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Infusion of UVB-treated splenic stromal cells induces suppression of β cell antigen-specific T cell responses in NOD mice

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Abstract

Our previous study has demonstrated that transfusion of UVB-irradiation-induced apoptotic β cells effectively prevents type 1 diabetes (T1D) in non-obese diabetic (NOD) mice. However, the limitation of β cell source would preclude the clinical application of this approach. Therefore, in the present study, we have attempted to establish a more practical approach by utilizing apoptotic non- β cells to prevent T1D. We find that apoptotic splenic stromal cells significantly suppress β cell antigen-reactive T cell proliferation *in vitro* and *in vivo*. Moreover, β cell antigen-specific T cells primed by β cell antigens in the presence of apoptotic stromal cells have markedly reduced responsiveness to the re-stimulation of the same β cell antigen. We also find that β cell antigen-specific IL-10-producing CD4⁺ T cells are induced in the presence of apoptotic splenic stromal cells. As expected, transfusion of apoptotic stromal cells effectively protected NOD mice from developing T1D. Furthermore, the proliferation of adoptively transferred β cell antigen-specific TCR-transgenic T cells in pancreatic draining lymph nodes is markedly suppressed in UVB-stroma-treated mice, indicating that UVB-stroma treatment induces immune tolerance to multiple β cell antigens. This study provides an effective and convenient approach for managing T1D by utilizing apoptotic non- β cells.

Keywords

Apoptosis; Immune tolerance; Type 1 diabetes; Regulatory T cell; Cell therapy

1. Introduction

Steady state cell apoptosis is a physiological process during which phagocytes immediately process apoptotic cells without causing inflammation and maintain self-tolerance [1,2]. Evidence has shown that impaired function of phagocytosing apoptotic cells is associated with development of autoimmune diseases [1–5], suggesting that processing of apoptotic cells through phagocytosis plays an important role in the maintenance of self-tolerance. It has been documented that steady state apoptotic cells phagocytosed by dendritic cells can render dendritic cells to be tolerogenic and subsequently tolerize CD4⁺ T cells and CD8⁺ T cells through direct antigen presentation and antigen cross-presentation, respectively [6]. It has also been shown that steady state apoptotic cells can trigger phagocytes to secrete immunosuppressive cytokines, such as IL-10 and TGF- β [7,8], which in turn facilitate the development of regulatory T cells [9]. Due to this unique relationship between apoptotic cells

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and immune tolerance, apoptotic cells have been employed with success in inducing immune tolerance to allogeneic antigens [10] and other exogenous antigens [11].

We have utilized ultraviolet B (UVB) irradiation-induced apoptotic immature dendritic cells to successfully induce immune tolerance across major histocompatibility complex (MHC) barriers [10]. Recently, we effectively induced β cell antigen-specific immune tolerance in the autoimmune diabetes mouse model, non-obese diabetes (NOD) mouse by transfusion of UVB-irradiated apoptotic β cells [12]. We found that three weekly transfusions of UVB-irradiated apoptotic β cells significantly delayed and prevented T1D in NOD mice when these mice were treated at 10 weeks of age (late stage of insulinitis). Nevertheless, limited β cell sources in humans deter the extensive application of this approach in T1D management. Although the studies on stem-cell-derived insulin-producing cells bring hope for this approach in clinical applications, there are still a variety of issues to be resolved in making stable insulin-producing cells [13]. Thus, it is necessary to find alternative techniques in utilizing apoptotic cells to induce β cell antigen-specific T cell tolerance. Recently, it was reported that intravenous injection of apoptotic syngeneic splenocytes along with administration of a hapten, 2,4-dinitro-fluorobenzene (DNFB) to the skin induced immune tolerance specific to DNFB [11]. This study suggests that it is possible to utilize apoptotic non- β cells, such as splenocytes or peripheral blood mononuclear cells to induce immune tolerance to β cell antigens with the exposure of endogenous β cell antigens during the process of autoimmunity, thereby preventing autoimmune diabetes.

