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## Inhibition of Matrix Metalloproteinase Activity Prevents Increases in Myocardial Tumor Necrosis Factor- $\alpha$

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### Abstract

**Aim**—TNF- $\alpha$  is known to cause adverse myocardial remodeling. While we have previously shown a role for cardiac mast cells in mediating myocardial TNF- $\alpha$ , matrix metalloproteinases (MMP) activation of TNF- $\alpha$  may also be contributory. We sought to determine the relative roles of MMPs and cardiac mast cells in the activation of TNF- $\alpha$  in the hearts of rats subjected to chronic volume overload.

**Methods**—Interventions with the broad spectrum MMP inhibitor, GM6001, or the mast cell stabilizer, nedocromil, were performed in the rat aortocaval fistula (ACF) model of volume overload.

**Results**—Myocardial TNF- $\alpha$  levels were significantly increased in the ACF. This increase was prevented by MMP inhibition with GM6001 ( $p \leq 0.001$  vs. ACF). Conversely, myocardial TNF- $\alpha$  levels were increased in the ACF + nedocromil treated fistula groups ( $p \leq 0.001$  vs. sham). The degradation of interstitial collagen volume fraction seen in the untreated ACF group was prevented in both the GM6001 and nedocromil treated hearts. Significant increases in LV myocardial ET-1 levels also occurred in the ACF group at 3 days post-fistula. Whereas administration of GM6001 significantly attenuated this increase, mast cell stabilization with nedocromil markedly exacerbated the increase, producing ET-1 levels 6.5 fold and 2 fold greater than that in the sham-operated control and ACF group, respectively.

**Conclusion**—The efficacy of the MMP inhibitor, GM6001, to prevent increased levels of myocardial TNF- $\alpha$  is indicative of MMP-mediated cleavage of latent extracellular membrane bound TNF- $\alpha$  protein as the primary source of bioactive TNF- $\alpha$  in the myocardium of the volume-overload heart.

### Keywords

MMP; TNF- $\alpha$ ; ventricular remodeling; aorto-caval fistula; cytokines; GM6001

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## Introduction

The induction of heart failure, regardless of etiology (i.e., chronic pressure overload, sustained volume overload, or myocardial infarction), is associated with increased expression of multiple pro-inflammatory cytokines.(1–5) TNF- $\alpha$  is chief among these inflammatory mediators and undergoes acute elevations in the remodeling heart, with TNF- $\alpha$  having been shown to mediate pathologic events.(6–11) Cardiac mast cells have been identified as the predominant source of TNF- $\alpha$  in the unstressed myocardium,(12–14) while Reil et al.(15) and others(13) demonstrated a biphasic pattern in which the initial release of myocardial TNF- $\alpha$  in response to ischemia and reperfusion was mast cell mediated, whereas, the subsequent increase in TNF- $\alpha$  protein expression was mast cell independent. Furthermore, we recently found that TNF- $\alpha$  was undetectable by ELISA and there were no volume overload-induced increases in TNF- $\alpha$  in the hearts of mast cell deficient rats.(16) However, subsequent findings have indicated other sources are also important.(17), and Diwan et al.(9) implicated enzymatic processing of TNF- $\alpha$  as a critical event initiating adverse ventricular remodeling. The A Disintegrin And Metalloproteinase (ADAM) family of enzymes encompassing ADAM 17, otherwise known as TNF- $\alpha$  converting enzyme (TACE),(18–20) is capable of generating soluble TNF- $\alpha$  by proteolytically cleaving membrane-bound pro-TNF- $\alpha$  (21;22) Therefore, the purpose of this study was to determine the predominant mechanism of the pathologic increases in myocardial TNF- $\alpha$  secondary to chronic cardiac volume overload. To this end, TNF- $\alpha$  levels were determined following interventions with the broad spectrum matrix metalloproteinase inhibitor, GM6001 or the mast cell stabilizer, nedocromil, three days after creating an aortocaval fistula (ACF) in the male rat.

