



# ATF3-dependent cross-talk between cardiomyocytes and macrophages promotes cardiac maladaptive remodeling☆



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## ABSTRACT

**Rationale:** Pressure overload induces adaptive remodeling processes in the heart. However, when pressure overload persists, adaptive changes turn into maladaptive alterations leading to cardiac hypertrophy and heart failure. ATF3 is a stress inducible transcription factor that is transiently expressed following neuroendocrine stimulation. However, its role in chronic pressure overload dependent cardiac hypertrophy is currently unknown.

**Objective:** The objective of the study was to study the role of ATF3 in chronic pressure overload dependent cardiac remodeling processes.

**Methods and results:** Pressure overload was induced by phenylephrine (PE) mini-osmotic pumps in various mice models of whole body, cardiac specific, bone marrow (BM) specific and macrophage specific ATF3 ablations. We show that ATF3-KO mice exhibit a significantly reduced expression of cardiac remodeling markers following chronic pressure overload. Consistently, the lack of ATF3 specifically in either cardiomyocytes or BM derived cells blunts the hypertrophic response to PE infusion. A unique cross-talk between cardiomyocytes and macrophages was identified. Cardiomyocytes induce an ATF3 dependent induction of an inflammatory response leading to macrophage recruitment to the heart. Adoptive transfer of wild type macrophages, but not ATF3-KO derived macrophages, into wild type mice potentiates maladaptive response to PE infusion.

**Conclusions:** Collectively, this study places ATF3 as a key regulator in promoting pressure overload induced cardiac hypertrophy through a cross-talk between cardiomyocytes and macrophages. Inhibiting this cross-talk may serve as a useful approach to blunt maladaptive remodeling processes in the heart.

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## 1. Introduction

Cardiac remodeling, such as hypertrophic growth of cardiac muscle, is an adaptive response to various mechanophysical, metabolic and genetic stresses. However, following sustained overload, the heart develops maladaptive remodeling processes resulting in contractile dysfunction and heart failure [1,2]. Therefore, cardiac hypertrophy is a

leading predictor for the development of serious complications such as the development of arrhythmias, sudden death and heart failure [3, 4]. Current medical treatments for the prevention of pathological hypertrophy rely on the pharmacological blockade of key membrane-bound receptors that respond to neuroendocrine stimuli. Yet, despite the capacity for a near-complete pharmacological blockade of the neurohumoral axis, the incidence of heart failure, associated with morbidity and mortality, are still on the rise [5].

Cardiac remodeling is regulated by multiple G-protein coupled receptor signaling pathways that converge on a limited number of transcription factors [6]. These signaling pathways, including calcineurin, calcium/calmodulin-dependent protein kinase, PI3K, JAK and mitogen activated protein (MAP) kinases, are all sufficient to induce cardiac hypertrophy. The activation of the above mentioned signaling pathways stimulate transcription factors such as AP-1, NFAT4, MEF2C, STAT3, EGR1 as well as chromatin remodeling enzymes such as proteins from the class II histone deacetylases (HDACs) ultimately leading to the conversion of the adaptive response into a hypertrophic program

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[6–8]. Thus, targeting the activity of key transcriptional regulators emerges as a primary strategy for the development of new therapeutic approaches aimed at preventing myocardial dysfunction.

Transcriptional regulation is one major control point in the process of cardiac hypertrophy and heart failure progression. Members of the basic leucine zipper (bZIP) family of transcription factors play a decisive role in these processes. Several lines of evidence suggest that the transcription factor complex, Activating Protein 1 (AP-1), plays a major role in myocardial hypertrophy and apoptosis. AP-1 is composed of dimeric combinatorial complexes of Jun homodimers (c-Jun, JunD or JunB) or Jun heterodimers with Fos family members (c-Fos, Fos B, Fra1, 2), or with some members of the ATF and CREB protein families, reviewed in [9].

Several models have shown that hypertrophic growth in the heart is accompanied by an increase in the expression of c-Fos and c-Jun transcription factor family members [10–12]. AP-1 inhibition using decoy-oligonucleotides prevents cardiomyocyte growth response to phenylephrine (PE) [13]. On the other hand, JunD protects cardiomyocytes from undergoing apoptosis and hypertrophy by inhibiting AP-1 activity [14,15]. More recently, c-Jun was found to play a protective role in pressure overload maladaptive cardiac hypertrophy [16]. Collectively, these studies demonstrate that the AP-1 transcription factor targets gene programs that both induce and suppress apoptosis and hypertrophy, depending on AP-1 complex composition, expression level and the specific insult exerted.

Previously, we identified two closely related bZIP repressor proteins, the c-Jun dimerization 2 (JDP2) [17] and Activating Transcription 3 (ATF3) [18] that play a role in cardiac hypertrophy [19]. ATF3 is an adaptive immediate early gene, the transcription of which is highly induced following various stress stimuli [18]. In contrast, JDP2 is constitutively expressed in all cells tested [17]. JDP2 and ATF3 are able to associate with numerous bZIP protein family members and either inhibit transcription [17,20–22] or potentiate transcription depending on their interacting partner [23]. Importantly, ATF3 expression is highly induced specifically in cardiomyocytes following acute challenge with isoproterenol, PE and angiotensin II [24]. Interestingly, cardiac-specific expression of both JDP2 and ATF3 during embryonic period results in atrial enlargement and premature death in transgenic mice [25–27]. In contrast, adult ATF3 expression is sufficient to induce ventricular hypertrophy even in the absence of additional insults such as pressure overload [27]. In line with these studies, ATF3 was found to be required for cardiomyocytes growth response to endothelin-1 stimulation [28]. In contrast, using transverse aortic constriction (TAC), the lack of ATF3 was found to promote cardiac hypertrophy [29,30]. In addition, ATF3 overexpression in the left ventricle of adult rat, by adenovirus infection, did not result in an apparent change in hypertrophic markers [31]. Thus, collectively, the role of ATF3 in pressure overload cardiac hypertrophy is controversial. Here, we used phenylephrine (PE), an  $\alpha$ 1-adrenergic agonist, infusion in mice model to increase blood pressure and induce hypertrophic cardiac remodeling processes [7,32]. We show that ATF3 plays a role in promoting cardiac remodeling and heart hypertrophy in response to chronic PE pressure overload. Furthermore, we describe an ATF3-dependent cross-talk between cardiomyocytes and macrophages that induces maladaptive remodeling processes in the heart.

