Expanded T cells from pancreatic lymph nodes of type 1 diabetic subjects recognize an insulin epitope

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In autoimmune type 1 diabetes, pathogenic T lymphocytes are associated with the specific destruction of insulin-producing β-islet cells[1,2]. Identification of the autoantigens involved in triggering this process is a central question. Here we examined T cells from pancreatic draining lymph nodes, the site of islet-cell-specific self-antigen presentation[3]. We cloned single T cells in a non-biased manner from pancreatic draining lymph nodes of subjects with type 1 diabetes and from non-diabetic controls. A high degree of T-cell clonal expansion was observed in pancreatic lymph nodes from long-term diabetic patients but not from control subjects. The oligoclonally expanded T cells from diabetic subjects with DR4, a susceptibility allele for type 1 diabetes[4], recognized the insulin A 1–15 epitope restricted by DR4. These results identify insulin-reactive, clonally expanded T cells from the site of autoinflammatory drainage in long-term type 1 diabetics, indicating that insulin may indeed be the target antigen causing autoimmune diabetes.

One of the major issues in understanding the pathophysiology of human autoimmune diseases is to identify the target antigens that drive the clonal expansion of autoreactive T cells. One difficulty in identifying potentially pathogenic T cells and their target antigens arises from observations in experimental animal models of autoimmune disease, which indicate that these pathogenic autoreactive T cells become activated and undergo clonal expansion in the draining lymph nodes of the inflamed target organ[5–8]. In human autoimmune disease, T cells from the inflammatory sites in rheumatoid arthritis and Graves' disease have been shown to recognize autoantigens[9,10]. Although autoreactive T cells may enter the circulation for short periods of time, it has not been possible to identify T cells undergoing local clonal expansion in peripheral blood[11].

Type 1 diabetes is an autoimmune disease in which autodestructive T cells infiltrate the pancreatic islets and specifically destroy insulin-producing β-islet cells[1,2,12]. Studies using the non-obese diabetes (NOD) model of autoimmune diabetes have shown that dendritic cells migrate from the pancreas to the pancreatic draining lymph nodes, where islet-cell antigens are presented to the pathogenic T cells that mediate the disease[3]. These investigations led to a study in which clonally expanded T cells were cloned directly from the draining lymph nodes of NOD mice and examined for antigen reactivity. Notably, these investigations revealed that insulin (an islet-cell-specific antigen) was the major target antigen for $CD8^+$ T cells in this mouse model of diabetes[13].

Together, these experiments indicated that clonally expanded, autoreactive T cells could be cloned directly from the pancreatic draining lymph nodes of NOD mice. However, the adaptation of this experimental approach to humans with autoimmune diabetes presents almost insurmountable problems. First, fresh draining lymph nodes from patients with autoimmune diabetes would need to be obtained, and single-cell lymphocyte cloning would have to be performed with relatively high efficiency. Second, unlike in the NOD model, humans will almost always have received insulin injections, which could potentially affect the results. Finally, owing to the long time course of the disease and the rarity of obtaining tissue, it will be virtually impossible to perform any type of kinetic analysis of the T-cell response to self-antigens from draining lymph nodes.

With these caveats in mind, pancreatic draining lymph nodes were obtained from three subjects with autoimmune diabetes and three non-autoimmune subjects. The disease state, age, gender and human leukocyte antigen (HLA) information for each subject are summarized in Table 1. Among the three control subjects, two are normal controls with no detectable immunologic defects, and one is a subject with long-standing type 2 diabetes. Two out of three pancreatic lymph node (PLN) samples (diabetic subjects 1 and 2) were from subjects with long-term disease (Table 1). Both of these subjects expressed the DRB1*0401 and DRB1*0301 class II major histocompatibility complex (MHC) alleles, and one expressed the DQ*0302 class II MHC allele, which are the three alleles highly associated with type 1 diabetes[4,14]. Subject 3 had a recent onset of clinical disease (1.5 yr). Immunohistochemical examination of the pancreatic tissue sample from this subject showed the remnants of islets, with lymphocytic infiltrate and $CD4^+$ T cells within the islets (see Supplementary Fig. 1). This is a rare case compared with most diabetic pancreases, in which usually no islets or T-cell infiltrates are left at the time of organ examination many years after disease onset. For example, examination of the pancreas from long-term diabetic subject 2 showed no infiltrate or cellular islet structure (Supplementary Fig. 2).

