

Review

Regulation of Immune Responses by Natural Killer T Cells

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Abstract. Natural killer T (NKT) cells, which comprise a minor population of T cells in primary and secondary lymphoid organs, possess phenotypic characteristics of both NK and T cells. NKT cells respond to various external stimuli by an early burst of cytokines, including IL-4 and IFN- γ . Thus, a key immunoregulatory role has been attributed to them. Autoimmune diseases, especially type I diabetes (T1D), may be caused by dysregulation of the immune system, which leads to hyporesponsiveness of regulatory T helper 2 (Th2) cells and promotion of autoimmune Th1 cells. Furthermore, several lines of evidence exist to support the notion that an NKT cell deficiency in individuals at risk of T1D may be causal to T1D. As a result, targeting NKT cells using immunotherapeutic agents may prove beneficial in the prevention or recurrence of T1D. Indeed, our data demonstrate that stimulation of NKT cells with a specific ligand prevents the onset and recurrence of T1D in nonobese diabetic (NOD) mice.

Key words: immunoregulation; autoimmunity; type I diabetes; α -galactosylceramide.

Introduction

The role of natural killer T (NKT) cells in the induction and regulation of immune responses has been the focus of many investigations. Due to their peculiar immunoregulatory characteristics, these cells may be a potential target for novel immunotherapeutic interventions aimed at the prevention or treatment of infectious disease, cancer and autoimmune disease. The main focus of this review is the role of NKT cells in autoimmune type I diabetes (T1D).

Phenotypic Characteristics and Development of NKT Cells

NKT cells were first discovered during the characterization of KLH-specific mouse suppressor T cell hy-

bridomas⁴⁷, which were found to express an invariant T cell receptor (TCR) α chain consisting of the V α 14-J α 281 gene segments²⁵. Subsequently, a subset of CD4⁺CD8⁺ thymocytes was identified as having a bias in their TCR β gene usage, as they frequently used the V β 8 followed by V β 7 and V β 2 gene segments¹⁸. Further characterization of the phenotypic markers of these cells revealed the expression of various NK cell-related surface markers, including NK1.1². Therefore, the term NKT cell was coined to delineate a cell population with both T and NK cell surface phenotypic characteristics. NKT cells may be detected in primary (thymus) and secondary (spleen) lymphoid organs, and are abundant in the liver and bone marrow. Cells with similar phenotypic characteristics have also been described in humans, preferentially using the invariant V α 24-J α Q TCR gene segments⁴².

Phenotypic markers expressed on the surface of NKT cells closely resemble those presented on acti-

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vated T cells, such as CD122 (IL-2R β), CD44 and CD69⁶. The majority of NK1.1⁺ T cells are either CD4⁺ or CD4⁻CD8⁻. Although CD8⁺ NK1.1⁺ T cells were reported to be undetectable, recent evidence, using a pan-NK cell marker (DX5), suggests the presence of CD8⁺ NKT cells^{23, 44}. The splenic DX5⁺TCR $\alpha\beta$ ⁺ subpopulation may differ from the conventional V α 14-J α 281 TCR $\alpha\beta$ ⁺ T cells, since transgenic expression of CD8 on T cells inhibits the development of V α 14-J α 281⁺ NKT cells³⁵.

The fetal development of V α 14 NKT cells starts at day 9 of gestation in mice, before the fetal thymus is formed, and their development can occur independently of the yolk sac³⁸. NKT cells appear to pass through a CD4⁺CD8⁺ double positive stage, as evidenced by the presence of a precursor cell population in day 12 fetal thymic cultures that differentiates into NK1.1⁺CD4⁺CD8⁺ T cells³. The number of NKT cells is very low at birth; subsequently, they accumulate in various tissues, including primary and secondary lymphoid organs, and become most abundant in liver and bone marrow. The dependence of NKT cell development on the thymus is presently unclear. Some NKT cell subpopulations appear to be thymus-dependent, whereas other subpopulations may develop extra-thymically, e.g. in the liver³⁶.

The origin of NKT cells is controversial, as it is not known whether NKT cells are derived from a unique, committed precursor cell or a subset of conventional T cells which, during some stages of their normal development, branch off to become NKT cells. Nevertheless, it is evident that these cells comprise a unique subset of T cells whose development is differentially regulated from mainstream T cells. This notion is supported by the observation that deletion of the V α 14 gene segment, which is involved in the formation of the NKT invariant TCR, selectively depletes the formation of NKT cells, but all other T cells remain virtually intact⁵⁰.

