Interferon-γ Ablation Exacerbates Myocardial Hypertrophy in Diastolic Heart Failure

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Abstract

Diastolic heart failure (HF) accounts for up to 50% of all HF admissions with hypertension being the major cause of diastolic HF. Hypertension is characterized by left ventricular (LV) hypertrophy (LVH). Pro-inflammatory cytokines are increased in LVH and hypertension but it is unknown if they mediate the progression of hypertension-induced diastolic HF. We sought to determine if IFNγ plays a role in mediating the transition from hypertension-induced LVH to diastolic HF. Twelve-week old BALB/c (WT) and IFNγ deficient (IFNγKO) mice underwent either saline- (n=12) or aldosterone-infusion (n=16), uninephrectomy and fed 1% salt water for 4-weeks. Tail-cuff blood pressure, echocardiography and gene/protein analyses were performed. Isolated adult rat ventricular myocytes were treated with IFNγ (250 U/ml) and/or aldosterone (1 μM). Hypertension was less marked in IFNγKO-aldosterone mice than in WT-aldosterone mice (127±5 vs. 136±4 mmHg; P<0.01), despite more LVH (LV/BW ratio: 4.9±0.1 vs. 4.3±0.1 mg/g) and worse diastolic dysfunction (E/A ratio: 3.1±0.1 vs. 2.8±0.1). LV ejection fraction was no different between IFNγKO-aldosterone vs. WT-aldosterone mice. LV end systolic dimensions were significantly decreased in IFNγKO-aldosterone vs. WT-aldosterone hearts (1.12±0.1 vs. 2.1±0.3 mm). Myocardial fibrosis and collagen expression were increased in both IFNγKO-aldosterone and WT-aldosterone hearts. Myocardial autophagy was greater in IFNγKO-aldosterone than WT-aldosterone mice. Conversely TNF-α and IL-10 expressions were increased only in WT-aldosterone hearts. Recombinant IFNγ attenuated cardiac hypertrophy in vivo and modulated aldosterone-induced hypertrophy and autophagy in cultured cardiomyocytes. Thus IFNγ is a regulator of cardiac hypertrophy in diastolic HF and modulates cardiomyocyte size possibly by regulating autophagy. These findings suggest that IFNγ may mediate adaptive
downstream responses and challenge the concept that inflammatory cytokines mediate only adverse effects.

Key Words: diastolic heart failure, aldosterone, IFNγ, cardiac hypertrophy, autophagy
**Introduction**

Diastolic heart failure (HF) accounts for up to 50% of all clinical HF presentations (43) but, unlike systolic HF, there are no evidence-based therapies for diastolic HF despite increasing morbidity and mortality (45). This is due, in part, to an incomplete understanding of mechanisms that underlie diastolic HF (45), coexisting co-morbidities and limited experimental models that can successfully replicate human diastolic disease (43).

Hypertension remains the most important cause of diastolic HF and may be characterized by left ventricular (LV) hypertrophy (LVH) (29). Resistant hypertension increases the propensity to developing diastolic HF (31). Importantly relative increases in aldosterone levels occur in both hypertension and diastolic HF (5; 12), with elevated aldosterone levels being present in resistant hypertension even in the absence of primary aldosteronism (36). In order to study potential mechanisms mediating diastolic dysfunction and diastolic HF, we developed and characterized a murine model of aldosterone-induced hypertension with LVH, diastolic dysfunction and diastolic HF (20; 34; 50). This experimental model of hypertension-induced diastolic HF encompasses the myocardium, effects of the vasculature and these mice demonstrate exercise impairment (50). Circulating aldosterone levels (5-6 ng/mL) are in the physiological range in this chronic aldosterone infusion model (39; 50). Aldosterone binds to the mineralocorticoid receptor (MR), which is present on cardiomyocytes (33). Others have shown that MR activation promotes increased diastolic stiffness and also the transition from hypertensive heart disease to diastolic HF in aged canines (38). Conversely selective inhibition of aldosterone prevents diastolic dysfunction and modulates reactive oxygen species (ROS) in hypertensive experimental models (25).
Antihypertensive therapy improves cardiac remodeling, LV systolic and diastolic performance with no added risk of cardiac deterioration should blood pressure rise subsequently (46). However, when LVH regression is absent, LV diastolic dysfunction does not improve (46). Although LVH is often an intermediary between hypertension and diastolic HF (40), hypertensive heart disease may progress to HF independent of LVH (22). Thus the mechanisms of LVH regression in hypertension-induced diastolic HF and the transition from LVH to diastolic HF are poorly understood.

Autophagy is a cell survival mechanism, that is upregulated during cell starvation and nutrient deprivation (42). Basal autophagy is adaptive and critical to the maintenance of cellular homeostasis and function (14). However, exaggerated autophagy is maladaptive and may lead to the transition from compensated LV remodeling to decompensated HF (27; 55). It is unknown if this autophagy is an epiphenomenon or a causative factor in the transition from adaptive LVH to maladaptive LV remodeling.

Pro-inflammatory cytokines (e.g. IFNγ and TNF-α) are increased in both hypertension and diastolic HF (20; 28; 34; 50) with a pro-inflammatory milieu possibly mediating the progression from hypertension and cardiac remodeling to subsequent LV dysfunction (24; 49). Likewise immune dysfunction, an imbalance between T-helper (Th1) and Th2 cells, is evident in human HF (51). Th1 cells produce IFNγ and other Th1-related cytokines (e.g., TNF-α, IL-12, IL-18) (6). Although TNF-α infusion directly impairs LV function (4), the role of IFNγ in HF and cardiac remodeling are not clear. On the one hand, IFNγ overexpression induces a cardiomyopathy with severe systolic and diastolic dysfunction (32); whereas IFNγ is cardioprotective in murine autoimmune myocarditis and HF (1) and suppresses inflammation in experimental autoimmune disease (2). We previously demonstrated increased myocardial IFNγ
gene and protein expression in mice with hypertension-induced diastolic HF, suggesting that IFNγ may play a pathophysiological role in mediating cardiac remodeling in LVH and diastolic HF (34; 50). We thus tested the hypothesis that IFNγ plays a pivotal role in the transition from hypertension-induced LVH to diastolic HF.

