Parasite-Derived Neurotrophic Factor/*trans*-Sialidase of *Trypanosoma cruzi* Links Neurotrophic Signaling to Cardiac Innate Immune Response

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The Chagas' disease parasite *Trypanosoma cruzi* elicits a potent inflammatory response in acutely infected hearts that keeps parasitism in check and triggers cardiac abnormalities. A most-studied mechanism underlying innate immunity in *T. cruzi* infection is Toll-like receptor (TLR) activation by lipids and other parasite molecules. However, yet-to-be-identified pathways should exist. Here, we show that *T. cruzi* strongly upregulates monocyte chemoattractant protein 1 (MCP-1)/CCL2 and fractalkine (FKN)/CX3CL1 in cellular and mouse models of heart infection. Mechanistically, upregulation of MCP-1 and FKN stems from the interaction of parasite-derived neurotrophic factor (PDNF)/*trans*-sialidase with neurotrophic receptors TrkA and TrkC, as assessed by pharmacological inhibition, neutralizing antibodies, and gene silencing studies. Administration of a single dose of intravenous PDNF to naive mice results in a dose-dependent increase in MCP-1 and FKN in the heart and liver with pulse-like kinetics that peak at 3 h postinjection. Intravenous PDNF also augments MCP-1 and FKN in TLR signaling-deficient MyD88-knockout mice, underscoring the MyD88-independent action of PDNF. Although single PDNF injections do not increase MCP-1 and FKN receptors, multiple PDNF injections at short intervals up the levels of receptor transcripts in the heart and liver, suggesting that sustained PDNF triggers cell recruitment at infection sites. Thus, given that MCP-1 and FKN are chemokines essential to the recruitment of immune cells to combat inflammation triggers and to enhance tissue repair, our findings uncover a new mechanism in innate immunity against *T. cruzi* infection mediated by Trk signaling akin to an endogenous inflammatory and fibrotic pathway resulting from cardiomyocyte-TrkA recognition by matricellular connective tissue growth factor (CTGF/CCN2).

Chagas' disease, a major cause of morbidity and mortality in Latin America (1–3), is commonly transmitted by blood-sucking reduviid bugs, which, during a blood meal, release *Trypanosoma cruzi*. As a metacyclic trypomastigote, *T. cruzi* gains access to the body through mucosal surfaces or discontinuity in the skin, where it elicits a pronounced inflammatory response. In the conjunctiva, inflammation and edema may last many weeks, and in areas of endemicity, the unilateral eye inflammation (Romaña's sign) serves as a diagnostic tool. After a few days in the transmission site, *T. cruzi* spreads throughout the body and in the heart triggers severe inflammation and tissue damage that often give rise to ventricular repolarization, ejection defects, pleural effusion, and cardiomegaly (4–6). This response wanes in most (>95%) infected patients, who progress to a symptomless and pathology-free indeterminate phase that can last years or a lifetime. However, <5% of acute chagasic patients progress to widespread and fulminating myocarditis.

Host survival and *T. cruzi* growth are controlled by innate and acquired immunity (1–3). During the initial stage of infection, members of the Toll-like receptor (TLR) family of pattern recognition proteins play an important role in *T. cruzi* recognition, as they are activated by parasite molecules, such as DNA, RNA, or glycosylphosphatidylinositol (GPI) anchors, initiating a signaling cascade dependent on the adaptor molecule myeloid differentiation factor 88 (MyD88), which mediates upregulation of proinflammatory genes pivotal to the resistance to *T. cruzi* infection. In addition, *T. cruzi* activates Nod1 and inflammasome (NLRP3) proinflammatory pathways, and the end result is an increase in the levels of interleukin-1 β (IL-1 β) (7), IL-6 (7, 8), IL-12 (9–13), tu-

mor necrosis factor alpha (10, 12–14), beta interferon (IFN- β) and IFN- γ (7, 12, 13, 15–17), and monocyte chemoattractant protein 1 (MCP-1)/CCL2 and other chemokines essential to the recruitment of inflammatory cells to infection sites (18, 19).

The *T. cruzi* outer membrane parasite-derived neurotrophic factor (PDNF) and a short form that contains receptor-binding sites (sPDNF) (20, 21) bind and activate nerve growth factor (NGF) receptor TrkA (22) and neurotrophin-3 (NT-3) receptor TrkC (23), triggering host cell survival events and parasite host cell entry (24, 25). PDNF was identified in 1983 in our labs as neuraminidase (26) consequent to the discovery of sialic acid in *T. cruzi* (27, 28). Nine years after its discovery, the neuraminidase was found to catalyze the covalent transfer of sialyl residues to β -galactosidase acceptors from glycoconjugates, hence its renaming to *trans*-sialidase (29, 30), even though the molecule has both neuraminidase and *trans*-sialidase activities (31). However, neurotrophic receptor activation is independent of sialic acid binding because (i) the neuraminidase/*trans*-sialidase does not lose Trk receptor recognition after it is mutated to contain point mutations that sharply reduce neuraminidase/*trans*-sialidase activities (32),

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(ii) Trk-binding activity is mimicked by a 24-mer synthetic peptide (33), and (iii) the neuraminidase/*trans*-sialidase binds and activates Trk receptors in sialic acid-deficient cells (25). Furthermore, after phosphorylation by Akt kinase, the enzyme promotes cell survival in the cytosol, where sialyl-conjugate substrates are absent (34). Neuraminidase/*trans*-sialidase is called PDNF to underscore the autocrine/paracrine growth factor function independent of sialic acid binding. Binding to TrkC leads to *T. cruzi* entry into cardiomyocytes and cardiac fibroblasts, whereas binding to TrkA triggers cardioprotection by autocrine and paracrine mechanisms (35, 36).

