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Myeloid-Derived Suppressor Cells Prevent Type 1 Diabetes in Murine Models

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Effective immunotherapy for type 1 diabetes (T1D; diabetes mellitus) is an insulin-dependent disorder characterized by kidney failure, blindness, heart disease, and chronic ulcers (1). It is now clear that the chronic inflammatory response against specific autoantigens, mainly insulin, leads to the eventual destruction of insulin-producing endocrine β cells. Daily injection of insulin cannot match the naturally precise timing and dosing of insulin secretion of the pancreas in response to hyperglycemia. Even well-managed diabetic patients, therefore, can experience severe treatment side effects and worsening of their disease (2). More recently, a variety of strategies has been developed, aimed at re-establishing physiological insulin production in diabetic patients (3). Despite these advancements, devising a means capable of restoring self-tolerance or specifically down-modulating autoimmune responses via diverse mechanisms (5–9). Accumulating evidence has implicated a potential broad application of MDSCs as a novel cell-based immunotherapy within the fields of transplantation and autoimmune diseases (10–12). To date, transfer of MDSCs has been shown to be capable of inducing immune tolerance in allogeneic bone marrow transplantation (13), prolonging the survival of allo-skin transplants via inhibitory receptor Ig-like transcript 2 mediated tolerance (14), participating in anti-CD28–mediated tolerance in allo-kidney transplantation (15), and amelioration of symptoms in the inflammatory bowel disease model (10). In this study, we have shown that adoptive transfer of CD11bGr-1—MDSCs plus hemagglutinin (HA) peptides efficiently prevents the onset of HA-specific TCR T cell-induced diabetes in INS-HA/RAG−/− recipient mice. Further, MDSCs prevented diabetes onset in NOD/SCID mice and maintained these mice diabetes-free for the long term.

Materials and Methods

Mice

CD4-HA-TCR transgenic mice (BALB/c, H-2b), a gift from Dr. Constantin A. Bona (Mount Sinai School of Medicine, New York, NY), express the 14.3d HA-specific TCR, which recognizes the influenza hemagglutinin (HA, 110-120) of A/PR/8/34 influenza virus in association with I-EK. INS-HA/RAG−/− mice (B10.D2, H-2b) express the HA protein in pancreatic β cells under the control of the rat insulin promoter. MHC class I KO, MHC class II KO, and actin-OVA transgenic mice were purchased from...
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The Jackson Laboratory (Bar Harbor, ME). All animal experiments were performed in accordance with the animal guidelines of the Mount Sinai School of Medicine.

Peptide and Abs
HA peptide (110SFERFEIFPKE120) and OVA peptide (323ISQAVHAA-HAEINEAGR339) were purchased from Washington Biotechnology (Baltimore, MD). All fluorescence-conjugated monoclonal Abs were purchased from eBioscience (San Diego, CA).

Isolation of MDSCs
BALB/c mice bearing syngeneic colon cancer MCA26 and C57BL/6 mice bearing syngeneic Lewis lung carcinoma were used as the source of MDSCs. The detailed Percoll protocol for MDSC isolation has been described previously (6). The fraction II Gr-1+CD11b+ MDSCs were sorted by flow cytometry, and cells with a purity of 98% were used in the INS-HA diabetic model.

Treg expansion and suppression of CD4+CD25+ T cell proliferation by MDSCs
CFSE-labeled, purified CD4+CD25+ Tregs and CD4+CD25- T cells from naive CD4-HA-TCR transgenic mice (BALB/c background) or CD4 OVA-specific TCR transgenic (OT-II) mice (B6 background) were cocultured with MDSCs, at a 4:1 ratio, in the presence of HA or OVA peptides (0.5 μg/ml) and IL-2 (100 U/ml) for 4 d. Proliferation was assessed by flow cytometry (CFSE dilution) using fluorescent-labeled Abs against CD4, CD25, and Foxp3 or isotype controls.

Diabetes model
CD4-HA-TCR transgenic T cells were enriched by T cell enrichment columns (R&D Systems, Minneapolis, MN) and adaptively transferred into INS-HA/RAG-2/- mice by tail vein injection (1 x 105 per mouse). Mouse glucose levels were measured daily using a blood glucose meter (Bayer, Pittsburgh, PA) to determine the onset of diabetes. Mice were considered diabetic when their blood glucose level was ≥200 mg/dl for two consecutive measurements. Sorted MDSCs (5 x 106 cell/mouse) were transferred twice, on the first and fourth days after T cell transfer.

