Placenta-Derived Adherent Stromal Cells Improve Diabetes Mellitus-Associated Left Ventricular Diastolic Performance

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Abstract

Left ventricular (LV) diastolic dysfunction is among others attributed to cardiomyocyte stiffness. Mesenchymal stromal cells (MSC) have cardioprotective properties. We explored whether intravenous (i.v.) application of Placenta-exPanded (PLX) MSC-like cells (PLX) improves LV diastolic relaxation in streptozotocin (STZ)-induced diabetic mice and investigated underlying mechanisms. Diabetes mellitus was induced by STZ application (50 mg/kg body weight) during five subsequent days. One week after the first STZ injection, PLX or saline were i.v. applied. Two weeks later, mice were hemodynamically characterized and sacrificed. At this early stage of diabetic cardiomyopathy with low-grade inflammation and no cardiac fibrosis, PLX reduced LV vascular cell adhesion molecule-1, transforming growth factor-β1, and interferon-γ mRNA expression, induced the percentage of circulating regulatory T cells, and decreased the splenic pro-fibrotic potential in STZ mice. STZ + PLX mice exhibited higher LV vascular endothelial growth factor mRNA expression and arteriole density versus STZ mice. In vitro, hyperglycemic PLX conditioned medium restored the hyperglycemia-impaired tube formation and adhesion capacity of human umbilical vein endothelial cells (HUVEC) via increasing nitric oxide (NO) bioavailability. PLX further induced the diabetes-downregulated activity of the NO downstream protein kinase G, as well as of protein kinase A, in HUVEC versus from STZ mice, which was associated with a raise in phosphorylation of the titin isoforms N2BA and N2B. Concomitantly, the passive force was lower in single isolated cardiomyocytes from STZ + PLX versus from STZ mice, which led to an improvement of LV diastolic relaxation. We conclude that i.v. PLX injection improves diabetes mellitus-associated diastolic performance via decreasing cardiomyocyte stiffness. STEM CELLS TRANSLATIONAL MEDICINE 2017;00:000–000

Introduction

Chronic heart failure in diabetic patients can be caused by a clinical entity referred as “diabetic cardiomyopathy”, which takes place in the absence of hypertension and coronary artery disease, and is among other features characterized by interstitial inflammation [1], interstitial and perivascular fibrosis [2], impaired vascularization [3], endothelial dysfunction [4], and cardiomyocyte stiffness [5]. Several lines of evidence demonstrate that impaired left ventricular (LV) diastolic relaxation represents the earliest preclinical manifestation of diabetic cardiomyopathy and indicate that it can progress to symptomatic heart failure [6, 7]. Due to the impairment in LV diastolic performance, diabetic patients are more prone to heart failure after myocardial infarction [8] and have a poorer prognosis on heart failure development [9, 10]. Impaired LV diastolic performance is attributed to excessive collagen deposition [11, 12] and cardiomyocyte stiffness [13, 14], of which

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Significance Statement

Impaired left ventricular (LV) diastolic relaxation represents the earliest preclinical manifestation of diabetic cardiomyopathy, a cardiac disorder with changes in cardiac structure and function in the absence of coronary artery disease and hypertension. Mesenchymal stromal cells (MSC) are well known for their cardioprotective features. This study demonstrates that intravenous application of placenta-derived MSC-like cells (PLX) improves LV diastolic relaxation and cardiomyocyte stiffness at an early stage of diabetic cardiomyopathy with low-grade inflammation and no cardiac fibrosis through increased titin phosphorylation. These findings offer new insights into the cardioprotective effects of PLX, advancing their potential use for cardiac therapies.
the latter has recently been shown to be sufficient to induce dia-
stolic dysfunction without any involvement of the extracellular
matrix [15]. Evidence shows that endothelial dysfunction [16] and
vascular rarefaction [17] underlie impaired diastolic relaxation and
particularly a systemic, low-grade inflammation contributes to
diastolic LV dysfunction [18] through coronary microvascular
endothelial activation, which alters paracrine signalling to cardio-
myocytes and predisposes them to high diastolic stiffness [19].

Mesenchymal stromal cells (MSC) are an attractive cell type
for cell therapy given their immunomodulatory [20–22], antifibri-
otic [21], proangiogenic [23], endothelial-protective features
[24] and their ability to be used allogenerically [25]. With respect to
diabetes mellitus, MSC have been shown to exert anti-diabetic
effects and to improve experimental diabetic cardiomyopathy [26,
27]. However, the effect of MSC on the onset of diabetic cardio-
myopathy and on its preclinical manifestation—diastolic dysfunc-
tion—HAS not been unraveled so far. Placental-derived MSC-like
cells, termed PLX, are stable adhesive stromal cells isolated from
full-term human placentas, cultured on carriers, and expanded in
a bioreactor [28]. They can be expanded in vitro without pheno-
typic or karyotypic changes [28] and have already been success-
fully used for the treatment of experimental hind limb ischemia
[24] and myocardial infarction [23].

The aims of the present study were to investigate whether
intravenous (i.v.) application of PLX could improve diabetes
mellitus-associated impaired LV diastolic relaxation at the early
onset of diabetic cardiomyopathy and to analyze underlying
mechanisms.

**Materials and Methods**

**PLX Cell Preparation**

The details of the PLX production process have been described
previously [28]. In brief, full-term human donor placentas were
processed at Pluristem Ltd., Haifa (Israel). Adherent cells were first
selected and grown in two-dimensional culture flasks for approxi-
mately 1 month and later expanded in a dedicated bioreactor
previously [23]. In brief, full-term human donor placentas were
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selected and grown in two-dimensional culture flasks for approxi-
mately 1 month and later expanded in a dedicated bioreactor
previously [23].

