A pro-fibrotic role for interleukin-4 in cardiac pressure overload

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Aims

The mechanisms underlying cardiac fibrosis in hypertension are yet to be defined, although inflammatory cells, fibroblasts, and cytokines have been implicated. Here, we investigated the role of interleukin-4 (IL-4) in cardiac fibrosis, which is elevated in the hypertensive heart. IL-4 has been shown to be pro-fibrotic in the liver and the lung, but its role in cardiac fibrosis has not been investigated.

Methods and results

Cardiac fibrosis was induced in mice by constricting the aorta between the two carotid arteries. Fourteen days later marked left ventricular fibrosis developed together with expression of IL-4. Anti-IL-4 neutralizing antibodies attenuated this fibrosis without affecting blood pressure or expression of the transforming growth factor-beta system. The reduction in fibrosis was associated with reductions in interstitial fibroblasts and macrophages together with reductions in proliferating cells and expression of monocyte chemoattractant protein-1 (MCP-1). Since mast cells are a source of IL-4, we also assessed their role in fibrosis. Cromolyn, a mast cell inhibitor attenuated mast cell degranulation as well as IL-4 mRNA expression and cardiac fibrosis without affecting blood pressure. Treatment with Cromolyn also reduced interstitial fibroblasts and macrophages in regions of developing fibrosis as well MCP-1 expression.

Conclusion

This study demonstrates for the first time that IL-4, most likely produced by mast cells in the heart during pressure overload, is a significant contributor to cardiac fibrosis. Targeting this cytokine may be a useful therapeutic strategy to limit cardiac fibrosis.

Keywords

Cardiac fibrosis • Hypertension • Interleukin-4 • Mast cells • Cromolyn

1. Introduction

Cardiac fibrosis is associated with diastolic dysfunction but preserved systolic function and is increasingly recognized as an important contributor to heart failure.1,2 It leads to increased stiffness of the heart and is a common feature of advanced cardiac failure regardless of the aetiology of cardiomyopathy.3 The accumulation of extracellular matrix in the cardiac interstitium also disrupts the coordination of myocardial excitation–contraction coupling in both systole and diastole which can result in profound functional impairment.3 Beyond these effects on cardiac function, fibrosis also promotes arrhythmogenesis through impaired anisotropic conduction and subsequent generation of re-entry circuits,5 cardiac fibrosis is thought to contribute to sudden death through ventricular tachyarrhythmias.6

The mechanisms underlying fibrosis are yet to be fully defined although it is recognized that fibroblasts and cytokines play a critical role.7,8 Cytokines implicated in fibrosis include transforming growth factor-beta1 (TGF-beta1), interleukin-13 (IL-13), and IL-4.9–11 With respect to IL-4 and cardiac fibrosis, this cytokine is up-regulated during the development of interstitial cardiac fibrosis in ageing mice10 and is also up-regulated in hearts of hypertensive spontaneously hypertensive rats during the development of fibrosis,12 suggesting it may exert pro-fibrotic effects on the heart. Other lines of evidence also suggest a pro-fibrotic role for IL-4. IL-4 is highly effective in stimulating collagen biosynthesis in human fibroblasts.13–15 It can stimulate and/or enhance the proliferation of fibroblasts, a key event in the development of fibrosis.16,17 Also, IL-4 can stimulate macrophages in regions of developing fibrosis to produce the insulin-like growth factor-1 (IGF-1) that protects myofibroblasts from apoptosis, thereby contributing to their persistence during fibrosis.18 There is also evidence that suggests a pro-fibrotic role for IL-4 in vivo. IL-4-deficient mice develop less pulmonary fibrosis than wild-type

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litter mates and anti-IL-4 neutralizing antibodies attenuate liver fibrosis. On the basis of this evidence, we hypothesized that IL-4 may contribute to fibrosis in hypertensive hearts. Thus, in the present study, we examined the functional significance of elevations in IL-4 during the development of cardiac fibrosis. We confirm elevations in IL-4 during the development of cardiac fibrosis following aortic coarctation in mice and demonstrate that its neutralization attenuates fibrosis, providing the first direct evidence for IL-4 being an important contributor to cardiac fibrosis. We also examine how IL-4 is up-regulated in pressure overloaded hearts and mechanisms by which it mediates pro-fibrotic effects on the heart.