## 2. 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 [33], ATF3-KO Flox [34] and  $\alpha$ MHC-CRE mice [35], ATF3-Tg and  $\alpha$ MHC-tTA [27]. Male mice were used in all the experiments performed in this study unless otherwise specified.

Genotyping was performed on genomic mouse tail DNA that was extracted using the RedExtract-N-AMP tissue PCR kit (Sigma, St. Louis, MO, USA).

### 2.2. Mice injections

C57BL/6 mice were injected intraperitoneally with 2.5 mg/kg of PE (Sigma P6126). At the indicated time following injection, the mice were anesthetized using ketamine and xylazine mixture prior to heart removal.

### 2.3. 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). Eight weeks 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 four parts that were used for protein extraction, RNA purification, heart sections and single cell suspensions.

### 2.4. Bone marrow transplantation

Recipient C57BL/6 mice were total body irradiated with a 6 MeV electron beam using Elekta Precise (Elekta Oncology Systems) linear accelerator at a total dose of 10 Gy at room temperature (Department of Radiation Therapy, Rambam Medical Center, Haifa Israel).

Bone marrow donor mice were either C57BL/6 or ATF3-KO mice (C57BL/6 background).

Bone marrow cells were harvested from mice by gently flushing their femurs with phosphate-buffered saline (PBS). Cells were centrifuged at 300 g for 5 min, resuspended in PBS and counted. Cells ( $10^7$  cells in 0.2 ml) were then intravenously injected into C57BL/6 recipient mice immediately after irradiation.

Following 6–8 weeks of bone marrow reconstitution, mice were implanted with micro-osmotic pumps.

### 2.5. Thioglycollate stimulation and macrophage harvest

C57BL/6 or ATF3-KO mice were intraperitoneally injected with 3 ml of 4% Thioglycollate medium brewer modified (BD) 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. Cells were centrifuged at 300 g for 5 min, resuspended in PBS, counted and diluted to a concentration  $10^6$  cells in 0.2 ml and intravenously injected into C57BL/6 three days post micro-osmotic pump implantation.

### 2.6. Flow cytometry acquisition and analysis

Single cell suspensions in Hanks' balanced salt solution (HBSS) from cardiac tissue were prepared using a MACS dissociator along with the manufacturer's instructions. Cells were immuno-stained for macrophages using Gr1<sup>+</sup>CD11b<sup>+</sup>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).

### 2.7. mRNA extraction

mRNA was purified using an Aurum total RNA fatty and fibrous tissue kit (#732-6830, Bio-Rad) according to the manufacturer's protocol. mRNA was quantified by measuring absorbance at 260 nm with a nanodrop spectrophotometer (ND-1000, NanoDrop Technologies, Rockland, DE, USA).

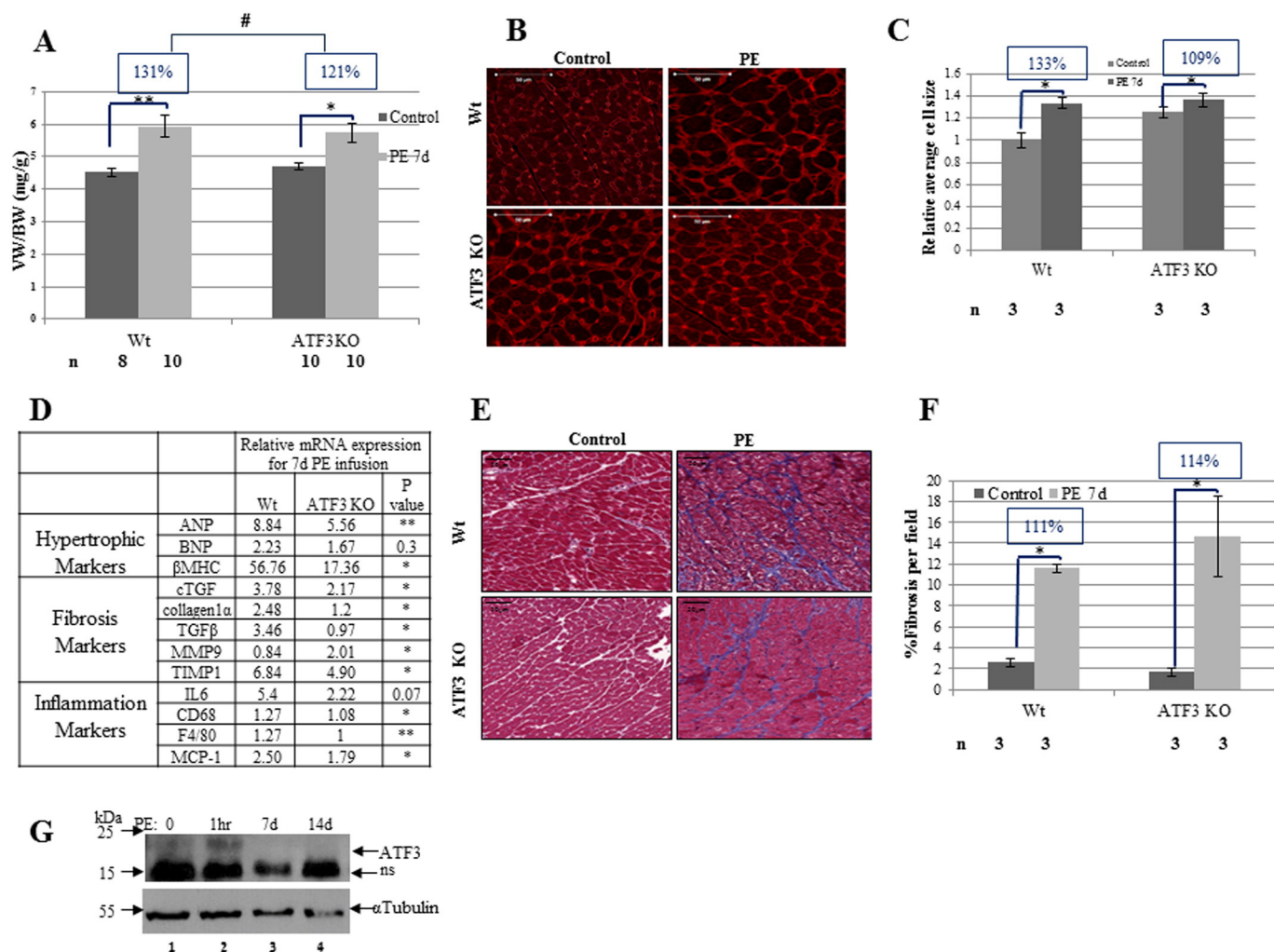
### 2.8. Quantitative real time PCR (qRT-PCR)

cDNA was synthesized from mRNA samples using 800 ng of RNA in a 20  $\mu$ l 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. The primer sequences are shown in supplementary Table 1.