Four years ago it was reported that connective tissue growth factor (CTGF), also known as CCN2, a matricellular protein implicated in fibrosis and inflammation in several disorders, such as diabetic neuropathy (37), augments the expression of various pro-inflammatory cytokines, including the chemokine MCP-1, following binding and activation of neurotrophic receptor TrkA on cardiomyocytes (38, 39). Given that the CTGF/CCN2 receptor is TrkA, the same receptor that sPDNF interacts with to promote downstream signaling and cell survival in host cells, including cardiac cells (35, 36), we hypothesized that *T. cruzi* PDNF activation of TrkA and/or TrkC on cardiomyocytes and cardiac fibroblasts upregulates mediators of innate immunity. The results reported here support the validity of the hypothesis.

MATERIALS AND METHODS

Parasites. Experiments were performed with the *T. cruzi* Tulahuen strain, which was propagated in Vero cells. Free-swimming infective trypomastigotes were harvested from supernatants (3 to 5 days after infection) by initial low-speed centrifugation ($500 \times g$, 5 min) to remove host cells and debris, followed by high-speed centrifugation ($1,200 \times g$, 10 min) to pellet the parasites, which were resuspended in Dulbecco modified Eagle medium (DMEM)–0.1% fetal calf serum (FCS). For *in vivo* experiments, the parasites were resuspended in phosphate-buffered saline (PBS) prior to injection into mice.

Mice. Female C57BL/6 mice (age, 6 to 8 weeks) and C57BL/6 breeding pairs were from The Jackson Laboratory (Bar Harbor, ME). MyD88-knockout mice were a generous gift from Thereza Imanishi-Kari, Tufts Medical School (40). All mouse experiments were approved by the Institutional Animal Care and Use Committee (IACUC) and the Division of Laboratory Animal Medicine (DLAM) of the Tufts University School of Medicine.

Cell lines and primary cell cultures. (i) **Cell lines.** H9c2 cells (ATCC CRL-1446) (rat cardiomyocytes) and HEK 293 cells (used to generate lentiviral particles) were maintained in DMEM–10% FCS, and Vero cells (used to grow *T. cruzi*) were maintained in DMEM–1% FCS. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

(ii) **Primary cardiomyocytes and cardiac fibroblasts.** Primary cardiomyocytes and cardiac fibroblasts were isolated using a modified version of a previously described procedure (35, 36, 41). In short, neonatal C56BL/6 mice (age, 1 to 3 days) were sacrificed by decapitation, and their hearts were excised, washed twice in PBS, and then kept in 20 mM HEPES, 130 mM NaCl, 1 mM NaH₂PO₄, 4 mM glucose, 3 mM KCl, pH 7.6, for 10 min on ice. The hearts were minced and digested in 0.25% trypsin-EDTA (Gibco) 3 to 4 times at 37°C with periodic mixing. Dissociated cells were pooled, and digestion was stopped with DMEM–10% FCS. The cells were then filtered through a 100- μ m-pore-size cell strainer, centrifuged at $500 \times g$, and plated for 3 h on 1% gelatin-coated plates in DMEM (Gibco)–Ham's F-12 medium (Sigma) at 50:50 containing 20% FCS (PAA Laboratories, Inc.), 5% horse serum (PAA), 2 mM L-glutamine (Gibco), 0.1 mM nonessential amino acids (Gibco), 3 mM sodium pyruvate (Gibco), and 1 μ g/ml bovine insulin (Sigma) with 1 $\times$ penicillin-streptomycin (Gibco). Nonadherent cells (cardiomyocytes) were re-

moved and plated onto new gelatin-coated plates, while adherent cells (cardiac fibroblasts) remained in the initial plate in DMEM–10% FCS until needed. Cardiomyocytes contained <5% cardiac fibroblasts and cardiac fibroblasts contained <5% cardiomyocytes, as determined by immunofluorescence using antibodies against the cardiomyocyte marker myosin heavy chain (MHC) and cardiac fibroblast marker vimentin, as described earlier (35).

Purification of recombinant sPDNF. PDNF was cloned from the *T. cruzi* Silvio X-10/4 strain (GenBank accession number AJ002174), and an N-terminal short form of PDNF (sPDNF) that contains Trk-binding sites was expressed in *Escherichia coli* BL21(DE3) bacteria and purified by Ni-affinity chromatography, as described previously (22, 24, 25, 35). Buffers were prepared in sterile endotoxin-free water, and sPDNF preparations were found to have undetectable levels of endotoxin, as determined by the *Limulus* amebocyte assay. sPDNF protein was buffer exchanged into PBS (0.01 M, pH 7.2), filter sterilized (pore size, 0.22 μ m), and kept at 4°C. sPDNF migrates as a 68-kDa protein on an SDS-polyacrylamide gel. PDNF was quantified by scanning densitometry (GS-800; Bio-Rad) of SDS-polyacrylamide gels stained with Coomassie brilliant blue.

