RESEARCH ARTICLE

Chronic treatment of mice with leukemia inhibitory factor does not cause adverse cardiac remodeling but improves heart function

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ABSTRACT. Recent evidence suggests that the IL-6 family cytokine, leukemia inhibitory factor (LIF) is produced by cardiac cells under stress conditions including myocardial infarction and heart failure. Additionally, short-term delivery of LIF has been shown to have preconditioning effects on the heart and to limit infarct size. However, cell culture studies have suggested that LIF may exert harmful effects on cardiac myocytes, including pathological hypertrophy and contractile dysfunction. Long-term effects of LIF on the heart *in vivo* have not been reported and were the focus of this study. Adult male mice were injected daily with LIF (2 μg/30 g) or saline for 10 days. LIF treatment caused an approximate 11% loss in body weight. Cardiac function as assessed by echocardiography was improved in LIF-treated mice. Ejection fraction and fractional shortening were increased by 21% and 32%, respectively. No cardiac hypertrophy was seen on histology in LIF-treated mice,, there was no change in the heart-to-tibia length ratio, and no cardiac fibrosis was observed. STAT3 was markedly activated by LIF in the left ventricle. Different effects of LIF were seen in protein levels of genes associated with STAT3 in the left ventricle: levels of SOD2 and Bcl-xL were unchanged, but levels of total STAT3 and MCP-1 were increased. There was a trend towards increased expression of miR-17, miR-21, and miR-199 in the left ventricle of LIF-treated mice, but these changes were not statistically significant. In conclusion, effects of chronic LIF treatment on the heart, although modest, were positive for systolic function: adverse cardiac remodeling was not observed. Our findings thus lend further support to recent proposals that LIF may have therapeutic utility in preventing injury to or repairing the myocardium.

Key words: cardiac remodeling, cytokine, cardiac dysfunction, JAK STAT signaling, cardiac repair, cardiac hypertrophy

Leukemia inhibitory factor (LIF) is a member of the interleukin 6 (IL-6) family of cytokines that signal through the transmembrane protein gp130. LIF is produced by cardiac myocytes and is reported to have protective effects on heart cells. For example, pretreatment of adult or neonatal cardiac myocytes with LIF protected against hypoxia-reoxygenation or doxorubicin-induced injury [1-4]. LIF treatment was also shown to protect against myocardial IR injury or infarction [5, 6]. These beneficial actions of LIF are attributed in part to the stimulation of angiogenesis and upregulation of SOD2 (MnSOD), Bcl-xL, and VEGF [2, 3, 5-8]. In cultured cardiac myocytes, LIF was observed to protect against oxidative stress by inducing activation and translocation of Akt/protein kinase B (PKB) to mitochondria [9, 10]. Akt is a serine/threonine-specific protein kinase involved in both the mediator and effector phases of cardiac ischemic preconditioning [11]. One target of Akt in response to LIF stimulation is hexokinase II (HK-II), which is phosphorylated and translocates to mitochondria [9]. In this way, HK-II stabilizes mitochondria against oxidative stress-induced depolarization and limits mitochondrial ROS production [12].

On the other hand, LIF was also shown to have effects on the growth, metabolism, contractility, and Ca^{2+} handling of cardiac myocytes that could be characterized as disadvantageous; however, these studies relied on cultured cells or isolated muscle and thus the physiological significance of these effects is uncertain [13-22]. Numerous reports have implicated IL-6 in the adverse cardiac remodeling associated with hypertension and myocardial infarction, as well as heart failure [23-26]. However, while IL-6 signals through gp130 homodimers, LIF signals through gp130 heterodimers with the LIF receptor, with notable consequences, such as a more sustained activation of STAT3 with LIF [27].

From the preceding discussion, it is clear that uncertainty surrounds the long term consequences of LIF exposure on

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the heart. As a first step in establishing whether LIF may have therapeutic utility in preventing injury to the heart or repairing the injured myocardium, we carried out an *in vivo* study giving mice daily, intraperitoneal (IP) injections of LIF (2 μg/30 g) over 10 days. Our hypothesis was that chronic treatment of mice with LIF would not adversely affect cardiac function or induce adverse cardiac remodeling.

