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The use of genomewide ENU mutagenesis screens to unravel complex mammalian traits: identifying genes that regulate organ- specific and systemic autoimmunity

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Summary: T-cell development is perhaps one of the best understood processes of mammalian cell differentiation, as many of the genes and pathways have been identified. By contrast, relatively little is known about the genes and pathways involved in immunological tolerance to self-antigens. Here, we describe the challenges associated with a genome-wide screen designed at identifying new immune regulatory genes that uses a model of organ-specific autoimmunity leading to type 1 diabetes. The successful propagation and identification of the new gene variants will shed light on the various developmental checkpoints in lymphocyte development that are crucial for establishing tolerance to self-antigens.

Introduction

The adaptive immune system comprises a large repertoire of antigen-specific T and B lymphocytes that have the potential to respond to any microbial pathogen. The immune system is faced with an enormous task of ensuring that all of the T and B cells it generates express antigen receptors that have high affinity for foreign proteins and do not respond to self-proteins. The functional silencing and removal of 'forbidden' self-reactive lymphocytes is referred to as immunological tolerance. Failure of self-tolerance leads to life-threatening autoimmune diseases.

During their development, immature T and B cells must negotiate several developmental checkpoints that ensure that they do not express an antigen receptor with high affinity for self-antigen that occurs in the thymus for T cells and the bone marrow for B cells (1). Forbidden self-reactive clones are instructed to die by apoptosis, and this process is referred to as clonal deletion. It is understood that more than 95% of thymocytes fail to progress to the final stages of T-cell maturation. However, not all self-reactive lymphocytes are deleted,

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and a small percentage of forbidden clones escape and enter the periphery. The fact that <7% of the population develops autoimmune disease illustrates that the immune system has developed a number of effective mechanisms to control the fate of self-reactive lymphocytes (1).

Cellular checkpoints in immunological tolerance

The immune system relies on a range of cell-intrinsic and -extrinsic mechanisms to limit the activation and differentiation of self-reactive lymphocytes. The discussion in this review is focused mostly on the mechanisms related to T-cell tolerance.

Clonal deletion

Clonal deletion is the primary mechanism used to remove self-reactive T cells during their development. The T-cell receptor (TCR) recognizes processed peptides presented in association with major histocompatibility complex (MHC) class I or class II molecules on the surface of cortical epithelial cells, and the strength of the signal delivered to the thymocytes will determine whether the cell dies or is allowed to complete its maturation (2). Most CD4⁺CD8⁺-double-positive (DP) thymocytes die by neglect, as they fail to express a TCR that can bind to self-MHC molecules. Less than 5% of the T cells survive beyond the DP stage, and following positive selection on self-peptide/MHC complexes, thymocytes migrate to the thymic medulla where their TCRs are continually assessed for self-reactivity by binding to self-peptide/MHC complexes expressed on medullary thymic epithelial cells (MTECs) or myeloid-derived dendritic cells (3). These specialized antigen-presenting cells (APCs) express the B7 molecules CD80 and CD86, and delivery of a strong TCR signal triggers death of the T cell (3). The importance of TCR and CD28 signaling in mediating negative selection and the generation of regulatory T cells in the thymus has only been recently appreciated. Thus, CD28 co-stimulation enhances DP cell apoptosis to low levels of TCR stimuli *in vitro* (4), and deficiency of CD28 dramatically exacerbates autoimmunity in a genetically susceptible background (5). In addition, NOD (non-obese diabetic) mice that lack expression of CD86 are protected from type 1 diabetes (T1D) but instead develop an autoimmune neuropathy, which is thought to arise from a failure to delete a population of neural-specific auto-reactive T cells in the thymus (6).

A specialized subset of MTECs has been identified in the thymus that can ectopically express tissue-specific antigens in the thymus, and the peptide/MHC ligands they present at the

cell surface are critical for ensuring tolerance to organ-specific antigens during T-cell development (7, 8). The ectopic expression of organ-specific antigens is controlled by the autoimmune regulator gene (AIRE), and loss of AIRE in humans causes autoimmune polyendocrine syndrome type 1 (APS-1), a rare monogenic disorder that results in widespread autoimmunity affecting a number of target organs (9–11). AIRE is expressed in a subset of thymic epithelial cells, and loss-of-function mutation in the AIRE gene in mice leads to a milder autoimmune disease phenotype and results in a failure to eliminate forbidden self-reactive T cells that enter the periphery to cause disease (12–15).

Defects in negative selection may also arise by a reduction in thymic expression of individual tissue-specific antigens due to variation in the promoter sequence of the gene, as illustrated by the human insulin-dependent diabetes mellitus 2 (IDDM2) locus, where susceptibility to anti-insulin autoimmunity is associated with a promoter variant that decreases thymic insulin expression by several fold (16, 17). Inherited variation in the efficiency of thymic presentation of tissue-specific peptides, determined by particular MHC allelic variants such as I-A^{g7} in NOD mice or HLA-DQ in humans (18), may also diminish thymic deletion. Thus, an accumulation of various genetic defects such as those affecting AIRE, MHC, and tissue expression of target antigens may act synergistically to disrupt negative selection of forbidden self-reactive T cells and thus precipitate autoimmune disease.