**PLX Characterization**

PLX cells were thawed, washed, resuspended in FACS buffer
(potassium buffered saline (PBS); Biochrom, Berlin, Germany)
with 1% FBS), and aliquoted for immunostaining (~200,000 cells
per tube). Manufacturer recommended concentrations of fluo-
rescein (FITC)- or phycoerythrin (PE)-conjugated mouse anti-human
monoclonal antibodies (mAbs) (Becton Dickinson, Heidelberg,
Germany) of interest (mesenchymal markers endothelial and leu-
cocyte markers or their isotypes) were added, and cells were incu-
bated at room temperature for 15 minutes. Cells were washed
twice before they were subjected to flow cytometry in a Beckman
Coulter FC-500 system. Representative flow cytometry charts of
PLX are illustrated in Supporting Information Figure 1.

**Animals**

Diabetes mellitus was induced in 8- to 9-week-old male C57BL6/J
mice from the Forschungseinrichtung für Experimentelle Medizin
(FEM; Berlin) by streptozotocin (STZ; Sigma Aldrich, St. Louis, MO)
application (i.p.; 50 mg/kg body weight) during 5 subsequent
days. 10⁵ PLX or saline were randomly i.v. injected 1 week after
the first STZ injection. Nondiabetic controls received PBS instead
of STZ. Fourteen days after PLX application or saline injection,
mice were hemodynamically characterized and subsequently sac-
ificed. After euthanization of mice, LV tissues were excised,
immediately snap frozen in liquid nitrogen and stored at −80°C
for molecular biology and immunohistological analyses. For the
evaluation of immunomodulatory mechanisms, blood was col-
lected and the spleen isolated. To evaluate the engraftment of PLX
after i.v. injection, the left ventricle, spleen, lung, kidney, liver, and
pancreas were collected, snap frozen in liquid nitrogen and stored at
−80°C for molecular biology. The investigation conformed to
the Guide for the Care and Use of Laboratory Animals published
in the European legislation (Directive 2010/63/EU) and was
approved by the local ethics committee (Landesarzt für Gesund-
heit und Soziales, Tierschutz, Berlin).

**Blood Glucose**

Blood glucose levels were measured with a commercial kit (MBL
International, Woburn, MA) according to the manufacturer’s
protocol.

**Assessment of PLX Engraftment**

The engraftment of PLX in the left ventricle, lung, kidney, liver,
spleen, and pancreas after i.v. application was determined according
to the method of McBride et al. [29], slightly modified, as described
previously [30]. In brief, genomic DNA was extracted from frozen
tissues as described previously [31]. A standard curve was generated
using human genomic DNA obtained from HUVEC serially diluted
over a 100,000-fold dilution range, into murine spleen genomic
DNA. Real-time PCR was performed with 800 ng of target DNA, Alu
specific primers and a fluorescent probe [29]. Values are expressed
as percentage of human DNA per 800 ng (phosphate buffered saline
(PBS); Biochrom, Berlin, Germany) of murine tissue.

**Immunohistochemistry**

Immunohistochemistry was carried out on 5 μm-thick cryo-
sections using following antibodies: anti-collagen I (Chemicon,
Limburg/Lahn, Germany), anti-collagen III (Merck Millipore,
Darmstadt, Germany), anti-CD68 (Abcam, Cambridge, U.K.), anti-
CD3 (Santa Cruz Biotechnology, Heidelberg, Germany) anti-CD4
(BD Biosciences, San Jose, CA), and anti-CD8a (BD Biosciences).

Analysis of stained sections was made in a blinded fashion by digi-
tal image analysis on a Leica DM2000 microscope (Leica Microsys-
tems, Wetzlar, Germany) at ×200 magnification. Artery and
arteriole density was measured using immunohistochemistry with
an anti-α-smooth muscle actin (SMA) antibody (1:200, Abcam).

Microscopy was performed on a Leica DM2000 light microscope.
Arteries and arterioles were counted in 10 high power fields (hpf)
per animal at a magnification of ×100. The density is presented
as number of arteries or arterioles per hpf.

**Gene Expression Analysis**

RNA was isolated using the RNasea Mini Kit according to the
manufacturer’s protocol (Qiagen GmbH, Hilden, Germany), followed
by cDNA synthesis. To assess the mRNA expression of the target genes
vascular cell adhesion molecule (VCAM)-1, transforming growth
factor (TGF)-β1, interferon (IFN)-γ, vascular endothelial growth
factor (VEGF), and L32, real-time polymerase chain reaction (PCR)
(Eppendorf Mastercycler epgradient replex, Hamburg, Germany)
was performed using gene expression assays for VCAM-1: Mm01320970_m1, TGF-β: Mm00441724_m1, interferon-γ (IFN-γ): Mm00801778_m1, and VEGF: Mm01281447_m1 from Applied Biosystems. mRNA expression was normalized to the housekeeping gene L32 and relatively expressed with the control group set as 1.

**Cell Culture**

PLX were cultured at a density of 6,000 cells per cm² in DMEM medium containing 10% fetal bovine serum (FBS) (Biochrom, Berlin, Germany). Human cardiac fibroblasts (Cell Applications, Inc. San Diego, CA) were propagated and cultured in Lung/Cardiac Fibroblasts Basal Medium (Cell Applications, Inc. San Diego CA) plus supplements (Cell Applications). Cardiac fibroblasts at passage 4 or 5 were plated at a cell density of 10,000 cells per 96-well or 135,000 cells per 6-well in Lung/Cardiac Fibroblasts Basal Medium (Cell Applications) plus supplements (Cell Applications). Twenty-four hours later, cells were washed, and cells were cultured in the presence of standard or conditioned PLX medium with or without 10 ng/ml of transforming growth factor-β1 (TGF-β1) (R&D Systems) for 24 hours for crystal violet, Sirius Red staining, or α-SMA flow cytometry.

