Granule cargo release from bone marrow-derived cells sustains cardiac hypertrophy

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1Division of Cardiovascular Medicine, Gill Heart Institute, University of Kentucky, Lexington, Kentucky; 2Laboratory of Blood and Vascular Biology, Rockefeller University, New York, New York; and 3Lexington Veterans Affairs Medical Center, Lexington, Kentucky

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Yang F, Dong A, Ahamed J, Sunkara M, Smyth SS. Granule cargo release from bone marrow-derived cells sustains cardiac hypertrophy. Am J Physiol Heart Circ Physiol 307:H1529–H1538, 2014. First published September 19, 2014; doi:10.1152/ajpheart.00951.2013.—Bone marrow-derived inflammatory cells, including platelets, may contribute to the progression of pressure overload-induced left ventricular hypertrophy (LVH). However, the underlying mechanisms for this are still unclear. One potential mechanism is through release of granule cargo. Unc13-4Jinx (Jinx) mice, which lack Munc13-4, a limiting factor in vesicular priming and fusion, have granule secretion defects in a variety of hematopoietic cells, including platelets. In the current study, we investigated the role of granule secretion in the development of LVH and cardiac remodeling using chimeric mice specifically lacking Munc13-4 in marrow-derived cells. Pressure overload was elicited by transverse aortic constriction (TAC). Chimeric mice were created by bone marrow transplantation. Echocardiography, histology staining, immunohistochemistry, real-time polymerase chain reaction, enzyme-linked immunosorbent assay, and mass spectrometry were used to study LVH progression and inflammatory responses. Wild-type (WT) mice that were transplanted with WT bone marrow (WT→WT) and WT mice that received Jinx bone marrow (Jinx→WT) developed LVH and a classic fetal reprogramming response early (7 days) after TAC. However, at late times (5 wk), mice lacking Munc13-4 in bone marrow-derived cells (Jinx→WT) failed to sustain the cardiac hypertrophy observed in WT chimeric mice. No difference in cardiac fibrosis was observed at early or late time points. Reinjection of WT platelets or platelet releasate partially restored cardiac hypertrophy in Jinx chimeric mice. These results suggest that sustained LVH in the setting of pressure overload depends on one or more factors secreted from bone marrow-derived cells, possibly from platelets. Inhibiting granule cargo release may represent a novel target for preventing sustained LVH.

Granule cargo release from bone marrow-derived cells sustains cardiac hypertrophy; granule secretion; Munc13-4; platelet; bone marrow-derived cells

CHRONICALLY ELEVATED AFTERLOAD, as occurs with hypertension or aortic stenosis, stimulates an increase in left ventricular (LV) mass or hypertrophy (LVH) and fibrosis, with resultant LV diastolic and/or systolic dysfunction. Hypertensive LVH is accompanied by perivascular inflammation peaking within the first week, which may contribute to cardiac remodeling later on, as described in Kai et al. (19). In animals, LV pressure overload can be modeled by transverse aortic constriction (TAC). Using immunohistochemistry (IHC), we and others have reported that inflammatory cells, including macrophages, T lymphocytes, and platelets accumulate along and within the coronary arterioles as early as 1 day after TAC and reach their peak at 7 days after TAC (35, 37). Platelet accumulation has also been reported along coronary arteries after angiotensin infusion (18). Early accumulation of these cells may influence subsequent arterial and ventricular remodeling. Moreover, the P2Y12 antagonist clopidogrel, a widely used antiplatelet agent, reduces hypertension-related cardiac inflammation and fibrosis formation elicited by angiotensin infusion in mice (18), suggesting a potential proinflammatory role of platelets in cardiac remodeling (10).

In addition to direct cell-to-cell interactions, inflammatory cells communicate with surrounding tissue by releasing cargo stored within granule compartments. Platelets, for example, have three distinct types of secretory vesicles: α-granules, dense granules, and lysosomes, which are a rich source of cytokines, chemokines, and growth factors (32). Components of platelet releasate [e.g., serotonin, transforming growth factor-β1 (TGF-β1), and sphingosine-1-phosphate (S1P)] have been implicated as regulators of pathological cardiac remodeling (3, 20, 22, 33). Thus it is possible that platelet deposition, activation, and granule content release could influence the tissue response in the setting of LV pressure overload. To investigate the role of granule secretion in LVH and cardiac remodeling, we made use of mice that lack the ability to release cargo from granules.