Cell stimulation. (i) ***T. cruzi*.** Primary cardiomyocytes and cardiac fibroblasts were seeded at 6×10^4 to 8×10^4 cells per well in DMEM–10% FCS and allowed to adhere overnight. The medium was changed to DMEM–5% FCS, and cells were infected with *T. cruzi* at a multiplicity of infection (MOI) of 10 for 10 to 24 h, at which time parasites were washed off. At 3, 24, and 72 h postinfection, cell monolayers were collected in TRIzol reagent (Invitrogen) to isolate RNA to generate cDNA for quantitative real-time PCR (qPCR).

(ii) **sPDNF.** Primary cardiomyocytes and cardiac fibroblasts and H9c2 cardiomyocytes were seeded at 6×10^4 to 8×10^4 cells per well in DMEM–10% FCS, allowed to adhere overnight, serum starved (in DMEM–0.1% FCS overnight), and treated with sPDNF (0.5 ng/ml to 8 μ g/ml, 0 to 16 h). Cell supernatants were collected and stored at –80°C, and cell monolayers were rinsed with PBS and collected in TRIzol for qPCR analysis.

Blocking Trk receptors. (i) **Pharmacological inhibitor.** H9c2 cells were pretreated with 0.1% dimethyl sulfoxide vehicle as a control or 200 nM K252a for 1 h prior to addition of sPDNF (1 μ g/ml). Supernatants and cell monolayers were collected for quantification of secreted chemokines, and cells were harvested for mRNA quantification by qPCR.

(ii) **Neutralizing antibodies.** H9c2 cardiomyocytes were pretreated for 30 min with 1.0, 1.5, or 2.0 μ g/ml neutralizing antibodies against TrkA (anti-TrkA; catalog no. SC-118; Santa Cruz), TrkB (anti-TrkB; catalog no. SC-8316; Santa Cruz), TrkC (anti-TrkC; catalog no. SC-14025; Santa Cruz), or combinations thereof, followed by stimulation with 1 μ g/ml sPDNF. Chemokine transcripts were quantified by qPCR, and MCP-1 protein was measured by enzyme-linked immunosorbent assay (ELISA; R&D Systems) (see below).

(iii) **Lentiviral transfection and Trk knockdown by shRNA.** Lentiviral vectors encoding short hairpin RNA (shRNA) constructs targeting TrkA (clones 1 to 4), TrkC (clones 1 to 5), or green fluorescent protein (GFP) mRNA (Open Biosystems) were generated from transfected HEK293 cells following the manufacturer's instructions, and aliquots were frozen at –80°C until use. After pretreatment with 8 μ g/ml Polybrene (Sigma-Aldrich) in DMEM–10% FCS, lentiviral infection of H9c2 cells was performed at a dose of 200 μ l vector-containing supernatant/2 ml medium in 6-well plates. Lentiviral particles were removed 24 h after infection, and 7 to 8 days later, cells were serum starved in serum-free DMEM for 2 h and stimulated without or with sPDNF (4 μ g/ml, 3 h).

qPCR. RNA was isolated from TRIzol lysates of cell monolayers or tissue samples that had been snap-frozen in liquid nitrogen and dissociated by a Tissue-Tearor mechanical homogenizer (Biospec Products, Inc.). cDNA was synthesized using a Quantitect reverse transcription kit (Qiagen) according to the manufacturer's instructions. Chemokine and chemokine receptor transcripts were amplified using specific primers, and their levels were normalized to those of hypoxanthine phosphoribosyltransferase (HPRT) using SYBR green (Qiagen) and, if needed, expressed

relative to those from control-unstimulated cells. The primers specific for the following were used: MCP-1 (forward primer, TCTCTCTCCACC ACTATGCA; reverse primer, GGCTGAGACAGCACGTGGAT), FKN (forward primer, GCGCGCCGAATTCCTGCACT; reverse primer, CAA TGGCAGGTTGCCGAG), CCR2 (forward primer, AATGAGAAGAA GAGGCACAGGGCT; reverse primer, ATGGCCTGGTCTAAGTGCTT GTCA), CX3CR1 (forward primer, CGACATTGTGGCCTTTGGAAC CAT; reverse primer, AGATGTCAGTGATGCTCTTGGGCT), and HPRT (forward primer, CAGCGTCGTGATTAGCGATGATG; reverse primer, CGAGCAAGTCTTTCAGTCCTGTC).

ELISA. For the MCP-1 ELISA, 96-well Maxisorp plates (Nunc) were coated overnight with relevant supernatants or MCP-1 standards (catalog no. 479-JE/CF; R&D Systems) in coating buffer (50 mM NaHCO₃/Na₂CO₃, pH 9.6, 0.02% NaN₃), blocked in 5% bovine serum albumin-PBS-Tween 20, and reacted with a MCP-1 detection antibody (1:200; 2 h; catalog no. SC-28879; Santa Cruz) and then alkaline phosphatase (AP)-conjugated secondary antibody (1:1,000, 1 h; catalog no. A3687; Sigma). Between each step, plates were washed 2 to 4 times with PBS-Tween 20. Wells were then incubated with colorogenic AP substrate (1 mg/ml in 100 mM glycine, 1 mM MgCl₂, 1 mM ZnCl₂, pH 10.4; catalog no. N9389; Sigma), and the absorbance at 405 nm was read on an EMax precision microplate reader (Molecular Devices). MCP-1 concentrations were calculated relative to the concentrations on a 4-parameter standard curve using the SOFTmax Pro program. MCP-1 ELISA (catalog no. DY479; R&D Systems) was used to analyze the supernatants from neutralizing antibody experiments. MIP-2 α (catalog no. DY452; R&D Systems) and IL-6 (catalog no. DY406; R&D Systems) ELISAs were performed according to the manufacturer's protocols.

