International Journal of Cardiology 228 (2017) 394–400

Contents lists available at ScienceDirect

International Journal of Cardiology

journal homepage: www.elsevier.com/locate/ijcard

The cardiac maladaptive ATF3-dependent cross-talk between cardiomyocytes and macrophages is mediated by the IFNγ-CXCL10-CXCR3 axis

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ARTICLE INFO

Article history:
Received 4 September 2016
Accepted 6 November 2016
Available online 10 November 2016

Keywords:
Cardiac remodeling
Pressure overload
Phenylephrine
ATF3
Macrophages
Cardiomyocytes

ABSTRACT

Rational: Pressure overload induces adaptive and maladaptive cardiac remodeling processes in the heart. Part of the maladaptive process is the cross-talk between cardiomyocytes and macrophages which is dependent on the function of the Activating Transcription Factor 3, ATF3. Yet, the molecular mechanism involved in cardiomyocytes-macrophages communication leading to macrophages recruitment to the heart and cardiac maladaptive remodeling is currently unknown.

Methods and results: Isolated peritoneal macrophages from either wild type or ATF3-KO mice were cultured in serum free medium to collect conditioned medium (CM). CM was used to probe an antibody cytokine/chemokine array. The interferon γ induced protein 10 kDa, CXCL10, was found to be enriched in wild type macrophages CM. Wild type cardiomyocytes treated with CXCL10 in vitro, resulted in significant increase in cell volume as compared to ATF3-KO cardiomyocytes. In vivo, pressure overload was induced by phenylephrine (PE) infusion using micro-osmotic pumps. Consistently, CXCL11 (CXCL10 competitive agonist) and CXCL10 receptor antagonist (AMG487) attenuated PE-dependent maladaptive cardiac remodeling. Significantly, we show that the expression of the CXCL10 receptor, CXCR3, is suppressed in cardiomyocytes and macrophages derived from ATF3-KO mice. CXCR3 is positively regulated by ATF3 through an ATF3 transcription response element found in its proximal promoter. Finally, mice lacking CXCR3 display a significant reduction of cardiac remodeling processes following PE infusion.

Conclusions: Chronic PE infusion results in a unique cardiomyocytes-macrophages cross-talk that is mediated by IFNγ. Subsequently, macrophages that are recruited to the heart secrete CXCL10 resulting in maladaptive cardiac remodeling mediated by the CXCR3 receptor. ATF3-KO mice escape from PE-dependent maladaptive cardiac remodeling by suppressing the IFNγ-CXCL10-CXCR3 axis at multiple levels.

1. Introduction

Cardiac remodeling occurs following various cardiac stress conditions such as volume and pressure overload. Although, remodeling initiates as an adaptive process, it deteriorates into maladaptive changes leading to heart failure [1–4]. Understanding the molecular mechanisms that are responsible for the switch to maladaptive process will enable to design novel therapeutic means to block heart failure. A case in point, is the cardiac maladaptive change that involves the cross-talk between the heart and macrophages in a pressure overload mice model [5]. The Activating Transcription Factor 3, ATF3, plays a key role in macrophage recruitment and activation. ATF3 is an immediate early gene from the basic leucine zipper (bZIP) family. Ectopic cardiac-specific expression of ATF3 during embryonic phase results in atrial enlargement and premature death in transgenic mice [6]. Consistently, adult ATF3 expression is sufficient to induce ventricular hypertrophy even in the absence of additional insults such as pressure overload. ATF3 expression is highly induced specifically in cardiomyocytes following acute challenge with endothelin, isoproterenol, PE and angiotensin II and following ischemia reperfusion challenge [7–9]. In aortic banding model, mimicking pressure overload, ATF3 expression was found to be cardiac protective [10]. In contrast, cardiomyocytes specific ATF3 transgene was sufficient for promoting maladaptive cardiac remodeling processes [6]. Consistently, following PE infusion, as a pressure overload model in mice, cardiac ATF3 expression was found to promote cardiac hypertrophy [5,6]. In the latter, we have identified a unique ATF3-dependent cross-talk between cardiomyocytes and macrophages that contribute to maladaptive cardiac remodeling [5]. Yet, the molecular mechanism involved in cardiomyocytes-macrophages communication leading to macrophages recruitment to the heart and cardiac maladaptive remodeling is unknown.

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http://dx.doi.org/10.1016/j.ijcard.2016.11.159
0167-5273/

Here, we studied the molecular mechanisms involved in cardiomyocytes-macrophages cross-talk and describe the role of IFNγ-CXCL10-CXCR3 axis in mediating the maladaptive cardiac remodeling processes in response to pressure overload. ATF3 appears as the master regulatory gene controlling the expression of all the components along the axis in the two communicating cells; cardiomyocytes and macrophages.

2. Materials and methods

2.1. Mice

This study was carried out in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Institute of Health. The protocol was approved by the Committee of the Ethics of Animal Experiments of the Technion. Surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. The animals were fed standard chow containing 0.5% NaCl and tap water ad libitum. All mice genotypes were backcrossed to C57Bl/6 background for at least 8 generations. Mice used in this study: ATF3-KO [11] and CXCR3-KO (Jackson Laboratory strain # 005796) [12]. Male mice were used in all the experiments performed in this study.

2.2. Micro-osmotic pump implantation

Alzet micro-osmotic pumps (#1002, Alzet) were filled with either PE (100 mg/kg/day, 0.06% acetic acid in saline), CXCL10 (2.4 μg/25 g/day), CXCL11 (2.5 μg/25 g/day, cat#250-29, Peprotech) or AMG487 (2.5 mg/25 g/day, cat # 4487, Tocris). Eight-week-old male mice were anesthetized with sodium pentobarbital and were subcutaneously implanted with pumps. The procedure was performed under sterile conditions. The mice were weighed and sacrificed 7–14 days following implantation as indicated. The genotypes and treatments were blinded to the person who sacrificed the mice and harvested the hearts. Ventricle weights were determined after separation from the atria (referred to hereafter as "ventricle weight", VW). The ventricles were then divided into three parts that were used for protein extraction, RNA purification and single cell suspensions.