Platelet granule secretion requires vesicular docking, tethering, priming, and membrane fusion. Vesicular fusion is driven by soluble NSF attachment protein receptor (SNARE) complexes composed of a VAMP, a syntaxin, and SNAP-23 proteins (6, 13). Members of the Rab family of Ras guanosine 5’-triphosphatases are essential for membrane tethering that occurs prior to fusion (12). The Rab27 effector protein Munc13-4 appears to play an important role in granule secretion (5, 11, 12, 17, 25–27, 31). Several mutations, including a single point mutation in Munc13-4, can result in type 3 familial hemophagocytic lymphohistiocytosis (FHL3), which is characterized by hepatosplenomegaly, anemia, and thrombocytopenia (11). Unc13-4Jinx (Jinx) mice carry a mutation in Unc13d, the murine homolog of Munc13-4, and display secretion defects in natural killer cells, cytotoxic T lymphocytes, neutrophils, mast cells, and platelets (5, 7, 17, 25–27) and features of FHL3 (9). Neutrophils from Jinx mice have alterations in the secretion of matrix metalloproteinase-9 (MMP-9) and myeloperoxidase (MPO), whereas overexpression of Munc13-4 enhances MMP-9 secretion in human neutrophils (26). Jinx platelets display defects in dense granule, α-granule, and lysosome secretion upon thrombin stimulation. Addition of Munc13-4 protein restores secretion to mutant platelets and increases secretion in wild-type (WT) platelets (27). The relatively low levels of Munc13-4 in platelets and the partial

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http://www.ajpheart.org
secretion defects in heterozygous mice have led to the suggestion that the protein may be the rate-limiting factor in platelet granule secretion (27).

In this report, we used Jinx mice to determine the contribution of granular secretion to LV remodeling following TAC surgery. Chimeric mice were created by bone marrow transplantation to identify a role for cargo release from bone marrow-derived cells. Although the loss of Munc13-4 did not affect the initial development of LVH, sustained hypertrophy did not occur in the absence of Munc13-4 in bone marrow-derived cells. Cardiac hypertrophy could be partially restored by administration of platelets or platelet releasate to chimeric mice. These results indicate that sustained LVH in the setting of pressure overload depends on one or more factors released from bone marrow-derived cells, including platelets.

**MATERIALS AND METHODS**

**Mice.** All procedures conformed to the recommendations in the Guide for the Care and Use of Laboratory Animals (Department of Health, Education, and Welfare publication NIH 78-23, 1996) and were approved by the Institutional Animal Care and Use Committees of the University of Kentucky and the Lexington Veterans Affairs Medical Center. C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). Unc13-4^{plus} (Jinx) mice were generously provided by Dr. Sidney W. Whiteheart. The Jinx mutation was originally identified in a mouse cytomegalovirus susceptibility screen performed in offspring of C57BL/6 mice that had been subject to random N-ethyl-N-nitrosourea-induced germline mutagenesis (8). Mice were housed in cages filtered with high-efficiency particulate air in rooms on 12-h:12-h light-dark cycles and fed Purina 5058 rodent chow ad libitum.

**Bone marrow transplantation.** Bone marrow cells were isolated from 8- to 12-wk-old donor mice by flushing femurs and tibias with 5 ml of ice-cold media [RPMI, 10 mM HEPES, 25 U/ml heparin, and 5% fetal bovine serum (FBS)] using a 27.5-gauge needle. The cells were washed by centrifugation (453 g at 4°C for 10 min), red blood cells were lysed in hypotonic saline, and the remainder of the cells were suspended in phosphate-buffered saline (PBS) containing 2% heat-inactivated FBS at 5 × 10^7 cells/ml. Recipient mice (6- to 8-wk old) were irradiated twice (450 rad), 3 h apart, and then injected with 5 × 10^6 cells within 4 h after the second irradiation.

**Aortic banding.** Pressure overload of the left ventricle was induced by TAC in mice aged 8–12 wk as previously described (2, 29). Briefly, the aorta was ligated between the innominate and left common carotid arteries by tying a 7-0 silk suture around a tapered 27-gauge needle placed on top of the aorta. The tapered needle was removed later, leaving the suture to produce a defined constriction of the vessel. The skin was closed with sutures, and buprenorphine was administered for analgesia. Sham operation was performed using the same procedure but without ligation of the aortic arch.

**Doppler studies.** Velocity of blood flow in the left and right carotid arteries was measured as previously described (15, 16) using a handheld 20-MHz Doppler probe (Indus Instruments) before and after surgery and at additional time points after surgery.

**Echocardiography.** Two-dimensional short- and long-axis views of the left ventricle were obtained by transthoracic echocardiography performed 5 wk after TAC using a 45 MHz probe (77B) and the Vevo 770 Imaging System (VisualSonics) under 1.5% isoflurane inhalation as previously described (2). M-mode tracings were recorded and used to determine LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), and LV posterior wall thickness in diastole (LVPWtd) over three cardiac cycles. LV fractional shortening (FS) was calculated using the formula % FS = (LVEDD − LVESD)/LVEDD.

