Congenic mesenchymal stem cell therapy reverses hyperglycemia in experimental type 1 diabetes

Running title: MSC therapy for T1D

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Objective: A number of clinical trials are underway to test whether mesenchymal stem cells (MSC) are effective in treating various diseases, including type 1 diabetes (T1D). Although this cell therapy holds great promise, the optimal source of MSC has yet to be determined with respect to MHC matching. Here, we examine this question by testing the ability of congenic MSC, obtained from the NOR mouse strain, to reverse recent-onset T1D in NOD mice, as well as determine the immunomodulatory effects of NOR MSC in vivo.

Research Design and Methods: NOR MSC were evaluated with regard to their in vitro immunomodulatory function in the context of autoreactive T cell proliferation and dendritic cell (DC) generation. The in vivo effect of NOR MSC therapy on reversal of recent-onset hyperglycemia and on immunogenic cell subsets in NOD mice was also examined.

Results: NOR MSC were shown to suppress diabetogenic T cell proliferation via PD-L1 and to suppress generation of myeloid/inflammatory DC predominantly through an IL-6-dependent mechanism. NOR MSC treatment of experimental T1D resulted in long-term reversal of hyperglycemia, and therapy was shown to alter diabetogenic cytokine profile, to diminish T cell effector frequency in the pancreatic lymph nodes, to alter antigen-presenting cell frequencies, and to augment the frequency of the plasmacytoid subset of DC.

Conclusions: These studies demonstrate the inimitable benefit of congenic MSC therapy in reversing experimental T1D. These data should benefit future clinical trials using MSC as treatment for T1D.

Mesenchymal stem cell (MSC) therapy has in recent years emerged as a promising treatment modality for diseases with immune etiology, particularly given the increasing appreciation for the morbidity associated with immunosuppression. MSC have been demonstrated to exhibit profound immunomodulatory effects in vitro and in vivo, and these immunomodulatory capabilities have been shown to be exerted through both direct contact as well as production of soluble markers (1-4). Moreover, upregulation of B7-H1/PD-L1 by IFN-γ has been shown to play a central role in the immunosuppressive properties of MSC via direct contact with activated T cells (5; 6). In vitro studies have also demonstrated the ability of MSC to regulate the function of T cell effector pathways through promotion of regulatory dendritic cell (DC) generation, due to MSC-modulated alteration of DC cytokine profiles as evidenced by increased production of regulatory cytokines such as IL-10 and reduction of inflammatory cytokines including IFN-γ, IL-12, and TNF-α, thereby inducing a more anti-inflammatory or tolerant DC phenotype (7; 8). These immunomodulatory effects as well as an extensive capacity for in vitro–expansion of MSC have prompted launch of numerous clinical trials (1). MSC therapy has yielded promising results in the treatment of GVHD as well as in the resolution of Crohn’s disease-associated fistulas, in stabilization of refractory progressive multiple sclerosis, and in reversal of multiorgan dysfunction in patients with systemic lupus erythematosus (9-12). However, while the therapeutic value of MSC for attenuating the autoimmune disorder type 1 diabetes (T1D) has logical potential, MSC treatment of this particular disease remains largely unexplored. Trials utilizing MSC therapy in patients with T1D
are underway, yet these efforts have been initiated in the near absence of preclinical data. In this regard, we and others have recently demonstrated delayed onset of experimental T1D as well as reversal of recent-onset diabetes in response to allogeneic MSC therapy, while in our study administration of autologous diabetic MSC showed no beneficial effect (13; 14). Our previous work also indicated that congenic NOR MSC imparted the greatest benefit in preventing T1D. The NOR/LtJ strain, while resistant to insulitis due to the protective Idd alleles (15), shares the diabetogenic H2\textsuperscript{g7} complex with the NOD/LtJ strain. NOR mice are 85% homogolous to spontaneously diabetic NOD mice and are thus somewhat analogous to non-diabetic siblings of T1D patients. Here, we sought to further examine the therapeutic efficacy of NOR MSC on reversal of recent-onset diabetes and to elucidate the mechanisms by which NOR MSC may act to ameliorate diabetes pathogenesis.

MATERIALS AND METHODS

Mice. NOR/LtJ, NOD/LtJ, and NOD.Cg-Tg(TcraBDC2.5)1DoiTg(TcrrBDC2.5)2Doi/DoiJ (BDC2.5) were purchased from the Jackson Laboratories (Bar Harbor, ME). All procedures used in animal experiments were in accordance with the standards set forth in the Guidelines for the Care and Use of Laboratory Animals at Harvard University.

MSC culture and differentiation. To generate NOR MSC, bone marrow mononuclear cells were isolated from the femurs and tibiae of NOR/LtJ mice. Cells were seeded in tissue culture flasks at a concentration of 1x10\textsuperscript{6}/cm\textsuperscript{2} as previously described (13) and were trypsinized at 80% confluence and consolidated 2:1 until passage 4 (P4); from P4 to P6, cells were used for injection, characterization, or in vitro assays. 7.5x10\textsuperscript{5} MSC/well were cultured for 48h in 6-well plates with 0.05, 0.5 or 5 ng/ml recombinant murine IFN-γ (Peprotech, Rocky Hill, NJ). MSC differentiation to mesodermal tissues was performed as previously described (13).