Although a lot is known about the cellular events arising from positive and negative selection in the thymus, there is still relatively little known about the intrinsic cellular signaling events that result in clonal deletion of self-reactive T cells (3). A role for ζ -associated protein of 70 kDa (ZAP-70) was identified through identification of a mouse strain carrying a point mutation in the Src homology 2 (SH2) domain of ZAP-70 (19). The mutation leads to defective deletion of arthritogenic T cells, resulting in autoimmune arthritis and joint destruction. There is no single mitogen-activated protein kinase (MAPK) pathway associated with either positive or negative selection of thymocytes (20). However, a rapid and transient burst of extracellular signal-regulated kinase (ERK) signaling as well as sustained activity of p38 and c-Jun N-terminal kinase (JNK) kinases acting downstream of TCR favors negative selection that is dependent on the SH2 adapter protein Grb2 and misshapen–Nck interacting kinase (NIK)-related kinase (MINK) (20–22). Exactly how the TCR is able to regulate ERK distinctly from the p38 and JNK kinases during positive and negative selection is not known. The BH3-only protein BIM is an important effector protein mediating clonal

deletion of thymocytes. Bim is upregulated during negative selection and antagonizes the prosurvival effects of Bcl-2 (23). Loss of Bim expression leads to a failure in negative selection, and this loss has been postulated as the basis of the negative selection defect in thymocytes of NOD mice (24). The nuclear transcription factor Nur77 is also involved in mediating negative selection of thymocytes, and a dominant negative Nur77 transgene in mice leads to a failure of clonal deletion (25).

Once autoreactive T cells leave the thymus, it appears that they undergo apoptosis via pathways that are in part distinct from those used by thymocytes. Peripheral T cells use the death receptor protein FAS and tumor necrosis factor receptors (TNFRs). These proteins contain death receptor domains and associate with adapter proteins such as Fas-associated death domain (FADD). These adapter proteins can activate downstream caspases to initiate apoptosis. The FAS/FAS ligand (FASL) signaling is not absolutely required for negative selection of thymocytes, but it can facilitate negative selection to high-affinity antigens (26–28). Deletion of numerous caspases has no effect on negative selection of thymocytes (3). However, the loss of FAS or FASL in both mice and humans has highlighted the essential role these molecules have in peripheral immune regulation, as mice lacking these genes develop severe autoimmune syndromes (29–31).

Antigen receptor tuning

There are several cell-intrinsic mechanisms used to regulate autoreactive cells in both primary and secondary lymphoid tissues. The development of T-cell anergy is associated with downmodulation of TCR expression at the cell surface and a distinct pattern of signaling downstream of the TCR involving activation of nuclear factor of activated T cells (NFAT) but not JNK, activator protein-1 (AP-1), or NF- κ B. Thus, chronic, suboptimal antigen receptor stimulation can induce an inhibitory feedback mechanism that selectively uncouples the TCR from downstream immunogenic signaling pathways by acting at the level of the early TCR signalosome (32). This process is thought to be critical in the establishment of anergy to help dampen or tune down the TCR signaling in response to chronic stimulation to self-antigens. A family of ubiquitin ligases, including CBL-B, ITCH, GRAIL, and NEDD4, are involved in the internalization of TCR from the cell surface and the targeting of specific signaling proteins for ubiquitin-mediated degradation (33). Studies in mice have identified that loss of *cbl-b* can uncouple the co-stimulatory requirements for naïve T-cell activation, and the dual loss of *cbl-b* and the related *c-cbl* can lead to a large number of activated T cells that can predispose to the development of autoimmunity in mice

(34, 35). The important immune regulatory function of CBL-B identified in murine T cells facilitated its identification as a diabetes susceptibility gene in the Komeda diabetes-prone rat strain (36). Further work is required to understand how CBL proteins function with the other ubiquitin ligase proteins to mediate T-cell anergy and also how the early growth response genes 2 and 3 (*egr2*, *egr3*) facilitate the induction of the anergic program in T cells (37). There is no clear indication that mutations in the different ubiquitin ligase genes predispose to autoimmunity in humans. It could be that an accumulation of heterozygous mutations in a number of key proteins that regulate TCR tuning could be sufficient to disable the anergy program and cause autoimmunity.