2.3. Macrophage extraction

Thioglycollate stimulation and macrophage harvest - C57Bl/6 or ATF3-KO mice were intraperitoneally injected with 3 ml of 4% Thioglycollate in brewer modified (BD) medium diluted in saline. Following 3 days, mice were euthanized and 5 ml of cold PBS was injected into the peritoneal cavity through the exposed peritoneal membrane using a sterile syringe and 22-gauge needle. The macrophage enriched fluid from the peritoneal cavity was carefully removed with a 22-gauge needle.

2.4. Conditioned medium preparation

Peritoneal macrophages were cultured in a concentration of 10^6 cells/ml in DMEM (Dulbecco's modified Eagle's medium, Sigma-Aldrich) supplemented with 100 i.u./ml penicillin and 0.1 μg/ml streptomycin and grown at 37 °C and 5% CO_2 for 48 h. Conditioned medium was removed, centrifuged at 1500 rpm for 5 min and the supernatant was aliquoted and kept at −20 °C.

2.5. Adult cardiomyocyte cell culture

Adult cardiomyocytes were isolated from 12 to 20-week-old C57Bl/6 wild type or ATF3-KO mice as described [13]. Mice were injected intraperitoneally with heparin (100 USP units/mouse), followed by a mixture of 100 mg/g ketamine and 8 mg/g xylazine. After 20 min, the heart was removed, cannulated via the ascending aorta, and mounted on a modified Langendorff perfusion apparatus. Isolated cells were plated in culture medium (MEM-Eagle Hanks' salts base (biological industries, Israel) supplemented with penicillin 100 U/ml, glutamine 2 mM, $NaHCO_3$ 4 mM, HEPES 10 mM, bovine serum albumin 0.2%, and 25 mM blebbistatin (cat # B0560, Sigma-Aldrich). Plating was performed on 22-mm^2 glass coverslips coated with 100 μl/coverslip of 56 nM mouse laminin (#L2020, Sigma-Aldrich) for 30–40 min. The plating density was 20,000 cells/coverslip, 60 cells/mm^2.

2.6. Immunofluorescence

Adult cardiomyocytes were plated on glass cover-slips coated with laminin. After cellular stimulation, cells were fixed with 4% formaldehyde for 10 min. After washing with PBS, the cells were permeabilized with 0.1% Triton X-100 for 5 min and incubated in a blocking solution of 5% FCS in PBS for 30 min. The cells were then incubated with anti-α-actinin (A7811, Sigma) antibody overnight in PBS containing 1% FCS. The cells were washed three times with PBS and incubated with a secondary fluorescent antibody mix (Jackson Laboratories) for 1 h in PBS containing: 2% BSA, 2% FCS, and 0.1% Tween-20. The cells were washed twice with PBS and processed for nuclear staining using DAPI (#D9542, Sigma-Aldrich) at a final concentration of 1 μg/μl in PBS. The stained cells were then washed twice with PBS and mounted in Fluoromount-G (Southern Biotechnology, Birmingham, AL, 0100-01).

2.7. Confocal microscopy

Fluorescence microscopy was performed using the Zeiss LSM 700 upright confocal microscope (Thornwood, NY) equipped with an X40/1.3 NA oil objective, and solid-state lasers (wavelengths of 405 to 639 nm). Images were acquired in z-stack in order to quantify cell volume using the Imaris software.

2.8. Flow cytometry acquisition and analysis

Single cell suspensions in Hanks' balanced salt solution (HBSS) from cardiac tissue were prepared using a MACS dissociator according to the manufacturer's instructions. Cells were stained for Macrophages using Gr1-$CD11b^+F4/80^+$surface markers. All antibodies were purchased from BioLegend (San Diego, CA). Fluorescence activated cell sorting (FACS) analysis was performed using Cyan-ADP flow cytometer (Beckman Coulter, Nyon, Switzerland) and analyzed with Summit Version 4.3 (Beckman Coulter).

2.9. mRNA extraction

mRNA from total hearts was purified using an Aurum total RNA fatty and fibrous tissue kit (#732-6830, Bio-Rad) according to the manufacturer's protocol. mRNA from mice cardiomyocytes and peritoneal macrophages was purified using a high purity RNA isolation kit (#11828665001, Roche) according to the manufacturer's protocol mRNA was quantified by measuring Ab260 nm with a nanodrop spectrophotometer (ND-1000, NanoDrop Technologies, Rockland, DE, USA).

2.10. Quantitative real time PCR (qRT-PCR)

cDNA was synthesized from mRNA samples using 800 ng of RNA for hearts, 100 ng RNA for macrophages, and 50 ng for cardiomyocytes in a 20 μl total reaction mix of high-capacity cDNA reverse transcription kit (#1708891, Bio-Rad). Real-time PCR was performed using Rotor-Gene 6000TM (Corbett) equipment with absolute blue SYBER green ROX mix (#1725125, Bio-RAD). Serial dilutions of a standard sample were included for each gene to generate a standard curve. Values were normalized to GAPDH expression levels. Primer sequences used for qRT-PCR are shown in Table 1.

2.11. Western blotting

Cells were lysed in whole-cell extract (WCE) buffer (25 mM HEPES, pH 7.7, 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM DTT, 20 mM β-glycerolphosphate, 0.1 mM Na_2VO4, 100 μg/ml PMSF, protease inhibitor cocktail 1:100; Sigma-Aldrich, P8340).

Harvested tissues were homogenized in RIPA buffer (1% NP-40, 5 mg/ml Na-deoxycholate, 0.1% SDS in PBS) supplemented with protease inhibitor cocktail (P-8340, Sigma-Aldrich) and 1 mM DTT, 2 mM PMSF, 20 mM β-glycerolphosphate, 0.1 mM Sodium vanadate, 20 mM PNPP, and PhosStop (cat # 04906837001, Roche). Homogenization was performed at 4 °C using the Bullet Blender homogenizer (BBX24; Next advance) according to the manufacturer's instructions. Next, lysates were centrifuged at maximal speed for 10 min and supernatants were frozen at −80 °C.

