of antigen presence underscores the importance of memory. Memory T-cells are primed to life-long recognize a specific antigen and have the ability to fuel destruction of cells carrying this antigen at any moment. Strategies that interfere with memory in particular or with (re)migration to pancreatic islets therefore are worthwhile exploring too.

The development of an antigen-specific therapy, selectively targeting pathogenic autoreactive T cells, is viewed by many as the best chance to restore immunological self-tolerance [10]. In NOD mice antigen restoring tolerance involves the generation and expansion of antigen-specific regulatory T-cells. In T1DM patients the aim of antigen-specific therapy is to regulate a single islet antigen which will subsequently regulate ongoing autoimmune responses against other islet antigens, via linked suppression [10].

Antigen-specific prevention studies with oral administration of insulin and injection of islet antigens/ peptides, as well as intervention studies in established T1DM patients, have shown limited, and not always consistent results [10]. However, collectively these studies have been reassuring in terms of safety. Specifically, administrating the autoimmune target does not fuel the course of the disease, as was feared [11]. In a recent published antigen-specific intervention study, T1DM patients within 5 years of their T1DM diagnose received a DNA vaccine (BHT-3021), encoding proinsulin [12]. In preclinical studies, this DNA vaccine was capable of preventing and reversing active insulitis in hyperglycemic

NOD mice. Upon weekly intramuscular injections, C-peptide levels improved at 15 weeks with the 1 mg dose, an effect that waned after discontinuation of DNA vaccination in patients. A reduction in antigen-specific CD8+ T-cells targeting proinsulin only was found, suggesting this gene therapy indeed affects CD8+ T-cells selectively under some conditions in some patients. Clinical relevance of this pilot study remains to be confirmed.

The development of antigen-specific therapies faces many challenges, such as patient selection, timing of intervention and optimizing dosing and administration strategies, yet there is a clear rationale for this type of intervention [10,11]. Our findings that T-cell recruitment to inflammatory sites in T1DM is antigen-specific, could act as argument in favour of further pursuing antigen-specific therapies, suggesting that inhibition of islet entry/retention may have a more significant therapeutic benefit that previously appreciated [13].

Beta-cell regeneration

Several therapies haven been able to temporarily preserve beta-cell function in newly onset diabetic patients and to actually restore normoglycemia in NOD mice [14,15,16,17]. However, the mechanism behind restoration of the functional beta-cell mass, either through replication of pre-existing beta-cells or through neogenesis from progenitor cells or differentiated non-beta-cells, remains unclear and highly controversial [18,19,20,21]. Functional recovery of pre-existing beta-cells by immunological remission and glycemic control is another mechanism that could significantly contribute to restoration of function beta-cell mass, comparable to the temporarily insulin-free honeymoon state in newly insulin-treated T1DM patients. After partial or near-total destruction of the mouse endocrine pancreas in a non-auto-immune environment, regeneration of the beta-cells is seen as to different extends. Nir *et al.* showed beta-cell replication as the main source of new beta-cells after 70-80% chemical beta-cell ablation by diphtheria toxin in a cell lineage tracing model [18]. Their labelling percentage was 30%, requiring statistical assumptions in results interpretation. Xu et al showed that endogenous beta-cell progenitors can be activated in the mouse pancreas after partial duct ligation [19]. Near-total chemical ablation by diphtheria toxin in an alternative cell lineage tracing model however revealed evidence for alpha-cell dedifferentiation [20]. Differences in outcome in these studies may be related to differences in experimental models, labelling percentages and in particular differences concerning the percentage of beta-cell destruction as regenerative stimuli used in different models may be insufficient to trigger a neogenesis pathway. Furthermore, none of these studies were performed in mice developing autoimmune diabetes or having insulitis, hence the role of both ongoing inflammation, autoimmunity and restored tolerance in this process is unknown.

In ***Chapter 3*** we describe the development of cell lineage tracing models in mice that spontaneously develop autoimmune diabetes. The individual transgenes that were introduced to facilitate Red Fluorescent Protein expression, showed not to interfere with diabetes susceptibility. The RFP labelling of pre-existing beta cells in our two different models (NOD.RIP-tTA/tet07-Cre.ROSA-tdRFP and NOD.RIPCreER.ROSA-tdRFP) showed

to be bright, cell type specific and achieved high labelling percentages (93.0% ± 1.3; 94.5% ± 1.3). Expression of RFP in pre-existing alpha cells in NOD.GluCre.ROSA-tdRFP mice was somewhat lower (58.0% ± 6.0).

Reliable cell lineage tracing models might help to distinguish between replication, functional recovery or neogenesis of beta-cells as predominant mechanism behind restoration of the functional beta-cell mass. Bright inheritable labelling of pre-existing islet cells in mice that spontaneously develop an autoimmune form of diabetes and that are successfully treated with immune therapy could help to unravel regeneration mechanisms, especially when combined with Bromodeoxyuridine (BrdU) labelling. This thymidine analogue can be incorporated in the newly-synthesized DNA of replicating cells. Upon successful immune-intervention in either NOD.RIP-tTA/tet07-Cre.ROSA-tdRFP or Tamoxifen-treated NOD.RIPCreER.ROSA-tdRFP mice, finding RFP+/ insulin+/ BrdU+ cells would suggest replicating pre-existing beta-cells as the predominant insulin producing source. RFP+/ insulin+/ BrdU- cells on the contrary would imply recovery of pre-existing beta-cells whereas RFP-/ insulin+ cells would point in the direction of a non-beta-cell source. In addition, finding RFP+/ insulin+ cells in NOD.GluCre.ROSA-tdRFP mice after successful immune intervention would suggest alpha-cells as the predominant source of new beta-cells, whereas RFP-/insulin+ cells would make this unlikely. Our current cell lineage tracing models only enable us to trace beta- and alpha-cells, if non-beta, non-alpha-cells are suggested as predominant source of new beta-cells, additional studies would be required.

We successfully generated cell lineage tracing NOD models with bright and specific labelling of a high percentage of their pre-existing beta- or alpha-cells. As spontaneous development of an autoimmune form of diabetes in these mice was not affected, these models could be used to address the origin of insulin producing cells after immune-intervention in preclinical studies. Furthermore these models could help revealing the role of ongoing autoimmunity on beta-cell regeneration.

Although our model adds a novel preclinical tool in diabetes beta-cell regeneration research, a caveat in clinically translating rodent study findings are the possible differences in regenerative pathways and regeneration capacity between species. For instance, an increase in beta-cell duplication has been consistently observed in rodent pregnancies, a strong physiological stimulus for postnatal beta-cell growth [22]. Although similar findings have been described in older autopsy studies of pregnant women [23], a more recent study suggested formation of new islets as the main source of beta-cell mass increase during pregnancy, as opposed to beta-cell duplication. This was based on the higher number of small islets and single beta-cells that were not associated with islets in pancreata of pregnant women compared to non-pregnant women [24]. A recent study in pregnant NOD mice however, using our NOD.RIP-tTA/tet07-Cre.ROSA-tdRFP cell lineage tracing model, suggests that duplication of pre-existing beta-cells is not the sole source of new beta-cells during pregnancy after all,

as the percentage of labeled beta-cells dropped from 97% prior to pregnancy to 87% at mid-pregnancy, an argument in favour of beta-cell replication and neogenesis being not mutually exclusive [25,26].

Evidence for beta-cell neogenesis in diabetes in humans is scarce and outcomes vary. Remaining beta-cells have been histologically demonstrated in individuals suffering from T1DM, even for as long as 50 years. This emphasizes the heterogeneous course of the disease and could also be an argument in favour of beta-cell regeneration, in whatever form [4, 27]. A prospective phase 1-2 study in recent onset T1DM patients undergoing autologous non-myeloablative hematopoetic stem cell transplantation showed prolonged insulin independency compared to the natural course of the disease [28]. The mechanism behind the increased beta-cell function allegedly involves recovery of pre-existing beta-cells, but this interpretation may not be sufficient to explain remission lasting more than seven years. A recent study in T2DM patients found double positive endocrine cells suggesting beta-cell neogenesis as a compensatory mechanism in newly diagnosed T2DM patients [29]. Although the classical pathophysiology of T2DM clearly differs from T1DM, some connexion in beta-cell inflammation with subsequent apoptosis of beta-cell between the two conditions is being found.

At the same time, no evidence for beta-cell regeneration was found in a study performed on human pancreatic tissue collected from 13 patients who underwent partial (50%) pancreatectomy. Differences in outcome may be related to differences in the percentage of beta-cell destruction, which might be insufficient to trigger a neogenesis pathway. In addition, chronic pancreatic inflammation was the underlying cause in the majority of the patients, which might influence beta-cell regeneration capacity. Another explanation however could be that regeneration capacity and regeneration pathways might differ between mice and men, emphasizing precaution in translation of results. Why use animal models to study beta-cell regeneration? First of all, there is limited accessibility to human pancreases during the course of the disease. And even if pancreas material is obtained, evidence of regeneration will be indirect, as opposed to cell lineage tracing studies. As cell lineage tracing cannot be performed in human beings, animal models have been elected. Furthermore, reliable biomarkers for regenerative pathways currently do not exist. Moreover, currently there is no cure for diabetes in humans and therefore it is unknown what happens to beta-cells when the immune attack is terminated. But despite similarities between mice and men in the development of autoimmune diabetes, these are by far outnumbered by differences. Awareness of model limitations and prudence in translation is therefore in order. However preclinical studies might give some clues and guidance as to what regenerative pathways may be considered.

Identifying the source of insulin producing cells after future successful immune intervention could have significant clinical implications. If pre-existing beta-cells are the only source of restored beta-cell mass, the residual beta-cell mass at the time of intervention is expected to predict the outcome of any immune intervention. However,

if other cells serve as beta-cell precursors, outcome should be independent of residual beta-cell mass [30]. Currently, different strategies are being undertaken to create insulin producing beta-cells from stem cells (either human embryonic stem cells or induced pluripotent stem cells) and from endocrine progenitors [31]. In addition to this, identifying regeneration pathways could eventually result in developing strategies capable of enhancing effectiveness of promising immune therapies.