The cell surface molecule cytotoxic T-lymphocyte antigen-4 (CTLA-4) is a potent inhibitor of T-cell activation through its ability to bind with high affinity to the B7 ligands CD80 and CD86 and to deliver an inhibitory signal to the T cell (38). The loss of CTLA-4 has a profound effect on immune tolerance, leading to a massive lymphoproliferative disease and neonatal death (39). The CTLA-4 molecule is expressed constitutively on the surface of regulatory T cells (40, 41), and the widespread autoimmunity observed in CTLA-4 knockout mice is thought to arise from the loss of inhibitory signaling in activated T cells as well as the loss of regulatory T-cell function.

Regulatory T cells

Regulatory T cells play a crucial role in the maintenance of immune homeostasis and self-tolerance, and they are highly enriched within the CD4⁺CD25⁺ population, which constitutes approximately 5% of the mature CD4⁺ cell progeny (42). The recent discovery that the *foxp3* gene is a master gene that directs the formation of CD4⁺CD25⁺ cells *in vivo* and that expression of this transcription factor is exclusive to this cell population has provided genetic evidence that they represent a unique and specialized subset of CD4⁺ T cells that function to suppress immune responses (43, 44). CD4⁺CD25⁺ T cells were first defined in mice by Sakaguchi and colleagues (42), and these cells are found in the peripheral circulation of humans. In both humans and mice, CD4⁺CD25⁺ cells play a critical role in maintaining the long-term health of the individual (42). Patients with X-linked autoimmunity-allergic dysregulation syndrome (XLAAD) or immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) as well as the *scurfy* mouse have a common loss-of-function mutation in *foxp3* that leads to a failure of CD4⁺CD25⁺ cell differentiation, and affected males develop a severe lymphoproliferative disease with a multiorgan inflammatory response

and autoimmunity (42). Although IPEX and XLAAD affect only a small proportion of people throughout the world, the fact that these individuals develop life-threatening autoimmune diseases highlights the importance of CD4⁺CD25⁺ T cells to human health. Despite their significant role in immune homeostasis, many questions remain as how regulatory T cells are selected in the thymus in response to high-affinity self-antigens and more importantly how they mediate suppression *in vivo* (45).

Genomic approaches to identify new autoimmune regulatory genes

The formation of T cells is perhaps one of the best understood processes in mammalian cell differentiation, as many genes and proteins that regulate this process have been identified (46). Relatively little is known, however, about the genes and proteins that regulate immunological tolerance. As described above, the identification of mouse strains with spontaneous mutations in key immune regulatory genes has been central by providing insight into the mechanisms of immune tolerance and defining autoimmune susceptibility genes in humans. Progress hinges upon new mouse strains with illuminating variants in autoimmune susceptibility genes, yet this approach is limited, because the rate of natural variation in animal colonies is low. Targeted mutations by homologous recombination in embryonic stem cells or RNA interference have been used to introduce germline mutations, but this strategy relies upon prior knowledge of the targeted gene that may be of immunological significance. Moreover, the complete ablation of a protein can obscure insights into its autoimmune regulatory actions, as illustrated by the contrasting effects of Zap70 knockout and point mutation alleles (19, 47, 48). To correct the deficit of new strains, we need to be able to produce new Mendelian mouse variants that either develop autoimmunity or display altered immune regulation, without depending upon prior knowledge about candidate genes or the way their sequence needs to be altered to reveal their function.

The alkylating agent N-ethyl-N-nitrosourea (ENU) is a very efficient chemical mutagen of mouse spermatogonial stem cells that is able to introduce single-point mutations at a rate between 1/100 000 and one in two million base pairs that usually affects single loci (49, 50). More than 80% of ENU-induced mutations are either AT to TA transversions or AT to GC transitions (51). Two-thirds of the variants arising from ENU treatment result in a change in a single codon resulting in an amino acid replacement, while the rest of the variants arise due to splicing errors or null alleles. The rate of mutation induced by ENU is about 100-fold higher than the rate of spontaneous mutations and about three-fold higher than that achieved by X-irradiation. ENU treatment

yields a new loss-of-function allele in any given gene, on average, one per 1000 gametes, and therefore screening 1000 first-generation (G1) offspring should yield at least one heterozygous loss-of-function mutation in the majority of immune regulatory genes (52, 53).

The gene knockout approach tends to result in the loss of expression of the whole protein. This loss can obscure key immunoregulatory functions of the protein, especially if it must interact with other proteins to mediate its function. Alternatively, naturally occurring mutations that give rise to missense alleles can disrupt important functional domains within proteins and lead to 'separation-of-function' alleles. These new variant alleles can reveal more information about a protein that would not have been previously identified had the gene been knocked out. There are now several examples where single-base substitutions in the mouse genome sequence, by causing amino acid substitutions in ZAP-70, Ikaros, linker for activated T cells (LAT), and Carma-1, have revealed inhibitory roles of these proteins in immune regulation that were obscured by complete protein ablation (19, 54–57).