The proteins (30 μg of cell lysate or 80 μg of tissue lysate) were separated by 12.5% SDS-PAGE, followed by Western blot analysis. Nitrocellulose membranes were blocked in 5% dry milk in PBS and washed three times for 5 min in PBS. Primary antibodies used were anti-α-tubulin (T-9026, Sigma-Aldrich) 1:2000, CXCR3 1:500 (ab-71864,abcam) and anti-ATF3 1:100 [9]. Primary antibodies were incubated for at least 1 h at 4 °C. Membranes were washed and then incubated with HRP-conjugated secondary antibodies (Sigma-Aldrich). Detection was performed using ECL.

2.12. Mouse cytokine array

Conditioned medium was applied onto mouse cytokine array (Proteome Profiler Mouse Cytokine Array Kit, Panel A, cat # ARY006,R&D systems), according to the manufacturer's instructions. Results were quantified using TotalLab Quant program software.

2.13. Evaluation of murine CXCL10 protein levels in the plasma

Thawed conditioned medium was applied onto a specific Mouse CXCL10/IP10/CRG-2 DuoSet ELISA kit (cat # DY466, R&D systems), according to the manufacturer's instructions.

CXCL10-IgG used was purified according to [14,15].

2.14. Tissue culture and cell lines

HEK-(human embryonic kidney) 293T cells were grown in DMEM (Dulbecco's modified eagle's medium) supplemented with 4.5 g/l D-glucose containing 10% fetal bovine serum, 100 unit/ml penicillin and 100 μg/ml streptomycin, grown in a 5% CO_2 incubator.

2.15. Transfection

HEK-293T cells were co-transfected with the appropriate expression plasmid (PCEFL ATF3-HA, 2 μg) and the appropriate reporter plasmid (PGL3 promoter, PGL3 CXCR3 promoter or PGL2-1351 ATF3 [16], 2 μg using the calcium phosphate (Ca_2PO_4) method. The

total amount of plasmid DNA was adjusted to 10 μg with an empty PCEFL-HA vector. The culture medium was replaced with fresh medium 4 h post-transfection and harvested 24 h thereafter.

2.16. Plasmids

pCEFL HA-ATF3 a mammalian expression plasmids encoding the wild-type ATF3.

pGL3 promoter luciferase reporter plasmid (Promega Inc.) was used to insert KpnI-XhoI fragments derived from the -500-1 CXCR3 promoter as indicated. The primers used in this study are shown:

−500-(−1) KpnI Top
5′ GCGG-GGTACC-AAATGGGATGCGGGACACAATGG 3′
−500-(−1) XhoI Bottom
5′ CCGCTCGAGTTGAGAGTTCATGCTCCTCTTGCC 3′
−250-(−1) KpnI Top
5′ GCGGGGTACCACACACAGCTCCTGACCTCGTCTA 3′
−500-(−1) XhoI Bottom
5′ CCGCTCGAGTTAGGAATCAGGGGAGGTA 3′
−325-(−280) KpnI TOP
5′GCGGGGTACCCTCTATAGTCAGTCAAGTGCCAAGTCATCTACATCCTGACTGGCGCTCG AGCGG 3′
−325-(−280) XhoI Bottom
5′CCGCTCGAGCGCCAGTCAGGATGTAGATGACTTGGCACTTGACTGACTATAGAGGGTAC CCCGC 3′

2.17. Reporter assay

Luciferase reporter assays were performed with the luciferase kit (Catalog # E1500 Promega Inc.) according to the manufacturer's instructions.

2.18. Statistical analysis

Data is presented as means ± SEM in (n) number of experiments.

Differences were analyzed using one-tailed Student's *t*-test, with an assumption of equal variance.

The ratio calculation was analyzed by a χ^2 test for expected-versus-observed ratio.

P values <0.05 were considered significant unless otherwise indicated.

3. Results

3.1. Identification of CXCL10 in Wt. conditioned medium

Previously we have shown that PE infusion recruits macrophages to the heart mediating a maladaptive cardiac remodeling process [5]. Here, we sought to study the molecular mechanism involved in the cardiomyocytes-macrophages cross-talk. Towards this end, wild type and ATF3-KO peritoneal macrophages were isolated 3 days following thioglycolate injection. Macrophages were cultured for 48 h in serum free media and conditioned medium (CM) was collected. We used wild type primary cardiomyocytes culture to examine the effect of macrophages CM. The addition of wild type CM resulted in a significant 20% increase in cardiomyocytes cell size as compared to serum free medium alone (Fig. 1A). This result suggested that macrophages secret a soluble factor that is able to promote cardiomyocytes growth. To identify putative macrophages soluble factor/s, we probed antibody cytokine/chemokine array nitrocellulose filters containing 40 different antibodies with CM purified from either wild type or ATF3-KO macrophages. We identified a single cytokine, CXCL10, that is present at 4.7-fold higher levels in macrophages CM derived from wild type as compared to ATF3-KO (Fig. 1B). Using an ELISA assay, we validated the existence of higher levels of CXCL10 in wild type derived macrophages CM (Fig. 1C). CXCL10 is a cytokine that is induced in response to IFNγ [17,18]. To examine whether IFNγ is induced following PE treatment, mice were challenged with either an acute PE injection (1 h) or chronic PE infusion (14 days) and the mRNA levels of IFNγ were analyzed by qRT-PCR (Fig. 1D). The IFNγ mRNA levels in the heart of untreated mice were modestly higher in ATF3-KO mice as compared to wild type mice. Acute PE treatment highly induced the levels of IFNγ by 4-fold in wild type but not in ATF3-KO mice (Fig. 1D). In contrast, following 14 days PE infusion, IFNγ levels remained high as compared to the basal levels in wild type mice, while in ATF3-KO mice the IFNγ levels were low compared to wild type levels (Fig. 1D).

Serum CXCL10 levels are known to correlate with heart failure [17,18], however, its direct role in cardiac remodeling is controversial. To examine the role of CXCL10, we treated wild type cardiomyocytes with recombinant CXCL10 for 24 h and found a significant 18% increase in cardiomyocytes volume (Fig. 1A). The increase in cardiomyocytes volume was abrogated in the presence of CXCL10 neutralizing antibodies. Importantly, CM derived from wild type mice pre-incubated with CXCL10 neutralizing antibody failed to result in an increase in cardiomyocytes volume (Fig. 1A).

Collectively these results suggest that macrophages secrete CXCL10 in response to PE treatment that results in cardiomyocytes remodeling.