**Organ weights measurement.** Mice were weighed and euthanized by an overdose of isoflurane at 7 days or 5 wk postsurgery. Hearts, lungs, livers, and kidneys were harvested and washed and in × 1 PBS. The excessive PBS was absorbed by cotton gauze.

**Histology.** Hearts were sectioned at the level of the papillary muscle. The base of the heart was immersed in 4% paraformaldehyde for 24 h, subsequently transferred to 70% ethanol, and embedded in paraffin. Multiple serial 5-μm sections were taken at the papillary muscle level of the heart. Slides from each heart were stained with hematoxylin and eosin, Masson’s trichrome, and periodic acid-Schiff (PAS) staining using laboratory protocols. The remaining slides were archived for later use.

**IHC was performed on frozen sections using CD68 (1:200, MCA1957; Serotec), CD8 (1:50, MCA2690; Serotec), CD19 (1:50, TM 550284; BD Pharmingen), MPO (1:50, 17111-500; Abcam), and platelet antibody (1:1,000, Intercell). Briefly, the heart bases were directly embedded in OCT and frozen at −20°C. Blocks were cut into 10-μm sections and fixed with chilled acetone at −20°C. To block endogenous peroxidases, slides were immersed in 1% H2O2 in methanol for 2 min at 40°C. Nonspecific sites were blocked using 1.5% serum from the secondary antibody-derived animal for 15 min at 40°C. Slides were then incubated with primary antibodies for 15 min at 40°C, followed by 15 min of biotinylated secondary antibody incubation, and 10 min of avidin-biotin complex detector application (ABC Kit; Vector Laboratories) at 4°C. A horseradish peroxidase substrate-chromogen (AEC) (Biomedia) kit was used as the chromogen, and hematoxylin (Accurate Chemical & Scientific) was used for counterstaining. Slides were mounted with aqueous mounting medium (Vector H-5501) and covered by glass covers. Immunostaining of targeted proteins was quantified in tissue sections from TAC mice and their respective sham-operated controls. For each antibody, isotope-matched nonreactive IgG served as the negative control. The percentage of the vessel area occupied by inflammatory cells was measured from digital images using Metamorph software by personnel who were blinded to the treatment. Perivascular and interstitial fibrosis was measured from slides stained with Masson’s trichrome. Cardiomyocyte size was measured by Metamorph software.

**Quantitative polymerase chain reaction.** RNA samples were extracted from the apex of the heart (stored in RNAlater, −80°C) using...
TRIzol (Invitrogen) as previously described (21). RNA concentration and quality were tested using an OD 260/280 ratio by Biomate3 (Thermo Electron) and also by examining the product on a 1% agarose gel. Complementary DNA synthesis was performed using the High-Capacity cDNA Archive kit (Applied Biosystems) starting with 1/10 g of RNA in a 20-/10 l reaction system. Quantitative real-time polymerase chain reaction (PCR) was carried out using Taqman Universal Master Mix and Assays-on-Demand primers and probes (Applied Biosystems), and the ABI 7500 system. 18S RNA was used as an endogenous control. An embryo RNA standard was used as a positive control. Results were expressed as mean fold changes of gene expression relative to sham-operated C57Bl/6 mice or control antibody-injected TAC mice using the 2−ΔΔCt method (21).

Washed platelet preparation. Blood was collected into ACD (2.5% sodium citrate, 0.4% citric acid, and 1.5% D-glucose) anticoagulant supplemented with 1/100 M prostaglandin E1 (PGE1) and diluted with normal saline before centrifugation (201 g for 10 min at room temperature) to obtain platelet-rich plasma/saline. After red blood cells were washed an additional time, supernatants were pooled, supplemented with 1/100 M PGE1, and centrifuged to pellet the platelets (974 g for 10 min at room temperature). The platelet pellet was washed by resuspension in CGS buffer (120 mM sodium chloride, 12.9 mM trisodiumcitrate, and 30 mM D-glucose, pH 6.5) and centrifuged. Washed platelets were resuspended at 1/100000000 ml in Tyrode buffer (NaHCO3 12 mM, NaCl 138 mM, D-glucose 5.5 mM, KCl 2.9 mM, MgCl2·6H2O 2 mM, NaH2PO4·H2O 0.42 mM, and HEPES 10 mM) pH 7.4.

