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Inhibition of Autoimmune Diabetes by TLR2 Tolerance

Do-Hoon Kim,* June-Chul Lee,* Sunshin Kim,† Seung Hoon Oh,* Moon-Kyu Lee,* Kwang-Won Kim,* and Myung-Shik Lee*‡

We have reported that apoptotic β cells undergoing secondary necrosis, called “late apoptotic (LA) β cells,” stimulated APCs and induced diabetogenic T cell priming through TLR2, which might be one of the initial events in autoimmune diabetes. Indeed, diabetogenic T cell priming and the development of autoimmune diabetes were significantly inhibited in TLR2-null NOD mice, suggesting the possibility that TLR2 blockade could be used to inhibit autoimmune diabetes. Because prolonged TLR stimulation can induce TLR tolerance, we investigated whether repeated TLR2 administration affects responses to LA β cells and inhibits autoimmune diabetes in NOD mice by inducing TLR2 tolerance. Treatment of primary peritoneal macrophages with a TLR2 agonist, Pam3CSK4, suppressed cytokine release in response to LA insulinoma cells or further TLR2 stimulation. The expression of signal transducer IRAK-1 and -4 proteins was decreased by repeated TLR2 stimulation, whereas expression of IRAK-M, an inhibitory signal transducer, was enhanced. Chronic Pam3CSK4 administration inhibited the development of diabetes in NOD mice. Diabetogenic T cell priming by dendritic cells and upregulation of costimulatory molecules on dendritic cells by in vitro stimulation were attenuated by Pam3CSK4 administration in vivo. Pam3CSK4 inhibited diabetes after adoptive transfer of diabetogenic T cells or recurrence of diabetes after islet transplantation by pre-existing sensitized T cells. These results showed that TLR2 tolerance can be achieved by prolonged treatment with TLR2 agonists, which could inhibit priming of naive T cells, as well as the activity of sensitized T cells. TLR2 modulation could be used as a novel therapeutic modality against autoimmune diabetes. The Journal of Immunology, 2011, 187: 5211–5220.

Previous papers have provided evidence suggesting that activation of dendritic cells (DCs) by apoptotic β cells during organogenesis of the pancreas and subsequent priming of autoreactive T cells by DCs in the pancreatic lymph nodes (PLNs) are the initial events in the development of type 1 autoimmune diabetes of NOD mice (1, 2). We recently showed that apoptotic β cells undergoing secondary necrosis, called “late apoptotic” (LA) β cells, could stimulate APCs through TLR2, contributing to the priming of diabetogenic T cells and to the development of autoimmune diabetes (3). These results suggest the possibility that cellular materials from LA cells could act as endogenous ligands for TLR2 and induce the release of inflammatory cytokines from macrophages (MΦs) or DC maturation. Those results also suggest that modulation of TLR2 could affect the course of autoimmune diabetes. Indeed, the development of autoimmune diabetes was significantly inhibited in TLR2-null NOD mice, suggesting the therapeutic potential of TLR2 blockade in autoimmune diabetes (3).

In contrast to TLR blockade, TLR stimulation could have more complex consequences. Short-term stimulation with TLR agonists induces inflammation and contributes to the initiation of immune responses; however, repeated stimulation with TLR agonists could lead to the abrogation of further inflammatory/immune responses, which was exemplified in the case of prolonged stimulation with LPS, a TLR4 ligand. This phenomenon of LPS tolerance could be an adaptive process that dampens excessive inflammation during the course of Gram-negative sepsis (4). Because of the potential clinical and scientific values of the LPS-tolerance phenomenon, significant efforts have been made to elucidate the mechanism underlying LPS tolerance and its application. Because tolerance to TLR stimulation may occur in response to TLR agonists other than TLR4 agonists (5), we investigated whether repeated TLR2 stimulation could affect further response of APCs, such as MΦs or DCs, to LA β cells and chronic administration of TLR2 agonists could affect the course of autoimmune diabetes. Our results showed that TLR2 agonists could inhibit the development of autoimmune diabetes or the recurrence of diabetes after islet transplantation, suggesting the potential therapeutic value of TLR2 agonists.

Materials and Methods

Cells and reagents

The culture conditions for MIN6N8 insulinoma and primary islet cells have been described (6). Pam3CSK4 was from EMC Microcollections. Polyinosinic-polycytidylic acid (poly [I:C]) and CpG oligonucleotide were purchased from InvivoGen. All other chemicals were from Sigma, unless indicated otherwise.

Mice

C57BL/6, NOD/Lt, and OT-II TCR-transgenic mice were from The Jackson Laboratory. BDC2.5/NOD TCR-transgenic mice were kindly provided by C. Benoist and D. Mathis (Harvard University). BDC2.5 mice were typed by flow cytometry using mAbs against CD4 and Vβ4 (eBioscience). Diabetes was monitored by testing urine glucose on a weekly basis and was confirmed

*Department of Medicine, Samsung Medical Center, Seoul 135-710, Korea; †Carcinogenesis Branch, Korean National Cancer Center, Goyang 410-769, Korea; and ‡Samsung Advanced Institute for Health Science and Technology, Sungkyunkwan University School of Medicine, Seoul 135-710, Korea

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Address correspondence and reprint requests to Prof. Myung-Shik Lee, Department of Medicine, Samsung Medical Center, 50 Irwon-dong Kangnam-ku, Seoul 135-710, Korea. E-mail address: msel0923@skku.edu

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Abbreviations used in this article: BCG, bacillus Calmette-Guerin; DC, dendritic cell; LA, late apoptotic; MΦ, macrophage; MFI, mean fluorescence intensity; MLN, mesenteric lymph node; PLN, pancreatic lymph node; poly [I:C], polyinosinic-polycytidylic acid; Treg, regulatory T cell.