To perform a genomewide screen with single-base substitutions, male founder mice are treated with ENU and mated with wildtype mice to yield G1 progeny. The G1 progeny themselves or their second-generation (G2) offspring can be screened for dominant variants. Alternatively, the progeny of individual G1 mice can be inbred for two generations so that recessive variants can come to homozygosity in their G3 progeny. Large-scale dominant screens have been carried out by several centers around the world that have focused on phenotypes ranging from visible phenotypes to immunological, biochemical, and behavioral phenotypes (58, 59). Other groups like our own have taken a more focused approach to address a key biological process such as immune regulation. The Beutler group (60–63) has used a genome mutagenesis screen to make remarkable advances in our understanding of the genes involved in regulating innate immune mechanisms. We have used genomewide screens designed to identify genes involved in lymphocyte differentiation as well as screens for systemic autoimmune diseases, immunization responses (54, 57, 64–66), and as we discuss here, a sensitized screen that encompasses a model of organ-specific autoimmune disease resulting in the development of T1D.

Rationale for a sensitized screen to identify autoimmune regulatory genes in diabetes

While entirely novel immune regulatory mechanisms are revealed by sequence variants induced in a simple inbred

strain such as C57BL/6 (65, 66), two factors constrain this approach. First, for diseases such as T1D, loss of function in important regulatory genes will often be insufficient to precipitate diabetes on their own. This point is illustrated by the Komeda diabetes-prone rat model, where homozygosity for a stop codon in *cbl-b* must be combined with a susceptible MHC haplotype and other loci for autoimmune diabetes to manifest (36). Similarly, AIRE deficiency in mice only leads to autoimmune gastritis or T1D when combined with a sensitized/susceptible genetic background (15, 67). Thus, a solution to this constraining factor is to perform ENU mutagenesis in an already diabetes-sensitized strain, where defects in individual regulators will be obvious.

The second constraining factor stems from the fact that many genes that contribute to protecting us from autoimmune diabetes will also perform other essential functions. Homozygous loss of function in these genes will often cause embryonic or neonatal lethality and hence fail to be detected as diabetes regulators. A solution to this constraint has been established in *Drosophila*, by performing mutagenesis in a sensitized strain where the process of interest (e.g. eye patterning) has already been partially crippled (68, 69). In these sensitized stocks, loss of a single copy of a gene supporting the process is now sufficient to disrupt that process, while remaining sufficient for development and viability. As described here, we have established a diabetes-sensitized indicator strain of mice, which reveals heterozygous loss-of-function variants in single genes that normally would be recessive, lethal, or undetected.

The TCR:InsHel model

The 3A9 TCR transgenic mouse is the basis of our sensitized screen to identify autoimmune regulatory genes in mice. The TCR encoded by this transgene recognizes with high affinity the immunodominant peptide of hen egg lysozyme (HEL) 46-61 presented in association with I-A^k. Several partner strains of transgenic mice have been engineered to express the HEL protein as a neo-self-antigen under different tissue-specific promoters (Table 1). We have focused on the well-characterized TCR:InsHel double transgenic combination for the purposes of the sensitized screen (70, 71), because it is on the brink of developing T1D, such that heterozygous defects in single genes (e.g. in *Roquin*, *Aire*, and *cbl-b*) are sufficient to precipitate overt diabetes (15, 65, GH and CG, unpublished data), as are single-gene defects in thymic deletion, regulatory T-cell formation, and peripheral anergy.

The TCR:InsHel strain is ideally suited as a model to the study of the development of immune tolerance to an organ-specific self-antigen. The neo-self-antigen HEL is highly expressed on pancreatic β cells and is expressed in an AIRE-dependent manner in thymic medullary epithelium. While there is no insulinitis or autoimmunity in single transgenic insHEL or TCR animals, in TCR:insHEL-double-transgenic (Dbl) mice, the pancreatic islets become infiltrated with leukocytes within 3–4 weeks of age yet do not progress to diabetes in the majority of animals. This preclinical phase of insulinitis occurs only with the TCR:InsHel mice and is similar to the preclinical disease process that occurs in NOD mice (72). To generate Dbl (TCR:InsHel) mice, 3A9 TCR mice are mated with InsHel transgenic mice. Both transgenes must be kept in a hemizygous state, requiring one of two breeding pair combinations: (i) TCR $\times$ InsHel or (ii) Dbl $\times$ non-transgenic mice. As the transgenes segregate independently at each generation, offspring are of four equally frequent genotypes: non-transgenic, TCR, InsHel, or Dbl. Only the Dbl offspring are sensitized to diabetes, allowing new diabetes susceptibility gene variants to be propagated from healthy carriers among the non-sensitized siblings.