A total of 515 independent T-cell clones were generated by single-cell cloning with mitogen in the presence of a blocking anti-Fas antibody. The single-cell cloning efficiency varied between 5% and 20% of the single T cells plated. Polymerase chain reaction with reverse transcription (RT–PCR) was performed for each of the T-cell clones to determine the degree of clonal expansion, and to ask specifically whether the T cells cloned from the draining lymph nodes showed characteristics of T cells undergoing antigen-driven clonal expansion. All of the T cell clones isolated from the three

Table 1 **Subject information**

Subjects	Age (yr)	Gender	Disease duration (yr)	HLA type
Control				
1	64	M	n.a.	A2, A23 B62, B49 DRB1*0101, *1101 DQB1*0301, *05
2	52	F	n.a.	A1, A3 B7, B37 DR1, DR2
3	45	F	n.a.	A2, A24 B7, B62 DRB1*0401, DR9
Diabetic				
1	39	F	29	A2, A2 B49, B50 DRB1*0401, *0301 DQ 2, DQ3
2	24	F	15	A1, A30, B18, B57 DRB1*0401, *0301 DQB1*0302, *0201
3	27	M	1.5	A3, A24 B7, B35 DRB1*0101, *0101 DQB1*05, *05

Control and diabetic pancreatic draining lymph nodes were from multi-organ donors except for diabetic sample 1, which was collected from a type 1 diabetic undergoing pancreas transplant removal. All samples were obtained with approval for research. Control sample 1 is from a type 2 diabetic. All lymph nodes were processed into single-cell suspensions and frozen in 10% dimethylsulphoxide in FBS within 72 h of harvest. DQ tissue typing was not performed for all samples. n.a., not applicable.

Table 2 **T-cell clones isolated from control subjects express few identical V_β chains**

Control subject	No. of T-cell clones sequenced	No. of identical sequenced V_β chains
1	19	2; 2; 2
2	26	2; 2
3	22	2

T-cell clones were isolated from control subject pancreatic draining lymph nodes, and T-cell receptor V_β chains were sequenced. In control subject 1, three sets of two identical V_β chains were found from 19 clones. In control subject 2, two sets of two identical V_β chains were found in 26 clones. In control subject 3, two identical V_β chains were seen in 22 T-cell clones. In all three subjects, each V_β chain was represented in more than one clone in approximately 10% of isolated clones.

control subjects express heterogenous T-cell antigen receptors, with only a small portion (~10%) isolated from a single sample expressing identical V_β chains (Table 2). These results indicate that there is little restriction of V_β families used by T cells isolated from pancreatic lymph nodes of control subjects, and that these cells are representative of a polyclonal repertoire.

In contrast, T-cell clonal expansion was clearly observed in pancreatic lymph nodes of diabetic subjects 1 and 2 (Table 3 and Supplementary Tables 1 and 2), where over half of the T-cell clones expressed identical V_β chains. T-cell clones isolated from diabetic subject 3 showed heterogeneous T-cell receptor (TCR)β chains. Analysis of TCRα chains of T-cell clones from diabetic subjects 1 and 2 expressing identical TCRβ chains revealed that almost half of the T-cell clones also expressed identical TCRα chains, suggesting that these clones were expanded from the same progenitor cell. Another three clones from diabetic subject 2 also expressed identical TCRα chains. The remaining sequenced clones had different TCRα chains.

To confirm whether T cells with common TCR sequences were derived from the same progenitor T cells in the thymus, rearrangement of TCRγ chains was examined in a panel of clones with identical TCRβ sequences. Oligoclonally expanded T cells from diabetic subject 2 containing identical TCR V_β and V_α sequences also possessed identical TCR V_γ chain sequences, demonstrating that these cells are of the same lineage (data not shown).