METHODS

Materials

Antibodies for total STAT3 (Cat. #9139), pY705 STAT3 (Cat. #9131), MCP-1 (Cat. #2029), and BcL-XL (Cat. #2764) were from Cell Signaling Technology (Danvers, MA, USA). Antibodies for SOD2 (sc-137254) and GAPDH (sc-25778/sc-166545) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Murine recombinant LIF was from Millipore (Billerica, MA, USA). Kinase extraction buffer (BP-116K) and activated vanadate were purchased from Boston BioProducts (Worcester, MA, USA). Protease inhibitor cocktail (P8340) was from Sigma-Aldrich (St. Louis, MO, USA).

Experimental protocol

We used male C57BL/6 mice (2-3 months old), which were divided into two groups: saline or control group (n = 4) and LIF-treated group (n = 4). Mice were injected IP with 200 μL LIF (2 μg per 30 g body weight) or saline. Mice received one injection daily for 10 days. This dosing protocol was shown to produce a sustained increase in plasma LIF of >1,000 U/mL for approximately three hours that remained above baseline for longer than six hours in mice (28). Cardiac function was assessed by echocardiography at day 0 and 10. On day 10, mice were weighed, echocardiography was done, mice were injected, and two hours later, hearts were harvested and weighed. The tibias were collected. Cardiac hypertrophy was assessed by measuring the heart to body weight and the heart weight-to-tibia length ratios; the left ventricles were processed for Western blot analysis, microRNA expression, and histology

Echocardiography

Assessment of cardiac function was performed with a Vevo 770 high resolution *in vivo* imaging system (VisualSonics). Measurements were made with a 707B RMV scanhead, with a center frequency of 25 MHz and a frequency band ranging from 12.5 to 37.5 MHz. The probe used was a single-element, mechanical vector probe. For the procedure, mice were maintained at 37°C and anesthesia induced with 1.5% isoflurane and oxygen (3 L/min). Mice were placed on a prewarmed EKG transducer pad and body temperature monitored. Heart rate was monitored via the EKG transducer pad. Two-dimensional B-mode parasternal long axis views were obtained to visualize the aortic and mitral valves. The transducer was then rotated clockwise 90° to obtain a parasternal short axis view. Ejection fraction (EF) and fractional shortening (FS) were analyzed and calculated using the VisualSonics advanced cardiovascular measurements package. Left ventricular (LV) wall dimensions were determined from M-mode images.

MicroRNA analysis

Total RNA was isolated from hearts and purified using miRNeasy Mini Kit from Qiagen (Cat. # 217004). To quantify miRNA levels, cDNA was reverse transcribed from total RNA samples using specific miRNA primers from the TaqMan MicroRNA Assays, and reagents from the Taq Man MicroRNA Reverse Transcription kit (Applied Biosystems). The resulting cDNA was amplified by PCR using TaqMan MicroRNA Assay primers with the TaqMan Universal PCR Master Mix and analyzed with Bio-Rad's CFX96 Touch^{TM} Real-Time PCR Detection System. Relative levels of miRNA expression were calculated using the ΔΔCt method and normalized to the signal of microRNA U6.

Histology

Hearts preserved in 10% formalin were embedded in paraffin, sectioned (5 μm thickness), and stained with Masson's Trichrome stain for collagen and myocardial morphology. Both cross and longitudinal sections (3-4 each) were prepared for each heart. Regions of the left and right ventricle were imaged with a Nikon E-600 and images collected with a 40X (NA = 0.75) objective.

Western blots

Whole-cell lysates were prepared by scraping cells into ice-cold RIPA-based buffer with 10 mM vanadate and protease inhibitor cocktail. Lysates were cleared by centrifugation at 100,000 g for 20 min at 4°C. Samples containing equal amounts of protein in Laemmli's-SDS reducing buffer were separated by SDS-PAGE. Separated proteins were blotted onto nitrocellulose membranes and the immunoreactive bands quantified using the Li-COR Odyssey infrared imaging system.

Statistical analysis

Results are expressed as mean ± SEM for n number of mice. Statistical significance involving single comparisons was determined by Student's t-test. For multiple comparisons, ANOVA followed by an appropriate *post hoc* test as noted was performed. A $P<0.05$ was taken as significant.