### 2.9. Western blotting

Intraperitoneal macrophage cells were lysed in whole-cell extract (WCE) buffer (25 mM HEPES, pH 7.7, 0.3 M NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM DTT, 20 mM  $\beta$ -glycerolphosphate, 0.1 mM Na<sub>2</sub>VO<sub>4</sub>, 100  $\mu$ g/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 PBSX1) supplemented with protease inhibitor cocktail (P-8340, Sigma Aldrich) and 1 mM DTT, 2 mM PMSF, 20 mM  $\beta$ -glycerolphosphate, 0.1 mM



**Fig. 1.** The ATF3 paradox – ATF3-KO mice exhibit reduced cardiac hypertrophy in response to PE infusion while ATF3 expression is only transiently induced in the heart. Wild type and ATF3-KO C57Bl/6 mice were sacrificed 7/14 days following PE infusion and hearts were analyzed. **A.** Ventricle/body weight (VV/BW) ratio is shown. The results represent the mean and SEM of the indicated number of animals (n). The percentage (%) of ventricle growth in PE infused compared to vehicle control for each genotype is indicated. Asterisks (\*\*/\*\*) indicate P value < 0.05 or 0.01 respectively of a one-tailed t-test compared to vehicle treated control for each genotype. Pound (#) indicates P value < 0.05 of a  $\chi^2$  test for expected-versus-observed ratio comparing the percentage (%) of ventricle growth in Wt to ATF3-KO of seven day PE versus control infused mice. **B.** Representative wheat-germ agglutinin TRITC conjugated staining (red) of left ventricle heart sections derived from control and PE treated mice for 14 days. **C.** Cross-sectional area analysis of heart sections described in **B.** **D.** The expression levels of the indicated genes were measured by qRT-PCR, normalized to GAPDH. The expression ratio in PE-treated to vehicle control-treated mice is shown. Results are expressed as means (number of animals (n) is indicated in **A**). Asterisks (\*\*/\*\*) indicate P value < 0.05 or 0.01 respectively of one-tailed t-test comparing wild type to ATF3-KO genotypes. **E.** Representative Masson's trichrome staining of left ventricle heart sections described in **B.** **F.** Quantitative analysis of Masson's trichrome stainings from **E.** **G.** Western blot analysis with anti-ATF3 and anti- $\alpha$ -tubulin of heart lysate treated with PE as indicated. Molecular weight marker is indicated in kDa. Non-specific bands are indicated (n.s.).

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 –70 °C.

The proteins (30  $\mu$ g of cell lysate or 80  $\mu$ g of tissue lysate) were then 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. The primary antibodies used were anti- $\alpha$ -tubulin (T-9026, Sigma Aldrich) 1:2000, and anti-ATF3 1:100 [24]. 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.

## 2.10. IHC

Heart tissues were fixed in 4% formaldehyde at least overnight, then embedded in paraffin, serially sectioned at 10  $\mu$ m intervals, and mounted on slides. Sections were processed for deparaffinization (xylene, 20 min), dehydration (Isopropanol) and rehydration (H<sub>2</sub>O). Masson's trichrome staining was performed according to standard protocol.

Images were acquired by using Virtual Microscopy (Olympus). Quantification of Masson's trichrome staining was performed with Image Pro Plus software. Five fields of each slide were quantified. Stained areas were segmented and quantified following

calibration using Image Pro Plus software. The results represent the mean and SEM from three mice.

## 2.11. Cardiomyocytes size analysis

Sections were stained following deparaffinization with Wheat-germ agglutinin TRITC-conjugated (Sigma Aldrich Cat# L5266) diluted 1:100 in phosphate-buffered saline (PBS). Sections were washed three times with PBS and mounted in Fluoromount-G (Southern Biotechnology, Birmingham, AL, 0100-01). Sections were viewed using a Zeiss LSM700 confocal microscope (Thornwood, NY) equipped with a 40 $\times$  oil objective and solid state 555 nm laser.

Quantification of the cell size was performed with Image Pro Plus software. Five fields in each slide were photographed. Unstained areas were then identified and segmented using Image Pro Plus software. In each stained area, the mean cell perimeter and area was calculated and the number of cells was measured.

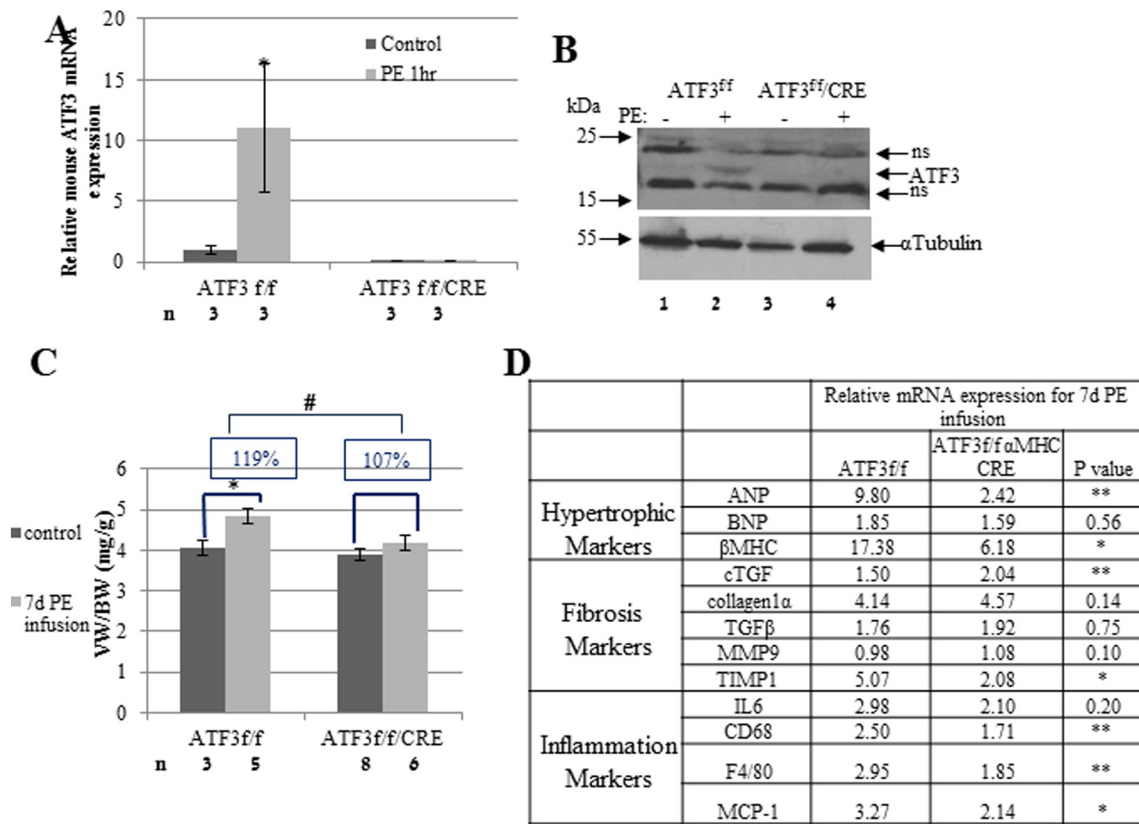
The results represent the mean and SEM from three mice.