**Methods and Materials**

**Animals.** Twelve-week old male IFNγ deficient (IFNγKO) mice on a BALB/c genetic background and wild-type BALB/c littermates (WT) were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were maintained on a 12-h light/dark cycle in a temperature-controlled (19–21°C) room. Mice were fed standard rodent chow *ad libitum*.

**Aldosterone infusion.** Mice were anesthetized with 80-100 mg/kg ketamine and 5-10 mg/kg xylazine intraperitoneal. All mice (25-27 g) underwent uninephrectomy and then received either a continuous infusion of saline (control) or d-aldosterone (0.15 μg/hour) (Sigma-Aldrich Co.) for 4-weeks via osmotic minipumps (Alzet, Durect Corporation) (20). All mice were maintained on 1% NaCl drinking water. The groups studied were: (a) WT-saline, n=6; (b) WT-aldosterone, n=8; (c) IFNγKO-saline, n=6 and (d) IFNγKO-aldosterone, n=8.

A 2nd group of mice underwent the same surgical procedures as outlined above; however 14 days after aldosterone infusion only, mouse recombinant IFNγ (rIFNγ; Prepro Tech, Rocky Hill, NJ) at 80 μg/kg or 5% BSA (bovine serum albumin) was given twice daily subcutaneously for the remaining 14 days (a) WT-saline, n=3; (b) WT-aldosterone, n=4; (c) IFNγKO-saline, n=3 and (d) IFNγKO-aldosterone, n=4. The Boston University School of Medicine Institutional Animal Care and Use Committee approved all the study procedures related to handling and surgery of animals.
Physiological measurements. Non-invasive heart rate (HR) and tail cuff blood pressure (BP) were measured weekly (BP-2000, VisiTech) as previously described (50).

Echocardiography determinants for LV dimensions. Transthoracic echocardiography was performed in conscious mice using an Acuson Sequoia C-256 echocardiography machine and a 15-MHz probe as previously described (34; 50). Total wall thickness (TWT) was derived from an average of the interventricular septum and posterior wall thickness. As previously validated in mice, left ventricular (LV) mass was calculated from the formula described by Devereux et al (7; 9).

Doppler echocardiography for diastolic function. Using the Vevo 770 High-Resolution In Vivo Imaging System (VisualSonics,Toronto) mitral Doppler flow studies were performed as previously described (50). Images were acquired using a 30 MHz transducer. Because diastolic function measurements are sensitive to HR and loading conditions (41; 52), HR was maintained around 350 beats/min by using isoflurane (0.5-1.5%) with minimal effects on diastolic function (34; 50; 54). Images were recorded for 30–40 cardiac cycles and measurements were made from representative cycles. The apical four-chamber view was used to record the mitral Doppler flow spectrum. Peak early (E) and late (A) mitral inflow velocities, deceleration time (DT) of early filling and isovolumetric relaxation time (IVRT) were measured.

Hemodynamic measurements. Measurements were obtained, immediately after the mitral Doppler flow studies under general anesthesia, which was maintained with 1.5% isoflurane during cannulation. The right carotid artery was dissected and cannulated with a 1.4F catheter tip micromanometer (ARIA, Millar Instruments), which was advanced retrogradely into the LV cavity under echocardiographic guidance. After hemodynamic indices were stable for ~10-15 minutes, LV end-diastolic pressure (LV EDP), and maximal rates of LV pressure rise
and fall (LV dP/dt<sub>max</sub> and dP/dt<sub>min</sub>) were recorded at a minimum sampling rate of 1,000 Hz on a Powerlab 4SP data recorder (AD Instruments) and stored for later analysis with PVAN software, version 3.0 (Millar Instruments). The time constant of isovolumic relaxation (τ) was derived using the PVAN data analysis software using the Weiss method (48). At the end of the experiment, mice were killed and organs were rinsed and blotted and body weights (BW), heart weights (HW) and LV weights were measured. The LV was snap-frozen in LN₂ for molecular and biochemical analyses.

**Organ weight and tissue analysis.** After 4-weeks mice were sacrificed, at which time BW, HW and LV weights were also determined. Hearts were either: (a) arrested in diastole by KCl (30mmol/l), weighed, perfused with 10% buffered formalin and sliced horizontally for histology or (b) snap-frozen in liquid nitrogen. Trichrome-stained sections (5 μm) were visualized by light microscopy to measure fibrosis and the entire section was quantified using Bioquant Image analysis software. The wet-to-dry lung weight ratios were determined as an index of pulmonary congestion (20). To determine cardiomyocyte cross-sectional area (Area=πr²), sections from the hearts were examined and quantified using the Bioquant Image analysis software (50).

**Determination of mRNA.** Total RNA from the LV of the 4 groups mice were extracted with PureLink™ Micro-to-Midi Total RNA purification system (Invitrogen). Total RNA from the LV were extracted with PureLink™ Micro-to-Midi Total RNA purification system (Invitrogen). cDNA synthesis from total RNA was performed using a Thermoscript™ RT-PCR system (Invitrogen) according to the manufacturer’s instructions. Transcript expression levels of collagen I, collagen III and GAPDH were quantified by iCycler iQ Real-Time PCR Detection
Systems (Bio-Rad) using FastStart Universal SYBR Green Master Mix (Roche, IN, USA). Transcript levels were adjusted relative to the expression of GAPDH.

Primer sequence for mouse.