A series of β-islet-associated candidate T-cell antigens have been identified, including β-cell-specific insulin in humans and NOD mice[15–18]. These candidates are potentially important in the autoimmune process and are of putative predictive clinical value with respect to type 1 diabetes. We used these observations to examine the antigen specificity of the clonally expanded T cells: MHC-matched, transformed B cell lines were cultured with peptides from candidate antigens and a panel of T-cell clones derived from the draining lymph nodes.

Clonally expanded $CD4^+$ clones from the two long-term diabetic subjects, but not from a control subject, recognized the insulin A 1–15 peptide (Fig. 1a–f). This recognition was specific, as the T-cell clones did not recognize insulin A 5–21 or any other epitope of proinsulin, or glutamic acid decarboxylase 65 (GAD65) peptide 274–286, GAD65 peptide 555–567 or a myelin basic protein (MBP) peptide (residues 85–99) that binds DRB1*1501 and DRB1*0401 (refs 19–21). The amount of insulin peptide required to induce proliferation and cytokine secretion followed a standard dose–response curve. T-cell clones responded to insulin A 1–15 and anti-CD3 stimulation with thymidine incorporation and interleukin (IL)-13 secretion, but no interferon (IFN)-γ secretion (not shown). It should be noted that cytokine secretion from long-term, mitogen-stimulated human T-cell clones in culture might not reflect *in vivo* secretion. Therefore, it is not possible to extrapolate the *in vivo* functional programme of these insulin-reactive T cells from these data.

Two of the subjects with diabetes and one control subject inherited the DRB1*0401 genotype. The other two control subjects and the subject with diabetes for 1.5 yr inherited the DRB1*0101 genotype (DR1). We were unable to expand any T-cell clones from diabetic subject 3, and analysis of antigen reactivity awaits construction of TCR-expressing hybridomas[21]. The T-cell clones investigated from diabetic subjects 1 and 2 expressed CD4. In these experiments, monoclonal antibodies that block MHC DR, but not DQ presentation of antigen, inhibited thymidine incorporation and cytokine secretion in response to insulin peptide (Fig. 1b–f). Use of cell lines homozygous for HLA*DRB1 alleles showed that the insulin-reactive T cells were DRB1*0401- and not DRB1*0301-restricted in the diabetic subjects expressing those genotypes (Fig. 2a). This is of interest because DR4 is strongly associated with the incidence of type 1 diabetes[4].

To be certain that the insulin A 1–15 peptide itself was non-mitogenic and that peptide reactivity was specific, more T-cell clones and peptides were tested. T-cell clones from diabetic subjects 1 and 2 responded only to insulin A 1–15 (in a dose-dependent

Table 3 **Clonally expanded T cells isolated from subjects with type 1 diabetes**

$V_\beta J_\beta$ usage	V_β/NDN/J_β junctional sequence*	Clone frequency	$V_\alpha J_\alpha$ usage	V_α/N/J_α junctional sequence	Clone frequency
Diabetic 1.Mi					
V_β29-1*03	DSSIYLCS / VEATRA / DTQYFGPGTRLTVL	(10/20) 50%	V_α8-3*02	YFCA / VGALA / GTASKLTFGTGTRL	(5/10) 25%
J_β2-3*01			J_α44*01		
V_β7-9*03	DSAMYLCASS / LAVIR / TDTQYFGPGTRLTVL	(2/20) 10%	n.d.		
J_β2-3*01					
Diabetic 2.Ba					
V_β5-1*01	DSALYLCASSL / ATSGGGS / DTQYFGPGTRL	(14/27) 51.8%	V_α39*01	YFCA / VVNM / DSNYQLIWGAGTKL	(7/14) 25.9%
J_β2-3*01			J_α33*01		
			V_α22*01	YFCA / DAGGTSYKL / FGQGTIL	(3/14) 11.0%
			J_α52*01		
			V_α26-2*01	YYCI / PGSEE / YGNKLVFGAGTIL	(1/14) 3.7%
			J_α47*01		
Diabetic 3					
V_β4-1*02	DSALYLCASSQ / VRLAGGG / EQFFGPGTRLTV	(2/43) 4.65%			
J_β2-1*01					
V_β5-1*01	DSALYLCASSL / GQGE / TEAFFGQGTRL	(2/43) 4.65%			
J_β1-1*01					
V_β14*01	DSGVYFCAS / RNLGL / NTEAFFGQGARL	(2/43) 4.65%			
J_β1-1*01					
V_β28*01	HTSMYLCASS / FRRV / TDTQYFGPGTRL	(2/43) 4.65%			
J_β2-3*01					