2. Methods

2.1 Animal experiments

Fifty male C57BL6 mice at 10 weeks of age, from the Precinct Animal Facility, AMREP, Melbourne, were used in the study. All the mice underwent surgery, either a sham-operation or a trans aortic coarctation (TAC). Briefly, mice were anaesthetized with a mixture of ketamine/xylazine/atropine (KXA, 80/20/0.06 mg/kg, ip) and subjected to tracheal intubation and ventilation. A midline incision was made at the upper sternum and with the aid of a dissecting microscope, the transverse aorta (between the left and right carotid), was dissected and then narrowed to a lumen size of 0.44 mm using a 4/0 suture. Sham-operated mice were subjected to the same procedure, except that the aortic arch was not narrowed. After closure of the chest, the mice were administered carprofen (5 mg/kg, sc) and allowed to recover on a heated pad. During surgery the adequacy of the anaesthesia was monitored by the disappearance of the pedal withdrawal reflex.

Five groups of mice (10 per group) were used in the studies and received the following treatments: Group 1, no treatment, sham-operation; Group 2, control rat non-immune IgG (1 mg, iv) starting at 1 day prior to TAC and continuing every third day until Day 14; Group 3, neutralizing rat IL-4 monoclonal antibody (11B11; 1 mg, iv) starting at 1 day prior to TAC and continuing every third day until Day 14; Group 4, cromolyn (50 mg/kg/day, ip) treatment starting at 1 h post-TAC and continuing until Day 14; and Group 5, vehicle (PBS) treatment starting at 1 h post-TAC and continuing until Day 14. Two independent individual studies were performed, the first involving groups 1–3 and the second involving groups 4 and 5. At the end of the study (Day 14) mice were killed with an overdose of pentobarbitone (120 mg/kg, ip), hearts were excised and placed in cold PBS solution before being divided into three transverse sections for histology, immunohistochemistry, and molecular studies. All experiments were approved by the Alfred Medical Research Education Precinct (AMREP) Animal Ethics Committee and the investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2 Blood pressure

Blood pressure was measured at 14 days post-surgery using a 1.4 F Millar microtipped transducer catheter (Millar Instruments, Houston, TX, USA). The mice were anaesthetized with KXA (80/20/0.06 mg/kg, ip) and their body weights recorded before being placed in a supine position, intubated, and ventilated. The catheter was then inserted via the right carotid artery into the aorta and resting aortic blood pressure was recorded using a computer and AD Instrument software (ADInstruments, Australia). The hearts were then excised, cleaned of fat/connective tissue, and subsequently weighed.

2.3 Anti-IL-4 neutralizing antibodies

Rat anti-mouse IL-4 neutralizing antibodies were prepared from the culture medium of 11B11 hybridoma (ATCC). Briefly, 11B11 hybridoma cells were cultured in RPMI 1640 medium containing L-glutamine and 10% heat-inactivated foetal bovine serum. Culture medium was collected from cells (5 x 10^5 cells/mL) twice weekly and IgG1 anti-IL-4 neutralizing antibodies were purified using protein G column chromatography.

2.4 Tissue processing

For histological measurements, transverse sections of the left ventricle (LV) were post-fixed in 10% buffered formalin and embedded in paraffin. The rest of the LV was either frozen at −70 °C for molecular studies or frozen in OTC at −70 °C for cryosectioning and immunohistochemistry.

2.5 Cardiac fibrosis

Sections, 6 μm in thickness, from transverse slices of the heart were used to assess collagen accumulation. The amount of collagen deposited in the LV was measured after staining the sections with picrosiris red (Picrosirius Red F3BA, 0.1% solution in saturated aqueous picric acid) using a computer-interfaced colour imaging system (Optimas Bioscan 2, Thomas Optical Measurement System, Inc.). Ten randomly selected fields per section were analysed and results expressed as per cent of the stained area.