RESULTS

LIF treatment increased STAT3 activation and total STAT3 protein levels

LIF is a potent activator of STAT3 in cardiac myocytes. As may be seen from *figure 1*, the left ventricles of mice receiving LIF showed marked levels of STAT3 activation noted as the phosphorylation of either Y705 or S727, normalized to total STAT3 protein levels. In addition, LIF-treated mice expressed higher levels of total STAT3 in the left ventricle (*figure 1*), which agrees with reports that activated STAT3 induces its own expression (29). However, no changes were seen in the expression levels of two cardiac protective proteins that others have linked to STAT3 activation in the heart, SOD2 and Bcl-xL (*figure 2*).

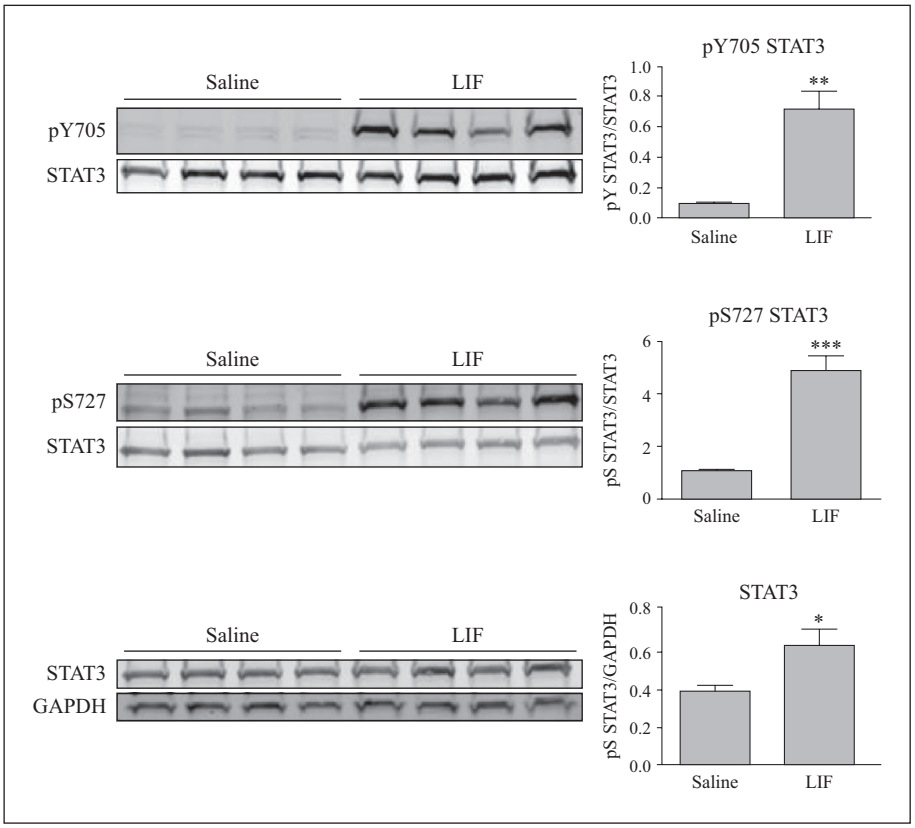

Figure 1

LIF treatment activated STAT3 in the left ventricles and increased total STAT3 levels. Mice received either 200 μL LIF (2 μg per 30 g body weight) or saline, daily by IP injection for 10 days (four mice per group). On the last day, hearts were extracted and the left ventricles processed for Western blot analysis for STAT3 Y705 and S727 phosphorylation, indicative of activation. Results were quantified by the Li-COR Odyssey system and normalized to STAT3 protein levels determined on the same blot (upper two panels). In a separate blot, STAT3 expression levels were normalized to GAPDH protein levels. *P<0.05, **P<0.01, ***P<0.001 (Student's t-test).