Mouse model of acute Chagas' disease and intravenous (i.v.) sPDNF administration into naive mice. (i) **Mouse model.** Female C57BL/6 mice (age, 6 to 8 weeks) were infected subcutaneously in the left hind footpad with 5×10^3 trypomastigotes in 30 μ l while the mice were under isoflurane anesthesia, and the mice were sacrificed on various days postinfection by CO₂ asphyxiation and cervical dislocation. Organs were perfused by intracardiac injection of 5 ml ice-cold PBS, and the heart (the atrium and ventricle were sometimes collected separately) and other organs were collected, flash frozen in liquid nitrogen, and stored at -80°C . Tissue parasitism was quantified using a qPCR method previously described (24, 25, 42).

(ii) **i.v. administration.** sPDNF was diluted in sterile endotoxin-free PBS in 200 μ l and injected via the tail vein into 6- to 8-week-old female C57BL/6 mice. Mice receiving single i.v. injections of 150 μ g sPDNF per mouse were sacrificed at 3, 6, 9, and 12 h postinjection, and mice receiving various doses of sPDNF (100 ng to 150 μ g) were sacrificed at 3 h postinjection. Mice receiving multiple injections of sPDNF or PBS vehicle control (0, 3, and 24 h, 100 μ g per injection) were sacrificed 24 h after the last injection (i.e., 48 h after the first injection). Mice were perfused with PBS, and organs were harvested as described in the previous paragraph.

Statistical analyses. Statistical analyses were performed using GraphPad Prism software (version 5.0) and Student's *t* test (for comparing two samples) or one-way analysis of variance with Tukey's posttest (for comparison of three or more samples).

RESULTS

***T. cruzi* and sPDNF augment expression of MCP-1 and FKN in cardiomyocytes and cardiac fibroblasts.** We first determined whether *T. cruzi* and sPDNF augment the expression of chemokines in cardiomyocytes and cardiac fibroblasts, focusing on MCP-1 and fractalkine (FKN). In line with and extending the findings of an earlier study (43), we found that *T. cruzi* infection of primary cardiomyocytes and cardiac fibroblasts upregulates MCP-1 and FKN transcripts in a time-dependent manner (Fig. 1a and b). In addition, the stimulatory activity of *T. cruzi* is mimicked by sPDNF, as it stimulates an increase in MCP-1 and FKN tran-

FIG 1 Both *T. cruzi* and sPDNF increase MCP-1 and FKN transcript levels in primary cultures of cardiomyocytes and cardiac fibroblasts. (a and b) *T. cruzi* infection. Primary cultures of mouse cardiomyocytes (a) and cardiac fibroblasts (b) were plated in 6-well plates and infected with the *T. cruzi* Tulahuen strain (MOI = 10) for 3, 24, and 72 h, and MCP-1 and FKN transcripts were quantified by qPCR. Chemokine gene expression was normalized to HPRT gene expression, and the fold change in expression relative to the level of expression for uninfected cells was calculated. Points represent the mean $\pm$ SD fold change in expression for triplicate samples. The experiment was repeated once, and the results were similar to those shown here. (c and d) sPDNF stimulation. Primary cardiomyocytes (c) and cardiac fibroblasts (d) were plated in 6-well plates, serum starved overnight, and then stimulated with the indicated concentration of sPDNF (3 h), and MCP-1 and FKN transcripts were quantified by qPCR. Chemokine gene expression was normalized to HPRT gene expression, and the fold change in expression relative to the level of expression for vehicle (Veh)-treated cells was calculated. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

scripts in both primary cardiomyocytes and cardiac fibroblasts in a dose-dependent manner (Fig. 1c and d).

In the cardiomyocyte cell line H9c2, sPDNF dramatically increased the secretion of MCP-1 dose dependently (Fig. 2a) and specifically, as sPDNF did not alter the secretion of another chemokine, macrophage inflammatory protein 2 α (MIP-2 α)/CXCL2, or the cytokine IL-6 (Fig. 2a). Similar to the results obtained with primary cardiomyocytes, the increase in MCP-1 and FKN mRNA in H9c2 cardiomyocytes is dose dependent (Fig. 2b). Furthermore, time course analysis revealed that the levels of the MCP-1 and FKN transcripts increase maximally 3 h after sPDNF stimulation and drop sharply to levels similar to those for unstimulated cells after 8 h in a typical pattern of pulse-like kinetics (Fig. 2c and d).

