Tolerogenic nanoparticles inhibit T cell–mediated autoimmunity through SOCS2

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Type 1 diabetes (T1D) is a T cell–dependent autoimmune disease that is characterized by the destruction of insulin-producing β cells in the pancreas. The administration to patients of ex vivo–differentiated FoxP3+ regulatory T (Treg) cells or tolerogenic dendritic cells (DCs) that promote Treg cell differentiation is considered a potential therapy for T1D; however, cell-based therapies cannot be easily translated into clinical practice. We engineered nanoparticles (NPs) to deliver both a tolerogenic molecule, the aryl hydrocarbon receptor (AhR) ligand 2-(1’H-indole-3’-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE), and the β cell antigen proinsulin (NPITE+Ins) to induce a tolerogenic phenotype in DCs and promote Treg cell generation in vivo. NPITE+Ins administration to 8-week-old nonobese diabetic mice suppressed autoimmune diabetes. NPITE+Ins induced a tolerogenic phenotype in DCs, which was characterized by a decreased ability to activate inflammatory effector T cells and was concomitant with the increased differentiation of FoxP3+ Treg cells. The induction of a tolerogenic phenotype in DCs by NPs was mediated by the AhR-dependent induction of Socs2, which resulted in inhibition of nuclear factor κB activation and proinflammatory cytokine production (properties of tolerogenic DCs). Together, these data suggest that NPs constitute a potential tool to reestablish tolerance in T1D and potentially other autoimmune disorders.

INTRODUCTION

Type 1 diabetes (T1D) is a T cell–mediated autoimmune disease characterized by the destruction of insulin-producing β cells in the pancreas (1, 2). Therefore, the reestablishment of immune tolerance is a major goal for the treatment of T1D. Immunological tolerance is mediated by a number of mechanisms including the removal and inactivation of self-reactive pathogenic T cell clones, as well as their active regulation by specialized regulatory cells. Several regulatory T (Treg) cell subsets mediate immune tolerance. One such subset of particular importance is the FoxP3+ Treg cell population (3, 4). Deficits in pancreatic Treg cells in both number and functionality have been described in recent-onset T1D patients (5, 6). Conversely, therapies that increase Treg cell numbers or functions prevent and treat T1D in preclinical models and are currently under investigation in clinical trials (7–9).

The administration of ex vivo–expanded (increased in number) Treg cells or tolerogenic dendritic cells (DCs) that promote the proliferation of Treg cells in vivo is a potential therapeutic approach for T1D, but cell-based therapeutic approaches are hard to translate into clinical practice (10, 11). Alternatively, antibody- and cytokine-based therapies have been developed to restore immune tolerance and suppress autoimmunity in TID (12, 13). However, these approaches can lead to generalized immune suppression. Thus, there is an unmet clinical need for therapeutic approaches to generate functional antigen-specific Treg cells in vivo as a method for the reestablishment of immune tolerance in T1D and other autoimmune disorders.

To generate tolerogenic DCs and promote Treg cell generation in vivo, we used nanoparticles (NPs) to co-deliver a tolerogenic molecule, the aryl hydrocarbon receptor (AhR) ligand 2-(1’H-indole-3’-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE), and the β cell antigen proinsulin (NPITE+Ins). We found that NPITE+Ins administration to 8-week-old nonobese diabetic (NOD) mice suppressed the spontaneous development of T1D. NPITE+Ins induced a tolerogenic phenotype in DCs characterized by decreased activation of inflammatory effector T cells and was concomitant with the increased differentiation of FoxP3+ Treg cells. Tolerogenic DC induction by these particles was dependent on the AhR-dependent induction of Socs2, which interfered with nuclear factor κB (NF-κB) activation and the production of proinflammatory cytokines. Collectively, these data suggest that NPs constitute a potential tool to reestablish tolerance in T1D and other autoimmune disorders.

RESULTS

Generation of NPs containing β cell antigens and the tolerogenic AhR ligand ITE

We (14, 15) and others (16–20) showed that AhR activation induces a tolerogenic phenotype in DCs, which promotes the differentiation of naïve CD4+ T cells into Treg cells. Insulin harbors epitopes that are targeted by diabetogenic and regulatory CD4+ and CD8+ T cells (7, 21, 22), and insulin has been identified as an initiating autoantigen in T1D (23). On the basis of these results and the need for methods to reestablish immune tolerance in T1D, we constructed NPs containing the tolerogenic AhR ligand ITE and a β cell–related recombinant antigen, such as proinsulin (NPITE+Ins) (Fig. 1A) or a mimotope peptide (MIMO) that activates the diabetogenic CD4+ T cell clone BDC2.5 (NPITE+MIMO) (24). As a substrate, we used gold NPs because they show minimal toxic or immunogenic activity in humans (25, 26). Unloaded NPs (NP), NPs loaded with ITE alone (NPITE), or NPs loaded with antigen alone (for example, proinsulin [NPITE+Ins]) were used as controls. NP solubility and stability were improved by adding a layer of thiol-polyethylene...
Development of T1D was monitored. We found that NPITE+Ins administration to female NOD mice was treated weekly with NPs, and the spontaneous diabetes (CAD) (Fig. 2A). Conversely, Foxp3 expression, which is required for Treg cell generation, was increased under these conditions (Fig. 2D).

