SHORT COMMUNICATION

Tracing the action of IL-2 in tolerance to islet-specific antigen

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Genetic variants of interleukin 2 (IL-2) and its receptor are associated with murine and human susceptibility to Type 1 diabetes, yet the role of IL-2 in controlling pancreatic islet-reactive T cells is unknown. Here, we develop a model where IL-2 deficiency precipitates a breakdown of self-tolerance and progression to diabetes, and its action upon diabetogenic islet-specific CD4 T cells can be tracked. We find that IL-2 is not required for *Aire*-dependent thymic clonal deletion of high-avidity diabetogenic clones, but is essential for thymic formation of islet-specific Foxp3-expressing CD4 T cells. The absence of IL-2 results in the expansion of low-avidity Foxp3[−] islet-reactive CD4 T cells. The mechanism by which IL-2 prevents diabetes is therefore through the establishment of a repertoire of islet-reactive Foxp3⁺ T cells within the thymus, and limitation of the peripheral activation of low-avidity islet-reactive T cells that normally escape thymic negative selection.

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Interleukin 2 (IL-2) has a well-established function promoting T-cell proliferation. Susceptibility to Type 1 diabetes is associated in humans with an inherited polymorphism in the gene encoding, one of the receptor subunits for IL-2, CD25/*IL2RA*,¹ and with variants of the *Il2* locus in the non-obese diabetic (NOD) mouse;² yet, little is known about how variation in IL-2 activity affects the control of islet-specific T cells.

Contradictory data exist about the role of IL-2 in tolerance. Lymphoproliferation and systemic immunopathology develop in knockout mice lacking IL-2 or the IL-2 receptor chains, IL-2R α (CD25) or IL-2R β (CD122).^{3–5} A role for IL-2 in negative selection has been proposed from results indicating reduced thymic apoptosis in *Il2*^{0/0} mice injected with anti-CD3 antibody.⁶ Other results limit this role to the major histocompatibility complex (MHC) class II-restricted lineage,⁷ with no effect on MHC class I-restricted lineages.^{7,8} Chimeric mice constructed with mixtures of wild type and *Il2r*-deficient bone marrow do not develop lymphoproliferation or immunopathology.^{9,10} Whereas suppression of this systemic disorder could result from IL-2-driven homeostatic T-cell proliferation, IL-2 is also needed for the development or peripheral maintenance of CD25⁺CD4⁺ regulatory T cells (T_R).^{11–13} However, use of CD25 as a marker to trace T_R is problematic since CD25 expression is promoted by IL-2 signalling. The X-chromosome encoded transcription factor, Foxp3, drives the development of CD25⁺CD4⁺ T_R^{14–16} and provides a definitive marker for this subset of cells. Opposite results have been published recently on the role of IL-2 in Foxp3⁺ T_R generation and maintenance.^{17,18} In a polyclonal repertoire, loss of

IL-2 had its primary effect on thymic formation of T_R.¹⁷ By contrast, parallel studies in TCR^{HA}:pgkHA double transgenic mice expressing a hemagglutinin (HA)-specific T-cell antigen receptor (TCR) and ubiquitously expressed HA antigen found that thymic T_R generation was independent of IL-2, whereas almost no HA-reactive T_R cells survived after thymic emigration in the absence of IL-2.¹⁸ Because the first study traced T cells of unknown specificity and the second T cells recognizing with low affinity, a ubiquitous antigen, the action of IL-2 in CD4 T cell tolerance to islet-specific antigens – which are displayed in the thymus by a specialized *Aire*-dependent pathway – remains unresolved. Here we examine the action of IL-2 in islet-specific tolerance.

RESULTS AND DISCUSSION

IL-2 is essential for controlling potentially diabetogenic CD4 T cells

To determine the role of IL-2 in CD4 T-cell tolerance to pancreatic islet antigens, *Il2*^{0/0} mice¹⁹ were crossed to 3A9 TCR^{HEL} × insHEL double transgenic mice. The 3A9 TCR transgene encodes a high-avidity TCR recognizing 46–61 of hen egg lysozyme (HEL) in complex with I-A^k that can be traced with the clonotype-specific antibody 1G12. The insHEL transgene is controlled by the rat insulin promoter and mirrors expression of insulin itself: high expression of HEL occurs in pancreatic islet β -cells, whereas expression in thymic epithelial cells depends upon *Aire*. In TCR:insHEL double transgenic mice, thymic expression deletes most clonotype-high insHEL-reactive CD4⁺ cells during the transition from CD4⁺CD8⁺ double positive (DP) to CD4⁺CD8[−]CD69[−] single positive (SP) mature thymocytes.²⁰ The

remaining clonotype-low CD4 cells are normally unable to destroy the HEL-expressing pancreatic islet β -cells and precipitate diabetes, although they promote subclinical insulinitis from an early age.^{21,22}

Homozygous *Il2* deficiency precipitated diabetes in 100% of TCR:insHEL animals (Figure 1). By contrast, insulinitis remained subclinical in the majority of TCR:insHEL animals on a wild-type background and in *Il2*^{+/-} heterozygous littermate controls, so that less

than 20% progressed to autoimmune diabetes. In the absence of pancreatic HEL expression, TCR *Il2*-deficient animals remained healthy and indistinguishable from wild-type TCR animals.