NKT-CD1 Interactions

Although natural ligands of NKT cells are not well characterized, NKT cell restriction to the CD1 molecule, whose structure resembles that of the major histocompatibility complex class I (MHC-I), is well established. Unlike expression patterns of MHC-I, the CD1 molecule is primarily expressed on hematopoietic cells such as B cells, macrophages and dendritic cells. The development of NKT cells is CD1-dependent, as the number of NKT cells is markedly reduced in *CD1* gene

knock-out mice³⁹. The absent NKT cells in *CD1*^{-/-} mice are those that preferentially use the invariant V α 14-J α 281 TCR, whereas another subpopulation of NKT cells may develop in the absence of CD1, which possesses a diverse TCR repertoire as well as phenotypic characteristics of naive T cells. The former population is predominantly present in the thymus and liver and the latter population is present in the spleen and bone marrow¹⁶. The CD1 crystallography findings indicate the presence of a deep, narrow and hydrophobic antigen-binding groove in this molecule, which can accommodate lipid antigens⁶³. The natural ligand of CD1-restricted NKT cells appears to be a glycosphosphatidylinositol (GPI)²⁷. Recently, a glycolipid, α -galactosylceramide (AGC), was identified by Kirin Brewery (Japan) from marine sponges as a potential anti-cancer compound. Further investigations determined that this compound is a ligand for both human and mouse NKT cells^{7, 31}. Presentation of AGC by the CD1 molecule is transporter-associated protein (TAP)-independent and NKT cells respond vigorously to this compound by proliferation, expression of activation molecules, increase in their cytotoxic activity and secretion of various cytokines³¹.

Function of NKT Cells

Upon stimulation, NKT cells exert various functions, ranging from cytotoxic activity to cytokine production. These cells have a unique property that enables them to produce large amounts of cytokines upon primary stimulation. For instance, production of a mixture of T helper 1 (Th1) and Th2 cytokines is detectable in splenocytes harvested from mice treated *in vivo* with an anti-CD3 mAb 90 min earlier, and the responsible cell population has the phenotypic characteristics of NKT cells⁶². More recently, the CD1-restricted NKT cell response to AGC was shown to be phylogenetically conserved, as human and mouse CD1 molecules can bind to AGC and activate NKT cells from both species⁷. NKT cells respond after interaction with CD1-bearing dendritic cells loaded with AGC. This interaction requires cell-cell contact through CD40-CD40L³³. Secretion of Th1 or Th2 type cytokines appears to be differentially regulated by the number of *in vivo* doses of treatment with AGC. While a single *in vivo* dose of AGC encourages expression of IFN- γ and other characteristics of a Th1 response¹², a multiple-dose regime will promote a Th2 response⁹. It is noteworthy that, although NKT cells are primarily triggered by AGC, bystander cells, including NK, CD8, CD4 and B cells,

may also become non-specifically activated as a consequence of NKT cell activity^{11, 34}. The peculiar property of NKT cells, which enables them to produce large amounts of Th1- and Th2-type cytokines very early after stimulation, renders them ideal candidates for the regulation of an immune response.

Effector activity

Due to the wide range of activities, NKT cells may play effector or regulatory roles in different immunological settings. AGC-activated human NKT cells confer anti-tumor activity via an elevated perforin-dependent cytotoxicity³². Similarly, mouse NKT cells activated with AGC-pulsed dendritic cells inhibit the metastasis of experimental melanoma⁵⁴. NKT cells may also have effector functions in response to various pathogenic microorganisms, e.g. resistance to infection with *Toxoplasma gondii*¹⁴ and *Plasmodium yoelli*⁴¹. Thus, it is tempting to speculate that NKT cells are intimately involved in providing the first layer of host immunity against invading pathogens. However, paradoxically, inhibition of CD1-NKT interaction in *Listeria monocytogenes*-infected mice reduced the expression of TGF- β 2 and, as a result, the survival of these mice was significantly increased⁴⁸. This suggests a regulatory function for NKT cells under certain cir-

cumstances in the course of a bacterial infection. NKT cells may also possess other effector functions during the development of concanavalin A-induced hepatitis²⁸, abortion in mice²⁶ and hyperacute graft-versus-host-disease (GVHD) in humans⁴⁹.