To further investigate the effects of NPs on the β-cell–specific T cell response in vivo, we used BDC2.5 T cell receptor (TCR) transgenic NOD mice (24). The BDC2.5 TCR was isolated from NOD diabeticogenic CD4+ T cell clones that target β-cell antigens expressed in both NOD mice and humans (30–32). We found that NPITE+MIMO administration to BDC2.5 transgenic NOD mice decreased the percentage of interferon-γ-producing (IFN-γ) and interleukin-17–producing (IL-17) CD4+ T cells in pancreatic lymph nodes (Fig. 2E). This decrease was concomitant with an increase in Foxp3 expression and in the percentage of FoxP3+ Treg cells in the pancreas (Fig. 2, F and G). Together, these data suggest that NPs loaded with ITE and a β-cell–related antigen limit the diabeticogenic T cell response.

We then investigated the effects of NPITE+MIMO on DCs in vivo. Treatment with NPITE+MIMO did not affect the recruitment to the pancreas of classic DCs linked to T cell activation (Fig. 2H). However, NPITE+MIMO administration enhanced the expression of the AhR-responsive gene Cyp1a1 in DCs (Fig. 2I). Moreover, after activation with the Toll-like receptor 4 (TLR4) agonist lipopolysaccharide (LPS) ex vivo, DCs isolated from NPITE+MIMO–treated mice showed decreased expression of Il6 and Il12a (Fig. 2J), which encode the cytokines IL-6 and IL-12, suggesting that NPITE+MIMO administration induced tolerogenic DCs in vivo.

To study the relevance of these tolerogenic DCs for the arrest of T1D by NPITE+Ins administration, we performed transfer experiments with NP-loaded DCs. Bone marrow–derived DCs (BMDCs) were incubated for 24 hours with NPs, washed, and injected intravenously into 6-week-old naïve NOD mice. This treatment was repeated three additional times, once every 4 days, and the development of spontaneous T1D was monitored. We found that treatment with NPITE+Ins–loaded DCs reduced the development of spontaneous T1D in NOD mice, such that 40% of the NOD recipients treated with BMDCs loaded with empty NP developed diabetes by the age of 22 weeks as compared to 10% of the mice that received DCs that had been loaded with NPITE+Ins (Fig. S2; P < 0.001). Together, these data suggest that NPITE+Ins administration induces tolerogenic DCs that suppress spontaneous T1D development in NOD mice.

AhR-targeting NPs induce tolerogenic DCs
On the basis of our findings on the effects of AhR-targeting NPs on T1D development and DC activation in NOD mice, we studied the effects of NPs on autoimmune T1D in NOD mice. We first used the cyclophosphamide-accelerated model of diabetes (CAD) (27, 28) to study the effects of NPs on T1D development. NPs were administered weekly (6 μg per mouse) starting 1 week before CAD induction in 8-week-old naïve NOD mice as described previously (27, 28). NPITE+Ins suppressed the development of T1D 4 weeks after CAD induction (fig. S1), whereas NPins had no effect on CAD development, and NPins stimulated a trend toward amelioration of T1D symptoms that was not statistically significant (fig. S1). Hence, in follow-up studies, we focused on the effects of NPITE+Ins on spontaneous T1D in NOD mice. naïve 8-week-old female NOD mice were treated weekly with NPs, and the spontaneous development of T1D was monitored. We found that NPITE+Ins administration arrested T1D development as determined by measurement of blood glucose concentrations and the histological analysis of pancreatic samples (Fig. 2, A to C). β-Cell–specific effector T cells drive T1D immune pathogenesis (1, 2, 29). Consistent with the arrest of T1D development, we detected decreased expression of the genes Tbx21 and Rorc [which encode the transcription factors Tbet and RoRyt, which are required for the generation of the proinflammatory T helper 1 (Th1) and Th17 subsets, respectively] in T cells isolated from the pancreatic lymph nodes of NPITE+Ins–treated mice (Fig. 2D). Conversely, Foxp3 expression, which is required for Treg cell generation, was increased under these conditions (Fig. 2D).