2.6 Immunohistochemistry

Three 6 μm cryo-sections from similar transverse slices of the LV were used for immunohistochemistry to assess macrophage accumulation (CD68), myofibroblasts (alpha-SM actin), MCP-1, and proliferating nuclear antigen staining (PCNA). Briefly, sections were fixed in cold (−20 °C) acetone for 20 min. The sections were then sequentially incubated in 3% hydrogen peroxide in PBS, 10% normal serum/PBS, and biotin/avidin-blocking reagents (Vector Laboratories). Then the sections were incubated (1 h) with primary antibodies in serum, rat anti-mouse IL-4 (1–20; BD Pharmingen: cat#554387), rat anti-mouse CD68 (1–100; Serotec: cat#MCA1957), rabbit anti-alpha smooth muscle actin (1–100; Abcam: #ab5694), rabbit anti-IL-4 neutralizing antibodies (1 mg, iv) starting at 1 h post-mouse anti-IL-4 monoclonal antibody (11B11; 1 mg, iv) starting at 1 day prior to TAC and continuing every third day until Day 14; Group 4, cromolyn (50 mg/kg/day, ip) treatment starting at 1 h post-TAC and continuing until Day 14; and Group 5, vehicle (PBS) treatment starting at 1 h post-TAC and continuing until Day 14. Two independent individual studies were performed, the first involving groups 1–3 and the second involving groups 4 and 5. At the end of the study (Day 14) mice were killed with an overdose of pentobarbitone (120 mg/kg, ip), hearts were excised and placed in cold PBS solution before being divided into three transverse sections for histology, immunohistochemistry, and molecular studies. All experiments were approved by the Alfred Medical Research Education Precinct (AMREP) Animal Ethics Committee and the investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.7 Reactive oxygen species

Superoxide [reactive oxygen species (ROS)] generation was measured in non-fixed frozen sections of both Sham-operated and TAC-injured mouse heart tissue on Day 14 using dihydroethidium (DHE), an oxidative fluorescence dye. Briefly, 30 μm thick frozen transverse heart sections were placed on glass slides before being incubated in DHE (10 μM in Krebs bicarbonate buffer), in a light protected humidified chamber at 37 °C for 30 min. Fluorescence images of the sections were then obtained using a fluorescence microscope and the excitation/emission characteristics for ethidium bromide (488 and 610 nm, respectively), detecting, and measuring fluorescence via a 585 nm long-pass filter.

In summary, the present study confirms that IL-4 is an important contributor to cardiac fibrosis. We also examine how IL-4 is up-regulated in pressure overloaded hearts and mechanisms by which it mediates pro-fibrotic effects on the heart.
2.8 Mast cell staining
Transverse LV paraffin (6 μm thick) sections from mice 14 day post-TAC + vehicle treated and TAC + cromolyn treated were subjected to mast cell analysis using acidic toluidine blue (TB) staining solution. Briefly, the sections were deparaffinized in xylene and hydrated through alcohol to distilled water. They were then incubated in an aqueous solution of 0.20% TB O (Sigma) acidified to pH 2.3 for 3–5 min. After several washes in distilled water, they were quickly dehydrated through 95% and two changes of 100% alcohol, before being cleared in xylene and mounted with Depex. Mast cells stained a violet/dark blue colour with a blue/light blue background. As TB specifically marks mast cells by reacting with acid mucopolysaccharides in granules to form metachromtic complexes, we identified degranulated mast cells as cells in which granules were substantially reduced (~70–90%). Results were expressed as per cent of degranulated mast cells.