## Methods

### Animal Welfare

All experiments were performed using 9 week (200–250 g) male Sprague-Dawley rats housed under standard environmental conditions and maintained on commercial rat chow and tap water *ad libitum*. All studies conformed to the principles of the National Institutes of Health “Guide for the Care and Use of Laboratory Animals,” and were approved by University of South Carolina School of Medicine Animal Care and Use Committee. The anesthetic agent used for all experimental procedures was sodium pentobarbital (50 mg/kg, i.p.).

### Experimental Design

In order to determine if mast cell stabilization or inhibition of MMPs would limit TNF- $\alpha$  release, *in vivo* experiments utilized either: 1) the mast cell stabilizer, nedocromil sodium, administered three days prior to fistula surgery via a 21 day time release pellet (Innovative Research of America, FL) placed subcutaneously to achieve a delivered dosage of 10mg/kg/day as previously published.(23;24); or 2) the broad spectrum matrix metalloproteinase inhibitor, GM6001, (5 mg/kg/day, Calbiochem®), dissolved in ethanol and phosphate buffered saline and delivered via a once daily subcutaneous injection initiated three days prior to surgery and continued for the duration of the experiment.(25) The dissociation constant for the binding of GM6001 to collagenases (MMP-1, MMP-8, MMP-3) and gelatinases (MMP-2, MMP-9) has been extensively characterized *in vitro* and *in vivo*.(19;26;27)

### Animal Groups

Four separate *in vivo* study groups were analyzed at 3 days post-surgery as follows: sham-operated control (Sham, n=6), untreated aortocaval fistula (ACF, n=5), nedocromil treated ACF (ACF + Nedocromil, n=5), and MMP inhibitor treated ACF (ACF + GM6001, n=6). Nedocromil and GM6001 were administered as described above and continued until the

experimental endpoint. This time point was chosen based on extensive prior characterization performed by our laboratory demonstrating a significant increase in cardiac mast cell density and concomitant MMP activation that resulted in marked collagen degradation and changes in ventricular morphology.(20;24;28) Drug treated sham operated controls were also performed, however, no differences between the treated and untreated sham-operated control groups were noted (data not shown).

At the experimental endpoint, aortocaval fistula was confirmed visually at sacrifice based upon the pulsatile flow of oxygenated blood into the vena cava. Additionally, a marked increase of ~50% or greater in cardiac output, as determined by measuring aortic flow (Aortic flow probe, Transonic Systems Inc<sup>®</sup>, Itacha NY), was indicative of a patent fistula. Under deep anesthesia, the heart was excised; the atria and great vessels removed, and the LV (including septum) and right ventricle were separated and weighed. A complete transmural section of the LV at the midventricular level was placed in 4% paraformaldehyde, and the remaining tissue was minced into 1 mm cubes and snap-frozen in liquid nitrogen for storage at  $-80^{\circ}\text{C}$ .

### **Infrarenal Abdominal Aorta-Inferior Vena Cava (AV) Fistula**

Infrarenal AV fistula was created in rats as described previously.(20;28) Briefly, a ventral abdominal laparotomy was performed to expose the aorta and caudal vena cava approximately 1.5 cm below the renal arteries. Both vessels were temporarily occluded, and an 18 gauge needle was inserted into the exposed abdominal aorta and advanced through the medial wall into the vena cava. The needle was withdrawn and the puncture site sealed with surgical glue.

### **Protein extraction from LV tissue**

100mg of LN<sub>2</sub> frozen LV tissue from each heart was maintained on ice and minced. Tissue was placed into homogenization tubes with 800  $\mu\text{l}$  PBS/protease inhibitor cocktail solution. Tissues were homogenized on ice prior to sonication. 50 $\mu\text{l}$  of 10% Triton-X 100 was added to each sample and vortexed. Samples were then incubated on ice for 30 min and vortexed after the first 15 min. Samples were then centrifuged @ 16,000 rpm ( $4^{\circ}\text{C}$ ) for 30min and the supernatant collected and frozen at  $-80^{\circ}\text{C}$ .