Human umbilical vein endothelial cells (HUVEC) were propagated and cultured in endothelial basal medium (EBM)-2 basal medium (Lonza Walkersville, Walkersville, MD) with endothelial cell growth medium (EGM)-2 supplements (Lonza). HUVEC at passage 4 or 5 were plated at a cell density of 125,000 cells per 6-well (adhesion assay), 150,000 cells per 6-well (flow cytometry, Western blot) or 10,000 cells per 96-well (caspase 3/7 activity assay) in EBM-2 Medium (Lonza) plus supplements (Lonza). Twenty-four hours later, cells were washed and cultured in the presence of standard or conditioned normo- or hyperglycemic PLX medium for 24 hours for DAF-FM (NO) or CMH2DCFDA (reactive oxygen species; ROS) flow cytometry, Western blot, or collected for adhesion assay.

**Preparation of PLX-Conditioned Medium**

PLX were plated at a density of 150,000 cells per 6-well in dulbecco’s modified eagle’s medium (DMEM) medium containing 10% FBS. After 24 hours, the medium was removed, cells were washed with PBS, and 1 ml of normo- or hyperglycemic EBM-2 medium or supplements (PLX-conditioned medium for endothelial cells) or 1 ml of standard PLX medium with or without 10 ng/ml of TGF-β1 (PLX-conditioned medium for cardiac fibroblasts) was added on the cells. In parallel, normo- or hyperglycemic EBM-2 medium or standard PLX medium with or without 10 ng/ml of TGF-β1 was added to wells without cells. These media served as respective controls. Twenty-four hours later, the medium was collected, centrifuged to discard cellular debris, and stored at −80°C until use.

**Coculture of Fibroblasts with Splenocytes**

Murine C4 fibroblasts were plated at a cell density of 10,000 cells per 96-well in Basal Iscove medium supplemented with 10% FBS and 1% penicillin/streptomycin. Twenty-four hours after plating, medium was removed and splenocytes isolated from control + PBS, control + PLX, STZ + PBS, and STZ + PLX mice, according to Van Linthout et al. [20], were added to the fibroblasts at a ratio of 10 splenic cells to 1 fibroblast in RPMI medium 10% FBS, 1% penicillin/ streptomycin, as described previously [21]. After 3 days, splenocytes were removed and Sirius Red staining was performed.

**Sirius Red Staining**

Sirius Red staining of fibroblasts was performed as described previously [32]. In brief, fibroblasts were fixed in methanol overnight at −20°C, washed once with PBS and incubated in 0.1% Direct Red 80 (Sirius Red; Sigma Aldrich) staining solution at room temperature for 1 hour. After a second wash with PBS, the Sirius red staining of the fibroblasts was eluted in 0.1N sodium hydroxide at room temperature for 1 hour on a rocking platform. The optical density representative for the accumulation of collagen I and III was measured at 540 nm on a SpectraMax 340PC microplate reader (Molecular Device GmbH, Biberach an der Riß, Germany).

**Western Blot**

Twenty-four hours after culture in standard or conditioned normo- or hyperglycemic PLX medium, HUVEC were lysed in lysis buffer (Thermo Fisher Scientific, Waltham, MA) supplemented with proteinase inhibitors (Roche). An equal amount of protein (40 µg) was loaded into 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels. Total endothelial nitric oxide synthase ([Enos] BD Biosciences) and β tubulin (Santa Cruz Biotechnology) were detected with each specific antibody, followed by incubation with an IR dye secondary antibody (LI-COR Biosciences, Lincoln, NE). The blots were visualized with Odyssey (LI-COR Biosciences). Quantitative analysis of the intensity of the bands was performed with Odyssey V3.0 software, as described previously [33, 34].

**Caspase 3/7 Activity Assay**

Twenty-four hours after culture in 100 µl of standard or conditioned normo- or hyperglycemic PLX medium, caspase 3/7 activity was determined with a Caspase Glo 3/7 activity kit (Promega, Madison, WI) according to the manufacturer’s protocol. Luminescence was measured in a microplate-reading luminometer (Mithras LB 940, Berthold Technologies GmbH & Co KG, Germany) as described previously [33].

**Adhesion Assay**

HUVEC, 24 hours cultured in standard or conditioned normo- or hyperglycemic PLX medium, were plated at a cell density of 40,000 cells/96-well in PBS for 2 hours on 96-well plates pre-coated with 2.5 µg/ml fibronectin (Sigma Aldrich). Medium and cells were removed and wells were next twice washed with warm PBS, followed by fixation with paraformaldehyde and crystal violet staining. The absorbance was next measured at 495 nm on a SpectraMax 340PC microplate reader (Molecular Device GmbH).

**Tube Formation**

HUVEC (20,000 cells per well) were resuspended in normo- or hyperglycemic EBM-2 medium, or conditioned full normo- or hyperglycemic PLX medium (see preparation of conditioned medium) and plated on top of a matrigel (BD Biosciences). Twenty-four hours after seeding, pictures of eight fields per condition were taken at magnification ×5 and send to Wimasis for analysis via WimTube software (Wimasis GmbH, München, Germany).