**Collagen I:** (F): 5′-CAG AAG ATG TAG GAG TCG AG-3′; (R): 5′-GGA CCC AAG GGA GAC CCT GG-3′; **Collagen III:** (F): 5′-GTG GAC TGC CTG GAC CTC CA-3′; (R): 5′-GGT ATC AAA GGC CCA GCT GG-3′; **GAPDH** (F): 5′-TCA CCA CCA TGG AGA AGG-3′, GAPDH (R): 5′-GCT AAG CAG TTG GTG GTG CA-3′.

Isolation and treatment of adult rat ventricular myocytes (ARVM). ARVM (90-95% purity) were isolated from the hearts of adult Sprague–Dawley rats as described previously (33). ARVM were plated at a nonconfluent density of 30 to 50 cells/mm² on plastic culture dishes (Fisher) precoated with laminin (1 µg/cm², Invitrogen), and maintained in ACCT medium (DMEM; BSA, 2 mg/mL; L-carnitine, 2 mmol/L; creatinine, 5 mmol/L; taurine, 5 mmol/L; penicillin, 100 IU/mL; and streptomycin, 10 g/mL) for 16-hours before drug treatment. ARVM were treated with aldosterone (1 µM, Sigma) for 18 hrs. Recombinant IFNγ (250 U/ml, Sigma) was added 30 minutes before aldosterone stimulation.

Western blot analysis. LV tissue was collected in lysis buffer. Protein concentration was determined using the Bradford assay (Bio-Rad). Membranes were probed with the following antibodies: LC3 and ATG5 (Novus Biologicals, Littleton CO); Beclin-1 (Cell Signaling, Danvers, MA); TNF-α and IL-10 (R&D Systems, Minneapolis, MN); p62, collagen I collagen III and GAPDH (Abcam, Cambridge, MA). Chemiluminescence was quantified by densitometry (Molecular Analyst, Bio-Rad). Blots were normalized to GAPDH or with Coomassie® Brilliant Blue staining of the gels (Sigma Aldrich). Chemiluminescence was quantified by densitometry (Molecular Analyst, Bio-Rad).
**3H-Leucine Incorporation.** 3H-leucine incorporation was measured over the final 4-hours of a 24-hour period of aldosterone treatment described previously (10). The activity of 3H-leucine was determined by scintillation counting of samples harvested and precipitated. Total cellular protein content was determined by the Bradford method using Coomassie Blue (BioRad Laboratories) as previously described (10). Cellular protein content was normalized to DNA content, which was measured by fluorometric assay after cell lysis.

**Statistical analysis.** Results are presented as mean±s.e.m. Statistical analyses of the data were carried out using the Student’s t test (2-sided). When necessary, 1- or 2-way ANOVA (followed by Student-Newman-Keuls post-hoc tests when appropriate) was applied for multiple comparisons. A value of P<0.05 was considered statistically significant.

**Results**

**Exacerbated LVH despite a diminished hypertensive response.** There was 100% survival in the mice. Aldosterone infusion significantly increased systolic BP over 4-weeks in both WT and IFNγKO mice. However, the aldosterone-induced hypertensive response was not as robust in IFNγKO vs. WT-aldosterone mice by the end of 4-weeks (127±5 mmHg vs. 136±4 mmHg, P<0.05; **Fig. 1A; Table**). As expected, chronic aldosterone increased LV mass (measured by echocardiography) in WT mice. However LV mass was greater in IFNγKO-aldosterone mice over the 4-weeks (**Fig. 1B; P<0.05**). Similarly LV/ body weight (BW) ratio increased in WT-aldosterone (4.3±0.1) and was more marked in IFNγKO-aldosterone mice (4.9±0.1; P<0.001 vs. WT-aldosterone). Chronic aldosterone increased cardiomyocyte cross-sectional area in WT-aldosterone but was greater in IFNγKO-aldosterone hearts (P<0.05; **Table**).
HR was lower in both groups of aldosterone infused mice but was no different between WT and IFNγ mice. BW was comparable between all groups of mice. Chronic aldosterone increased pulmonary congestion (as measured by wet-to-dry lung ratio) but no different between WT and IFNγ aldosterone-infused mice (Table).

**Mice lacking IFNγ had smaller LV cavity size (Figs. 2A-D).** In WT mice, chronic aldosterone had negligible effects on LV end diastolic dimensions (LVEDD) and end systolic dimensions (LVESD) vs. WT-saline mice (3.5±0.4 vs. 3.4±0.2 mm and 2.1±0.1 vs. 1.8±0.2 mm, respectively). However, aldosterone infusion in IFNγKO mice, significantly decreased LVESD (1.1±0.1 mm) vs. WT-aldosterone mice. LVEDD was unchanged. LV ejection fraction (EF) was within the normal range but significantly increased in IFNγKO-aldosterone vs. WT-aldosterone hearts (81±2% vs. 70±2%). Total wall thickness (TWT) was greater in IFNγKO-aldosterone than WT-aldosterone mice (1.4±0.1 vs. 1.2±0.1 mm; P<0.05). There was no intracavitary gradient or obstruction.