Table 1
RT-PCR primers used.

Primer name	*Sequence*
mATF3	F-GCTGCTGCCAAGTGTCGAAA
	R-TACATGCTCAACCTGCACCG
CXCR3	F-TACCTTGAGGTTAGTGAACGTCA
	R-CGCTCTCGTTTTCCCCATAATC
CXCL10	F-CCAAGTGCTGCCGTCATTTTC
	R-GGCTCGCAGGGATGATTTCAA
GAPDH	F-TTGCCATCAACGACCCCTTCAT
	R-AGACTCCACGACATACTCAGCA
Nppa (ANP)	F-GCTTCCAGGCCATATTGGAG
	R-GGGGGCATGACCTCATCTT
Nppb (BNP)	F-GAGGTCACTCCTATCCTCTGG
	R-GCCATTTCCTCCGACTTTTCTC
Myh7 (βMHC)	F-TGCAAAGGCTCCAGGTCTGA
	R-CTTGAACCTGTCCAACCACAA
Connective Tissue Growth Factor (cTGF)	F-AGACCTGTGGGATGGGCAT
	R-GCTTGGCGATTTTAGGTGTCC
Collagen type 1α (col1α)	F-CTGGCGGTTCAGGTCCAAT
	R-TTCCAGGCAATCCACGAGC
Transforming Growth Factorβ (TGFβ)	F-CCTGGCCCTGCTGAACTTG
	R-GACGTGGGTCATCACCGAT
Interleukin 6 (IL-6)	F-TAGTCCTTCCTACCCCAATTTCC
	R-TTGGTCCTTAGCCACTCCTTC
CD68	F-TGTCTGATCTTGCTAGGACCG
	R-GAGAGTAACGGCCTTTTTGTGA
F4/80	F-CCCCAGTGTCCTTACAGAGTG
	R-GTGCCCAGAGTGGATGTCT

3.2. CXCL10 affects cardiac remodeling in vivo

To assess the contribution of CXCL10 in vivo, mice were infused with either PE or CXCL10 for 7 days. Both PE and CXCL10 infusion resulted in a significant increase (20% and 9% respectively) in ventricle/body weight ratio (Fig. 2A). CXCL10 signaling is mediated through the CXCL10 receptor, designated; CXCR3. Infusion of the CXCR3 antagonist AMG487 completely abrogated the increase in ventricle/body weight in mice infused with CXCL10. In addition, about 30% attenuation in ventricle/body weight is observed when combined with PE infusion (Fig. 2A). The significant decrease in ventricle/body weight was also accompanied by the decrease in the molecular hypertrophic marker (ANP) fibrosis markers (collagen type I and TGFβ) and all inflammatory markers examined (IL-6, CD68, F480 and IFNγ) (Fig. 2B). Consistently, macrophage recruitment was significantly reduced when PE infused mice were treated with the CXCR3 antagonist, AMG487 (Fig. 2C).

CXCL11 is a CXCR3 agonist that competes with CXCL10 binding to the receptor and displays a different biological response [15]. To examine the ability of CXCL11 to counteract CXCL10 cardiac remodeling, we treated PE infused mice with CXCL11. CXCL11 infusion resulted in a 30% reduction of ventricle/body weight as compared to PE infusion alone (Fig. 2D).

Taking together, the results suggest that the IFNγ-CXCL10 axis is activated in response to PE infusion. Since we observed that both

IFNγ and CXCL10 expression levels are reduced in ATF3-KO mice, we hypothesized that similar to wild type mice, CXCL10 treatment of cardiomyocytes derived from ATF3-KO mice will lead to an increase in cardiomyocytes cell size. Surprisingly, CXCL10 treatment resulted in no increase in cardiomyocytes size (Fig. 3A). Similarly, CM derived from wild type macrophages failed to increase ATF3-KO derived cardiomyocytes volume (Fig. 3A). One possible explanation for the non-responsiveness to CXCL10 treatment is the lack of CXCR3 receptor. Thus, we examined CXCR3 expression levels in Wt. and ATF3-KO mice using qRT-PCR. In the heart, CXCR3 expression levels were only 50% lower in ATF3-KO mice as compared to wild type (Fig. 3B). Interestingly, in cultured cardiomyocytes and isolated macrophages derived from ATF3-KO mice, the CXCR3 levels were undetectable as compared to the corresponding wild type mice derived cells (Fig. 3C–D). Western blot analysis with macrophages lysate confirmed the absence of CXCR3 protein in ATF3-KO mice (Fig. 3E). In addition, PE infusion resulted in elevation of CXCR3 expression in the hearts derived from wild type mice, however, no significant rise in CXCR3 levels is observed in the hearts derived from PE infused ATF3-KO mice (Fig. 3B). Consistently, a significant increase in CXCR3 transcription was observed in cardiomyocytes treated with PE, while only modest rise was observed in ATF3-KO mice. Collectively, the results show a link between CXCR3 transcription with ATF3 expression.

3.3. CXCR3 is a bona fide ATF3 target gene

To explore the possibility that CXCR3 is an ATF3 bona fide target gene, we designed a luciferase reporter plasmid driving the expression of the luciferase gene under the control of the CXCR3 proximal promoter region (−1)-(−500). HEK-293 cells were co-transfected with the CXCR3-luciferase reporter plasmid in the absence or presence of ATF3 expression plasmid. ATF3 expression resulted in a significant 60% increase in CXCR3-luciferase activity (Fig. 4A). As a control for ATF3 transcription repression activity, we used a luciferase-reporter plasmid under the control of the ATF3 promoter (−1351)-(+60) [19]. Consistent with previous reports, under the same conditions, ATF3 expression strongly suppressed the transcription of the ATF3-luciferase reporter plasmid (Fig. 4B). We further mapped the ATF3 responsive element within the CXCR3 promoter to be located between (-250)-(-500) that displayed a 2.5-fold increase in the reporter activity in the presence of ATF3 (Fig. 4A). Within this domain, we identified a putative ATF3 binding domain, composed of 45 bp, that was sufficient to recapitulate the transcription activity obtained with the (-250)-(-500) fragment derived from the CXCR3 promoter (Fig. 4A).