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by nonfasting blood glucose levels >300 mg/dl. Blood-chemistry profiles were determined using a Fujif Dri-Chem 3000 blood chemistry analyzer (Fujif), according to the manufacturer’s instructions. All mice were maintained under specific pathogen-free conditions at Samsung Biomedical Research Center Facility. All animal experiments in this work were performed in accordance with the institutional guideline of the Samsung Medical Center Animal Facility, an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited institution.

**MOF preparation and culture**

Primary peritoneal MOFs were collected from 2–3-mo-old mice 3 d after i.p. injection of 3.85% thioglycolate (Difco), plated on plastic tissue culture plates, and incubated at 37°C for 16 h (7). Nonadherent cells were removed by washing three times with fresh RPMI 1640, and adherent MOFs were cultured overnight in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine for further use.

**Generation of LA cells**

To obtain LA cells with features of secondary necrosis, MIN6N8 insulinoma cells were irradiated at 20 mJ/cm² with a UV Statelinker 2400 (Stratagene) and left for 30 h. The mode of cell death was confirmed by flow cytometry after staining with Annexin V-FITC reagent (PharMingen) and propidium iodide (3).

**ELISA**

After treatment of primary peritoneal MOFs with TLR agonists, supernatant was harvested. In the case of LA cells, 2 × 10⁵ cells were added to the same number of peritoneal MOFs, and the supernatant was collected after culture. TNF-α, IL-12p40, or IL-10 in the culture supernatants was measured by ELISA, according to the manufacturer’s recommendation (R&D Systems).

**Western blot analysis**

MOFs were lysed in a buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 0.5 mM NaVO₄, 50 mM NaF, 1 mM PMSE, and protease inhibitor mixture [Roche]) for 20 min on ice. The protein concentrations in cell lysates were determined using a Bio-Rad protein assay kit. An equal amount of protein for each sample was separated by SDS-PAGE and subsequently transferred to Hybond ECL nitrocellulose membranes (Amersham). After blocking with 5% skim milk, the membranes were sequentially incubated with the indicated primary Ab and horseradish peroxidase-conjugated secondary Ab (Amersham). Abs to IRAK-1, IRAK-4, -M, and -b-actin were purchased from Santa Cruz Biotechnology. Ab to IRAK-M was from Cell Signaling. Intensity of bands in Western blot was quantified by densitometry using ImageJ Program (National Institutes of Health) and normalized to the bands of housekeeping genes.

**RT-PCR**

The expression levels of IRAK-1, -4, -M, and -b-actin mRNA were determined by RT-PCR using specific primer sets (IRAK-1 forward, 5’-CTGAAAGA-GCCGTGAGTGACC-3’; reverse, 5’-GATGTCCTCCTCCTGGTACA-3’; IRAK-4 forward, 5’-CAACAACCTTGCAAGCACA-3’; reverse, 5’-GCAGAAAACCTGATGCCATT-3’; and IRAK-M forward, 5’-TCTTCTCA-GGTGTCCTCTCCTCAGT-3’; reverse, 5’-CCCTCTCCCTGTTGCT-GCTC-3’). Intensity of RT-PCR bands was quantified by densitometry, as above.

**Insulitis scoring**

Paraffin-embedded sections of the pancreas were stained with H&E for light microscopy. To determine the severity of insulitis, 30–50 pancreatic islets from three or more parallel sections of different cut levels, >40 μm apart, were analyzed/mouse. The degree of insulitis was classified into the following four categories: 0, no insulitis; 1, peri-insulitis with or without minimal lymphocytic infiltration in islets; 2, invasive insulitis with islet destruction ≤50%; and 3, islet destruction >50% (6).

**In vivo T cell priming**

Priming of diabeticogenic T cells was assessed using CFSE (Molecular Probes)-labeled BDC2.5 CD4⁰ T cells, as described (2), with modifications (3). CD4⁰ T cells were prepared from the pooled spleens of BDC2.5/NOD mice by the negative-selection method using a MACS CD4⁺ T cell isolation kit (Miltenyi Biotec). Purity of CD4⁺ T cells was confirmed by FACS analysis (>95%). CFSE-labeled CD4⁺ T cells (2 × 10⁵) were transferred by tail vein injection into recipient NOD mice, to which Pam3CSK₄ was administered three times a week for 1–3 wk, beginning at 3 wk of age, or control NOD mice of the same age. The PLNs, mesenteric lymph nodes (MLNs), and spleens were harvested 66 h after transfer, and single-cell suspensions were analyzed for CFSE dilution by flow cytometry gated on CD4⁺ and Vβ4⁺ cells.

**In vitro T cell proliferation**

To study the effect of chronic administration of Pam3CSK₄ on Ag presentation in vitro, splenocytes from NOD mice treated with 100 μg Pam3CSK₄, three times a week for 3 wk were spun through 16% Accudenz (Accurate Chemical & Scientific) to enrich the DC fraction. Splenic DCs were then positively selected using a MACS DC cell isolation kit (Miltenyi Biotec), and 2 × 10⁴ DCs were cocultured with CFSE-labeled BDC2.5 CD4⁺ T cells (1 × 10⁵) in the presence of 100 ng/ml BDC2.5 mimotope (RVRPLWLRME (Peptron) (8). Splenic DCs were also isolated by the same method from C57BL/6 mice that were treated with 100 μg Pam3CSK₄, three times a week for 3 wk. Splenic DCs from C57BL/6 mice (2 × 10⁴) were cocultured with CFSE-labeled OT-II CD4⁺ T cells (2 × 10⁴) from OT-II transgenic mice in the presence of 100 ng/ml OVA123–33 peptide (Peptron) (9). CD4⁺ T cell proliferation was determined by measuring CFSE dilution in CD4⁺ T cells using flow cytometry.