2.9 Analysis of gene expression by real-time and RT–PCR
Total RNA was extracted from LVs snap frozen in liquid nitrogen using RNeasy kits (Qiagen) and quantitated by measuring absorbance at 260 nm. Real-time PCR was performed using a Quantifast SYBR One-step RT-PCR kit (Qiagen; cat#:204154) and quantitative gene expression analysis was performed on an ABI PRISM 7500 fast real-time PCR system (Applied Biosystems) using SYBR Green technology (Applied Biosystems). Primers were designed using Primer Express (Applied Biosystems) and PeriPrimer software packages.24 The following oligonucleotide primer pairs were used: TßR-1, sense: 5′-CATCTATGCAATGGGTCTAGTT-3′ and antisense, 5′-AGGCCAAGTGTAGTCTTCTGGATT-3′; TßR-II, sense 5′-ATGGAAGATGTGCAACGATTAC-3′ and antisense 5′-ACACCTGCTATGTGAAATAAGC-3′; TGF-b1, sense 5′-AGCCCTGTATGCAACTTGC-3′ and antisense 5′-TCCAACCCAGTGTCCCTCTC-3′; PDGF-B, sense 5′-TCGAGGAACTGATGTAGAAAGTCT-3′ and antisense 5′-CCATCTTCTACGGGTCTTC-3′; 18S, sense 5′-CAGGCTCAACATTCAAGAGGC-3′ and antisense 5′-GCATTATCGGCGGTCTGTC-3′; IL-4, sense 5′-GTTTGCTCTCCTTGCC-3′ and antisense 5′-CTGTGTTGCTCTTCTGGCC-3′. Optimum concentrations of primers were determined in the amplifications and the specificity of the oligonucleotides for amplifications was confirmed by subjecting the PCR products to agarose gel electrophoresis. Expression of genes was calculated as fold increases using the 2−ΔΔCt as recommended by the manufacturer.

RT–PCR was performed as previously described,25 using oligonucleotide primers to detect IL-4, sense: 5′-CAACGGAAACCAACAGAG-3′ and antisense: 5′-AGGAAAGTCTTTCAGTGTG-3′ and housekeeping gene (L7, sense: 5′-CCTGAGAAAGATGTGC-3′ and antisense: 5′-CCTGTGAGTCTTCAAAAGGGG-3′) and one-step RT–PCR kit [SuperScript RT-PCR system (Life Technologies Invitrogen)].

2.10 Statistical analyses
All grouped data are expressed as mean ± SEM. Comparisons between two groups were made using unpaired t-tests. Multiple grouped data comparisons were made using one-way ANOVA using GraphPad Prism 5 software. When a significant F test (P < 0.05) was obtained, intergroup comparisons were analysed post hoc using Tukey’s multiple comparison test. The results were considered statistically significant at P < 0.05.

3. Results
3.1 Interleukin-4 and cardiac fibrosis
Since cardiac fibrosis has been associated with increased IL-4 expression,10,12 we examined its significance for fibrosis by treating mice subjected to aortic coarctation either with a control IgG or an anti-IL-4 neutralizing antibody (clone: 11B11) throughout the 14 day study period. At the end of the study, mice subjected to aortic coarctation and treated with a control IgG exhibited elevated levels of IL-4 mRNA compared with sham-operated mice and IL-4 protein expression was also evident in regions of developing fibrosis (Figure 1A and B), confirming previous associations of IL-4 with cardiac fibrosis.10,12 Anti-IL-4 antibody treatment did not affect blood pressure and only marginally affected cardiac hypertrophy (P < 0.05; Figure 1C), but greatly attenuated the left ventricular fibrosis, by nearly 57% (P < 0.05; Figure 1D). Since IL-4 has the potential to enhance the proliferation of fibroblasts16 and also augment survival of fibroblasts,18 we next examined whether fibroblast numbers and the proliferation of cells in regions of developing fibrosis were affected. Following anti-IL-4 neutralizing antibody treatment, the number of interstitial alpha-SM positive fibroblasts was reduced by >50% (P < 0.05; Figure 1E) and PCNA positive interstitial cells by nearly 60% (P < 0.05; Figure 1F).

3.2 IL-4 and tissue macrophages
Tissue macrophages play an important role in fibrosis by metabolizing arginine and proline, an essential amino acid for collagen biosynthesis.26 Since IL-4 has been shown to drive accumulation of tissue macrophages through self-renewal,27 we performed immunohistochemistry to determine whether IL-4 also influences the number of macrophages in regions of developing fibrosis. Treatment of the mice with anti-IL-4 neutralizing antibodies attenuated the increased macrophage numbers in fibrotic regions by 70% (P < 0.05; Figure 2A). Since IL-4 can stimulate expression of monocyte chemoattractant protein-1 (MCP-1),28,29 a potent chemotactic factor for monocytes/macrophages,30 we also examined whether IL-4 affected its expression. MCP-1 was highly expressed in fibrotic regions of the LV and in anti-IL-4 antibody-treated mice expression was reduced by ~25% (P < 0.05; Figure 2B).