**Fig. 1. Characterization of NPs.** (A) Schematic representation of NPITE+MIMO. (B) TEM analysis of the indicated NPs. (C) MIMO incorporation into NPs was quantified with a fluorescence-based peptide quantification kit as described in Materials and Methods. (D) Human embryonic kidney 293 cells transfected with a reporter plasmid encoding luciferase under the control of an AhR-responsive promoter were incubated with the indicated NPs, and luciferase activity was measured after 24 hours. Cotransfection with a thymidine kinase–Renilla construct was used for normalization purposes. Data are means ± SEM of one experiment representative of at least three independent experiments. Each experiment was performed with at least two biological samples. *P < 0.05 and **P < 0.01.
Fig. 2. NPI_TET-Ins administration suppresses T1D development in NOD mice. (A and B) Female NOD mice were left untreated (none) or were treated intraperitoneally weekly for 1 month with the indicated NPs. Treatment was started when the mice were 8 weeks old. Diabetes incidence (A) and glucose amounts (B) were measured at the indicated times. Data are means ± SEM of 12 to 15 mice per group from one experiment that is representative of three independent experiments. (C) Left: Histopathologic analysis of pancreas samples from the untreated and NP-treated mice described in (A) and (B). Analysis was performed when mice were 22 weeks old. Right: Analysis of the histological scores of mice treated with the indicated NP types. Data are means ± SEM of one experiment representative of at least three independent experiments. (D) Pancreatic lymph nodes from 22-week-old NOD mice treated with the indicated NP types were subjected to quantitative polymerase chain reaction (qPCR) analysis of the abundances of Rorc, Tbx21, and Foxp3 mRNAs, which were normalized to the abundance of Gapdh mRNA. (E to G) BDC2.5 NOD mice were treated weekly for 1 month with the indicated NPs. One week after the last NP administration, pancreatic lymph nodes were isolated and the percentages of IFN-γ+ and IL-17+ CD4+ T cells were analyzed by flow cytometry (E), the abundance of Foxp3 mRNA normalized with Gapdh mRNA was determined by qPCR analysis (F), and the percentage of FoxP3+ CD4+ T cells was determined by flow cytometric analysis (G). Numbers in the representative dot plots in (G) show the percentages of cells in the indicated gates. (H) NOD mice were treated once a week with the indicated NPs for 1 month. One week after the last NP administration, the percentages of DCs within pancreatic lymph nodes were analyzed by flow cytometry. cDC, classical dendritic cell. (I) DCs isolated from the NP-treated mice described in (H) were subjected to qPCR analysis of the abundance of Cyp1a1 mRNA normalized to that of Gapdh mRNA. (J) DCs isolated from the indicated NP-treated mice were treated with LPS for 24 hours and then were subjected to qPCR analysis of the abundances of Il6 and Il12a mRNAs normalized to that of Gapdh mRNA. Data are means ± SEM from one representative experiment of at least three independent experiments. All experiments were performed with at least three biological samples. *P < 0.05, **P < 0.01, and ***P < 0.001.
Fig. 3. NP_{ITE+MIMO} administration induces the generation of tolerogenic DCs. (A) TEM analysis of NP_{ITE+MIMO} uptake by splenic DCs after the indicated times in culture. Boxed areas in the images on the top row are shown under higher magnification in the bottom row. Images are from one representative experiment of two independent experiments, with each experiment performed with three biological samples. (B) Splenic DCs were incubated with the indicated NPs for 24 hours and then subjected to qPCR analysis of Cyp1a1 mRNA abundance normalized to that of Gapdh mRNA. (C) Splenic DCs incubated in vitro with the indicated NPs and activated with LPS for 24 hours were subjected to flow cytometric analysis of the relative abundances of the indicated cell surface markers. The red lines indicate the staining obtained from the control antibody, whereas the blue lines indicate specific antibody staining. Numbers inside the plots represent the percentages of cells within the indicated gates. (D and E) Splenic DCs were incubated in vitro with the indicated NPs and activated with LPS for 24 hours. The cells were then subjected to qPCR analysis of the abundances of Il6 and Il12a (D) and Il10 and Ido1 (E) mRNAs relative to that of Gapdh mRNA. (F to J) Splenic DCs were stimulated with LPS in the presence or absence of NPs for 24 hours and then cocultured with naïve BDC2.5+ CD4+ T cells for 3 days. T cell proliferation was evaluated by [3H]thymin incorporation (F); IFN-γ and IL-17 production in culture medium was measured by enzyme-linked immunosorbent assay (ELISA) (G); Ifnγ and Il17 mRNA abundances were normalized to that of Gapdh mRNA (H); and the percentages of FoxP3, IFN-γ, and IL-17 expression in the gate of CD4+ T cells were analyzed by flow cytometry (I). Ratios of FoxP3+ to IFN-γ+ T cells (left) and of FoxP3+ to IL-17+ T cells (right) (J). Data in (B) to (H) are means ± SEM from one representative experiment of at least three independent experiments. Each experiment was performed with at least three biological samples. *P < 0.05, **P < 0.01, and ***P < 0.001.
of Il12a and Il6, which encode the cytokines IL-12 and IL-6 that promote the differentiation of naïve CD4^+ T cells into Th1 and Th17 cells, respectively (Fig. 3D). Conversely, NPITE+MIMO administration increased the expression of Il10, which encodes the anti-inflammatory cytokine IL-10, but did not alter the expression of Ido1, which has been linked to the anti-inflammatory effects of AhR in other experimental systems (Fig. 3E) (17, 37, 38).

To study how the NP-induced modulation of DCs affected T cell responses, we treated splenic DCs from NOD mice with NPs and then used these cells to activate naïve CD4^+ T cells from BDC2.5 mice. We found that NP-MIMO-treated DCs induced the proliferation of BDC2.5 T cells (Fig. 3F), as well as the generation of IFN-γ and IL-17 mRNA and protein by these cells without the need to add exogenous MIMO (Fig. 3, G to I), which suggests that MIMO in the NPs was delivered to the DCs and then presented to the T cells. Consistent with a tolerogenic role for AhR signaling in DCs, NP-MIMO-treated DCs were less potent at inducing the proliferation of BDC2.5 T cells and their production of IFN-γ and IL-17 (Fig. 3, F to I). Concomitantly, the generation of Foxp3^+ CD4^+ T cells was increased under conditions in which BDC2.5 T cells were incubated with NPITE-MIMO-treated DCs (Fig. 3I), which increased the ratio of Foxp3^+ to IL-17^+ T cells and the ratio of Foxp3^+ to IFN-γ^+ T cells (Fig. 3J). Together, these results suggest that AhR-targeting NPs induce a tolerogenic phenotype in DCs.