**Mice lacking IFNγ had more severe diastolic dysfunction.** As in our prior studies (20; 34; 50), chronic aldosterone caused diastolic HF (defined as preserved LV EF with pulmonary congestion) in both WT and IFNγKO mice (Table, Fig. 2D). There was evidence of aldosterone-induced LV diastolic dysfunction using both hemodynamic monitoring and Doppler echocardiography (Table). Peak rates of LV pressure development (LV dP/dt\textsubscript{max}) increased with aldosterone infusion in both WT and IFNγKO mice indicating improved LV contractile function. LV dP/dt\textsubscript{min} in the IFNγKO-aldosterone mice was significantly less negative compared to IFNγKO-saline and WT-aldosterone mice, indicating markedly worse myocardial relaxation. Although Tau was elevated in both groups of aldosterone-infused mice it was comparable between IFNγKO-aldosterone and WT-aldosterone hearts. However the impaired LV relaxation
(LV dp/dt\textsubscript{min}) and increased LV EDP suggested worse diastolic dysfunction in IFN\gamma KO-aldosterone than WT-aldosterone mice. Similarly mitral valve inflow velocity (with Doppler echocardiography) also showed impaired diastolic function in IFN\gamma KO-aldosterone mice (Table). Because normal physiological HRs in mice are rapid (>600bpm) and results in the loss of the A-velocity by Doppler (41), these measurements were made at comparable (although non-physiological) HRs using isoflurane which lowered HRs to comparable levels (~350bpm) to control for loading conditions (41). In sedated mice HR were lowered in all 4 groups and were no different (Table). In WT mice, aldosterone significantly increased mitral E velocity by 55\pm 2\% (early diastolic filling velocity) whilst there was minimal change in mitral A wave velocity (late diastolic filling). The resultant E/A ratio (2.8\pm 0.1) was increased in WT-aldosterone mice, indicating reduced LV compliance (increased LV stiffness). Aldosterone also significantly increased mitral E velocity by 75\pm 5\% in IFN\gamma KO mice. The resultant E/A ratio (3.1\pm 0.1) –a measure of diastolic dysfunction, was greater in IFN\gamma KO-aldosterone than WT-aldosterone hearts (P<0.05). Similar to humans, the increased E/A ratio suggests a restrictive mitral inflow velocity profile. Deceleration time (DT) was non-significantly shortened consistent with the elevated E/A ratio and diastolic dysfunction in both groups of aldosterone-infused hearts. Aldosterone caused prolongation of isovolumetric relaxation time (IVRT) in only IFN\gamma KO-aldosterone hearts (P<0.05 vs. respective saline control). Collectively, these hemodynamic and Doppler measurements demonstrate that diastolic function is significantly impaired in IFN\gamma KO mice with hypertension-induced diastolic HF.

**Cardiac fibrosis was not different (Figs. 3A-D).** The extracellular matrix plays an important role in diastolic HF and diastolic dysfunction (23), thus cardiac fibrosis was quantified after Masson trichrome staining. Total fibrosis (interstitial and perivascular fibrosis) was
increased but no different between WT-aldosterone (3.0-fold \(P<0.05\) vs. WT-saline) and IFN\(\gamma\)KO-aldosterone mice (5.0-fold \(P<0.05\) vs. IFN\(\gamma\)KO-saline). Myocardial collagen transcripts and QRT-PCR were performed with specific primer sets with normalization to GAPDH. Chronic aldosterone increased both collagen I and collagen III transcripts in WT mice (4.3-fold and 2.5-fold, respectively) and in IFN\(\gamma\)KO mice (4.8-fold and 2.6-fold, respectively). Aldosterone also increased both collagen I and III protein expression in WT and IFN\(\gamma\)KO hearts vs. respective saline controls (data not shown). Similar to gene expression, protein expression was no different between WT-aldosterone or IFN\(\gamma\)KO-aldosterone hearts suggesting that changes in fibrosis or collagen were unlikely to contribute to the progression of diastolic dysfunction and diastolic HF in IFN\(\gamma\)KO mice.

**Total deletion of IFN\(\gamma\) increases myocardial autophagic protein expression (Figs. 4A-C).** Autophagy serves as a cell survival mechanism and is upregulated during cell stress (such as nutrient deprivation) (42), but autophagy in excess may mediate adverse cardiac remodeling (55). It is unknown if this autophagy plays a role in diastolic dysfunction and diastolic HF. We thus measured LV autophagic protein expression. The ratio of LC3-II and LC3-I protein expression is used as an indicator of autophagosome formation and as a measurement of autophagy (26). Aldosterone increased the LC3-II/LC3-I ratio expression in WT hearts by 76±4\% vs. WT-saline hearts (\(P<0.001\)) and in IFN\(\gamma\)KO hearts by 163±16\% vs. IFN\(\gamma\)KO-saline (\(P<0.01\)) and was significantly greater in IFN\(\gamma\)KO-aldosterone vs. WT-aldosterone hearts (\(P<0.05\)). Chronic aldosterone also increased p62 protein expression in WT hearts (2.5-fold) and in IFN\(\gamma\)KO hearts (3.2-fold) (\(P<0.05\) for both vs. respective saline controls). There was no significant difference in p62 expression between WT-aldosterone and IFN\(\gamma\)KO-aldosterone.
hearts. Neither myocardial Beclin-1 nor ATG5 protein expression was altered by chronic aldosterone or IFNγ deficiency (data not shown).

**No increase in myocardial pro-inflammatory cytokine expression with total deletion of IFNγ (Figs. 4D-F).** We previously showed that aldosterone increases myocardial TNF-α and IFN-γ expression in hypertension-induced cardiac remodeling (34). TNF-α and IL-10 expression were increased 85±8% ($P<0.001$ vs. WT-saline) and 35±2% ($P<0.05$ vs. WT-saline), respectively in WT-aldosterone hearts. Lack of IFN-γ in aldosterone-induced diastolic HF did not induce myocardial TNF-α and IL-10 expression. Thus IL-10 expression was lower than WT-aldosterone mice ($P<0.01$). Lack of myocardial Th2 cytokine induction, in particular IL-10 in the absence of IFNγ, may contribute to the progression of hypertension-induced diastolic dysfunction and diastolic HF.