Beta-cell replacement

Currently islet transplantation is an accepted therapy for patients with complete insulin deficiency, unstable glycemic control and repeated severe hypoglycemia despite optimal diabetes management and compliance [32,33]. Challenges in clinical islet transplantation remain manifold: there is scarcity of donor material, significant islet cell loss occurs during the transplantation procedure and current immune suppressive regimens have significant side effects, including intrinsic beta-cell toxicity [34]. In addition, there is a need to specifically address ongoing islet autoimmunity [35].

In ***Chapter 4*** we tested whether T-cell recruitment in an islet transplantation model is comparable to T-cell recruitment into endogenous islets. We monitored recruitment of CD8+ T-cells reactive to the $IGRP_{206-214}$ epitope into epitope competent- or epitope invisible grafts transplanted either in diabetic wildtype NOD mice (harboring both naive and memory epitope specific T-cells) or epitope invisible hosts (harboring only naive epitope specific T-cells). All four host-donor combinations had development of recurrent diabetes within two weeks, indicating that IGRP contributes to but is dispensable for graft destruction in diabetic epitope competent hosts. Wildtype hosts recruited epitope specific T-cells into epitope competent, but not epitope invisible grafts. In epitope invisible hosts, there was no recruitment of epitope specific T-cells, regardless of donor type.

The "non physiological" lymphatic and vascular anatomy of islet cells grafted under the kidney capsule could conceivably render these permeable to bystander T-cells. We demonstrate however that absence of an auto-antigen in syngeneic extra pancreatic islet grafts in diabetic hosts, renders the grafts 'invisible' to cognate memory (and naive) T-cells. Local auto-antigen expression is a requirement for accumulation of antigen-specific T-cells into islet grafts, comparable to T-cell recruitment to endogenous islets.

We furthermore specifically addressed the contribution of memory T-cell to islet graft failure. Tracking of naive splenic CFSE labelled epitope specific T-cells from (8.3) T-cell transgenic NOD mice in wildtype NOD hosts transplanted with epitope competent- or epitope invisible islets showed vigorous proliferation in the lymph nodes draining epitope competent grafts as opposed to the lymph nodes draining epitope invisible grafts. There were however very few donor 8.3-CD8+ T-cells in both epitope competent and epitope invisible grafts. Therefore we conclude that graft derived IGRP did activate naive epitope specific CD8+ T-cells, but graft destruction by memory T-cells invariably predated their recruitment. Our results indicate that recurrent diabetes in the absence of allo-immunity, is driven by auto reactive T-cells primed during the primary immune response.

Our syngeneic rodent islet transplantation model enables partial unravelling of the complex processes of T-cell recruitment and/ or accumulation in transplanted islets. Our findings that previously primed autoreactive T-cells drive recurrent autoimmunity underscores the importance of developing immune strategies to tackle autoreactive T-cell memory after beta-cell replacement therapy. Indeed, studies in clinical islet transplantation from our group had previously demonstrated that reactivation of memory islet autoreactive T-cells is a paramount hurdle to achieve or preserve insulin-independence in transplanted T1D patients, implying that current immune suppressive strategies remain insufficient to deal this autoimmune memory and point to the need of novel immune suppressive therapies targeting memory T-cells [36,37,38]. We contend that our preclinical model may be of service for validation studies here.

Interfering with memory T-cell recruitment by bio-protecting/ encapsulating transplanted islets, could be another possibility [39]. And perhaps, in parallel to the argument favouring the pursuit of antigen-specific therapies in (recent onset) T1DM, our findings that T-cell recruitment to graft sites in autoimmune diabetes is antigen-specific could lead to pursuing antigen-specific therapies as induction therapy in clinical islet transplantation on the long run. But we are not there yet.

The identification of immune markers as correlates for autoimmunity peri-islet transplantation has been a major step forward. The pre-transplant peripheral frequencies of autoreactive T-cells in diabetic islet recipients proved to be predictive of allograft fate: presence of autoreactive T-cells against one or more autoantigens before transplantation was associated with delayed insulin-independence and lower circulating C-peptide levels during the first year after transplantation. Also, post-transplant increases of auto-reactive T-cell frequencies were associated with loss of graft function, suggesting that recurrent autoimmunity plays a paramount role in the outcome of allograft islet transplantation. [36,37,38]. Furthermore, the amount of transplanted beta-cell mass combined with pre-transplant autoreactivity associates with clinical outcome [37]. This once more emphasizes the role of recurrent autoimmunity in islet transplantation and has possible implications for the selection and treatment of T1DM candidate islet recipients.

Transplantation of genetically immune protected islets could be one approach to improve clinical outcome, by avoiding reactivation of islet-antigen specific memory T-cells [40,41,42,43,44]. In ***Chapter 5*** we show that primary human islet cells can be efficiently transduced by lentiviral vectors. To enhance transduction efficiency, islets were dispersed. These 'pseudo-islets', formed by self-reaggregation, proved to be histological and functional comparable to wildtype islets, as confirmed by insulin secretion upon glucose stimulation. The protective effect of combined compromised immune recognition by down-regulation of MHC-I expression (antigen recognition) and inhibition of the cytotoxic granzyme pathway (beta-cell destruction) was demonstrated in surrogate beta-cells and human primary beta-cells by co-culturing these cells with

cytotoxic T-cells directed against an epitope located in the signal peptide of the pre-pro-insuline (PPI) molecule. These autoreactive T-cells are derived from a recent onset T1DM patient. Insulin release upon glucose stimulation was maintained by immune protected beta-cells as opposed to insulin release in non-protected beta-cells. As *in vivo* proof of concept, immune protected human islets were co-transplanted with patient-derived PPI-directed T-cells under the kidney capsule of mice lacking innate immunity and NK cell activity (NOD.SCID. IL-2R$^{-/-}$; NSG mice). Human insulin release and C-peptide levels were monitored following intra-peritoneal glucose-tolerance tests. In agreement with the *in vitro* results, immune protected cells maintained insulin secretion as opposed to non-protected controls, indicating that genetically engineered US2/Serpin 9 expression does not impact islet viability *in vivo*, but instead protects beta-cells from autoimmune T-cell attack.

Our *in vivo* strategy to measure beta-cell toxicity and protection from autoreactive T-cell mediated killing by inserting a luciferase reporter gene specifically in beta-cells proved successful. A killing assay using the autoreactive T-cells isolated from a recent onset T1DM patient directed against PPI was not affected by the quality of the islet isolate. Thus, we engineered a novel assay to assess specific, auto-immune mediated destruction of primary human beta-cells *in vitro*. This approach facilitates the creation of a screening platform for identification of new compounds that inhibit the interplay between beta-cells and autoreactive T-cells. More specifically, this screening platform could be used for the in vitro testing of both efficacy and toxicity of new immune interventions. Furthermore, we designed a preclinical humanized mouse model to allow assessment of the fate of primary human beta-cells in an autoimmune environment. And finally we showed that lentiviral vectors represent an efficient system for gene transfer into human islet cells that can be subsequently reaggregated into functional pseudo-islets. The latter offers new possibilities for genetic modifications to protect human islet cells against the effect of autoreactive and possibly allo-reactive T-cells. By targeting two molecular pathways (MHC class I synthesis and the perforin cell death pathway) we could reinforce human beta-cells to recurrent autoimmunity.

Immune evasion is a possible strategy worth exploring in improving outcome of clinical islet transplantation, be it still far from clinical application. First of all, allo-immunity has not yet been addressed in our study. Furthermore, technical difficulties such as the scaling up of gene transfer under clinically applicable procedures, the stability of transgene expression and the efficacy of down regulation of different HLA haplotypes as well as the risk of tumour development remain to be addressed before any translational research [45]. Yet, experimental clinical gene transfer studies, using lentiviral vectors are currently being pursued in other fields of medicine, delivering the first encouring clinical proof of principle [46,47].

In order to optimize clinical outcome, simultaneous optimizing various 'influenceables' in islet transplantation will be necessary. Among these are islet graft size, the choice of

the engraftment site, encapsulation of islets to protect from host inflammatory reactions while ensuring sufficient oxygen supply during the revascularization period and as mentioned above, choosing the most appropriate and specific immune suppression strategies [39]. Immune evasion could be a strategy to contribute to clinical outcome.

Aspects of immune-intervention

Targeted immune therapies, such as anti-CD3 therapy, have shown encouraging results in the treatment of T1DM, especially in subgroups [14,15,16]. A major safety concern in the use of any immune modulating agent in T1DM is the preservation of anti-tumour immunity and recall immunity (the immune reaction towards pathogens to which patients have been exposed). In the successful European Phase II Otelixizumab (humanized anti-CD3 antibody; ChAglyCD3) trial in recent onset T1DM patients the chosen antibody dosage was considerably higher than the dosage elected for the Phase III DEFEND1 study, which did not reach its primary endpoint of preserved beta-cell function [48]. The dosage had been reduced for safety reasons: during the Phase II Otelixizumab trial, 75% of the treated patients showed transient and self-limiting EBV reactivation [49]. Although the number of EBV copies returned to normal levels within 1-3 weeks in all patients, comparable with that observed in individuals following infectious mononucleosis in general, this finding emphasizes the importance of addressing recall immunity.

In ***Chapter 6*** we demonstrate in a sub cohort of the Phase II placebo-controlled trial with humanized anti-CD3 antibody in recent onset T1DM patients that recall immunity is preserved in spite of high-dose anti-CD3 treatment. Proliferative responses towards common pathogens upon in vitro stimulation with different recall antigens were preserved in anti-CD3 treated patients and were highly similar to those in placebo-treated T1DM patients. An additional concern in the treatment of T1DM is the recurrence of auto-immunity actually caused by immune intervention. Monti *et al* [50] proposed homeostatic expansion of auto reactive T cells in T1DM patients receiving islet allografts under anti-IL-2 receptor antibody induction therapy, followed by low dose tacrolimus and rapamycin maintenance therapy. Homeostatic expansion of auto reactive T-cells could lead to exacerbation of autoimmunity and precipitation of the disease. We showed that T-cell responses towards auto-antigens are not significantly altered after high dose anti-CD3 therapy, which means we did not find evidence for reduced or enhanced and fuelled autoimmunity. Furthermore, the proliferative response upon stimulation with the human suppressor protein p53 was invariably high in both the anti-CD3 and the placebo-treated patients underlining preserved desired anti-tumour immunity in spite of anti-CD3 treatment. This observation is in line with the 48 month clinical follow-up where no lymphoma or other malignancies were observed [15]. Although clinical end points were not met in subsequent Otelixizumab studies testing a much lower dose (Phase III DEFEND1 and 2 studies), it seems premature to disqualify anti-CD3 antibodies as potential therapy is recent onset T1DM. Currently a dose-finding Phase I/ Phase II study to investigate Otelixizumab in new-onset autoimmune T1DM patients is ongoing. We demonstrate in this subcohort of recent onset T1DM patients

treated with Otelixizumab, that recall immunity is preserved in spite of high-dose anti-CD3 treatment, adding to the safety of high dose anti-CD3 treatment as an immune modulatory agent in the treatment of T1DM.