**Flow Cytometry**

Flow cytometry analysis of blood mononuclear cells (MNCs) isolated with Histopaque 1083 (Sigma Aldrich) and cardiac fibroblasts was performed using directly conjugated monoclonal antibodies. The α-SMA antibody was purchased from BD Biosciences (Franklin Lakes, NJ). The mouse Treg detection kit containing CD4, CD25,
FoxP3 antibodies, fixation, and wash solutions was purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Surface staining was performed according to the manufacturer’s instructions. MNCs were first stained with anti-mouse CD4 and CD25 antibodies, and after fixation, intracellular FOXP3 staining was performed. MNC samples were acquired on a MACSQuant Analyzer (Miltenyi Biotec), and cardiac fibroblast samples on fluorescence-activated cell sorting (FACS) BD FACSCanto II with BD FACS Diva software version 6.1.3. For the analysis of NO and ROS in HUVEC, cells were stained with 5 μM of DAF-FM Diacetate (4-amino-5-methylamino-2’,7’-diluorofluorescein diacetate, Thermo Fisher Scientific) or of CM-H2DCFDA (5-(and-6)-chloromethyl-2’,7’-dichlorodihydrofluorescein diacetate, acetyl ester) (Thermo Fisher Scientific) in PBS, respectively, and incubated for 30 minutes at 37°C, as described previously [20]. Next, cells were washed with 200 μl of PBS and after resuspending in fresh PBS, incubated for an additional 30 minutes at 37°C. Flow cytometry analysis was performed on a MACSQuant Analyzer (Miltenyi Biotec). Analysis of flow cytometry data was performed using FlowJo software version 8.8.6. (Tree Star Inc.).

Titin Isoform Separation

Titin isoforms were separated as described previously [13]. Tissue samples were solubilized in 50 mM Tris SDS buffer (pH 6.8) containing 8 μg/ml leupeptin (Peptin Institute, Japan) and phosphatase inhibitor cocktail (PIC [P2880], 10 μl/ml; Sigma Aldrich). Samples were heated for 3 minutes at 96°C and centrifuged. Then, samples (20 μg) were separated on agarose-strengthened 1.8% sodium dodecyl sulfate-polyacrylamide gels. The gel was run at 5 mA constant current for 16 hours.

All-Titin Phosphorylation by Pro-Q Diamond Stain

The phosphorylation state of cardiac titin isoforms, N2BA and N2B, was determined using Pro-Q Diamond phosphoprotein stain (Thermo Fisher Scientific). To preserve the endogenous phosphorylation state of the proteins, frozen tissues from LV mouse hearts were solubilized and treated as described above in the Titin Isoform Separation section. The gels were stained for 1 hour with Pro-Q Diamond phosphoprotein stain. Fixation, washing, and destaining were performed according to the manufacturer’s guidelines (Thermo Fisher Scientific). To assess total protein content, gels were stained overnight with SYPRO Ruby (Thermo Fisher Scientific). To preserve the endogenous phosphorylation state, frozen tissues were homogenized in sample buffer containing 15% glycerol, 62.5 mM Tris-HCl (pH 7.4), 20 mM magnesium acetate, 0.2 mM EGTA, 0.05% Triton X-100, and protease inhibitor cocktail (Sigma Aldrich) and centrifuged for 5 minutes. Supernatants containing equal amounts of total protein were analyzed for titin in control samples is 100%.

Myocardial Protein Kinase G Activity

Myocardial protein kinase G (PKG) activity was assessed in homogenized tissue samples. Tissues samples were homogenized in 25 mM Tris (pH 7.4), 1 mM EDTA, 2 mM EGTA, 5 mM dithiothreitol (DTT), 0.05% Triton X-100, and protease inhibitor cocktail (Sigma Aldrich) and centrifuged for 5 minutes. Supernatants containing equal amounts of total protein were analyzed for PKG activity. The reaction mixture contained (at final concentration): 40 mM Tris-Cl (pH 7.4), 20 mM magnesium acetate, 0.2 mM EGTA [32P] ATP (500–1,000 c.p.m. pmol-1) (GE Healthcare LifeScience, Little Chalfont, U.K.), 113 mg/ml heptapeptide (RKRSRAE), 3 mM/ml cGMP (Promega, Madison, WI) and a highly specific inhibitor of cAMP-dependent protein kinase (5–24, Merck Millipore). The reaction mixtures were incubated at 30°C for 10 minutes, followed by termination of the reaction by spotting 70 μl of the reaction mix onto Whatman P-81 filters, which were then soaked with 75-mmol/l H3PO4 for 5 minutes and washed three times with 75-mmol/l H3PO4 to remove any unbound [32P] ATP. Filters were rinsed with 100% ethanol and air dried before quantification. For quantification of PKG activity, counts were taken in a Wallac 1409 Liquid Scintillation Counter using universal scintillation cocktail. Specific activity of PKG was expressed as pmol of [32P] incorporated into the substrate (pmol/minute/mg protein).

Ca2+ Calmodulin Dependent Protein Kinase II Activity

Ca2+ calmodulin dependent protein kinase II (CaMkII) activity was determined using a CycLex CaMkII assay kit (CY-1173; MBL Corporation, MA) according to the manufacturer’s guidelines. Briefly, frozen tissues were homogenized in sample buffer containing 15% glycerol, 62.5 mM Tris; pH 6.8; 1% (wt/vol) SDS, protease inhibitor, and protein phosphatase inhibitor, all prepared in distilled H2O. Homogenates were centrifuged at 10,000g for 15 minutes at 4°C. The supernatant was removed and stored at −80°C. Protein samples were loaded onto microtiter wells (concentration, ~2.0 μg per well) coated with CaMkII specific substrate, syntide-2, along with kinase reaction buffer with or without Ca2+ /calmodulin. To quantify CaMkII activity, a standard curve correlating the amount of active CaMkII and the level of phosphorylation of syntide-2 was constructed.