On a wildtype B10.BR background, we observe a background incidence of diabetes at a frequency of 20% of the Dbl mice (72). Those animals that develop diabetes do not appear to have a complete breakdown of tolerance to insHEL, as they do not produce immunoglobulin G (IgG) antibodies to the HEL protein. By contrast, TCR:InsHel mice bred on the NOD-H2^k background do exhibit a complete breakdown in T-cell tolerance, with >90% of Dbl animals developing diabetes, formation of germinal centers in the pancreatic islets, and secretion of high titers of anti-HEL IgG in the serum (72). A further advantage of the 3A9 model is that we can track the fate of the Hel-reactive CD4⁺ T cells in vitro and in vivo with the use of a TCR clonotype-specific monoclonal antibody (1G12) to determine the frequency of Hel-reactive T cells in either TCR or TCR:InsHel mice using flow cytometry (14, 72). This analysis provides an early insight into the cellular mechanisms that may be disrupted by new gene variants identified in the screen.

Pedigree structure to screen for gene variants that precipitate T1D

The majority of sequence variants that are generated by ENU treatment will be recessive mutations, and to identify new sequence variants in ENU-treated gametes, we use a three-generation breeding structure to bring recessive mutations to

Table 1. Properties of T-cell deletion in different TCR × Hel transgenic mouse strains

Strain	Transgenic promoter	Tissue expression	Stage of negative selection
TCR: InsHel	Rat insulin	Pancreatic β- cells and thymus	Early and late CD4 SP
TCR: thyr:hel	thyroglobulin	thyroid	DP and early CD4 SP
TCR: Membrane:Hel	Class I MHC (H2 ^k)	All somatic cells	DP
TCR: Soluble:Hel	metallothionein	Peripheral circulation	Early and late singel positive CD4

homozygosity (Fig. 1). By establishing large numbers of G3 pedigrees, it is possible to simultaneously screen hundreds of genes for their autoimmune regulatory potential. The C57BL/6J strain is routinely used in ENU mutagenesis. Their spermatogonial stem cells are exquisitely sensitive to the alkylating agent ENU, and the male mice readily regain their fertility (52, 53). Following ENU treatment, founder G0 B6 male mice (prepared in batches of 20–50) are bred with female B10.BR (H2^k) TCR:InsHel transgenic mice (Fig. 1). Each G1 mouse is estimated to carry an independent set of approximately 30 loss-of-function variants on the paternal chromosomes, based on the average of new loss-of-function variants per gamete (1/1000) and the number of mammalian genes (approximately 30 000 genes). The TCR, *InsHel*, and H2 genes, as well as the variant alleles induced by ENU, all segregate independently within the offspring at each generation. All G1, G2, and G3 mice are genotyped for HEL, TCR, and H2 by polymerase chain reaction (PCR) tests performed on ear-punch biopsies prepared in bar-coded 96-well plates. As we are restricted to breeding TCR:InsHel × non-transgenic or

TCR × InsHel mice, we use a brother × sister pairing with the complementary genotype at each generation until we reach G3. The G3 mice are screened for the onset of T1D, which is identified by the presence of a wet cage with a cutoff at 24 weeks of age.

Diabetic animals in the ENU screen are first identified by the presence of a wet cage. The affected mouse is tested for urine glucose by diastix, and the levels of blood glucose are also monitored. Unless treated, diabetic mice become unwell and begin to lose weight within several weeks, precluding any possibility of breeding to transmit the desired gene variants that they carry. This problem has been solved neatly by establishing a diabetes curative regime that enables the long-term health and breeding of individual diabetic mice that carry variant alleles of autoimmune regulatory genes. Once the diagnosis of T1D is made, the affected animals initially receive daily subcutaneous insulin injections to stabilize their blood glucose. This treatment prevents weight loss and allows growth, but they usually do not breed. To cure the animals of diabetes permanently, they receive a pancreatic islet transplant

Fig. 1. Pedigree structure for the ENU type 1 diabetes screen. The three-generation breeding structure is designed to bring recessive mutations to homozygosity in G3 animals. G1 mice that carry about 100 loss-of-function alleles are intercrossed with female G1 mice by breeding TCR:InsHel × non-transgenic or TCR × InsHel mice. ENU, N-ethyl-N-nitrosourea.

from syngeneic non-transgenic donor mice, given under the kidney capsule. Following surgery, the animal's blood glucose levels return within the normal range within 1–2 weeks (Fig. 2). As the transplanted islet cells are derived from non-transgenic donors, they do not express HEL antigen and are ignored by the transgenic autoreactive T-cell repertoire. It should be noted that the diabetic animals also receive no other form of immune suppression after transplant. Our experience is that this maneuver cures diabetes long-term, so that the mice begin to breed within 2–4 weeks of surgery and thrive to 12–18 months of age.