**DC tolerance**

To study DC tolerance by in vivo administration of Pam3CSK₄, mononuclear cells were isolated from the spleen or PLNs of NOD mice treated with 100 μg Pam3CSK₄, three times a week for 3 wk and incubated with Pam3CSK₄ ex vivo for 1 d. The expression of CD80, CD83, and CD86 costimulatory molecules on DCs was determined by flow cytometry gated on CD11c⁺ cells after incubation with specific Abs (eBioscience). To study possible DC activation by chronic Pam3CSK₄ administration in vivo, splenocytes were freshly isolated from NOD mice that were treated with Pam3CSK₄ for 3 wk, and the expression of CD80, CD83, and CD86 costimulatory molecules on DCs was determined as above, without ex vivo Pam3CSK₄ treatment. For the study of signaling molecules, bone marrow-derived DCs were prepared, as previously described (3), and the expression of IRAK-1, -4, -M was evaluated with the same protocols that were used for MOFs. The expression of IRAK-1, -4, -M was also examined in splenic DCs that were prepared as above.

**Th1/Th2 differentiation**

Th1/Th2 differentiation of naive T cells was carried out, as described (3), with modifications. Briefly, CD4⁺ T cells prepared from the spleens using a MACS CD4⁺ T cell isolation kit were subjected to multiparameter FACS using CD25, CD44, and CD62L Abs (eBioscience). Naive CD4⁺CD25⁺ CD44⁺ CD62L⁻ T cells (purity >99%) were stimulated with 5 μg/ml immobilized anti-CD3e Ab (145-2C11) and 1 μg/ml soluble anti-CD28 Ab (PharMingen) in the presence of 5 ng/ml IL-12 (R&D Systems) and 10 μg/ml anti-IL-4 Ab (11B11) for Th1 differentiation or 1000 U/ml IL-4 (Endogen), 5 μg/ml anti-IFN-γ Ab (XMG1.2), and 3 μg/ml anti-IL-12 Ab (C17.8) for Th2 differentiation. After 1 d of culture, 50 U/ml IL-2 (R&D Systems) was added for an additional 6 d of culture. Cells were then extensively washed, and an equal number of cells was restimulated with immobilized anti-CD3e Ab for 1 d. Supernatants were harvested for measurement of IFN-γ and IL-4 release by ELISA (R&D Systems). For intracellular cytokine staining, naive CD4⁺CD25⁻ CD44⁺ CD62L⁻ T cells were cultured in a polarizing medium containing IL-2 for 6 d, as above. Cells were then stimulated with 50 ng/ml PMA and 500 ng/ml ionomycin for 5 h in the presence of brefeldin A (eBioscience). After surface staining with anti-CD4 Ab, cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences). Subsequently, cells were stained intracellularly with anti-IFN-γ and anti-IL-4 Abs. Samples were acquired on a FACS Calibur with CellQuest software (BD Biosciences), and data were analyzed using FlowJo software (Tree Star).

**Regulatory T cells**

The percentage of CD4⁺CD25⁺ regulatory T cells (Tregs) among the total cell population was analyzed by flow cytometry, as previously described (3). Foxp3 staining of Tregs was done using a kit (eBioscience), according to the manufacturer’s recommendation. Briefly, mononuclear cells from the spleen, PLNs, and MLNs (1 × 10⁶) were incubated with 0.125 μg anti-CD4 and 0.06 μg anti-CD25 Abs in 100 μl FACs-staining buffer at 4°C for 30 min. After permeabilization and Fc blocking, cells were incubated with 0.5 μg anti-mouse/rat Foxp3 (FJK-16s) Ab or isotype control for triple-colored flow cytometry. For in vitro suppression assay, CD4⁺CD25⁻ Tregs and CD4⁺CD25⁺ cells were purified from the pooled spleens by a MACS Regulatory T cell isolation kit (Miltenyi Biotec), according to the manufacturer’s recommendation. Briefly, CD4⁺ T cells were purified by depletion of non-CD4⁺
T cells using a negative-selection method. Cells were labeled with PE-conjugated anti-CD25 Ab plus anti-PE microbeads and passed through a magnetic column twice to fractionate CD4+CD25+ and CD4+CD25- cells. The purity of the resulting cell fractions was 92–94%. CD4-depleted spleen cells were irradiated at 3000 rad and used as APCs (3). For thymidine-uptake assays, graded numbers of CD4+CD25+ Tregs were cocultured with 5 x 10^5 CD4+CD25- T cells in the presence of 1 μg/ml soluble anti-CD3e Ab (145-2C11) and 5 x 10^4 irradiated APCs for a total of 3 d. Proliferation of CD4+CD25+ T cells was determined by measuring incorporation of [3H]thymidine (0.5 μCi/well) for the last 18 h.

**Adoptive transfer**

Adoptive transfer of diabetes was conducted according to a previous report, with modifications (10). In brief, 5 x 10^6 splenocytes from diabetic female or male NOD mice were infused into the tail vein of each 8–10-wk-old NOD mouse of the same sex. Recipient mice were sublethally irradiated at 770 rad using a Cesium irradiator (IBL 457; EIES bio international) 16 h before transfer of splenocytes. The incidence of diabetes was >90% at 4 wk after adoptive transfer in our previous experiments.

**β cell mass**

The relative β cell mass was measured by point counting after insulin immunohistochemistry of pancreatic sections, as previously described (11).

