REVIEW ARTICLE

Treatment of Autoimmune Diabetes by Inhibiting the Initial Event

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Recent papers have shown that the initial event in the pathogenesis of autoimmune type 1 diabetes (T1D) comprises sensing of molecular patterns released from apoptotic β-cells by innate immune receptors such as toll-like receptor (TLR). We have reported that apoptotic β-cells undergoing secondary necrosis called 'late apoptotic' β-cells stimulate dendritic cells (DCs) and induce diabetogenic T cell priming through TLR2. The role of other innate immune receptors such as TLR7 or TLR9 in the initiation of T1D has also been suggested. We hypothesized that TLR2 blockade could inhibit T1D at the initial step of T1D. Indeed, when a TLR2 agonist, Pam3CSK$_4$ was administered chronically, the development of T1D in nonobese diabetic (NOD) mice was inhibited. Diabetogenic T cell priming by DCs was attenuated by chronic treatment with Pam3CSK$_4$, indicating DC tolerance. For the treatment of established T1D, immune tolerance alone is not enough because β-cell mass is critically reduced. We employed TLR2 tolerance in conjunction with islet transplantation, which led to reversal of newly established T1D. Dipeptidyl peptidase 4 (DPP4) inhibitors are a new class of anti-diabetic agents that have beneficial effects on β-cells. We investigated whether a combination of DPP4 inhibition and TLR2 tolerization could reverse newly established T1D without islet transplantation. We could achieve normoglycemia by TLR2 tolerization in combination with DPP4 inhibition but not by TLR2 tolerization or DPP4 inhibition alone. β-cell mass was significantly increased by combined treatment with TLR2 tolerization and DPP4 inhibition. These results suggest the possibility that a novel strategy of TLR tolerization will be available for the inhibition or treatment of established T1D when combined with measures increasing critically reduced β-cell mass of T1D patients such as DPP4 inhibition or stem cell technology.
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INTRODUCTION

While the last step in the development of type 1 diabetes (T1D) is death of β-cells producing insulin, the initial step or "the initial event" of T1D has been unclear. Recent papers have shown the role of β-cell death in the initial course of T1D, suggesting that β-cell death or apoptosis is α and ω in the pathogenesis of T1D. Such findings provide not only innovative scientific clues to the understanding of T1D but also a novel strategy for the inhibition of treatment T1D. In this review, we will summarize recent findings regarding the relationship between β-cell death and autoimmunity, and their application to the prevention or treatment of T1D.

β-CELL APOPTOSIS AS A MECHANISM OF DIABETOGENIC T CELL SENSITIZATION IN T1D

In T1D, apoptosis of pancreatic β-cells appears to be the

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Abbreviations: T1D, type 1 diabetes; TLR, toll-like receptor; DC, dendritic cell; NOD, nonobese diabetic; DPP4, dipeptidyl peptidase, Mφ, macrophage; DAMP, death-associated molecular pattern; APC, antigen-presenting cell; CRAMP, cathelicidin-related antimicrobial peptide; GLP-1, glucagon-like peptide 1; iPSc, induced pleuripotent stem cell

last step in the development of diabetes. After a long sequence of autoimmune processes, finally β-cell apoptosis occurs and clinically overt diabetes ensues when β-cell mass is lowered below a certain threshold (1,2). In contrast, the initial event in the development of T1D has been unclear, while the initial event could be more important both scientifically and clinically compared to the last event. Clues to the initial event came after the elucidation of the cell death mechanism and cloning of innate immune receptors. Suggestion that cell death and the initiation of autoimmunity are interrelated was from the observation that there is a transient wave of physiological β-cell apoptosis peaking at 2~3 weeks of age during the pancreas organogenesis (3,4), which coincides with the onset of autoimmunity to β-cell autoantigens in nonobese diabetic (NOD) mice developing classical T1D. Apoptotic cells are rapidly engulfed by macrophages (MΦs), thus no inflammation ensues after apoptosis. In contrast, necrotic cells release death-associated molecular pattern (DAMP) and can stimulate antigen-presenting cells (APCs) such as MΦs or dendritic cells (DCs), which acts as an alarm signal for immune stimulation. MΦs of NOD mice have been reported to have defect in their phagocytic capability, which may allow apoptotic cells to undergo secondary necrosis. Such secondary necrotic β-cells can induce inflammation or immunity through innate immune receptors. Indeed, we observed that insulinoma cells undergoing secondary necrosis can stimulate MΦs in a TLR2-dependent pathway, inducing inflammatory cytokine release from MΦs (5). We also found that and priming of diabetogenic T cells by DCs *in vivo* that occurs selectively in pancreatic lymph nodes due to β-cell death is also dependent on TLR2. *In vivo* role of TLR2 in the development of T1D was further supported by a marked decrease in the incidence of diabetes in two types of T1D animal models—multiple low-dose streptozotocin model and spontaneous T1D model of NOD mice (5). Role of TLRs other than TLR2 in T1D has also been suggested in a recent paper reporting that activation of TLR9 of plasmacytoid DCs by a complex of self DNA, anti-double-strand DNA antibody released from B-1a lymphocytes in response to β-cell apoptosis and DNA-binding cathelicidin-related antimicrobial peptide (CRAMP) released from neutrophils in the initiation of T1D (6).

