Ablation of a small subpopulation of diabetes-specific bone marrow-derived cells in mice protects against diabetic neuropathy

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Keywords

Bone marrow-derived cells, Diabetic peripheral neuropathy, Diphtheria toxin, Diphtheria toxin receptor, Dorsal root ganglion, Proinsulin, TNF-α,

Abbreviations

AGE, advanced glycation end-products; BMDCs, bone marrow-derived cells; BMT, bone marrow transplantation; CMAP, compound muscle action potential; Cre, Cre recombinase; DM, diabetes mellitus; DPN, diabetic peripheral neuropathy; DRG, dorsal root ganglion; DT, diphtheria toxin; DTR, diphtheria toxin receptor; iDTR-f, inducible diphtheria toxin receptor-floxed mice; iDTR-f/RIP-Cre, inducible diphtheria toxin receptor-floxed/rat insulin promoter-Cre recombinase double transgenic mice; PI, proinsulin; MNCV, motor nerve conduction velocity; RIP-Cre, rat insulin promoter-Cre recombinase mice; SNAP, sensory nerve action potential; SNCV, sensory nerve conduction velocity; STZ, streptozotocin; WT, wild-type

Highlight
Selective ablation of abnormal bone marrow cells protects against diabetic neuropathy
Abstract

Diabetic peripheral neuropathy (DPN) is a major diabetic complication. We previously showed that hyperglycemia induces the appearance of proinsulin (PI)-producing bone marrow-derived cells (PI-BMDCs), which fuse with dorsal root ganglion neurons, causing apoptosis, nerve dysfunction and DPN.

In this study, we have devised a strategy to ablate PI-BMDCs in mice in vivo. Use of this strategy to selectively ablate TNF-α-producing PI-BMDCs in diabetic mice protected these animals from developing DPN. The findings provide powerful validation for a pathogenic role of PI-BMDCs, and identify PI-BMDCs as an accessible therapeutic target for the treatment and prevention of DPN.
Introduction

Diabetic peripheral neuropathy (DPN) is the most common chronic complication of diabetes mellitus (1; 2; 6; 17; 23; 28). Multiple factors have been implicated in its pathogenesis, which include oxidative stress (16; 24), the production of advanced glycation end-products (AGE) and other ligands for AGE receptors (11; 20; 21), protein kinase C activation (25), stimulation of polyol pathways (12; 26) and inflammatory cytokines (4). About a decade ago, our laboratory observed that hyperglycemia in diabetes induces the appearance of proinsulin-positive bone marrow derived cells (PI-BMDCs) in multiple tissues in diabetes (9). We subsequently reported that the diabetes-specific PI-BMDCs play a key role in the pathogenesis of DPN by directly damaging dorsal root ganglion (DRG) neurons in diabetic rodents (19). Most of these abnormal PI-BMDCs co-express TNF-α; the TNF-α-expressing PI-BMDCs fuse with DRG neurons, leading to their malfunction and premature apoptosis, culminating in DPN (8; 18; 22; 27).
Studies to date strongly suggest that PI-BMDCs are heavily involved in the pathogenesis of DPN. In this investigation, we sought to determine if PI-BMDCs play an essential role in DPN development, and whether this cell type could be a target for therapeutic intervention. Notably, despite their central role in pathogenesis, PI-BMDCs account for only 2-3% of the total BM cells in diabetes (8). To address these hypotheses, we developed an innovative method to ablate this minor BMDC subset in diabetic mice and determined whether the maneuver impacts the development of DPN in the treated animals. The results suggest that PI-BMDCs are readily accessible therapeutic targets for the treatment of DPN.
MATERIALS AND METHODS

Animals

C57BL/6J and C57BL/6-Gt(ROSA)26Sor<sup>tm1(HBEGF)</sup>Awai/J mice (strain number 007900) were purchased from Jackson Laboratories (Bar Harbor, ME, USA).