**Islet transplantation**

Islets were isolated, using the collagenase-digestion technique (6), from overnight-fasted 4-wk-old prediabetic NOD mice or adult NOD mice pretreated with CFA to inhibit the development of diabetes (12). After 1 d of culture, isolated pancreatic islets appeared circumscribed and apparently free of detectable associated lymphocyte aggregates. Four hundred islets were grafted under the kidney capsule of diabetic NOD mice.

**Statistical analysis**

The incidence of diabetes was plotted according to the Kaplan-Meier method. The incidence between two groups was compared using the log-rank test. For other experiments, the statistical significance was determined by a two-tailed Student t test. Experimental data in the figures are presented as the mean ± SE. The p values < 0.05 were considered to represent statistically significant differences.

**Results**

**TLR2 tolerance in vitro**

In an attempt to determine whether repeated TLR2 stimulation could affect APC responses to LA β cells, which could be one of the initial events in the development of autoimmune diabetes (1, 2), we first studied the release of inflammatory cytokines in response to LA MIN6N8 insulinoma cells or TLR agonists from Mφs that were pretreated with TLR2 agonists. When Mφs from 2–3-mo-old NOD mice were first stimulated for 16 h with 100 ng/ml Pam3CSK4, a prototypic TLR2 agonist (13), and then incubated with LA insulinoma cells that activate APCs in a TLR2-dependent manner (3) for 10 h, the TNF-α release in response to LA insulinoma cells was significantly decreased compared with Mφs preincubated with culture medium alone (p < 0.005) (Fig. 1A). TNF-α release in response to 100 ng/ml Pam3CSK4 treatment for 10 h was also significantly decreased by Pam3CSK4 pretreatment for 16 h (p < 0.005), indicating the induction of TLR2 tolerance by a specific agonist (Fig. 1A) (5). TNF-α release in response to LA insulinoma cells was also decreased by pretreatment with LPS, a TLR4 agonist; however, the decrement was not statistically significant (p > 0.05). TNF-α release in response to Pam3CSK4 was significantly decreased by LPS pretreatment (p < 0.05), whereas the decrement was much less than that resulting from Pam3CSK4 pretreatment (Fig. 1A). The pattern of IL-12 release was similar. IL-12 release in response to LA insulinoma cells or 100 ng/ml Pam3CSK4 treatment for 10 h was significantly decreased by Pam3CSK4 pretreatment for 16 h (p < 0.005, p < 0.005) but not by LPS pretreatment (p > 0.1, p > 0.05) (Fig. 1B). This pattern of TLR2 tolerance was also observed when the preincubation time varied between 6 and 16 h (data not shown). When we pretreated Mφs with LA insulinoma cells for 16 h and then treated with Pam3CSK4 or LA insulinoma cells for 10 h, release of TNF-α or IL-12 was significantly decreased by pretreatment with LA insulinoma cells compared with control pretreatment (p < 0.005 for all comparisons) (Fig. 1C, 1D), supporting the fact that LA insulinoma cells act on TLR2, and prolonged TLR2 stimulation leads to tolerance. In contrast, release of TNF-α or IL-12 in response to poly[I:C] (a TLR3 agonist), LPS, or CpG oligonucleotide (a TLR9 agonist) was not significantly reduced by pretreatment with LA insulinoma cells (p > 0.1 for all comparisons) (Fig. 1C, 1D). TLR2 tolerance by Pam3CSK4 was observed in Mφs from C57BL/6 mice in a similar manner, except that the decrease in TNF-α release in response to

**FIGURE 1. TLR2 tolerance of NOD Mφs in vitro.** A. Primary peritoneal Mφs isolated after thioglycolate injection into NOD mice were pretreated with LPS or Pam3CSK4 for 16 h and then stimulated with LPS, Pam3CSK4, or LA insulinoma cells for 10 h. TNF-α release was measured by ELISA. B. Primary peritoneal Mφs were treated as in A, and release of IL-12 (p40 subunit) was measured by ELISA. C and D. Mφs were pretreated with LA insulinoma cells for 16 h and then treated with LA cells, Pam3CSK4, poly I:C, LPS, or CpG oligonucleotide for 10 h. Release of TNF-α (C) or the IL-12 p40 subunit (D) was determined by ELISA. The results are representative of more than four independent experiments, showing similar tendencies. *p < 0.05, **p < 0.005.
Pam3CSK₄ after pretreatment with LPS was statistically insignificant, probably due to differences in genetic background (Supplemental Fig. 1A, 1B).

We next studied the intracellular mechanism of TLR2 tolerance by examining changes in the signal transduction molecules downstream of TLR. Because previous papers reported decreased expression of IRAK-1 in TLR tolerance (5, 14), we studied whether IRAK-1 expression is changed by Pam3CSK₄. Western blotting showed that the expression of IRAK-1 protein was notably decreased after treatment of primary peritoneal MΦs from 2–3-mo-old NOD mice with 100 ng/ml Pam3CSK₄ for 2–24 h (Fig. 2A), suggesting that decreased IRAK-1 expression plays a role in TLR2 tolerance, which is in agreement with a previous study (5). In addition to IRAK-1, the expression of IRAK-4 protein, another signal transducer downstream of TLR, was decreased by Pam3CSK₄ treatment of MΦs from NOD mice for 2–24 h (Fig. 2A). In contrast to Western blotting, RT-PCR analysis showed that IRAK-1 or -4 mRNA expression was not changed by Pam3CSK₄ treatment for 2–24 h (Fig. 2B), suggesting that Pam3CSK₄ reduces IRAK-1 or -4 protein expression by posttranslational mechanisms. The decreased expression of IRAK-1 or -4 protein by Pam3CSK₄ treatment for 6 h was dose dependent between 10 and 100 ng/ml (Fig. 2C, 2D). Because a previous paper suggested degradation of IRAK proteins by ubiquitin-proteasome machinery (15), we studied the effect of proteasomal inhibitors on the level of IRAK protein. Pretreatment with 20 μM MG132 or lactacystin for 2 h reversed the decrease in the expression of IRAK-1 and -4 proteins after Pam3CSK₄ treatment for 6 h (Fig. 2E), showing a role for proteasomal degradation of IRAK-1 and -4 proteins in TLR2 tolerance.