Targeting cardiomyocyte neurotrophin receptors TrkA and TrkB for MCP-1 and FKN upregulation. We used three distinct criteria to determine whether *T. cruzi* PDNF exploits TrkA or

H9c2 Cardiomyocytes

FIG 2 sPDNF ups MCP-1 and FKN expression in H9c2 cardiomyocytes dose dependently and in a pulse-like manner. (a) MCP-1 secretion and dose-response. H9c2 cardiomyocytes were plated in 6-well plates, grown in low serum (0.1% FCS) overnight, and stimulated with the indicated doses of sPDNF for 3 h. The levels of secreted MCP-1, MIP-2α, and IL-6 were determined by ELISA and were not found to be increased by sPDNF stimulation. (b) MCP-1 and FKN transcripts and dose-response. H9c2 cardiomyocytes were plated in 6-well plates, grown in low serum (0.1%) overnight, and stimulated with the indicated doses of sPDNF for 3 h, and the MCP-1 transcript was quantified by qPCR. Chemokine gene expression was normalized to HPRT gene expression, and the fold change in response relative to the response of vehicle-treated cells was calculated. Points represent the mean $\pm$ SD fold change in expression for triplicate samples. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$. (c, d) Time course of MCP-1 and FKN transcripts. H9c2 cardiomyocytes were plated in 6-well plates (triplicate), grown in low serum (0.1%) overnight, and stimulated with sPDNF (1 μg/ml) for the indicated times, and MCP-1 and FKN transcripts were quantified by qPCR. Three experiments gave similar results.

TrkC, or both, to increase MCP-1 and FKN production in cardiac cells.

First, we preincubated (1 h) H9c2 cardiomyocytes with the Trk antagonist K252a, which blocks Trk signaling by inhibiting Trk autophosphorylation (44), followed by stimulation (3 h) with sPDNF (1 μg/ml) or vehicle (PBS), and quantification of MCP-1 and FKN transcripts was carried out by qPCR. We found that K252a completely abrogates the stimulatory effect of sPDNF (Fig. 3a and b), indicating that the agonistic effect of sPDNF requires Trk signaling.

Second, we designed experiments to reduce Trk expression with short hairpin mRNA (shRNA) to determine whether enhanced expression of MCP-1 and FKN results from the binding of sPDNF to TrkA and/or TrkC. For this purpose, we transfected H9c2 cardiomyocytes with lentivirus encoding shRNA for control green fluorescence protein (shGFP), TrkA (shTrkA; four distinct vectors), or TrkC (shTrkC; five distinct vectors), stimulated untransfected and transfected myocytes with sPDNF (1 μg/ml, 3 h), and assessed the inhibitory effect of gene silencing by comparing secreted MCP-1 levels in sPDNF-stimulated cells with those in PBS vehicle-treated cells. Compared to GFP-transfected cardiomyocytes or untransfected cardiomyocytes, cardiomyocytes transfected with shTrkA or shTrkC constructs significantly reduced their MCP-1 secretion in response to sPDNF stimulation (Fig. 3c).

Third, to further validate the conclusion that sPDNF triggers MCP-1 and FKN expression through both TrkA and TrkC receptors, we preincubated (0.5 h) cardiomyocytes with neutralizing neurotrophin receptor antibodies against neurotrophin receptors prior to stimulation with sPDNF (1 μg/ml, 3 h). The rationale for this experiment is that, if sPDNF uses the Trk receptors to augment chemokine expression, then neutralizing the receptors with antibodies against the extracellular domain of the receptors will prevent access of sPDNF to the receptors and block sPDNF stimulation of MCP-1 and FKN expression. We found that antibodies against TrkA (anti-TrkA plus sPDNF) significantly block sPDNF-induced upregulation of MCP-1 (29.2% $\pm$ 3.5% inhibition) and FKN (29.5% $\pm$ 5.3% inhibition), as do antibodies against TrkC

(MCP-1 inhibition, 53% $\pm$ 2.3%; FKN inhibition, 47.5% $\pm$ 1.3%) (Fig. 3d and e). In contrast, neutralizing antibodies against TrkB (anti-TrkB plus sPDNF) are ineffective in preventing sPDNF upregulation of both MCP-1 and FKN (Fig. 3d and e), consistent with our earlier finding that TrkB is not recognized by sPDNF, thus serving as a negative control for the anti-TrkA and anti-TrkC inhibition experiments (22, 23). Of note, under the conditions used here, Trk antibodies have no demonstrable effect on MCP-1 and FKN upregulation (Fig. 3d and e). In addition, the conclusion that sPDNF uses TrkA and TrkC to augment chemokine expression was yet further validated by preincubating cardiomyocytes with a mixture of anti-TrkA and anti-TrkC antibodies, as the results showed that the mixture has an additive/synergistic effect in reducing the secretion of MCP-1 protein compared to the effect of either anti-TrkA or anti-TrkC alone (Fig. 3f).

Thus, on the basis of these three criteria that operate by distinct mechanisms, we conclude that sPDNF uses both TrkA and TrkC to upregulate MCP-1 and FKN, akin to the utilization of TrkA by the matricellular protein CTGF/CCN2 to increase the MCP-1 level in cardiomyocytes (38). Notably, dual usage of TrkA and TrkC to upregulate MCP-1 and FKN is analogous to neuronal cell invasion, which depends on both TrkA (24) and TrkC (25), and contrary to the mechanism of *T. cruzi* entry into cardiac fibroblasts or cardiomyocytes, which preferentially depends on TrkC (36), and to sPDNF-induced NGF secretion by cardiomyocytes, which is selective for TrkA (35).