Flow cytometric analysis. MSC were analyzed for surface expression of a battery of markers at P4. Anti-mouse antibodies purchased from BD Biosciences (San Jose, CA) included CD45, Ly-6A/E/Sca-1, CD44, CD90.2, and CD73. Antibodies purchased from eBioscience (San Diego, CA) were CD105, CD29, CD106/VCAM-1, PD-1, B7-H1/PD-L1, and B7-H2/PD-L2. For ex vivo studies, the spleen and pancreatic lymph nodes (PLN) were harvested and subjected to analysis for CD4 effectors, CD8 effectors, and Tregs as previously described (13). Splenocytes or DC cultures were also stained with CD11c, CD11b, F4/80, CD45R/B220, CD40, CD80, CD86, and Ly-6c (BD Biosciences). The biotinylated lineage panel was purchased from Miltenyi Biotec (Auburn, CA), and cells were secondarily stained with streptavidin (BD Biosciences).

Immunohistochemistry. Adherent NOR MSC were fixed on slides and stained with hematoxylin-eosin (H&E) for morphological evaluation, as previously described (13). Immunohistochemistry was also performed as previously described (13).

TCR-stimulated proliferation. NOD CD4\textsuperscript{+} T cells were isolated by magnetic bead separation using CD4 microbeads (Miltenyi Biotec). 1x10\textsuperscript{5} CD4\textsuperscript{+} T cells were stimulated with 1 \mu g/ml anti-mouse CD3 and anti-mouse CD28 (BD Biosciences) alone or in combination with 1x10\textsuperscript{4}, 2x10\textsuperscript{4}, or 4x10\textsuperscript{4} control or IFN-γ-challenged (0.05, 0.5, or 5 ng/ml for 48h) NOR MSC or NOR splenocytes in 96-well plates for 48h, followed by pulsing with 1 \mu Ci tritiated thymidine (Perkin Elmer, Waltham, MA) for 16h. Tritium uptake was assessed using a MicroBeta FilterMate-96 Harvester and a 1450 MicroBeta TriLux (both from Perkin Elmer).
Autoreactive T cell proliferation. BDC2.5 CD4+ cells were extracted from isolated splenocytes using magnetic bead separation (Miltenyi Biotec, Auburn, CA), and the BDC2.5 autoreactive T cell assay was performed as previously described (13). In experiments with siRNA knockdown, PD-L1 or non-targeting pool siRNA were added to MSC using the Accell platform (Dharmacon, Lafayette, CO) for 3 days prior to culturing with BDC2.5 T cells, NOD DC, and BDC2.5 islet peptide, followed by pulsing with 1 μCi tritiated thymidine as above.

ELISPOT. The autoreactive T cell assay was performed as above using NOR MSC in an ELISPOT assay as previously described (16). ELISPOT kits (BD Biosciences) to assess IFN-γ and IL-6 production were used according to the manufacturer’s instructions.

Luminex and ELISA. To assess cytokine production of murine serum and culture supernatant samples, a 21-plex cytokine kit (Millipore, St. Charles, MO) was used according to the manufacturer’s instructions and as previously described (16). To assess production of IFN-α, M-CSF, and Flt3L, murine ELISA kits (R&D Systems, Minneapolis, MN) were used according to the manufacturer’s instructions.

DC culture. Bone marrow-derived NOD DC were generated as previously described (16). In DC and MSC co-culture, 1x10^5 NOR MSC were plated at day 0 of NOD DC culture. For blocking studies, 10 μg/ml anti-IL-6 (eBioscience) was added at day 0 and replaced on days 3 and 6 at the same time as media additions or changes.

Giemsa staining. Cytospins of DC cultures at day 8 were stained with Giemsa stain (Sigma-Aldrich, St. Louis, MO) according to the manufacturer’s instructions, and images were obtained using a Nikon TE300 system.

Reversal studies. Female NOD mice were monitored beginning at 10 weeks of age, and on day 2 of hyperglycemia (>240 mg/dL), a sustained-release insulin pellet (LinBit, LinShin Canada, Inc., Ontario, Canada) was placed subcutaneously into the dorsum. The initial MSC injection (1x10^6 cells i.v.) was injected within 24h of pellet placement, and 1x10^6 NOR MSC were injected intravenously twice per week thereafter for 4 weeks. Normoglycemia was maintained as needed during the last two weeks of treatment by 250 ng/dL insulin (Lantus, Sanofi-Aventis, Bridgewater, NJ). Mice were monitored daily by measuring blood glucose until the time of sacrifice, and measurements were performed by tail bleeding according to NIH guidelines.

RESULTS
NOR MSC phenotype is consistent with mesenchymal lineage. NOR MSC were evaluated by immunohistochemical and flow cytometric analysis for their expression of classical MSC markers as well as costimulatory molecules. Cultured cells were shown to be positive for the MSC markers CD29, CD105, and CD44, but were negative for the hematopoietic lineage-restricted marker CD34 following immunohistochemical staining (Figure 1a). Surface staining revealed substantial expression of CD29, CD44, and CD105, with moderate expression of CD73 and Sca-1, and MSC were found to be negative for the leukocyte antigen CD45 (Figure 1b). Our NOR MSC were additionally found to be capable of differentiating into cells of mesodermal lineage (Figure 1c). These data confirm that our cultured NOR MSC are phenotypically and lineally mesenchymal as well as functionally multipotent.