Epicrise

As most PhD students, I started my journey as the 'quest for the Holy Grail' [51]. At the near finish of this thesis I wonder whether there is *one* Holy Grail in T1DM research. I contend that an immune intervention can be successful, but in order for a patient to become insulin independent, beta-cell destruction and restoration of beta-cell function need to be addressed simultaneously. Likewise, there will be no successful beta-cell replacement therapy without the necessary immune protection. Furthermore, there is a need to address safety concerns in the development of any immune therapy, while at the same time one has to be aware of the health risks of diabetes itself with current (often suboptimal) insulin treatment regimes. These quests all interconnect just as the projects in this thesis interconnect.

What do I have to offer to a patient with new onset Diabetes Mellitus Type I that was not there before I started this Ph.D.? I wish I could offer a cure, but unfortunately the diabetes field is not there yet, at least in the vast majority of cases. Instead, we talk about T1DM pathophysiology, insulin therapy and blood glucose self-management, and I explain that despite all efforts, unfortunately treatment targets are not met in the vast majority of patients, underscoring the need for alternative treatments. Next, we talk about changing technologies. We talk about the developing closed loop artificial pancreas, aiming at automatically controlling blood glucose levels by providing the substitute endocrine functionality of a healthy pancreas and the (un)safety aspects involved [52]. We talk about a new device developed to 'scan' your subcutaneous blood glucose levels and philosophize if this device will eventually replace the numerous burdensome fingerpricks currently needed [53]. We talk about the several times a year a cure for diabetes is being found in mice, and how no cure currently exists for most humans, hence how this information should be interpreted with prudence in the clinical situation. At the same time, I explain why we use animal models: to gain knowledge on autoimmune diabetes in general that we cannot obtain otherwise.

And perhaps at subsequent visits, we talk about the projects summarized in this thesis, their results and implications. I explain that we show that T-cell recruitment in both spontaneous autoimmune diabetes and islet transplantation requires presence of a cognate antigen, which could be used as an argument in favour of further pursuing antigen-specific therapies. I explain that we show that recurrent diabetes in an islet transplantation model is driven by memory auto reactive T-cells and that this latter finding has contributed to the present testing of immune suppressive drugs that indeed address recurrent autoimmunity, to improve outcome in clinical islet transplantation. I also explain the many current caveats of clinical islet transplantation and that this therapy is currently only available for a specific subset of patients. I explain that we designed

and tested a novel autoimmune diabetes line tracing model for future testing of the regenerative capacity of islet cells. Furthermore, I explain that we showed that immune evasion protects beta-cells from autoimmune T-cell attack in vivo. I explain that currently different immune evasion techniques, such as islet encapsulation are being tested in the clinic [54,55]. I explain that we show that recall immunity is preserved in spite of high dose anti-CD3 treatment, adding to the safety of high dose anti-CD3 treatment as an immune modulator agent in the treatment of T1DM. I also explain that studies testing a much lower dose of anti-CD3 were terminated early as these failed to meet the clinical endpoints and that we are awaiting the results of a dose finding anti-CD3 study. I explain that any intervention therapy most likely will have side-effects, but underscore that the same applies for plainly having T1DM considering the need for frequent insulin injections, the risk of hypo- and hyperglycemia and the almost certain development of micro- and macrovascular complications in time. If we want to cure diabetes, health risks are unfortunately involved and it is only fair to discuss this.

We talk about the combined Holy Grail in T1DM research: the necessity to halt or elude the immune attack in T1DM in a safe manner, while simultaneously restoring beta cell function. We talk about the fact that we are not there yet, but that the diabetes research field is ever developing in its search for a cure. And as an old Chinese proverb states: "*be not afraid of going slow, be afraid only of standing still*".

Gonnie Alkemade

REFERENCES

1. Roep BO. The role of T-cells in the pathogenesis of Type 1 diabetes: from cause to cure. Diabetologia 2003;46:305-321
2. Bottazzo GF, Dean BM, McNally JM et al. In situ characterization of autoimmune phenomena and expression of HLA molecules in the pancreas in diabetic insulitis. N Engl J Med 1985;313;353-360
3. Itoh N, Hanafusa T, Miyazaki A et al. Mononuclear cell infiltration and its relation to the expression of major histocompatibility complex antigens and adhesion molecules in pancreas biopsy specimens from newly diagnosed insulin-dependent diabetes mellitus patients. J Clin Invest 1993;92:2313-2322
4. Coppetiers M, Dotta F, Amirian N et al. Demonstration of islet-autoreactive CD8 T cells in insulitic lesions from recent onset and long-term type 1 diabetes patients. J Exp Med 2012;209:51-60
5. Chen AM, Khanna N, Stohlman SA et al. Virus-specific and bystander CD8 T- cells recruited during virus-induced encephalomyelitis. J Virol 2005;79:4700-4708
6. Peterson JD, Berg R, Piganelli JD. Analysis of leukocytes recruited to the pancreas by diabetic T cell clones. Cell Imunol 1998;189:92-98
7. Ostler T, Pircher H, Ehl S. "Bystander recruitment of systemic memory T cells delays the immune response to respiratory virus infection. Eur J Immunol 2003;33:1839-1848
8. Rhode A, Pauza ME, Barral A et al. Islet-specific expression of CXCL10 causes spontaneous islet infiltration and accelerates diabetes development. J Immunol 2005;175:3516-3524
9. Bluestone JZ. Regulatory T-cell therapy: is it ready for the clinic? Nat Rev Immunol 2005;5:343-349
10. Von Herrath M, Peakman M, Roep B. Progress in immune-based therapies for type 1 diabetes. Clin Exp Immunol. 2013;172:186-202
11. Abreu JRF, Roep BO. Targeting proinsulin-reactive CD8+ T cells: a new direction for Type 1 diabetes treatment. Expert Rev Clin Immunol 2013;9:1001-1004
12. Roep BO, Solvason N, Gottlieb PA et al. Plasmid-encoded proinsulin preserves C-peptide while specifically reducing proinsulin-specific CD8(+) T cells in type 1 diabetes. Sci Transl Med 2013;191:1-9
13. Lennon GP, Bettini M, Burton AR et al. T cell islet accumulation in type 1 diabetes is a tightly regulated, cell-autonomous event. Immunity 2009;31:643-53
14. Keymeulen B, Vandemeulebroucke E, Ziegler AG et al. Insulin needs after CD3-antibody therapy in new-onset type 1 diabetes. N Engl J Med 2005; 352:2598-2608
15. Keymeulen B, Walter M, Mathieu C et al. Four-year metabolic outcome of a randomized controlled CD3-antibody trial in recent-onset type 1 diabetic patients depends on their age and baseline residual beta cell mass. Diabetologia 2010; 53:614-623

16. Herold KC, Hagopian W, Auger JA et al. Anti-CD3 monoclonal antibody in new-onset type 1 diabetes mellitus. N Engl J Med 2002; 346:1692-1698

17. Chatenoud L, Thervet E, Primo J et al. Anti-CD3 antibody induces long-term remission of overt autoimmunity in nonobese diabetic mice. Proc Natl Acad Sci USA 1994;91:123-127

18. Nir T, Melton DA, Dor Y. Recovery from diabetes in mice by beta cell regeneration. J Clin Invest 2007;117:2553-2561

19. Xu X, D'Hoker J, Stangé G et al. Beta cells can be generated from endogenous progenitors in injured adult mouse pancreas. Cell 2008;132:197-207

20. Thorel F, Népote V, Avril I et al. Conversion of adult pancreatic alpha-cells to beta-cells after extreme beta-cell loss. Nature 2010;464:1149-1154.

21. Granger A, Kushner JA. Cellular origins of beta-cell regeneration: a legacy view of historical controversies. J Intern Med 2009;266:325–338

22. Bouwens L, Rooman I. Regulation of pancreatic beta-cell mass. Physiol Rev. 2005;85:1255-1270

23. Van Assche FA, Aerts L, De Prins F. A morphological study of the endocrine pancreas in human pregnancy. Br J Obstet Gynaecol. 1978;85:818-820

24. Butler AE, Cao-Minh L, Galasso R et al. Adaptive changes in pancreatic beta cell fractional area and beta cell turnover in human pregnancy. Diabetologia. 2010;53:2167-2176

25. Toselli C, Hyslop CM, Hughes M et al. Contribution of a non-β-cell source to β-cell mass during pregnancy. PLoS One. 2014;9:e100398

26. Bonner-Weir S, Li W, Ouziel-Yahalom L et al. Beta-cell growth and regeneration: replication is only part of the story. Diabetes 2010;59:2340-2348

27. Foulis AK, Liddle CN, Farquharson MA et al. The histopathology of the pancreas in type 1 (insulin-dependent) diabetes mellitus: a 25-year review of deaths in patients under 20 years of age in the United Kingdom. Diabetologia 1986;29:267-274

28. Couri CE, Oliveira MC, Stracieri AB et al. C-peptide levels and insulin independence following autologous nonmyeloablative hematopoietic stem cell transplantation in newly diagnosed type 1 diabetes mellitus. JAMA 2009;301:1573-1579

29. Yoneda S, Uno S, Iwahashi H et al. Predominance of β-cell neogenesis rather than replication in humans with an impaired glucose tolerance and newly diagnosed diabetes. J Clin Endocrinol Metab 2013;98:2053-2061