Cardiac MCP-1 and FKN are upregulated in a mouse model of acute Chagas' myocarditis. Groups of three mice were infected with *T. cruzi* (Tulahuen strain) and sacrificed at several days postinfection to quantify the cardiac MCP-1 and FKN mRNAs and the cardiac parasite burden by qPCR (24, 25, 35, 36, 42). In line with the findings of a previous study (45), our results show that the levels of MCP-1 and FKN mRNA parallel the degree of heart parasite burden (Fig. 4a and b). However, unlike the expression of MCP-1 transcripts, the expression of FKN transcripts remained significantly elevated for at least 20 days after cardiac parasitism became barely detectable (Fig. 4b), in agreement with the

FIG 3 Neurotrophic receptors TrkA and TrkC are targeted by sPDNF for upregulation of MCP-1 and FKN in cardiomyocytes. (a and b) Pharmacological inhibition. H9c2 cardiomyocytes were stimulated with sPDNF (2 μ g/ml, 3 h) without pretreatment (sPDNF) or after pretreatment with K252a (200 nM, 1 h) (K252a + sPDNF), and transcripts of MCP-1 (a) and FKN (b) were quantified by qPCR. Chemokine gene expression was normalized to HPRT gene expression, and the fold change in expression relative to the level of expression for vehicle-treated cells was calculated. Bars represent the mean $\pm$ SD fold change in expression for duplicate samples. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$. The experiment was performed three times, with similar results obtained each time. (c) RNA silencing. H9c2 cardiomyocytes were transfected with lentiviral particles containing shRNAs targeting GFP (control), TrkA (clones 1 to 4), and TrkC (clones 1 to 5). After 7 to 8 days, cells were serum starved (2 h) and stimulated with vehicle or sPDNF (4 μ g/ml, 3 h), and the concentration of MCP-1 secreted in the supernatants was determined by ELISA. ns, not significant relative to shGFP-transfected cells; *, $P < 0.05$ relative to shGFP-transfected cells; **, $P < 0.01$ relative to shGFP-transfected cells; ***, $P < 0.005$ relative to shGFP-transfected cells. (d to f) Neutralizing antibodies. (d and e) H9c2 cardiomyocytes were stimulated with sPDNF (1 μ g/ml, 3 h) without pretreatment (+ sPDNF) or after pretreatment with neutralizing antibodies (1 μ g/ml, 30 min) against TrkA (α -TrkA + sPDNF), TrkB (α -TrkB + sPDNF), and TrkC (α -TrkC + sPDNF) followed by sPDNF; MCP-1 and FKN expression was assessed by real-time PCR. (f) H9c2 cardiomyocytes (duplicate wells) were pretreated for 30 min with neutralizing anti-Trk antibodies (2 μ g/ml for each type of antibody or the combination of anti-TrkA and anti-TrkC [α -TrkA + α -TrkC]) and then stimulated with sPDNF (1 μ g/ml). After 3 h, the MCP-1 secreted in the cardiomyocyte culture supernatants was quantified by ELISA (R&D Systems). The experiment was repeated three times using antibodies at individual concentrations of 1.0, 1.5, and 2.0 μ g/ml; in each instance, anti-TrkA and anti-TrkC combined significantly reduced the level of MCP-1 secretion compared to that achieved with anti-TrkA or anti-TrkC alone. ns, not significant; *, $P < 0.05$; **, $P < 0.01$.

view that FKN plays an important role in recruiting blood monocytes that promote cardiac healing after myocardial injury (46).

A single intravenous dose of sPDNF increases MCP-1 and FKN expression in the heart dose dependently and with pulse-like kinetics in both naive wild-type and MyD88^{-/-} mice. The robust MCP-1 increase in acutely infected hearts (Fig. 4a) is likely due to the stimulation of TLRs and other canonical proinflammatory pathways by *T. cruzi* molecules, exemplified by glycosylphosphatidylinositol-anchored mucin-like glycoproteins, which increase MCP-1 levels and mediate leukocyte recruitment in the pleural cavity of mice primed with an IFN- γ -inducing agent (18). If *T. cruzi* PDNF is also a mechanism responsible for triggering

MCP-1 expression in *T. cruzi*-infected hearts, then intravenous (i.v.) administration of sPDNF should upregulate chemokines MCP-1 and FKN in the heart of uninfected, naive mice. A previous pharmacokinetics study showed that sPDNF administered i.v. into naive mice has a half-life of $\sim$ 15 min in the blood and that its level peaks in the myocardium at $\sim$ 15 min postinjection, triggering cardiac responses such as NGF upregulation hours after injection (35).

We injected groups of naive wild-type C57BL/6 mice with vehicle (PBS) or a single dose of i.v. sPDNF, sacrificed the mice at various time points postinjection, and measured heart MCP-1 and FKN levels by real-time PCR. We found that i.v. administered

FIG 4 MCP-1 and FKN mRNAs increase in acute chagasic hearts in proportion to the parasite burden. (a and b) Kinetics of cardiac MCP-1 and FKN mRNAs in response to acute *T. cruzi* infection in relation to the heart parasite burden. Groups of C57BL/6 mice (3 per group) were subcutaneously infected with *T. cruzi*, and at the indicated time points, the mice were sacrificed and MCP-1 and FKN levels and the parasite burdens in the hearts were quantified by qPCR.

sPDNF significantly upregulates both cardiac MCP-1 and FKN transcripts with pulse-like kinetics, as both chemokines peaked shortly after administration (3 h postinjection) and quickly went down to the levels in uninjected mice (Fig. 5a). We then screened for the agonist effect of i.v. sPDNF in various organs using the time at which the response was optimum (3 h postinjection) and the same dose used in the experiment whose results are presented in Fig. 5a. We found that sPDNF upregulated MCP-1 in the heart, liver (where the response was the most robust), and spleen but not in the bone marrow, aorta, esophagus, colon, or skin (Fig. 5b). The organ distribution of MCP-1 expression in response to i.v. sPDNF was similar to that of FKN expression (data not shown). The selective increase in MCP-1 and FKN in various organs likely reflects the uneven expression of the PDNF receptors TrkA and TrkC in neural and nonneural tissues (47, 48). Furthermore, i.v. sPDNF-triggered MCP-1 and FKN upregulation is dose dependent in the heart (Fig. 5c) and liver (Fig. 5d), in agreement with the hypothesis that sPDNF stimulates MCP-1 and FKN through a receptor (TrkA and TrkC)-mediated process, akin to the results obtained with cardiac cells in culture (Fig. 3).