In the present study, we attempted to determine the effect of UVB-irradiated splenic stromal cells derived from NOD mice on induction of immune tolerance to β cell antigens as well as on T1D prevention. We found that UVB-irradiated NOD splenic stromal cells significantly suppressed β cell antigen-reactive T cell response and induced IL-10-producing CD4⁺ T cells. Three weekly transfusions of UVB-irradiated apoptotic stromal cells significantly prevented NOD mice from developing T1D.

2. Materials and methods

2.1. Animals

Female NOD and NOD-SCID mice were purchased from the Jackson Laboratory. NOD.BDC2.5 mouse breeders were kindly provided by Dr. David Serreze (Jackson Laboratory). All mice were housed in a specific pathogen-free facility of the Mouse Colony of the Department of Pathology, Immunology and Laboratory Medicine at the University of Florida. Animal studies were performed in accordance with the guidelines of University of Florida Institutional Animal Care and Use Committee.

2.2. Culture media and reagents

RPMI 1640 supplemented with 10% fetal calf serum (Cambrex Bio Science, Walkersville, MD) was used for the culture of splenic stromal cells and *in vitro* T cell functional studies. HL-1 media (Cambrex Bio Science, Walkersville, MD) were used for the culture of splenocytes stimulated with β cell antigens. Mouse IL-4, IL-10 and IFN- γ Luminex kits were purchased from Upstate (Temecula, CA). Fluorescence-conjugated antibodies (anti-IFN- γ and anti-IL-10) and isotype control antibodies, Annexin-V-FITC, leukocyte activation cocktail kits, intracellular cytokine staining kits and dead cell removal kits were purchased from BD-PharMingen (San Diego, CA). The fluorescent dye, CFSE was obtained from Invitrogen Molecular Probes (Eugene, OR). Peptides B9-23 (sequence: SHLVEALYLVCGERG) and BDC2.5 TCR-specific mimotope 1040-55 (sequence: RVRPLWVRME) were synthesized by Peptide International (Louisville, KY). The purity of these peptides was in the range of 95–

97%. Mouse splenic CD4⁺ T cell isolation kits were purchased from Stem Cell Biotech (Vancouver, Canada). CD11c-microbeads were obtained from Miltenyi (Auburn, CA).

2.3. NOD splenic stromal cell culture

NOD splenic stromal cell line was generated using the method as previously described [14] with some modifications. In brief, whole splenocytes from four-week-old mice without any cell depletion and enrichment were cultured in six-well culture plates in RPMI 1640–10% FCS at 37 °C with 100% humidity and 5% CO₂. After two to three weeks, when the stromal cells had formed a monolayer with 80% confluence, the cells were dispersed with 0.25% trypsin containing 5 mM EDTA. The stromal cells were maintained in long-term culture in RPMI 1640–10% FCS by weekly passage to new plates.

2.4. Preparation of UVB-irradiated stromal cells

The stromal cell line was maintained in culture with RPMI–10% FCS media. Stromal cells were harvested after incubation with 0.25% trypsin–5 mM EDTA for 5 min at room temperature. Cells were washed twice with PBS and resuspended in 0.5 ml of PBS. Then, the cell suspension was placed in a 3-cm Petri dish and irradiated with UVB (1200 mJ/cm²) for 3 min. After irradiation, the cells were harvested and enumerated using a hemacytometer under a microscope. The UVB-irradiated cells were immediately placed on ice until injection. The sensitivity of stromal cells to UVB-irradiation-induced apoptosis was the same as that of NIT1 cells, an NOD β cell line used in our previous study [12]. We consistently found that >90% cells became apoptotic after 24 h incubation in media post UVB-irradiation.

2.5. Cell isolation

Mouse CD4⁺ T cells were isolated by negative selection using StemCellSep™ kits following instructions from the manufacturer. The purity of CD4⁺ T cells was in the range of 95–97%. Splenic DCs were purified by positive selection using CD11c-microbeads according to instructions from the manufacturer. The purity of CD11c⁺ cells was in the range of 90–95%.