## 2.12. Statistical analysis

Data is presented as means  $\pm$  SEM in (n) number of experiments.

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





**Fig. 2.** Cardiac specific ATF3-KO mice exhibit reduced cardiac hypertrophy. A. The expression level of ATF3 was measured by qRT-PCR, normalized to GAPDH. The ratio of expression levels in PE-treated (1 h) to vehicle control-treated ATF3<sup>fl/fl</sup> mice is shown. Results are expressed as means of the indicated number of animals (n). B. Western blot analysis with anti-ATF3 antibodies and anti-α-tubulin of heart cell lysate described in A. Molecular weight marker is indicated in kDa. Non-specific bands are indicated (n.s.) C. Mice were sacrificed seven days following PE infusion and VW/BW was calculated. The results represent the mean and SEM of the indicated number of animals (n). Asterisk (\*) indicates P value < 0.05 of a one-tailed t-test compared to vehicle treated control for each genotype. The percentage (%) of ventricle growth in PE infused mice compared to vehicle control is indicated for each genotype. Pound (#) indicates P value < 0.05 of a  $\chi^2$  test for expected-versus-observed ratio comparing the percentage (%) of ventricle growth in ATF3<sup>fl/fl</sup>/CRE to ATF3<sup>fl/fl</sup> of PE versus control infused mice D. The expression levels of the indicated genes were measured by qRT-PCR, normalized to GAPDH. The ratio of expression levels in PE-treated to vehicle control-treated mice is shown. Results are expressed as means of the indicated number of animals (n) in C. Asterisks (\*/\*\*) indicate P value < 0.05 or 0.01 respectively of a one-tailed t-test comparing ATF3<sup>fl/fl</sup> with ATF3<sup>fl/fl</sup>/CRE genotypes.

The ratio calculation was analyzed by a  $\chi^2$  test for expected-versus-observed ratio. P values < 0.05 were considered significant unless otherwise indicated.

### 3. Results

#### 3.1. ATF3-KO mice displays reduced cardiac remodeling

To investigate the role of ATF3 in cardiac hypertrophy, we compared the effect of pressure overload in wild type C57Bl/6 mice to counterpart mice harboring a complete loss of function mutation in the ATF3 gene (ATF3-KO) [33]. To induce pressure overload cardiac hypertrophy, mice were implanted with Alzet mini-osmotic pumps filled with either PE or vehicle (control). After one week, the extent of cardiac hypertrophy was evaluated by calculating the ventricle/body weight ratio (VW/BW). As expected, wild type mice exhibited a significant increase in VW/BW in response to PE-induced pressure overload (31%). Interestingly, ATF3-KO mice also exhibited an increase in VW/BW ratio, but to a lesser extent than wild type mice (21%, Fig. 1A). Cardiomyocytes cross-sectional area (CSA) analysis was performed by wheat-germ hemagglutinin staining of heart sections. This analysis revealed that PE treatment resulted in a significant increase in CSA of left ventricle cardiomyocytes derived of wild type mice (33%), while only a modest increase (9%) in CSA was observed in left ventricle cardiomyocytes derived from ATF3-KO mice (Fig. 1B–C). No increase in CSA was observed for right ventricle cardiomyocytes (supplementary Fig. 1). In addition, we examined

the expression levels of various hypertrophic and fibrosis markers by qRT-PCR. Both the hypertrophic and fibrosis markers were significantly increased in mice subjected to PE-induced pressure overload, with wild type mice exhibiting a greater increase than ATF3-KO mice (Fig. 1D). Masson's trichrome staining analysis of heart sections revealed a strong interstitial staining in the left but not right ventricles of both wild type and ATF3-KO heart sections (Fig. 1E, Supplementary Fig. 2). No significant difference was observed between genotypes (Fig. 1F). The lack of difference in fibrosis between wild type and ATF3-KO mice may be due to the increased expression of tissue inhibitor metalloproteinase (TIMP1) and reduced expression of MMP9 following PE treatment in wild type mice as compared to ATF3-KO mice (Fig. 1D).

#### 3.2. ATF3 protein expression paradox

ATF3 is an adaptive stress response immediate early gene. ATF3 expression is transiently induced in the heart following various neuro-hormonal stimuli, including PE [24]. To compare the effect of acute and chronic pressure overload on ATF3 expression in the heart, mice were either injected with PE and sacrificed 1 h later or infused with PE using the mini-pumps and sacrificed after 1 or 2 weeks. Hearts were extracted, ventricle lysates were prepared and proteins were resolved by SDS-PAGE. Western blot analysis revealed that acute pressure overload efficiently induced the expression of ATF3. However, ATF3 expression was not detected in the hearts of mice infused with PE for 1 or

**Table 1**

qRT-PCR indicates inflammatory program one hour following acute PE injection. The expression levels of the indicated genes measured by qRT-PCR, normalized to GAPDH. The expression ratio in PE-treated to vehicle control-treated wild type and ATF3-KO mice is shown. Asterisks (\*/\*\*) indicate P value < 0.05 or 0.01 respectively of a one-tailed t-test comparing the expression ratio obtained following 1 h PE treatment between wild type to ATF3-KO mice.