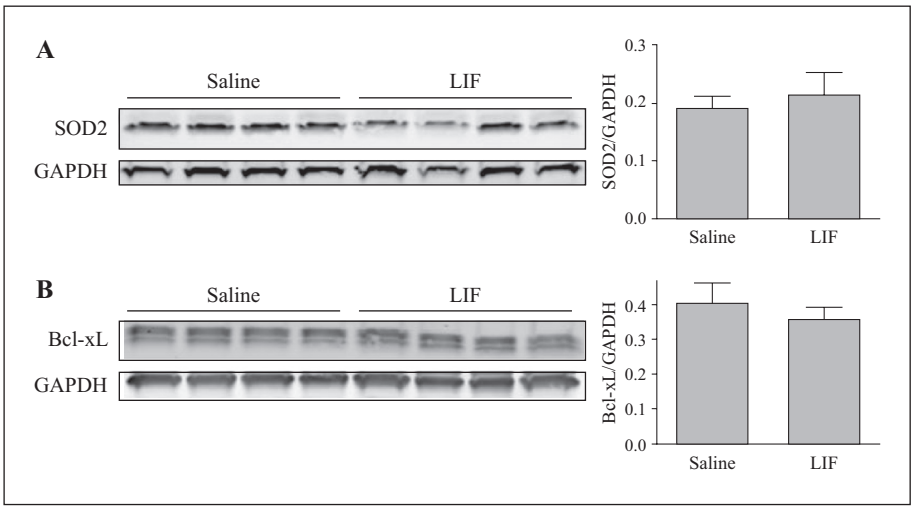

Figure 2

LIF treatment did not change expression levels of SOD2 or Bcl-xL. Mice received either 200 μL LIF (2 μg per 30 g body weight) or saline, daily by IP injection for 10 days (four mice per group). On the last day, hearts were extracted and the left ventricles processed for Western blot analysis for (A) SOD2 and (B) Bcl-xL. Results were quantified by the Li-COR Odyssey system and normalized to GAPDH protein levels determined on the same blot. No difference was seen between saline- and LIF-treated mice.

LIF treatment improved cardiac function but did not cause hypertrophy

As seen from *table 1*, mice receiving LIF for 10 days lost approximately 11% of body weight, which is consistent with reports that LIF, like other IL-6 family cytokines, is associated with cachexia (30). However, we did not see any abnormalities in the physical appearance or behavior of mice receiving LIF. No cardiac hypertrophy was noted as indexed by the heart-to-body weight ratio or the heart weight-to-tibia length ratio (*table 1*). Moreover, we did not find any changes in the left ventricular dimensions measured by echocardiography: LVIDd, left ventricular internal dimension at diastole; LVPWd, left

Table 1
Chronic LIF treatment of mice.

	Saline	**LIF**
Body weight day 0 (g)	29.0 ± 0.6 (4)	28.0 ± 0.0 (4)
Body weight day 10 (g)	28.8 ± 0.5 (4)	24.8 ± 0.2 (4)***
Heart weight/body weight, mg/g	5.07± 0.09 (4)	5.66 ± 0.17 (4)
Heart weight/tibia length, mg/mm	7.69 ± 0.34 (4)	6.86 ± 0.27 (4)
LVIDd, Day 0 (mm)	4.05 ± 0.18 (4)	3.75 ± 0.35 (3)
Day 10 (mm)	4.30 ± 0.15 (4)	3.74 ± 0.07 (3)
LVPWd, Day 0 (mm)	1.00 ± 0.11 (4)	1.08 ± 0.16 (3)
Day 10 (mm)	0.76 ± 0.04 (4)	1.13 ± 0.11 (3)
LVIDs, Day 0 (mm)	2.77 ± 0.24 (4)	2.46 ± 0.18 (3)
Day 10 (mm)	2.83 ± 0.11 (4)	2.15 ± 0.14 (3)
LVAWd, Day 0 (mm)	0.86 ± 0.04 (4)	0.70 ± 0.06 (3)
Day 10 (mm)	0.77 ± 0.08 (4)	0.88 ± 0.04 (3)

LVIDd, left ventricular internal dimension at diastole; LVPWd, left ventricular posterior wall dimension at diastole; LVIDs, left ventricular internal dimension at systole; and LVAWd, left ventricular anterior wall dimension at diastole. Values are mean ± SEM for (n) number of mice. ***P<0.001 *versus* LIF body weight day 0 (paired t-test).

ventricular posterior wall dimension at diastole; LVIDs, left ventricular internal dimension at systole; and LVAWd, left ventricular anterior wall dimension at diastole. On the other hand, our echocardiography results show that mice who received LIF treatment presented a significant increase in EF and FS for both individual and grouped data (*figure 3*). These findings indicate that, compared to what has already been described for IL-6 (23-26), LIF did not induce hypertrophy or adverse remodeling of the heart, but had more beneficial effects on heart function and contractility.