We produced inducible diphtheria toxin receptor-floxed / rat insulin promoter-Cre recombinase (iDTR-f/RIP-Cre) mice by breeding transgenic iDTR floxed (iDTR-f) mice with RIP-driven Cre (RIP-Cre) mice (3; 14). For bone marrow transplantation (BMT), we irradiated (10 Gy) 8-week-old C57BL/6J female wild type (WT) mice and then injected 4 × 10<sup>6</sup> bone marrow (BM) cells from male iDTR-f and iDTR-f/RIP-Cre mice (iDTR-f→WT and iDTR-f/RIP-Cre→WT). At 4 weeks after BMT, we induced diabetes in mice by intraperitoneal injection of streptozotocin (STZ; 150 mg/kg). On day 7 after STZ injection, we determined the blood glucose and included in our study only diabetic mice that showed glucose levels of > 250 mg/dl. Starting from 1-2 weeks after STZ injection, we administered diphtheria toxin (DT) (Sigma) (25ng/g body weight) by intraperitoneal injections every 3 days for a
period of 10-11 weeks (10). All animals were housed, fed mouse chow ad
libitum, and maintained under a 12-h light-dark cycle. All experiments were
performed according to the guidelines of the Institutional Animal Care and
Use Committee at Baylor College of Medicine.

**Analysis of tissue mRNA and quantitative RT-PCR**

We extracted total RNA using RNeasy Kit (Qiagen, Valencia, CA, USA), and
treated them with DNase I (Life Technologies, Carlsbad, CA, USA) digestion.
After reverse transcription using oligo-dT primer (Life Technologies), we
quantified the levels of *Ins1* mRNA using PerfeCta qPCR SuperMix (Quanta
Biosciences, Gaithersburg, MD, USA) and *Ins2* and *Tnf* mRNA using
PerfeCta SYBR Green SuperMix, Low ROX (Quanta Biosciences) by
real-time PCR. The primer sequences are listed in Table 1. We used
Mx3005P QPCR system (Stratagene, La Jolla, CA, USA), and results were
analysed by MxProQPCR software Ver4.10 (Stratagene), using β-actin as
control.
Immunohistochemical and immunocytochemical analysis

After exsanguination, we fixed the mice in 4% paraformaldehyde by perfusion and isolated the dorsal root ganglia (DRG) at L3–L5. We incubated 10-20 μm-thick frozen sections from fixed tissues with anti-proinsulin (mouse monoclonal; Fitzgerald Industries International, Acton, MA, USA) (guinea pig polyclonal; PROGEN Biotechnik GmbH, Heidelberg, Germany), anti-TNF-α antibody (goat polyclonal; Santa Cruz Biotechnology, Dallas, TX, USA) and anti-cleaved caspase-3 (rabbit polyclonal; Cell Signaling Technology, Danvers, MA, USA). Next we incubated the sections with species-matched fluorescence-labelled second antibodies and with NeuroTrace 435/455 Blue Fluorescent Nissl Stain (Molecular Probes, Eugene, OR, USA), and observed the sections under a fluorescence or light microscope (Zeiss, Thornwood, NY, USA). We counted 350-500 DRG neurons per mouse in at least three sections separated by 50 μm intervals. The number of immunopositive cells was counted in three dimensional pictures.
and was normalised to the total number of neurons.

**TUNEL staining**

We fixed DRG in 4% paraformaldehyde at L3–L5, cut them into 20 μm-thick frozen sections and labelled the apoptotic nuclei using a TUNEL labelling kit (Roche, Mannheim, Germany). Positive and negative controls were generated according to the kit protocol. We examined sections of DRG every 10 serial sections at 100 μm intervals and evaluated 400–600 neurons/mouse in three dimensional pictures; we calculated the prevalence of TUNEL-positive cells in data generated from 5-10 individual mice.