	Relative mRNA expression for 1hr PE		P value
	Wt	ATF3 KO	
mATF3	37.81	0.17	**
CD74	1.91	1.17	**
H2-Aa	1.95	0.98	**
CCl7	1.95	0.90	**
Tnfrsf12a	2.86	2.61	*
CCL4	5.32	0.23	**
CXCL1	5.28	0.19	**
CD14	2.12	1.59	**
Socs3	3.02	0.93	*
F4/80	1.19	0.68	*
CD68	1.15	0.49	*
CD72	1.52	0.19	**
IL-6	10.1	4.46	**
TNF $\alpha$	3.11	1.72	

2 weeks (Fig. 1G). This result was surprising because, under these conditions of chronic pressure overload, ATF3-KO mice exhibited reduced cardiac hypertrophy compared to wild type mice (Fig. 1A–C).

### 3.3. Cardiac specific ATF3 ablation blunts cardiac remodeling processes

To address this seeming paradox, we sought to examine whether ATF3 expression in cardiomyocytes is necessary for the maladaptive cardiac remodeling following PE infusion. Towards this end, we generated cardiac-specific ATF3-KO mice by serial crossing mice harboring a floxed ATF3 allele (ATF3<sup>f/f</sup>) [34] with  $\alpha$ MHC-CRE mice [35] (ATF3-CRE, ATF3<sup>f/f</sup>/CRE). To confirm the ablation of ATF3 expression, ATF3<sup>f/f</sup> and ATF3<sup>f/f</sup>/CRE mice were injected with PE and sacrificed after one hour. Lysates were prepared from extracted hearts and ATF3 mRNA and protein levels were analyzed by qRT-PCR and Western blot analysis, respectively. While ATF3 expression was efficiently detected in the heart lysate derived from ATF3<sup>f/f</sup> mice one hour after PE injection, no ATF3 expression was detected in ATF3<sup>f/f</sup>/CRE mice (Fig. 2A–B). Next, the VW/BW ratio of control-treated and PE-infused mice was analyzed. Whereas ATF3<sup>f/f</sup> mice displayed a 19% increase in VW/BW, ATF3<sup>f/f</sup>/CRE mice displayed only 7% increase in heart growth in response to one

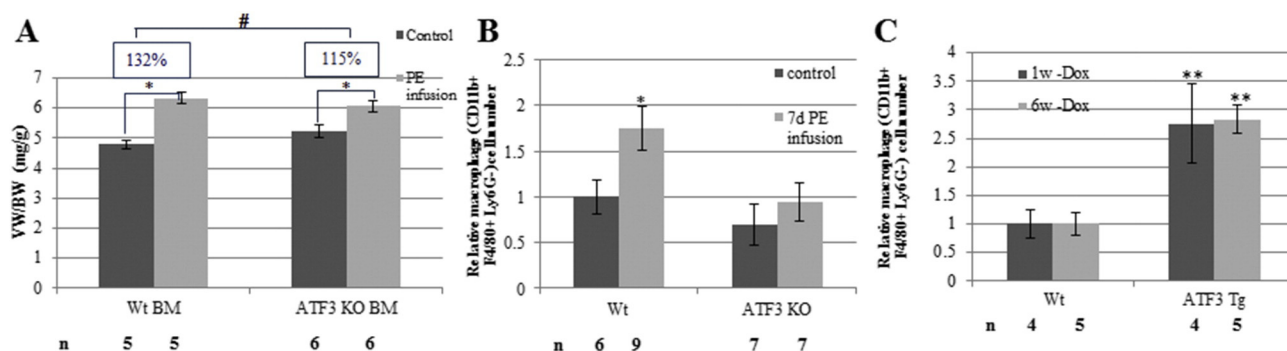
week of PE treatment (Fig. 2C). The increase in VW/BW ratio of PE infused ATF3<sup>f/f</sup> mice was accompanied by an increased level of multiple hypertrophic markers (Fig. 2D). These results suggest that ATF3 expression in the heart is absolutely necessary for the induction of cardiac remodeling processes following pressure overload stress. However, since ATF3 expression is absent in ventricles already after one week of PE exposure (Fig. 1G), we were unable to exclude the possible involvement of other non-cardiomyocyte cells residing in the heart. Importantly, we observed a significant increase in the transcription level of inflammatory gene marker, IL-6, which was higher in wild type as compared with ATF3-KO mice. In addition, macrophage markers, CD68 and F4/80 were also found to be elevated along with the increase in monocyte chemoattractant protein 1 (MCP-1) in the heart derived from wild type mice as compared with ATF3-KO mice following one week PE infusion (inflammation markers, Figs. 1D and 2D), suggesting that an ATF3-dependent inflammatory response may contribute to cardiac maladaptive remodeling.