Criteria for screening for T1D

A challenge posed by most sensitized screens is the inevitable existence of a background level of phenocopies due to non-genetic sources of variation. In our case, this background is manifest by a 20% incidence of diabetes in wildtype TCR:InsHel mice, with onset around 8–10 weeks of age (72) (Fig. 3). We employ four strategies to filter out this source of false-positive results. First, high priority is given to animals that develop diabetes at <7 weeks of age, as this time of onset is very rare in the wildtype stock (Fig. 3). Second, high priority is given to animals that are found by enzyme-linked immunosorbent assay (ELISA) to have IgG antibodies against HEL in their serum, as this does not occur as part of the background diabetes in the wildtype stocks (72). In addition to testing each mouse that develops diabetes, healthy G3 Dbl mice are routinely screened for the presence of anti-HEL IgG antibodies at 8–10 weeks of age to predict those animals that may be at risk of developing T1D. Any animals scoring positive in the primary screen are re-bled to confirm their

positive test, and upon confirmation, the mice are used for breeding. Third, we give high priority to Dbl animals of H2^{kb} genotype that develop diabetes, as there is <3% incidence of diabetes in wildtype H2^{kb} Dbl mice. Positive selection of the 3A9 TCR is inefficient in H2^{kb} heterozygous animals, so that there is a larger repertoire of other TCR α chains that normally prevents any progression to diabetes. Fourth, animals with a high frequency of Dbl siblings that develop diabetes are given higher priority, as this familial clustering suggests heritable variants, especially for dominant or semidominant alleles. Finally, the diabetic animals are progeny-tested after being cured by islet transplantation, with the expectation that 50% of their Dbl offspring will be diabetic. This frequency is expected either if they carry a dominant trait that has been outcrossed to B10.BR or if the index diabetic animal was homozygous for a recessive variant and was intercrossed with a G3 sibling that is likely to be a heterozygous carrier.

As the same criteria must also be able to be applied to map the variants in an intercross with another inbred strain, we also have established the background incidence in wildtype stocks of Dbl (TCR:insHEL) mice intercrossed and backcrossed to the CBA/H strain. To date, the B10.BR $\times$ CBA/H Dbl mice backcrossed four generations on the CBA background display an identical disease incidence (Fig. 3) to the B6 $\times$ B10.BR Dbl animals, and the background incidence of diabetes on the mixed or backcross background is also not accompanied by secretion of anti-HEL IgG antibodies.

Further improvements to the sensitized screen

The establishment of a phenotypically matched counterpart of CBA/H TCR:insHEL animals, as described above, has in

Fig. 2. Monitoring of blood glucose in Dbl (TCR:insHEL) diabetic variant mice following non-transgenic islet transplant. The figure shows the blood glucose measurements of 12 different diabetic Dbl animals identified through the ENU screen. Typically, the diabetic animals have a blood glucose level of >20. Following transplant under the kidney capsule of syngeneic islet cells, the blood glucose returns to a normal level (<10) within a week of the transplant and remains normal for the life of the animal without any immuno-suppression. ENU, N-ethyl-N-nitrosourea.

Fig. 3. Background incidence of diabetes in wildtype Dbl (TCR:InsHel) mice. The figure shows the diabetes incidence curves for Dbl mice on the B10.BR background and different generations of backcrossing the transgenes to the CBA background. On average, the disease onset occurs between 8 and 10 weeks of age with a peak incidence of 20–25%, and none of the diabetic animals generate anti-Hel IgG antibodies. Thus, in the mutagenized stocks, high priority is given to variant animals with diabetes onset at <7 weeks old and/or accompanied by anti-HEL IgG.

turn enabled two additional improvements in the screen to accelerate each step of the screen. As illustrated in Fig. 4, rather than constructing libraries as an intercross between ENU-treated B6 mice and TCR:insHEL transgenic B10.BR mice, the latter are now substituted by CBA/H TCR:insHEL animals. As a result, the G1 founders of each pedigree are (B6 × CBA)F1 hybrids, which has a powerful effect on their breeding performance by introducing a high level of hybrid vigor. These G1 founders are backcrossed with wildtype CBA/H animals of complementary TCR and insHEL genotype. Their G2 offspring are (B6>CBA)N2 backcross animals, retaining strong hybrid vigor when they are in turn mated to their G1 parent, so that the yield of G3 mice from each mating is greatly enhanced compared to B6 × B10.BR pedigrees. This mating enables pedigrees to reach the target number of G3 Dbl indicator offspring much more quickly and efficiently. The second advantage of this strategy is that the G3 animals are already at a mapping generation when they are discovered, enabling a chromosomal assignment either at this point or in their immediate offspring from progeny testing. A variation on this approach, which has also proved successful (used in a library called ENU 6CAT), has been to induce single-nucleotide substitutions in CBA/H mice and then intercross with B10.BR TCR:insHEL animals to yield pedigrees with vigorous breeding and early mapping in the same way.