3.3 IL-4 and the transforming growth factor-beta system
IL-4 has been reported to either increase the expression of TGF-beta15 or directly stimulate fibroblasts to produce collagen. Consequently, we assessed whether anti-IL-4 neutralizing antibody treatment affected the expression of TGF-beta1 and its receptors by real-time PCR. Following aortic coarctation and treatment of mice with control non-immune IgG, mRNA encoding TGF-beta1 in the LV was twice the levels observed in sham-operated mice (P < 0.05; Figure 3); TGF-beta1 mRNA levels were unaffected following treatment with anti-IL-4 neutralizing antibodies (Figure 3). Similarly, TGF-beta receptors, ALK-5 and TßR-II, were elevated after aortic coarctation but unaffected by treatment with the anti-IL-4 neutralizing antibody as was PDGF-B (Figure 3).

3.4 Aortic coarctation and cardiac reactive oxygen species
Oxidative stress stimulates mast cells to increase IL-4 gene transcription and secretion.32 To determine whether an increase in oxidative stress could contribute to the elevation in IL-4 in fibrotic LVs, we examined using dihydroethidium whether ROS were elevated in the LVs of mice subjected to aortic coarctation.32 ROS in the LV of mice subjected to aortic coarctation was on average, nearly three times greater than in sham-operated mice (P < 0.05; Figure 4).
Figure 1  (A) IL-4 mRNA expression in the hypertensive left ventricle 14 days after aortic coarctation (AC) and control IgG or sham operation (S).  
(B) IL-4 protein expression in fibrotic regions of the hypertensive heart 14 days after aortic coarctation and control IgG.  
(C) Body weights, blood pressures, and heart weight/body weight ratios in mice treated with control IgG or IL-4 neutralizing antibody  
(D) Fibrosis in the left ventricle 14 days after aortic coarctation (left panel) and its attenuation by treatment with anti-IL-4 neutralizing antibodies (right panel).  
(E) Increase in alpha-SM actin expressing myofibroblasts in fibrotic regions of the left ventricle 14 days after aortic coarctation and attenuation by anti-IL-4 neutralizing antibody treatment.  
(F) Increase in proliferating nuclear antigen staining (PCNA) expressing cells in fibrotic regions of the left ventricle 14 days after aortic coarctation and attenuating by treating mice with anti-IL-4 neutralizing antibody.  *P < 0.05 from IgG-treated mice.  
Bar on photomicrographs represents 50 μm.
3.5 Cardiac fibrosis and mast cell activation

Cells of several lineages produce IL-4 including CD4+ and CD8+ T cells and mast cells. Since only low numbers of CD4+ T cells could be detected in regions of developing fibrosis (not shown), we hypothesized that mast cell activation might be responsible for the IL-4 stimulated cardiac fibrosis. Previously, it has been shown that the treatment of spontaneously hypertensive rats with the mast cell inhibitor, Nedocromil, attenuates IL-4 protein expression in the heart.12 Thus we also treated mice subjected to aortic coarctation with the mast cell inhibitor cromolyn. Cromolyn also attenuated IL-4 expression by 56% ($P < 0.05$; Figure 5B) and mast cell activation in the LV by 60%, assessed by the degranulation of mast cells ($P < 0.05$; Figure 5A); it reduced the LV/BW ratio by 17% ($P < 0.05$; Figure 5B) but did not affect either body weight or blood pressure ($P > 0.05$; Figure 5B). Mast cell inhibition did, however, attenuate developing cardiac fibrosis by 70% ($P < 0.05$; Figure 5C). As with anti-IL-4 antibody treatment inhibition of mast cell degranulation also attenuated myofibroblast accumulation in regions of developing fibrosis by 75% ($P < 0.05$; Figure 5D).

Figure 2 (A) CD68 expressing macrophages accumulating in fibrotic regions of the left ventricle 14 days after aortic coarctation and attenuation by anti-IL-4 neutralizing antibody treatment. (B) Increase in MCP-1 protein expression in fibrotic regions of the left ventricle 14 days after aortic coarctation and attenuation by treatment with anti-IL-4 neutralizing antibody. *$P < 0.05$ from IgG-treated mice. Bar on photomicrographs represents 50 μm.