Normal negative selection of diabetogenic CD4 cells in *Il2*^{0/0} mice

To determine if IL-2 deficiency precipitated diabetes by interfering with *Aire*-dependent negative selection, thymic positive and negative selection was measured (Figure 2). HEL-specific T cells were positively selected with equal efficiency in homozygous *Il2*-deficient TCR and control animals, generating equivalent numbers and proportions of fully mature 1G12^{hi} CD69⁻ SP thymocytes (Figure 2a, b and d). Negative selection in TCR:insHEL double transgenic mice was also equally efficient in homozygous *Il2*-null mice and controls (Figure 2a, b and d). Decreased cell surface expression of TCR V β 8.2 and CD3 and increased expression of CD5 were also equivalently induced in *Il2*^{0/0} and control double transgenic thymocytes (Figure 2, and data not shown).

The lack of any requirement for IL-2 in thymic negative selection in TCR:insHEL animals provides strong evidence against a role for this cytokine in this major mechanism of organ-specific self-tolerance. The insHEL transgene has been demonstrated to mimic endogenous expression of insulin, which appears to be a major self-antigen initiating and controlling autoimmune diabetes.²³ Deletion of 3A9 TCR transgenic thymocytes in double transgenic mice is sensitive to minor changes in antigen expression and thymocyte responsiveness,²⁴ so that subtle quantitative actions of IL-2 in negative selection would have been detected. Previous data for a putative role of IL-2 in thymic negative selection have been conflicting and was performed in models

Figure 1 IL-2 deficiency causes TCR:insHEL mice to develop diabetes. TCR:insHEL double transgenic mice of B10^k ($n=40$), *Il2*^{+/-} ($n=16$) and *Il2*^{0/0} ($n=13$) genotypes were tracked for diabetes from weaning to 24 weeks of age. The incidence of diabetes in *Il2*^{0/0} double transgenic mice was greater than that in B10^k (*Il2*^{+/+}) and *Il2*^{+/-} double transgenic mice ($P<0.0001$; $P<0.0001$).

Figure 2 IL-2 deficiency does not affect thymic negative selection but cripples thymic generation of Foxp3⁺ T cells bearing an islet-specific TCR. Thymocytes from B10^k TCR transgenic and TCR:insHEL double transgenic mice with *Il2*^{+/+}, *Il2*^{+/-}, or *Il2*^{0/0} genotypes were analysed by flow cytometry. Representative profiles show staining for (a) CD4 vs CD8; (b) 1G12 (3A9 TCR clonotype) vs CD69, gated on CD4⁺CD8⁻ SP thymocytes; (c) Foxp3 vs 1G12 clonotype, gated on CD4⁺CD8⁻ SP thymocytes. (d) Efficiency of thymic negative selection in individual TCR:insHEL double transgenic mice (squares) compared with TCR transgenic controls (diamonds). Plots show the absolute number of mature CD69⁻1G12^{hi} SP thymocytes and immature CD4⁺CD8⁺ DP thymocytes in *Il2*^{+/+} (black), *Il2*^{+/-} (gray) and *Il2*^{0/0} (white) animals. All individual mice tested are represented, for TCR $n=9,6,5$, for TCR:insHEL $n=10,8,10$. (e) Individual percentage of CD4⁺CD8⁻1G12⁺ thymocytes that are Foxp3⁺, for TCR ($n=7,5,4$) and TCR:insHEL ($n=5,5,6$) mice of *Il2*^{+/+} (black), *Il2*^{+/-} (gray) and *Il2*^{0/0} (white) genotypes.

that do not reflect organ-specific tolerance. IL-2-independent negative selection was demonstrated with ubiquitous high-affinity antigens (endogenous superantigens and the male H-Y antigen^{7,25}), whereas IL-2-dependent negative selection was indicated in models which may reflect nonspecific apoptosis as a result of peripheral T-cell activation (anti-CD3 antibody or injection of peptide in transgenic mice^{6,7}).