Yield from the sensitized diabetes screen

We have screened 261 G3 pedigrees and identified 42 strains with demonstrated heritable increase in diabetes susceptibility (Table 2). This finding represents a 16% hit rate per pedigree, which is higher than the 10% hit rate obtained for screens on lymphocyte abnormalities in blood lymphocyte subsets and lower than the 30% hit rate obtained with screens for an early surrogate indicator of susceptibility to systemic autoimmunity based on presence of anti-nuclear antibodies in serum (46, 66). The majority of strains 36/42 (or 86%) identified in the T1D screen behave as recessive mutations, whereas 6/42 or (14%) are dominant traits, which means that the loss of a single copy of the variant allele is sufficient to induce diabetes (Table 2). Approximately 30% of the T1D strains produce anti-HEL IgG antibodies, possibly indicating mutations in genes that control peripheral regulation of HEL-reactive CD4⁺ T cells.

Mapping and sequencing the new diabetes-susceptible variants

Once a true breeding diabetes variant is identified, the process of linkage mapping through intercrosses follows a strategy of outcrossing the mice to the CBA/H strain to provide the genetic polymorphisms that will allow the use of simple sequence length polymorphisms (SSLPs) or single-nucleotide polymorphisms (SNPs) to initially identify chromosome linkage (66, 73). The choice of the mapping strain is dictated by the necessity to maintain the correct H2 haplotype to enable the 3A9 TCR to undergo efficient positive selection. It is for this reason that the CBA/H strain has been used as the mapping partner for the ENU 4AT, 5AT, and 6AT libraries, as mutations in each of these libraries are transmitted on chromosomal DNA originating from the founder C57BL/6 (H2^b) parent. For the ENU 6CAT library, we use the B10.BR strain, as the mutations are carried on a CBA chromosomal segment originating from the G0 parent.

We can determine in the first backcross to the mapping strain whether or not the mutation is dominant, and if so, we continue to backcross the F1 mice onto CBA mice to generate an N2 generation. If no diabetes is observed in the F1 progeny, we assume that the mutation is recessive, and the F1 siblings are mated as TCR:InsHel × non-transgenic or TCR × InsHel. The F2 progeny are scored for T1D. We expect 25% of the F2 offspring to be homozygous for the mutation and only 25% of the offspring to be a TCR:insHEL mouse. Therefore, we expect only 1/16 F2 mice to be a TCR:InsHel mouse and to carry a homozygous mutation. We need to

ENU8CAT pedigrees founded by G1 male

Fig. 4. An improved pedigree structure to enhance the identification and mapping of new autoimmune variant strains in the latest ENU library. ENU, N-ethyl-N-nitrosourea.

Table 2. Summary of diabetes screening in multiple G3 progeny

Disease		4AT	5AT	6AT	6CAT	Total
Type 1 diabetes	Number of G3 pedigrees screened	105	54	62*	40*	261
	Number of diabetic strains confirmed	10	3	15	14	42
	Number of recessive mutations	9	3	13	12	36
	Number of dominant mutations	1	0	2	3	6
	Number of anti-Hel IgG ⁺ strains	5	0	5	3	13
Type 2 diabetes		0	0	2	0	2

* These libraries have not finished G3 screen

breed at least 64 F2 progeny from one breeder pair to be confident that we score enough F2 TCR:InsHel mice to ensure that the mutation is heritable.

We have used two different strategies to map mutations in the ENU-induced variant mice. The first strategy uses a pooled DNA approach. DNAs from 15–20 affected F2 TCR:InsHel

mice are tested for a high proportion of B6-derived chromosomal regions by PCR amplification and agarose gel of SSLP markers spaced at about 20 cM, which gives between three and four markers per chromosome. Alternatively, if there are fewer than 10 affected TCR:InsHel F2 mice, we can screen the individual mice for the various SSLP markers at the ends of the

chromosome using the method of Beier (49). The linked interval is confirmed and narrowed by individually typing each mouse sample with additional SSLP makers that span the region of linkage. We have also developed a mapping method based on SNPs that naturally occur between the different strains (e.g. B6, B10, and CBA/H). This latter technique is a fluorescence-based assay and takes away the requirement of agarose gel electrophoresis completely. The fluorescent SNP analysis can be used in exactly the same way as the original SSLP markers, i.e. as a pooled strategy or screening of individual affected mice.

Once a region of linkage is identified, it is necessary to reduce the interval to within 1–2 Mb. This reduction requires screening subsequent F2 or N2 mice for two flanking markers that span the region of interest to identify useful recombinants that can help narrow the interval. It is possible to use a candidate gene approach to examine genes within the interval that might be expected to give a phenotype similar to that of the mutant animal, and it is possible to sequence the gene(s). Once the region has been reduced to 1–2 Mb, the region will hopefully contain <30 genes, and all of the mRNAs encoded in the interval may be sequenced. PCR amplification is used to generate overlapping 700-bp fragments of the candidate gene sequence, and these are gel-purified and sequenced. Typically 75% of ENU variants have single-nucleotide substitutions that lead to amino acid substitutions in the primary amino acid sequence. These can disrupt functional domains on the protein, and 25% can be alterations in mRNA splicing.