LIF treatment did not lead to fibrosis

No gross morphological differences were observed between the saline and LIF hearts (*figure 4*). There was no

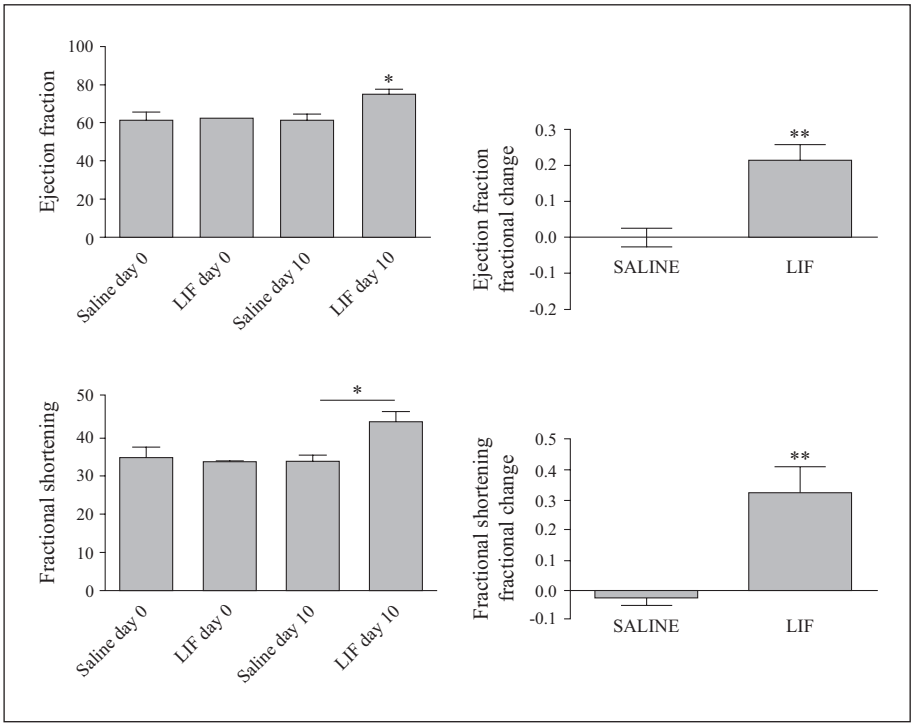

Figure 3
LIF treatment increased ejection fraction and fractional shortening. Mice received either 200 μL LIF (2 μg per 30 g body weight) or saline, daily by IP injection for 10 days. Echocardiography was performed the day before the first injection (Day 0) or immediately prior to the last injection (Day 10). Group data (mean ± SEM) are shown in the left panels and were analyzed by one-way ANOVA followed by Newman-Keuls (ejection fraction; *P<0.05 LIF Day 10 *versus* all other groups) or Tukey's (fractional shortening; *P<0.05 Saline Day 10 *versus* LIF Day 10) *post hoc* test. Fractional changes for individual mice are shown in the right panels. **P<0.01, Student's t-test.

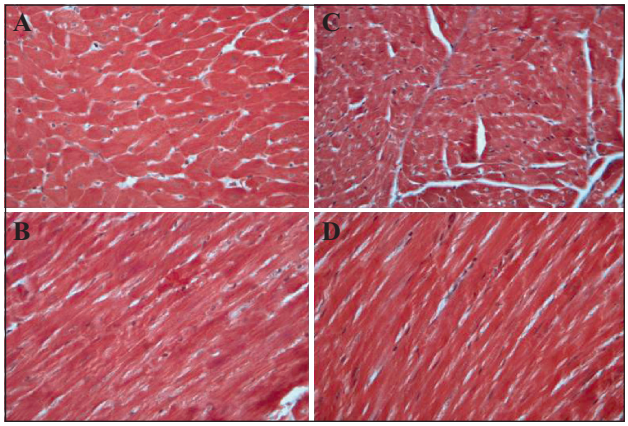

Figure 4
Trichrome staining of the myocardium of mice treated with saline or LIF. Saline control: **(A)** typical cross-section and **(B)** longitudinal-section morphology of the myocardium in the left ventricle. LIF-treated: **(C)** typical cross-section and **(D)** longitudinal-section morphology of the myocardium in the left ventricle.

fibrosis present in the LIF samples or saline controls. Also, there appeared to be no differences in cardiac myocyte morphology or myofibrillar structure between the controls and LIF-treated mice.