AhR activation with NPs inhibits NF-κB signaling in DCs
To investigate the mechanisms involved in the tolerogenic effects of NPITE+Ins we used NanoString nCounter arrays to analyze the transcriptional programs of splenic DCs isolated from NP-treated NOD mice and of DCs treated in vitro with NPs. When compared with empty NPs and NPins, NPITE+Ins decreased the expression of genes whose products are associated with DC activation and maturation, as well as the production of proinflammatory mediators and molecules linked to T1D pathogenesis (Fig. 4A). Among these pathways, we detected statistically significant effects of NPITE+Ins on signaling by p38 mitogen-activated protein kinase (MAPK), extracellular signal–regulated kinase 1 (ERK1) and ERK2, and NF-κB, all of which play an important role in the regulation of inflammation (Fig. 4, A and B). To validate these findings, we analyzed the effects of NPs on these pathways using Western blotting. We found that NPITE+Ins administration resulted in reduced activation and translocation of the p65 subunit to the nucleus in splenic DCs (Fig. 4C). No statistically significant effects on p38 MAPK activation by the NPs were detected, but we observed a trend toward inhibition of ERK1/2 activation by NPITE+Ins administration (Fig. 4C).

The AhR ligand 2,3,7,8-tetrachlorodibenzodioxin (TCDD) induces Socs2 expression in B cells (39). Moreover, SOCS2 interferes with NF-κB activation by suppressing the activation of tumor necrosis factor (TNF) receptor–activating factor 6 (TRAF6) (40). Thus, we analyzed the effects of NPs on Socs2 expression in DCs. Consistent with our transcriptional analysis, we detected a statistically significant increase in the expression of Socs2, but not Socs1 or Socs3, in DCs treated in vitro with NPITE+Ins (Fig. 4D and fig. S4A). Concomitantly, when compared to NP administration, NPITE+Ins administration reduced the abundance of TRAF6 in LPS-activated DCs (Fig. 4E). A bioinformatics analysis of the Socs2 promoter identified three potential AhR-binding sites (xenobiotic responsive elements; XRE-1, XRE-2, and XRE-3) (Fig. 4F). Chromatin immunoprecipitation (ChIP) studies showed a substantial recruitment of AhR to the Socs2 locus in DCs treated with NPITE+Ins (Fig. 4F). To study the role of AhR in the induction of Socs2 expression by NPITE+Ins administration, we used DCs carrying the AhR-d hypomorphic allele, which shows reduced sensitivity to AhR agonists (41). We found that deficient AhR signaling in AhR-d DCs abrogated the induction of Socs2 expression by NPITE+Ins administration (Fig. 4G). Thus, AhR activation by NPITE+Ins increases Socs2 expression in DCs.

AhR activation with NPs induces human tolerogenic DCs
To evaluate the translational potential of NPs, we studied their effects on human monocyte-derived DCs (hDCs). For these studies, we used NPs carrying ITE and glutamic acid decarboxylase (GAD_{55, 65}) (44), a major target of autoimmune T cells in human T1D (NPITE+GAD) (1, 2, 29). As expected, NPITE+GAD administration activated AhR in hDCs, increasing the expression of the AhR target gene Cyp1a1 (Fig. 5A). Treatment with NPITE+GAD also modulated the expression of genes involved in the activation and function of antigen-presenting cells (APCs), including genes whose products participate in NF-κB signaling (Fig. 5, B and C). Treatment of hDCs with NPITE+GAD decreased the cell surface abundances of the costimulatory molecules CD40 and CD86 (Fig. 5D), which suggests that NPITE+GAD administration induced a tolerogenic phenotype in hDCs. To investigate the functional relevance of these observations, we used NP-treated hDCs to activate the 325 GAD-reactive CD4^+ T cell line, which was isolated from a T1D patient (42). We found that treatment of mature or immature hDCs with NPITE+GAD decreased their ability to stimulate IFN-γ production by the T cells (Fig. 5E). Together, these data suggest that NPITE+GAD promote the generation of tolerogenic hDCs that have an impaired ability to stimulate the activation of human diabeticogenic T cells.

DISCUSSION
The reestablishment of antigen-specific tolerance is a potential therapeutic approach for T1D. We found that the NP-based coadministration of a β cell antigen and the tolerogenic AhR ligand ITE suppressed the spontaneous development of T1D in NOD mice. These protective effects involved the control of the diabeticogenic immune response by tolerogenic DCs and Treg cells. Given that treatment in our studies was initiated when mice were 8 to 10 weeks old, a time at which insulitis is already detectable (43), our results suggest that NPs provide a therapeutic avenue to reestablish antigen-specific tolerance in T1D and perhaps in other immune-mediated diseases.