30. Ablamunits V, Sherry NA, Kushner JA et al. Autoimmunity and beta cell regeneration in mouse and human type 1 diabetes: the peace is not enough. Ann N Y Acad Sci 2007;1103:19-32

31. Borowiak M, Melton DA. How to make beta cells. Curr Opinion Cell Biollogy 2009;21:727-732

32. Ludwig B, Ludwig S, Steffen A et al. Islet versus pancreas transplantation in type 1 diabetes: Competitive or complementary? Curr Diab Rep 2010; 10:506-511

33. Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, Kneteman NM, Rajotte RV. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl Med 2000;343:230-238

34. Robertson RP. Islet transplantation as a treatment for diabetes- a work in progress. N Engl J Med 2004;350:694-705

35. Abreu JRF, Roep BO. Immune monitoring of islet and pancreas transplant recipients. Curr Diab Rep 2013;13:704-712

36. Roep BO, Stobbe I, Duinkerken G, Van Rood JJ, Lernmark A, Keymeulen B, Pipeleers D, Claas FH, De Vries RR. Auto- and allo-immune reactivity to human islet allografts transplanted into type 1 diabetic patients. Diabetes 1999;48:484-490

37. Huurman VA, Hilbrands R, Pinkse GG et al. Cellular islet autoimmunity associates with clinical outcome of islet cell transplantation. PLoS One 2008;3: e2435

38. Hilbrands R, Huurman VA, Gillard P et al. Differences in baseline lymphocytes counts and autoreactivity are associated with differences in outcome of islet cell transplantation in type 1 diabetic patients. Diabetes 2009;58:2267-2276

39. Veriter S, Gianello P, Dufrane D. Bioengineered sites for islet cell transplantation. Curr Diab Rep 2013;13:745-755

40. Katz J, Benoist C, Mathis D. Major histocompatibility complex class I molecules are required for the development of insulitis and non-obese diabetes mice. Eur J Immunol 1993;23:3358-3360

41. Serreze DC, Leiter EH, Christianson GJ et al. Major histocompatibility complex class I-deficient NOD-B2Mnull mice are diabetes and insulitis resistant. Diabetes 1994;43:505-509

42. Wicker LS, Leiter EH, Todd JA et al. Beta 2-microglobulin-deficient NOD mice do not develop insulitis or diabetes. Diabetes 1994;43:500-504

43. Kagi D, Odermatt B, Ohashi PS et al. Development of insulitis without diabetes in transgenic mice lacking perforin-dependent cytotoxicity. J Exp Med 1996;183:2143-2152

44. Thomas HE, Trapani JA, Kay TW. The role of perforin and granzymes in diabetes. Cell Death Differ 2010;17:577-585

45. Zaldumbide A, Hoeben RC. How not to be seen: immune-evasion strategies in gene therapy Gene Ther 2008;15:239-246

46. Biffi A, Montini E, Lorioli L et al. Lentiviral hematopoietic stem cell gene therapy benefits metachromatic leukodystrophy. Science 2013;341:doi 10.1126/1233158

47. Cartier N, Hacein-Bey-Abina S, Bartholomae CC et al. Lentiviral hematopoietic cell gene therapy for X-linked adrenoleukodystrophy. Methods Enzymol 2012;507:187-198

48. Aronson R, Gottlieb PA, Christiansen JS et al. Low-Dose Otelixizumab Anti-CD3 Monoclonal Antibody DEFEND-1 Study: Results of the Randomized Phase III Study in Recent-Onset Human Type 1 Diabetes. Diabetes Care 2014;37:2746-2754

49. Keymeulen B, Candon S, Fafi-Kremer S et al. Transient Epstein-Barr virus reactivation in CD3 monoclonal antibody-treated patients. Blood 2010;115:1145-1155.

50. Monti P, Scirpoli M, Maffi P et al. Islet transplantation in patients with autoimmune diabetes induces homeostatic cytokines that expand autoreactive memory cells. J Clin Invest 2008;118:1806-1814

51. Graham Chapman, John Cleese, Terry Gilliam et al. Monty python and the holy grail. BBC 1975

52. Peyser T, Dassau E, Breton M et al. The artificial pancreas: current status and future prospects in the management of diabetes. Ann N Y Acad Sci 2014;1311:102-123

53. Abbott Freestyle Libre 2014

54. Ludwiga B, Reichela A, Steffena A et al. Transplantation of human islets without immunosuppression. Proc Natl Acad Sci 2013;110:19054-19058

55. Viacyte Inc. Clinical Trial of VC-01™ Candidate Cell Replacement Therapy for Type 1 Diabetes 2014

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Summary

Nederlandse samenvatting

Acknowledgement/
Dankwoord

Publications

Curriculum Vitae

SUMMARY

Type 1 diabetes mellitus (T1DM) is a chronic disease caused by autoimmune mediated destruction of insulin producing beta-cells in the islets of Langerhans in the pancreas. Currently there is no cure; treatment consists of frequent or continuous insulin administration to mimic beta-cell function. Despite intensive insulin therapy, T1DM still contributes substantially to morbidity and mortality.

Over the last decades, much progress has been made in our understanding of the pathophysiology and possible starting points of treatment of T1DM. In spite of this progress, many questions remain unanswered. We know that T1DM is a T-cell mediated autoimmune disease, but the prerequisites for T-cell recruitment to the endogenous pancreatic islets are not fully understood. Specifically, it is unknown whether antigen presence is a prerequisite for T-cell recruitment. The first part of this thesis addresses beta-cell destruction. There is evidence that functional beta-cell mass can be restored after successful immune intervention in diabetes rodent models but we do not know the source of new insulin producing cells nor the role of ongoing autoimmunity in this process. The second part of this thesis hence addresses beta-cell regeneration. Clinical islet transplantation is an accepted treatment in a small subset of T1DM patients. Yet, little is known about T-cell recruitment to islet transplants either. Furthermore there is a growing interest in the contribution of memory T-cells to recurrent autoimmunity in graft failure. T-cell antigen recognition and beta-cell destruction pathways have been largely unraveled, but it is unclear whether we can protect beta-cells by compromising these processes simultaneously, hence whether we can evade the immune system. The third part of this thesis addresses aspects of beta-cell replacement. Clinical trials have shown that anti-CD3 antibodies can temporarily preserve beta-cell function in recent onset T1DM patients, but it is not known how safe this treatment is in terms of recall immunity (the immune reaction towards pathogens to which patients have been exposed) and preservation of desired immunity against tumours. The fourth and final part of this thesis addresses aspects of immune intervention.

A major rodent model in T1DM research is the non-obese diabetes (NOD) mouse, originally developed in Japan during the selection of a cataract-prone strain. The NOD mouse spontaneously develops an autoimmune form of diabetes, sharing genetic and immuno-pathological features with human T1DM.

Yet, despite the similarities between mice and men in the development of autoimmune diabetes there are many more differences, emphasizing prudence in translating results. Because accessibility to human pancreases during the course of the disease is limited and because reliable biomarkers of both disease activity and aspects of intervention such as efficacy and safety are not readily available, many studies currently rely on animal models.

Beta-cell destruction

The prerequisites for T-cell recruitment to the pancreas that precedes beta-cell destruction are not fully determined. Clinical autopsy studies of recent onset T1DM patients compellingly indicate *antigen*-specific infiltration of auto reactive CD8+ T-cells into insulitic pancreas lesions. However, studies in a number of infection and autoimmune disease models have suggested additional (bystander) T-cell recruitment in a *non*-antigen specific manner, for instance via cytokines and chemokines.

In ***Chapter 2*** we monitored the recruitment of CD8+ T-cells, specific for the prevailing diabetogenic epitope *islet-specific glucose-6-phosphatase catalytic subunit-related protein* $(IGRP)_{206\text{-}214}$ in gene targeted non-obese diabetic (NOD) mice expressing a T cell 'invisible' $IGRP_{206\text{-}214}$ epitope. The aim of our study was to ascertain whether local expression of cognate pMHC is a prerequisite for the recruitment to and/ or the accumulation of CD8+ T-cells in the pancreas during the development of spontaneous autoimmune diabetes. These mice with IGRP epitope invisible islets developed insulitis and autoimmune diabetes with the same incidence and kinetics as wildtype NOD mice. As opposed to wildtype NOD mice, however, the islet-associated T-cells of these pre-diabetic mice did not contain IGRP epitope reactive CD8+ cells. Additional adoptive transfer experiments revealed that recruitment of both naïve and pre-activated IGRP reactive CD8+ T-cell to the inflamed islets of pre-diabetic NOD mice expressing the T-cell invisible IGRP epitope was severely impaired. Furthermore, pre-activated IGRP specific cytotoxic T lymphocytes failed to home to the insulitic lesions of these gene-targeted NOD mice. These data indicate that local expression of a specific antigen is a prerequisite for the recruitment to and/ or accumulation of their cognate memory CD8+ T-cells in the pancreatic islets during the development of spontaneous autoimmune diabetes. Our data also show that removing a major diabetogenic epitope in NOD mice, such as IGRP, will not suffice in preventing diabetes nor will it influence the course of the disease. This was illustrated by the significant increase in recruitment of other auto reactive T cell specificities such as insulin-$B_{15\text{-}23}$ reactive CD8+ T-cells. However, inducing tolerance via the induction of regulatory T-cells may be a possibility to interfere with T-cell recruitment. The rationale behind this treatment is that regulation of one specific attack might subsequently cause regulation of attacks against other beta cell targets. Furthermore the importance of antigen presence underscores the importance of memory. Strategies that interfere with memory in particular or with (re)migration to pancreatic islets therefore are worthwhile exploring too. Contributions to understanding the complex process of T-cell recruitment to endogenous islets during development of T1DM could bring us a step closer to intervening in this process. Our findings specifically could be an argument in favour of further pursuing antigen-specific therapies.

Beta-cell regeneration

It is estimated that at diagnose of T1DM 40-90% of the beta-cells have been destroyed. Among the number of issues that need to be addressed in T1DM research, finding a way to restore the severely compromised beta-cell mass/ function is one of these. The

availability of therapies that decrease insulin requirements and normalize glycemia in T1DM patients and that can fully reverse hyperglycemia in newly diagnosed diabetic NOD mice, raises the question of how functional beta cell mass could be restored after successful immune intervention. In addition, the worldwide scarcity of pancreas/ islet allografts as an alternative for restoration of beta-cell mass/ function, fuels the exploration of the beta-cell regenerative capacity.