NOD/SCID diabetic model
CD4+ T cells were purified from the spleens of diabetic NOD females using a CD4 isolation kit. To generate MDSCs, mouse mammary tumor T6A cells (106) were implanted into the fat pad of NOD/SCID mice. Two to three weeks later, MDSCs (CD11b+Gr-1+ cells) were positively selected from bone marrow using Ab-conjugated Miltenyi beads (Miltenyi, Auburn, CA). Female NOD/SCID mice aged 6 wk received a tail vein injection of PBS, CD4+ T cells from diabetic NOD mice (2 x 105), and control CD4+ T cells from normal mice plus a single various dose response of MDSCs (0, 0.5, or 1, or 2 x 106).

Using an Elite glucometer (Bayer), fasting blood glucose concentrations were measured daily starting postinjection (days 0 to 100). Mice with fasting blood glucose concentrations over 200 mg/dl for two consecutive measurements. Sorted MDSCs (5 x 106 cell/mouse) were transferred twice, on the first and fourth days after T cell transfer.

Histopathological analysis
Pancreatic tissue was fixed in 10% buffered formalin solution and embedded in paraffin. Prepared sections were stained by Mayer’s H&E. For each organ, 10 sections were analyzed. Staining for intracellular insulin was performed using polyclonal rabbit anti-insulin (Santa Cruz Biotechnology, Santa Cruz, CA) in conjunction with HRP-goat–anti-rabbit conjugate (Southern Biotechnologies, Birmingham, AL). Additional tissue samples were frozen at −80°C, and frozen sections were cut in stepwise fashion (8 μm per section). Staining for CD4 T cells in islets was performed using monoclonal anti-CD4 (eBioscience) in conjunction with HRP-goat–anti-mouse conjugate (Southern Biotechnologies).

Cytokine detection by ELISA
The concentrations of mouse IFN-γ, IL-10, and TGF-β in the culture supernatants were measured by ELISA (R&D Systems).

Suppression/proliferation assay
Column-enriched Thy-1+ T cells (1 x 105) from TCR transgenic mice or treated mice were cocultured with HA peptide (5 μg/ml) alone or in the presence of escalating numbers of CD25+ T cells recovered from treated nondiabetic mice in 96-well microplates. Irradiated (2500 rad) naive splenocytes (5 x 104) were used as APCs. [3H]Thymidine was added during the last 8 h of 72 h culture.

Quantitative real-time PCR
Total RNA was extracted using TRIzol reagent (Invitrogen). The reverse transcription-PCR (RT-PCR) procedure (One-step RT-PCR kit; Qiagen, Valencia, CA) using primers for Foxp3 and for GAPDH (internal control) has been previously described (9). For quantitative real-time PCR, reverse-transcribed cDNA was amplified by real-time PCR with SYBR Green Universal PCR Mastermix (Bio-Rad, Richmond, CA). Samples were analyzed in duplicate with the IQ-Cycler (Bio-Rad). The normalized signal level was calculated based on the ratio to the corresponding GAPDH signal.

Results
MDSCs can act as APCs for Treg expansion and suppress effector T cell proliferation in vitro
Our laboratory and others have found that MDSCs from tumor-bearing mice can suppress T cell proliferation and induce Treg expansion (5–8, 9, 17). We have reported that MDSC-mediated Treg activation requires the presence of IFN-γ and IL-10 (6). We then studied whether MDSCs can act as APCs to mediate Treg activation and, therefore, lead to the development of a better strategy for cell-based tolerogenic therapy using MDSCs.

To determine whether MDSC-mediated Treg expansion was Ag-dependent and MHC-restricted, CD4+CD25+ splenic T cells purified from naive OT-II mice were labeled with CFSE and cocultured with wild-type (WT) MDSCs or MHC class I or II KO MDSCs in the presence or absence of OVA peptides, or with MDSCs isolated from tumor-bearing, constitutive actin promoter-driven, OVA-expressing, transgenic mice. Based on FACS analysis for the presence of CFSE-labeled cells (Fig. 1A) on four reproducible and separate experiments, we observed a significantly higher level of CD4+CD25+Foxp3+ Treg expansion (i.e., CFSE dilution) in the presence of WT MDSCs and OVA peptide (80.1 ± 2.7%; Fig. 1B) than in the presence of WT MDSCs alone (62 ± 4.5%) or OVA peptide alone (46.1 ± 4.7%) (Fig. 1B; t test, p < 0.001). Treg expansion was similarly mediated by actin-OVA-expressing MDSCs (67.4 ± 2.1%; p < 0.001), which contain endogenous OVA. Furthermore, Treg expansion in the presence of MHC class II KO MDSCs and OVA peptide (46.9 ± 6.9%) was significantly decreased to levels similar to those observed when cultured in the presence of OVA peptide alone. However, even when cultured with MHC class I KO MDSCs in the presence of OVA peptide, Treg expansion was not significantly decreased (77.0 ± 10.0%; p < 0.001). Taken together, these data suggest that MDSC-mediated Treg expansion is Ag-specific and MHC class II dependent.