Although our sPDNF preparations did not have detectable levels of endotoxin (as assessed by the *Limulus* amoebocyte assay) and were nontoxic in recombinate-activating gene 2 (RAG-2^{-/-})-deficient mice (data not shown), which are highly susceptible to endotoxin shock (49), we sought to further ensure that the response observed in wild-type mice was not due to TLR activation by assessing the MCP-1 and FKN response to i.v. sPDNF in TLR signaling-deficient MyD88^{-/-} mice (50). Accordingly, our results showed that i.v. sPDNF gives rise to a sharp increase in the expression of both MCP-1 and FKN in the heart and liver of MyD88^{-/-} mice (Fig. 5e to h), further supporting the concept that sPDNF acts on Trk receptors independently of MyD88 signaling.

Multiple doses, but not a single dose, of i.v. sPDNF increase the expression of MCP-1 and FKN receptors CCR2 and CX3CR1 in the mouse heart and liver. One of the functions of MCP-1 and FKN is to attract cells expressing their receptors (CCR2 and CX3CR1, respectively) to tissue sites. We found that CCR2 and CX3CR1 transcript levels did not increase in cardiac and hepatic tissues following a single i.v. sPDNF dose (Fig. 6a and b), consistent with the finding that the MCP-1 and FKN increase is transient and pulse-like (Fig. 5a). However, sustained tissue exposure to

sPDNF, obtained by multiple and closely spaced systemic sPDNF injections into naive mice (i.v. sPDNF at times of 0, 3 and 24 h, with chemokine levels measured 24 h after the last injection), resulted in a significant increase in CCR2 and CX3CR1 in the liver and heart (ventricle and atrium) (Fig. 6c and d). Thus, the possibility exists that sPDNF-induced MCP-1 and FKN promote the migration of cells bearing CCR2 and CX3CR1 receptors.

DISCUSSION

The results presented here demonstrate a novel mechanism underlying the innate immune response to *T. cruzi*, which, via outer membrane-bound PDNF (51), ups the expression of the chemokines MCP-1 and FKN by activating neurotrophic receptors TrkA and TrkC. In this mechanism, *T. cruzi* exploits a pathway used by CTGF/CCN2, an inflammatory/fibrotic matricellular growth factor that promotes chemokine and inflammatory cytokine expression through the activation of TrkA (38, 39). Interestingly, earlier studies identified a relationship between *T. cruzi* infection of cultured cells and expression of CTGF/CCN2 (52). However, it remains to be determined whether alteration of CTGF/CCN2 expression by *T. cruzi* relates to our results reported here.

Alteration of MCP-1 expression in infected inflamed tissues is traditionally interpreted in the context of recruitment of proinflammatory immune cells for microbicidal purposes, including in *T. cruzi* infection (18, 43, 45). Indeed, a first wave of immune cells to reach infected tissues is composed of Ly-6C^{high} monocytes, a major subset of monocytes in the bloodstream that are attracted by MCP-1–CCR2 signaling (53). Ly-6C^{high} monocytes exhibit phagocytic, proteolytic, and inflammatory activities and are thus thought to control the digestion of damaged tissue. It should be noticed that recruitment of microbicidal cells by MCP-1 is a mutually beneficial action, as it keeps infection in check, preventing parasite overgrowth, which would kill both the host and *T. cruzi*.

However, microbicidal cells are not the only ones recruited by MCP-1, as it also attracts anti-inflammatory cells; reparative immune cells, exemplified by regulatory T cells (54); and neural crest-derived cardiac stem/progenitor cells that differentiate into cardiomyocytes after myocardial infarction (55). It may therefore very well be that *T. cruzi* PDNF-induced MCP-1 expression in cardiomyocytes and cardiac fibroblasts (Fig. 1 to 3) and in the

FIG 5 Intravenous sPDNF upregulates MCP-1 and FKN in the heart and other organs of wild-type and MyD88^{-/-} mice. (a to d) Wild-type C57BL/6 mice. (a) A single i.v. dose of sPDNF increases MCP-1 and FKN transcript levels in the heart with a pulse-like response. Groups of mice (2 per group) were injected with vehicle (Veh) or sPDNF (150 μ g/mouse) and sacrificed at 3, 6, 9, and 12 h postinjection, and cardiac MCP-1 and FKN transcripts were quantified by qPCR. Points represent the mean $\pm$ SD fold change in transcript levels relative to those in vehicle-injected mice. The experiment was repeated three times with similar results each time; the liver also responded with similar pulse-like kinetics (data not shown). (b) Organ distribution of the MCP-1 increase following a single i.v. dose of sPDNF. The experimental design was analogous to that summarized in the legend to panel a. (c and d) A single i.v. dose of sPDNF augments MCP-1 and FKN transcripts dose dependently in the heart (c) and liver (d). Groups of three mice were injected with various doses of sPDNF, and the heart and liver were harvested at 3 h postinjection to quantify MCP-1 and FKN by qPCR. The results are plotted as a composite of the results of three distinct experiments. The increase in hepatic FKN mRNA was not statistically significant. PI, postinfection. (e to h) TLR signaling-deficient MyD88^{-/-} mice. MyD88^{-/-} mice (two per group) were injected i.v. with vehicle (PBS) or sPDNF (100 μ g per mouse) and sacrificed at 3 h postinjection, and MCP-1 mRNA was quantified in the heart (e) and liver (g) by qPCR, as was FKN mRNA (f and h, respectively). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

myocardium (Fig. 4 and 5) reflects cardioprotection and cardiac repair in acutely infected hearts.