Proposed mechanisms of beta-cell regeneration are replication of pre-existing beta cells, islet neogenesis from progenitor/ stem cells or non-beta-cells (via transdifferentiation for instance from alpha cells) and recovery of exhausted beta cells. However, the true origin of new insulin-producing cells in preclinical mousemodels remains difficult to prove.

In ***Chapter 3*** we describe the development of cell lineage tracing models in mice that spontaneously develop autoimmune diabetes. The technique of cell lineage tracing is based on the inheritable labelling of individual islet cells. The introduced individual transgenes to facilitate Red Fluorescent Protein (RFP, the tracer) expression did not interfere with diabetes susceptibility. The RFP labelling of pre-existing beta-cells in our two different models (NOD.RIP-tTA/tet07-Cre.ROSA-tdRFP and NOD.RIPCreER.ROSA-tdRFP) showed to be bright, cell type specific and achieved labelling percentages beyond 90%. Expression of RFP in pre-existing alpha-cells in NOD.GluCre.ROSA-tdRFP mice was somewhat lower (around 60%). Until this thesis, beta-cell regeneration studies had been performed in non-autoimmune cell lineage tracing models. As the mice in our cell lineage tracing models spontaneously develop an autoimmune form of diabetes, these could be used to address the origin of insulin producing cells after immune-intervention in preclinical studies, especially when combined with BrdU labeling, which indicates cell replication. Furthermore these novel models could reveal the role of ongoing autoimmunity on beta-cell regeneration, which most likely will suppress any form of beta-cell regeneration. A caveat in clinically translating rodent study findings are the possible differences in regenerative pathways and regenerative capacity between species. Yet, with model limitations in mind, cell lineage tracing studies might give some clues and guidance as to what regenerative pathways may be considered. Identifying regenerative pathways could eventually result in developing strategies capable of enhancing effectiveness of promising immune therapies.

Beta-cell replacement

Currently islet transplantation is an accepted therapy for patients with complete insulin deficiency, unstable glycemic control and repeated severe hypoglycemia in spite of optimal diabetes management and compliance, however challenges in clinical islet transplantation are plenty.

There is a growing interest in the role of recurrent or ongoing autoimmunity in the outcome of allograft islet transplantation. In ***Chapter 4*** we tested whether T-cell recruitment in an islet transplantation model is comparable to T-cell recruitment into endogenous islets as described in Chapter 2. We monitored recruitment of epitope $IGRP_{206-214}$ specific

CD8+ T-cells into epitope competent or epitope invisible grafts transplanted either in diabetic wildtype NOD mice (harboring both naive and memory IGRP epitope specific T-cells) or epitope invisible hosts (harboring only naive IGRP epitope specific T-cells). All four host-donor combinations had development of recurrent diabetes within two weeks, indicating that IGRP contributes to, but is dispensable for, graft destruction in diabetic $IGRP_{206-214}$ competent hosts. Wildtype hosts recruited epitope specific T-cells into epitope competent, but not epitope invisible grafts. In epitope-invisible hosts, there is no recruitment of epitope specific T-cells, regardless of donortype. We demonstrated that absence of an auto-antigen in syngeneic islet grafts in diabetic hosts renders the grafts 'invisible' to cognate memory (and naive) T-cells, comparable to T cell recruitment to endogenous islets, as described in Chapter 2. Graft derived $IGRP_{206-214}$ did activate naive $IGRP_{206-214}$ $CD8^+$ T-cells, but graft destruction by memory T-cells invariably predated their recruitment. Our results indicate that recurrent diabetes in the absence of allo-immunity, is driven by autoreactive T-cells primed during the primary immune response. Our findings that previously primed auto-reactive T-cells drive recurrent autoimmunity underscores the importance of developing immune strategies to tackle autoreactive T-cell memory after beta-cell replacement therapy and has possible implications for the selection and treatment of T1DM candidate islet recipients. Indeed, studies in clinical islet transplantation from our group had previously demonstrated that reactivation of memory islet autoreactive T-cells is a paramount hurdle to achieve or preserve insulin-independence in transplanted T1D patients, implying that current immune suppressive strategies remain insufficient to deal this autoimmune memory and point to the need of novel immune suppressive therapies targeting memory T-cells. We contend that our preclinical model may be of service for validation studies here.

Transplantation of genetically immune protected islets to elude host immune responses could be one approach to improve clinical outcome. In ***Chapter 5*** we tested whether compromised immune recognition by down-regulation of MHC-I expression (a molecule necessary for antigen presentation to $CD8^+$ T-cells), combined with inhibition of the cytotoxic granzyme B activity (an enzyme involved in the actual beta-cell killing) via genetically engineered US2/Serpin 9 expression, protects human beta-cells from acute recurrent islets autoimmunity. We showed *in vitro* and *in vivo* that dispersed primary human islet-cells could be efficiently transduced by lentiviral vectors into self-reaggregating pseudo-islets, histologically and functionally comparable to wild type islets. The protective effect of these strategies was demonstrated in surrogate beta-cells and human primary beta cells in which insulin release upon glucose stimulation was maintained despite co-culturing these cells with cytotoxic T-lymphocytes (CTL) against pre-pro-insulin (PPI). These auto-reactive T-cells were derived from a recent onset T1DM patient. We furthermore designed a preclinical humanized mouse model to allow assessment of the fate of primary human beta-cells in an autoimmune environment. Upon co-transplantation with PPI-directed CTLs under the kidney capsule of NOD.SCID. $IL\text{-}2R^{-/-}$; (NSG) mice, immune protected islets maintained insulin secretion as opposed to non-protected controls, indicating that US2/ Serpin 9 expression does not impact islet

viability *in vivo* and does protects beta-cells from autoimmune CTL attack. Our findings indicate that immune evasion using lentiviral vectors does not negatively affect islet quality and that it protects beta-cells against auto-immunity.

In order to asses immune protection, reliable measurements of beta-cell toxicity is required. Beta-cell toxicity measurements however are influenced by the quality of the isolated islet fraction, as islet preparations contain a mixture of cell types, including beta-cells, alpha-cells, duct cells and endothelial cells. In ***Chapter 5*** we additionally describe a successful strategy to measure beta-cell toxicity and protection from autoimmune T-cell destruction in vitro, using a destabilized luciferase reporter gene, expressed under the human insulin promoter. A killing assay using PPI-directed CTLs as previously described, demonstrated that the luciferase assay was not affected by the quality of the isolated islet fraction. This approach facilitates the creation of a screening platform for identification of new compounds that inhibit the interplay between beta-cells and autoreactive T-cells.

Aspects of immune therapy

Targeted immune therapies, such as anti-CD3 therapy, have shown encouraging results in intervention treatment of T1DM, especially in subgroups. A major safety concern in the use of any immune modulating agent in type 1 diabetes mellitus is how well the immune reaction towards pathogens patients have prior been exposed to (recall immunity) and desired tumour immunity are preserved. In the successful European placebo-controlled Phase II Otelixizumab (humanized anti-CD3 antibody; ChAglyCD3) trial in recent onset T1DM patients the chosen antibody dosage was considerably higher than the dosage elected for the Phase III DEFEND1 and 2 studies, which did not reach its primary endpoint of preserved beta-cell function. The dosage had been reduced for safety reasons in light of temporary EBV reactivation. In ***Chapter 6*** we demonstrate in a subcohort of the European phase II Otelixizumab trial that proliferative responses towards common pathogens upon in vitro stimulation with different recall antigens were preserved in anti-CD3 treated patients and were highly similar to those in placebo-treated T1DM patients. We furthermore showed that T-cell responses towards auto-antigens were not significantly altered after high dose anti-CD3 therapy, which means we did not find evidence for reduced or enhanced and fuelled auto-immunity. The proliferative response upon stimulation with the human suppressor protein p53 was invariably high in both the anti-CD3 and the placebo-treated patients underlining preserved desired anti-tumour immunity in spite of anti-CD3 treatment. Although clinical endpoints were not met in subsequent Otelixizumab studies testing a much lower dose, it seems premature to disqualify anti-CD3 antibodies as potential therapy in recent onset T1DM. We demonstrated in this subcohort of recent onset T1DM patients treated with Otelixizumab, that recall immunity is preserved in spite of high-dose anti-CD3 treatment, adding to the safety of high dose anti-CD3 treatment as an immune modulatory agent in the treatment of T1DM.

Epicrise

As most PhD students, I started my journey as the "quest for the Holy Grail". At the near finish of this thesis I wonder if there is *one* Holy Grail in T1DM research. I contend that an immune intervention can be successful, but in order for a patient to become insulin independent, beta-cell destruction and restoration of beta-cell function need to be addressed simultaneously. Likewise, there will be no successful beta-cell replacement therapy without the necessary immune protection. Furthermore, there is a need to address safety concerns in the development of any immune therapy, while at the same time one has to be aware of the health risks of diabetes itself with current (often suboptimal) insulin treatment regimes. These quests all interconnect just as the projects in this thesis interconnect.

In this thesis, in close collaboration with my research colleagues, I have shown that T-cell recruitment in both spontaneous autoimmune diabetes and islet transplantation requires presence of a cognate antigen, which could be used as an argument in favour of further pursuing antigen-specific therapies. We have shown that recurrent diabetes in an islet transplantation model is driven by memory autoreactive T-cells and this latter finding has contributed to the present testing of immune suppressive drugs that indeed address recurrent autoimmunity, to improve outcome in clinical islet transplantation. We have designed and tested a novel auto-immune diabetes cell lineage tracing model for future testing of the regenerative capacity of islet-cells. We have furthermore shown that immune evasion protects beta-cells from autoimmune T-cell attack *in vivo*. Currently different immune evasion techniques, such as islet encapsulation are being tested in the clinic. And we have shown that recall immunity is preserved in spite of high dose anti-CD3 treatment, adding to the safety of high dose anti-CD3 treatment as an immune modulator agent in the treatment of T1DM.