Both Th11 and Th17 cells can induce diabetes in NOD recipient mice (44); however, the induction of diabetes by Th17 cells is associated with the coexpression of IFN-γ (44). The expression in T cells is required for T1D development in NOD mice (45), and although loss of IL-17 ameliorates autoimmunity in the central nervous system, it fails to affect T1D development in NOD mice (46). Together, these data suggest that Th11 cells play a dominant role in the diabeticogenic response in NOD mice. Furthermore, the arrest of diabetes with NPITE+Ins was linked to a stronger reduction in the frequency of Th11 cells than in the frequency of Th17 cells.
Because NPITE+Ins inhibited the production of both Th1- and Th17-polarizing cytokines by DCs in vitro, these observations likely reflect the increased susceptibility of Th1 cells to inhibition in vivo, the differential targeting of specific Th1-inducing APCs, or both. In addition, given that T1D in NOD mice is also driven by CD8+ T cells (2), our data suggest that NPITE+Ins also control the CD8+ T cell response; however, additional studies are needed to investigate the specific effects of NPITE+Ins on the diabetogenic CD8+ T cell response and the mechanisms involved.

**Fig. 4.** AhR activation by NPITE+Ins inhibits DC activation by inducing Socs2 expression. (A) Splenic DCs from NP-treated NOD mice and NP-treated BMDCs were subjected to gene expression analysis with NanoString nCounter arrays. Signaling pathways were targeted by NPITE+Ins in DCs. (B) Proposed effects of NPITE+Ins administration on NF-κB signaling in DCs. (C) Left: Splenic DCs were treated with the indicated NPs in the absence (−) or presence (+) of LPS, fractionated into cytoplasmic and nuclear fractions (Nu/Cyt), and then subjected to Western blotting analysis with antibodies specific for the indicated proteins. Western blots are from a single experiment and are representative of three independent experiments. Right: Densitometric analysis of the relative abundances of the indicated proteins. (D) Splenic DCs were treated with the indicated NPs and then subjected to qPCR analysis of Socs2 mRNA abundance relative to that of Gapdh mRNA. (E) Splenic DCs were treated with the indicated NPs in the absence or presence of LPS and then were analyzed by Western blotting with antibodies against the indicated proteins. Right: Densitometric analysis of the abundance of TRAF6 relative to that of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) in the indicated cells. (F) Left: AhR-responsive elements (XRE-1, XRE-2, and XRE-3) in the mouse Socs2 promoter. Right: ChIP analysis of the interaction of AhR with XRE-1, XRE-2, and XRE-3 at the mouse Socs2 promoter in cells treated with the indicated NPs. (G) Splenic DCs from wild-type (WT) and AhR-d mice were treated with the indicated NPs and then were subjected to qPCR analysis of Socs2 mRNA abundance relative to that of Gapdh mRNA. (H) Left: Splenic DCs transfected with control siRNA or with Socs2-specific siRNA (siSocs2) were treated with the indicated NPs in the presence of LPS. The cells were fractionated and analyzed by Western blotting with a p65-specific antibody. Western blots are from one experiment and are representative of three experiments. Right: Densitometric analysis of the ratio of nuclear to cytoplasmic p65 abundance. (I) BMDCs transfected with control siRNA or with Socs2-specific siRNA were treated with the indicated NPs and then subjected to qPCR analysis of the abundances of Il6 and Il12a mRNAs normalized to that of Gapdh mRNA. (J) BMDCs transfected with control siRNA or with Socs2-specific siRNA were treated with NPITE+Ins and then cocultured with CD8+ T cells for 3 days. The T cells were then subjected to flow cytometric analysis of the percentages of IL-17+ and IFN-γ+ cells. Numbers in the plots represent the percentages of cells in the indicated gates. For all panels, a single experiment that is representative of three independent experiments is shown. Each experiment was performed with three biological samples. *P < 0.05, **P < 0.01, and ***P < 0.001.
Fig. 5. NPITE+GAD administration induces the generation of tolerogenic human DCs. (A to E) hDCs were incubated with NP, NPITE, NP_GAD, or NPITE+GAD for 24 hours. (A) The cells were subjected to qPCR analysis of the abundance of CYP1A1 mRNA relative to that of GAPDH mRNA. (B and C) Gene expression was analyzed with NanoString nCounter arrays. Genes whose expression was increased in cells treated with NPITE+GAD compared to that in cells treated with NP are shown in blue, whereas genes whose expression was increased in cells treated with NPITE+GAD compared to that in cells treated with NP are shown in red. (D) hDCs treated with the indicated NPs were subjected to flow cytometric analysis of the relative abundances of the indicated cell surface markers. The red lines indicate the staining obtained from the control antibody, whereas the blue lines indicate specific antibody staining. Numbers inside the plots represent the percentages of cells within the indicated gates. (E) Cells of the human CD4+ T cell clone 325GAD were cocultured with hDCs prepared as in (D), and the amounts of IFN-γ in the cell culture medium were determined by ELISA. Data are from one representative experiment of at least three independent experiments, with each experiment performed with three biological samples. *P < 0.05 and ***P < 0.001.
Several types of T<sub>reg</sub> cells control T1D development, and among these, FoxP3<sup>+</sup> T<sub>reg</sub> cells play an important role. Deficits in T<sub>reg</sub> cells are associated with T1D in mice and humans (47), and the loss or removal of FoxP3<sup>+</sup> T<sub>reg</sub> cells results in the acceleration of T1D onset in NOD mice (48). Conversely, the transfer of FoxP3<sup>+</sup> T<sub>reg</sub> cells or their induction in vivo blocks T1D development in NOD mice (49, 50). Our data suggest that NPs increased the numbers of FoxP3<sup>+</sup> T<sub>reg</sub> cells, which can transfer protection from T1D to naive NOD recipients. It is possible, however, that the protective effects of NPs also involve other T<sub>reg</sub> cell populations, such as IL-10<sup>+</sup> CD4<sup>+</sup> Tr1 cells and CD8<sup>+</sup> T<sub>reg</sub> cells. Antigen-specific anti-diabetogenic CD8<sup>+</sup> T<sub>reg</sub> cells induced by NPs arrest T1D in NOD mice (51), although it should be noted that the induction of CD8<sup>+</sup> T<sub>reg</sub> cells by NPs is dependent on FoxP3<sup>+</sup> T<sub>reg</sub> cells (52, 53). Given reports that describe that effector T cells in T1D patients are resistant to regulation by certain T<sub>reg</sub> cells (7–9), the induction of multiple T<sub>reg</sub> cell populations is likely to boost the therapeutic potential of NPs. Gold NPs similar to those used in our studies are reported to be taken up by T cells in the context of tumors (54), suggesting that AhR activation in T cells by NPs may directly promote the differentiation of T<sub>reg</sub> cells (41, 55, 56). However, the molecular and functional data presented in this study suggest that the tolerogenic effects of NPs are mediated, at least partially, by DCs.