### **Assessment of Mast Cell Density and Fibrillar Collagen Concentration**

At the end of the experimental period, a transmural section of LV was taken from the mid ventricle, formalin-fixed and processed for routine histopathology. A 5 micron paraffin-embedded section was stained with pinacyanol erthrosinate for the visualization of mast cell morphology. Mast cell density was determined from the total number of mast cells in the LV cross section normalized for tissue area determined from a digitized image (ImageQuant, Molecular Dynamics). The percentage of mast cell degranulation was determined based upon 400 $\times$  microscopic examination of the entire LV slide section of four randomly selected hearts from control and treated groups. Degranulation was defined as the presence of disseminated granules external to the cell membrane and/or loss of membrane integrity as previously described.(29)

LV interstitial collagen volume fraction (CVF) was determined by analysis of picrosirius red-stained sections as previously described.(20;29) Sections were incubated with 0.2% phosphomolybdic acid (2 min) to reduce background staining before staining with picrosirius red (0.1% Sirius Red F3BA in picric acid). Twenty randomly-chosen fields per LV section were analyzed in a blinded fashion using a Biorad MRC-1024 confocal laser-scanning microscope under 400 $\times$  magnification. Image analysis was performed using Scion-image software. For each field, the percent of collagen was obtained by dividing the pixel count of fluorescent interstitial collagen fibers in the field by the total number of pixels present in the

field. The individual collagen fraction values were then averaged to obtain the representative CVF for the LV.

### Enzyme-Linked ImmunoSorbent Assay

Myocardial tissue levels of ET-1 and TNF- $\alpha$  were analyzed using commercially available kits (Alpco<sup>®</sup> and R&D Systems Quantikine, respectively). Briefly, precoated wells were incubated with 100 $\mu$ l of extracted protein sample with assay buffer for one hour, followed by washing 5 times with wash buffer. Wells were then incubated with appropriate monoclonal antibody and washed again before incubation with HRP conjugated secondary antibody. The plates were then read by spectrophotometry at 450nm, in accordance with manufacturer's specifications.

### Flow Cytometry

Cardiac mast cells were isolated as previously described.(20;30) Isolated cells were washed and blocked against non-specific staining with PBS/1% BSA for 30 min before incubation for 1 hr at 37°C with AR32AA4 (mast cell; mouse monoclonal, 1:100; BD Pharmingen) followed by conjugation for 1 hr at 37°C with PE secondary antibody (rabbit, 1:500; Abcam). After washing, the cells were fixed with 4% paraformaldehyde and permeabilized using saponin (0.1%) for 20 min at 4°C. The cells were then incubated with anti-TNF- $\alpha$  antibody (rabbit monoclonal, 1:500; Abcam) and conjugated with Alexa Fluor 488 secondary antibody. Cell preparations were analyzed using an Epics XL FACS system equipped with a 15mW argon ion laser operating at 488nm and data analyzed with EXPO 32 software.

### Statistical analysis

Statistical analyses were performed with Graphpad Prism 5.0 (GraphPad Software, Inc. San Diego, CA). All grouped data are expressed as means  $\pm$  SEM unless otherwise noted. Grouped data comparisons were made by one-way analysis of variance with intergroup comparisons analyzed using Bonferroni post-hoc testing. Statistical significance was taken to be  $p \leq 0.05$ .

## Results

### In Vivo TNF- $\alpha$ Elaboration and Neurohormone Cytokine Interaction

A substantial 5 fold elevation in myocardial TNF- $\alpha$  levels relative to that in the sham-operated group occurred in the untreated rats at 3 days post-fistula (Table 1). GM6001 treatment prevented the volume overload induced increase in TNF- $\alpha$  levels post-fistula ( $p \leq 0.01$  vs. ACF). In contrast, mast cell stabilization with nedocromil failed to attenuate the significant increase in LV TNF- $\alpha$  levels. In figure 1, flow cytometry of mast cells stained for TNF- $\alpha$  shows that the percentage of mast cells containing TNF- $\alpha$  was unchanged between sham, fistula and fistula treated with nedocromil. Additionally, mean fluorescent intensity indicated that there was no difference in the amount of TNF- $\alpha$  in mast cells isolated from sham, fistula or fistula treated with nedocromil.