NOR MSC suppress autoantigen-specific and nonspecific T cell proliferation. To further characterize NOR MSC, we performed cytokine studies of NOR MSC cultures, which demonstrated the presence of various cytokines, with IL-6 most notable in its production (n=4, Figure 2a). As MSC are defined by their immunomodulatory ability as
well as their surface marker profile and multipotent potential, we added increasing numbers of NOR MSC previously challenged with increasing concentrations of IFN-γ to an anti-CD3/-anti-CD28-stimulated proliferative assay using NOD CD4+ T cells to assess the capacity of NOR MSC to suppress T cell proliferation. NOR MSC potently inhibited TCR-stimulated proliferation in a dose-dependent manner (Figure 2b, n=5, p<0.027 for indicated conditions), and pretreatment with IFN-γ enhanced suppression of proliferation dose-dependently. To examine whether NOR MSC are able to specifically suppress autoreactive T cells, MSC were added to an autoreactive T cell assay, in which isolated BDC2.5 CD4+ T cells, or H2g7-restricted diabetogenic T cells, are co-cultured with NOD DC and BDC2.5 islet peptide. As shown in Figure 2c, NOR MSC significantly suppressed autoreactive T cell proliferation, as assessed by CFSE dilution and calculation of proliferation index (n=5, p=0.047). Addition of NOR MSC was also able to suppress production of IFN-γ by BDC2.5 T cells, which has been described previously as a characteristic of proinflammatory autoreactive T cells (17), and the production of IL-6 was shown to be enhanced in the presence of NOR MSC (Figure 2d, n=6, p=0.0024 for IFN-γ, p=0.0005 for IL-6). These data demonstrate that NOR MSC are capable of not only suppressing T cell proliferation stimulated through the TCR via anti-CD3/-CD28 stimulation, but that in the specific context of diabetogenic T cells, NOR MSC have a potent immunomodulatory effect on autoreactive T cell proliferation and production of IFN-γ. NOR splenocytes were also tested for their suppressive ability in all of the assays above in order to assess the effects of congenicity, and no effects on anti-CD3/CD28-stimulated T cell proliferation, autoreactive T cell proliferation, or autoreactive T cell IFN-γ production were observed (Figure 2e [n=4, p<0.0001 for NOR MSC, p=ns for NOR splenocytes] and data not shown, respectively).

**Suppression of autoreactive T cell proliferation by NOR MSC is mediated by PD-L1.** Recent studies have highlighted the central role of the negative costimulatory PD-1 pathway ligand PD-L1 in suppressing the proliferation of autoreactive T cells and in consequently halting the progression of T1D in NOD mice (18-21). Given these data, we hypothesized that inhibition by PD-L1 serves as a mechanism by which NOR MSC exert their immunomodulatory effects on diabetogenic T cells. We thus examined the expression of PD-L1 on resting NOR MSC and following activation with IFN-γ to evaluate expression of components of the PD-1 pathway in the context of inflammation. Although NOR MSC expressed PD-L1 at insubstantial levels at baseline, stimulation with increasing doses of recombinant murine IFN-γ resulted in prodigious upregulation of PD-L1 expression in a dose-dependent manner, while PD-1 and PD-L2 expression underwent modest changes following IFN-γ challenge (Figure 2f, n=3, p=0.0002 for PD-L1 expression of baseline compared to 5 ng/ml IFN-γ). We also performed experiments to examine whether addition of unstimulated NOR MSC to the BDC2.5 autoreactive assay would result in upregulation of PD-L1 expression in a dose-dependent manner, while PD-1 and PD-L2 expression underwent modest changes following IFN-γ challenge (Figure 2f, n=3, p=0.0002 for PD-L1 expression of baseline compared to 5 ng/ml IFN-γ). We also performed experiments to examine whether addition of unstimulated NOR MSC to the BDC2.5 autoreactive assay would result in upregulation of PD-L1, as substantial IFN-γ was detected in the BDC2.5 autoreactive T cell assay (Figure 2d); indeed, co-culture of NOR MSC with autoreactive T cell assay components resulted in considerable NOR MSC expression of PD-L1 (Figure 2g, n=5, p<0.0001), indicating that an autoreactive inflammatory milieu induces marked upregulation of PD-L1 on MSC. To determine the functionality of PD-L1 expression in the suppression of autoreactive T cell proliferation by NOR MSC, we treated NOR MSC with PD-L1 siRNA prior to adding MSC to our autoreactive T cell assay. Indeed, treatment of NOR MSC with PD-L1
siRNA abrogated the suppressive effect of MSC on BDC2.5 T cell proliferation (Figure 2h, n=6; p=0.0034 for control vs. non-targeting siRNA, p=ns for control vs. PD-L1 siRNA for representative experiment shown). Addition of greater numbers of PD-L1 siRNA-treated NOR MSC (4 and 8x10^4; n=4) to the autoreactive T cell assay did not result in restoration of the suppressive effect of NOR MSC (data not shown), perhaps due to the fact that MSC have been shown to exert their immunomodulatory effects through cell contact. To confirm that our siRNA treatment resulted in efficient knockdown of PD-L1 expression, siRNA-treated MSC were examined for PD-L1 copy number by real-time PCR. As shown in Figure 2g, PD-L1 transcripts were significantly decreased by PD-L1 siRNA treatment of MSC (n=4, p=0.016), demonstrating effective suppression of PD-L1. These data indicate that PD-L1 plays a significant role in the specific context of MSC-mediated suppression of diabetogenic T cells.