*NDN: N, N-region additions; D, diversity region additions.
From diabetic subject 1, 50% of T-cell clones expressed identical V_β chains, and of these clones, five expressed identical V_α rearrangements (25%). From diabetic subject 2, 14/27 (51.8%) of T-cell clones expressed identical rearranged V_β chains, and of these 14, seven were identical for TCR V_α (25.9%). Another four clones used the same V_β chain, but were paired with two other V_α chains. From diabetic subject 3, V_β chains were expressed at low frequency (4.65%), and each of these sets of clones expressed different V_α chains (not shown). n.d., not determined.

manner) and not to other peptides reported to bind to DRB1*0401 (Fig. 2b, c). Additionally, T-cell clones derived from the cerebrospinal fluid of a patient with multiple sclerosis (DRB1*0401, DRB1*0301) or clones from an DRB1*0401 subject named NC3 (including one of the expanded clones, NC3.13; see Table 2) did not recognize the peptide when presented by the MHC DRB1*0401 allele (Fig. 2d). T-cell clones from diabetic subject 2 with the same V_β chain but a different β-chain CDR3 region and different V_α chain from the clones reported in Fig. 1 did not respond to the insulin A 1–15 peptide presented by the MHC DRB1*0401 allele (Clone Ba.32, Fig. 2d). A T-cell clone recognizing the self-antigen MBP was examined, and this clone did not respond to the insulin peptide by proliferation or cytokine secretion (Fig. 2e).

NOD mouse studies have generally examined islet-infiltrating lymphocyte, draining lymph node or splenic cell populations for TCR repertoires in mice with recent disease onset or established disease[22–25], but not at the equivalent long-term point of diabetic subjects 1 and 2 because NOD mice are not generally maintained on exogenous insulin for extended periods after disease onset. In contrast, diabetic subject 3 had a relatively shorter disease duration of 1.5 yr. For this subject, there were scattered $CD4^+$ T lymphocyte infiltrates in the pancreatic islets (Supplementary Fig. 1). As one might suspect, the timing of a TCR repertoire assay after disease onset is crucial. Examination of the TCR repertoire in islet infiltrates of 2–3-week-old NOD mice showed enrichment of a few TCRα and β sequences with similar sequences to those seen in the pancreatic draining lymph nodes[26]. However, there are a number of reports indicating a heterogenous TCR repertoire in islet-infiltrating cells, spleen and lymph nodes of 7–11-week-old NOD mice[23–25].