**Recombinant IFNγ attenuates cardiac hypertrophy in vivo (Figs. 5A-C).** To examine whether IFNγ supplementation ameliorated aldosterone-induced cardiac hypertrophy in vivo, both IFNγKO-aldosterone and WT-aldosterone mice were treated with either mouse recombinant IFNγ (rIFNγ) or 5% BSA (control) for 14 days post–surgery. Recombinant IFNγ or BSA treatment had no effect on BP in either WT-aldosterone or IFNγKO-aldosterone mice (data not shown). Recombinant IFNγ attenuated the aldosterone-induced changes in LVH (increased total wall thickness) observed in IFNγKO (-20±3% vs. BSA-treated IFNγKO-aldosterone mice; $P<0.05$) and WT (-22±2% vs. BSA-treated WT-aldosterone mice; $P<0.05$) mice. Recombinant IFNγ also decreased the cardiomyocyte cross-sectional area in WT-aldosterone ($P<0.05$) and IFNγKO-aldosterone hearts ($P<0.01$). There was a significant reduction in LV EDP in WT-aldosterone and IFNγKO-aldosterone hearts with rIFNγ treatment.
Recombinant IFNγ modulates cardiomyocyte hypertrophy and autophagy in vitro (Figs. 6A-C). To further investigate the reduction in cardiomyocyte cross-sectional area, \(^3\)H-leucine incorporation and total protein were measured in ARVM exposed to aldosterone. Cultured ARVM treated with aldosterone (1 \(\mu\)M) increased \(^3\)H-leucine incorporation and total protein content by 23±4% and 20±2%, respectively \((P<0.05\) vs. control for both). Recombinant IFNγ (250 U/ml) alone had no effect on both \(^3\)H-leucine incorporation and total protein content \((P=NS\) vs. control). Pretreatment with rIFNγ 30 min before aldosterone stimulation significantly decreased \(^3\)H-leucine incorporation and total protein content \((P<0.001,\) for both). To examine the effects of rIFNγ on cardiac hypertrophy-associated autophagy, ARVM were stimulated with aldosterone (1 \(\mu\)M) with or without rIFNγ (250 U/ml). Aldosterone stimulation induced LC3-II protein expression and thus increased the ratio of LC3-II/LC3-I. This increase was ameliorated by pretreatment with rIFNγ \((P<0.01\) vs. aldosterone alone). Recombinant IFNγ alone had no effect on LC3-II/LC3-I expression.

Collectively, these data suggest that IFNγ modulates cardiac hypertrophy via regulation of the autophagic protein, LC3.

Discussion

In hypertension-induced diastolic HF, complete deletion of IFNγ was associated with marked LVH. This LVH was accompanied by a worsening of diastolic dysfunction, despite a less robust hypertensive response in the aldosterone-infused IFNγKO mice. There was an increased LC3-II/LC3-I ratio (suggesting an increased accumulation of autophagosomes) and a lack of cytokine expression in the LV of these hypertrophied hearts. Recombinant IFNγ
modulated the aldosterone-induced LVH, reduced LV EDP in vivo and attenuated the increased LC3-II/LC3-I ratio in isolated cultured cardiomyocytes stimulated with aldosterone.

LVH is a common finding in hypertension (22). In humans, hypertension may proceed directly to clinical HF without LVH (40). Despite LVH being compensatory in response to myocardial stress, progressive LVH poses an increased risk for HF (22). We previously showed that reducing BP, in hypertension-induced diastolic HF, improved diastolic dysfunction without LVH regression (50). In the present study, independent of BP, rIFNγ reduced LVH, cardiomyocyte hypertrophy and decreased LV filling pressures. IFNγ acts directly on cardiomyocytes to inhibit pathological growth, thereby contributing to the beneficial effects of IFNγ on diastolic dysfunction in diastolic HF. Transcripts for IFNγ receptors are expressed on all cells and thus mediate IFNγ signaling (8). IFNγ has previously been shown to exert potential benefits in hypertensive heart disease (37). In our study the lower BP in the IFNγKO-aldosterone mice at the end of 4-weeks initially suggested that rIFNγ repletion might raise BP. On the contrary there was no effect on BP, thus raising the possibility that the elevated BP and LVH seen in the IFNγKO-aldosterone mice might not be temporally related. Diastolic dysfunction, like LVH (21), may be an intermediary between hypertension and diastolic HF (40) and an independent risk factor for HF and cardiovascular death (3). Similar to a human study by Watchell et al. (46), we previously showed that independent of blood pressure, increased LVH was associated with worsening of diastolic dysfunction. In the present study, the development of LVH may have exacerbated diastolic dysfunction in IFNγKO-aldosterone mice. Conversely diastolic dysfunction may have preceded the increase in LVH. Others have also shown that IFNγ abrogates cardiomyocyte hypertrophy (18), thus these data suggest that IFNγ may be a therapeutic target for diastolic dysfunction and diastolic HF.
Cardiac fibrosis is an important mediator in the pathophysiology of diastolic HF (43; 44; 49). Despite the exacerbation of LVH and diastolic dysfunction in IFNγKO-aldosterone mice, we saw no differences in myocardial fibrosis and collagen expression. Although regression of LVH is associated with collagen gene expression pattern reversal (11), we did not investigate this since there was no initial differences between the increase in collagen between both groups of aldosterone-infused mice. The quality of collagen (specifically cross-linking and glycation) and not the quantity may play a key role in translating quantity into mechanical stiffness in diastolic HF (44). Metalloproteinase activation may also influence stiffness independent of changes in collagen thus accelerating diastolic dysfunction (34).

The presence of autophagy in IFNγKO-aldosterone hearts suggests that it may play a pathophysiological role in LV cardiac remodeling and diastolic dysfunction in hypertension-induced diastolic HF. LC3-II and p62 expression were increased in both groups of aldosterone-induced diastolic HF hearts, with only LC3-II expression being greater in IFNγKO mice. The increased LC3-II/LC3-I ratio suggests autophagosome accumulation whereas the increased p62 expression suggests defects in the lysosomal end of the pathway. Although not explored in our study, p62 has been reported to mediate a protective role in cardiomyocytes against increased misfolded proteins: aggresome formation and the activation of selective autophagy (53). Pretreatment of aldosterone-stimulated isolated cardiac myocytes with rIFNγ resulted in the suppression of LC3-II protein expression indicating that pro-inflammatory cytokines may modulate aldosterone-induced autophagy and autophagosome accumulation.