Islet-specific T_R cells fail to develop in the thymus of *Il2*-deficient mice

A subset of CD4 cells expressing the HEL-reactive TCR (1G12⁺) resist deletion in TCR:insHEL mice (Figure 3a). Unlike the peripheral 1G12⁺ cells in TCR transgenic mice, most (~75%) of the peripheral islet-specific CD4 cells in TCR:insHEL double transgenic mice also express Foxp3, accounting for ~0.2% of peripheral lymphoid cells (Figure 3c–e). The Foxp3⁺ population of islet-specific CD4 T cells is minor (~0.05% of peripheral lymphoid cells) and their frequency does not shift in double transgenic mice that develop autoimmune diabetes.

Homozygous *Il2* deficiency had a profound effect on the formation of the 1G12⁺CD4⁺Foxp3⁺ cells in the thymus of TCR:insHEL double transgenic mice. In IL-2-sufficient controls, a substantial population of 1G12⁺CD4⁺ thymocytes expresses Foxp3 in thymi expressing insHEL (Figure 2c and e). By contrast, the frequency of 1G12⁺Foxp3⁺ CD4 thymocytes was reduced to 20% of control levels in IL-2-deficient TCR:insHEL thymi (Figure 2e), although small numbers of clonotype-low and clonotype-negative Foxp3⁺ cells remained (Figure 2c). These results demonstrate that thymic generation of islet-specific Foxp3⁺ T_R cells is exquisitely IL-2-dependent.

Despite the thymic defects, Foxp3⁺1G12⁺ CD4 cells were present in the periphery of *Il2*^{0/0} double transgenic mice, and the overall frequency of these potentially insHEL-reactive T_R cells was not reduced relative to *Il2*-sufficient controls (filled bars in Figure 3e). However, the majority of clonotype-expressing CD4 cells that were present expressed lower levels of 1G12, and little or no Foxp3 and CD25 (Figure 3b and c). This result demonstrates that peripheral islet-specific Foxp3⁺ T_R cells are able to survive and persist in the absence of IL-2.

Although clonotype-positive insHEL-reactive CD4 cells were eliminated intrathymically from both Foxp3⁺ and Foxp3⁺ subsets of *Il2*-deficient TCR:insHEL mice, there was nevertheless a 250% increase in the peripheral population of clonotype-positive CD4 cells when compared with *Il2*^{+/+} and *Il2*^{+/-} double transgenic control animals (Figure 3a and e). This expansion occurred in the Foxp3⁺ subset of peripheral islet-reactive T cells, with an eightfold increase from ~0.05% of the peripheral lymphoid pool to ~0.4%. The reduction

in surface 1G12 was not due to activation-induced downregulation, as it was not observed in the HEL-specific T-cell population in diabetic control mice, and the levels did not increase after *in vitro* culture of *Il2*^{0/0} splenocytes in the absence of cognate antigen (data not shown). The low to negative expression of Foxp3 in these islet-specific cells (Figure 3c) could have two non-exclusive explanations. First, these cells may simply represent the subset of T cells in the conventional

Figure 3 IL-2 deficiency causes peripheral expansion of Foxp3⁺ CD4 T cells with low expression of islet-reactive TCR. Splenocytes from B10^k TCR transgenic and TCR:insHEL double transgenic mice of *Il2*^{+/+}, *Il2*^{+/-} and *Il2*^{0/0} genotypes were analysed by flow cytometry. (a) Staining for CD4 vs 1G12 (3A9 TCR clonotype). (b) Histograms for CD25 gated on CD4⁺1G12⁺Foxp3⁺ splenocytes from *Il2*^{+/+} TCR:insHEL double transgenic mice (shaded), *Il2*^{0/0} TCR:insHEL double transgenic mice (dotted line) and *Il2*^{+/+} TCR transgenic mice lacking HEL (thin line). (c) Staining with 1G12 clonotypic-specific antibody vs Foxp3, gated on CD4⁺ splenocytes from *Il2*^{+/+} TCR transgenic, *Il2*^{+/+} TCR:insHEL double transgenic, *Il2*^{0/0} TCR:insHEL double transgenic and non-transgenic control. Plots are representative of six experiments. (d and e) Mean and standard deviation of the percentage of CD4⁺1G12⁺ splenocytes for *Il2*^{+/+}, *Il2*^{+/-} and *Il2*^{0/0} genotypes in TCR (d; n=7,5,4) and TCR:insHEL (e; n=6,7,6) mice, divided into Foxp3⁺ (black) and Foxp3⁻ (white) cells. Gross splenic cellularity is unchanged in all strains, thus percentage change reflects increases in absolute number.