**Motor and sensory nerve conduction measurements**

We measured motor nerve conduction velocity (MNCV), sensory nerve conduction velocity (SNCV), compound muscle action potential (CMAP) and sensory nerve action potential (SNAP) using a Sierra 6200A instrument (Cadwell, Kennewick, WA, USA) in anesthetised mice at 37°C core body
temperature. We used sciatic nerve for motor nerve conduction measurements. For measurement of sensory nerve function, we stimulated sural nerves in the distal site at ankle joint level and recorded in the proximal site.

Statistical analysis

Results are presented as means ± SD. We performed statistical analysis using SPSS Statistics 19 software (IBM SPSS, Chicago, IL, USA) and used Student’s t test to compare two independent groups and one-way ANOVA followed by the multiple comparison test to compare three or more groups. Statistically significant difference is defined as a p value of < 0.05.
RESULTS

We followed the experimental protocol described in Methods (summarized in Figure 1a, b) to determine the effect of selective ablation of PI-BMDCs on DPN in diabetic mice. We bred floxed transgenic inducible diphtheria toxin receptor (iDTR-f) mice into rat insulin promoter (RIP)-driven Cre (RIP-Cre) mice to produce bigenic iDTR-f/RIP-Cre mice. To exclude the pancreatic $\beta$ cells from iDTR expression, we transplanted the BM of the double transgenic mice to WT mice, such that in the recipient mice only BMDCs that expressed iDTR in the presence of hyperglycemia-induced proinsulin would be selectively ablated by DT injections (Fig. 1a).

We induced diabetes by STZ treatment in mice 4 weeks after BMT. One week after STZ-induced diabetes, we administered DT by intraperitoneal injections every 3 days for a period of 10-11 weeks to produce persistent ablation of PI-BMDCs during this time (as described in Methods, Fig. 1b). STZ produced similar degrees of hyperglycemia and similar reductions in body weight in both iDTR-f→WT and iDTR-f/RIP-Cre→WT mice (Fig. 1c, d).
Twelve weeks after STZ-induced diabetes, we measured the peripheral motor and sensory nerve conduction velocities, compound muscle action potential (CMAP) and sensory nerve action potential (SNAP) to assess the effects of PI-BMDC ablation (Fig. 2a, b, c, d). CAMP did not differ between groups, but motor nerve conduction velocity (MNCV), sensory nerve conduction velocity (SNCV), as well as SNAP were significantly decreased in DM compared to the values measured in the same mice immediately before STZ (denoted non-DM in all figures) in the iDTR-f→WT group. In contrast, in the iDTR-f/RIP-Cre→WT group, the MNCV and SNCV were comparable in the DM and non-DM groups, which indicates that ablation of diabetes-induced PI-BMDCs protects against the development of motor and sensory nerve dysfunction that characterises diabetic neuropathy in mice (Fig. 2a, b, d).