3.4. CXCR3-KO mice display attenuated maladaptive cardiac remodeling

Finally, to assess the role of CXCR3 in PE-dependent pressure overload cardiac remodeling, mice lacking CXCR3 (CXCR3-KO) were infused with PE for 2 weeks. CXCR3-KO mice displayed a reduced cardiac remodeling as shown by the attenuated ventricle/body weight ratio as compared to Wt. mice (Fig. 5A). Reduction in heart hypertrophy in CXCR3-KO mice was also accompanied by significant lower levels of hypertrophic marker (ANP), fibrosis markers (CTGF and TGFβ) and inflammatory gene markers (IL-6, CD68 and CXCL10) as compared to wild type mice (Fig. 5B).

Collectively, ATF3-KO mice suppress cardiac maladaptive cardiac remodeling by attenuation of the IFNγ, CXCL10 and CXCR3 signaling pathway.

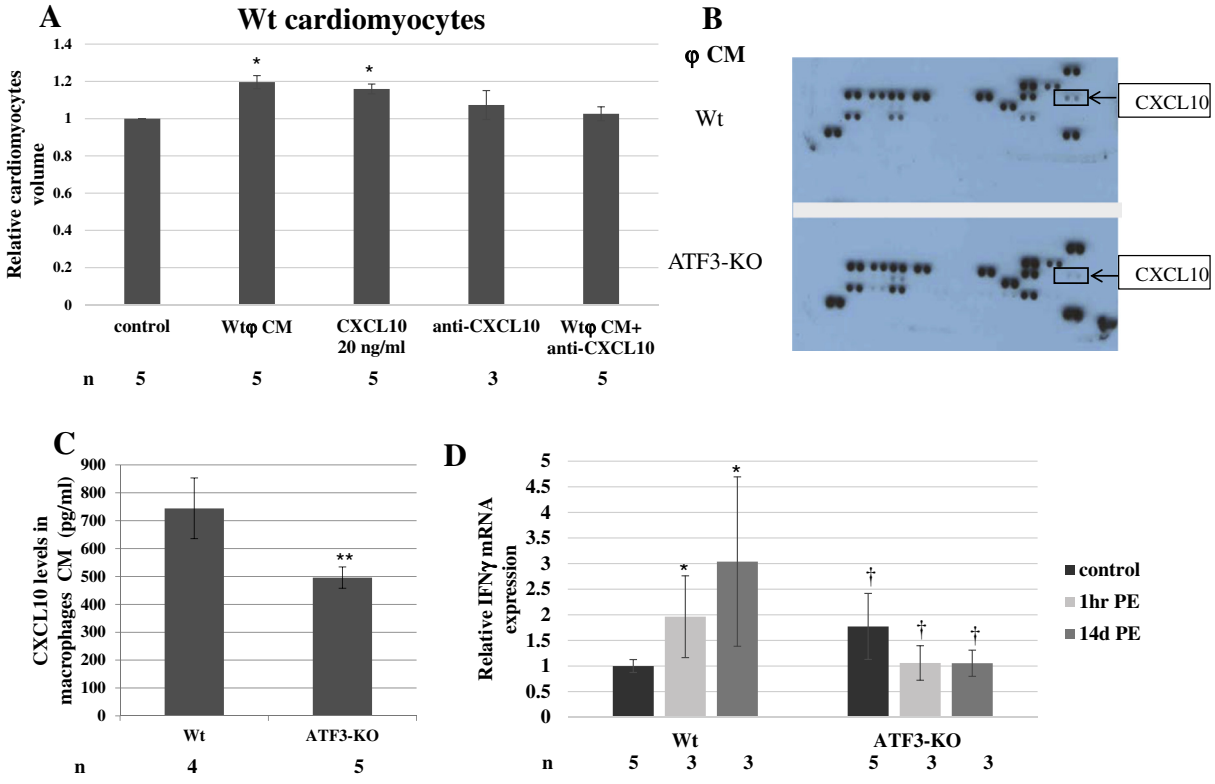
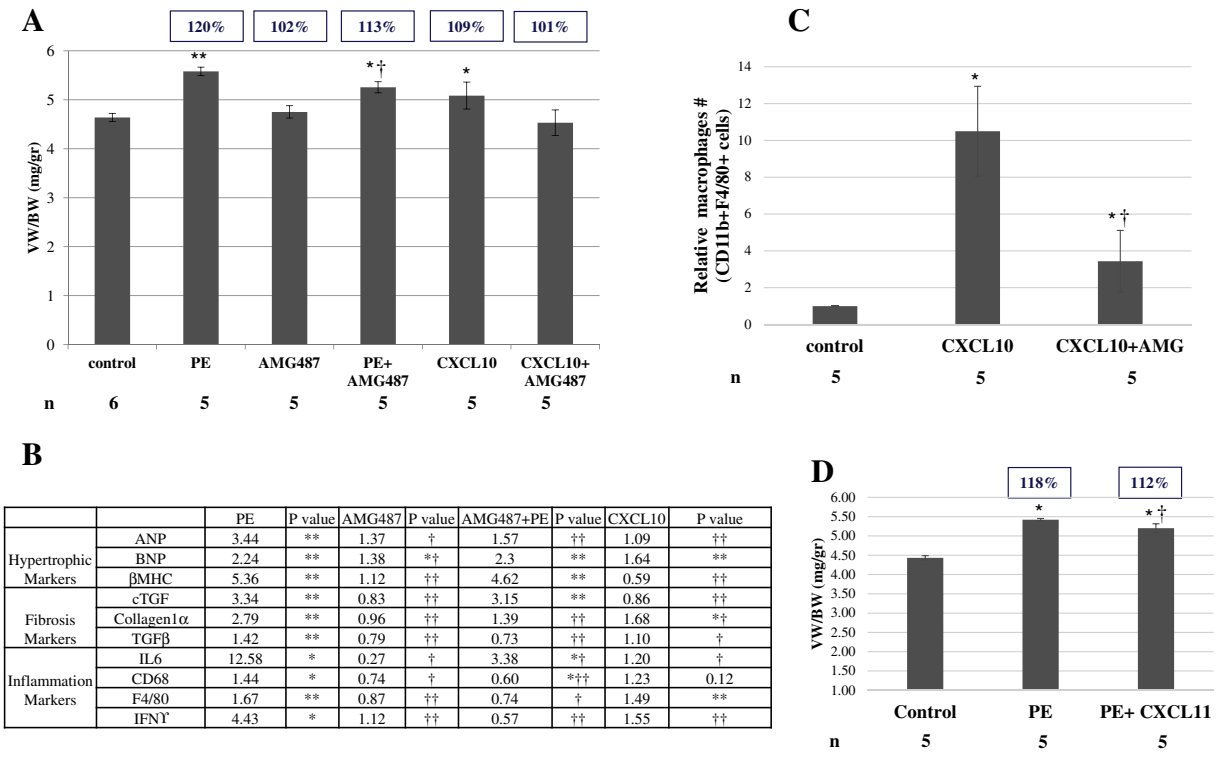