Passive Force

Force measurements were performed on single skinned cardiomyocytes as described [35, 36]. Briefly, samples were defrozen in relaxing solution (in mmol/l: free Mg, 1; KCl, 100; EGTA, 2; Mg-ATP, 4; imidazole, 10; pH 7.0), mechanically disrupted and incubated for 5 minutes in relaxing solution supplemented with 0.5% Triton X-100. The cell suspension was washed 5 times in relaxing solution. Single cardiomyocytes were selected under an inverted microscope (Zeiss Axiovert 135, ×40 objective, Oberkochen, Germany) and attached with silicone adhesive a force
transducer and a piezoelectric motor as part of a “Permeabilized Myocyte Test System” (1600A; with force transducer 403A; Aurora Scientific, Aurora, Ontario, Canada). Cardiomyocyte Fpassive was measured in relaxing buffer at room temperature within a sarcomere-length range between 1.9 and 2.4 μm. Force values were normalized to myocyte cross-sectional area calculated from the diameter of the cells, assuming a circular shape. As a test of cell viability, each cardiomyocyte was also transferred from relaxing to maximally activating solution (pCa4.5), at which isometric force developed. Once a steady state force was reached, the cell was shortened within 1 ms to 80% of its original length to determine baseline force. Only cells developing active forces >20 kN/m² were included in the analysis. The passive tension values shown were measured at the end of each stretch step and thus consisted of both the viscous and the elastic force component.

**Hemodynamic Measurements**

Fourteen days after i.v. PLX injection, mice were anesthetized (0.8–1.2 g/kg urethane and 0.05 mg/kg buprenorphine i.p.), intubated, and artificially ventilated with a rodent ventilator type 7025 (Ugo Basile, Comerio VA, Italy). A 1.2F micro-tip catheter (Sisence Inc., Ontario, Canada) was positioned in the LV through the apex in an open chest model. Heart rate and LV contractility, measured by the peak rate of LV pressure rise dp/dtmax, were measured. Diastolic performance was measured by the peak rate of LV pressure decrease dp/dtmin, and the time constant of LV relaxation Tau (τ).

**Statistical Analysis**

Statistical analysis was performed using Prism 7 for Mac OS X (GraphPad Software, Inc., La Jolla, CA). Ordinary one-way analysis of variance (ANOVA) was used for statistical analysis of the data with correction for multiple comparisons via the Tukey test or as stated elsewhere. Data are presented as mean ± SEM. Differences were considered to be significant when the two-sided p value was lower than .05.

## RESULTS

**Engraftment of PLX After Intravenous Injection**

We evaluated the presence of PLX in the left ventricle, lung, kidney, liver, spleen, and pancreas at the day of sacrifice, 14 days after i.v PLX application in control and STZ-induced diabetic mice via the detection of human Alu sequences (Fig. 1). PLX were retrieved in all analyzed tissues. Interestingly, STZ-induced diabetes mellitus raised the engraftment of PLX to the left ventricle, lung, kidney, pancreas, and tendentiously to the spleen as obviated by 4.5-fold (p < .005), 19-fold (p < .005), 4.7-fold (p < .005), 47-fold (p < .005), and 2.4-fold (p = .1694) higher PLX presence in respective tissues of diabetic compared with control mice.

**PLX Exert Anti-Inflammatory/Immunomodulatory Effects in Streptozotocin-Induced Diabetic Mice**

Given the immunomodulatory properties of MSC [37] and the importance of cardiac inflammation in diabetic cardiomyopathy [38], we next evaluated whether i.v. PLX application decreased cardiac inflammation in STZ-induced diabetic mice. In this STZ diabetic model of low-grade inflammation with no increased cardiac presence of CD68 macrophages, CD3+, CD4+, and CD8+ T lymphocytes (Supporting Information Fig. 2), PLX application downregulated the 1.8-fold (p < .0001) STZ-induced increase in LV VCAM-1 expression to levels not significantly different from controls (Fig. 2A) and led to a 1.8-fold (p < .05) and 3.1-fold (p < .05) decrease in STZ-induced transforming growth factor-β1 (TGF-β1) and IFN-γ mRNA expression (Fig. 2B, 2C), respectively. Systemically, PLX induced the percentage of circulating regulatory T cells (CD4 + CD25 + FoxP3+ cells) by 1.6-fold in STZ mice (Fig. 2D). PLX application did not reduce blood glucose levels (control: 7.5 ± 0.85; STZ: 24 ± 3.0; control PLX: 4.6 ± 0.72; STZ PLX: 24 ± 2.9; p < .0001 STZ vs. control).

**PLX Exert Antifibrotic Effects**

Given the relevance of cardiac fibrosis in diabetic cardiomyopathy [1] and the antifibrotic potential of MSC [21], we further evaluated whether PLX also exert antifibrotic effects. Therefore, we first examined in vitro whether PLX decrease collagen deposition and the expression of the myofibroblast marker α-SMA in cardiac fibroblasts in a paracrine manner. Addition of the pro-fibrotic factor TGF-β1 to cardiac fibroblasts induced collagen deposition and α-SMA expression by 1.2-fold (p < .001) and 1.5-fold (p < .001), respectively. In contrast, collagen deposition and α-SMA expression was 1.7-fold (p < .0001) and 2.3-fold (p < .0001) lower in cardiac fibroblasts supplemented with PLX + TGF-β1 conditioned medium compared with TGF-β1 medium (Fig. 3A, 3B), respectively. Given the link between inflammation and fibrosis [39] and the relevance of the cardioplegic axis, that is, the homing of immune cells from the spleen toward the heart and their subsequent involvement in cardiac remodeling [40], we next evaluated whether the PLX-mediated immunomodulatory effect could influence fibroblast collagen production. Therefore, splenocytes of all in vivo experimental groups were cocultured with murine fibroblasts and their impact on collagen deposition of murine fibroblasts was determined (Fig. 3C). Supplementation of splenocytes from control mice did not induce collagen production compared with monocultured fibroblasts. In contrast, addition of STZ splenocytes to fibroblasts raised collagen production by 16% (p < .001) versus monocultured fibroblasts. STZ + PLX splenocytes did not induce collagen production compared with monocultured fibroblasts or fibroblasts cocultured with control splenocytes (Fig. 3D).