We also examined the changes in IRAK-M, a negative regulator of TLR signaling (16), which was reported to be induced by LPS (17). In contrast to IRAK-1 or -4, the expression of both IRAK-M mRNA and protein was increased by treatment of MΦs from NOD...
mice with Pam3CSK4 for 6–24 h in a dose-dependent manner (Fig. 2B–D), suggesting that induction of IRAK-M also contributes to the TLR2 tolerance after treatment with specific agonists. Real-time RT-PCR analysis, which was performed to obtain more accurate and quantitative data, also demonstrated that the expression of IRAK-M mRNA was significantly increased by Pam3CSK4, whereas the expression of IRAK-1 or -4 mRNA was not significantly changed (Fig. 2F). Pam3CSK4 treatment of MΦs from C57BL/6 mice also induced a similar decrease in the expression of IRAK-1 and -4 proteins and an increase in the expression of IRAK-M mRNA and protein (Supplemental Fig. 1C, 1D).

We also studied the changes in IRAK proteins in DCs, which are critical in the sensitization of naive diabetogenic T cells by pancreatic β cells undergoing physiological death during pancreas organogenesis in a TLR2-dependent manner (3). The expression of IRAK-1 and -4 proteins in bone marrow-derived DCs (Fig. 2G) or splenic DCs (Fig. 2H) was reduced, whereas that of IRAK-M was enhanced by Pam3CSK4 treatment for 6 h (Fig. 2G, 2H), which was similar to the changes in MΦs.

We further investigated whether TLR2 tolerance induced by prolonged treatment with TLR2 agonists affects intracellular signaling downstream of IRAK, such as NF-κB activation. We examined IκBα degradation as an estimate of NF-κB activation after TLR2 stimulation. Degradation of IκBα by Pam3CSK4 was markedly blunted by pretreatment of MΦs with Pam3CSK4 for 16 h (Fig. 2I), indicating that prolonged treatment with TLR2 agonists dampens intracellular signal transduction after TLR2 activation.

Next, we examined whether other mechanisms could be involved in the TLR2 tolerance, such as induction of IL-10, as reported by other investigators (18). However, we did not detect significant changes in the IL-10 level after treatment of MΦs from NOD mice with LPS, Pam3CSK4, or LA insulinoma cells, eliminating the role of IL-10 in the induction of TLR2 tolerance in our model (Supplemental Fig. 2).

**Prevention of autoimmune diabetes by repeated TLR2 administration**

Because Pam3CSK4 treatment inhibited MΦ responses to LA insulinoma cells, we next investigated whether Pam3CSK4 affects the development of autoimmune diabetes in vivo by modulating APC function. An i.p. injection of 100 μg Pam3CSK4 three times a week for 3 wk, beginning at 3 wk of age, significantly decreased the incidence of diabetes in NOD mice (p < 0.005) (Fig. 3A), suggesting modulation of APC function by repeated stimulation with Pam3CSK4. The insulitis score was also significantly decreased by 100 μg Pam3CSK4 treatment three times a week for 3 wk (p < 0.005) (Fig. 3B). However, weekly administration of 300 μg Pam3CSK4 for 3 wk failed to decrease the incidence of diabetes in NOD mice, suggesting that continuous treatment with Pam3CSK4 is required for inhibition of diabetes (Supplemental Fig. 3).

We next investigated the in vivo mechanism of the decreased incidence of diabetes by Pam3CSK4 administration. Using the BDC2.5 CD4+ T cell-transfer technique (2), we studied whether Pam3CSK4 administration affects the Ag-presenting function of DCs that are more relevant to the sensitization of T cells than MΦs. BDC2.5 CD4+ T cells harboring transgene-encoded TCR-α and -β genes derived from a diabetogenic CD4+ T cell clone proliferate in the draining PLNs in vivo, reflecting autoreactive T cell priming by DCs following β cell apoptosis. When we assessed in vivo proliferation of transferred BDC2.5 CD4+ T cells in control NOD mice by flow cytometry, successive dilution of the CFSE label in BDC2.5 CD4+ T cells was observed in the PLNs of control NOD mice but not in the MLNs or spleens, suggesting proliferation of diabetogenic CD4+ T cells specifically in the PLNs, as previously reported (Fig. 4A) (2). In NOD mice treated with Pam3CSK4 for 3 wk, proliferation of BDC2.5 CD4+ T cells, as measured by CFSE dilution, was significantly reduced compared with untreated mice, indicating that priming of autoreactive T cells by DCs was impaired by chronic Pam3CSK4 treatment, probably as a result of TLR2 tolerance (p < 0.005) (Fig. 4A). To further confirm tolerance of DCs after chronic Pam3CSK4 administration, we conducted an in vitro proliferation assay. We isolated splenic DCs from Pam3CSK4-treated NOD mice and cocultured with BDC2.5 CD4+ T cells in the presence of a BDC2.5 mimotope (8). Proliferation of BDC2.5 CD4+ T cells in response to mimotope was significantly decreased by in vivo treatment with Pam3CSK4 for 3 wk, supporting DC tolerance by Pam3CSK4 treatment (Supplemental Fig. 4). Treatment of OT-II transgenic mice of C57BL/6 background with Pam3CSK4 for 3 wk also significantly reduced proliferation of OT-II CD4+ T cells in response to OVA peptide (Supplemental Fig. 4). We next studied the mechanism of the reduced DC-mediated T cell proliferation in mice treated with Pam3CSK4 in vivo. We isolated splenocytes from Pam3CSK4-treated NOD mice, incubated the splenocytes with 100 ng/ml Pam3CSK4 in vitro for 1 d, and assessed activation of DCs. DC activation represented by increases in the mean fluorescence intensities (MFIs) of costimulatory molecules, such as CD80, CD83, or CD86, were significantly less in DCs from NOD mice treated with Pam3CSK4 in vivo for 3 wk compared with control NOD mice (p < 0.05 for all three
NOD mice that were treated with Pam3CSK4 (dilution by flow cytometry gated on CD4⁺ and V cell proliferation in the PLNs and MLNs was measured by analyzing CFSE NOD mice with Pam3CSK4 for 3 wk, CD4⁺ T cells prepared from CFSE and transferred into recipient mice by tail vein injection. CD4⁺ T BDC2.5/NOD mice by the negative-selection method were labeled with representative graph is shown (left panel right panel from four independent experiments are presented (Fig. 4C). We also examined the expression of costimulatory molecules in DCs from the PLNs of Pam3CSK₄-treated NOD mice, because DC-mediated BDC2.5 CD4⁺ T cell proliferation occurs specifically in the PLNs. The induction of CD80, CD83, or CD86 in DCs by in vitro treatment with Pam3CSK₄ was significantly less after in vivo treatment with Pam3CSK₄ for 3 wk compared with control treatment (p < 0.05–0.01) (Supplemental Fig. 5).