Significant increases in LV myocardial ET-1 levels also occurred in the ACF group at 3 days post-fistula (Table 1). Whereas administration of GM6001 significantly attenuated this increase in ET-1 levels post-fistula, mast cell stabilization with nedocromil markedly exacerbated the increase, producing ET-1 levels 6.5 fold and 2 fold greater than that in the sham-operated control and ACF group, respectively.

### In Vivo Mast Cell Density and Percent Degranulation

The influence of *in vivo* mast cell stabilization and MMP inhibition on volume overload induced increases in LV mast cell density was also determined at 3 days post-fistula. As can be seen in Table 1, volume overload induced a significant increase in LV mast cell density in

both the untreated and GM6001 treated fistula groups (79% and 98%, respectively, relative to control). Conversely, administration of nedocromil prevented the AV fistula-induced increase in myocardial mast cell density. Consistent with the increased mast cell density, the percentage of activated cells was significantly greater in the untreated fistula group ( $19 \pm 4\%$  degranulated,  $p \leq 0.05$ ), relative to the small number of degranulated cells seen in the sham ( $10 \pm 2\%$ ) and nedocromil ( $11 \pm 4\%$ ) groups. MMP inhibition with GM6001 resulted in substantially greater mast cell activation above that in the untreated fistula group ( $32 \pm 5\%$  degranulated,  $p \leq 0.05$  vs. ACF).

### Extracellular Matrix Content and Composition

Compared to control, untreated fistula hearts underwent a marked loss of ventricular collagen at 3 days post-fistula ( $1.8 \pm 0.1\%$  vs.  $1.3 \pm 0.09\%$  percent of myocardial tissue; Sham vs. ACF,  $p \leq 0.05$ , Table 1). However, interventions with nedocromil or GM6001 were equally efficacious in preventing the fistula associated decrease in myocardial CVF.

### Discussion

The increase in TNF- $\alpha$  in the stressed or injured myocardium has been well documented.(2; 12–15) We recently found significant elevations in myocardial TNF- $\alpha$  after 5 days of chronic LV volume overload.(28) Bozkurt et al.(31) found that chronic administration of TNF- $\alpha$  in normal rats produced significant LV dilatation along with a striking reduction in interstitial collagen. In this study we utilized a broad spectrum matrix metalloproteinase inhibitor, GM6001 and treated male adult rats for three days after creating an ACF. GM6001 possesses a broad spectrum MMP inhibitory capacity which includes the A Disintegrin And Metalloproteinase (ADAM) family of enzymes encompassing ADAM 17, otherwise known as TNF- $\alpha$  converting enzyme (TACE).(27;32) ADAM 17 is capable of generating soluble TNF- $\alpha$  by proteolytically cleaving membrane-bound pro-TNF- $\alpha$ .(21;22) However, it must be noted that redundant mechanisms for generating myocardial TNF- $\alpha$  have also been described.(15; 17;33;34) Specifically, other MMPs have also been shown to mediate cleavage of pro-TNF.(35;36) In the chronic *in vivo* scenario, MMP inhibition with GM6001 was efficacious in preventing the increase in myocardial TNF- $\alpha$  in the ACF. GM6001 also prevented ACF-induced collagen degradation. The significantly decreased level of total myocardial collagen in the untreated fistula group (28% less than that in sham-operated control), reflecting degradation by MMP activation is consistent with previous reports.(29;37) Of further interest is the observation that GM6001 inhibition of TNF- $\alpha$  cleavage resulted in similar results to those observed using the TNF- $\alpha$  inhibitor, etanercept, where collagen degradation was also prevented in the same model of volume overload.(38)