Finally, we evaluated the impact of i.v. PLX application on cardiac fibrosis in STZ mice via immunohistochemistry. Concomitant with
the low cardiac inflammation, cardiac fibrosis was not prominent at this stage of diabetic cardiomyopathy, as indicated by no STZ-induced accumulation of collagen I and III (Supporting Information Fig. 3).

PLX Improve Vascularization in Streptozotocin-Induced Diabetic Mice

Endothelial inflammation and impaired vascularization underlie diabetic dysfunction [12, 41]. On the other hand, PLX have endothelial-protective and proangiogenic properties [23, 24]. Therefore, we next explored the endothelial-protective and proangiogenic potential of PLX in STZ-induced diabetic mice and under hyperglycemic conditions. In STZ mice, LV mRNA expression of VEGF was 2.1-fold ($p < .001$) lower versus nondiabetic control mice, whereas STZ + PLX mice exhibited 2.2-fold ($p < .05$) higher LV VEGF mRNA expression compared with STZ mice (Fig. 4A). In parallel, LV arteriole density was 1.4-fold ($p < .0001$) lower in STZ versus control mice and 1.2-fold ($p < .05$) higher in the STZ mice receiving PLX compared with untreated STZ mice (Fig. 4B, 4C). Artery density was comparable in STZ versus control mice (Fig. 4B–4D). In vitro, hyperglycemia resulted in a 1.6-fold ($p < .01$) increase in eNOS expression, which was not altered in HUVEC cultured with conditioned hyperglycemic PLX medium (Fig. 4E). The hyperglycemia-induced eNOS expression did not affect NO production in HUVEC (Fig. 4F), but led to a 1.2-fold ($p < .05$) increase in ROS production compared with control values (Fig. 4G). This increase in oxidative stress was not associated with prominent endothelial apoptosis (Fig. 4H), but reflected in a 1.1-fold ($p < .05$) lower caspase 3/7 activity (Fig. 4H), a 1.2-fold ($p < .0001$) decreased adhesion capacity of HUVEC (Fig. 4I) and impaired tube formation (Fig. 4J–4M) as shown by 1.9-fold ($p < .05$), 1.4-fold ($p < .01$), and 1.3-fold ($p < .05$) lower branching points, total tubes, and tube length, respectively. PLX abrogated the hyperglycemia-triggered ROS in a paracrine manner by 1.2-fold ($p < .0001$), which was paralleled by a 1.2-fold ($p = 0.13$) lower caspase 3/7 activity (Fig. 4H), a 1.2-fold ($p < .001$) higher adhesion capacity (Fig. 4I) and improved tube formation (Fig. 4J–4M) as shown by 1.9-fold ($p < .05$), 1.4-fold ($p < .01$), and 1.3-fold ($p < .05$) higher branching points, total tubes, and tube length in HUVEC cultured in hyperglycemic PLX condition medium compared with hyperglycemic medium alone, respectively.

PLX Decrease Cardiomyocyte Stiffness and Improve Diastolic Relaxation in Streptozotocin-Induced Diabetic Mice

Given the importance of cardiac titin dysregulation and cardiomyocyte stiffness in diastolic dysfunction [13], the impact of PLX on cardiac titin phosphorylation and cardiomyocyte passive tension was investigated. PLX elevated the STZ-impaired phosphorylation state of titin isoforms N2BA and N2B by 3.3-fold ($p < .0005$) and 6.1-fold ($p < .0001$), respectively, to values not different from controls (Fig. 5A–5C). Concomitantly, PLX restored the diabetes-downregulated PKA and PKG activity by 1.2-fold ($p < .05$) and 1.8-fold ($p < .05$) to control values (Fig. 5D, 5E), respectively, and tendentially increased the diabetes-downregulated CaMKII activity (Fig. 5F). Protein kinase C activity was not different among the groups (Fig. 5G). In parallel, passive force in single isolated cardiomyocytes from STZ + PLX mice was comparable to control mice and was lower than diabetes-induced passive force of cardiomyocytes from STZ mice (Fig. 5H). In vivo, STZ + PLX mice showed an improved diastolic function compared with STZ diabetic mice as indicated by the heart-rate independent 1.2-fold ($p < .005$) lower time constant of LV relaxation parameter Tau and the 1.2-fold ($p < .05$) increase of the relaxation parameter $dp/dt_{max}$ (Fig. 5I, 5J). As known for the early time point in the STZ animal model [42], LV contractility was slightly reduced in both STZ-groups versus controls (STZ: 15% and STZ + PLX: 13% versus controls; $p = NS$), but did not differ significantly between the four groups.