To determine the effect of DT-mediated ablation of PI-BMDCs on the DRGs, we performed immunocytochemical staining of tissue sections of DRGs in non-DM and DM mice for the presence of proinsulin and TNF-α in
iDTR-f→WT and iDTR-f/RIP-Cre→WT mice before (non-DM) and after STZ-induced DM (Fig. 3a). Before diabetes induction, none of the mouse DRGs displayed detectable PI or TNF-α immunostaining. In agreement with previous observations (8; 18; 22; 27), we noted a large number of immunoreactive PI-positive and TNF-α-positive cells in the DRGs of the diabetic (DM) iDTR-f→WT control mice. Importantly, we found a significantly reduced number of PI- and TNF-α-positive cells in the DRGs of DM DT-ablated iDTR-f/RIP-Cre→WT mice (Fig. 3a). Quantification of the number of PI-positive and TNF-α-positive cells in the DRGs of DM mice in both the control iDTR-f→WT and iDTR-f/RIP-Cre→WT groups (Fig. 3b) revealed the presence of PI-positive (~13%) and TNF-α-positive (~15%), and double positive (~10%) neurons in DM iDTR-f→WT mice, whereas the numbers of PI-positive, TNF-α-positive and double positive cells were substantially lower in DM the iDTR-f/RIP-Cre→WT vs. iDTR-f→WT mice (Fig.3b, PI-positive, TNF-α-positive and double positive cells: $p<0.01$), consistent with successful ablation of PI-BMDCs in these mice. We next
analysed mRNA expression level of *Ins1*, *Ins2* and *Tnf* mRNA by quantitative RT-PCR in the DRGs of non-DM and DM mice and compared the values in iDTR-f→WT and iDTR-f/RIP-Cre→WT mice (Fig. 3c). The data showed that the level of *Ins2* and *Tnf* mRNA expression in DM DRGs was significantly higher than that in non-DM DRGs in iDTR-f→WT mice. In contrast, however, the mRNA expression level of these mRNAs was not significantly different in the DRGs of non-DM and DM iDTR-f/RIP-Cre→WT mice. Therefore, DT ablation of PI-BMDCs substantially reduced the level of PI-BMDCs (marked biochemically by *Ins1* and *Ins2* expression) in the DRGs of iDTR-f/RIP-Cre→WT mice; ablation also substantially suppressed the level of *Tnf* mRNA expression, a gene product known to occur in PI-BMDCs that had also been shown to play a pathogenic role in DPN (8; 18; 22; 27). We visualized the number of apoptotic cells in the DRGs in the different groups of mice by TUNEL staining and observed TUNEL-positive cells mainly in the DRGs of the DM iDTR-f→WT mice, which occurred with much higher frequency than those in the non-DM iDTR-f→WT mice (Fig. 4a).
Direct quantification showed significantly higher TUNEL-positive cells in the DM vs. non-DM iDTR-f→WT or iDTR-f/RIP-Cre→WT mice (Fig. 4b). In contrast, the number of TUNEL-positive cells in the DM iDTR-f/RIP-Cre→WT mice was significantly lower than that in the DM iDTR-f→WT mice (Fig. 4a, b). We also stained for proinsulin, cleaved caspase-3 and Nissl stain in the DRG in iDTR-f→WT or iDTR-f/RIP-Cre→WT mice (Fig. 4c) and observed a similar pattern of significantly reduced number of cells that were positive for both proinsulin and cleaved caspase 3 co-expression (Fig. 4d). Therefore, DT ablation of PI-BMDCs in DM iDTR-f/RIP-Cre→WT mice significantly reduced the number of apoptotic cells in their DRGs compared to those in control DM iDTR-f→WT mice (Fig. 4d).
PI-BMDCs were first identified about a decade ago as an abnormal cell type induced by hyperglycemia that appeared to play a central role in the pathogenesis of DPN (19), a common diabetic complication. These abnormal BMDCs express PI as well as TNF-α, a proinflammatory cytokine shown to be necessary for DPN development. Furthermore, PI-BMDCs were also found to be fusigenic under both in vitro and in vivo conditions (8; 19). They fuse with DRG neurons, a process whereby they ‘fix’ the TNF-α production to within the confines of the DRG, adversely affecting all neurons in the immediate neighborhood. As a consequence, PI-BMDC-neuronal cell fusion causes premature apoptosis of affected neurons, propagating the nerve dysfunction (8; 19).

We recently showed strong evidence for an essential role of TNF-α in mediating the ill effects of PI-BMDCs (8; 18; 22; 27). The fact that PI-BMDCs are the major source of the TNF-α on PI-BMDC-neuron fusion cells prompted us to develop a strategy to target these abnormal BMDCs. We
took advantage of the diphtheria toxin (DT) ablation strategy (3) by generating transgenic mice with insulin promoter-driven DT receptor (DTR) expression to confer hyperglycemia-dependent DTR expression in these mice. The analogous strategy had previously been used successfully to selectively ablate B cells (5), dendritic cells (13), mast cells (7; 15) and macrophages (10) in mice. As we used the insulin promoter for creating the DTR mice, we had to circumvent the production of DTR by pancreatic β cell by using these mice as BMT donors. Indeed, pilot experiments indicated that DT injections every 3 days in iDTR-f/RIP-Cre→WT BMT recipients using a published protocol (10) was highly effective in selectively ablating the PI-producing subset of BMDCs in STZ-diabetic animals in the absence of obvious toxic complications.

