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

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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 cytokines (4). About a decade ago, our laboratory observed 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. The use of this strategy to selectively ablate TNFα-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.

Animals. C57BL/6J and C57BL/6-Gt(Rosa)26Sortm1(HBEGF)Awai (strain no. 007900) were purchased from Jackson Laboratories (Bar Harbor, ME). 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-wk-old C57BL/6J female wild-type (WT) mice and then injected 4 × 10^6 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 wk 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 levels of >250 mg/dl. Starting from 1 to 2 wk after STZ injection, we administered diphtheria toxin (DT; 25 ng/g body wt) (Sigma) by intraperitoneal injections every 3 days for a period of 10-11 wk (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 the RNeasy Kit (Qiagen, Valencia, CA) and treated the RNA with DNase I (Life Technologies, Carlsbad, CA) digestion. After reverse transcription using oligo(dT) primer (Life Technologies), we quantified the levels of Ins1 mRNA using PerfeCta quantitative PCR SuperMix (Quanta Biosciences, Gaithersburg, MD) and Ins2 and Tnfα mRNA using PerfeCta SYBR Green SuperMix, Low ROX (Quanta Biosciences), by real-time PCR. The primer sequences are listed in Table 1. We used the Mx3005P QPCR system (Stratagene, La Jolla, CA), and the results were analyzed by MxProQPCR software version 4.10 (Stratagene), using β-actin as control.

Immunohistochemical and immunocytochemical analysis. After exanguination, we fixed the mice in 4% paraformaldehyde by perfusion and isolated the DRG at L3–L5. We incubated 10- to 20-μm-thick frozen sections from fixed tissues with anti-proinsulin (mouse monoclonal; Fitzgerald Industries International, Acton, MA), guinea pig polyclonal (PROGEN Biotechnik, Heidelberg, Germany), anti-TNFα

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antibody (goat polyclonal; Santa Cruz Biotechnology, Dallas, TX), and anti-cleaved caspase-3 (rabbit polyclonal; Cell Signaling Technology, Danvers, MA). Next, we incubated the sections with species-matched fluorescence-labeled second antibodies and with NeuroTrace 435/455 Blue Fluorescent Nissl Stain (Molecular Probes, Eugene, OR) and observed the sections under a fluorescence or light microscope (Zeiss, Thornwood, NY). 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 normalized 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 labeled the apoptotic nuclei using a TUNEL-labeling 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 per mouse in three-dimensional pictures; we calculated the prevalence of TUNEL-positive cells in data generated from five to 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) in anesthetized 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) and used Student’s t-test to compare two inde-
pendent groups and one-way ANOVA, followed by the multiple-
comparison test to compare three or more groups. Statistically signif-
icant difference is defined as a P value of <0.05.

RESULTS

We followed the experimental protocol described in MATERIAls and METHODS (summarized in Fig. 1, A and B) to
determine the effect of selective ablation of PI-BMDCs on
DPN in diabetic mice. We bred iDTR-f mice into RIP-Cre
mice to produce bigenic iDTR-f/RIP-Cre mice. To exclude the
pancreatic β-cells from iDTR expression, we transplanted the
BM of the double-transgenic mice to the WT mice such that in
the recipient mice only BMDCs that expressed iDTR in the
presence of hyperglycemia-induced proinsulin would be selec-
tively ablated by DT injections (Fig. 1 A).

We induced diabetes by STZ treatment in mice 4 wk after
BMT. One week after STZ-induced diabetes, we administered
DT by intraperitoneal injections every 3 days for a period of
10–11 wk to produce persistent ablation of PI-BMDCs during
this time (as described in MATERIAls and METHODS; see Fig. 1 B).
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. 1, C and D).