As said, I wonder if there is one Holy Grail in T1DM research. The combined Holy Grail in T1DM research in my opinion can be summarized as: the necessity to halt or elude the immune attack in T1DM in a safe manner, while simultaneously restoring beta-cell function.

NEDERLANDSE SAMENVATTING

Diabetes mellitus type 1 (T1DM) is een chronische ziekte die veroorzaakt wordt doordat het immuun systeem, dat geprogrammeerd is om ons te beschermen tegen aanvallen van buitenaf, de eigen insuline producerende bètacellen in de eilandjes van Langerhans in de alvleesklier bij vergissing vernietigt. Dit proces wordt *auto*-immuniteit genoemd. Op dit moment bestaat er geen genezing voor T1DM: de behandeling bestaat uit frequente toediening van het hormoon insuline om de bètacelfunctie na te bootsen. Ondanks intensieve insuline behandeling, lukt het slechts bij een klein gedeelte van de T1DM patiënten om de bloedsuikers voldoende stabiel te houden. Het grootste gedeelte van de patiënten ontwikkelt complicaties aan ogen, nieren, lange zenuwbanen, hart en grote bloedvaten. Hierdoor draagt T1DM, ondanks insuline behandeling, nog steeds bij aan een hoge ziektelast, vermindering van kwaliteit van leven en een kortere levensverwachting.

De laatste decennia is veel kennis beschikbaar gekomen over het ontstaan van T1DM en mogelijke aanknopingspunten voor behandeling. Er zijn echter nog steeds veel vragen onbeantwoord. Het is bekend dat specifieke cellen van het afweersysteem, de zogenaamde CD8+ T-cellen, bètacellen herkennen en aanvallen, maar het is niet volledig bekend *waardoor* T cellen naar alvleesklier migreren om daar vervolgens bètacellen te vernietigen. Specifiek is het de vraag of het antigen of doelwit waartegen T-cellen reageren, noodzakelijkerwijs aanwezig moet zijn voor T-cel rekrutering. Het eerste deel van deze thesis richt zich op onderzoek naar bètacel vernietiging ofwel bètacel destructie. Diabetes kan *in muizen* genezen worden. Dit betekent dat het eerdere tekort aan insuline wordt opgeheven en dat er weer voldoende insuline geproduceerd wordt. Maar het is onduidelijk waar nieuwe insuline producerende cellen vandaan komen. Kunnen aangeslagen of uitgeputte bètacellen herstellen als ze niet meer worden aangevallen en/ of overvraagd worden? Delen reeds bestaande bètacellen zich? Worden nieuwe bètacellen gevormd uit voorloper- of stamcellen? Ook de invloed van het auto-immuun systeem op dit proces is onvoldoende bekend. Het tweede deel van deze thesis richt zich op bètacel herstel ofwel bètacel regeneratie. Klinische eilandjes transplantatie is een geaccepteerde behandeling in een zeer kleine subgroep van T1DM patiënten. Het succes hiervan wordt onder andere beïnvloed door T-cellen die naar het transplantaat migreren om de (getransplanteerde) bètacellen aan te vallen. Maar wederom is het onvoldoende bekend *waardoor* T-cellen naar de (getransplanteerde) bètacellen migreren. Omdat getransplanteerde eilandjes meestal afkomstig zijn van een donor met een andere genetische achtergrond dan de ontvanger, zal een ontvanger een afstotingsreactie ontwikkelen, zogenaamde *allo*-immuniteit. Patiënten worden zo veel mogelijk tegen dit proces beschermd via afweer onderdrukkende medicijnen. Een nadeel is dat deze medicijnen zelf vaak een negatief effect op bètacellen hebben. Naast allo-immuniteit is er een groeiende aandacht voor de rol van *auto*-immuniteit in eilandjes transplantatie. T-cellen die tijdens het T1DM ziekteproces eigen bètacellen aanvallen, blijven vervolgens in het lichaam aanwezig

als memory of geheugen cellen die in staat blijven getransplanteerde eilandjes te herkennen en aan te vallen. Het is bekend dat T-cellen bètacel antigenen of doelwitten herkennen, een proces dat in T1DM uiteindelijk leidt tot bètacel destructie. Het is de vraag of bètacellen beschermd kunnen worden door deze processen van antigen presentatie en bètacel destructie gelijktijdig te beïnvloeden. Hiermee proberen we feitelijk bètacellen te camoufleren en het immuunsysteem om de tuin kunnen leiden. Specifiek is het de vraag of getransplanteerde eilandjes hierdoor beschermd kunnen worden. Het derde deel van deze thesis richt zich op verschillende aspecten van bètacel transplantatie ofwel bètacel vervanging. Klinisch onderzoek richt zich veelal op patiënten die net gediagnostiseerd zijn met T1DM. Een genezing voor T1DM is er op dit moment nog niet maar er zijn wel verschillende studies die laten zien dat het ziekteproces vertraagd kan worden. Gerichte immuuntherapieën, zoals Otelixizumab ofwel anti-CD3 therapie, hebben bemoedigende resultaten laten zien in de behandeling van T1DM, in het bijzonder in subgroepen van patiënten. Dit medicijn grijpt breder in op het afweersysteem dan alleen op het T1DM ziekteproces. Het is niet bekend wat het effect van deze therapie is ten aanzien van behoud van *gewenste* afweerreacties zoals tegen infecties en kanker. Het vierde en laatste deel van deze thesis richt zich op aspecten van immuun interventie.

Een veelgebruikt muismodel in T1DM onderzoek is de non-obese diabetes (NOD) muis. Deze muis ontwikkelt spontaan een auto-immuun vorm van diabetes en vertoont genetische en immunologische overeenkomsten met T1DM in patiënten. Naast overeenkomsten, zijn er grote verschillen tussen muizen en mensen, wat voorzichtigheid in het vertalen van resultaten gebied. Er is echter beperkte toegang tot de alvleesklier van patiënten tijdens het T1DM ziekteproces. Daarnaast is er een beperkte beschikbaarheid van betrouwbare biomarkers van zowel het beloop van het T1DM ziekteproces als van aspecten van interventie zoals effect en veiligheid. Daarom wordt in een groot aantal studies gebruikt gemaakt van diermodellen.

Bètacel destructie

Het is bekend dat T-cellen bètacellen herkennen en aanvallen. De omstandigheden die nodig zijn voor het rekruteren van T-cellen naar de alvleesklier zijn niet volledig bekend. Klinische autopsie studies van recent gediagnostiseerde T1DM patiënten wijzen overtuigend op de aanwezigheid van T-cellen in de alvleesklier die heel specifiek een auto-immuun antigen of doelwit herkennen. Met andere woorden, deze studies wijzen erop dat T-cel rekrutering een *antigen*-specifiek proces is. Echter, studies in verschillende infectie- en auto-immuun ziekte modellen suggereren dat T-cellen ook op *niet-antigeen*-specifieke wijze gerekruteerd kunnen worden en bijvoorbeeld aangetrokken worden door chemische stoffen die vrijkomen tijdens het onstekingsproces.

In ***Hoofstuk 2*** beschrijven we de rekrutering van T-cellen, specifiek voor het veelvoorkomende en bij de ontwikkeling van diabetes betrokken epitoop of doelwit *islet-specific glucose-6-phosphatase catalytic subunit-related protein* $(IGRP)_{206-214}$.

We volgden de rekrutering van deze specifieke T-cellen naar de eilandjes van zowel gewone (wildtype) NOD muizen, als naar de eilandjes van genetisch gemodificeerde NOD muizen die een "onzichtbaar" IGRP doelwit tot expressie brengen. Hiermee testten we of T-cellen, die specifiek tegen het doelwit IGRP reageren ook tijdens het auto-immuun diabetes ziekteproces naar de alvleesklier worden gerekruteerd, als dit doelwit onzichtbaar voor hen is. Of anders gezegd, we testten of aanwezigheid van dit doelwit noodzakelijk is voor rekrutering van deze T-cellen naar de eilandjes van Langerhans. Muizen met gemodificeerd en dus onherkenbaar eilandjes-doelwit IGRP ontwikkelden even snel en even vaak auto-immuun diabetes als wildtype NOD muizen. In tegenstelling tot in wildtype NOD muizen echter, vonden we in de eilandjes van deze onherkenbaar IGRP doelwit muizen geen IGRP specifieke T-cellen. Aanvullende studies lieten zien dat rekrutering van zowel naïeve als van geactiveerde IGRP specifieke T-cellen naar de onherkenbare IGRP doelwit-eilandjes van deze muizensterk verstoord was.

Deze resultaten laten zien dat lokale expressie van een specifiek antigen wel degelijk een voorwaarde is voor de migratie van specifieke T-cellen naar de eilandjes van Langerhans. Onze resultaten laten ook zien, dat het simpelweg camoufleren van één specifiek antigen het T1DM ziekteproces niet zal beïnvloeden. We zien namelijk in de onherkenbare IGRP doelwit-eilandjes een toename van specifieke T-cellen, gericht tegen een ander doelwit. Echter, het opwekken van tolerantie van T-cellen tegen een specifiek bètacel doelwit zou mogelijk wel het ziekteproces kunnen beïnvloeden en hier is verder onderzoek naar nodig. Rationale hierachter is dat het reguleren van één specifieke aanval zou leiden tot ook het reguleren van aanvallen tegen andere bètacel doelwitten. Het belang van aanwezigheid van een antigen benadrukt bovendien het belang van memory of geheugencellen. Deze cellen kunnen hun specifieke antigen te allen tijde opnieuw herkennen en aanvallen. Strategieën die aangrijpen op memory of die aangrijpen op (re)migratie naar eilandjes van Langerhans zijn de moeite van het exploreren waard. Inzicht in het complexe proces van T-cel rekrutering naar eilandjes van Langerhans tijdens het T1DM ziekteproces zou ons een stap dichter bij een interventie kunnen brengen. Specifiek zouden onze bevindingen een argument kunnen zijn voor het verder ontwikkelen van antigen-specifieke therapieën.