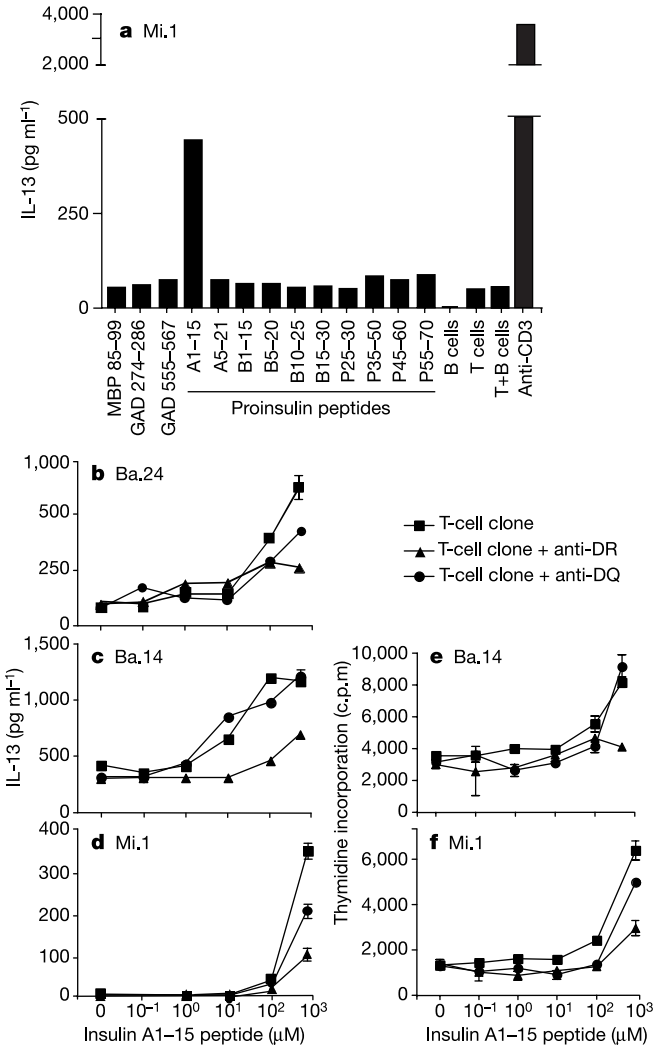

Figure 1 Expanded T-cell clones from diabetic PLN recognize insulin A 1–15 presented by the MHC DRB1*0401 allele. **a**, T-cell clone Mi.1 from diabetic subject 1 responded to insulin A 1–15 peptide in the context of Priess B cells (homozygous for DRB1*0401) but not other proinsulin peptides or other peptides reported to bind to DRB1*0401. All peptides were present at 250 μM. **b**–**f**, Clones Ba.24 (**b**), Ba.14 (**c**, **e**) and Mi.1 (**d**, **f**) recognize insulin A 1–15 peptide in a dose-dependent manner, as shown by IL-13 secretion levels (**b**–**d**) and by thymidine incorporation (**e**, **f**). This insulin-reactivity was blocked by an anti-DR antibody, but not by an anti-DQ antibody. Ba.24 and Ba.14 are expanded clones from diabetic subject 2 and possess identical TCR V_β and V_α chains. Error bars represent the s.e.m. of triplicates; one representative experiment out of three similar experiments is shown.

In the diabetic subjects, we found MHC-restricted recognition of the insulin A 1–15 peptide with $CD4^+$ clones that represented almost half of the clonable T cells in the draining pancreatic lymph nodes. This allowed us to calculate a minimal frequency of insulin A 1–15 antigen-reactive T cells of 1.04% for diabetic subject 1 and 0.86% for diabetic subject 2, on the basis of the cloning frequency for each lymph node. Although we do not know the autoantigen(s) potentially driving any clones not reactive to insulin and the cells we could not clone, this nevertheless represents an extremely high frequency of antigen-reactive $CD4^+$ cells generated without *in vitro* exposure to antigen.

There are a number of caveats to be considered in the interpretation of our presented clinical data. First, it is important to determine whether the clonally expanded, insulin-A-1–15-reactive CD4 cells in the pancreatic draining lymph nodes were found specifically in the draining lymph nodes. In addition, the diabetic subjects had high blood glucose levels and were subjected to insulin injections over a long period of time; it is possible that daily administration of exogenous insulin could initiate and sustain a systemic anti-insulin T-cell response.

A series of experiments was performed to examine these questions. As the clonally expanded CD4 cells from diabetic subject 2 expressed the $V_\beta5.1$ TCR, we either plated $CD4^+V_\beta5.1^+$ T cells from the subject's spleen at a density of one cell per well and expanded them in the presence of irradiated allogeneic peripheral blood mononuclear cells (PBMCs), mitogen and IL-2, or instead directly amplified the $V_\beta5.1$ CDR3 sequence from the single-cell $CD4^+V_\beta5.1^+$ genomic DNA and sequenced the PCR product (see Supplementary Table 3). None of the four independent $CD4^+V_\beta5.1^+$ clones recognized any pro-insulin peptide (data not shown). Moreover, out of the 70 independent TCR V_β sequences obtained from 160 wells, none showed similarity to the expanded, insulin-reactive T-cell clone Ba.14, found in the pancreatic draining lymph nodes of this long-term type 1 diabetic subject. We also examined the panel of T-cell clones generated from the pancreatic draining lymph nodes from NC1, a long-term type 2 diabetic subject with dysregulated, non-autoimmune insulin responses. Using autologous lymph node cells as antigen-presenting cells, none of the T-cell clones from this subject's pancreatic lymph node recognized any peptide of proinsulin (Fig. 2f).