Fig. 1. ATF3 deficiency attenuates INFγ-CXCL10 axis. A. Adult Wt. cardiomyocytes were either left untreated or treated as indicated. Treatment included: Wt. macrophages conditioned medium (CM), 20 ng/ml CXCL10, in the presence or absence of anti-CXCL10 antibody (1 μg/ml). Cell volume was determined by confocal microscopy and 3D-Imaris analysis program. The results represent indicated number (n) of independent cardiomyocyte preparations with at least 20 cells per assay. B. A cytokine array panel A (R&D Systems) was probed with CM derived from 48 h cultures of either Wt. (upper panel) or ATF3-KO (lower panel) peritoneal macrophages. Cytokine array spots were normalized to reference spots and quantified. C. CXCL10 level was measured in macrophages CM derived from either Wt. or ATF3-KO by ELISA. The results represent the mean and SEM of the indicated number of CM samples (n). D. Wt. and ATF3-KO mice treated with PE for either 1 h or 14 days. mRNA was extracted from ventricles and the expression level INFγ was measured by qRT-PCR, normalized to GAPDH. The ratio of normalized INFγ expression levels of PE treated to vehicle treated mice is shown. Results are expressed as relative mean and SEM ratio of the indicated number of animals (n). Asterisks (*, **) indicate *P* value <0.05 or 0.01 respectively. Cross (†) represent *P* value <0.05 between genotypes.



		PE	P value	AMG487	P value	AMG487+PE	P value	CXCL10	P value
Hypertrophic Markers	ANP	3.44	**	1.37	†	1.57	††	1.09	††
	BNP	2.24	**	1.38	*†	2.3	**	1.64	**
	βMHC	5.36	**	1.12	††	4.62	**	0.59	††
Fibrosis Markers	cTGF	3.34	**	0.83	††	3.15	**	0.86	††
	Collagen1α	2.79	**	0.96	††	1.39	††	1.68	*†
	TGFβ	1.42	**	0.79	††	0.73	††	1.10	†
Inflammation Markers	IL6	12.58	*	0.27	†	3.38	*†	1.20	†
	CD68	1.44	*	0.74	†	0.60	*††	1.23	0.12
	F4/80	1.67	**	0.87	††	0.74	†	1.49	**
	IFNγ	4.43	*	1.12	††	0.57	††	1.55	††

Fig. 2. CXCL10 receptor antagonists suppress PE-induced cardiomyocyte hypertrophy. A. Wt. mice were implanted with Alzet micro-osmotic pumps containing: vehicle control, PE 100 mg/kg/day purified CXCL10-IgG 2.4 μg/25 g/day and AMG487 1.25 mg/25 g/day, as indicated. Mice were sacrificed seven days following implantation and hearts were analyzed. VW/BW ratio is shown. The results represent the mean and SEM of the indicated number of animals (n). The percentage (%) of ventricle/body weight relative to sham operated mice is indicated. B. mRNA was extracted from ventricles of Wt. mice treated as described in A. The expression levels of the indicated genes were measured by qRT-PCR, normalized to GAPDH. Shown is the mean ratio of expression levels in treated to sham-treated mice of the indicated number of animals (n) in A. C. Ventricles were prepared as single cell suspensions followed by FACS analysis using $CD11b^{+}$, $Gr1^{-}$ (Ly-6G) and $F4/80^{+}$ cell surface antibodies. The results represent the mean and SEM of the indicated number (n) of animals relative to sham-control considered as 1. D. Wt. mice were implanted with Alzet micro-osmotic pumps containing: vehicle control, PE 100 mg/kg/day and CXCL11 2.5 μg/25 g/day as indicated. Mice were sacrificed seven days following implantation and hearts were analyzed. VW/BW ratio is shown. The results represent the mean and SEM of the indicated number of animals (n). The percentage (%) of ventricle growth relative to sham operated mice is indicated. Asterisks (*/**) indicate *P* value <0.05 or 0.01 respectively of a one-tailed *t*-test compared to vehicle treated wild type mice. Cross (†/††) indicates *P* value <0.05 or 0.01 respectively, of χ^2 test for expected-versus-observed ratio comparing to PE infusion results.

4. Discussion

The heart undergoes adaptive and maladaptive changes following various stresses leading to heart failure. Mice treated with PE infusion display maladaptive cardiac remodeling processes which are partially dependent on cardiac ATF3 expression [5]. Using this model, we identified ATF3 transient expression in cardiomyocytes that turns-ON a program that promotes macrophages recruitment to the heart leading to maladaptive cardiac remodeling. Macrophages activation is dependent on ATF3 expression in both cardiomyocytes and macrophages [5]. However, the molecular mechanisms that mediate the cross-talk between cardiomyocytes to macrophages were not explored. Here, we used a limited cytokine/chemokine antibody array to screen for macrophages secreted factor involved in cardiac remodeling. We identified, CXCL10, also called interferon γ induced protein 10 kDa, IP-10, that belongs to the CXC family of chemokines [17,18]. CXCL10 is an IFNγ responsive gene [18]. Indeed, we found that IFNγ is expressed in the heart in an ATF3 dependent manner. Moreover, we identified sustained levels of IFNγ mRNA following 14 days PE treatment in wild type mice but not in the ATF3-KO mice counterpart. Elevation of IFNγ promotes macrophages to secrete CXCL10. Thus, in ATF3-KO mice lower levels of CXCL10 are secreted. Therefore, the IFNγ-CXCL10 axis is significantly impaired. In addition, cardiomyocytes lacking ATF3, did not respond to excess of exogenous recombinant CXCL10. This was found to be due to the lack of the CXCL10 receptor, CXCR3. Interestingly, cardiac CXCR3 expression levels are attenuated in ATF3-KO mice by only 50%, but in isolated adult cardiomyocytes, CXCR3 levels are barely detectable. Although cardiomyocytes are the major cell component in the heart volume, multiple cardiac resident cells such as fibroblasts, endothelial, immune cells and progenitor cells contribute to CXCR3 expression in an ATF3 independent manner. Thus, collectively, the IFNγ-CXCL10-CXCR3 axis is significantly abrogated in ATF3-KO mice.