Autophagy is differentially regulated by numerous cytokines (15), with IFNγ and TNF-α inducing autophagy, while IL-4 and IL-10 are inhibitory (15). Moreover, autophagy itself can regulate the production and secretion of cytokines, including TNF-α, and IFNγ. In hypertension-
The greatest myocardial expression of autophagic proteins was seen in IFNγKO mice, but was noticeable for the lack of expression of the anti-inflammatory, IL-10 and the pro-inflammatory cytokine, TNF-α. Although IFNγ has been shown to induce autophagy (15), we show that IFNγ inhibits aldosterone-induced LC3-II expression in cardiac myocytes. IFNγ, produced mainly by NK and T cells, stimulates Th1 T-cell development, activates macrophages, and is pro-inflammatory. However, IFN-γ has been shown to suppress inflammation in animal models of autoimmune disease (2). Even as basal levels of autophagy function to maintain cell homeostasis, autophagy can be rapidly increased in response to stress or elevated levels of ROS induced by aldosterone-induced diastolic HF. Our lab has shown that aldosterone induces ROS in cardiomyocytes (33) and increasing amounts of ROS may activate signaling pathways that lead to autophagic-induced cell death (19). Similarly elevated ROS can contribute to p62 induction and cell damage (13). P62 interacts with LC3 and this interaction is required for recruiting autophagosomes thus leading to autophagy (30). P62 expression has been shown to be downregulated during autophagy (17), but recently, similar to our findings, p62 was transcriptionally upregulated in hearts that overexpress misfolded proteins (53) and acts as a central player in regulating NF-κB activation (35). Similarly p62 increases LC3-II in cardiomyocytes (53). P62 expression suggests that oxidative stress plays a role in diastolic HF but may not play a role in the worsening of structural remodeling and diastolic dysfunction in aldosterone-infused IFNγKO mice.

Although LVH and diastolic dysfunction were more impaired in IFNγKO-aldosterone mice –possibly a restrictive pattern, it is unknown if the severe diastolic dysfunction is a reversible or fixed restrictive pattern. Diastolic HF was no different between both groups of aldosterone mice. It is possible that if mice were followed for a longer duration they may have
developed worsening of diastolic HF i.e., LVH and diastolic dysfunction precede the development of diastolic HF. It is also possible that diastolic dysfunction and LVH are independent of diastolic HF and does not lead to diastolic HF. In the human Irbesartan in HFpEF trial (I-PRESERVE), structural remodeling (59% had LVH) and diastolic dysfunction (69%) was present in the majority of patients with diastolic HF also known as “HF with preserved EF” (HFpEF) (56). Structural remodeling and diastolic dysfunction predicted adverse clinical outcomes in HFpEF. Our data suggest that reversing structural remodeling and improving function may improve outcomes and could reduce morbidity and mortality in HFpEF patients. Taken together, the finding that IFNγ is as a regulator of cardiomyocyte hypertrophy serves to enhance our understanding of the pathophysiology underlying diastolic heart failure (or HFpEF), possibly via the regulation of autophagy.

**Limitations:** Firstly, our findings do not suggest a causal relationship between cytokines and autophagy and will require further investigation. There is cross-talk between pro-death pathways and we did not explore the role of IFNγ in apoptosis and autophagy. Secondly, although IFNγ inhibits cardiomyocyte hypertrophy, the reduction in LC3-II expression may be an epiphenomenon and simply reflect the reduction in hypertrophy. We did not explore the mechanisms by which IFNγ modulates cardiomyocyte hypertrophy. Potential mechanisms include nitric oxide independent mechanisms as seen in the inhibition of PGF2α-induced cardiac hypertrophy (18). Additional pathways will be explored in future studies. Increased LC3 expression suggests that autophagy contributes to hypertension-induced cardiac remodeling. Whether this is a pro-survival response or one that contributes to clinical decompensation likely depends on the level of stress in the pathologic setting. Thirdly, the normal physiological HR in mice is rapid (>600bpm) and results in the loss of the A-velocity by echocardiography (41; 52),
thus in order to measure diastolic function parameters specifically A-velocity (late diastolic filling) all groups of mice were subjected to lower, but non-physiological, HR as we described previously (34; 50). Although others have shown comparable anesthesia-induced hemodynamic changes (16; 47), our measurements were standardized at lower and comparable HR for all 4 groups of mice. Finally, alterations in the vasculature and the kidney are evident in both human and experimental diastolic HF (34; 50). We did not determine if these extra-cardiac changes were altered with IFNγ deficiency in diastolic HF.

In conclusion, deficiency in IFNγ increases structural remodeling, diastolic dysfunction and diastolic HF suggesting that Th1 cytokines play a beneficial role in hypertension-induced diastolic HF. This is associated with autophagy which may be an important effector of Th1/Th2 polarization. IFNγ modulates aldosterone-induced cardiac hypertrophy independent of blood pressure. These findings challenge the concept that all inflammatory cytokines are deleterious. It may be particularly important in humans with resistant hypertension and human diastolic HF where aldosterone levels are relatively elevated (5; 12) suggesting that IFNγ may represent a novel target for hypertension-induced heart disease and cardiac hypertrophy with diastolic dysfunction.
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Disclosures

None
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antihypertensive drugs on diastolic function in patients with hypertension and diastolic

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Figure 1. Blood pressure and LVH. (A) Systolic blood pressure. There was a significant and progressive rise in tail-cuff blood in IFNγKO-aldosterone ($P<0.05$) and WT-aldosterone ($P<0.01$) over 4 weeks. †$P<0.05$ vs. WT-saline; ‡$P<0.01$ vs. WT-saline; *$P<0.05$ vs. IFNγKO-saline (B) LV mass measurements from echocardiography were made weekly. LV mass progressively increased in IFNγKO-aldosterone ($P<0.01$) and WT-aldosterone ($P<0.05$) over 4 weeks. *$P<0.05$ vs. IFNγKO-saline; †$P<0.01$ vs. IFNγKO-saline; ‡$P<0.001$ vs. IFNγKO-saline; §$P<0.05$ vs. WT-saline; #$P<0.01$ vs. WT-saline. Results are presented as mean±s.e.m; n=6-8/group.