The lack of a systemic response to insulin peptides, together with the absence of the potentially pathogenic T-cell clones in the spleen of the diabetic subject, suggest that the parenteral administration of insulin is not the explanation for the pancreatic lymph-node-specific responses. Moreover, it has been demonstrated that for islet-cell transplants in patients with no insulin secretion (similar to the diabetic subjects in this investigation), there is a highly pathogenic T-cell response to islet cells. Their transplantation, even into an identical twin, results in tissue destruction with lymphocytic infiltrate within days of engraftment[27]. The clinical transplant data provide strong evidence that there is persistence of T cells, probably residing in the pancreatic draining lymph nodes, and (considering the kinetics of the islet cell rejection) capable of recognizing islet-cell

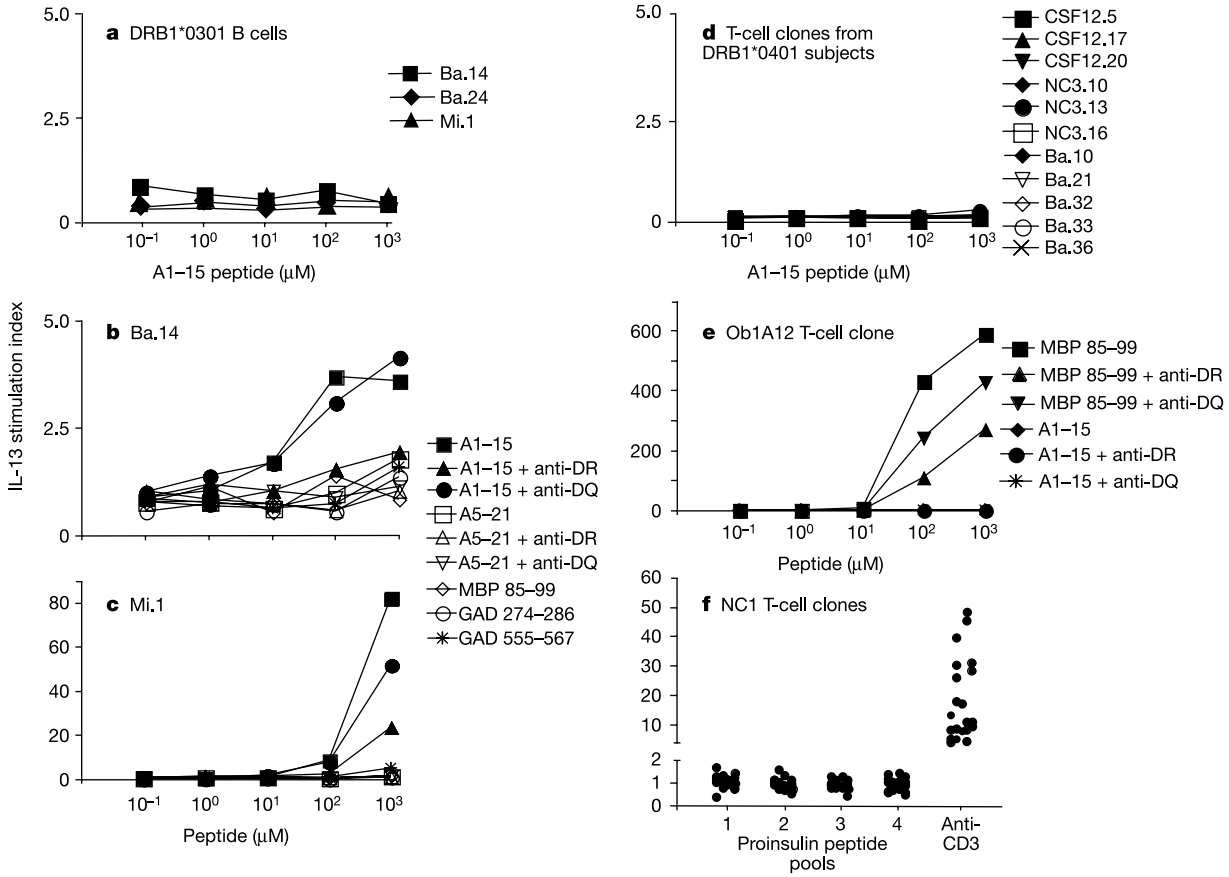

Figure 2 Specificity of peptide- and T-cell-clone-reactivity. **a**, Expanded T-cell clones from diabetic subjects 1 and 2 do not recognize insulin A 1–15 peptide in the context of QBL B cells homozygous for DRB1*0301. **b**, **c**, Expanded T-cell clones Ba.14 (**b**) and Mi.1 (**c**) did not respond to increasing doses of insulin A 5–21, MBP 85–99, or GAD65 peptides 275–286 or 555–567 presented by the MHC DRB1*0401 allele (Priess B cells). **d**, T-cell clones derived from the cerebrospinal fluid of a multiple sclerosis patient (CSF12.5, 12.17 and 12.20) or from the PLN of a DRB1*0401-expressing normal control subject (NC3.10, 3.13, 3.16) did not recognize insulin A 1–15 peptide presented by DRB1*0401. Five non-oligoclonally expanded T-cell clones from diabetic subject 2 were not reactive with insulin A 1–15 peptide, including clone Ba.32 (which has the same V_β chain as Ba.14 and Ba.24, but with a different CDR3 region and another V_α chain, V_α25.1). **e**, A T-cell clone of known specificity derived from the peripheral blood of a multiple sclerosis patient (Ob1A12 recognizing MBP 85–99 presented by DRB1*1501 and DRB1*0401) did not respond to insulin A 1–15 peptide presented by DRB1*0401. **f**, None out of 21 T cell clones (including three expanded clones) from the PLN of the type 2 diabetic subject NC1 (Table 2) recognized pools of proinsulin peptides in the context of autologous pancreatic lymph node cells. However, they did incorporate thymidine and secrete IFN-γ and IL-13 in response to anti-CD3 stimulation (proliferation and IFN-γ secretion not shown). Pool 1, insulin A chain peptides 1–15 and 5–21. Pool 2, insulin B chain peptides 1–15 and 5–20. Pool 3, insulin B chain peptides 10–25 and 15–30. Pool 4, proinsulin-specific peptides P25–30, P35–50, P45–60 and P55–70). Each peptide was present at 250 μM.