**NOR MSC inhibit in vitro DC differentiation via IL-6.** Aberrant DC development and imbalance in APC subsets have been reported to be responsible for the lack of tolerance mechanisms in NOD mice (22; 23). To examine the effect of NOR MSC on DC generation, we performed in vitro studies of NOR MSC and NOD DC using an established method of DC culture (16; 24). Due to the substantial production of IL-6 in our NOR MSC cultures (Figure 2a) as well as the fact that IL-6 has been demonstrated to both suppress and alter DC differentiation (2; 7; 25; 26), we performed IL-6 blocking studies in conjunction with co-culture of MSC and DC. The presence of MSC strikingly reduced CD11c and CD11b expression of DC, so that the predominant population induced by MSC was CD11c<sup>low</sup>CD11b<sup>low</sup> (Figure 3a, CD11c<sup>+</sup>CD11b<sup>+</sup> cells=40.7±2.6% and 22.1±2.4% for control and NOR MSC-treated, respectively, n=5, p=0.0007). Addition of anti-IL-6 somewhat abrogated the change in phenotype observed in DC co-culture with NOR MSC (Figure 3a, n=4, p=ns in comparison to control [-/-] and in comparison to MSC alone [-/+]). Of note, costimulatory molecule expression in the CD11c<sup>+</sup> population was not found to be significantly different in the presence or absence of MSC (data not shown). The CD11b<sup>+</sup> population was also evaluated with respect to Ly-6c expression, as both CD11b<sup>+</sup>Ly-6c<sup>high</sup> and CD11b<sup>+</sup>Ly-6c<sup>int</sup> cells have been demonstrated to be inflammatory monocytes recruited to sites of inflammation (22; 27). Co-culture with NOR MSC resulted in downregulation of both the CD11b<sup>+</sup>Ly-6c<sup>high</sup> and CD11b<sup>+</sup>Ly-6c<sup>int</sup> populations (Figure 3a, n=4, p=0.0053 and p=0.02, respectively), and this difference was again abrogated by blockade of IL-6. We then assessed the number of lineage-negative cells as a function of progenitor frequency or lack of differentiation, and NOR MSC treatment was shown to increase lineage-negative cells as well as increase the expression of Sca-1 within the lineage-negative population (Figure 3b, n=4, p=0.004 and p=0.0085, respectively). Treatment with anti-IL-6 was somewhat efficacious in abrogating the suppression of differentiation observed in response to MSC co-culture, suggesting that other factors may be involved in the effect of MSC on DC differentiation. We therefore examined the supernatants of DC and MSC co-cultures at day 8 for cytokine production. As shown in Figure 3c, co-culture with MSC significantly enhanced IL-6 levels (n=4, p=0.0074), and addition of IL-6 blocking antibody efficiently suppressed IL-6 production. Moreover, Flt3L and M-CSF production was increased in response to MSC (n=4, p=0.03 and p=0.04, respectively), and IL-6 blockade had no effect on increased levels of these cytokines (p=0.013 and p=0.018, respectively, in comparison to DC alone, Figure 3c). Conversely, production of
TNF-α, a growth factor involved in the maturation of DC as well as a cytokine secreted by mature DC (16; 28) was reduced in the presence of MSC (Figure 3c, n=4, p=0.0056), and blocking of IL-6 resulted in abrogation of this effect. To examine the morphology of DC in response to MSC, we performed Giemsa staining of day 8 DC cultures and found that the nuclear to cytoplasmic ratio appeared to decrease following co-culture with MSC, a feature commonly associated with earlier stages of differentiation (29), and IL-6 blockade appeared to partially reverse this effect (Figure 3d). Examination of side scatter of DC by FACS revealed that co-culture with MSC resulted in a dramatic reduction in the degree of granularity (data not shown), again demonstrating a lack of differentiation in response to MSC (30). Addition of MSC to plasmacytoid DC cultures resulted in enhanced plasmacytoid DC frequency, and this effect was fully reversed by IL-6 blockade (Supplemental Figure 1 in the online appendix at http://diabetes.diabetesjournals.org), as IL-6 has been previously demonstrated to be important for plasmacytoid DC generation (31). Taken together, these data demonstrate a marked effect of NOR MSC on DC phenotype, differentiation, and cytokine production, which is in large part mediated by IL-6.

NOR MSC efficiently reverse recent-onset experimental autoimmune T1D. Given our previous data in which congenic NOR MSC were found to be most effective in preventing onset of diabetes in the NOD mouse model in comparison to autologous or allogeneic MSC treatment (13), we sought to determine the efficacy of NOR MSC in reversing recent-onset hyperglycemia in NOD mice. Our previous work demonstrated that reversal of hyperglycemia in response to BALB/c MSC therapy, while effective, was only temporary, perhaps due to eventual rejection of the allogeneic cells (13). Using a treatment protocol identical to that of our previous study, we observed reversal of recent-onset hyperglycemia in 8 out of 9 NOD mice treated with NOR MSC at the 5-week point of post-treatment observation (Figure 4a). Of note, only 1 mouse in the NOR MSC-treated group exhibited blood glucose levels greater than 600 mg/dL (* in Figure 4a) at approximately 5 weeks into treatment. While 2 NOD mice treated with NOR MSC succumbed to unexplained deaths with no evidence of remission break, the other mouse was sacrificed. Although several weeks of observation suffices for most reversal studies, the remaining 5 mice were observed for an extended period to ensure that their reversal was maintained (i.e. with no return to hyperglycemia). At 12 weeks post treatment, their average blood glucose measurement was 222±13.4 mg/dL. All NOD mice (both NOR MSC-treated and controls) were provided a slow-release insulin pellet to allow for a limited period of metabolic recovery, yet in contrast to NOR MSC-treated mice, all control NOD mice (n=9) re-displayed hyperglycemia almost immediately following dissolution of the insulin pellet. Weekly mean blood glucose measurements of treated mice were significantly reduced compared to controls from 2 to 12 weeks; notably, 8 out of 9 hyperglycemic untreated mice died within 6 weeks after the onset of diabetes (Figure 4b, p<0.001 for all time points, 1 surviving control is shown beyond week 6.5). No treatment bias was present, as glucose measurements at the initiation of treatment did not differ between groups (Figure 4b, p=ns). These data demonstrate efficient and long-term reversal of recent-onset hyperglycemia in response to NOR MSC therapy.