Several pathways control the anti-inflammatory functions of DCs. For example, IL-27 signaling and the cell surface expression of CD39 in DCs limit T cell–mediated inflammation (34). The tolerogenic effects of NPs used in our studies were mediated by an AhR-SOCS2 axis and are consistent with previous reports of the anti-inflammatory effects of AhR signaling in DCs (14, 15, 17, 19, 20). SOCS protein production is induced in innate immune cells by the activation of pattern recognition receptors, which results in the control of inflammation. For example, SOCS1 and SOCS3 inhibit the activation of the Janus-activated kinase (JAK)–signal transducer and activator of transcription (STAT) pathway in DCs, limiting the production of proinflammatory cytokines (57, 58). Moreover, SOCS1 inhibits the NF-κB signaling pathway by stimulating the ubiquitination and degradation of the Toll–interleukin-1 receptor (TIR) domain–containing adaptor protein (TIRAP) in the cytoplasm, p65 in the nucleus, or both (59, 60). SOCS2 signaling in T cells is linked to the induction of FoxP3<sup>+</sup> T<sub>reg</sub> in response to treatment with anti-CD3 antibody (61) and the maturation of HDCs (62, 63). Our work identifies SOCS2 as a mediator of the tolerogenic effects of AhR in DCs through the control of TRAF6 abundance and the inhibition of NF-κB activation. At this point, it is unclear whether the inhibitory effects of the AhR-SOCS2 axis are mediated solely by control of TRAF6 abundance, or whether they also involve the direct inhibition of p65. However, these data are consistent with the AhR-dependent decrease in TRAF6 abundance and the inhibition of NF-κB triggered by lipoxin A<sub>4</sub> in DCs (40, 64). Thus, together with the production of retinoic acid and kynurenine that have been previously linked to AhR (14, 17, 37, 38), increasing the abundance of SOCS2 is a mechanism by which AhR controls DC activation. Future studies should determine whether these or other AhR-dependent immunomodulatory mechanisms are associated with specific APC types.

Several strategies have been pursued to modulate antigen-specific responses in vivo. Tolerogenic antigen administration arrests antigen-specific T cell responses, but its success in the arrest of T1D has been limited (65). DNA vaccines showed promising results in experimental models of autoimmunity and human clinical trials (27, 66). More recently, it was reported that NP-administered antigens induce tolerance (15, 51, 67). The strategy that we describe here enhances the induction of tolerance by coadministering autoantigens and the tolerogenic AhR ligand ITE with NPs. In combination with methods for the identification of the specific autoantigens targeted in each T1D patient (28, 68), NPs offer a new therapeutic avenue for the treatment of T1D and potentially of other autoimmune diseases (15).

MATERIALS AND METHODS

Mice and reagents
NOD and BDC2.5 mice (24) were purchased from The Jackson Laboratory and kept in a pathogen-free facility at the Harvard Institutes of Medicine. All experiments were performed in accordance with the guidelines of the standing committee of animals at Harvard Medical School. In all of the studies described here, the mice were randomly assigned to each of the experimental groups. Blood glucose was measured weekly, and mice with blood glucose concentrations ≥200 mg/dl were deemed diabetic. For the induction of CAD, 4 mg of cyclophosphamide monohydrate (Sigma-Aldrich) was injected intraperitoneally into mice twice, 7 days apart.