Platelet aggregation. Platelets were diluted to 1–3 × 10⁸/ml in Tyrode buffer and allowed to rest for at least 30 min. Aggregation was

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**Table 1. Echocardiography parameters of bone marrow transplanted mice before surgery**

<table>
<thead>
<tr>
<th></th>
<th>WT&gt;WT</th>
<th>Jinx&gt;WT</th>
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<tbody>
<tr>
<td>LVIDs, mm</td>
<td>3.1 ± 0.3</td>
<td>3.0 ± 0.1</td>
</tr>
<tr>
<td>LVPWs, mm</td>
<td>1.0 ± 0.2</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>LVIDd, mm</td>
<td>4.2 ± 0.2</td>
<td>4.2 ± 0.1</td>
</tr>
<tr>
<td>LVPWd, mm</td>
<td>0.7 ± 0.2</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>EF, %</td>
<td>52 ± 5</td>
<td>54 ± 5</td>
</tr>
<tr>
<td>FS, %</td>
<td>26.5 ± 3.7</td>
<td>28.0 ± 3.4</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>436 ± 31</td>
<td>442 ± 16</td>
</tr>
</tbody>
</table>

EF, ejection fraction; FS, fractional shortening; Jinx, Unc13-dJinx mice; LVIDd, left ventricular internal diameter in diastole; LVIDs, left ventricular internal diameter in systole; LVPWd, left ventricular posterior wall thickness in diastole; LVPWs, left ventricular posterior wall thickness in systole; WT, wild-type mice; No significant differences were observed between the two groups (n = 6/group) by Student’s t-test. (P < or = 0.05 is considered statistically different.)

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**Fig. 2. Transverse aortic constriction (TAC)-induced cardiac hypertrophy 7 days after TAC. A: heart weight-to-body weight ratio (HW:BW) of chimeric mice. In the box plot, the median with 25th and 75th percentiles are shown as vertical boxes with bars; 10th and 90th percentiles are indicated by stars. WT→WT sham-operated mice n = 8; WT→WT TAC mice n = 11; Jinx→WT sham-operated mice n = 7; Jinx→WT TAC mice n = 12. *P < 0.001 by two-way ANOVA.**

**B: quantification of cardiomyocyte area demonstrated increased cell size in both WT→WT and Jinx→WT mice 7 days after TAC. Histological quantification was performed using Metamorph software; n = 5–7 mice/group for TAC, n = 3 mice/group for sham-operated. *P < 0.05 by two-way ANOVA.**

**C: cardiomyocyte representative images from periodic acid-Schiff (PAS)-stained heart sections (×40, bar = 70 μm).**

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performed at 37°C in a Chronolog dual chamber lumi-aggregometer in silicon-coated cuvettes with stir bars.

Platelet releasate collection. Washed platelets (1 x 10^9/ml) suspended in Tyrode buffer were preincubated at 37°C for 10 min and stimulated with thrombin (0.125 U/ml) for 10 min at 37°C. To measure TGF-β1, the suspension was centrifuged (14,000 g for 20 min in 4°C) to collect the supernatant and platelet pellets. Releasate to inject into mice was obtained by centrifugation at 3,000 g for 10 min at 4°C. Hirudin (0.125 U/ml) was applied after the stimulation to neutralize thrombin effect.

Platelet or platelet releasate reconstitution. Washed platelets were isolated from WT mice. Platelets (5 x 10^8) or the releasate from the same amount of platelets were injected to the recipient Jinx→WT mice through the retro-orbital vein 1 day before TAC surgery. The administration was repeated every 5 days for 5 wk.

Measurement of TGF-β1. Total TGF-β1 levels in platelet releasates, serum, and plasma were measured using an enzyme-linked immunosorbent assay (R&D Systems) following published methods (1, 23). In brief, blood was collected by retro-orbital puncture into an Eppendorf tube containing 10 mM EDTA in combination with 10% (vol/vol) citrate-theophylline-adenosine-dipyridamole (CTAD). The anticoagulated samples were centrifuged at 12,000 g for 5 min at room temperature to separate plasma. Mice blood was collected into a glass tube without any anticoagulant and incubated at 37°C for 4 h. The clot was removed gently using a wooden stick, and serum was collected by centrifuging it at 14,000 g for 20 min at 4°C.

Lipid extraction and S1P determinations were performed as previously described (30). Methanol (2 ml), 1 ml of chloroform, and 450 μl of 0.1 M HCl were added to an 8-ml borosilicate glass tube with the addition of 50 μl of plasma sample from each mouse. Fifty microliters of C17 S1P was also added as an internal standard. Analysis of S1P, sphingosine, and hexadecenal (as its semicarbazone derivative) were accomplished using Shimadzu ultrafast liquid chromatography cou-
pleaded with an ABI 4000-Qtrap hybrid linear ion trap, triple quadrupole mass spectrometer in multiple reaction monitoring mode.