INHIBITION OF T1D BY TLR2 TOLERANCE

These results showing the sensing of DAMP from β-cells by TLR2 on DCs in the initial step of the development of T1D
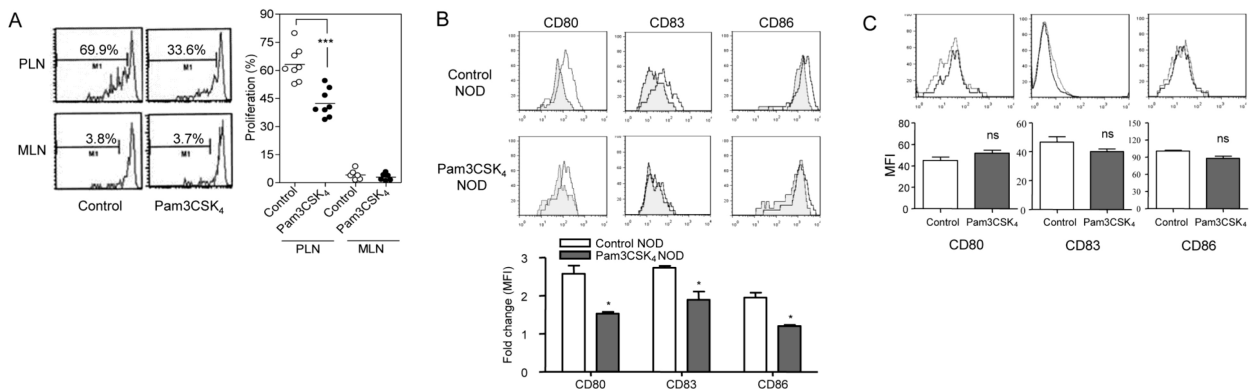

Figure 1. TLR2 tolerance induced by prolonged treatment with Pam3CSK$_4$ *in vivo*. (A) When CD4$^+$ diabetogenic T cells from BDC2.5/NOD mice were labeled with carboxyfluorescein succinimidyl ester (CFSE) and transferred into control recipient NOD mice, CD4$^+$ T cell proliferation measured by analyzing CFSE dilution by FACS analysis gated on CD4$^+$ and Vβ4$^+$ cells was observed specifically in the pancreatic lymph nodes but not in the mesenteric lymph nodes, as previously reported (16). In NOD mice treated with Pam3CSK$_4$ *in vivo* for 3 weeks, CD4$^+$ diabetogenic T cell proliferation was significantly attenuated, indicating DC tolerance. Pooled results (right). A representative histogram (left). (B) Splenocytes prepared from NOD mice to which Pam3CSK$_4$ was administered for 3 weeks, were further treated with Pam3CSK$_4$ (black line) or PBS (gray filled histogram) *in vitro* for 1 day. The induction of costimulatory molecules on DCs by in vitro treatment with Pam3CSK$_4$ that was determined by FACS analysis gated on CD11c$^+$ cells was significantly lower compared to control NOD mice, suggesting DC tolerance. Fold changes of mean fluorescent intensities (MFI) (lower). (C) No significant difference in the expression of costimulatory molecules on splenic DCs between NOD mice that were treated with Pam3CSK$_4$ *in vivo* (black line) and control NOD mice (gray line), suggests that *in vivo* Pam3CSK$_4$ treatment for 3 weeks does not activate DCs. (***p<0.005; *p<0.05; ns, not significant) (Originally published in *The Journal of Immunology*. Kim D. H., J-C. Lee, S. Kim S, S. H. Oh, M.-K. Lee, K.-W. Kim, M.-S. Lee. 2011. Inhibition of autoimmune diabetes by TLR2 tolerance. *J. Immunol*.187: 5211-5220. Copyright © [2011] The American Association of Immunologists, Inc.)