Human T cell clone
The 325GAD T cell clone was generated by H. Reijonen (Benaroya Research Institute at Virginia Mason University, Seattle, WA) from the peripheral blood of a T1D patient by sorting and inducing the proliferation of CD4<sup>+</sup> T cells that bound to a human leukocyte antigen (HLA)–DRB1*04:04 tetramer loaded with hGAD65<sub>555–567</sub>(557F<sub-></sub>1) (42). The substituted peptide stabilizes binding to the tetramer, and the T cell clone recognizes the native peptide (NFFRMVISNPAAT) and the substituted peptide in the context of HLA-DRB1*04:01 and DRB1*04:04. The native peptide was used in all of the experiments described here.

NP preparation
NPs were produced with ultrapure water, 60-nm tannic acid-stabilized gold particles at a concentration of 2.6 × 10<sup>10</sup> particles/ml (Ted Pella Inc.), methoxypolyethylene glycol (mPEG)–SH (with a molecular mass of 5000 kD; Nektar Therapeutics), ITE (Toctri Bioscience), MIMO peptide (MEVGWYRSPFSRVVHLYRNGK, cat. #62756; AnaSpec Inc.), and proinsulin. Freshly prepared solutions of 3.5 mM ITE, protein, or peptide (1 mg/ml) were added dropwise to a rapidly mixing gold colloid at 1:6 ITE solution/colloid volume ratio, which facilitated even distributions of the molecules on the gold particle surface (69). After a 30-min incubation at room temperature, 10 mM mPEG-SH was added dropwise to the colloids, with a minimum ratio of 30,000 PEG-SH molecules per 60-nm gold particle. The resulting PEG monolayer stabilized gold colloids against aggregation under various conditions (90). Note that the addition of a 10- to 20-fold excess of PEG-SH does not affect colloid stability or the thickness of the polymer-coating layer (69). After an additional 30-min incubation at room temperature, the colloids were pelleted by centrifugation, resuspended in ultrapure water, and characterized by ultraviolet-visible spectroscopy and TEM. The incorporation of peptide or protein onto the NPs was assessed with the fluorescence-based LavaPep Peptide Quantification Kit (FLUOROtech).

Flow cytometric analysis
To detect intracellular cytokines, cells were stimulated in culture medium containing phorbol 12-myristate 13-acetate (50 ng/ml; Sigma-Aldrich), ionomycin (1 µg/ml; Calbiochem), and GolgiStop (BD Biosciences) for 4 hours. After the staining of cell surface markers, cells were fixed, permeabilized, and incubated with cytokine-specific antibodies (at a 1:100 dilution) at 25°C for 30 min before the cells were analyzed by flow cytometry according to standard protocols.

DC isolation and generation
Murine DCs were purified from the spleens of naïve NOD mice with CD11c<sup>+</sup> magnetic beads (Miltenyi) according to the manufacturer’s instructions. Cells were incubated with NP and used to stimulate BDC2.5<sup>+</sup> CD4<sup>+</sup> T cells. To generate BMDCs, bone marrow cells were isolated from the...
overnight in culture with rhTNF-α, male AB serum (Omega Scientific) for 3 hours, and then nonadherent cells were harvested. DCs were then treated with NPs overnight. The DCs were washed and transferred intravenously into 8-week-old female NOD recipient mice four times, once every 4 days. Blood glucose concentrations were measured weekly. Mice with glycemia >200 mg/dl were considered to be diabetic.

Mouse T cell differentiation in vitro

BDC2.5+ CD4+ T cells were activated by coculture with BMDCs or splenic DCs at a 3:1 (100,000:30,000) T cell to DC ratio and MIMO beads. DCs (1 × 106 to 2 × 106 cells per mouse) were then extensively washed and transferred intravenously into 8-week-old female NOD recipient mice four times, once every 4 days. Blood glucose concentrations were measured weekly. Mice with glycemia >200 mg/dl were considered to be diabetic.

BMDc transfer experiments

BMDCs were generated as described earlier. On day 7, NPs were added to the cells, and 24 hours later, the cells were purified with CD11c+ magnetic beads. DCs (1 × 10^6 to 2 × 10^6 cells per mouse) were then extensively washed and transferred intravenously into 8-week-old female NOD recipient mice four times, once every 4 days. Blood glucose concentrations were measured weekly. Mice with glycemia >200 mg/dl were considered to be diabetic.

Histology

Pancreata were fixed in 4% PFA, cut, and stained with standard hematoxylin and eosin, and the average degree of insulitis was assessed over 20 islets scored per pancreas by an investigator blinded to treatment. Each islet was classified as “clear” if no infiltrate was detected, “mildly infiltrated” if peri-insulitis or an intra-islet infiltrate occupied less than 25% of the islet, or “heavily infiltrated” if 25 to 50% or >50%, respectively, of the islet was occupied by inflammatory cells.

**BMDC transfer experiments**

BMDCs were generated as described earlier. On day 7, NPs were added to the cells, and 24 hours later, the cells were purified with CD11c+ magnetic beads. DCs (1 × 10^6 to 2 × 10^6 cells per mouse) were then extensively washed and transferred intravenously into 8-week-old female NOD recipient mice four times, once every 4 days. Blood glucose concentrations were measured weekly. Mice with glycemia >200 mg/dl were considered to be diabetic.