Figure 2. In vivo transthoracic echocardiography measurements at 4 weeks. (A) LV end-diastolic diameter (LVEDD), (B) LV end-systolic diameter (LVESD), (C) Total wall thickness (TWT), (D) LV ejection fraction (LVEF). WT-saline (black bar), WT-aldosterone (white bar), IFNγKO-saline (grey bar), and IFNγKO-aldosterone (cross-hatched bar), mice. Results are presented as mean±s.e.m., n=6-8/group. §$P<0.05$ vs. WT-saline; ‡$P<0.001$ vs. IFNγKO-saline; †$P<0.01$ vs. IFNγKO-saline.

Figure 3. Cardiac fibrosis and collagen expression. (A) Qualitatively there is more fibrosis in both WT and IFNγKO hearts subjected to chronic aldosterone. (B) Percent (%) myocardial fibrosis is increased in both WT-aldosterone and IFNγKO-aldosterone hearts (*$P<0.05$ and †$P<0.01$ vs. respective saline for both). There was no difference in fibrosis between WT and IFNγKO -aldosterone hearts. Data are mean±s.e.m and reflects ten measurements from three sections each for WT and IFNγKO (n=3) hearts. (C) Collagen I and
Collagen III mRNA expression were increased in both WT-aldosterone and IFNγKO-aldosterone hearts. (*P<0.05; †P<0.01 and ‡P<0.001 vs. respective saline for both) but were no different between aldosterone–infused WT and IFNγKO hearts. Results are presented as mean±s.e.m., n=4/group.

Figure 4. Autophagic protein and cytokine expression in the LV. (A) LC3-II expression was increased relative to LC3-I resulting in an increase in the LC3-II/LC3-I ratio in WT-aldosterone (‡P<0.001) and IFNγKO-aldosterone hearts (§P<0.01) vs. respective controls. (B) P62 expression was increased in WT-aldosterone hearts (*P<0.05) and in IFNγKO-aldosterone hearts (†P<0.05) vs. respective controls. (C) Representative immunoblots of LC3, P62 and GAPDH expression in the LV. (D) TNF-α expression was increased in WT-aldosterone hearts (**P<0.01) but was non-significantly increased in IFNγKO-aldosterone hearts (P=NS) vs. respective controls. (E) IL-10 expression was increased in WT-aldosterone hearts (*P<0.05) and not expressed in IFNγKO-aldosterone hearts vs. respective controls. IL-10 expression was greater in WT-aldosterone than IFNγKO-aldosterone hearts (P<0.01). (F) Representative immunoblots of TNF-α, IL-10 and GAPDH expression in the LV.

Figure 5. Recombinant IFNγ supplementation protects against the development of cardiac hypertrophy. rIFNγ supplementation in IFNγKO and wild-type (WT) mice attenuated cardiac hypertrophy in response to aldosterone infusion as shown by echocardiography. rIFNγ or BSA was given i.p. for 14 days post surgery. LV total wall thickness (TWT is the average of IVS and LVPW) was determined at 28 day after surgery (or 14 after rIFNγ or BSA). (A) IFNγKO mice showed an increased cardiac hypertrophy following aldosterone infusion relative to wild-type mice (n=3). Fourteen days of rIFNγ supplementation in WT (n=4) and IFNγKO (n=4)...
attenuated TWT in response to chronic aldosterone as shown by echocardiography and was determined 28 days after aldosterone infusion. (B) Cardiac myocyte cross-sectional area in WT-aldosterone and IFNγKO-aldosterone (n=4) treated with rIFNγ or BSA (control) were measured at 28 day after surgery. There was a significant reduction in cardiomyocyte size with rIFNγ in both WT-aldosterone and IFNγKO-aldosterone hearts. (C) LV end-diastolic filling pressure (LVEDP) was determined using a 1.4F Millar catheter tip in WT-aldosterone and IFNγKO-aldosterone treated with 14 days of rIFNγ or BSA. LVEDP was significantly reduced with rIFNγ therapy in both WT-aldosterone and IFNγKO-aldosterone hearts. Results are presented as mean±s.e.m., n=3-4/group.

Figure 6. (A) Effects of IFNγ and aldosterone on \(^{3}\)H-leucine incorporation. \(^{3}\)H-leucine incorporation increased in ARVM after 24 hr exposure to 1μM aldosterone (P<0.01 vs. control). \(^{3}\)H-leucine incorporation was abrogated when aldosterone-stimulated ARVM were pretreated with rIFNγ (250U/ml) (P<0.05 vs. aldosterone). IFNγ alone had no effect on \(^{3}\)H-leucine incorporation. Results are presented as mean±s.e.m., n=6 experiments. (B) Aldosterone (1μM) for 18hr increased LC3II/I protein expression ratio in ARVM by 85±5% (‡P<0.001 vs. control), which was attenuated by pretreatment with rIFNγ (250U/ml) by 92±11% (P<0.01 vs. aldosterone-treated cells). (C) Representative Western blot analysis of LC3-II expression. Results are presented as mean±s.e.m., n=4 experiments.
Figure 1

- Systolic blood pressure (mmHg)

- WT - aldosterone
- IFNγKO - aldosterone
- WT - saline
- IFNγKO - saline

Week 1, Week 2, Week 3, Week 4

P < 0.05
Figure 1
**Figure 3**

(A) Histological images showing differences in fibrosis between WT-aldosterone, IFN\(_\gamma\) KO-aldosterone, WT-saline, and IFN\(_\gamma\) KO-saline samples. Images are labeled as X100.