2.6. T cell suppression by UVB-stromal cells

NOD.BDC2.5 splenic CD4⁺ T cells (1×10^5) were stimulated with purified NOD splenic dendritic cells (1×10^4) pulsed with β cell antigenic mimotope 1040-55 in the presence of different concentrations of UVB-irradiated stromal cells as indicated for four days. Then, ³H-thymidine (1 μCi/well) (Amersham Biosciences) was added to the cultures for an additional 16 h. Cells were washed and harvested onto a glassfiber filter using an automated cell harvester (Perkin Elmer). T cell proliferation was determined by liquid scintillation counting.

2.7. T cell tolerance assay

Responder CD4⁺ T cells (1×10^5) purified from NOD.BDC2.5 mouse splenocytes primed for three days by splenic dendritic cells and pulsed with BDC2.5 mimotope in the presence or absence of UVB-stroma (1×10^5) were re-stimulated with 1×10^4 BDC2.5 mimotope-pulsed splenic DCs for four days in a U-bottom 96-well plate. In some cultures, anti-IL-10 (10 μg/ml) was added. ³H-thymidine (1 μCi/well) was added to each well for an additional 16 h. The ³H-thymidine incorporation was examined by liquid scintillation counting.

2.8. T cell cytokine assay by Luminex

Splenocytes (1×10^6) from NOD mice with different treatments as indicated were stimulated with 12.5 μM insulin B9-23 or NOD insulinoma cell line (NIT1) lysates (50 μg/ml) for four days. Supernatants were pooled from triplicate cultures and assessed for the presence/quantity of IL-4, IL-10, and IFN-γ using the Beadlyte Mouse Multi-Cytokine Detection System 1 Kit

(Upstate Signaling). Cytokine concentrations were analyzed using the Luminex instrumentation (Austin, TX) and Upstate Signaling Beadlyte software (Charlottesville, VA).

2.9. Intracellular cytokine staining

NOD.BDC2.5 mouse spleen CD4⁺ T cells (1×10^5) were primed with purified NOD spleen DC (1×10^4) pulsed with BDC2.5 mimotope peptide in the absence or presence of apoptotic spleen stroma (1×10^5) for four days. Thereafter, dead cells were removed using the dead cell removal kit (BD-Phar-Mingen). The viable cells were incubated with leukocyte activation cocktails (PMA + ionomycin + GolgiPlus) (BD-PharMingen) for 4 h according to instructions from the manufacturer. Intracellular IL-10 and IFN- γ were stained using intracellular cytokine staining kits (BD-PharMingen). IL-10- and IFN- γ -producing T cells were examined by flow cytometry.

2.10. Measurement of Th polarization induced in vivo by transfusion of UVB-irradiated stromal cells

Eight-week-old NOD mice were treated with three weekly transfusions of UVB-irradiated stroma (1×10^5 /mouse) or PBS. In the week following the last transfusion, mice were sacrificed and spleens were processed according to previously reported methods [15]. Splenocytes (10^6 /well) were cultured in 96-well plates in HL-1 media and stimulated with insulin B9-23 peptide (12.5 μ M) or NIT1 lysates (50 μ g/ml) for four days. Culture supernatants were collected and measured for T cell cytokine production using Luminex.

2.11. In vivo suppression of β cell antigen-specific T cell proliferation by UVB-irradiated stromal cells

Eight-week-old NOD mice received intravenous injections of CFSE-labeled BDC2.5 splenocytes (1×10^7 /mouse) along with UVB-irradiated stromal cells (1×10^6 /mouse) or PBS. Four days later, the proliferation of CFSE-labeled CD4⁺ T cells in pancreatic draining lymph nodes and other lymphoid tissues were examined by flow cytometry.

2.12. T1D prevention

Eight-week-old NOD mice were treated with three weekly transfusions of UVB-irradiated NOD splenic stromal cells (1×10^5 /mouse). Mice were monitored for diabetes weekly using urinalysis test strips (Clinistix, Bayer) and a blood glucose meter (Accucheck Advantage) when elevated levels of urine glucose were detected. Diabetes was diagnosed when blood glucose was >250 mg/dL on two consecutive days.