Stimulation with TLR2 agonists could have effects on the immune system other than APC stimulation, such as Th1/Th2 differentiation (19) or Treg function (20). Furthermore, the development of autoimmune diabetes in NOD mice could be modulated by the Th1/Th2 environment (21) or suppressor function of CD4⁺CD25⁺ Tregs (22). Hence, we investigated whether TLR2 stimulation with Pam3CSK₄ affects Th1/Th2 development or Tregs. When CD4⁺CD25⁺CD44⁺CD62L⁺ naïve T cells from Pam3CSK₄-treated or control NOD mice were differentiated with anti-CD3 and anti-CD28 Ab in the presence of IL-12 (Th1) or IL-4 (Th2) and restimulated with anti-CD3 Ab, IFN-γ production from Th1 cells and IL-4 production from Th2 cells were not different between the two groups (p > 0.1, p > 0.1), suggesting that the prevention of diabetes in NOD mice by Pam3CSK₄ treatment is not due to its effect on Th1/Th2 differentiation (Fig. 5A). Intracellular cytokine staining also demonstrated that the percentages of IFN-γ⁺CD4⁺ T cells and IL-4⁺CD4⁺ T cells under Th1- and Th2-polarizing conditions, respectively, were not different between Pam3CSK₄-treated and control NOD mice (Fig. 5B), further supporting that the prevention of diabetes by Pam3CSK₄ treatment is not related to Th1/Th2 balance. When we examined Tregs that could affect the development of autoimmune diabetes, the percentages of CD4⁺CD25⁺ or CD4⁺Foxp3⁺ Tregs in the PLNs, MLNs, and spleens of Pam3CSK₄-treated NOD mice were similar to control NOD mice (Fig. 5C, 5D). MFI of CD25 among CD4⁺Foxp3⁺ Tregs, reflecting their peripheral function (23), was also not affected by Pam3CSK₄ treatment (Fig. 5E). Furthermore, in vitro suppressor function of CD4⁺CD25⁺ Tregs from NOD mice treated with 100 μg Pam3CSK₄ three times a week for 3 wk was not significantly different from that of Tregs from control NOD mice (p > 0.1 at all ratios) (Fig. 5F), suggesting that the effect of Pam3CSK₄ on diabetes of NOD mice is unrelated to Treg modulation.

Treatment of autoimmune diabetes by TLR2 tolerance induction

Because these results suggested that repeated TLR2 stimulation prevents the development of autoimmune diabetes in NOD mice by inhibiting sensitization of diabetogenic naïve T cells, we next investigated whether the same repeated TLR2 stimulation could inhibit in vivo activity of sensitized diabetogenic effector T cells, which is more relevant to the treatment of established autoimmune diabetes than is the inhibition of naïve T cell sensitization. When