(B) Graph showing the percentage of total fibrosis. Data are expressed as mean ± SD. P=NS indicates no significant difference.

(C) Graph showing the ratio of Collagen I/GAPDH for WT-saline, IFN\(_\gamma\) KO-saline, WT-aldosterone, and IFN\(_\gamma\) KO-aldosterone. Symbols indicate significance: * indicates P<0.05, † indicates P<0.01, ‡ indicates P<0.001.

(D) Graph showing the ratio of Collagen III/GAPDH for WT-saline, IFN\(_\gamma\) KO-saline, WT-aldosterone, and IFN\(_\gamma\) KO-aldosterone. Symbols indicate significance: * indicates P<0.05, † indicates P<0.01, ‡ indicates P<0.001.
Figure 4
Figure 4

**Figure 4**

**Panel D**
- Graph showing TNFα expression levels.
- Saline and aldosterone treatment groups for both WT and IFNγKO mice.
- P-value indicated as P=NS (not significant).

**Panel E**
- Graph showing IL-10 expression levels.
- Saline and aldosterone treatment groups for both WT and IFNγKO mice.
- P-value indicated as P<0.01.

**Panel F**
- Western blot images for TNFα, IL-10, and GAPDH.
- Saline and aldosterone treatment groups for both WT and IFNγKO mice.
Figure 5
Figure 5

C

<table>
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<tr>
<th>LVEDP (mmHg)</th>
<th>BSA</th>
<th>rIFN-γ</th>
<th>BSA</th>
<th>rIFN-γ</th>
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<tr>
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<tr>
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<tr>
<td>IFN-γKO</td>
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Aldosterone

P<0.05
Figure 6

3H-leucine incorporation

% Control

Control Aldo IFN\gamma IFN\gamma + Aldo

P<0.05
Figure 6

**B**

![Bar chart showing LC3 II protein expression](chart.png)

- Aldo
- rIFNγ
- Aldo + rIFNγ
- Control

Statistical significance: P<0.01

**C**

![Western blot images](image.png)

- Aldo
- rIFNγ
- Aldo + rIFNγ
- Control

Proteins: LC3II, GAPDH

*Figure 6*
### Table. Characteristics, LV hemodynamics and LV Doppler measurements from WT and IFNγKO mice 4-weeks after saline- or aldosterone-infusion

<table>
<thead>
<tr>
<th>Groups</th>
<th>Wild-type (WT)</th>
<th>Interferon-γ (IFNγ) KO</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>saline</td>
<td>aldosterone</td>
</tr>
<tr>
<td>Number</td>
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<td>8</td>
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<tr>
<td>Body weight (g)</td>
<td>25.5 ± 0.5</td>
<td>26 ± 0.7</td>
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<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>110 ± 4</td>
<td>136 ± 4**</td>
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<tr>
<td>Heart rate (bpm)</td>
<td>636 ± 18</td>
<td>531 ± 22**</td>
</tr>
<tr>
<td>LV/ BW (mg/g)</td>
<td>3.9 ± 0.1</td>
<td>4.3 ± 0.1*</td>
</tr>
<tr>
<td>Lung (wet / dry) ratio</td>
<td>4.3 ± 0.07</td>
<td>4.6 ± 0.05*</td>
</tr>
<tr>
<td>Cardiomyocyte C/S area (μm²)</td>
<td>183 ± 35</td>
<td>378 ± 23¶</td>
</tr>
</tbody>
</table>

#### LV Hemodynamics

| Heart rate (bpm) | 409±30 | 433±21 | 395±39 | 403±25 |
| LV EDP (mmHg) | 4 ± 0.9 | 10 ± 1.1¶ | 5 ± 0.8 | 16 ± 2.3‡§ |
| LV dp/dt_max (mmHg/sec) | 8,125 ± 504 | 10,690 ± 754* | 8,380 ± 409 | 11,005 ± 776† |
| Tau τ (ms) | 10 ± 1.0 | 26 ± 2.3¶ | 11.2 ± 0.8 | 25 ± 2.6‡ |
| LV dp/dt_min (mmHg/sec) | -5,992 ± 204 | -4,218 ± 245¶ | -5,905 ± 465 | -3,029 ± 367‡§ |

#### LV Diastolic Function

| Heart rate (bpm) | 384±45 | 359±30 | 375±40 | 366±25 |
| Mitral E velocity (mm/sec) | 699 ± 44 | 1083 ± 36¶ | 538 ± 21 | 945 ± 37‡§ |
| Mitral A velocity (mm/sec) | 388 ± 32 | 399 ± 21 | 322 ± 17 | 305 ± 25§ |
| E/A ratio | 1.7 ± 0.2 | 2.8 ± 0.1¶ | 1.7 ± 0.1 | 3.1 ± 0.1‡§ |
| IVRT (ms) | 19 ± 0.9 | 20 ± 0.8 | 17 ± 0.8 | 21 ± 1.0† |
| DT (ms)       | 17 ± 0.9 | 15 ± 0.9 | 16 ± 1.0 | 14 ± 1.0 |

*P<0.05 vs. WT-saline; **P<0.01 vs. WT-saline; ¶P<0.001 vs. WT-saline; †P<0.05 vs. IFNγKO-saline; ‡P<0.001 vs. IFNγKO-saline; §P<0.05 vs. WT-aldosterone; #P<0.001 vs. WT-aldosterone.

Data are represented as mean±s.e.m. Abbreviations: LV: left ventricular; BW: body weight; C/S: cross-sectional; EDP: end-diastolic pressure; IVRT: isovolumic relaxation time; DT: deceleration time.