LIF treatment tended to increase levels of STAT3-associated microRNAs

STAT3 has been linked to the expression of three 3 miRNAs: miR-17, miR-21, and miR-199 [31]. All three of these miRNAs have been implicated in cardiac function or remodeling [32, 33]. As seen from *figure 5*, there was a trend towards increased expression of miR-17, miR-21, and miR-199 in the left ventricle of LIF-treated mice; however, these changes did not reach statistical significance.

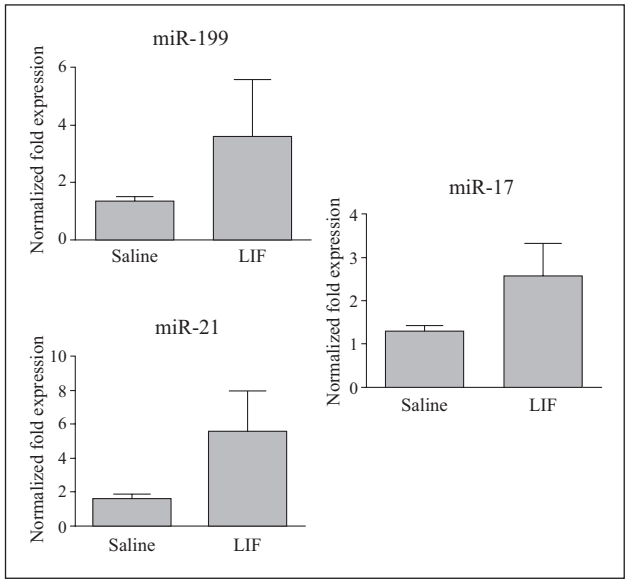

Figure 5
LIF treatment did not change expression levels of miR-17, miR-21, and miR-199. Mice received either 200 μL LIF (2 μg per 30 g body weight) or saline, daily for 10 days (four mice per group). On the last day, hearts were extracted and the left ventricles processed for micro-RNA extraction, cDNA synthesis, and real time PCR analysis. The values were normalized to the expression levels of micro-RNA U6.

LIF induced expression of MCP-1 protein

MCP-1 (monocyte chemoattractant protein-1), also known as chemokine (C-C motif) ligand 2 (CCL2), is a cytokine belonging to the CC chemokine family. MCP-1 is produced by macrophages and endothelial cells and plays an important role in the recruitment of inflammatory cells to sites of injury. This cytokine is upregulated in the heart during stress or injury. In the mouse, MCP-1 is heavily glycosylated and exists as a monomer or dimer [34, 35]. The anticipated molecular weight of the glycosylated monomer is ~30 kDa. MCP-1 can be extensively glycosylated on its C-terminus, which favors formation of the dimer. Our results show that mice who received LIF treatment expressed a higher order form of MCP-1 of ~66 kDa consistent with the glycosylated dimer (*figure 6*).

DISCUSSION

Several studies have reported that LIF induces a hypertrophic response in cultured cardiac myocytes [*e.g.,* 13]. However, to the best of our knowledge, whether or not LIF induces cardiac hypertrophy *in vivo* had not been determined. In the present study, we found that chronic administration of LIF did not induce cardiac hypertrophy in mice. This observation raises the possibility that the findings on cultured cells, which, after all, are obtained under contrived growth conditions (including serum-starvation), may not be readily transferable to the *in vivo* situation.
We observed an increase in EF and FS with LIF treatment of mice, suggesting that LIF positively affects cardiac contractility. This could have resulted from a direct effect on cardiac myocytes or an indirect effect on the adrenergic control of heart function. Consistent with the first possibility is the observation that LIF was reported to increase calcium entry through the L-type channel in