**ChIP assays**

Cells were cross-linked with 1% PFA and lysed with the appropriate lysis buffer [1% SDS, 10 mM EDTA, 50 mM tris-HCl (pH 8.1)] containing 1× protease inhibitor cocktail (Roche Molecular Biochemicals). Chromatin was sheared by sonication, and supernatants were collected after centrifugation and diluted in buffer [1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM tris-HCl (pH 8.1)]. Five milligrams of antibody was pre-bound for a minimum of 4 hours to protein A/G Dynal magnetic beads (Invitrogen), washed three times with ice-cold phosphate-buffered saline containing 5% bovine serum albumin, and then added to the diluted chromatin before being immunoprecipitated overnight. The magnetic bead-chromatin complexes were washed three times with radioimmunoprecipitation assay buffer [50 mM Heps (pH 7.6), 1 mM EDTA, 0.7% sodium deoxycholate, 1% NP-40, 0.5 M LiCl] and then twice with tris-EDTA buffer. Immunoprecipitated chromatin was extracted with 1% SDS, 0.1 M NaHCO3 and then heated at 65°C for at least 6 hours to reverse the PFA-induced cross-linking. DNA fragments were purified with a QIAquick DNA Purification Kit (Qiagen) and analyzed by SYBR Green real-time PCR (Takara Bio Inc.). Anti-SOCS2 antibody (cat. #2779; Cell Signaling Technology) was used for ChIP. The following primer pairs were used: AhR (XRE-1), 5′-GGAATGGAGGCCACAGGA-3′; (forward) and 5′-GGAATGGAGGCCACAGGA-3′ (reverse); AhR (XRE-2), 5′-ATGAGTCAACAGAGACAGGA-3′; (forward) and 5′-ATGAGTCAACAGAGACAGGA-3′ (reverse); AhR (XRE-3), 5′-TGCTGACCCTCGTTTTGGGAG-3′; (forward) and AhR (XRE-3), 5′-TGCTGACCCTCGTTTTGGGAG-3′ (reverse).

**Western blotting analysis**

DCs were lysed with the appropriate amount of lysis buffer (Cell Fractionation Kit, cat. #9038; Cell Signaling Technology), and cytoplasmic and nuclear fractions were saved for protein quantification (cat. #23235; Thermo Scientific). Lysates of DCs were resolved by electrophoresis through 4 to 12% bis-tris NuPAGE gels (Invitrogen) and were transferred onto polyvinylidene difluoride membranes (Millipore). The membranes were then
blocked with 5% milk for 1 hour and incubated overnight with shaking at 4°C with the following antibodies: anti-TRAf6 (cat. #ab33915; Abcam), anti-SOCS2 (cat. #2779; Cell Signaling Technology), anti–NF-κB p65 (cat. #8242; Cell Signaling Technology), anti–phosphorylated p38 MAPK (Thr180/Tyr182, cat. #9211; Cell Signaling Technology), anti–p38 MAPK (cat. #9212; Cell Signaling Technology), anti–p44/42 MAPK (Thr202/Tyr204, cat. #4376; Cell Signaling Technology), anti–p44/42 MAPK (cat. #9102; Cell Signaling Technology), anti–GAPDH (cat. #5174; Cell Signaling Technology), and anti–histone H3 antibody (cat. #07-690; Millipore). The next day, the membranes were washed with tris-buffered saline containing Tween 20 and incubated with horseradish peroxidase–conjugated anti-rabbit immunoglobulin G (cat. #7074; Cell Signaling Technology), and anti–rabbit immunoglobulin G (cat. #7074; Cell Signaling Technology), anti–GAPDH (cat. #5174; Cell Signaling Technology), and anti–histone H3 antibody (cat. #07-690; Millipore).

REFERENCES AND NOTES

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Fig. S2. BMDCs loaded with NPITE+Ins suppress T1D in NOD mice. Significance of the time-response curves. Values of <0.05 were considered to be statistically significant.


SUPPLEMENTARY MATERIALS

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Fig. S1. NPITE+Ins administration suppresses CAD in NOD mice. Fig. S2. BMDCs loaded with NPITE+Ins Suppress T1D in NOD mice. Fig. S3. NPs loaded with ITE activate AhR in BMDCs. Fig. S4. Effects of NPITE+Ins on Socs1 and Socs3 expression in DCs.

REFERENCES AND NOTES


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Nanoparticles restore tolerance

Type 1 diabetes (T1D) is caused by the destruction of pancreatic β cells by inflammatory T cells. One strategy to treat T1D involves using suppressive T regulatory (T_{reg}) cells that are grown in culture and then given back to patients to dampen the autoimmune response and induce tolerance. Yeste et al. used gold nanoparticles as a delivery mechanism to induce tolerance directly in a mouse model of T1D without having to grow immune cells ex vivo. The mice had increased numbers of T_{reg} cells and decreased disease severity when given nanoparticles coated with an antigenic peptide of unprocessed insulin and a ligand that promotes the ability of dendritic cells to induce tolerance. These results suggest that nanoparticle-based therapies may be useful in restoring tolerance not only in T1D but also in other autoimmune diseases.