Twelve weeks after STZ-induced diabetes, we measured the
peripheral motor and sensory nerve conduction velocities,
CMAP, and SNAP to assess the effects of PI-BMDC ablation
(Fig. 2). CAMP did not differ between groups, but MNCV and
SNCV, as well as SNAP, were decreased significantly in DM
compared with the values measured in the same mice imme-
diately before STZ (denoted as 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 characterizes diabetic neu-
ropathy in mice (Fig. 2, A, B, and 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 TNFα in iDTR-f→WT and
iDTR-f/RIP-Cre→WT mice before (non-DM) and after STZ-
induced DM (Fig. 3 A). Before diabetes induction, none of the
mouse DRGs displayed detectable PI or TNFα immunostaining.
In agreement with previous observations (8, 18, 22, 27),
we noted a large number of immunoreactive PI-positive and
TNFα-positive cells in the DRGs of the diabetic (DM) iDTR-
f→WT control mice. Importantly, we found a significantly
reduced number of PI- and TNFα-positive cells in the DRGs of
DM DT-ablated iDTR-f/RIP-Cre→WT mice (Fig. 3 A). Quantifi-
cation of the number of PI-positive and TNFα-positive cells
in the DRGs of DM mice in both the control iDTR-f→WT and
iDTR-f/RIP-Cre→WT groups (Fig. 3 B) revealed the presence of
PI-positive (~13%) and TNFα-positive (~15%) and double-
positive (~10%) neurons in DM iDTR-f→WT mice, whereas the numbers of PI-positive, TNFα-positive, and dou-
ble-positive cells were substantially lower in DM the iDTR-f/
RIP-Cre→WT vs. iDTR-f→WT mice (PI-positive, TNFα-
positive, and double-positive cells, P < 0.01; Fig. 3 B), con-

Fig. 2. Nerve conduction study in the iDTR-f/RIP-
Cre study. Motor nerve conduction velocity
(MNCV; A), sensory nerve conduction velocity
(SNCV; B), compound muscle action potential
(CMAP; C), and sensory nerve action potential
(SNAP; D) of mouse lower limbs before (non-DM)
and 12 wk after STZ administration (DM) in iDTR-
f→WT (n = 8, n = 10) or iDTR-f/RIP-Cre→WT
(n = 8, n = 9) mice. Data are means ± SD. *P <
0.05 and **P < 0.01 for the indicated comparison.
Consistent with successful ablation of PI-BMDCs in these mice. We next analyzed 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 or 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 and 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 numbers of cells that were positive for both proinsulin and cleaved caspase-3 coexpression (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 with those in control DM iDTR-f→WT mice (Fig. 4D).

**DISCUSSION**

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 TNFα, 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 TNFα 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 TNFα in mediating the ill effects of PI-BMDCs (8, 18, 22, 27).

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**Fig. 4.** TUNEL and cleaved caspase-3 staining in DRG neurons in the iDTR-f/RIP-Cre study. A: TUNEL (green) and DAPI (blue) staining in DRG before (non-DM) and 12 wk after STZ administration (DM) in iDTR-f→WT or iDTR-f/RIP-Cre→WT mice. Narrow images show transection of x- or y-axis (dashed line in iDTR-f→WT DM group), and circle shows TUNEL positive nuclei. Scale bar, 10 μm. B: population of TUNEL-positive neurons normalized to the total no. of nuclei in DRG before (non-DM) and 12 wk after STZ administration (DM) in iDTR-f→WT or iDTR-f/RIP-Cre→WT mice. Narrow images show transection of x- or y-axis (dashed line in iDTR-f→WT DM group), and circle shows TUNEL positive nuclei. Scale bar, 10 μm. C: immunohistochemistry of proinsulin (green), cleaved caspase-3 (red), and neuron marker (Nissl stain; blue) in DRG before (non-DM) and 12 wk 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 normalized to the total no. of neurons in DRG before (non-DM) and 12 wk after STZ administration (DM) in iDTR-f→WT or iDTR-f/RIP-Cre→WT mice. Scale bar, 50 μm. Data are means ± SD. *P < 0.05 and **P < 0.01 for the indicated comparison.
The fact that PI-BMDCs are the major source of the TNFα on PI-BMDC neuron fusion cells prompted us to develop a strategy to target these abnormal BMDCs. We took advantage of the 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. Because 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-/-RIP-Cre→WT BMT recipients using a published protocol (10) were 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 toward 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 our 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 diabetest.

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DISCLOSURES

The authors declare that there is no duality of interest associated with this article.

AUTHOR CONTRIBUTIONS


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manuscript. H.U., T.T., H.K., and L.C. interpreted results of experiments.