In humans CXCL10 levels contribute positively to the initiation and progression of atherosclerosis. In addition, CXCL10 has been recognized as a biomarker for cardiovascular risk prediction and right ventricular dysfunction [20]. Yet, the direct role of CXCL10 in cardiac maladaptive remodeling processes was not explored [17,18]. Here, we show that mouse adult cardiomyocytes treated with CXCL10 displayed enlarged volume. In addition, CXCL10 when infused into mice resulted in a significant increase in ventricle/body weight. Several lines of evidence suggest that CXCL10 is part of the macrophages maladaptive cardiac remodeling, first, in vitro neutralizing CXCL10 antibodies block the increase in cardiomyocytes growth when treated with macrophages CM. Second, in vivo, the use of CXCL10 receptor blocker (AMG487) or agonist (CXCL11), significantly suppressed cardiac remodeling. Third, mice lacking the CXCL10 receptor, CXCR3, displayed reduced hypertrophy. These results suggest that CXCL10 as a sole agent is sufficient to induce maladaptive cardiac remodeling.

5. Conclusion

This study places the IFNγ-CXCL10-CXCR3 axis as a crucial player in the cross-talk between cardiomyocytes and macrophages mediating maladaptive cardiac remodeling processes in a pressure overload model. We propose that pressure overload turns-ON an inflammatory response in cardiomyocytes regulating the expression of numerous cytokines,

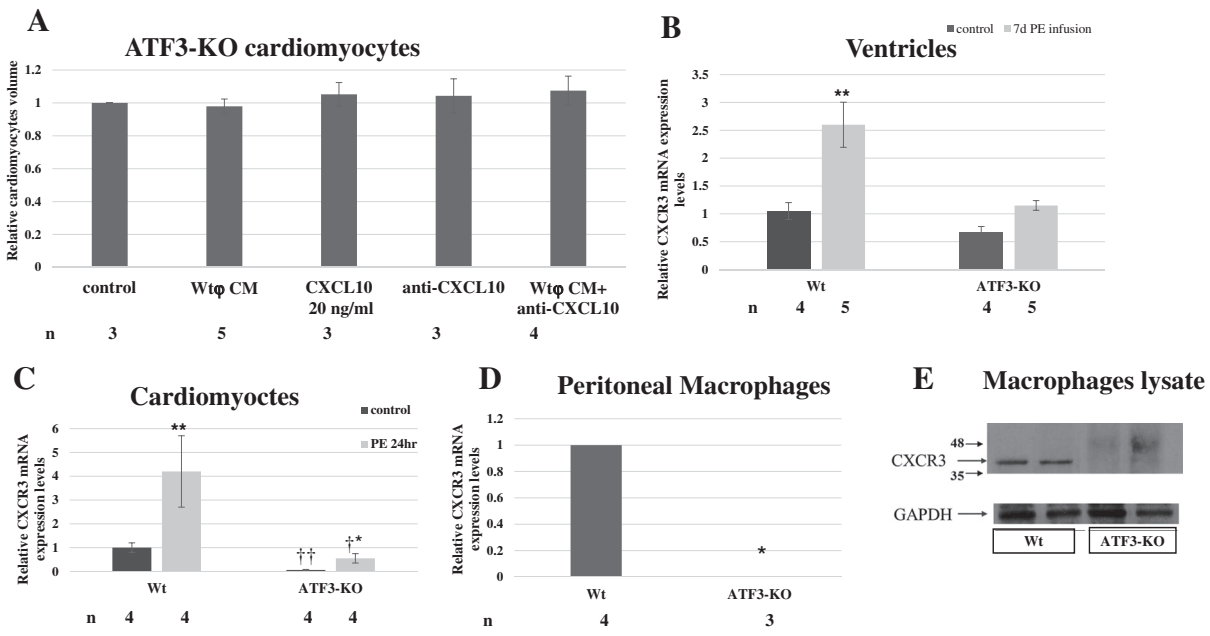


Fig. 3. CXCR3 receptor expression is suppressed in ATF3-KO mice. A. Adult ATF3-KO cardiomyocytes were either left untreated or treated as indicated. Treatment included: Wt. macrophages conditioned medium (CM), 20 ng/ml CXCL10, in the presence or absence of anti-CXCL10 antibody (1 μg/ml). Cell volume was determined by confocal microscopy and 3D-Imaris analysis program. The results represent the indicated number (n) of independent cardiomyocyte preparations with at least 20 cells. B–D. mRNA was extracted from ventricles (B), cardiomyocytes (C) and macrophages (D) of either Wt. or ATF3-KO mice. The expression levels of the CXCR3 gene were measured by qRT-PCR, normalized to GAPDH. Shown is the relative expression levels in treated to Wt. control mice considered as 1. Results are expressed as mean and SEM ratio of the indicated number of animals (n). E. Western blot analysis of peritoneal macrophage cell lysate isolated from either Wt. or ATF3-KO mice. Molecular weight marker is indicated in kDa. Asterisks (*/**) indicate *P* value <0.05 or 0.01 respectively of one-tailed *t*-test compared to sham-treated wild type mice. Cross (†) indicates *P* value <0.05 of χ^2 test for expected-versus-observed ratio comparing to PE treated Wt. cardiomyocytes.

among them IFNγ. Increased IFNγ levels in the blood results in an activation signal to macrophages that subsequently infiltrates to the inflamed heart. Macrophages activation is accompanied by elevation of CXCL10 secretion which affects maladaptive cardiac remodeling via CXCR3 signaling cascade. The ATF3 expression in both cardiomyocytes and macrophages plays a pivotal role in controlling IFNγ-CXCL10-CXCR3 axis allowing the communication between cardiomyocytes to macrophages and maladaptive cardiac remodeling. Therefore, we propose that ATF3 is a bona fide target for therapeutic intervention to attenuate pressure overload maladaptive cardiac remodeling.