### 3.4. PE induces an ATF3 dependent inflammatory response in the heart

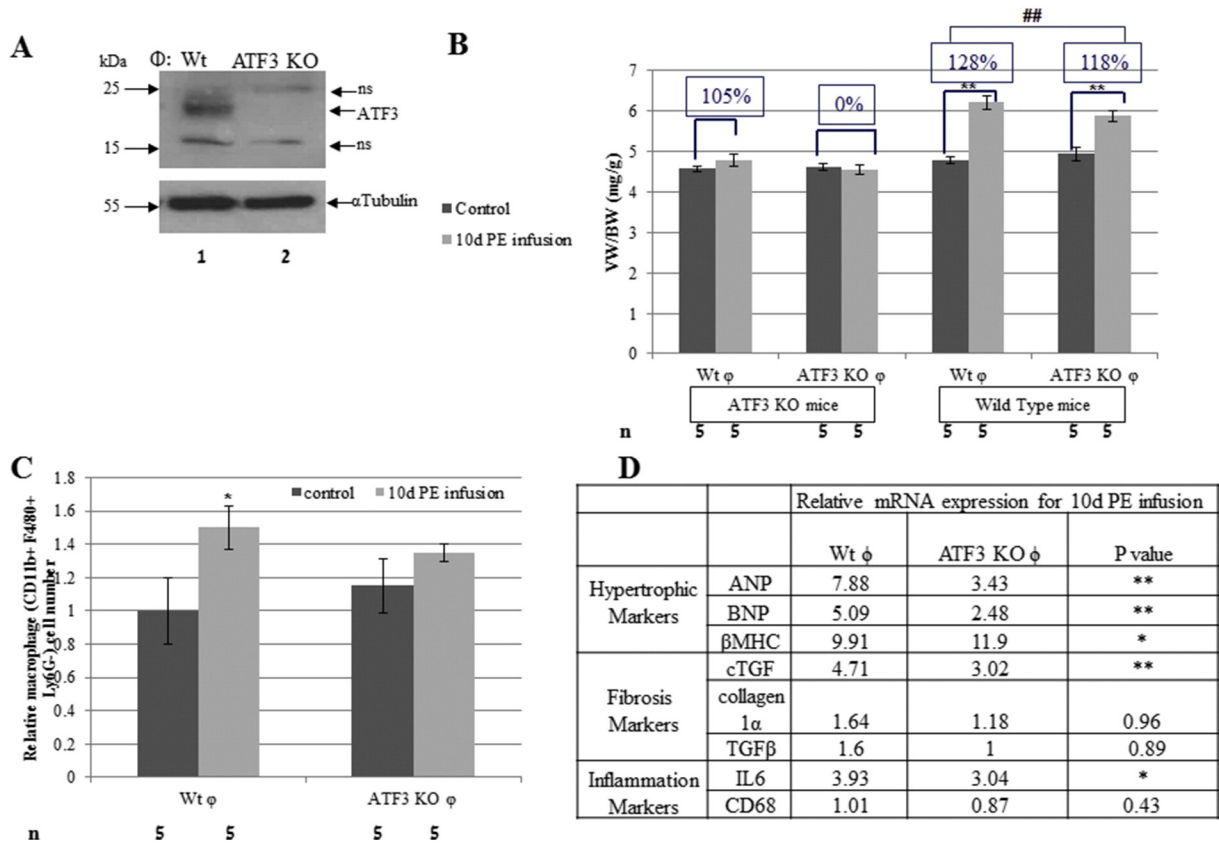
To examine whether transient ATF3 expression in response to PE may induce an inflammatory response, we assessed the mRNA levels of multiple cytokines and inflammatory markers one hour after PE injection in the hearts of wild type and compared it to the response of ATF3-KO mice. Indeed, PE treatment induced the transcription of ATF3 as well as multiple cytokines and inflammatory related genes in wild type mice (Table 1). In contrast, ATF3-KO mice exhibited a weaker transcriptional induction of the various cytokines and inflammatory genes, suggesting that ATF3 protein may be involved in the inflammatory response following PE-induced pressure overload (Table 1). Collectively, these results led us to hypothesize that expression of ATF3 in non-cardiomyocyte cells, possibly hematopoietic derived cells, may contribute to maladaptive cardiac remodeling processes.

### 3.5. Bone marrow transplantation

To evaluate the contribution of ATF3 expression in BM cells to cardiac remodeling processes, we performed a bone marrow transplantation experiment (BMT), in which the BM of wild type mice was replaced with BM derived from ATF3-KO mice. Briefly, C57Bl/6 mice were lethally irradiated and then injected intravenously with freshly prepared BM from either wild type or ATF3-KO mice. To verify complete replacement of the wild type BM with ATF3-KO derived BM, genomic DNA was



**Fig. 3.** ATF3 expression in the BM is required for pressure overload-induced hypertrophy. A. BM transplantation of C57Bl/6 female mice followed by vehicle control or PE treatment (2 weeks). VW/BW was calculated. The results represent the mean and SEM of the indicated number of animals (n). Asterisk (\*) indicates P value < 0.05 of a one-tailed t-test compared to vehicle treated controls. The percentage (%) of ventricle growth in PE infused mice compared to vehicle control for each BM genotype is indicated. Pound (#) indicates P value < 0.05 of a  $\chi^2$  test for expected-versus-observed ratio comparing the percentage (%) of ventricle growth in Wt BM to ATF3-KO BM of PE versus control infused mice. B. C57Bl/6 wild type and ATF3-KO mice were implanted with either vehicle control or PE mini-osmotic pumps for 10 days. Ventricles were separated to single cells followed by FACS analysis using CD11b, Gr1 (Ly-6G) and F4/80 cell surface markers. The level of infiltrating macrophages (CD11b<sup>+</sup>, F4/80<sup>+</sup>, Ly-6G<sup>+</sup> cells) was calculated relative to that in the hearts of wild type untreated mice, which was set as 1. The results represent the mean and SEM of the indicated number of mice (n). Asterisk (\*) indicates P value < 0.05 of a one-tailed t-test compared to vehicle treated control. C. Adult ATF3-Tg mice fed with normal chow for one or six weeks. Mice were sacrificed and ventricles were prepared as single cells followed by FACS analysis as described in B. The results represent the mean and SEM of the indicated number of animals. Asterisks (\*\*) indicate P value of 0.01 of one-tailed t-test relative to Wt one week control set as 1.



**Fig. 4.** Peritoneal wild type macrophages induce cardiac hypertrophy in wild type but not ATF3-KO PE-infused mice. **A.** Western blot analysis of peritoneal macrophage cell lysate isolated from either wild type or ATF3-KO mice. Molecular weight marker is indicated in kDa. Non-specific bands are indicated (n.s.). **B.** Peritoneal macrophages ( $10^6$  cells) isolated from either wild type or ATF3-KO mice were injected into the tail vein of either wild type or ATF3-KO mice three days after the implantation of osmotic pumps containing vehicle control or PE. One week later, mice were sacrificed and VW/BW was calculated. The results represent the mean and SEM of the indicated number of animals (n). Asterisk (\*\*) indicates P value < 0.01 of a one-tailed t-test compared to vehicle treated control. Pound (##) indicates P value < 0.01 of a  $\chi^2$  test for expected-versus-observed ratio comparing the percentage (%) of ventricle growth in Wt to ATF3-KO injected macrophages of PE versus control infused mice. **C.** Wild type mice injected with wild type or ATF3-KO macrophages were treated as described in B. Mice were sacrificed and ventricles were prepared as single cells followed by FACS analysis using CD11b, Gr1 (Ly-6G) and F4/80 cell surface markers. The level of infiltrating macrophages (CD11b<sup>+</sup>, F4/80<sup>+</sup>, Ly-6G<sup>+</sup>) was calculated relative to that in the hearts of untreated mice injected with wild type macrophages, which was set as 1. The results represent the mean and SEM of the indicated number of mice (n). Asterisk (\*) indicates P value < 0.05 of a one-tailed t-test compared to vehicle treated control. **D.** The expression levels in the hearts derived from the experiment described in B were measured by qRT-PCR, normalized to GAPDH. The ratio of expression levels in PE-treated to vehicle control-treated mice is shown. Results are expressed as means of the indicated number of animals (n) in B. Asterisks (\*/\*\*) indicate P value < 0.05 or 0.01 respectively of a one-tailed t-test comparing wild type to ATF3-KO macrophage injection.