We next asked whether MDSC-mediated suppression of naive CD4+CD25+ OT-II TCR transgenic T cell proliferation was Ag- and/or MHC-restricted. Using the same experimental design described in Fig. 1, we found that suppression of CD4+CD25+ T cell proliferation was both Ag and MHC class II dependent. We again performed FACS analysis for CFSE-labeled cells (Fig. 2A) in four separate and reproducible experiments and found a significant proportion of CD4+CD25+ OT-II T cells proliferated in the presence of OVA peptide and irradiated naive splenocytes (53.5 ± 5.4%) (CT, Fig. 2B). In the presence of WT MDSCs and OVA peptide or actin-OVA MDSCs, the proliferation of CD4+CD25+ OT-II T cells was significantly suppressed (9.4 ± 2.6% and 18.4 ± 5.0%; p < 0.001, compared with control splenocytes with OVA
Moreover, the results depicted in Figs. 1 and 2 indicate that both T cells and the lack of traceable markers for in vivo studies. Evaluation of autoimmune responses against natural autoantigens diabetes MDSCs prevent the onset of autoreactive T cell-mediated immune response against pancreatic islet cells expressing HA and, thus, prevent the development of diabetes in INS-HA/RAG<sup>−/−</sup> recipient mice.

**MDSCs prevent the onset of autoreactive T cell-mediated diabetes**

Evaluation of autoimmune responses against natural autoantigens has been impeded due to the polyclonal nature of autoreactive T cells and the lack of traceable markers for in vivo studies. Moreover, the results depicted in Figs. 1 and 2 indicate that both MDSC-mediated suppression of T cell proliferation and Treg expansion were Ag-dependent and MHC class II-restricted processes.

Collectively, these data indicate that both MDSC-mediated suppression of T cell proliferation and Treg expansion are Ag-dependent and MHC class II-restricted processes.

**MDSCs inhibit lymphocyte infiltration and insulitis**

Lymphocyte infiltration and the resultant inflammation is a hallmark feature of diabetes. To better understand the effect of MDSCs on the suppression of diabetes development, we examined the histological changes in the pancreata of treated mice. H&E staining MDSCs plus HA peptide can effectively suppress the HA-specific T cell-mediated immune response against pancreatic islet cells expressing HA and, thus, prevent the development of diabetes in INS-HA/RAG<sup>−/−</sup> recipient mice.

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indicated those recipients of MDSCs plus HA peptide had intact islets, and the severity of insulitis in these mice was markedly reduced (Fig. 4, upper left panel). In sharp contrast, examination of nontreated (PBS) mice showed massive peri-islet infiltration of mononuclear cells (Fig. 4, upper right panel). These observations were further verified by assessing CD4 T lymphocyte infiltration. MDSC plus HA peptide recipients showed only minimal staining (Fig. 4, middle left panel), whereas the islets recovered from PBS-treated mice contained large numbers of CD4 T cells (Fig. 4, middle right panel). Furthermore, immunohistochemical staining for insulin revealed strong insulin expression in the intact islets of mice treated with MDSCs plus HA (Fig. 4, lower left panel). On the contrary, insulin production was almost absent from the pancreata of mice that received transferred T cells and PBS (Fig. 4, lower right panel). These results indicate that transfer of MDSCs effectively inhibits the infiltration of T lymphocytes into islets, thus avoiding the harmful inflammatory response against islet cells and destruction of insulin-producing β cells.

**MDSCs mediate T cell tolerance**

We postulated that the profound suppression of T cell infiltration into the islets of treated mice likely resulted from potent induction of T cell tolerance by MDSCs. To test this idea, we reisolated autoreactive CD4-HA-TCR T cells from treated mice and examined their ability to proliferate in vitro. As shown in Fig. 5A, T cells recovered from mice receiving MDSCs + OVA, control cells from Fr3 + HA, or PBS proliferated vigorously upon restimulation with HA peptide. In the group treated with MDSCs + HA, T cells isolated from clinically diabetic mice still proliferated to some extent, but those from diabetes-free mice exhibited barely detectable proliferative potential, similar to that of naive mice (without HA-C4D T cell transfer). The inability of T cells to proliferate in response to specific Ag stimulation suggests that an anergic state was actively induced in the autoreactive T cells from the mice treated with MDSCs + HA.