NOR MSC treatment augments regulatory cytokine levels and induces regulatory DC. In order to elucidate the mechanisms by which reversal of recent-onset hyperglycemia
occurred in response to NOR MSC therapy, we performed serum cytokine studies following the completion of MSC administration at days 0, 7, 14, and 21. As shown in Figure 5a, NOR MSC treatment resulted in increased circulating levels of IL-6, IL-7, IL-10, and IL-12(p40) (n=3-5 samples, p<0.00065 where indicated). Of note, IL-12(p70) levels were decreased in serum of NOR MSC-treated mice, but the difference did not reach statistical significance (data not shown). Flow cytometric analysis of the spleen and PLN of treated and control mice at 2 weeks following the initiation of treatment demonstrated a reduction in the populations of CD4+CD44highCD62Llow and CD8+CD44highCD62Llow effector T cells (Figure 5b, n=5, p=0.041 and p=0.0022 for CD4 and CD8 effectors, respectively), while no effect on Tregs was detected. In studies in which NOD mice were treated for 2 weeks with NOR MSC and in which the proliferative capacity of isolated CD4 and CD8 T cells in response to concanavalin A and anti-CD3/-CD28 stimulation was examined, no differential results were observed in proliferation of CD4 or CD8 T cells isolated from splenocytes of NOR MSC-treated or control mice (data not shown). In light of our in vitro DC data, we analyzed the splenic APC populations in control and NOR MSC-treated mice. CD11c single-positive and CD11chighCD11b+ cells were found to be reduced in frequency in response to treatment with NOR MSC (Figure 5c, n=5, p=0.004 and p=0.015, respectively). Fewer macrophages, identified as F4/80+CD11c-, were also found in NOR MSC-treated mice (Figure 5c, n=5, p=0.015). Conversely, the CD11clowCD11b+ population was increased following NOR MSC therapy (Figure 5c, n=5, p=0.029). The CD11b+ population also showed reduced expression of Ly-6c following treatment with NOR MSC (Figure 5c, n=3-5, p=0.042). Further analysis of the CD11c single-positive DC population revealed a dramatic increase in B220 expression in this subset in response to NOR MSC treatment (Figure 5c, n=5, p=0.0094), and plasmacytoid DC of this phenotype have been shown to promote tolerance and to delay the onset of diabetes (32).

**DISCUSSION**

The incidence of T1D is steadily rising at a global level (33-35). The most common form of intervention seeking to reverse the disease in recently diagnosed patients has been that of immunosuppression through use of agents such as anti-CD3. However, as immunosuppressive regimens are commonly associated with acute morbidity, novel treatments to reduce the burden of immunosuppression are in dire need of development. MSC therapy is one such treatment modality that, due to the considerable immunomodulatory effects of these cells, has shown promising results in treating autoimmune diseases and has the potential to serve as a component of combination therapy to reduce immunosuppressive regimen morbidity (9-12). While MSC are capable of differentiating into a number of mesenchymal cell lineages, hematopoietic stem cells (HSC) are multipotent stem cells that give rise to all cells in the blood have been shown to have immunomodulatory roles as well; indeed, HSC transplantation in patients with newly diagnosed T1D has resulted in improved β cell function (36). Clinical trials examining the effects of MSC therapy have also been initiated for a multitude of disorders, including T1D. However, T1D trials have been initiated with a paucity of preclinical data, which are necessary to determine the type and course of MSC therapy as well as to elucidate the mechanisms by which MSC exert their immunomodulatory effects. We and others have previously demonstrated the benefit of MSC therapy in the specific setting...
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of T1D (13; 14; 37; 38). Importantly, our previous work demonstrated no therapeutic benefit of autologous MSC in vivo using an NOD mouse model (13). Conversely, allogeneic BALB/c MSC treatment was efficient in treatment of T1D, but reversal was short-lived, perhaps due to the eventual rejection of the allogeneic cells. Our previous work also indicated that the most significant preventative effect on experimental T1D occurred with congenic NOR MSC treatment. Given these data, we sought to examine the effect of NOR MSC on reversal of hyperglycemia as well as to elucidate the mechanisms responsible for NOR MSC immunomodulation.