Bètacel regeneratie

Bij het stellen van de diagnose T1DM is naar schatting reeds de meerderheid van de bètacellen vernietigd. Een van de hoofdthema's in T1DM onderzoek, is het vinden van een manier om deze bètacelmassa en -functie te herstellen. Er zijn therapieën beschikbaar voor T1DM patiënten die het ziekteproces vertragen. In muizen zijn er therapieën beschikbaar die leiden tot volledige genezing van auto-immuun diabetes. De beschikbaarheid van deze therapieën leidt tot de vraag hoe de functionele bètacel massa (tijdelijk) kan herstellen na succesvolle (immuun) interventie. Bovendien draagt het wereldwijde tekort aan alvleesklier donoren voor transplantatie als alternatief voor herstel van bètacelmassa en -functie bij aan de noodzaak van studies naar de regeneratieve of herstel capaciteit van de alvleesklier.

Veronderstelde mechanismen achter bètacel regeneratie zijn replicatie (het delen van reeds bestaande bètacellen), neogenese (nieuwvorming van bètacellen vanuit stamcellen of vanuit andere (alvleesklier) cellen) en herstel van uitgeputte bètacellen. Het blijft echter moeilijk de ware oorsprong van nieuwe insuline producerende cellen in preklinische modellen te herleiden.

In ***Hoofdstuk 3*** beschrijven we de ontwikkeling van zogenaamde celherkomst traceer modellen in NOD muizen. Deze techniek baseert zich op het blijvend en erfelijk overdraagbaar labellen van individuele eilandjes cellen. Deze genetisch gemodificeerde muizen brengen een Rood Fluorescerend Proteine (RFP) tot expressie in ofwel hun bètacellen ofwel hun alfacellen, wat de cellen traceerbaar maakt. Alfacellen bevinden zich ook in de eilandjes van Langerhans, zij produceren het hormoon glucagon. Glucagon is een soort tegenhormoon van insuline, dat beschermd tegen een te lage bloedsuiker. Alfacellen worden onder andere genoemd als mogelijke oorsprong voor bètacel regeneratie. We testten drie modellen, twee met RFP gelabelde bètacellen en één met RFP gelabelde alfacellen. De RFP labeling van bètacellen in onze eerste twee modellen was intens, cel type specifiek en bereikte labelings percentages van meer dan 90%. Expressie van RFP in alfacellen ons derde model was iets lager (circa 60%).

Wat nieuw is aan onze modellen is het feit dat deze muizen spontaan een auto-immuun vorm van diabetes ontwikkelen vanwege hun NOD achtergrond. Tot nu toe zijn bètacel regeneratie studies uitgevoerd in celherkomst traceermodellen zonder auto-immuun achtergrond, wat mogelijk effect heeft op de uitkomst. Onze modellen zouden gebruikt kunnen worden om de origine van insuline producerende cellen na succesvolle immuun interventie in preklinische studies te achterhalen. Bovendien kunnen deze modellen inzicht geven in de rol van aanhoudende auto-immuniteit op bètacel regeneratie. Voorzichtigheid is geboden in de klinische translatie van bevindingen uit muismodel studies, vanwege de mogelijke verschillen in regeneratie en regeneratief vermogen tussen muizen en mensen. Maar met model limitaties in het achterhoofd, zouden celherkomst traceer studies ons kunnen helpen ten aanzien van welke regeneratieve mogelijkheden verder onderzocht zouden kunnen worden. Mits relevant voor de mens, zou het identificeren van regeneratieve mogelijkheden kunnen leiden tot het ontwikkelen van strategieën die het effect van succesvolle immuun therapieën versterken.

Bètacel vervanging

Eilandjes transplantatie is een geaccepteerde therapie voor patiënten met complete insuline deficiëntie, die niet goed te reguleren zijn en die bij herhaling levensbedreigende lage bloedsuikers hebben. Klinische eilandjes transplantatie kent echter nog vele uitdagingen.

Er is toenemend aandacht voor de invloed van auto-immuniteit op het effect van eilandjes transplantatie. In ***Hoofstuk 4*** testten we of T-cel rekrutering in een eilandjes transplantatie model vergelijkbaar is met T-cel rekrutering naar eigen eilandjes zoals beschreven in hoofdstuk 2. We monitorden de rekrutering van dezelfde specifieke IGRP T-cellen naar

getransplanteerde eilandjes die ofwel herkenbaar ofwel onherkenbaar IGRP doelwit tot expressie brengen. Deze eilandjes werden getransplanteerd in ofwel diabetische wildtype NOD ontvangers ofwel in diabetische NOD ontvangers met genetisch gemodificeerd en dus onherkenbaar IGRP doelwit. Er waren dus in totaal vier donor-ontvanger combinaties. Wildtype NOD ontvangers bezitten zowel naïeve als memory IGRP specifieke T-cellen omdat deze reeds voor transplantatie, tijdens de ontwikkeling van diabetes, het IGRP doelwit zijn tegengekomen. Ontvangers met onherkenbaar IGRP doelwit bezitten alleen naïeve IGRP specifieke T-cellen, aangezien deze nooit eerder hun IGRP doelwit zijn tegengekomen. Alle getransplanteerde muizen ontwikkelden binnen twee weken opnieuw diabetes. Dit laat zien dat de ontspoorde afweerreactie tegen IGRP wel een rol speelt in maar niet noodzakelijk is voor transplantaat falen in NOD muizen met diabetes. Wildtype NOD ontvangers rekruteerden IGRP specifieke T-cellen naar transplantaten met herkenbaar IGRP doelwit, maar niet naar transplantaten met onherkenbaar IGRP doelwit. In onherkenbaar IGRP doelwit NOD ontvangers werd geen rekrutering van IGRP specifieke T-cellen gezien, onafhankelijk van de donor. We laten hiermee zien dat afwezigheid van een autoantigen of doelwit in eilandjes van Langerhans, de transplantaten 'onzichtbaar' maakt voor de memory (en naïeve) T-cellen die geprogrammeerd zijn om dit doelwit te herkennen. Deze resultaten laten zien dat lokale expressie van een specifiek antigen een voorwaarde is voor de migratie van specifieke T-cellen naar getransplanteerde eilandjes van Langerhans. Eerder hebben we in hoofdstuk 2 laten zien dat dit ook het geval is voor T cel rekrutering naar de eigen eilandjes. Doelwit IGRP afkomstig uit het transplantaat was in staat om naïeve IGRP specifieke T-cellen te activeren, maar transplantaat destructie door memory T-cellen trad sneller op dan rekrutering van nieuw geactiveerde cellen. In dit model kijken we niet naar allo-immuniteit, de immuunreactie tegen weefsel van donoren met een andere genetische achtergrond dan de ontvanger. Hoewel dit bij klinische eilandjes transplantatie wel degelijk een belangrijke rol speelt, kijken we in ons model specifiek naar auto-immuniteit in transplantatie. Onze resultaten wijzen erop dat auto-immuniteit in afwezigheid van allo-immuniteit gedreven wordt door T-cellen die geactiveerd zijn tijdens de oorspronkelijke immuun reactie, de zogenaamde memory T-cellen. Onze bevindingen benadrukken het belang van het ontwikkelen van immuun strategieën die zich richten op deze auto reactieve memory T-cellen na bètacel vervangende therapie en hebben mogelijk ook invloed op de selectie en behandeling van T1DM kandidaten voor eilandjes transplantatie. Studies naar klinische eilandjes transplantatie vanuit onze onderzoeksgroep hebben eerder laten zien dat reactivatie van memory autoreactieve T-cellen een enorm obstakel is voor het bereiken ofwel behouden van insuline-onafhankelijkheid in getransplanteerde T1DM patiënten. Dit impliceert dat de huidige immuun suppressieve strategieën niet voldoende aangrijpen op auto-immuun memory en wijst op de noodzaak van het ontwikkeling van nieuwe immuunsuppressieve therapieën die zich richten op memory T-cellen. Ons preklinische model zou gebruikt kunnen worden om validatie studies op dit gebied te verrichten.

Transplantatie van genetisch gemodificeerde en feitelijk gecamoufleerde eilandjes zou een manier kunnen zijn om bescherming te bieden tegen auto-immuniteit. Dit zou

mogelijk het klinisch effect van eilandjes transplantatie kunnen verbeteren. In ***Hoofstuk 5*** testten we of beïnvloeding van immuun herkenning door verminderde MHC-I expressie (een molecuul dat noodzakelijk is voor de presentatie van bètacel doelwit aan (CD8+) T-cellen) gecombineerd met remming van de cytotoxische granzyme B activiteit (een enzym dat een belangrijke rol speelt in de daadwerkelijke destructie van bètacellen) humane bètacellen beschermt tegen de invloed van auto-immuniteit. We hebben laten zien dat eilandjes cellen, losgeweekt uit de eilandjes, met behulp van lentivirale vectoren efficiënt genetisch gemodificeerd kunnen worden en vervolgens hergroepeerden tot pseudo-eilandjes. Deze humane pseudo-eilandjes bleken histologisch en functioneel vergelijkbaar met niet gemodificeerde eilandjes. Zij bleken echter beter beschermd tegen een auto-immuun aanval door specifieke T-cellen getraind om bètacellen aan te vallen, dan niet gemodificeerde eilandjes. De T-cellen waarmee we dit testten zijn afkomstig van een patiënt met recent gediagnostiseerde T1DM. Ook wanneer deze humane pseudo-eilandjes samen met de specifieke T-cellen onder het nierkapsel van immuun gecompromitteerde muizen werden getransplanteerd, behielden deze eilandjes hun natuurlijk vermogen tot afgifte van insuline in tegenstelling tot onbeschermde eilandjes. Dit preklinische gehumaniseerde muismodel biedt mogelijkheden om het lot van bètacellen in een ontstekingsomgeving te testen. Onze bevindingen wijzen erop dat beïnvloeding van immuun herkenning met behulp van lentivirale vectoren, ofwel immuun-evasie, de kwaliteit van eilandjes niet negatief beïnvloedt en dat het beta cellen beschermt tegen auto-immuniteit.