Figure 3 IL-4 and the transforming growth factor-beta system (ALK-5, TβRII, TGF-β1) and PDGF-B mRNAs in left ventricle of mice 14 days after aortic coarctation. *$P < 0.05$ from sham-operated mice.

Figure 4 Increased reactive oxygen species (ROS) in the left ventricle of mice 14 days after aortic coarctation compared with sham-operated mice. *$P < 0.05$ from sham-operated mice. Bar on photomicrographs represents 50 μm.
3.6 Mast cell activation, macrophages, and proliferation

Since anti-IL-4 neutralizing antibody treatment also attenuated the accumulation of macrophages in fibrotic regions and MCP-1 expression, we also assessed whether inhibiting mast cell degranulation had similar effects. Treatment of mice with cromolyn reduced the accumulation of macrophages by nearly 70% ($P < 0.05$; Figure 6A); similarly, MCP-1 expression was reduced by nearly 60% ($P < 0.05$; Figure 6B), effects similar to those observed after anti-IL-4 neutralizing antibody treatment. Cromolyn also reduced the number of proliferating PCNA + cells ($P < 0.05$; Figure 6C).

4. Discussion

Immune cells and TGF-beta1 have been implicated in the development of cardiac fibrosis. In the present study, we demonstrate that IL-4 is also important for the development of cardiac fibrosis stimulated by high blood pressure. IL-4 expression is increased in pressure overloaded hearts and its neutralization attenuates the development of cardiac fibrosis. The fibrotic effects of IL-4 appear to be independent of the TGF-beta system. Furthermore, mast cells appear to be an important source of IL-4 as the inhibiting of mast cell degranulation, which attenuates IL-4 expression, also attenuates the development of cardiac fibrosis.
Several pro-fibrotic factors have been implicated in the development of cardiac fibrosis including angiotensin, endothelin, connective tissue growth factor, platelet-derived growth factor, and TGF-beta1. Of these TGF-beta1 appears to be most important, accounting for much of the pro-fibrotic effects of angiotensin, connective tissue growth factor, and platelet-derived growth factor. Our findings also indicate a key role for IL-4 in the development of fibrosis in hypertensive hearts. Although little attention has been given to IL-4 in the development of cardiac fibrosis, IL-4 has been associated with cardiac fibrosis and remodelling in patients with heart failure. IL-4 has also been implicated as a pro-fibrotic agent in other tissues, including fibrosis of the liver and pulmonary fibrosis. Our findings definitely demonstrate that IL-4 contributes to fibrosis in the LV in response to sustained pressure overload. Not only is expression of IL-4 increased but its neutralization attenuates the development of left ventricular fibrosis. The magnitude of the reduction in fibrosis indicates that like TGF-beta1, IL-4 is also an important pro-fibrotic cytokine for cardiac fibrosis. While it has been suggested that IL-4 might stimulate collagen synthesis by augmenting TGF-beta1 production, neutralizing IL-4 in our study did not affect TGF-beta1 mRNA.

**Figure 6** Effects of mast cell inhibition with cromolyn on macrophage accumulation, MCP-1 expression and proliferating cells. (A) Cromolyn treatment attenuates macrophage numbers in regions of developing fibrosis. (B) MCP-1 expression is attenuated by cromolyn in regions of developing fibrosis. (C) PCNA positive cell numbers are reduced in regions of developing fibrosis by cromolyn treatment. *P < 0.05 from vehicle-treated mice. Bar on photomicrographs represents 50 μm.
expression during the development of fibrosis or other components of the TGF-beta signalling system, indicating TGF-beta1-independent effects on collagen production. In vitro IL-4 appears to be similar in potency to TGF-beta1 in stimulating collagen synthesis.\(^\text{13}\)