In this report, we first demonstrate that NOR MSC are functionally and lineally mesenchymal as well as confirm their immunomodulatory function in suppressing nonspecific TCR-stimulated proliferation. We next examined their immunosuppressive ability in the specific context of autoreactive T cell proliferation through use of the BDC2.5 autoreactive T cell assay; NOR MSC were shown to potently suppress diabetogenic T cell proliferation and IFN-γ production. Although NOR MSC expressed PD-L1 at low levels at baseline, treatment with recombinant IFN-γ resulted in abundant PD-L1 expression. Similarly, previous reports have demonstrated IFN-γ to be important for MSC-mediated immunosuppression or PD-L1-mediated MSC immunoregulation (6; 39; 40). In our model, IFN-γ production by autoreactive T cells may upregulate PD-L1 expression on MSC and thereby augment their immunomodulatory capability. Indeed, we show that addition of NOR MSC to the BDC2.5 autoreactive assay results in prodigious upregulation of PD-L1 expression on NOR MSC; we postulate that a similar upregulation occurs following in vivo administration of MSC to diabetic mice. With regard to functionality, the immunosuppressive effect of NOR MSC was shown to be mediated in part via PD-L1 through siRNA knockdown of MSC PD-L1 expression and consequent abrogation of the suppressive effect on autoreactive T cell proliferation. Given these results, further exploration of the effects of administering PD-L1-positive MSC and the consequent potential for reducing the MSC number injected to diabetic NOD mice is certainly of merit. We also investigated the role of MSC in regulating the phenotype of DC. Primary diabetic insult is identified by peri-insulitis of the pancreas following DC and macrophage infiltration, and MSC have been shown to both suppress and alter DC differentiation in other models (2; 7). Herein, we show that the presence of MSC reduces CD11c and CD11b expression, decreases inflammatory Ly-6c expression in the CD11b+ population, and suppresses differentiation as shown by increased frequency of lineage-negative and Sca-1+ cells. TNF-α production, a growth factor involved in DC maturation and produced by mature DC, was suppressed in MSC co-cultures, and DC morphology was similarly altered in the presence of MSC. Moreover, we demonstrate that these effects are in large part mediated by IL-6 through the use of a blocking IL-6 antibody and the consequent reversal of this effect, although other factors such as Flt3L and M-CSF may be involved due to incomplete abrogation of all phenotypic alterations.

Our reversal studies in the NOD mouse show a marked incidence in reversal of recent-onset hyperglycemia in 8 out of 9 mice in response to NOR MSC treatment, with prolonged reversal of 83% of mice treated with NOR MSC (i.e. in 5 out of 6 long-term survivors), while control mice reverted to hyperglycemia soon after cessation of insulin replacement. Serum cytokine studies of reversal mice showed changes in the cytokine profile with respect to IL-6, IL-7, IL-10, and IL-12(p40). IL-6 is a pleiotropic cytokine purported to have both anti- and pro-inflammatory roles; in T1D, reports of its
effects are conflicting, but it has been shown to protect β cells from apoptosis and impaired function as well as delay the onset of overt diabetes in NOD mice (41-43). Our in vitro data also demonstrate the central role of IL-6 in NOR MSC suppression of DC generation. IL-7, although important for effector memory T cell survival, has also recently been demonstrated to be necessary for common lymphoid progenitor development, from which plasmacytoid DC arise (44). IL-10 is an established immunoregulatory cytokine, while the homodimer IL-12(p40) inhibits action of the bioactive heterodimer IL-12(p70) (45; 46). Taken together, changes in cytokines appear to skew the inflammatory diabetogenic environment toward a more regulatory profile in response to NOR MSC treatment. Consistent with our in vitro data, we demonstrate altered DC phenotype in response to NOR MSC treatment, manifested by diverting the CD11c+ subset toward a more regulatory cell as well as possibly preventing myeloid APC differentiation, as demonstrated by reduced expression of CD11c and F4/80 following MSC injection in vivo. Interestingly, the CD11c+CD11b+ subset has been shown to prime autoreactive T cells, resulting in physiological β cell death (47), and our treatment, particularly given our in vitro data, appears to retard development of this subset. Moreover, plasmacytoid DC frequency was found to be markedly increased in response to NOR MSC treatment, and DC of this lineage have been shown to prevent acceleration of insulitis in NOD mice, as well as to suppress myeloid DC activation of effector cells (48; 49), which is consistent with the decreased frequency of CD4 and CD8 effectors we observed in response to NOR MSC treatment. As it has also been shown that T1D patients have a reduced pDC compartment (50), increased frequency of pDC following NOR MSC treatment may thus contribute to the reversal of hyperglycemia we observed.

Taken together, NOR MSC treatment resulted in efficient reversal of hyperglycemia, suppressed autoreactive T cell proliferation via PD-L1, and increased production of regulatory cytokines and frequency of plasmacytoid DC. This work is the first to demonstrate the distinct benefit of congenic MSC in reversing hyperglycemia and ameliorating diabetes pathogenesis. Further exploration to optimize and to confirm the safety and efficacy of MSC therapy is the subject of our future studies. These data should serve to shape future T1D clinical trials with regard to optimal MSC source and therapeutic regimen.

Author contributions. M.J. researched data, contributed to discussion, and wrote the manuscript. S.Y. researched data. A.A. researched data and contributed to discussion. J.G. researched data and contributed to discussion. R.M. researched data. J.A. researched data. P.F. contributed to discussion. M.A. reviewed and edited the manuscript. M.H.S. contributed to discussion and reviewed and edited the manuscript. R.A. contributed to discussion and wrote the manuscript.