2.13. Histology of pancreas and grading of insulinitis

Pancreata were immediately removed from the mice following CO₂ asphyxiation and fixed in 10% formalin for 4 h. Subsequent tissue processing, including sectioning and staining, was performed using services provided by the University of Florida's Molecular Pathology and Immunology Core. Briefly, sections through formalin-fixed pancreas were paraffin-embedded, cut and collected 100 μ m apart, then stained with hematoxylin and eosin. Four non-adjacent tissue sections from each animal were used for insulinitis grading. Islets were observed under light microscopy at 40 $\times$, enumerated and graded blindly by a single observer. We defined no infiltration as grade I, infiltration less than half area of an islet as grade II, infiltration more than half area of an islet as grade III. More than 40 islets were graded for each animal.

2.14. In vivo assessment of β cell antigen-specific tolerance

Eight-week-old NOD mice were treated with three weekly transfusions of UVB-irradiated stromal cells or PBS. In the following week, all mice received CFSE-labeled NOD.BDC2.5

splenocytes (1×10^7 /mouse). To ensure a similar exposure of β cell antigens to the injected NOD.BDC2.5 splenocytes, all mice received intraperitoneal injection of streptozotocin (40 mg/kg). Four days after the cell injection, the proliferation of CFSE-labeled CD4⁺ T cells in PLN or other lymphoid organs was examined using flow cytometry.

3. Results

3.1. Apoptotic splenic stromal cells markedly suppress T cell proliferation *in vitro* and *in vivo*

To determine the suppressive effect of apoptotic cells on T cell response, we tested the effect of different concentrations of UVB-irradiated splenic stromal cells *in vitro* on the proliferation of BDC2.5 CD4⁺ T cells stimulated by NOD splenic DCs pulsed with β cell antigen mimotope, 1040-55. We found that apoptotic splenic stromal cells suppressed CD4⁺ T cell proliferation in a cell dose-dependent fashion (Fig. 1A). Furthermore, we tested whether UVB-irradiated apoptotic stromal cells were able to suppress proliferation of β cell antigen-specific T cells from NOD.BDC2.5 mice *in vivo*. The results demonstrated that one injection of 1×10^6 UVB-irradiated apoptotic stromal cells, but not PBS, dramatically suppressed the proliferation of the simultaneously injected CD4⁺ T cells from NOD.BDC2.5 mice in pancreatic lymph nodes (PLN) (Fig. 1B). There was no proliferation of CFSE-labeled BDC2.5 cells in other lymphoid organs (data not shown), indicating that the CD4⁺ T cell proliferation in PLN was stimulated by natural β antigens. It was also noted that the frequency of CFSE-labeled non-CD4⁺ cells was lower in UVB-stroma-treated than in PBS-treated mice (Fig. 1B), for which the reason is yet unknown.

3.2. Apoptotic stromal cells induce IL-10-producing T cells *in vitro*

To examine the *in vitro* effect of apoptotic stromal cell pre-treatment on β cell antigen-specific T cell recall response, we incubated BDC2.5 CD4⁺ T cells with mimotope-pulsed DC in the presence or absence of apoptotic stromal cells for three days, then isolated the viable CD4⁺ cells to measure proliferation in response to four day re-stimulation with mimotope-pulsed DC. We found that pretreatment with apoptotic stromal cells markedly reduced the β cell antigen-specific T cell response to β cell antigen rechallenge (Fig. 2A) as compared to the response of T cells without pretreatment by apoptotic stromal cells. This reduction appeared to be attributable to IL-10 production, as incubation of the purified CD4⁺ T cell:DC coculture with anti-IL-10 partially restores the T cell response. As expected, flow cytometric analysis revealed that β cell antigen-specific IL-10 producing CD4⁺ T cells were induced with apoptotic stromal cell pretreatment (Fig. 2B).