Regulatory activity

The role of NKT cells in the regulation of an immune response has received much attention (Fig. 1). Initially, two V α 14-J α 281 TCR $\alpha\beta$ ⁺ KLH-specific T cell hybridomas were described that could each secrete a soluble suppressor factor. The suppressor factor from one hybridoma induced the other hybridoma ("inducible acceptor suppressor") to exert its KLH-specific functions^{25, 47}. More recently, DNP-specific T suppressor hybridomas were identified, one of which has a TCR V α 14-J α 281 rearrangement. These hybridomas also produce a soluble suppressor factor, which may be a soluble TCR⁴. However, these soluble suppressor factors could also be cytokines, such as TGF- β , IL-4 and IL-10, or other immunomodulators that enhance one type of immune response and suppress another type. In fact, early investigations pointed to the crucial role of NKT cells in triggering Th2 type responses⁶¹. Despite these findings, several groups have argued that NKT cells have a dispensable role in the development of

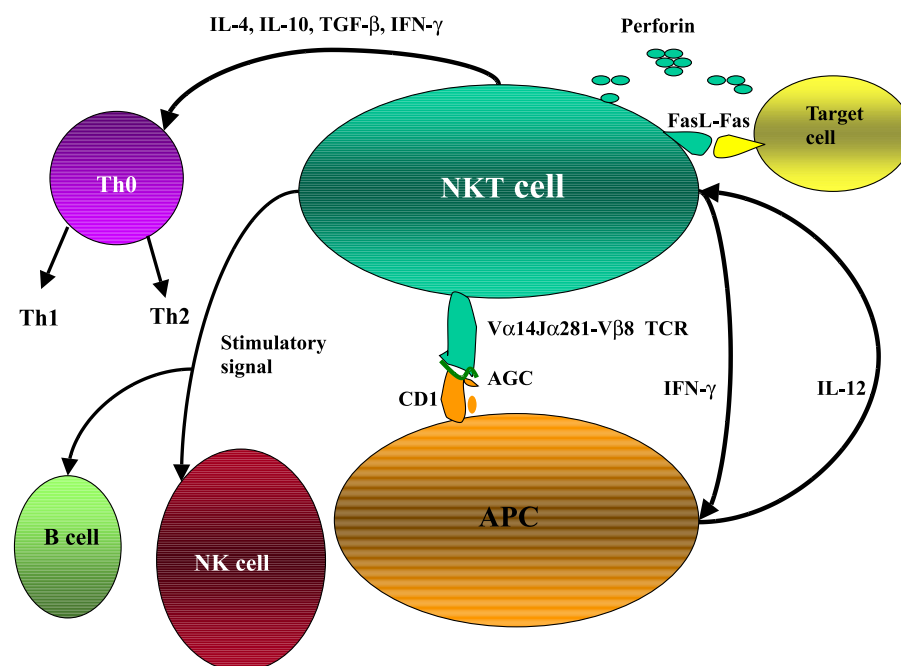


Fig. 1. Model of NKT activation and function. NKT cells are stimulated by CD1-bearing antigen-presenting cells (APC) that are loaded with AGC. NKT cells receive stimulatory signals from APC (via IL-12) and, in return, stimulate them by secretion of IFN- γ . Upon stimulation, NKT cells exert effector (e.g. cytotoxicity via Fas-FasL and perforin pathways) and immunoregulatory (e.g. secretion of cytokines: IL-4, TGF- β and IFN- γ) functions. The early burst of cytokine secretion by NKT cells may be critical in the differentiation of Th0 cells to Th1 or Th2 cells. B cells and NK cells may also become activated by NKT cells

a Th2 response⁸. The degree of NKT cell involvement in the development of a Th2 versus a Th1 response likely depends on the particular immunological setting. For example, NKT cells potentiate a Th1 response by mediating IL-12-induced IFN- γ production³⁰. Dendritic cells are stimulated by ligand-activated NKT cells through a direct CD40-CD40L interaction that increases their production of IL-12³³. Subsequently, the secreted IL-12 induces NKT cells to increase their surface expression of IL-12R as well as production of IFN- γ ³³. The AGC-induced IL-12 production is inhibited in V α 14-J α 281 knock-out mice, which selectively lack NKT cells, or by *in vivo* administration of an anti-CD40L mAb⁵¹. Thus, an intimate interaction between NKT cells and antigen-presenting cells (APCs) is critical for the development of a Th1 or a Th2 response.