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REFERENCES
8. Ryan JM, Barry FP, Murphy JM, Mahon BP: Mesenchymal stem cells avoid allogeneic rejection. *J Inflamm (Lond)* 2:8, 2005


43. Kristiansen OP, Mandrup-Poulsen T: Interleukin-6 and diabetes: the good, the bad, or the indifferent? Diabetes 54 Suppl 2:S114-124, 2005


**LEGENDS**

**Figure 1. Characterization of NOR MSC.** (A) Immunohistochemical staining of NOR MSC cultures demonstrates fibroblast cell morphology by H&E staining, substantial expression of the MSC markers CD44 and CD105, moderate expression of CD29, and lack of expression for the hematopoietic stem cell marker CD34. (B) Flow cytometric analysis of NOR MSC P4 cultures (n=5, data shown as mean±SEM) shows abundant expression of the classical MSC markers CD29, CD44, and CD105, while MSC were negative for the hematopoietic lineage-restricted markers CD45 and CD90.2. Sca-1, CD73, and VCAM (CD106) were expressed at substantial levels. (C) NOR MSC were shown to undergo osteogenesis and chondrogenesis following exposure to differentiation factors.

**Figure 2. NOR MSC suppression of diabetogenic autoreactive T cells via PD-L1.** (A) Cytokine studies of NOR MSC cultures revealed considerable levels of IL-6, with M-CSF and Flt3L produced at lesser but substantial levels in comparison to other growth factors (n=4). (B) NOR MSC suppress TCR-stimulated proliferation of NOD CD4+ cells in a dose-dependent manner, in which increasing numbers of IFN-γ-stimulated NOR MSC were added to 1x10^5 NOD CD4+ cells in the presence of 1 µg/ml anti-CD3 and anti-CD28 (n=5, p<0.027 for 1x10^4 MSC, p<0.0001 for 2x10^4 and 4x10^4 MSC), and IFN-γ challenge enhanced the suppressive effect of NOR MSC. (C) 2x10^4 NOR MSC were shown to significantly reduce autoreactive T cell proliferation (n=5, p=0.047), as evaluated by CFSE dilution and calculation of proliferation index when added to a BDC2.5 autoreactive assay containing BDC2.5 CD4+ T cells, NOD DC, and 100 ng/ml BDC2.5 islet peptide. (D) IFN-γ production was similarly suppressed in the presence of 2x10^4 NOR MSC by ELISPOT in the BDC2.5 autoreactive assay (n=5, p=0.0024), while IL-6 levels were enhanced (p=0.0005). (E) Addition of 1, 2, or 4x10^4 NOR splenocytes had no suppressive effect on anti-CD3/CD28 T cell proliferation as compared to addition of identical numbers of NOR MSC (n=4, p<0.0001 for NOR MSC, p=ns for NOR splenocytes). (F) NOR MSC stimulated with 0.05, 0.5, or 5 ng/ml recombinant murine IFN-γ show dose-dependent upregulation of PD-L1 expression by FACS (n=3, p<0.008 for 0.5 and 5 ng/ml), a minor increase in PD-1 (p=ns), and no increase in expression of PD-L2. (G) NOR MSC following co-culture with BDC2.5 CD4+ T cells, NOD DC, and BDC2.5 peptide exhibited marked upregulation of PD-L1 expression at 72h by flow cytometric analysis (n=5, p<0.0001). (H) siRNA knockdown of PD-L1 in MSC abrogated the suppressive effect observed on autoreactive T cell proliferation when 2x10^4 MSC were added (n=6, p=0.0034 for control vs. non-targeting siRNA, p=ns for control vs. PD-L1 siRNA for representative experiment shown), and (I) gene expression analysis of MSC demonstrated efficient suppression of PD-L1 transcripts in response to siRNA treatment (n=4, p=0.016). Experiments were performed between 3 and 6 times and data are displayed with means and SEM.
Figure 3. MSC suppression of DC differentiation. (A) Using an established model of DC generation from NOD bone marrow mononuclear cells, co-culture with NOR MSC was shown to markedly reduce the CD11c+CD11b+ population, so that the predominant cell phenotype was CD11clowCD11b+ (CD11c+CD11b+ cells=40.7±2.6% 22.1±2.4% for control and NOR MSC-treated, respectively, n=5, p=0.0007), while treatment with anti-IL-6 in large part abrogated this effect (CD11c+CD11b+ cells=30.9±4.7%, p=ns in comparison to control [−/−] and MSC alone [+/-]). Analysis of expression of Ly-6c in the CD11b+ fraction demonstrated that co-culture with NOR MSC resulted in a decrease in both the CD11b+Ly-6chigh and CD11b+Ly-6cintr populations (n=4, p=0.0053 and p=0.02, respectively), which was fully abrogated by blockade of IL-6 (p=ns). (B) The population of lineage-negative cells was evaluated in DC culture as a function of progenitor frequency; co-culture with MSC increased the percentage of Lin− cells (n=4, Lin− cells=8.94±0.87% and 13.73±1.08% for control and NOR MSC-treated, respectively, p=0.004), which was in part rescued by addition of anti-IL-6 (p=ns). Similarly, Sca-1 expression within the lineage-negative population was markedly increased in the presence of MSC (n=4, Lin−Sca-1+ cells=6.98±1.27% and 30.53±6% for control and NOR MSC-treated, respectively, p=0.0085). Although treatment with anti-IL-6 resulted in loss of significance of this effect, IL-6 blockade appeared to be incompletely effective in reducing Sca-1 expression in response to MSC. (C) Cytokine analysis of co-cultures of DC and NOR MSC demonstrated marked IL-6 production in the presence of MSC (n=4, p=0.0074) as well as efficient blockade of IL-6 in response to treatment with anti-IL-6. Both Flt3L and M-CSF levels were substantially increased in response to MSC co-culture (p=0.03 and p=0.04, respectively), and IL-6 blockade had no effect on these growth factors (p=0.013 and p=0.018, respectively, in comparison to DC alone). Conversely, TNF-α production was reduced in the presence of MSC (p=0.0056), and anti-IL-6 treatment resulted in abrogation of this effect (p=ns). (D) Giemsa staining of DC culture cytospins demonstrated a lower nuclear:cytoplasmic ratio in response to co-culture with MSC, and IL-6 blockade appeared to in large part abrogate this effect. Experiments were performed between 3 and 5 times, and data are displayed with means and SEM.