3.3. Transfusion of UVB-irradiated stromal cells prevents T1D in NOD mice

Given that our previous experiments demonstrated that apoptotic stromal cells suppressed β cell antigen-specific proliferation and induced IL-10 producing T regulatory cells, we attempted to investigate whether the treatment of UVB-irradiated stromal cells was able to prevent T1D in NOD mice, the autoimmune diabetes mouse model. We treated eight-week-old NOD mice with three weekly transfusions of UVB-irradiated apoptotic stromal cells or PBS. We found that transfusions of UVB-irradiated apoptotic stromal cells markedly prevented T1D in comparison to PBS treatment (Fig. 3). The diabetes-free rate by 28 weeks of age was significantly higher in UVB-stroma-treated than in PBS-treated mice (56% versus 20%, $p = 0.025$) (Fig. 3).

3.4. Transfusion of UVB-irradiated stroma alleviates inflammatory cell infiltration in islets

Eight-week-old NOD mice were given transfusions of UVB-stroma or PBS once a week for three weeks, then sacrificed at 15 weeks of age for histological assessment of pancreata for inflammatory infiltrate. We found that around 50% of total islets observed from UVB-treated

mice had no inflammatory infiltration, whereas only less than 5% were infiltration-free in PBS-treated mice (Fig. 4). Additionally, the severity of insulinitis was markedly reduced in UVB-treated mice since less than 10% of the observed islets were determined to be grade III as compared to around 60% in PBS-treated mice. Collectively, these data demonstrate that transfusion of UVB-irradiated stroma alleviates inflammatory cell infiltration in pancreatic islets.

3.5. Transfusion of UVB-irradiated stroma induces IL-10-producing T cells in vivo

To determine the β cell antigen-specific T cell response induced *in vivo* by UVB-stroma treatment, we treated eight-week-old NOD mice with three weekly transfusions of UVB-stroma (1×10^5 /mouse) or PBS, then observed their cultured splenocyte response to four day stimulation with insulin B9-23 peptide (12.5 μ M) or NIT1 lysates (50 μ g/ml). We detected higher levels of IL-10 in splenocyte cultures from UVB-stroma-treated mice as compared to PBS controls (Fig. 5A), suggesting that transfusion of UVB-irradiated stroma induces IL-10-producing T cells *in vivo*. IFN- γ production was comparable between both treatment groups, while IL-4 production was undetectable (Fig. 5B and C).

3.6. Proliferation of adoptively transferred β cell antigen-specific TCR-transgenic T cells from NOD.BDC2.5 mice is suppressed in UVB-stroma-treated NOD mice

We next attempt to assess whether UVB-stroma-treatment-induced regulatory T cells can suppress β cell antigen-specific T cells *in vivo*. NOD mice were treated with UVB-stroma or PBS as described above. Thereafter, CFSE-labeled β cell antigen-specific TCR-transgenic NOD.BDC2.5 mouse splenocytes were intravenously injected into UVB-stroma-treated or PBS-treated mice. In addition, all mice received an intraperitoneal injection of streptozotocin (40 mg/kg) to ensure similar endogenous β cell antigen exposure to the injected T cells in different treatment groups. We found that the proliferation of CFSE-labeled CD4⁺ T cells in the PLN of UVB-stroma-treated mice was significantly suppressed as compared to PBS-treated mice (Fig. 6). Dividing CFSE⁺ CD4⁺ cells were found in insignificant numbers in the inguinal lymph nodes of both groups.

4. Discussion

T1D is an autoimmune disease characterized by the breakdown of immune tolerance to β cell antigens, resulting in self-reactive effector T cells that destroy β cells in the presence of an ineffective regulatory response [16]. Therefore, intervention of the autoimmune process by suppressing effector T cells or converting self-reactive effector T cells into tolerant T cells is a promising strategy for T1D prevention and therapeutic management. In our recent study [12], we showed that transfusion of apoptotic β cells effectively prevented T1D in NOD mice. However, the limitation of a β cell source will restrict the clinical application of this approach. In the present study, we have developed a practical approach in T1D prevention by utilizing apoptotic non- β cells to prevent T1D in NOD mice.