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**FIGURE 4.** TLR2 tolerance in vivo. A. After treatment of recipient NOD mice with Pam3CSK₄ for 3 wk, CD4⁺ T cells prepared from BDC2.5/NOD mice by the negative-selection method were labeled with CFSE and transferred into recipient mice by tail vein injection. CD4⁺ T cell proliferation in the PLNs and MLNs was measured by analyzing CFSE dilution by flow cytometry gated on CD4⁺ and Vβ4⁺ T cells. Results pooled from four independent experiments are presented (right panel). A representative graph is shown (left panel). B, Splenocytes were isolated from NOD mice that were treated with Pam3CSK₄ (middle row) or PBS (top row; Control) for 3 wk in vivo. Splenocytes were further incubated with Pam3CSK₄ (black line) or PBS (gray filled graph) ex vivo for 1 d, and the expression of costimulatory molecules on DCs was determined by flow cytometry gated on CD11c⁺ cells. Changes in MFIs are shown (bottom panel). C, Fresh splenocytes were isolated from NOD mice that were treated with Pam3CSK₄ for 3 wk (black line) or from control NOD mice (gray line). The expression of costimulatory molecules on DCs was determined by flow cytometry as in B, without ex vivo Pam3CSK₄ treatment (top row). MFI values are shown (bottom row). Results are representative of three independent experiments, showing similar tendencies. *p < 0.05, ***p < 0.005.
5 × 10^6 splenocytes from diabetic NOD mice were transferred to the 6–8-wk-old NOD mice of the same sex after sublethal irradiation, >80% of the recipient mice became diabetic in 4 wk after the transfer. In contrast, only ∼20% of the recipient mice that were treated with 100 μg Pam3CSK4 three times a week for 2 wk beginning on the day of adoptive transfer (immediately prior to adoptive transfer) developed diabetes in 4 wk (Fig. 6A), suggesting that repeated TLR2 stimulation could abrogate the diabetogenic effect of sensitized effector T cells and that continuous interaction between effector T cells and APCs is required for full in vivo activity of diabetogenic T cells. The effect of Pam3CSK4 treatment for 4 wk, beginning 2 wk prior to adoptive transfer, on diabetes incidence was similar to Pam3CSK4 treatment beginning on the day of adoptive transfer for 2 wk, suggesting that Pam3CSK4 treatment ahead of adoptive transfer is not necessary for the inhibition of sensitized diabetogenic T cells (Supplemental

FIGURE 5. Unaltered Th1/Th2 polarization and Tregs after Pam3CSK4 treatment. A, Naive CD4^+ T cells, prepared as described in Materials and Methods, were stimulated with immobilized anti-CD3 Ab and soluble anti-CD28 Ab in the presence of IL-12 and anti–IL-4 Ab (Th1) or IL-4, anti–IFN-γ Ab, and anti–IL-12 Ab (Th2). After 6 d of culture in the presence of IL-2, cells were restimulated with anti-CD3 Ab, and the supernatants were collected for measurement of IFN-γ or IL-4 by ELISA. B, Naive CD4^+ T cells cultured in the polarizing medium containing IL-2 were restimulated with PMA and ionomycin in the presence of brefeldin A. After permeabilization, intracellular cytokine staining was done using specific Abs. C, Cells from the PLNs, MLNs, and spleens of NOD mice that were treated with Pam3CSK4 or PBS (Control) for 3 wk were stained with anti-CD4 and anti-CD25 mAbs for flow cytometry. Percentages of CD4^+CD25^+ cells were compared (n = 3 each; p > 0.1 for all three comparisons) (right panels). A representative scattergram is shown (left panels). The numbers in the top right quadrants represent the percentage of CD4^+CD25^+ Tregs among all cells analyzed. D and E, Triple-colored flow cytometry for Tregs was done using anti-CD4, -CD25, and -Foxp3 mAbs as described in Materials and Methods. The numbers in D represent the percentage of CD4^+Foxp3^+ Tregs. Those in E denote the MFI s of CD25 on the gated cells in D. F, CD4^+CD25^+ Tregs were cocultured with CD4^+CD25^− T cells from untreated NOD mice in the presence of anti-CD3 mAb and irradiated APCs. Proliferation of CD4^+CD25^− T cells was assessed by [3H]thymidine incorporation at the indicated T effector/Treg ratios. Control denotes an experimental condition without added Tregs. Results are representative of three or four independent experiments, showing similar tendencies.
PBS (administered three times a week for 2 wk) increased the duration of Pam3CSK4 treatment to 6 wk; however, overt diabetes recurred in 2–3 wk due to persistent diabetogenic T cells (n = 4) (Fig. 6B). When Pam3CSK4 was administered from the day of transplantation for 2 wk, recurrence of diabetes (mild) was not observed until 6 wk after transplantation, which was a significant delay (n = 2) (Fig. 6B). When we increased the duration of Pam3CSK4 treatment to >6 wk, recurrence of diabetes was not observed in most mice (4/5) as long as Pam3CSK4 treatment continued (Fig. 6B), suggesting the potential of Pam3CSK4 as a therapeutic agent against established autoimmune diabetes when combined with approaches increasing β cell mass, such as islet transplantation.

Because these results suggested the potential usefulness of Pam3CSK4 in the clinical setting, we examined whether it had systemic toxic effects that could hamper its clinical application. The laboratory profile did not reveal any abnormalities in blood cell counts or chemistries in NOD mice treated with 100 μg Pam3CSK4 for 3 wk, with the exception of the leukocyte count, which was increased compared with control NOD mice but still within normal limits (Supplemental Table I). Biopsies of the liver, muscle, kidney, heart, and other major organs conducted after 3 wk of 100-μg Pam3CSK4 treatment showed no detectable abnormalities (Supplemental Fig. 7), with the exception of splenomegaly and enlargement of lymph nodes (1.5–2-fold in weight), which resolved 2 wk after discontinuation of Pam3CSK4. The structure of lymphoid follicles (Supplemental Fig. 7) and proportions of CD4+ or CD8+ T cells, B cells, and NK cells, as determined by FACS analysis, were not all normal in the spleens and PLNs of Pam3CSK4-treated NOD mice (Supplemental Fig. 8). In addition, Pam3CSK4 treatment did not affect the blood glucose level, β cell mass, body weight, or rectal temperature in C57BL/6 mice (Supplemental Fig. 9), suggesting that 100 μg Pam3CSK4 for 3 wk has no significant toxicity in mice, and the effect of Pam3CSK4 on diabetes in NOD mice is not due to its direct effect on β cell mass.