prepared from white blood cells and used as DNA template for PCR with ATF3 primers designed to distinguish between wild type and ATF3-KO alleles. Indeed, this analysis showed a complete replacement of the wild type BM with ATF3-KO derived BM (supplementary Fig. 3). Mice transplanted with either wild type or ATF3-KO BM were treated with either vehicle control or PE infusion. Mice were sacrificed after one week and heart growth was assessed. The extent of heart growth in mice transplanted with ATF3-KO derived BM was significantly less than in mice transplanted with wild type BM (Fig. 3A), recapitulating the results obtained with mice harboring complete ATF3-KO (Fig. 1A). In addition, the level of BNP (hypertrophic marker) and  $\alpha$ -skeletal actin (fibrosis marker) were significantly higher in mice harboring wild type BM as compared with mice transplanted with ATF3-KO BM (supplementary Fig. 4). We failed to detect significant alterations in inflammatory gene markers in vehicle control treated mice, probably due to the strong inflammatory response following X-Ray irradiation (supplementary Fig. 4).

### 3.6. PE induces macrophage cell number in the heart in an ATF3 dependent manner

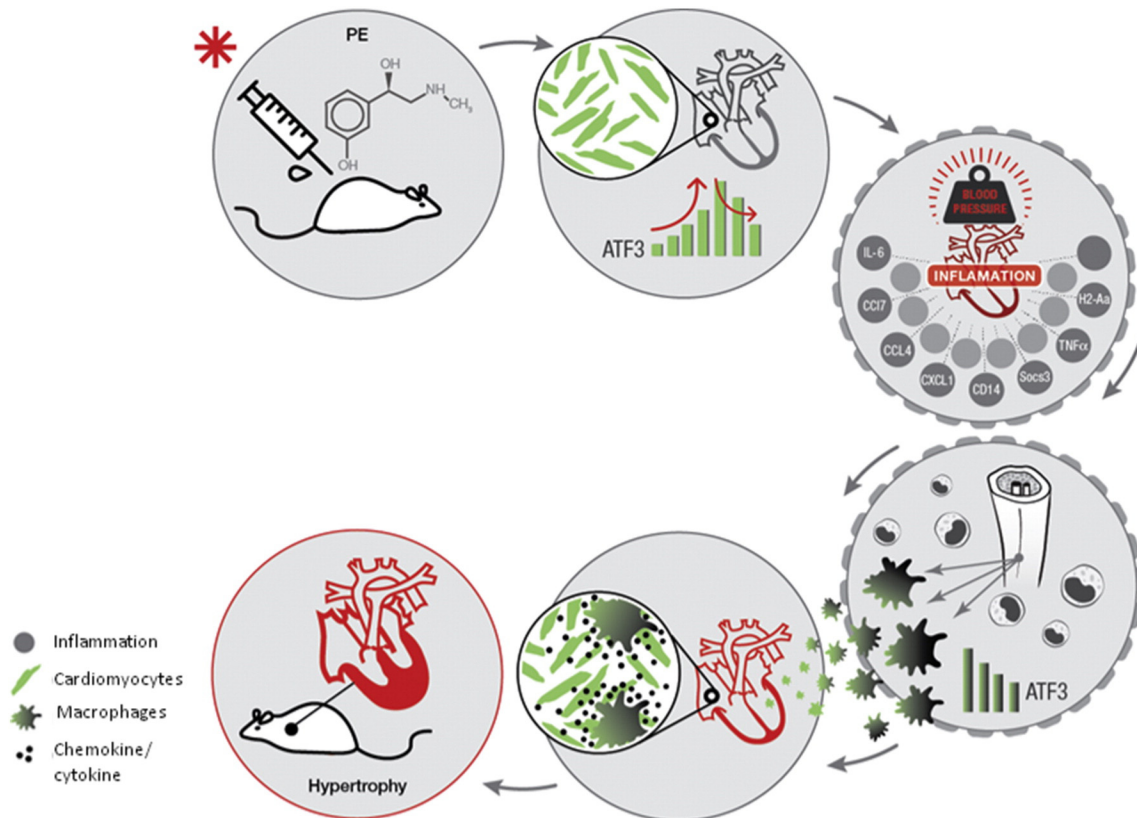
To identify the specific bone-marrow derived cell type involved in the cardiac remodeling effects following PE infusion, hearts derived from either vehicle control or one week PE treated mice were prepared as single cell suspensions. Cells were stained with antibodies against

various cell surface markers and analyzed by flow cytometry. The number of macrophages in the ventricles of wild type mice was increased by almost two folds following one week PE infusion. In contrast, there was no significant change in the number of macrophages in the ventricles of PE-infused ATF3-KO mice (Fig. 3B). Recruitment of various other cell types, such as dendritic cells, granulocytes, monocytes and T-cells, was not significantly changed in the hearts of wild type or ATF3-KO mice in response to PE infusion (supplementary Fig. 5). Consistently, ventricles derived from cardiac specific ATF3 transgenic mice exhibited a 3 fold increase in the number of macrophages one and six weeks following ATF3 expression (Fig. 3C). This suggests that cardiac ATF3 expression is sufficient to result in macrophage attraction independent of chronic pressure overload.

### 3.7. Wild type macrophages potentiates cardiac remodeling in wild type mice following PE infusion

To directly examine the role of macrophage infiltration into the heart, mice were injected with thioglycolate and activated-peritoneal-macrophages were isolated from either wild type or ATF3-KO mice. Western blot analysis revealed that ATF3 was highly expressed in peritoneal macrophage cells derived from wild type but not from ATF3-KO mice (Fig. 4A). Next, a macrophage adoptive transfer experiment was performed, namely, isolated peritoneal macrophages were injected intravenously ( $10^6$ ) into either vehicle treated or PE infused





**Fig. 5.** Schematic model for PE induced cardiac remodeling. Single PE injection induces a transient expression of ATF3 in cardiomyocytes. Upon chronic PE infusion, an ATF3 dependent inflammatory gene program is induced in the heart. This results in macrophage activation and recruitment to the heart and secretion of cytokines/chemokines, yet to be identified that induce cardiac maladaptive processes in an ATF3 dependent manner. Ablation of ATF3 expression in either cardiomyocytes or macrophages may be beneficial in suppressing the maladaptive response in cardiac remodeling processes following chronic pressure overload.

wild type or ATF3-KO mice three days after the implantation of osmotic pumps and mice were sacrificed a week later. ATF3-KO mice displayed no significant increase in VW/BW in response to PE treatment following injection of either wild type or ATF3-KO macrophages (Fig. 4B).