Phenotypic analysis of the T1D mutant mice

The primary aim following screening of affected G3 mice is to establish a true breeding strain. Until we have established the strain, minimal phenotypic analysis is performed on the variant strains. Once established, the mutant diabetic animals are screened by fluorescence-assisted cell sorting (FACS) analysis to determine what effect the mutation might have on negative selection in the thymus, using an approach similar to that used by Liston *et al.* (14, 72). Irradiation chimeras are employed to determine whether the mutation acts cell-autonomously, and this finding indicates whether the mutation is likely to be involved in signal reception or transduction. Alternatively, the mutation may behave non-autonomously, which may be the case if the mutation affected antigen presentation by APCs, regulatory T-cell action, or a failure to secrete a cell–cell acting cytokine.

An example is provided in Fig. 5 that shows analysis of wildtype and a diabetic mutant mouse arising in strain 4AT

24. Susceptibility to T1D in this strain is inherited in a dominant manner, but there is no significant expansion of the Hel-reactive (CD4⁺IG12⁺) T cells in the peripheral tissues of the diabetic mice. We conclude that thymic negative selection is normal in these animals and that the mutation instead affects some aspect of the peripheral regulation of the autoreactive T cells. This conclusion is supported by the finding that approximately 50% of the diabetic animals in this strain make high titers of anti-Hel IgG in their serum.

Translating the findings from mice to humans

The development of immunological tolerance is essential to prevent the development of autoimmune diseases. Although we understand much about the cellular aspects of tolerance induction to self-antigens, there is still a major gap in our knowledge about the number and identity of the genes involved in this process. Several genes that regulate clonal deletion of forbidden self-reactive lymphocytes (e.g. Aire, BIM, and MINK) and antigen receptor tuning (c-Cbl, Cbl-b, Grail, etc.) have already been defined, but hundreds more are likely to be involved in regulating self-tolerance to organ-specific and systemic self-antigens. By generating new mouse variants through controlled variation of the genome sequence with ENU, a rich resource for new animal models for the study of autoimmunity is provided that may allow us to probe more deeply into the mechanisms and pathways central to the induction and maintenance of tolerance. As the full sequence of both the mouse and human genomes is available, it should be possible to translate findings from mice to humans relatively quickly. As was the case for *foxp3* and *Fas*, defining autoimmune regulatory genes in mouse strains will illuminate orthologous human genes as candidates for sequence and functional variation that may explain human autoimmune susceptibility. As most human autoimmune diseases are likely to be polygenic, unlike that of APS-1 which is caused by a single-gene defect in *Aire* or XLAAD/IPEX caused by a mutation in *foxp3*, it may be that those patients with susceptibility to autoimmunity accumulate a number of heterozygous mutations in key regulatory genes that control a pathway or a process, and it is the sum of these mutations that act in combination to cause a breakdown in self-tolerance and autoimmunity. Thus, the information gained from analysis of each new variant mouse strain will begin to build a detailed cellular map of the key developmental checkpoints involved in lymphocyte development and in immunological tolerance, and this map should advance our understanding of the same process in humans.

Fig. 5. Example of a strain identified in the ENU 4AT library that carries a dominant mutation predisposing to T1D. (A) The genealogy tree shows only the TCR:InsHel mice born at each generation for simplicity. The filled symbols represent diabetic animals, and the striped symbols represent diabetic mice that also make anti-HEL IgG antibodies. The open symbols are non-diabetic offspring, and symbols with bars across represent TCR:InsHel offspring that died. (B) Phenotypic analysis of a wildtype TCR transgenic and a TCR:InsHel mouse compared to a mutant TCR:InsHel mouse. The top panel shows the analysis of CD4⁺ T-cell differentiation, and the lower panel shows the expression of the clonotype TCR⁺ cells at the late CD4-single-positive stage of development. The lower two panels show the frequency of CD4⁺1G12⁺ T cells in the spleen and the blood, respectively, for the different strains analyzed. (C) The diabetes incidence curve for N2 generation mice studied over a 24-week period. N2 mice with diabetes onset before 7 weeks old are used for mapping the susceptibility gene variant. ENU, N-ethyl-N-nitrosourea; T1D, type 1 diabetes.

versus CD8 T-cell differentiation, and the lower panel shows the expression of the clonotype TCR⁺ cells at the late CD4-single-positive stage of development. The lower two panels show the frequency of CD4⁺1G12⁺ T cells in the spleen and the blood, respectively, for the different strains analyzed. (C) The diabetes incidence curve for N2 generation mice studied over a 24-week period. N2 mice with diabetes onset before 7 weeks old are used for mapping the susceptibility gene variant. ENU, N-ethyl-N-nitrosourea; T1D, type 1 diabetes.

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