Our findings that IL-4 increases cell proliferation and the number of alpha-SM actin positive interstitial myofibroblasts during the development of fibrosis is consistent with earlier in vitro studies suggesting a role for IL-4 in cell proliferation.\(^\text{12,13}\) While IL-4 does not appear to directly stimulate myofibroblasts proliferation, it augments platelet-derived growth factor and fibroblast growth factor proliferative responses.\(^\text{17}\) IL-4 can also contribute to the increase in alpha-SM actin positive cells by stimulating macrophages to produce IGF-1, which protects myofibroblasts from apoptosis during growth factor withdrawal.\(^\text{18}\) IL-4 appears to contribute to \(\sim 50\)% of the increase in the myofibroblasts population during pressure-induced myocardial fibrosis. In addition to its effects on interstitial myofibroblasts, IL-4 also contributes to the increase in macrophages within developing regions of fibrosis. Neutralization of IL-4 markedly attenuated macrophage numbers suggesting a major role for IL-4 in regulating macrophage numbers within regions of developing fibrosis. This effect was only partially due to reductions in the expression of the macrophage chemoattractant MCP-1, which were small. This finding of IL-4 markedly affecting macrophage numbers is consistent with recent findings indicating that IL-4 increases local tissue macrophage numbers by stimulating their proliferation rather than stimulating the recruitment of circulating monocytes and their subsequent differentiation into macrophages.\(^\text{23}\) Neutralizing IL-4 reduced the number of proliferating cells in fibrotic regions. IL-4 also stimulates macrophages to polarize toward an M2 phenotype characterized by the expression of molecules such as Ym-1 and arginase.\(^\text{39}\) M2 macrophages have been shown to contribute to fibrosis.\(^\text{40}\)

Our finding that cromolyn markedly attenuated mast cell degranulation and attenuated IL-4 expression as well as cardiac fibrosis confirms previous studies in spontaneously hypertensive rats on the importance of mast cells in the development of pressure-induced cardiac fibrosis.\(^\text{12}\) Furthermore, earlier reports indicating that mast cells release IL-4 during degranulation\(^\text{41}\) and mast cell inhibition in the heart reduces IL-4 expression during the development of cardiac fibrosis further support our findings of an important role for IL-4 in the development of cardiac fibrosis. Inhibiting mast cell degranulation had similar effects to IL-4 neutralization on myofibroblasts and macrophage cell numbers and the chemokine MCP-1 during the development of fibrosis. Mechanisms responsible for the activation of mast cells during the development of cardiac fibrosis are unknown, although ROS could be involved. Hydrogen peroxide elevates IL-4 expression as well as the activity of the IL-4 promoter in mast cells.\(^\text{42}\) Furthermore, ROS such as superoxide anion and hydrogen peroxide stimulate the nuclear translocation of APE/Ref-1,\(^\text{32}\) a protein capable of inducing activator protein-1 DNA-binding activity,\(^\text{43}\) which is important for IL-4 expression in mast cells.\(^\text{32,44}\) Our finding of increased levels of ROS in the LV of the hypertensive mice support these in vitro findings of an important role for ROS in IL-4 expression in mast cells and developing fibrosis. However, further experimentation is required to confirm this hypothesis.

In summary, our study provides new insights into the fibrogenic properties of the Th2 cytokine IL-4 in the pressure overloaded heart. Our finding that neutralizing IL-4 reduces cardiac fibrosis together with myofibroblast and macrophage numbers, suggests that IL-4 may exert its pro-fibrotic effect via mechanisms that involve actions on both myofibroblasts and macrophages. IL-4 can directly stimulate fibroblasts to increase collagen production.\(^\text{13–15}\) It can also increase myofibroblast numbers in regions of developing fibrosis by stimulating macrophages to produce IGF-1, which protects myofibroblasts from apoptosis\(^\text{18}\) and augment myofibroblast proliferative responses to growth factors.\(^\text{17}\) We observed reductions in myofibroblast numbers in regions of developing fibrosis upon neutralizing IL-4. IL-4 also stimulates tissue macrophage proliferation,\(^\text{27}\) which is consistent with our observations on macrophage numbers in regions of developing fibrosis. It can also induce an M2 macrophage phenotype in which arginase-1 is elevated,\(^\text{39}\) increasing production of proline, which is essential for collagen biosynthesis. IL-4 apparently exerts its fibrotic effects independently of TGF-beta1 and appears to be mostly mast cell derived. Targeting IL-4 could be a useful therapeutic strategy for attenuating cardiac fibrosis associated with hypertension and its complications.

Conflict of interest: none declared.

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