Several additional immunomodulatory roles of NKT cells have been reported. During allogeneic GVHD, NKT cells can ameliorate disease⁶⁴. In this model, conventional T cells (TCR $\alpha\beta$ ⁺CD4⁺ or TCR $\alpha\beta$ ⁺CD8⁺) derived from bone marrow or blood elicit acute GVHD, whereas NK1.1⁺CD4⁺, NK1.1⁺CD8⁺ or NK1.1⁺CD4⁺CD8⁺ T cells suppress GVHD. Suppression of disease by NK1.1⁺ T cells is dependent on expression of IL-4, as NK1.1⁺ T cells from IL-4^{-/-} mice lose their modulatory activity⁶⁴. Paradoxically, in a hyperacute case of human allogeneic GVHD, the frequency of NKT cells in peripheral blood was very high and this result implicated their effector role in this response⁴⁹. An immunomodulatory role of NKT cells in immune privilege was recently described in an elegant study involving a model of anterior chamber-associated immune deviation (ACAID)⁴⁵. Induction of regulatory T cells in the spleen, which inhibit proliferation or the effector functions of CD4⁺, may initiate ACAID. However, induction of ACAID is severely perturbed in strains of mice, including SJL/J and β 2m knock-out mice, which have developmental and functional deficits in their NKT cell population⁴⁵. Further investigations proved more conclusively that ACAID is dependent on CD1 expression, as evidenced by the absence of ACAID in CD1^{-/-} mice and by an *in vivo* blockade of CD1-NKT interaction using anti-CD1 antibodies⁴⁵.

Other immunoregulatory functions of NKT cells may involve the induction of tolerance. In the trinitrobenzenesulfonic acid (TNBS)-induced model of colitis in mice, NK1.1⁺ liver-associated lymphocytes were found to be important in the induction of oral tolerance against the development of colitis⁵⁵. Tolerized mice had significantly higher serum concentrations of IL-4 and TGF- β 1, while the serum concentration of IFN- γ was significantly reduced⁵⁵. Furthermore, intra-graft NKT

cells may be important in the induction of tolerance in recipients of hepatic allografts⁴⁰.

NKT Cells and Autoimmunity

A potential role for NKT cells in the regulation of autoimmunity was initially recognized in SJL mice that are susceptible to experimental allergic encephalomyelitis (EAE), a Th1-mediated disease. SJL mice are deficient in their Th2-mediated responses, and their NKT cells are significantly reduced in number and function (TCR-stimulated rapid burst in cytokine production) in various tissues⁶⁰. A functional deficit in NKT cells, especially in the production of IL-4 and other regulatory cytokines, in autoimmune disease-prone mouse strains can lead to a dominant pathogenic autoreactive Th1 response. NKT cells have also been implicated in the regulation of susceptibility to autoimmune disease in humans. Patients with systemic sclerosis have a reduced frequency of peripheral blood NKT (V α 24-J α Q⁺) cells⁴⁶. Similarly, the number of NKT cells in the peripheral blood of patients with rheumatoid arthritis is reported to be significantly decreased⁵⁹.

NKT cells and type I diabetes

The strongest evidence in support of a role for NKT cells in the regulation of autoimmunity stems from studies of T1D in humans⁵⁷ and nonobese diabetic (NOD) mice²¹. NOD mice spontaneously develop a form of T1D that shares many genetic, immunologic and pathologic features with the human disease¹³. Pancreatic islet β cell destruction is mediated by infiltrating T cells, and both CD4⁺ and CD8⁺ T cells are required for the development of T1D. Although islet β cell destruction and the onset of T1D is attributed to the presence of autoimmune CD4⁺ Th1 effector cells²⁹, an important role for CD8⁺ T cells in islet β cell destruction has also been identified^{15, 58}.