In contrast, wild type mice injected with wild type macrophages displayed a significantly higher increase in VW/BW (28%) as compared to wild type mice that were injected with ATF3-KO macrophages (18%, Fig. 4B). Moreover, wild type macrophages infiltrated the hearts of PE-infused mice to a greater extent than ATF3-KO macrophages (Fig. 4C). Consequently, the increase in heart growth was accompanied by the rise in the level of multiple hypertrophic, fibrosis and inflammatory markers (Fig. 4D). Collectively, these results demonstrate that ATF3 expression in the heart is a critical primary step for subsequent macrophage infiltration and suggest that ATF3 expression in macrophage cells contribute directly to maladaptive cardiac remodeling processes.

#### 4. Discussion

Cardiac hypertrophy is an adaptive response to meet higher cardiac demand under various stress conditions such as volume and pressure overload. If the stress sustains, the adaptive response becomes pathological, leading to cardiac remodeling, heart hypertrophy and heart failure [6–8].

Here we studied the role of ATF3 in cardiac remodeling processes. ATF3 serves as a hub of cellular adaptive responses to signals that perturb homeostasis [36]. ATF3 typically dampens the response by functioning as a negative regulator and repressing transcription. However, ATF3 may act as an activator in different cellular contexts [36] and through the association with different protein partners [23]. We used a PE infusion model aiming to mimic chronic pressure overload [7,32]. Here, we demonstrate that acute PE treatment results in the activation

of an ATF3 dependent inflammatory response in the heart (Table 1). ATF3 induction is typically transient, however, when PE treatment persists and become chronic, ATF3 expression is induced in macrophages and is responsible for the maladaptive cardiac remodeling response. Two lines of evidence suggest that cardiac ATF3 expression is absolutely necessary to initiate cardiac remodeling. First, cardiac specific ATF3-KO completely abrogates cardiac hypertrophy response following PE infusion (Fig. 2C). Second, in the macrophages adoption experiment, wild type macrophages injection into ATF3-KO mice failed to induce cardiac remodeling processes (Fig. 4B). Although ATF3 is found to be necessary, it is not sufficient to induce cardiac remodeling. We showed that wild type mice harboring ATF3-KO BM display reduced cardiac remodeling (Fig. 3A). In addition, injection of ATF3-KO macrophages into wild type mice displayed significantly reduced maladaptive cardiac remodeling (Fig. 4B). Therefore, our results show that ATF3 expression in the heart is necessary but it is not sufficient to induce maladaptive cardiac remodeling processes. Cardiac ATF3 expression needs to be accompanied by simultaneous ATF3 expression in macrophages in order to obtain maximal cardiac remodeling outcome.

Under normal physiological conditions, several immune cell types reside in the myocardium. Upon pathological stress, additional immune cells are recruited to the heart and promote cardiac remodeling by releasing cytokines, growth factors and metalloproteinases (MMPs) [2]. Following PE infusion, we observed the majority of cardiac infiltration corresponds to M2 macrophages (supplementary Fig. 6). M2 macrophages are known to suppress the immune response and promote fibrosis and tissue remodeling [37]. It was shown that myocardial damage following type 1 diabetes mellitus resulted in macrophage cell recruitment to the myocardium. Macrophages recruitment to the heart is probably due to the secretion of MCP-1 in response to high glucose levels [38]. Thus, MCP-1 secretion by cardiomyocytes may

serve as a macrophage chemoattractant [39]. Consistently, MCP-1 neutralizing antibody was shown to attenuate cardiac macrophage cells accumulation and inhibits fibroblast proliferation and fibrosis in a pressure overload model [40]. Suggesting that macrophage infiltration following pressure overload participates in the maladaptive remodeling changes in the heart.

Here, we demonstrate that MCP-1 expression is elevated in the heart in response to PE treatment. Since MCP-1 expression is higher in wild type mice as compared with ATF3-KO mice, it may be part of the macrophage recruitment mechanism in response to pressure overload. We propose that the recruitment of activated macrophage contribute to the maladaptive remodeling process by the secretion of various cytokines/chemokines that are yet to be identified.

A previous study using transverse aortic constriction (TAC), suggests that the lack of ATF3 expression promotes the maladaptive cardiac remodeling and worsen cardiac function. Significantly, in the TAC model, ATF3 expression gradually increases with the progression of maladaptive cardiac remodeling [29,30]. In contrast, in the PE infusion model, we observe a loss of ATF3 expression with model progression. In addition, PE, an  $\alpha$ 1-adrenergic agonist may act by activation of signaling pathways beside the increase in blood pressure. Although ATF3 expression may be the basis for the apparent discrepancy, chronic adult ATF3 expression in transgenic mice is maladaptive either alone or in combination with chronic PE infusion treatment [27]. Therefore, other mechanisms are involved in the switch between ATF3-mediated induction and suppression of cardiac remodeling processes. Understanding the context difference between these two models is an important challenge towards revealing how pressure overload adaptive responses turn into maladaptive processes resulting in reduced cardiac function, heart failure and death.

## 5. Conclusion

We suggest that PE dependent cardiac maladaptive remodeling response is made up of two stages. This is described schematically in Fig. 5. The first, involves pressure overload that induces transient ATF3 expression specifically in cardiomyocytes. ATF3 induction turns-on an inflammatory program in the heart that attracts bone marrow derived macrophages to the heart. Macrophage recruitment promotes an ATF3 dependent gene expression program that includes secretion of cytokines/chemokines that results in a cardiac maladaptive remodeling response and poor cardiac outcome. Blocking ATF3 activity in either cardiomyocytes or macrophages may be beneficial in suppressing the maladaptive response to cardiac remodeling processes following chronic pressure overload.

## Disclosures

The authors declare no conflict of interests.

## Authors' contribution

Lilach Koren designed and performed all experiments and manuscript preparation; Dror Alishekevitz designed and performed bone marrow transplantation and guided in FACS experiments performance and analysis. Alex Nevelsky designed and performed bone marrow transplantation; Izhak Kehat performed IHC assessment and data analysis; Yuval Shaked designed, performed data analysis and manuscript preparation; and Tsonwin Hai designed, performed data analysis and manuscript preparation.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.ijcard.2015.06.099>.

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