antigens and destroying antigen-expressing cells in patients with long-standing diabetes.

Although we generated over 515 independent T-cell clones, another obvious caveat is that we examined three subjects. Other diabetic subjects might have different antigen reactivities driving the autoimmune response. It should be pointed out that intense investigation of each human subject effectively translates to examination of a single strain of mice. These experiments demonstrate the feasibility of non-biased T-cell cloning from the draining lymph node of an organ targeted in an autoimmune disease in order to identify a putative autoantigen. Our results with humans confirm those found in the NOD mouse model, identifying insulin as a critical autoantigen in diabetes.

Is insulin an important antigen—or perhaps the primary antigen—driving the autoimmune response in patients with type 1 diabetes? In the NOD model of diabetes, recent experiments have shown that gene disruption of insulin prevents the onset of diabetes[28,29]. In addition, induction of tolerance to pre-proinsulin 2 reduced the onset and degree of diabetes in NOD mice, indicating that insulin and its precursors are important in diabetes initiation. Second, experiments using a similar approach of non-biased T-cell cloning[13] similarly identified insulin as a critical T-cell antigen in the NOD model of diabetes. However, other islet antigens, such as GAD65, must be considered as crucial candidates in diabetes initiation[30]. These data, together with the results presented here, provide perhaps the most conclusive evidence possible from *in vitro* experiments that insulin is a critical antigen in the aetiology of human type 1 diabetes. Ultimately, to fulfill 'Koch's postulate' with regards to identifying autoantigens in T-cell mediated human autoimmune diseases, clinical experiments will be required in which tolerization to the antigen results in disease amelioration. □

Methods

Tissue

Pancreatic draining lymph nodes (PLN, superior and inferior pancreaticoduodenal and supra/infrapancreatic lymph nodes) and spleen were harvested from multi-organ donors or during surgery, with appropriate institutional review.