An intricate balance between autoimmune destructive T cells and immunoregulatory T cells regulates the development of T1D¹³. A consensus opinion suggests that the conversion from a non-destructive insulinitis to destructive insulinitis involves a disruption of the balance between destructive Th1 and regulatory Th2 autoimmune cells. Non-destructive insulinitis is associated with higher pancreatic levels of IL-4, whereas destructive insulinitis is associated with a higher intra-pancreatic concentration of IFN- γ ¹⁹. We have observed deficiencies in NOD T cells that may be causal to the occurrence of T1D, including T cell proliferative hypore-

sponsiveness following TCR stimulation⁶⁵ and reduced IL-4 and IL-2 production upon T cell activation⁴³. Stimulation with IL-4 fully restores proliferative hyporesponsiveness and a normal level of IL-4 production by NOD T cells, and treatment of prediabetic NOD mice *in vivo* with IL-4 prevents insulinitis and onset of T1D⁴³. In addition, young, 2-week-old, NOD mice treated with IL-4 for 10 weeks show a reduced incidence of T1D, which is attributed to the prevention of Th1 autoreactive T cells from entering the pancreas as well as the promotion of regulatory Th2 cell activity in the thymus, spleen and pancreatic islets¹⁰. Taken together, these findings support the notion that immunoregulatory CD4⁺ Th2 cells mediate protection of NOD mice from T1D¹³. Thus, it is conceivable that the proliferative hyporesponsiveness of regulatory Th2 cells promotes a dominant Th1 environment in the pancreas that leads to the breakdown of peripheral self tolerance.

Co-stimulatory pathways, such as those mediated by CD28-B7 interactions, may also contribute to NOD T cell hyporesponsiveness and, consequently, to the loss of tolerance and onset of T1D. Indeed, administration of an activating anti-CD28 mAb to young NOD mice of 2–4 weeks of age restores IL-2 and IL-4 secretion of thymic and splenic T cells and also completely protects them from T1D¹. These results and those highlighted above further support the idea that immune dysregulation is a principal causal factor of autoimmune T1D. Accordingly, correction of this state of immune dysregulation by an immunomodulatory agent that elicits minimal *in vivo* adverse effects offers a promising approach to prevent occurrence of T1D in high risk subjects and to inhibit recurrence of T1D in patients receiving islet transplants.

NKT cells provide a potentially interesting target for such an immunomodulator(s). As previously discussed, the critical role of NKT cells in the development of several Th2 responses is widely accepted^{9, 61}. Interestingly, there exist age-dependent deficiencies in the number and function of NKT cells in NOD mice and human T1D patients. In NOD mice between 3 and 5 weeks of age, the proportion of NKT-like thymocytes is reduced and TCR cross-linking-induced IL-4 production is significantly impaired²¹. Note that these quantitative and qualitative deficiencies first appear at an age that represents an important checkpoint in the progression to T1D, i.e. immediately prior to the development of insulinitis in 3- to 4-week-old mice. Subsequently, these deficiencies become virtually undetectable in 12- to 15-week-old NOD mice.

Deficient IL-4 production by NOD NKT-like thymocytes can be corrected by stimulation with IL-7²²,

raising the possibility that the developmental deficiency of NOD NKT thymocytes might be related to an insufficient availability of IL-7. The finding that the development of NKT-like thymocytes in the NOD thymus is impaired was later confirmed by GODFREY et al.²⁰, who also noted that transfer of thymocytes enriched for CD4⁺CD8⁺TCR $\alpha\beta$ ⁺ cells (i.e. NKT cells) into young NOD recipients prevents T1D⁵. The protection conferred by these thymic NKT cells is mediated by IL-4 and IL-10, as neutralization of these cytokines abrogates the protective effects of NKT cells²³. Analyses of different lines of transgenic NOD mice that express variable levels of the TCR V α 14-J α 281 chain have also confirmed a pivotal role for NKT cells in protection from T1D³⁷. The most protection from T1D was achieved in the mouse line that possessed the greatest number of NKT cells, highest level of IL-4 secretion by NKT cells induced by TCR stimulation, and best capacity to transfer protection with splenic T cells. We have shown that NKT cells in the thymus and spleen of NOD mice secrete normal amounts of IFN- γ , but are deficient in IL-4 production in response to *in vitro* stimulation with AGC⁴⁴. In contrast, TCR- or IL-12-stimulated NOD NKT cells were previously reported to be severely deficient in IFN- γ , but not IL-4 production¹⁷. In the latter studies, purified NOD NKT cells supplemented with IL-7 not only did not prevent T1D, but rather accelerated the onset of T1D¹⁷. Further experimentation is required to resolve these apparent discrepancies.