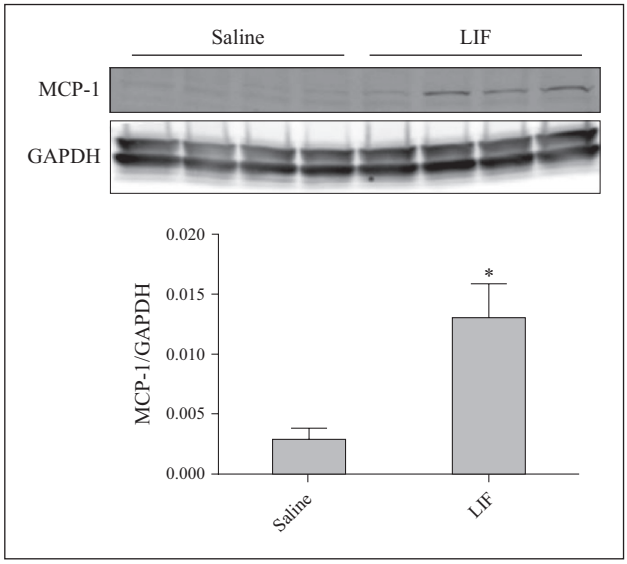

Figure 6
LIF treatment increased cardiac MCP-1 levels. Mice received either 200 μL LIF (2 μg per 30 g body weight) or saline, daily for 10 days (four mice per group). On the last day, hearts were extracted and the left ventricles processed for Western blot analysis for MCP-1. Results were quantified by the Li-COR Odyssey system and normalized to GAPDH protein levels determined on the same blot. Values are mean ± SEM, *P<0.05 (Student's t-test).

cultured cardiac myocytes [21]. Concerning the second possibility, it should be noted that cholinergic transdifferentiation of cardiac sympathetic nerves has been ascribed to LIF in rodents [36]. One may speculate that the pulsatile increases in blood levels of LIF expected in the present study [35] exerted a rebound effect on the sympathetic drive of the heart. Others have reported that IL-6 family cytokines have a negative effect on cardiac contractility due to an induction of nitric oxide synthase 2 (NOS2) [18]; however, in our study no induction of NOS2 was observed (data not shown). Our findings highlight an important point that is often overlooked with regard the IL-6 family cytokines. Although these cytokines share the gp130 signaling module and activate STAT3, they may exert disparate and even contrary effects on cells/tissues. We recently proposed that this is due not only to differences in intensity and duration of STAT3 activation, but to variability in the recruitment of concurrent signals [27].

As expected we found that STAT3 was activated in hearts of mice treated with LIF. Moreover, levels of STAT3 were increased, which is consistent with reports of STAT3-induced STAT3 expression [29]. Notably, no increase in expression levels of SOD2 or Bcl-xL was observed in the present study. In cultured cardiac myocytes, protective effects of LIF against oxidative stress induced by hypoxia/reoxygenation or doxorubicin were implicated by the upregulation of SOD2 and Bcl-xL, respectively [2, 3, 8]. SOD2 protects mitochondria against oxidative stress by eliminating superoxide, while Bcl-xL is a transmembrane mitochondrial protein that prevents apoptosis. LIF treatment did induce formation of MCP-1 (*figure 6*) and recent evidence indicates that MCP-1 may have direct, protective effects on cardiac myocytes [37, 38].

LIF treatment tended to increase levels of miR-17, miR-21, and miR-199a, although these changes did not reach statistical significance. Of the three, the evidence that STAT3 has a positive effect on the expression levels of miR-17 is perhaps the strongest; however, the role of miR-17 in the adult heart has not been well studied. There is evidence to suggest that miR-17 has a role in endothelial cell proliferation [39]. MiR-21 is highly expressed in cardiac fibroblasts [40]. This miRNA promotes cardiac growth [41, 42] and has been shown to play a role in blocking apoptosis by targeting programmed cell death 4 (PCDC4) protein in hydrogen peroxide-treated cardiac myocytes [43]. miR-199a has been implicated in maintaining cell size in cardiac myocytes and in regulating cardiac hypertrophy [44].

In conclusion, under these experimental conditions, the presence of LIF did not result in the formation of fibrosis in the myocardium. Additionally, LIF did not appear to have any effect on cardiac myocyte morphology or myofibrillar integrity. Rather, the effects of chronic LIF treatment on the heart, although modest, were positive for systolic function as demonstrated by an increase in EF and FS. In addition, we saw an increase in MCP-1, which may be protective, and a trend towards an increase in protective miRNAs. Our findings thus lend further support to recent proposals that LIF may have therapeutic utility in preventing injury to or repairing the myocardium.

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