Funds

This work was partially supported by: Mars Pittsburgh Fund for Medical Research to A.A. The authors wish to thank the D. Dan and Betty Kahn travel grant support to L.K.

Disclosures

The authors report no relationships that could be construed as a conflict of interest.

Authors' contribution

Lilach Koren – designed and performed all experiments, manuscript preparation; Uri Barash – CXCL10-IgG purification, Yaniv Zohar – discussion and data analysis, Nathan Karin – data analysis; Ami Aronheim – experimental design, data analysis and manuscript preparation.

Acknowledgments

The authors wish to thank Drs: Edith Suss-Toby and Ofer Shenker from the Biomedical Core Facility, Gizi Wildbaum for providing

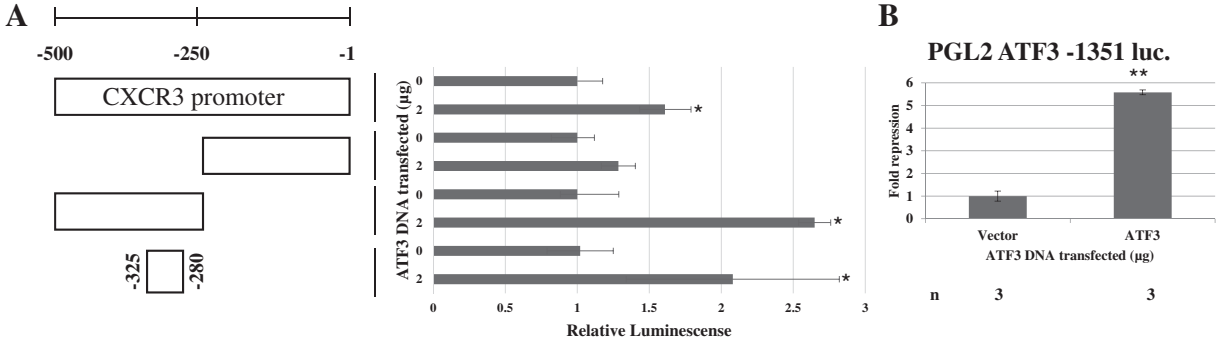
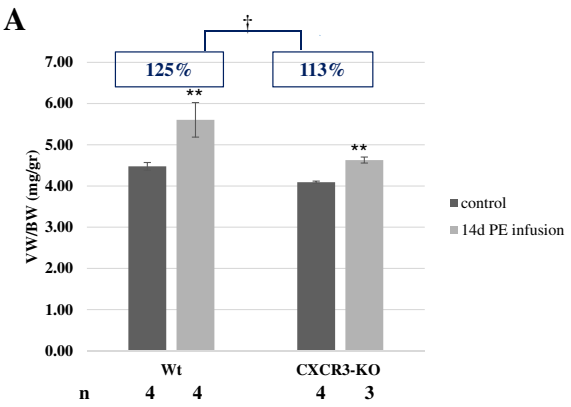


Fig. 4. CXCR3 is a bona fide ATF3 target gene: A. Schematic representation of the CXCR3 promoter deletion mutants used. DNA fragments were subcloned upstream of the luciferase gene in the PGL3 promoter luciferase reporter plasmid. Fragment length (bp) upstream (−) in respect to the mouse CXCR3 transcription start site. HEK-293T cells were co-transfected with 2 μg of PGL3 reporter plasmid in the presence or absence of 2 μg of HA-ATF3 expression plasmid. B. HEK-293T cells were co-transfected with 2 μg of PGL2-1351ATF3 reporter plasmid in the presence or absence of 2 μg of ATF3 expression vector. Luciferase activity was measured 24 h following transfection. Fold repression was calculated by dividing the luciferase activity obtained with the pCEFL empty expression plasmid to the luciferase activity obtained in the presence of ATF3 expression plasmid. The results represent the mean and SEM of at least three independent experiments. Asterisks (*/**) indicate *P* value <0.05 or 0.01 respectively of one-tailed *t*-test compared to luciferase activity in the absence of ATF3.

A



B

		Wt		CXCR3-KO		P value
		control	PE	control	PE	
Hypertrophic Markers	ANP	1.00	5.16	0.92	1.93	††
	BNP	1.00	1.42	0.61	1.42	
	βMHC	1.00	6.18	0.31	4.41	
Fibrosis Markers	cTGF	1.00	4.02	0.49	1.63	†
	Collagen1α	1.00	1.42	0.57	0.92	
	TGFβ	1.00	2.63	0.80	0.75	††
Inflammation Markers	IL6	1.00	6.24	0.39	0.73	†
	CD68	1.00	1.35	0.51	0.39	†
	CXCL10	1.00	3.02	1.17	1.54	

Fig. 5. CXCR3-KO mice display reduced cardiac hypertrophy following PE infusion. A. Wt. and CXCR3-KO mice were implanted with Alzet micro-osmotic pumps containing: vehicle control or PE 100 mg/kg/day. Mice were sacrificed 14 days following implantation and ventricles were analyzed. VW/BW ratio is shown. The results represent the mean and SEM of the indicated number of animals (n). The percentage (%) of ventricles growth relative to control is indicated. B. mRNA was extracted from ventricles of Wt. and CXCR3-KO mice treated as described above. The expression levels of the indicated genes were measured by qRT-PCR, normalized to GAPDH. Shown is the ratio of expression levels in treated to vehicle control-treated mice. Results are expressed as mean ratio of the indicated number of animals (n) in A. Asterisks (**) indicate P value <0.01 of one-tailed t-test compared to vehicle treated wild type mice. Cross (†/††) indicates P value <0.05 or 0.01 of χ^2 test for expected-versus-observed ratio comparing CXCR3-KO to the corresponding treatment in Wt. mice.

CXCR3-KO mice and Liat Linde for the Bioinformatics analysis. This work was partially supported by the Mars Pittsburgh Fund for Medical Research to A.A.

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