In addition to generating strong evidence for the pathogenic role of PI-BMDCs in DPN in diabetic mice, the selective ablation experiments have also identified the PI-BMDCs as a valid therapeutic target. These cells are undetectable in non-DM animals; they make up only 2-3% of the BMDCs in
diabetic rodents with DPN (8). It is clear that the DT ablation strategy that abrogates DPN development in diabetic mice is not directly applicable to humans who normally express DTR in most cells and not in a PI-BMDC-specific manner, and the use of lethal DT injections as a treatment is totally out of the question. On the other hand, the fact that ablation of PI-BMDCs in diabetic mice did not produce major toxic side effects suggests that we should devote our time and effort towards a full characterization of PI-BMDCs in people. A better knowledge of these abnormal cells in diabetic individuals may uncover molecules that are specific to human PI-BMDCs that can be used to advantage to design low molecular weight compounds or molecules, as well as large molecules such as monoclonal antibodies, which specifically target PI-BMDCs for modulation or destruction. The findings that we have uncovered in this investigation strongly support in-depth investigations into the possibility of an analogous therapeutic approach toward human PI-BMDCs in diabetes.
ACKNOWLEDGMENTS

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DISCLOSURES

The authors declare that there is no duality of interest associated with this manuscript.
Figure legend

Figure 1
Design of iDTR-f/RIP-Cre study, and blood glucose and body weight of animals. (a, b) Scheme of iDTR-f/RIP-Cre study. BM cells of male iDTR-f or iDTR-f/RIP-Cre mice were transplanted into female WT mice. Four weeks later, STZ was injected to induce diabetes. From one week after STZ injection, DT was administrated (25ng/g body weight) intraperitoneally every 3 days for a period of 10-11 weeks. (c, d) Blood glucose and body weight before (non-DM) and 12 weeks after STZ administration (DM) iDTR-f→WT (n=8, n=10) or iDTR-f/RIP-Cre→WT (n=9, n=9) mice. BMT, bone marrow transplantation; DT, Diphtheria Toxin; iDTR-f, inducible diphtheria toxin receptor floxed mice; RIP, rat insulin promotor; Cre, Cre recombinase; WT, wild-type; STZ, streptozotocin; BW, body weight; DM, diabetic; non-DM, non-diabetic. Data are mean ± SD. **p<0.01 for the indicated comparison.

Figure 2
Nerve conduction study in iDTR-f/RIP-Cre study. MNCV (a), SNCV (b), CMAP (c) and SNAP (d) of mouse lower limbs before (non-DM) and 12 weeks after STZ administration (DM) in iDTR-f→WT (n=8, n=10) or iDTR-f/RIP-Cre→WT (n=8, n=9) mice. MNCV, motor nerve conduction velocity; SNCV, sensory nerve conduction velocity; CMAP, compound muscle action potential; SNAP, sensory nerve action potential; iDTR-f, inducible diphtheria toxin receptor floxed mice; RIP, rat insulin promoter; Cre, Cre recombinase; WT, wild-type; DM, diabetic; non-DM, non-diabetic. Data are mean ± SD. *p<0.05 and **p<0.01 for the indicated comparison.