CD4 lineage that escaped thymic deletion due to lower expression of insHEL-reactive TCRs. Alternatively, these cells may be derived from the 1G12^{low} Foxp3⁺ population in the thymus, but have failed to sustain Foxp3 expression in the periphery. Both possibilities are supported by the observation of overlapping TCR repertoires in Foxp3⁺ T_R cells and autoimmune T cells in Foxp3⁻ animals,²⁶ and the evidence that IL-2 increases Foxp3 expression in peripheral T_R cells.¹⁷ This population of islet-reactive T cells appears unable to initiate diabetes in the majority of TCR:insHEL animals under normal conditions. In the absence of IL-2, these clonotype-low cells increase in frequency and presumably mediate development of diabetes in 100% of *IL2*^{0/0} TCR:insHEL animals. Their expansion could either be due to defects in the normal complement of islet-reactive Foxp3⁺ CD4 cells, or the absence of other *IL2*-dependent regulatory mechanisms, such as Foxp3⁻ independent regulatory populations.

The stringent requirement for IL-2 for thymic generation of islet-specific Foxp3⁺ CD4 cells defined here parallels studies tracking T cells of unknown specificity in a polyclonal repertoire that found between 50 and 80% of thymic CD4⁺Foxp3⁺ cells required IL-2.¹⁷ Based on the data here using a high-avidity islet-specific TCR transgene, we propose strongly that islet-reactive Foxp3⁺ cells depend on IL-2 for their formation or survival in the thymus. Nevertheless, 1G12^{low} and 1G12-Foxp3⁺ CD4 cells with low avidity for islet antigen are able to develop in the thymus, consistent with studies of low-avidity HA-specific T cells.²⁷ Whereas the behaviour of all possible diabetogenic clones cannot be predicted from TCR transgenic studies, the approach used here uniquely allows tracing of T-cell fate in the presence and absence of antigen. Moreover, the 3A9 clone is representative of a key set of autoimmunogenic CD4 cells: it recognizes a well-characterized, dominant epitope that is presented in the thymus of insHEL transgenic mice by an *Aire*-regulated mechanism like insulin itself. These data are therefore highly relevant to the subset of diabetogenic T cells that become pathogenic in the absence of IL-2. The depletion of islet-reactive Foxp3⁺ CD4 cells in the thymic repertoire of IL-2-deficient animals indicates a mechanistic overlap with diabetes pathogenesis in FOXP3-deficient children, and provides an explanation for the association between polymorphisms in *CD25/IL2RA* and human diabetes susceptibility,¹ and between the NOD allele of the *IL2/Id3* locus and the enhanced proliferation in pancreas-draining lymph nodes of islet-specific T-cells transferred into NOD mice.^{28,29} Although a mechanistic explanation for the diabetogenic impact of loss of IL-2 function is suggested in this study, the means by which partial reduction in function polymorphisms in *IL2* and *IL2RA* are translated into diabetes is of greater complexity. The change in *IL2RA* function in diabetogenic alleles is unclear and less severe than a known null truncated form,³⁰ whereas the *Id3* allele of *IL2* functions similar to *IL2*-haplo deficiency, with a twofold decrease in IL-2 production.³¹ It is notable that *IL2*-heterozygous mice in this study do not present a phenotype, demonstrating that on a resistant background subtle changes in IL-2 function are not sufficient to induce diabetes. Genetic analysis of both loci demonstrates that polymorphisms in *IL2* and *IL2RA* are not diabetogenic alone, indicating that the accumulated effects of multiple loci are required for subtle changes in IL-2 function to precipitate diabetes.

METHODS

Mice

3A9 TCR transgenic³² and ILK-3 insHEL transgenic mice²¹ produced in C57BL/6J mice were backcrossed more than seven generations to B10.Br/SgSnJ (B10^k JAX). 129P2/OlaHsd *IL2*^{0/0} mice backcrossed to C57BL/6 (¹⁹; JAX) were backcrossed (three generations) to insHEL:3A9 B10^k mice. Diabetes incidence

was measured using urine glucose testing with Testape/Glucostix at biweekly intervals or when cage was wet. Non-diabetic mice were culled at 24 weeks.

Flow cytometry

About 6–12-week-old mice were analysed using the following antibodies: 1G12 anti-clonotype³³ culture supernatant followed by rat anti-mouse IgG1-APC; anti-CD8 α -PerCP; anti-CD4-fluorescein isothiocyanate (FITC) or PE, anti-CD69-PE and anti-CD25-PE (Pharmingen, San Jose, CA, USA). Staining for Foxp3 used FITC-conjugated anti-Foxp3 (FJK-14s; eBioscience, San Diego, CA, USA).

Statistical analysis

Analysis was performed using Student's *t*-test (after testing for normality, *P*>0.95). Diabetes incidence was analysed by log-rank test.

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