imply that TLR2 blockade could be employed to inhibit auto-immune diabetes. Thus, we employed the strategy of TLR2 tolerance induction which is similar to the well-known LPS tolerance (7). Indeed, when we administered a TLR2 agonist, Pam3CSK$_4$ to NOD mice since 3 weeks of age, the incidence of diabetes was significantly suppressed, suggesting that TLR2 tolerance can inhibit the development of T1D (8). The inhibition of T1D by chronic treatment with Pam3CSK$_4$ could be attributed to TLR2 tolerance of DCs since diabetogenic T cell proliferation in pancreatic lymph nodes by DCs was significantly suppressed after prolonged treatment with Pam3CSK$_4$ (Fig. 1A). Induction of costimulatory signals on DCs by *in vitro* incubation with Pam3CSK$_4$ was also attenuated by chronic administration of Pam3CSK$_4$ *in vivo* for 3 weeks, again attesting the induction of DC tolerance (8) (Fig. 1B). On the other hand, the expression of costimulatory molecules on DCs was not changed by *in vivo* treatment with Pam3CSK$_4$ alone, suggesting that prolonged Pam3CSK$_4$ administration itself does not activate DCs (Fig. 1C). When we studied the molecular mechanism of TLR2 tolerance by chronic treatment with TLR2 agonist, decreased expression of IRAK-1 and -4, positive regulator of TLR signaling, and increased expression of IRAK-M, a negative regulator of TLR signaling were noted. Downregulation of IRAK-1 and -4 protein levels was due to proteasomal degradation as the reduced IRAK-1 and -4 protein levels were restored by proteasomal inhibitors. In this regard, a recent paper showed contribution of IRAK-4 to TLR2 tolerance but not to endotoxin tolerance (9), which can explain the selective TLR2 tolerance after treatment with Pam3CSK$_4$ without heterotolerance to endotoxin (8). Besides DC tolerance, other immune mechanisms such as changes in Th1/Th2 polarization or Treg cells can play a role in the inhibition of T1D after chronic treatment with Pam3CSK$_4$. However, we observed no changes in the Th1/Th2 polarity and the number or activity Treg cells after Pam3CSK$_4$ treatment, while we cannot totally eliminate the role of Th1/Th2 polarization or Treg cells in the inhibition of T1D by Pam3CSK$_4$ (8).

TREATMENT OF ESTABLISHED T1D BY TLR2 TOLERANCE IN CONJUNCTION WITH MEASURES INCREASING β-CELL MASS

While T1D of NOD mice could be inhibited by TLR2 tolerization, immune tolerance alone is not enough for the treatment of established T1D because β-cell mass is already critically

reduced below a certain threshold in clinically overt T1D. Immune tolerance in established T1D is also different from that in prediabetic animal models in that immune tolerization should be targeted to sensitized T cells rather than naïve T cells. To confirm that TLR2 tolerance could be induced in sensitized T cells, adoptive transfer experiment was conducted in which transfer of sensitized T cells from diabetic NOD mice rapidly induces diabetes in nondiabetic NOD mice. When we administered Pam3CSK$_4$ to recipient mice since just before adoptive transfer of T cells from diabetic mice, the development of diabetes in the recipient mice was significantly suppressed (Fig. 2), suggesting that TLR2 tolerization can block activity of sensitized T cells as well. Sustained interaction between DCs and diabetogenic T cells may be necessary for full *in vivo* activity of effector T cells. To attain another goal for the treatment of established T1D-replenishment of critically reduced β-cell mass, we employed a well-known strategy of islet transplantation. When islets were grafted under the kidney capsule of newly diabetic NOD mice, blood glucose was normalized immediately. However, diabetes recurred in 10 days because already established autoimmunity destroys islet graft. In contrast, when we administered Pam3CSK$_4$ since just before islet transplantation, blood glucose was maintained below diabetic level in most mice as long as Pam3CSK$_4$ was administered (8), suggesting
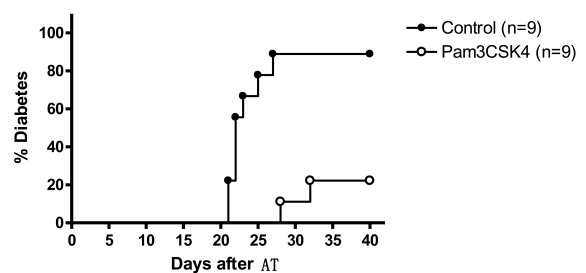