We also assessed cytokine expression in the culture supernatants harvested from the above T cell proliferation assays. Strikingly, although there was no substantial difference in IFN-γ production between all HA-CD4 T cell transferred groups, large amounts of IL-10 and TGF-β were detected only in samples from diabetes-free mice treated with MDSCs + HA (Fig. 5B; p < 0.001). This indicates that regulatory, rather than inflammatory, cytokines were generated in vivo in the presence of MDSCs, and, hence, these factors may play a critical role in the induction of T cell tolerance by MDSCs.

**MDSCs induce Treg development**

Previous studies from our group and others have demonstrated that MDSCs exhibit an exceptional ability to activate and expand Tregs (6, 17), a cell type shown to be a key component in the regulation of T1D and other autoimmune responses. To assess the potential involvement of Tregs in the control of T1D by MDSCs, Treg development in secondary lymphoid organs (i.e., spleen) was evaluated. As shown in Fig. 6A, T cells recovered from diabetes-free mice receiving MDSCs and HA peptide contained a significantly higher percentage (35.5%) of Foxp3+CD4+CD25+ T cells compared with that of T cells recovered from diabetic mice (15.0%) receiving the same treatment or control treatments. We further confirmed these findings by examining Foxp3 gene expression by both RT-PCR (Fig. 6B, upper panel) and real-time RT-PCR (Fig. 6B, lower panel), which demonstrated that the highest level of Foxp3 gene expression was detected in non-diabetic mice that received MDSC and HA treatment (lane 1). These results suggest that transfer of HA peptide pulsed MDSCs induced a considerable proportion of autoreactive T cells to become Tregs.

![FIGURE 5.](http://www.jimmunol.org/)

Proliferation and production of inhibitory cytokines by autoreactive T cells recovered from treated diabetes-free mice. A. A comparison of the proliferative capacity of T cells recovered from the different groups at 30 d posttreatment. Purified T cells and irradiated naive splenocytes (as APCs) were cocultured in the presence of HA peptide (5 μg/ml). [3H]Thymidine (1 μCi/well) was added for the last 8 h of a 72 h culture. Stimulation index is calculated as cpm in the presence of HA divided by cpm in the absence of HA (n = 3 replicates). B. Cytokine production by autoreactive T cells. Cytokine concentrations in the supernatants, harvested from the same cultures as described in A, were measured by ELISA (n = 3 replicates).

**FIGURE 4.** Lymphocyte infiltration, islets, and insulin expression in mice treated with MDSCs plus HA peptides. Serial sections of pancreas were prepared from treated mice at 4 wk after cell transfer. Sections were stained by H&E (upper panels; original magnification ×400) and immunohistochemically stained with anti-CD4 (middle panels; original magnification ×400) or rabbit polyclonal anti-insulin (lower panels; original magnification ×400). Left panels, Representative photomicrographs from MDSC + HA peptide–treated diabetes-free mice. Right panels, Representative photomicrographs from PBS-treated diabetic mice.
We then aimed to test whether MDSC-induced Tregs possess suppressive activity. T cells purified from naive CD4-HA-TCR transgenic mice and CD25+ T cells isolated from diabetes-free mice receiving MDSC plus HA peptide were cultured alone or in the presence of HA peptide at various ratios. The results indicate that the CD25+ T cells from nondiabetic mice mediated potent suppression of Ag-specific T cell proliferation in a dose-dependent manner (Fig. 6C).