Like the NOD insulinoma cell line, NIT1 [12], NOD splenic stromal cells were sensitive to UVB-irradiation-induced apoptosis (data not shown). For all *in vitro* and *in vivo* experiments, we utilized UVB-irradiated stromal cells immediately after irradiation, or placed them on ice until performing the experiments, so that we would be able to ensure that the early apoptosis of stromal cells occurred during the immune responses, and therefore, facilitated the tolerance formation. One could suggest using purified apoptotic cells instead. However, we were concerned about the homogeneity of the purified apoptotic cells. Some apoptotic cells might be in their early apoptotic phases while others might be in their late apoptotic or early necrotic phases. Thus far, there are yet no reliable markers for distinguishing apoptotic cells at different stages [17]. The feature of single phosphatidylserine (PS) positive has been used to distinguish

early apoptotic cells [18]; however, it was not applicable to UVB-irradiation-induced cell apoptosis, because we found that shortly after UVB-irradiation, the irradiated cells rapidly became PS⁺ and 7-AAD⁺ double positive [12] (data not shown), which was thought to be a feature of late apoptotic cells.

It has been demonstrated that apoptotic cells and necrotic cells play distinct roles in regulating immune responses [19,20]. Apoptotic cells induce immune tolerance whereas necrotic cells activate immune response [17]. Therefore, for tolerance induction, especially *in vivo* tolerance induction, the encounter of apoptotic cells at their early phase by immune system is critical. Transfusions of UVB-irradiated stromal cells immediately after irradiation would allow most of the apoptotic process to occur *in vivo* whereby tolerizing the antigen-responding T cells during the autoimmune responses.

In line with previous studies [21,22] showing immunosuppression by apoptotic cells, our studies demonstrated that apoptotic splenic stromal cells markedly suppressed the proliferation of β cell antigen-specific T cells *in vitro* and *in vivo* (Fig. 1). Several mechanisms underlying the direct immunosuppression induced by apoptotic cells may be involved. For example, the exposure of phosphatidylserine (PS) on apoptotic cells may play an important role as evidence has shown that PS-carried liposomes significantly suppress T cell responses [23]. Of interest, we found that the apoptotic stromal cells did not affect T cell activation induced by the activators, such as CD3 antibody or antigenic peptide (data not shown). The normal activation of T cells and the arresting of T cell proliferation in the presence of apoptotic cells suggest that responding T cells are rendered unresponsive following activation before they enter into the cell cycle, which may facilitate immune tolerance induction [24,25].

The results shown in Fig. 2 indicate the apoptotic stromal cells promote the induction of immune tolerance specific to the antigens used for stimulating T cells. We also found that the tolerance-inducing feature is not unique for UVB-stroma. UVB-irradiated bone marrow cells and splenocytes had similar effects. There is also no specific MHC requirement (data not shown). This finding suggests that the environment created by apoptotic cells, no matter which type of apoptotic cells are used, is critical for tolerance induction. This finding also underscores the possibility of utilizing a type of universal apoptotic cell, such as apoptotic PBMCs in clinics for inducing immune tolerance to the targeted antigens including self-antigens in autoimmune diseases and MHC antigens in allogeneic transplantations. Indeed, Maeda et al. [11] reported that intravenous injection of apoptotic syngeneic splenocytes led to immune tolerance specific to DNFB antigens simultaneously administered to the skin during induction of contact hypersensitivity. Recent evidence has also shown that T1D patients benefited from photopheresis, which is proposed to be associated with photopheresis-induced apoptosis of blood cells [26].

It appears that the β cell antigen-specific tolerance induced in the presence of apoptotic stroma is associated with the induction of IL-10-producing T cells (Figs. 2 and 5). IL-10-producing T cells (Tr1) are one type of regulatory T cells which play an important role in maintaining immune tolerance [27]. Induction of Tr1 cells is an important strategy for preventing autoimmune diseases and allograft rejection [28]. The antigen recall experimental data shown in Fig. 5 indicate that *in vivo* injection of apoptotic stroma induces IL-10-producing T cells which produce high levels of IL-10 upon β cell antigen challenge. The significant prevention of T1D in NOD mice and reduction of insulinitis by transfusion of UVB-stroma (Figs. 3 and 4) may be associated with the induced IL-10-producing T cells.