**DISCUSSION**

The salient finding of the present study is that i.v. PLX application reduces cardiomyocyte stiffness and improves LV diastolic relaxation in an early phase of STZ-induced diabetic mice. Endothelial dysfunction [16], impaired vascularization [3], microvascular inflammation [43], cardiac fibrosis [11, 12], and cardiomyocyte stiffness [15] all underlie impaired LV diastolic relaxation, one of the first clinical signs of diabetic cardiomyopathy. In this model of low-grade STZ-induced diabetes mellitus, i.e. PLX application...
improved LV diastolic relaxation and cardiomyocyte stiffness without affecting glucose levels. PLX reduced the diabetes-associated low-grade inflammation as indicated by the decrease in diabetes-induced LV TGF-β1 and IFN-γ expression, and the increase of blood regulatory T cells, which have on their own vascular-protective [44, 45] and antifibrotic features [46]. Cardiac fibrosis was not yet pronounced in this STZ-induced diabetes mellitus model. Nevertheless, splenocytes of STZ mice displayed already a higher pro-fibrotic potential compared with splenocytes of control mice, which follows from their higher capacity to induce collagen deposition in murine fibroblasts upon coculture relative to control splenocytes, an effect, which could previously also be observed with splenocytes from ob/ob versus control mice [47]. The pro-fibrotic splenocyte activity of STZ mice was abolished in STZ mice receiving PLX, indicating the immunomodulatory and antifibrotic potential of PLX under diabetic conditions. These findings are supported by observations in myocarditis mice, where splenocytes from Coxackievirus B3-infected mice injected with MSC induced less collagen production compared with splenocytes from Coxackievirus B3 mice [21], be it now in a diabetic setting. Given the importance of the cardiopleamic axis [40], these findings foresee that i.v. PLX application may counteract the cardiac fibrogenesis process during the pathogenesis of diabetic cardiomyopathy in this systemic manner, that is, independently from their, as also shown in vitro, direct paracrine antifibrotic potential.

In agreement with previously demonstrated endothelial-protective and proangiogenic features of PLX in experimental models of hind limb ischemia [24] and myocardial infarction [23], PLX further decreased the LV VCAM-1 expression and reinitiated the diabetes-downregulated LV VEGF expression and arteriole density in STZ mice. In vitro experiments with hyperglycemic PLX conditioned medium support that PLX restore the hyperglycemia-impaired vascularization in a paracrine manner as indicated by the increased tube formation (total branching points, tubules and tube length) of HUVEC cultured in hyperglycemic PLX conditioned medium versus hyperglycemic medium. In parallel, hyperglycemic PLX conditioned medium raised the adhesion potential of endothelial cells. These effects were found in the presence of a decrease in hyperglycemia-induced ROS levels and unaltered NO levels compared with hyperglycemic conditions, indicative for an improved NO bioavailability [33, 48]. In agreement with the potential of PLX to increase NO bioavailability, PLX administration in STZ mice raised the low activity of the NO-downstream target PKG in STZ mice to levels of control mice. Low PKG activity increases resting tension due to hypophosphorylation of titin [12, 13, 49]. In addition, low PKA [50] and low CaMKII [36] activities are both associated with hypophosphorylation of titin and increased cardiomyocyte stiffness. In contrast, increased PKC activity and subsequent PKC phosphorylation of titin’s PEVK element leads to myocardial stiffness [51]. STZ mice displayed reduced cardiac PKA and CaMKII activity compared with control mice, whereas PLX recovered the diabetes-downregulated PKA activity and tendentially raised CaMKII activity in STZ mice. PKC activity was not different among the groups. In parallel to the restored PKG and PKA activity, the hypophosphorylation of the titin isoforms N2B and N2BA found in STZ mice was repaired in STZ PLX mice and normalized to controls. Consequently, STZ PLX mice exhibited lower cardiomyocyte stiffness compared with STZ mice, which was further translated into ameliorated diastolic relaxation as shown by an improvement in Tau and the relaxation parameter dp/dt max. Our findings are consistent with the postulated paradigm that comorbidities including diabetes mellitus induce a systemic pro-inflammatory state, which triggers endothelial inflammation, which in turn reduces NO bioavailability and PKG activity in adjacent cardiomyocytes leading to cardiomyocyte stiffness [41] and/or provoke cardiac fibrosis. In obese, diabetic ZSF1 rats, diastolic dysfunction is present due to cardiomyocyte stiffness in the absence of cardiac fibrosis [13]. Similarly, at this early stage of diabetes in non-obese ZSF1/DI rats, negatively charged fibronectin and collagens may contribute to the stiffness of the cardiac tissue, which is reversed by PLX treatment.

Figure 3. PLX exert antifibrotic effects in vitro. PLX conditioned medium reduces TGF-β1-induced collagen deposition and α-SMA expression in cardiac fibroblasts. Bar graphs represent the mean ± SEM of (A) the absorbance at 540 nm detected after Sirius Red staining, depicting collagen deposition, and (B) α-SMA expression of cardiac fibroblasts cultured in PLX medium, PLX medium + TGF-β1, PLX conditioned (cond.) medium, or PLX cond. medium + TGF-β1 as indicated, with open bars representing without TGF-β1 and black bars with TGF-β1 conditions, and n = 6/group. Splenocytes derived from STZ PLX mice induce lower collagen deposition in murine fibroblasts upon coculture compared with splenocytes from STZ mice. (C): Schematic representation of the experimental set-up. (D): Bar graph represents the mean ± SEM of the absorbance at 540 nm detected after Sirius Red staining of fibroblasts cultured in monoculture or cocultured with splenocytes from control, STZ, control PLX, or STZ PLX mice as indicated, with n = 5–6/group. For all data: *, p < .05; **, p < .01; ***, p < .001; ****, p < .0001. Abbreviations: PLX, Placenta-eXpanded cell; STZ, streptozotocin; TGF-β1, transforming growth factor-β1.
stage of STZ-induced diabetic cardiomyopathy without signs of cardiac fibrosis, cardiomyocyte stiffness underlied the diastolic stiffness, which could be improved via i.v. PLX application. Conform to the capacity of MSC to home to damaged tissue upon i.v. application [21], cardiac engraftment of PLX was higher in STZ compared with control mice. In contrast to previous reports with MSC [52], no anti-diabetic effect of PLX could be observed in the STZ mice. We suggest that the cardioprotective outcome of PLX in STZ mice are a consequence of their paracrine endothelial-protective and proangiogenic effects upon cardiac engraftment.