While our results with GM6001 clearly show the importance of MMP cleavage of TNF- $\alpha$  in volume overload-induced remodeling, previous histological analysis of LV sections from normal hearts demonstrated concentrated localization of TNF- $\alpha$  within cardiac mast cells.(13;14;39) Consistent with mast cells regulating myocardial TNF- $\alpha$ , levels of TNF- $\alpha$  were undetectable by ELISA in mast cell deficient rats post-fistula.(16) However, in that same study we also observed diffuse labeling of TNF- $\alpha$  dispersed throughout the myocardium of the wild type control, indicating the possibility of cardiomyocyte membrane-bound TNF- $\alpha$  in normal rats. Further, a recent study by Reil et al. (15) indicates that, although the initial release of TNF- $\alpha$  following ischemia-reperfusion is cardiac mast cell-dependent, subsequent increases are derived from other sources. In fact, in a similar volume overload model of heart failure to that herein, Chen Y and coworkers(17) identified isolated myocytes as a potential source of TNF- $\alpha$ . These observations, together with our previous findings that mast cells activate MMPs in the ACF model, raise the possibility that increased levels of TNF- $\alpha$  in stressed or injured

myocardium are the result of mast cell mediated activation of MMPs cleaving latent extracellular membrane bound TNF- $\alpha$ .

The confounding factor in this study is that TNF- $\alpha$  levels in the myocardium remained significantly elevated after treatment with nedocromil. One limitation of this study is the inability to differentiate interstitial TNF- $\alpha$  from the cytokine content of cardiac mast cells in whole tissue homogenate, thus, flow cytometry was used to determine whether the increased levels of myocardial TNF- $\alpha$  following treatment with nedocromil was the result of retention and/or increases of TNF- $\alpha$  in mast cells due to membrane stabilization. Our results indicate that the percentage of mast cells containing TNF- $\alpha$  was unchanged between sham, fistula and fistula treated with nedocromil. Additionally, mean fluorescent intensity indicated that there was no difference in the amount of TNF- $\alpha$  in mast cells isolated from sham, fistula or fistula treated with nedocromil. Therefore, at this stage we are unable to explain the increased myocardial TNF- $\alpha$  in the ACF+nedocromil group. It is possible though that nedocromil prevents release of mast cell products that activate MMPs, thus, preventing the cleavage of cardiomyocyte-bound TNF- $\alpha$ . Since cardiomyocytes are known to upregulate TNF- $\alpha$  in the ACF (17) this may lead to an accumulation of membrane bound TNF- $\alpha$  in the ACF+nedocromil group.

We have also previously demonstrated that cardiac mast cells are responsive to the endogenous endothelin system.(28;40) Cardiac mast cell activation herein was also positively correlated with myocardial ET-1 levels. A similar relationship has been postulated between ET-1 levels and MMP activation,(41–44) Previous work by Podesser et al.(44) and Ozdemir and coworkers (45) indicated that MMP induction following myocardial infarction was an ET<sub>A</sub> receptor specific phenomenon, yet no particular cell type was implicated. Few have thus far considered that cardiac mast cells may broker the interaction.(28;29) A recent study by Deschamps et al. (43) found selective ET<sub>A</sub> receptor antagonism attenuated the ET-1 mediated increase in membrane type MMP (MT1-MMP) activity following ischemia/reperfusion and MT1-MMP is involved in the processing of bioactive cytokines.(46) Similarly, studies have documented that ET-1 is capable of inducing TNF- $\alpha$  expression in mucosal and connective tissue type mast cells.(47;48)

The marked increase in ET-1 levels in the ACF and ACF + Nedocromil groups may have been the outcome of an endogenous stimulus to mediate MMP activation. However, the substantially greater increase in the ACF + Nedocromil group could also be related to mast cell-derived chymase being essential for localized degradation of ET-1.(49;50) The reduction in myocardial ET-1 levels by GM6001 was in stark contrast to the increases seen in the ACF and ACF + Nedocromil group. However, Fernandez-Patron et al. recently reported that MMP-2 cleavage of big endothelin 1 yielded the vasoactive peptide ET-1,(51) therefore, this observation may reflect impaired MMP mediated generation of ET-1. Further, GM6001 would not be expected to alter mast cell activation (i.e. release of preformed serine proteases) which would still allow degradation of ET-1 via mast cell-derived chymase.