Figure 3
Immunohistochemistry and mRNA expression in DRG tissues in iDTR-f/RIP-Cre study. (a) Immunohistochemistry of proinsulin (green), TNF-α (red) and neuron marker (Nissl stain, blue) in DRG before (non-DM) and 12 weeks after STZ administration (DM) in iDTR-f→WT or iDTR-f/RIP-Cre→WT mice. Arrows indicate proinsulin- and TNF-α-positive cells. Scale bar, 50 μm. (b) Population of proinsulin or TNF-α
immunoreactive cells normalised to the total number of nuclei in DRG before (non-DM) and 12 weeks after STZ administration (DM) in iDTR-f→WT (n=5, n=12) or iDTR-f/RIP-Cre→WT (n=7, n=7) mice. DRG, dorsal root ganglion. Data are mean ± SD. (c) Expression levels of Ins1, Ins2 and Tnf mRNA quantified by real-time PCR in the DRG before (non-DM) and 12 weeks after STZ administration (DM) in iDTR-f→WT (n=3-5, n=5-10) or iDTR-f/RIP-Cre→WT (n=3-8, n=9-13) mice. Results were normalised to β-actin and shown as a ratio against non-DM condition in iDTR-f→WT mice. Total RNA was extracted before and 12 weeks after STZ administration. Data are mean ± SD. *p<0.05 and **p<0.01 for the indicated comparison.  

**Figure 4**

TUNEL and cleaved caspase-3 staining in DRG neurons in iDTR-f/RIP-Cre study. (a) TUNEL (green) and DAPI (blue) staining in DRG before (non-DM) and 12 weeks after STZ administration (DM) in iDTR-f→WT or iDTR-f/RIP-Cre→WT mice. Narrow pictures were transection of x- or y-axis broken line in iDTR-f→WT DM group, and circles showed TUNEL positive nuclei. Scale bar, 10 μm. (b) Population of TUNEL-positive neurons normalised to the total number of nuclei in DRG before (non-DM) and 12 weeks after STZ administration (DM) in iDTR-f→WT (n=5, n=10) or iDTR-f/RIP-Cre→WT (n=6, n=7) mice. (c) Immunohistochemistry of proinsulin (green), cleaved caspase-3 (red) and neuron marker (Nissl stain, blue) in DRG before (non-DM) and 12 weeks after STZ administration (DM) in iDTR-f→WT or iDTR-f/RIP-Cre→WT mice. Scale bar, 50 μm. (d) Population of cleaved caspase-3-positive neurons normalised to the total number of neurons in DRG before (non-DM) and 12 weeks after STZ administration (DM) in iDTR-f→WT (n=5, n=10) or iDTR-f/RIP-Cre→WT (n=5, n=7) mice. Data are mean ± SD. *p<0.05 and **p<0.01 for the indicated comparison.
References


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(a) Schematic diagram illustrating the Cre/loxP recombination system. iDTR 
^flox/flox mouse crossed with RIP-Cre mouse results in the deletion of iDTR in Insulin(+)
cells, leading to cell death.

(b) Timeline of experiments:
- 4 weeks: BMT
- 12 weeks: STZ 150mg/kg i.p.
- iDTR-f → WT
- iDTR-f/RIP-Cre → WT

(c) Blood glucose levels:
- non-DM: iDTR-f→WT, non-DM: iDTR-f/RIP-Cre→WT
- DM: iDTR-f→WT, DM: iDTR-f/RIP-Cre→WT

(d) Body weight:
- non-DM: iDTR-f→WT, non-DM: iDTR-f/RIP-Cre→WT
- DM: iDTR-f→WT, DM: iDTR-f/RIP-Cre→WT

** indicates statistical significance.
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<th>Gene</th>
<th>5’ upstream primer, 3’ downstream primer, hydrolysis probe</th>
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<tr>
<td>Ins1</td>
<td>5’-ACCAGCTATAATCAGAGAC-3, 3’-GATGCTGTTTGACAAAAAG-3, 5’-ATCAGCAAGCAGGTCATTGTTTCAAA-3</td>
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<tr>
<td>Ins2</td>
<td>5’-ATGGCCCTGTGGATGCGCTT-3, 3’-CTAGTTGCAGTAGTTCTCCAGCTGG-3</td>
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<td>Tnf</td>
<td>5’-TGCCCTATGTCTCAGCCTCTTTC-3, 3’-GGAGGCCATTTGGAACCT-3</td>
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<tr>
<td>β-actin</td>
<td>5’-ATGGATGACGATATCCTGCTGC-3, 3’-TCTGTCAGGTCCCGGCA-3</td>
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