The hypothesis of an anti-inflammatory and repair function for MCP-1 induced in response to PDNF is consistent with the findings of other studies that unequivocally demonstrate a cardioprotective effect of MCP-1 signaling and with those of studies showing a link between nervous system signaling and inflammation (56). Overexpression of MCP-1 in the heart, under the control of the α -cardiac myosin heavy chain promoter, gives rise to

transgenic mice that are significantly protected against cardiac dysfunction and remodeling after myocardial infarction (57) and severe heart injury caused by ischemia/reperfusion (58).

The hypothesis of an anti-inflammatory/repair function of *T. cruzi* PDNF via MCP-1 upregulation is consistent with the repair function of FKN, whose expression is also increased by sPDNF. FKN, in contrast to MCP-1, has rarely been studied in *T. cruzi* infection settings. A PubMed search in February 2014 using the words “fractalkine CX3CL1 *Trypanosoma cruzi*” revealed only one

FIG 6 Multiple intravenous injections of sPDNF increase the transcript levels of MCP-1 and FKN receptors CCR2 and CX3CR1 in the heart and liver. (a and b) Single i.v. doses of sPDNF. Mice (3 per group) were injected with various i.v. doses sPDNF and sacrificed at 3 h postinjection, and the transcript of the MCP-1 receptor CCR2 in the heart and liver was quantified by qPCR (a), as was the transcript of the FKN receptor CX3CR1 (b). Note that receptor transcript levels did not increase following a single i.v. dose of sPDNF at 3 h postinjection or 24 h postinjection (data not shown). (c and d) Multiple i.v. doses of sPDNF. Mice (3 per group) were injected i.v. with vehicle (Veh) or sPDNF (25 μg per injection) at 0, 3, and 24 h and sacrificed 24 h after the last injection. CCR2 mRNA in the liver, colon, and heart (ventricles and atria) was quantified by qPCR (c), as was CX3CR1 mRNA (d). Notice a statistically significant upregulation of MCP-1 and FKN receptors in the heart and liver 48 h after the start of three doses of i.v. sPDNF spaced within a 24-h span. *, $P < 0.05$; **, $P < 0.01$.

paper (59), yet FKN/CX3CR1 signaling is widely recognized as an important mechanism in tissue repair, including in the healing of the myocardium (46) and the brains of animal models of Parkinson's disease (60) and other neurodegenerative diseases (61).

FKN is widely accepted to be a pivotal mechanism in tissue repair, as it attracts reparative Ly-6C^{low} monocytes through CX3CR1 recognition in the second phase of inflammation, following the inflammatory Ly-6C^{high} monocyte waves (53). Ly-6C^{low} monocytes control inflammatory responses and tissue damage and promote healing via myofibroblast accumulation, deposition of collagen, and angiogenesis through the secretion of vascular endothelial growth factor (VEGF) (46). Thus, increased FKN expression in *ex vivo* cardiomyocytes and cardiac fibroblasts (Fig. 1 to 3), acutely infected hearts (Fig. 4b), or the myocardium

following i.v. sPDNF (Fig. 5) may reflect a tissue-healing effect of the PDNF-Trk receptor interaction. This hypothesis is consistent with the findings of our recently published work indicating that sPDNF is cardioprotective via a paracrine mechanism (NGF secretion via TrkC activation) and an autocrine mechanism (direct TrkA activation) (35). The concept that FKN controls tissue repair following myocardial injury is consistent with the observed sustained expression of FKN, but not that of MCP-1, in the absence of an overt cardiac parasite burden (Fig. 4).

Unlike broad expression-like kinetics, pulse-like expression of growth factors administered directly into tissues is a sought-after therapeutic property, as best exemplified by intracardiac injection of chemically modified VEGF-A mRNA, which is translated in a transient, pulse-like fashion and, as such, is much more effective in directing the fate of cardiac progenitor cells and in promoting vascular regeneration after myocardial infarction than intracardiac injection of VEGF-A DNA, which gives rise to broad and sustained VEGF-A expression (62). Therefore, like modified paracrine factor VEGF-A mRNA, pulse-like kinetics in the upregulation of cardioprotective growth factor NGF (35, 36) and of MCP-1 and FKN (Fig. 5) in response to sPDNF administration raise the possibility of an application for sPDNF as an effective therapeutic for injured myocardium, such as in chronic Chagas' cardiomyopathy, and other tissues where i.v. sPDNF triggers tissue repair mechanisms. Given that sPDNF effectively works following i.v. administration (see reference 35 and Fig. 5 and 6), it may be a better biological therapeutic than growth factors administered in the form of modified mRNA or DNA that require direct injection into the heart or relevant organ, which requires surgery.

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