Om immuun protectie te toetsen zijn betrouwbare metingen van bètacel toxiciteit ofwel bètacel destructie noodzakelijk. Deze metingen worden echter beinvloed door de kwaliteit van de geisoleerde eilandjes fractie, die naast bètacellen bestaat uit andere celtypen zoals alfacellen, duct cellen en endotheel cellen. In ***Hoofstuk 5*** beschrijven we een strategie om selectief bètacel toxiciteit en de bescherming tegen autoimmuun T-cel destructie te meten. Deze assay zou gebruikt kunnen worden als screeningsplatform om de effectiviteit en toxiciteit van nieuwe interventie strategieën in T1DM te testen.

Aspecten van immunotherapie

Gerichte immuuntherapieën, zoals Otelixizumab ofwel anti-CD3 therapie, hebben bemoedigende resultaten laten zien in de behandeling van T1DM, in het bijzonder in subgroepen van patiënten. Een belangrijke aspect ten aanzien van veiligheid in de toepassing van welk immuun modulerende therapie dan ook in T1DM is de mate van behoud van gewenste afweerreacties, zoals tegen infecties en kanker.

In ***Hoofdstuk 6*** testten we in een deel van de patiënten die deelnamen aan de Europese Fase II studie met Otelixizumab de immuunreactie tegen veelvoorkomende ziekteverwekkers (bacterie, virus en gist). We laten zien dat deze immuunreacties in tact zijn gebleven in anti-CD3 behandelde patiënten en dat deze vergelijkbaar zijn met immuunreacties bij met placebo behandelde T1DM patiënten. We tonen verder aan dat immuunreacties tegen bètacel doelwit niet anders was na hoge dosis anti-CD3 therapie

en dat hiermee geen aanwijzingen gevonden werden voor verhoogde auto-immuniteit, het ziekteproces bij T1DM. De immuunreactie tegen tumoreiwit was onveranderlijk hoog in zowel de anti-CD3 behandelde als de placebo controle groep, wat het wenselijk behoud van anti-tumor immuniteit benadrukt. Hoewel het doel van diabetesinterventie niet behaald werd in vervolgstudies met veel lagere dosis Otelixizumab (om reactivatie van Epstein-Barr virus te voorkomen), lijkt het voorbarig om anti-CD3 te diskwalificeren als potentiele therapie in recent gediagnostieerde T1DM patiënten. In dit subcohort van recent gediagnostieerde patiënten met T1DM behandeld met Otelixizumab laten we zien dat de gewenste afweerreactie tegen infecties en kanker intact blijft ondanks hoge dosis anti-CD3 behandeling. Deze bevindingen benadrukken de veiligheid van hoge dosis anti-CD3 als immuun modulator in de behandeling van T1DM.

Epicrise

Zoals de meeste promovendi, begon ik dit T1DM onderzoekstraject als 'de zoektocht naar de heilige graal'. Nu mijn thesis bijna af is, vraag ik me af of er één heilige graal bestaat binnen het T1DM onderzoek. Een immuun interventie kan succesvol zijn, maar zonder aandacht te schenken aan bètacel destructie en herstel van bètacel functie, zal een patiënt in de meeste gevallen afhankelijk blijven van insuline-injecties. Bètacel vervangende therapie kan alleen succesvol zijn met behulp van geschikte immuun protectie, anders zullen de getransplanteerde bètacellen opnieuw vernietigd worden. En het ontwikkelen van een immuun therapie brengt automatisch aandacht voor veiligheidsaspecten met zich mee, waarbij men zich wel moet realiseren dat het simpelweg hebben van T1DM met de huidige (suboptimale) insuline regimes, ook gezondheidsrisico's met zich mee brengt. Deze zoektochten zijn allemaal met elkaar verbonden, net zoals de projecten binnen mijn thesis met elkaar verbonden zijn.

In deze thesis heb ik, in nauwe samenwerking met mijn collega-onderzoekers, laten zien dat lokale expressie van een antigen een voorwaarde is voor de migratie van specifieke T-cellen naar de eilandjes van Langerhans, zowel tijdens het T1DM ziekteproces, als na eilandjes transplantatie. Dit zou een argument kunnen zijn voor het verder ontwikkelen van antigen-specifieke therapieën. We hebben in een transplantatiemodel ook laten zien dat auto-immuniteit in afwezigheid van allo-immuniteit gedreven wordt door T-cellen die geactiveerd zijn tijdens de oorspronkelijke immuun reactie, de zogenaamde memory T-cellen. Deze bevindingen hebben bijgedragen aan onderzoek dat op dit moment verricht wordt naar het effect van verschillende immuun strategieën op allo- en auto immuniteit, ter verbetering van de transplantaatfunctie. We hebben een nieuw auto-immuun celherkomst traceer model ontwikkeld en getest, wat gebruikt zou kunnen worden in onderzoek naar het regeneratieve vermogen van eilandjes cellen. We hebben verder laten zien dat immuun-evasie bètacellen tegen een aanval van auto-immuun T-cellen beschermt en op dit moment worden verschillende immune-evasie technieken klinisch getest. We hebben tot slot laten zien dat gewenste immuunreacties in tact zijn gebleven in anti-CD3 behandelde patiënten, informatie die bij draagt aan de veiligheid van hoge dosis anti-CD3 in de behandeling van T1DM.

Zoals gezegd, vraag ik me af of er één heilige graal bestaat binnen het T1DM onderzoek. De gecombineerde heilige graal in T1DM onderzoek is mijns inziens samen te vatten in de noodzaak om de immuun aanval in T1DM op een veilige manier te stoppen of af te leiden, met gelijktijdig aandacht voor herstel van bètacel functie.

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PUBLICATIONS GONNIE ALKEMADE

1. *Wolfraim LA,* ***Alkemade GM****, Alex B, Sharpe S, Parks WT, Letterio JJ:* Development and application of fully functional epitope-tagged forms of transforming growth factor-β. Journal of Immunological Methods 2002;266:7-18
2. ***Alkemade GM****, Willems JM:* Polycytemia Vera presenting as sudden onset cognitive impairment. Journal of the American Geriatrics Society 2008;56:2362-2363
3. *Wang J, Tsai S, Shameli A, Yamanouchi J,* ***Alkemade G****, Santamaria P:* In situ recognition of autoantigen as an essential gatekeeper in autoimmune CD8+ T cell inflammation. Proceedings of the National Academy of Sciences of the United States of America 2010;107:9317-9322
4. ***Alkemade GM****, Hilbrands R, Vandemeulebroucke E, Pipeleers D, Waldmann H, Mathieu C, Keymeulen B, Roep BO:* Preservation of recall immunity in anti-CD3-treated recent onset type 1 diabetes patients. Diabetes/ Metabolism Research and Reviews 2011;8:925-927
5. ***Alkemade GM****, Clemente-Casares X, Yu Z, Xu BY, Wang J, Tsai S, Wright JR Jr, Roep BO, Santamaria P:* Local autoantigen expression as essential gatekeeper of memory T-cell recruitment to islet grafts in diabetic hosts. Diabetes 2013;62:905-11
6. *Zaldumbide A,* ***Alkemade GM****, Carlotti F, Nikolic T, de Abreu J, Engelse M, Skowera A, de Koning EJ, Peakman M, Roep BO, Hoeben RC, Wiertz E:* Genetically engineered human islets protected from CD8 mediated autoimmune destruction *in vivo*. Molecular Therapy 2013;21:1592-1601
7. ***Alkemade GM****, Bakker M, Rikhof B, IJpma FFA, Van Ginkel RJ, Kluin PM, Van Doorn J, Dullaart RPF:* Hypoglycemia in a patient with a big "big"-IGF-II producing tumor. Journal of Clinical Endocrinology and Metabolism. 2013;87:3113-3114
8. *De Kruijf EJFM,* ***Alkemade GM****, Van Os R, Fibbe WE, Van Pel M:* Peripheral blood hematopoietic stem and progenitor cell frequency is unchanged in patients with alpha-1-antitrypsin deficiency. International Journal of Hematology, *2014;99:714-720*
9. *Brouwers MC, Linthorst GE, Karstens FP, Rennings A,* ***Alkemade G****, Meersseman W, Cassiman D, Thijs A, Wolffenbuttel BH, Hollak CE, Janssen MC, Langendonk J:* Adults with an inherited metabolic disorder: a rapidly growing population with unique challenges. Nederlands Tijdschrift voor Geneeskunde 2014;158:A7745.

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Gonnie Alkemade was born March 13th 1977 in Amersfoort, the Netherlands. She attended secondary school at 'Het Nieuwe Eemland College' in Amersfoort, the Netherlands and passed her gymnasium exam in 1995. From 1995-1996 she studied Medicine at the University of Leuven, Belgium. From 1996-1997 she studied Biomedical Health Sciences at the Radboud University of Nijmegen, the Netherlands. She subsequently started her Medicine study at this university, during which she spent 6 months at the National Institutes of Health in Bethesda, U.S.A. (Principle Investigator dr. J.J. Letterio, Laboratory of Cell Regulation and Carcinogenesis, supervisor prof. dr. P.H.M. De Mulder, Radboud University of Nijmegen). In 2003 she obtained her medical degree. After 18 months as resident Internal Medicine (ANIOS) at former 'Rode Kruis Ziekenhuis', the Hague, the Netherlands (currently Haga Ziekenhuis) she started her formal Internal Medicine training (AIOS) at this hospital in 2005 (Head of Department: dr. R.M. Valentijn) and subsequently at Leiden University Medical Center, the Netherlands (Head of Department: prof. dr. J.A. Romijn). In 2008 she started her Ph.D. as a collaboration between Leiden University Medical Center, the Netherlands (prof. dr. B.O. Roep, Department of Immune haematology en Blood Transfusion) and the University of Calgary, Canada (prof. dr. P. Santamaria, Julia McFarlane Diabetes Research Center and Department of Microbiology and Infectious Diseases). In 2012 she continued her Internal Medicine training as fellow Endocrinology at University Medical Center Groningen, the Netherlands (Head of Department: prof. dr. B.H.R. Wolffenbuttel). In December 2013 she registered as Internist-Endocrinologist and continued to work at the Endocrinology department of the University Medical Center Groningen. In January 2015 she and her family moved to Aberdeen, Scotland. In April 2015 she joined the Diabetes and Endocrinology Department of the Aberdeen Royal Infirmary, Scotland, U.K. (Head of Department: dr. P. Abraham).