Other mechanisms might also be involved in β cell antigen-specific tolerance induction by apoptotic stromal cells. It has been reported that apoptotic cells act on antigen-presenting cells such as dendritic cells and make the latter less sensitive to maturation stimuli [6,29–31], and

the steady state of immature dendritic cells is essential for tolerance maintenance [6]. Recently, Kim et al. [32] reported that apoptotic cells shut down IL-12 through cell-to-cell contact, and promoted IL-10 production by DCs. Additionally, apoptotic cells can trigger macrophages or dendritic cells to produce an anti-inflammatory cytokine, TGF- β [7,8]. TGF- β has recently been shown to be a key factor in converting CD4⁺ CD25⁻ Foxp3⁻ T cells into CD4⁺ CD25⁺ Foxp3⁺ regulatory T cells [33–35]. It has also been reported that apoptotic cells induced Mer tyrosine kinase-dependent blockade of NF- κ B activation and suppressed proinflammatory cytokine production by dendritic cells [36]. In addition, apoptotic cells can promote the development of CD8⁺ regulatory T cells [37]. The mechanisms underlying the apoptotic stroma-induced T1D protective effect need to be further elucidated.

The NOD.BDC2.5 mouse has a transgenic TCR on CD4⁺ T cells that recognizes a specific β cell antigen. Although the natural antigen for this TCR is unknown, several antigen mimotopes have been discovered including peptide 1040-55 [38] used in this study. This TCR-transgenic mouse model is useful for studying the immunopathogenic mechanisms of T1D. It is noted in the present study that the adoptively transferred TCR-transgenic NOD.BDC2.5 T cells exhibit reduced proliferation in pancreatic draining lymph nodes (PLN) in UVB-stroma-treated NOD mice as compared to PBS-treated NOD mice (Fig. 6), suggesting that immune tolerance to multiple β cell antigens might be induced with UVB-stroma transfusion. As described above, in NOD.BDC2.5 mice, only CD4⁺ T cells possess TCRs that respond to specific β cell antigens [38]. Consistent with this, only CFSE-labeled BDC2.5 CD4⁺ T cells were found with proliferation in our study (Fig. 6A). This finding suggests that the suppression of CD4⁺ T cell proliferation in PLN of UVB-stroma-treated mice was β cell antigen-specific, and that β cell antigen-specific regulatory T cells might be involved.

In summary, UVB-irradiated splenic stromal cells suppress T cell responses directly as well as through induction of IL-10-producing regulatory T cells *in vitro* and *in vivo*. The T1D protection observed in NOD mice after transfusion of apoptotic splenic stromal cells suggests that the use of apoptotic non- β cells is a potential clinical approach for the management of T1D in humans.

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Fig. 1.

Apoptotic stromal cells suppress β cell antigen-specific T cell proliferation *in vitro* and *in vivo*. (A). Purified splenic dendritic cells were pulsed with BDC2.5 TCR-specific antigenic peptide ($10 \mu\text{g}/10^6$ cells/ml) at 37°C and $5\% \text{CO}_2$ incubator for 2 h. Antigen-pulsed dendritic cells (1×10^4) were cultured with CD4+ T cells (1×10^5) isolated from BDC2.5 mouse spleen in $200 \mu\text{l}$ HL-1 medium in a U-bottom 96-well plate with different concentrations of UVB-irradiated stromal cells as shown in the figure for four days. Then, ^3H -thymidine ($1 \mu\text{Ci}/\text{well}$) was added for an additional 16 h. Triplicate wells were used for each culture condition. Results shown are representative of three separate experiments. (B). Eight-week-old NOD mice received intravenous injections of CFSE-labeled splenocytes ($1 \times 10^7/\text{mouse}$) from BDC2.5 mice with simultaneous injections of UVB-irradiated stromal cells ($1 \times 10^6/\text{mouse}$) or PBS. Four days later, the division of CFSE+ CD4+ T cells in pancreatic lymph nodes (PLN) and inguinal lymph nodes (not shown) was examined by flow cytometry after staining with anti-CD4-PE. The data shown are from one of three mice in each group.