Figure 2. Effect of *in vivo* treatment with Pam3CSK$_4$ on diabetes transfer by sensitized diabetogenic T cells. The incidence of diabetes after adoptive transfer (AT) of splenocytes from diabetic NOD mice to irradiated nondiabetic recipient NOD mice was significantly reduced by treatment of the recipient NOD mice with Pam3CSK$_4$ for 2 weeks before AT (p < 0.005). (Originally published in *The Journal of Immunology.* Kim D. H., J-C. Lee, S. Kim S, S. H. Oh, M.-K. Lee, K.-W. Kim, M.-S. Lee. 2011. Inhibition of autoimmune diabetes by TLR2 tolerance. *J. Immunol.*187: 5211-5220. Copyright © [2011] The American Association of Immunologists, Inc.)

successful treatment of newly established T1D by a combination of TLR2 tolerance and islet transplantation.

While established T1D could be treated by a combination of TLR2 tolerization and islet transplantation, supply of islets is too limited to cover clinical demand, which preclude the clinical application of islet transplantation for the treatment of established T1D. We thus employed another strategy of increasing reduced β-cell mass. Recently, glucagon-like peptide 1 (GLP-1) mimetics or dipeptidyl peptidase 4 (DPP4) inhibitors that inhibit degradation of GLP-1 by DPP4 *in vivo*, are being used as new classes of anti-hyperglycemic agents with β-cell trophic action not only in animal model of diabetes (10,11) but also in humans (12). We investigated whether a combination of DPP4 inhibition by DA1229, a novel inhibitor of DPP4 (13) and TLR2 tolerance could treat established T1D. Indeed, euglycemia was achieved in more than 80% of newly diabetic NOD mice after chronic treatment with Pam3CSK$_4$ in combination with DA1229, while treatment with Pam3CSK$_4$ or DA1229 alone was without effect (14). Consistent with the improved blood glucose level, β-cell mass was significantly increased after combined treatment with Pam3CSK$_4$ and DA1229 but not after treatment with Pam3CSK$_4$ or DA1229 alone. The increase in β-cell mass could be attributed to the enhanced proliferation of β-cells as evidenced by increased number of bromodeoxyuridine-incorporating β-cells and an increased number of small β-cell units that comprise less than four β-cells and represent β-cell neogenesis, which was observed in diabetic NOD mice treated with Pam3CSK$_4$ in combination with DA1229 but not in those treated with Pam3CSK$_4$ or DA1229 alone (14). These data suggest the possibility that a combination of TLR2 tolerization and DPP4 inhibition could employed as a new method for the treatment of established T1D without islet transplantation.

CONCLUSION

While it has been elusive for a long time, the real identity of the initial event of T1D is finally emerging, which was owing to the elucidation of the relationship between cell death and innate immune responses and characterization of innate immune receptors. Such a revelation is likely, to lead to the development of novel strategies for the prevention or treatment of T1D. Methods increasing β-cell mass is already available such as DPP4 inhibitors or GLP-1 mimetics. Better methods to increase β-cell mass will be available in the fu-

ture since the discovery of inducible pleuripotent stem cell (iPSc) technology (15). By combining such methods of increasing β-cell mass and inhibition of the initial event of T1D, clinically applicable methods of treating established T1D patients will be finally available.

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CONFLICTS OF INTEREST

The author has no financial conflict of interest.

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