**Discussion**

Our data showed that repeated TLR2 stimulation by Pam3CSK4 could suppress further responses of Mφs to LA cells or TLR2 agonists in vitro. We observed decreased expression of IRAK-1 or -4 protein and increased expression of IRAK-M, a negative regulator of TLR signaling, as potential mechanisms leading to TLR2 tolerance after repeated stimulation with Pam3CSK4, in agreement with previous reports (15, 24). Mechanisms other than IRAK modulation have been reported as causes of tolerance to TLR agonists, such as downregulation of TLR protein (25), increased p50 homodimer formation instead of the p65/p60 heterodimer (5), ST2 induction (26), selective histone deacetylation of the promoter (27), or induction of IL-10 (18). Indeed, such mechanisms might contribute to the TLR tolerance observed in this study, because decreased IRAK-1 or -4 protein and increased IRAK-M alone cannot adequately explain the much stronger homotolerance (unresponsiveness to the same TLR2 agonist after treatment with TLR2 agonist) compared with heterotolerance (unresponsiveness to TLR2 agonist after treatment with TLR4 agonist). However, we did not detect significant changes in the IL-10 level after Pam3CSK4 treatment of Mφs in vitro. The serum level of IL-10 was also not significantly changed after treatment of NOD mice in vivo with Pam3CSK4, suggesting that induction of IL-10 is not responsible for TLR2 tolerance in our model (D.-H. Kim, J.-C. Lee, and M.-S. Lee, unpublished observations).

In addition to the suppression of further inflammatory responses in vitro, the Ag-presenting function of DCs was decreased, and the development of autoimmune diabetes in NOD mice was inhibited by prolonged administration of Pam3CSK4 >3 wk in vivo. TLR2 tolerance in vivo has been studied in other disease models, such as sepsis (28, 29). Previous studies also reported a decreased incidence of diabetes resulting from the in vivo administration of LPS, poly IC (30), or CpG oligonucleotide (31); however, other studies reported discrepant results (32, 33), reflecting the complex nature of the immunostimulatory versus immunoinhibitory activities of immune modulators related to the method of administration.

Furthermore, the mechanism of inhibition of autoimmune diabetes by such TLR agonists was not clearly elucidated. Zymosan is a fungal cell wall component with TLR2 stimulatory activity, and it has been administered to NOD mice with a significant delay in hyperglycemia (34, 35). However, zymosan is not a specific TLR2 agonist and acts on dectin-1, a C-type lectin receptor (36), which is in contrast to Pam3CSK4, a specific agonist for the TLR2–TLR1 complex (13). Our results differ from other studies in that Pam3CSK4 was targeted to the initial priming of diabetogenic T cells by APCs, and our approach was based on the pathogenesis of autoimmune diabetes of NOD mice related to the APC responses toward apoptotic β cells. In contrast, the effect of zymosan was mostly directed to the modulation of Tregs (34, 35), whereas we observed no significant effect of prolonged Pam3CSK4 administration on Tregs. The difference between the effect of zymosan and that of Pam3CSK4 could be due to the activity of zymosan on receptors other than TLR2. Further, the side
effects of zymosan have not been investigated (34). Because zymosan has been used to induce peritonitis or arthritis and to activate inflammasomes or complements (37–39), its therapeutic potential against autoimmune diseases would not be considered unless the safety issue is clearly addressed.

Our results might also be related to the well-known inhibition of autoimmune diabetes by CFA or bacillus Calmette-Guérin (BCG) (12). Although our studies did not directly delineate the molecular identity of the responsible elements in CFA or BCG, it is very likely that agonists for TLR2 or other types of innate immune receptors are the substances responsible for the inhibition of autoimmune diabetes because mycobacteria or mycobacterial products, as essential components of both CFA and BCG, contain several agonists for TLR receptors, including TLR2 (40).

Our results showing the inhibition of diabetes transfer by Pam3CSK4 demonstrated that TLR2 tolerance by repeated stimulation with agonists inhibited the initial sensitization of diabetogenic T cells, as well as the in vivo activity of sensitized T cells. Those results also suggested the possibility that TLR2 tolerance by repeated administration of agonists could be used as a therapeutic option against established autoimmune diabetes when combined with modalities increasing β cell mass, such as islet transplantation, as was shown in this study.

Taken together, our results showed that repeated administration of TLR2 agonists could lead to tolerance instead of stimulation in vitro and prevention of autoimmune diabetes in vivo, suggesting the potential therapeutic value of Pam3CSK4 in autoimmune diabetes. Several TLR ligands, including TLR2 ligands, have been used as adjuvant against cancer or infectious diseases in humans (41–45). Among TLR2 agonists, Pam3CSK4 has been used for vaccination of cattle (46) or experimental animals (47, 48) against bacterial infection, culminating in lethal septic shock-like syndrome in mice (49). Pam3CSK4, TLR2 tolerance would affect innate immune response in major organs also were not observed, with the exception of splenomegaly and lymphadenopathy, which resolved 2 wk after discontinuation of Pam3CSK4. These results are in apparent contrast to the destruction of lymphoid follicles by CpG oligonucleotide, a TLR9 ligand (51). Although we did not observe significant adverse effects of prolonged administration of Pam3CSK4, TLR2 tolerance would affect immune response to microbes. Without intact TLR2 function, defense against acute infection with bacteria harboring TLR2 signature molecules would be weakened. In contrast, inappropriate harmful response to TLR2 agonists in the bacterial wall components released during bacterial infection, culminating in lethal septic shock-like syndromes, might be alleviated (52). Thus, TLR2 tolerance might have both positive and negative effects on host response to pathogens.

Because several clinical trials using potential TLR agonists or antagonists are in progress (52, 53), immunotherapy targeting cancer, infectious diseases, or autoimmune disorders in humans by TLR modulation will be possible; TLR2 agonists, such as Pam3CSK4, will be strong candidates for such indications.

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Disclosures
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