Fig. 2.

Apoptotic stromal cells promote the development of IL-10-producing T cells *in vitro*. (A). Purified BDC2.5 CD4⁺ T cells (2×10^6) were incubated with purified NOD splenic dendritic cells (2×10^5) pulsed with peptide 1040-55 in the presence or absence of UVB-irradiated stromal cells (1×10^6) in 1 ml culture medium in a 24-well plate. Three days later, dead cells were removed using a dead cell removal kit (BD). The viable CD4⁺ T cells (1×10^5) were purified by MACS and stimulated with dendritic cells (1×10^4) pulsed with 1040-55 peptide in a U-bottom 96-well plate for four days. ³H-thymidine (1 μ Ci) was added to each well and incubated for an additional 16 h. (B). The above purified CD4⁺ T cells were stimulated with a leukocyte activation cocktail reagent (BD) for 4 h. Intracellular staining for IFN- γ and IL-10 was performed using an intracellular cytokine staining kit (BD). These experiments were repeated three times yielding similar results.

Fig. 3.

Transfusion of UVB-irradiated stromal cells prevents T1D in NOD mice. Eight-week-old female NOD mice were treated with three weekly transfusions of UVB-irradiated stromal cells (1×10^5 /mouse) or PBS. Urine glucose was measured once a week for all mice and positive readings were confirmed by blood glucose measurement. Diabetes was diagnosed after blood glucose measured was greater than 250 mg/dL for two consecutive days. The data were analyzed by the log rank test. The difference is considered significant when $p < 0.05$.

Fig. 4.

Transfusion of UVB-irradiated stromal cells alleviates islet insulinitis. Eight-week-old female NOD mice were treated with three weekly transfusions of UVB-stroma or PBS (three mice in each group). All mice were sacrificed at 15 weeks of age. Pancreatic insulinitis was graded after H-E staining. (A). Pancreas from mice receiving UVB-stroma and PBS treatments, respectively. (B). The summaries of the percentages of different grades of insulinitis in two different groups are shown.

Fig. 5.

Transfusion of UVB-stroma induces *IL-10* producing *CD4*⁺*T* cells *in vivo*. Eight-week-old female NOD mice were treated with three weekly transfusions of UVB-stroma (1×10^5 /mouse), or PBS (three mice in each group). The following week, all mice were sacrificed and splenocytes were prepared as described elsewhere. Splenocyte cultures (1×10^6 /well) were stimulated with insulin B9-23 (12.5 μ M) or NIT1 lysates (50 μ g/ml) for four days. The production of IL-4, IL-10 and IFN- γ was measured using Luminex beads. (Comparison of UVB-stroma treated versus PBS-treated groups, * $p < 0.05$, ** $p < 0.01$.)

Fig. 6. Proliferation of adoptively transferred NOD.BDC2.5 T cells is suppressed in UVB-stroma-treated NOD mice. Eight-week-old female NOD mice were treated with three weekly transfusions of UVB-stroma (1×10^5 /mouse) or PBS (three mice in each group). The following week, mice received CFSE-labeled BDC2.5 splenocytes (1×10^7 /mouse). All mice received intraperitoneal injections of streptozotocin (40 mg/kg). Four days later, mice were sacrificed and cells from PLN and inguinal lymph nodes were obtained for analysis by flow cytometry. (A) Proliferation of CD4⁺ cells in PLN and inguinal lymph nodes was determined based on CFSE dilution by flow cytometry. Data shown were from one mouse representative of results obtained from treatment group (three mice/group). (B) The percentages of dividing CD4⁺ T

cells in total CFSE+ CD4+ cells are summarized and statistically analyzed. The difference is considered significant when $p < 0.05$.