A role for NKT cells in the pathogenesis of human T1D was recently reported⁵⁷. In studies of a group of twin/triplet sets discordant for T1D, the diabetic siblings had a lower frequency of CD4⁺CD8⁺TCRV α 14J α Q⁺ NKT cells in their peripheral blood in comparison with their non-diabetic non-progressor siblings. More importantly, CD4⁺CD8⁺V α 14J α Q⁺ T cell clones from these discordant sets differed appreciably in their production of IL-4 and IFN- γ . Whereas all clones from diabetic siblings only produced IFN- γ , 76 of 79 clones derived from at risk non-progressor siblings simultaneously produced IL-4 and IFN- γ . Thus, it is conceivable that correction of quantitative and qualitative deficiencies of NKT cells in individuals at risk for T1D may prevent occurrence of the disease. Alternatively, the up-regulation of NKT cell number and function may prevent/reduce the recurrent autoimmune destructive response and, as a result, prolong islet graft survival in newly transplanted diabetic patients.

Since AGC is a ligand for CD1-restricted mouse NKT cells and is a potent stimulator of these cells, we investigated whether treatment of NOD mice *in vivo* with AGC protects against T1D. Our results indicate

that female NOD mice are significantly protected against the onset of cyclophosphamide (CY)-induced T1D when treated with AGC on days 0, 2, 4, 6 and 8 relative to the time of CY challenge⁴⁴. Moreover, when female NOD mice are treated with AGC at 10, 11 and 14 weeks of age, the spontaneous development of T1D is delayed by 4–5 weeks and the incidence is reduced by 50%. A more comprehensive treatment regime beginning at 4 weeks of age also affords partial protection and a significant delay in disease onset. Our data indicate that these different modes of AGC-induced protection from T1D in NOD mice are mediated by the activation of NKT cells.

We have also explored whether AGC can modulate the onset of a recurrent autoimmune destructive response in overtly diabetic NOD recipients of syngeneic pancreatic islet transplants. In this model, engraftment is normal and euglycemia is restored for about 7–10 days post-transplantation, following which grafts generally fail owing to a recurrent autoimmune response. More recently, prolongation of pancreatic duodenal graft survival in non-recurrent spontaneous diabetic BB rats was associated with the proliferation of donor-derived NKT cells in hepatic and splenic tissues⁵³ and higher serum levels of IL-4⁵². We have obtained evidence in support of the hypothesis that stimulation of NKT cells by AGC prolong islet graft survival by down-modulation of the recurrent autoimmune response. Our data demonstrate that multiple doses of AGC administered pre- and post-transplantation in recipients prolong islet graft survival in newly diabetic NOD mice from the typical 7 days to as long as 22 weeks⁴⁴. Ongoing studies will determine whether NKT cells play a direct or indirect role in enhanced islet graft survival and homeostasis.

Conclusions

Failure of immune regulation in NOD mice or human subjects at risk of T1D, which stems from deficient T cell-mediated regulation, and Th2 anergy may be causal to the onset of autoimmune T1D and, conceivably, correction of this immune dysregulation may protect at risk individuals against T1D. Indeed, we have shown that cytokine-based (IL-4) and mAb-based (anti-CD28) immunotherapies can protect NOD mice from T1D. NKT cells, which are quantitatively and qualitatively deficient in diabetes-prone NOD mice and humans, provide an excellent target for future immunotherapeutic approaches aimed at the prevention or reversal of human T1D. Since these cells play a major

role in the early induction of Th1 and Th2 immune responses, appropriate activation of NKT cells, e.g. towards a Th2 response, may correct immune dysregulation and prevent the pathogenesis of T1D. The newly discovered specific ligand for NKT cells, AGC, has already been tested in phase I clinical anti-cancer trials and its lack of toxicity has been confirmed⁵⁶. Thus, it seems feasible to use this compound to protect individuals who are genetically and environmentally at risk for T1D. Alternatively, this immunomodulator may be used in diabetic patients that have received an islet transplantation to protect them from the recurrence of T1D.

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