**Figure 4.** PLX improve hyperglycemia-impaired vascularization and endothelial function. (A): Bar graphs represent the mean ± SEM of LV VEGF mRNA expression in control (open bars) and STZ (closed bars) mice injected with PBS or PLX, as indicated with n = 7/control and n = 6/STZ and PLX groups. (B): Representative pictures at magnification ×100 of arteries and arterioles in the LV of control, STZ, control + PLX, and STZ + PLX mice as indicated. Bar graphs represent the mean ± SEM of LV (C) arteriole and (D) artery density per high power field (hpf) in control (open bars) and STZ (closed bars) mice injected with PBS or PLX, as indicated with n = 8/control, n = 10/STZ, n = 5/control + PLX, and n = 11/STZ + PLX groups. In vitro, PLX-conditioned hyperglycemic medium modulate (E) eNOS expression, (F) NO, (G) ROS levels, and (H) caspase 3/7 activity in HUVEC, which is translated in improved (I), adhesion capacity and (J–M) tube formation versus HUVEC cultured under hyperglycemic conditions. Bar graphs represent the mean ± SEM of (E) eNOS expression normalized to β-tubulin and the control groups set as 100%, (F) % of DAF-positive, (G) % of DCF-positive gated cells, with all n = 4/condition, (H) caspase 3/7 activity depicted with the normoglycemia group set as 100% with n = 8–10/condition, and (I) adhered crystal violet-stained HUVEC depicted as the absorbance at 495 nm with n = 5–6/condition. (J): Representative pictures of HUVEC cultured in normo- (5 mM glucose), hyperglycemic (30 mM glucose) medium, or normo- (5 mM glucose), hyperglycemic (30 mM glucose) conditioned PLX medium, as indicated. Bar graphs represent the mean ± SEM of (K) total branching points, (L) total tubes, and (M) tube length analyzed from n = 7 to 8 fields/condition. For all data: *, p < .05; **, p < .01; ***, p < .001; ****, p < .0001. Abbreviations: Enos, endothelial nitric oxide synthase; NO, nitric oxide; ROS, reactive oxygen species, PLX, Placenta-eXpanded cell; ROS, reactive oxygen species; STZ, streptozotocin.
Figure 5. PLX restore cardiomyocyte stiffness and improve diastolic relaxation in streptozotocin-induced diabetic mice. (A): Representative images of titin N2BA and N2B phosphoproteins and total proteins in control, STZ, control PLX, and STZ PLX mice, as indicated. Bar graphs represent the mean ± SEM of (B) N2BA phosphorylation and (C) N2B phosphorylation depicted as % with the control set as 100% of control (open bars) and STZ (closed bars) mice injected with PBS or PLX, as indicated with n = 7–8/control, n = 5–7/control + PLX and n = 7–8/STZ + PLX groups and *, p < .05; **, p < .01; ***, p < .0005; ****, p < .00001 of (D) PKA (ng/μl) in control (open bars) and STZ (closed bars) mice injected with PBS or PLX, as indicated with n = 3/group and *, p < .05; (E) PKG (pmol/min/μg protein) in control (open bars) and STZ (closed bars) mice injected with PBS or PLX, as indicated with n = 4/PBS group, n = 3/PLX group and *, p < .05, (F) CaMKII activity (CaMKII; μU/ml) in control (open bars) and STZ (closed bars) mice injected with PBS or PLX, as indicated with n = 9/control group, n = 9/STZ group, and n = 4/PLX groups, and *, p < .05. (G) PKC (ng/μl) in control (open bars) and STZ (closed bars) mice injected with PBS or PLX, as indicated with n = 3/group and *, p < .05, (H) Passive tension F passive (kN/m²) versus sarcomere length (μm) in control (-•-), STZ (■), control + PLX (○), and STZ + PLX (□), as indicated, with n = 15–19 cardiomyocytes/group and *, p < .05 versus control and STZ + PLX and §, p < .05 versus control + PLX. Bar graphs represent the mean ± SEM of (I) dP/dt min and (J) Tau with n = 8 and n = 11 for the control and STZ groups, respectively, and *, p < .05; **, p < .01; ***, p < .0001 analyzed by one-way analysis of variance (ANOVA) without correction for multiple comparison. Abbreviations: PKA, protein kinase A; PKC, protein kinase C; PKG, protein kinase G; PLX, PLacenta-expanded cell; STZ, streptozotocin.
and their systemic extracardiac immunomodulatory effects [33], which may be of extra relevance in this model of systemic low-grade inflammation.

CONCLUSION

In conclusion, i.v. PLX application improves LV diastolic relaxation and cardiomyocyte stiffness at an early stage of diabetic cardiomyopathy through increased titin phosphorylation. These findings offer new insights into the cardioprotective effects of PLX, advancing their potential use for cardiac therapies.

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AUTHOR CONTRIBUTIONS

S.V.L.: conception and design, administrative support, collection and/or assembly of data, data analysis and interpretation, manuscript writing; N.H.: conception and design, collection and/or assembly of data; data analysis and interpretation; K.M., A.K., I.M., and L.P.: collection and/or assembly of data, data analysis and interpretation; Z.A.: financial support, final approval of manuscript; K.P.: collection and/or assembly of data; W.A.L.: data analysis and interpretation, final approval of manuscript; C.T.: conception and design, data analysis and interpretation, financial support, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

L.P. is an employee of Pluristem Therapeutics Inc. and holds stock and stock options. Z.A. is an employee and shareholder of Pluristem Therapeutics Inc. and has intellectual property rights. The other authors indicated no potential conflicts of interest.

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