In summary, the efficacy of the MMP inhibitor, GM6001, to prevent increased levels of myocardial TNF- $\alpha$  and subsequent myocardial remodeling is indicative of MMP-mediated cleavage of latent extracellular membrane bound TNF- $\alpha$  protein as the source of bioactive TNF- $\alpha$  in the volume-stressed myocardium. The results presented in Table 1 demonstrate that stabilization of mast cells failed to attenuate the local generation of TNF and ET-1, but that MMP inhibition significantly reduced TNF and ET-1 levels following acute AV fistula. The schematics depicted in Figure 2 further illustrate the involved pathways. In panel A, volume overload results in an increase in ET-1 which in turn causes mast cell degranulation and activation of membrane (memb) type MMPs. Mast cell-derived chymase feeds back to degrade ET-1 while other mast cell-released substances activate interstitial MMPs. These activated

MMPs are then responsible for interstitial collagen degradation and possibly contribute to additional activation of membrane type MMPs. The activated membrane type MMPs are responsible for the release of membrane bound TNF- $\alpha$  which is known to cause myocyte remodeling. The results of pretreatment with nedocromil are depicted in Panel B. By preventing mast cell degranulation, the negative feedback associated with chymase release is no longer present and serine proteinase activation of MMPs does not occur. As a result, ET-1 is further elevated, membrane type MMPs are activated and membrane bound TNF- $\alpha$  released. Thus, while interstitial collagen remains intact, myocyte remodeling will occur. As can be seen in Panel C, GM6001 inhibits the membrane type MMPs despite the fact that mast cells are degranulated. As a result collagen degradation and myocyte remodeling do not occur. Accordingly, the local source and conversion of TNF following the volume overload stimulus at this time point is not the mast cell per se, but that this induction is an MMP dependent process.