T-cell cloning and examination of antigen reactivity

Single-cell suspensions were made from PLN and spleen cells within 72 h of organ harvest, and were frozen in 10% dimethylsulphoxide in FBS and stored in liquid nitrogen until use. T cells were cloned from PLN at 0.3 cells per well with 3 μg ml^{-1} phytohaemagglutinin (PHA-P, Remel) and irradiated, allogeneic PBMCs and 20 U ml^{-1} recombinant human IL-2 (Tecin, NCI) in the presence of 10 μg ml^{-1} anti-Fas antibody (Boehringer Ingelheim) to prevent death of re-activated T cells from the PLN when activated with allogeneic feeders and PHA. Media for T-cell cultures contained 5% heat-inactivated human male AB

serum (Omega Scientific) in RPMI 1640 with 10 mM HEPES buffer, 2 mM l-glutamine, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (all from Cambrex BioScience). T-cell clones were expanded with IL-2, assayed on day 9 or 10 post-stimulation and re-stimulated as previously described[11].

Antigen reactivity was examined using irradiated (5,000 rads) Priess EBV-transformed B cells (homozygous for DRB1*0401) or QBL B cells (homozygous for DRB1*0301) pulsed with peptide (250 μM for Fig. 1a) in the presence or absence of antibody (10 μg ml^{-1} anti-DR LB3.1 and anti-DQ IVD12) for 2 h, washed and plated in triplicate at 50,000 cells per well with equal numbers of T-cell clones. Each T-cell clone was also plated onto plate-bound anti-CD3 antibody (OKT3; 0.05 μg per well) to assess the viability of each clone in each experiment. After 48 h, 20 U ml^{-1} IL-2 was added to each well. Supernatants were collected after a further 24 h for measurement by cytokine ELISA (BD PharMingen). For testing of T-cell clones from the type 2 diabetic subject NC1, irradiated, autologous pancreatic draining lymph node cells were pulsed with pools of peptides (each at 250 μM) for 2 h, washed and plated at 120,000 cells per well with 50,000 T cells. The remainder of the assay was as described above.

Sequencing of T-cell receptors

RNAs were isolated using the standard RNAzol method (Tel-Test). Complementary DNAs were synthesized using Superscript Reverse Transcriptase (Gibco) and oligo dT as the primer for reverse transcription. PCR primers for both V_α and V_β families were designed and grouped according to refs 31 and 32. PCR products were purified from agarose gel and subjected to DNA sequencing (Brigham & Women's Hospital Sequencing Facility). The V_α and V_β sequences obtained were aligned against the ImMunoGeneTics (IMGT) database.

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Enhancement of cellular memory by reducing stochastic transitions

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On induction of cell differentiation, distinct cell phenotypes are encoded by complex genetic networks[1–3]. These networks can prevent the reversion of established phenotypes even in the presence of significant fluctuations. Here we explore the key parameters that determine the stability of cellular memory by using the yeast galactose-signalling network as a model system. This network contains multiple nested feedback loops. Of the two positive feedback loops, only the loop mediated by the cytoplasmic signal transducer Gal3p is able to generate two stable expression states with a persistent memory of previous galactose consumption states. The parallel loop mediated by the galactose transporter Gal2p only increases the expression difference between the two states. A negative feedback through the inhibitor Gal80p reduces the strength of the core positive feedback. Despite this, a constitutive increase in the Gal80p concentration tunes the system from having destabilized memory to having persistent memory. A model reveals that fluctuations are trapped more efficiently at higher Gal80p concentrations. Indeed, the rate at which single cells randomly switch back and forth between expression states was reduced. These observations provide a quantitative understanding of the stability and reversibility of cellular differentiation states.

Complex gene and protein networks store cellular memory by creating two or many discrete, stable states of network activity[4–6]. The generation of bistability by simple feedback loops in synthetic circuits is well understood[7–10]. However, naturally occurring networks, in particular in eukaryotic organisms, possess a complex organization of multiple nested feedback loops, making an analysis of system dynamics disproportionately more complicated[11,12]. These networks are exemplified by the galactose signalling pathway