**Effects of MDSCs on the development of diabetes in NOD/SCID mice**

To test whether MDSCs could affect the development of T1D in another murine model, NOD/SCID mice were adoptively transferred with PBS, splenic CD4+ T cells isolated from NOD diabetic mice, or splenic CD4+ T cells from NOD diabetic mice along with MDSCs. NOD/SCID mice treated with PBS did not develop diabetes (nontreatment, Fig. 7A). In sharp contrast, NOD/SCID mice, which received autoreactive CD4+ T cells from diabetic NOD mice, did become diabetic around day 10, as evidenced by fasting blood glucose levels over 200 mg/dl (CD4, Fig. 7A). However, a single adoptive transfer of MDSCs effectively decreased the rate of diabetes development (CD4 + MDSC, Fig. 7A), and 60% of the animals remained diabetes-free as long as 100 d posttreatment. We also assessed the extent of leukocyte infiltration, a hallmark of insulitis, in the above mice at 15 d postinjection. We consistently found that adoptive transfer of CD4+ T cells, versus PBS control transfer, significantly increased the extent of leukocyte infiltration into the pancreata of NOD/SCID mice (nontreatment versus CD4, Fig. 7B). In contrast, inclusion of MDSCs remarkably decreased the extent of leukocyte infiltration (Fig. 7B). These findings regarding dysregulated glucose and insulitis suggest that MDSCs can suppress autoreactive T cells and thereby prevent T1D in the NOD system. As shown in Supplemental Fig. 1, this protective effect was maintained even at lower MDSC doses.

**Discussion**

In recent years, MDSCs have garnered increased interest. Previous studies have shown that MDSCs may be involved in down-modulating alloimmune responses in a variety of allogeneic transplant models (11–15). In this study we provide firm evidence...
that in vitro MDSC-mediated suppression of CD4\(^+\)CD25\(^-\) T cell proliferation and Treg expansion were Ag-dependent, MHC class II-restricted processes, and that MDSCs were effective at perturbing the autoimmune response in the two diabetic animal models tested. Using a transgenic murine model of autoimmune diabetes, we demonstrated that injection of CD115\(^+\)Gr-1\(^+\) MDSCs and HA peptide, a surrogate autoantigen, could efficiently protect INS-HA/RAG\(^-/-\) mice from HA-specific TCR T cell-mediated diabetes in an Ag-dependent fashion. In addition, we observed markedly reduced lymphocyte infiltration and insulitis in the islets of the MDSC-treated mice. We also confirmed the ability of MDSCs to prevent the natural autoimmune T cell response in NOD/SCID diabetic mice. These findings suggest that MDSCs may have great translational implications for treating T1D and other autoimmune diseases.

The protective effects afforded by MDSC treatment include hypoproliferation of autoreactive T cells and dramatically decreased infiltration of T lymphocytes into pancreatic islets in the diabetes-free treated mice. The latter likely results from an early inhibition of HA-specific T cell expansion in secondary lymphoid organs, as well as specific target organs (pancreas). This inference is supported by previous studies documenting a profound suppression of activated T cell proliferation by MDSC transfer in both tumor (6, 19) and allogeneic bone marrow transplantation models (13). Given the fact that peri-islet infiltration occurs prior to \(\beta\) cell destruction, as observed in NOD mice and TCR-transgenic mice (20), the suppression of autoreactive T cell responses by transferred MDSCs may account for the suppression of the autoimmune response and prevention of T1D onset.

Tregs are widely recognized as crucial players in the development of T1D. Previous reports have shown that defects in the frequency and/or function of Tregs are closely associated with the onset or progression of T1D in both NOD mice (21–23) and human patients with T1D (24, 25). Moreover, Treg-based therapies have been under extensive investigation. Two primary strategies for the therapeutic application of Tregs include in vivo induction (26, 27) and in vitro expansion (28, 29). Compared with the currently available approaches, MDSCs, which can be efficiently derived from embryonic and hematopoietic stem cells in vitro (13), may offer a unique opportunity for specific immune intervention in T1D and other autoimmune diseases by inducing Ag-specific Tregs, which are able to suppress directly autoreactive T cells.

Most interestingly, using the NOD/SCID model, we have demonstrated that a one-time injection of MDSCs can provide long-term protection against the onset of T1D. We and others have shown that monocytic MDSCs may play a key role in preventing graft-versus-host disease in allograft transplantation (12, 13). Using our Gr-1\(^+\)CD11b\(^+\) MDSC population, we observed a significant treatment effect even at doses as low as 1 \(\times\) 10\(^7\)/mouse and with only a diminutive monocytic population (4–6%; Supplemental Fig. 2). We continue to investigate the relative potency of monocytic versus granulocytic MDSCs. Quantifying the relative abilities of monocytic and granulocytic MDSCs to provoke immune suppression and give rise to Ag-specific immune tolerance is an area of continued investigation.

In conclusion, our findings clearly indicate that transfer of MDSCs conferred an exceptional capability to establish immune tolerance to self-Ag and protect against the onset of autoimmune T1D.

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Disclosures

The authors have no financial conflicts of interest.