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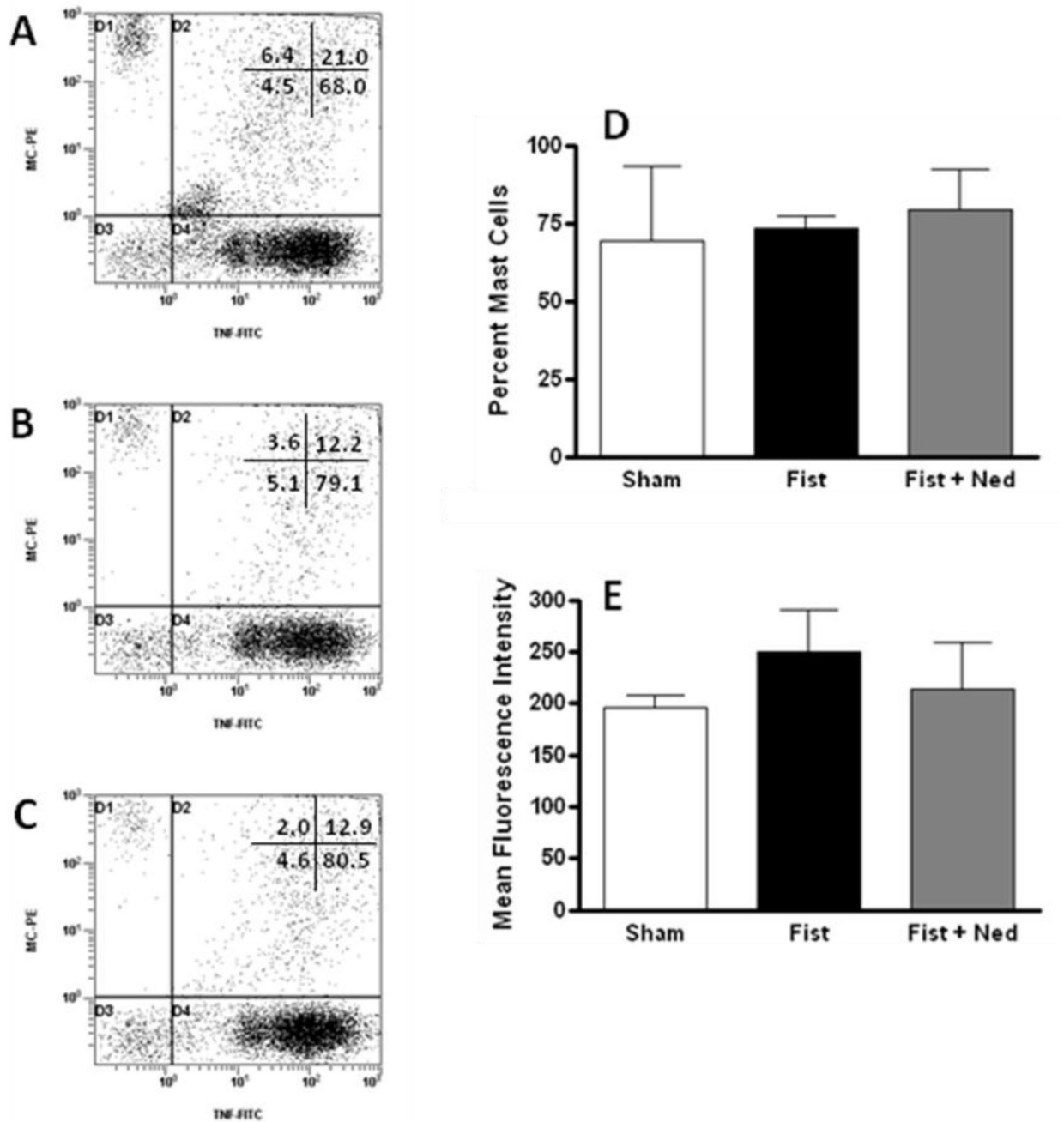
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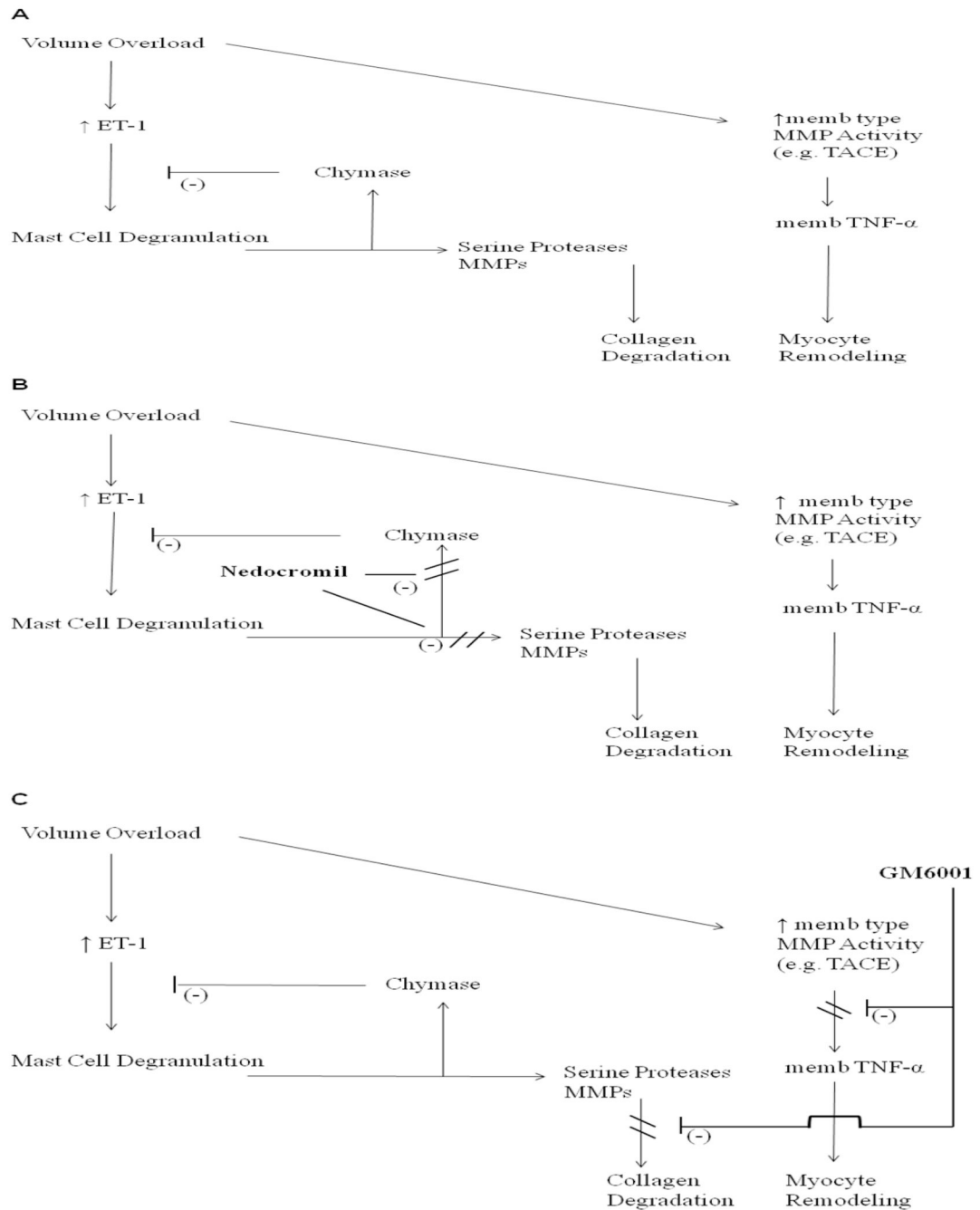