Figure 4. NOR MSC therapy induces long-term reversal of recent-onset hyperglycemia. (A) NOD mice were monitored beginning at 10 weeks of age, and on day 2 of hyperglycemia (blood glucose>240 mg/dL), an insulin pellet was inserted subcutaneously for maintenance of normal glycemia during treatment. Mice were randomized to control or NOR MSC-treated groups; for NOR MSC treatment, 1x10⁶ cells were injected i.v. twice per week for 4 weeks, and blood glucose measurements were taken daily. 8 out of 9 NOD mice treated with NOR MSC exhibited reversal of diabetes. 5 out of 6 treated mice followed for 12 weeks maintained their reversal, while controls reverted to hyperglycemia (greater than 600 mg/dL) soon after dissolution of the insulin pellet. (B) Means of cumulative blood glucose measurements demonstrate no difference in level of hyperglycemia at days -1 and 0 (p=ns), while weekly mean measurements beginning at week 2 following the initiation of treatment show significant decreases in blood glucose in response to NOR MSC therapy (p<0.001 for all from week 2 to week 12). Data are displayed with means and SEM.

Figure 5. NOR MSC therapy alters DC phenotype, cytokine profile, and effector cell frequency in vivo. (A) Serum cytokine studies of NOR MSC-treated and control mice were performed at days 0, 7, 14, and 21 following completion of our treatment protocol at 4 weeks (n=3-5 samples). NOR MSC therapy increased circulating levels of IL-6 at days 7 and 14 (p<0.0065), increased IL-7 levels at days 0 and 7 (p<0.00021), increased levels of IL-10 at days 0 and 7 (p<0.0052), and increased levels of IL-12(p40) at days 0 and 7 (p<0.00091). (B) CD4
and CD8 effector cell frequency (identified as CD44\textsuperscript{high}CD62L\textsuperscript{low}) was reduced in the PLN of NOR MSC-treated mice, while no difference was detected in Treg frequency (n=5, p=0.041 and p=0.0022 for CD4 and CD8 effectors, respectively). (C) CD11c single-positive cells and CD11c\textsuperscript{high}CD11b\textsuperscript{+} cells were reduced in response to NOR MSC therapy (n=5, p=0.004 and p=0.015, respectively) while the CD11c\textsuperscript{low}CD11b\textsuperscript{+} population was increased (n=5, 1.12±0.1% and 2.06±0.34%–for control and NOR MSC-treated, respectively, p=0.0294). Analysis of the CD11c single-positive population showed a marked increase in the plasmacytoid DC subset in NOR MSC-treated mice (gated on CD11c\textsuperscript{+}CD11b\textsuperscript{−}, followed by analysis of B220 expression, n=5, 21.9±3.18% and 37.5±3.31% for control and NOR MSC-treated, respectively, p=0.0094). The frequencies of CD11b\textsuperscript{+}Ly-6c\textsuperscript{+} monocytes (n=3-5, p=0.042) and F4/80\textsuperscript{+} macrophages (n=5, p=0.015) were also found to be reduced in response to NOR MSC. All data are displayed with means and SEM.

**Figure 1**
Figure 2

A

B

C

D

E

F

G

H

I

MSC therapy for T1D
Figure 3

A

-IL-6  +anti-IL-6

-MSC

+MSC

B

-anti-IL-6  +anti-IL-6

-MSC

+MSC

C

IL-6 production (pg/ml)

FN6 production (pg/ml)

M-CSF production (pg/ml)

TNF-α production (pg/ml)

D

-anti-IL-6  +anti-IL-6

-MSC

+MSC
MSC therapy for T1D

Figure 4

A

Blood glucose

Weeks

Control
NOR MSC-treated

B

Blood glucose (mg/dL)

Weeks

Control
NOR MSC-treated

Figure 5

A

IL-6

IL-7

IL-10

IL-12(p40)

Day 0
Day 7
Day 14
Day 21

Day 0
Day 7
Day 14
Day 21

Day 0
Day 7
Day 14
Day 21

Day 0
Day 7
Day 14
Day 21

B

% expression

CD4 eff
CD8 eff
Tregs

CD4 eff
CD11c
CD11b
CD45RB220

CD4 eff
CD11c
CD11b
CD45RB220

CD4 eff
CD11c
CD11b
CD45RB220

CD4 eff
CD11c
CD11b
CD45RB220

C

Control
NOR MSC-td
Control
NOR MSC-td

CD11b

CD11b

F4/80

F4/80

Ly-6c
CD11b

Ly-6c
CD11b

pDC

pDC