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**Figure 1.** Representative scatter plots of isolated cardiac mast cells containing TNF- $\alpha$  from (A) shams; (B) untreated fistula; and (C) fistula treated with nedocromil. The grouped data are presented as mean  $\pm$  SD. (D) Percent of cardiac mast cells containing TNF- $\alpha$ ; (E) mean intensity fluorescence for cardiac mast cells containing TNF- $\alpha$ .

**Figure 2.**

(A) Schematic diagram of resident cardiac mast cell mediated ventricular remodeling secondary to chronic volume overload. Panel B depicts the potential effects of mast cell stabilization (Nedocromil) on preformed product release from mast cells and how this would play a role in ET-1 and TNF- $\alpha$  levels. (C) Illustrates the possible effects of the broad spectrum inhibition of matrix metalloproteinases (GM6001) and subsequent attenuation of adverse myocardial remodeling.

**Table 1**

Analysis of Tumor Necrosis Factor-  $\alpha$  (TNF- $\alpha$ ), Endothelin-1 (ET-1), mast cell density, and interstitial collagen in left ventricular tissue in sham-operated, drug treated controls, untreated aortocaval fistula (ACF), ACF + Nedocromil and ACF + GM6001 treated fistula hearts.

	TNF- $\alpha$ (pg/ml)	ET-1 (fmol/ml)	Mast Cell Density (LV mast cell /mm <sup>2</sup> )	Collagen (% myocardial area)
Sham	5 $\pm$ 0.6	1 $\pm$ 0.2	2.2 $\pm$ 0.3	1.8 $\pm$ 0.1
Sham+ Nedo	4 $\pm$ 0.1	1 $\pm$ 0.1	1.4 $\pm$ 0.2	1.7 $\pm$ 0.1
Sham+ GM6001	6 $\pm$ 1	0.8 $\pm$ 0.03	2.9 $\pm$ 0.4 *	1.8 $\pm$ 0.05
ACF	27 $\pm$ 3 *	3 $\pm$ 0.6 *	3.5 $\pm$ 0.3 *	1.4 $\pm$ 0.1 *
ACF+ Nedoc	21 $\pm$ 2 *	6.5 $\pm$ 1.7 *	1.3 $\pm$ 0.1 †	2.2 $\pm$ 0.1 †
ACF+GM6001	5 $\pm$ 2 †	1.7 $\pm$ 0.2 †	3.8 $\pm$ 0.2 *	1.9 $\pm$ 0.05 †

Values are reported as average  $\pm$  SEM.

\*  $p \leq 0.01$  vs. sham.

† denotes  $p \leq 0.01$  vs. untreated ACF.