Statistical analysis. All results are expressed as means ± SD. Statistical significance within strains was determined using a Student’s t-test or two-way ANOVA with multiple pairwise comparisons as appropriate. In t-tests, if a sample failed the normality test, a rank t-test was used. In some cases of two-way ANOVA, data were log-transformed to be normally distributed. Statistical analysis was performed using Sigma-STAT software, version 3.5 (Systat Software). A value of P < 0.05 was considered significant.

RESULTS

Our previous findings indicated that inflammatory cells and platelets accumulate along coronary vessels and within ventricular tissue within days after pressure overload (37). Both cell types have been implicated as modulators of cardiac remodeling by us and others. Release of granule contents is one mechanism by which these cells could influence the response to pressure overload. Therefore, we created animals with impaired cargo release from hematopoietic cells by lethally irradiating WT C57BL/6 male mice and reconstituting them with bone marrow isolated from Unc13-dJinx (Jinx) mice, which lack functional Munc13-4 protein and manifest defects in granular secretion. WT mice that received WT marrow (WT→WT) were used as controls. Immunoblot analysis confirmed the absence of Munc13-4 in platelets from WT mice reconstituted with Jinx bone marrow (Jinx→WT) (Fig. 1).

Lack of Munc13-4 does not alter early response to pressure overload. At 6 to 7 wk after bone marrow transplantation, mice were subjected to sham or TAC surgery. Prior to surgery, echocardiography showed similar cardiac function in mice that had received WT marrow (WT→WT) and Jinx marrow (Jinx→WT) (Table 1). Bone marrow transplant did not affect the initial hypertrophic response 1 wk after TAC. The heart weight-to-body weight (HW:BW) ratio in WT→WT mice was 20% greater following TAC than in sham-operated controls (6.67 ± 0.69 vs. 5.48 ± 0.31 mg/g, P < 0.001, Fig. 2A) and

Fig. 4. TAC-induced perivascular fibrosis 7 days after TAC. A: gene expression of collagen 1a (col 1a) was measured by real-time polymerase chain reaction (PCR). No difference was observed by two-way ANOVA, P ≥ 0.05, was considered significant. B: quantification of perivascular collagen performed using Metamorph software. No significant difference in fibrosis 7 days after TAC was observed by two-way ANOVA; n = 6 mice/group for TAC. C: representative images of Picro-sirius red-stained heart sections (×20, bar = 145 μm).
similar to that previously reported for WT mice not subjected to irradiation and transplantation (2). Jinx→WT chimeric mice that underwent TAC also had significantly higher HW:BW ratios than did sham-operated counterparts (6.38 ± 0.85 vs. 5.21 ± 0.27 mg/g, P < 0.001, Fig. 2A). The extent of early cardiac hypertrophy in the Jinx chimeras was similar to that observed in WT→WT mice (Fig. 2A). Quantification of PAS-stained heart sections indicated that TAC induced significant increases in cardiomyocyte size in both WT→WT and Jinx→WT mice as early as 7 days after surgery (Fig. 2, B and C). Leukocyte count and hematocrit were similar in WT→WT and Jinx→WT TAC mice, whereas platelet count was significantly higher in Jinx→WT mice after TAC (Table 2). Immunohistochemical analysis of hearts suggested that early inflammatory cell accumulation was unaffected by the loss of Munc13-4 in hematopoietic cells, such as CD8+ T lymphocytes, macrophages (CD68), neutrophils (MPO), and platelets (Fig. 3). Real-time PCR detected an increased trend in collagen 1a expression in both WT→WT and Jinx→WT 7 days after TAC (Fig. 4A), although we were not able to detect a significant increase in perivascular collagen deposition by IHC as one might expect (Fig. 4, B and C).

Attenuated cardiac hypertrophy 5 wk after TAC in mice with impaired granular cargo release from hematopoietic cells. To determine whether granule secretion contributed to the progression of cardiac hypertrophy, the response in mice with WT or Jinx marrow was examined at 5 wk after TAC surgery. Blood counts for the mice at this time point are presented in Table 3. As expected, TAC-operated WT→WT mice had a significantly higher HW:BW ratio than did sham-operated WT→WT animals (7.6 ± 2.3 vs. 5.3 ± 0.3, P = 0.044, Table 4). WT→WT mice also had a significant increase in their LVPWd following TAC (1.3 ± 0.2 vs. 0.8 ± 0.1 mm in sham-operated controls, P = 0.004, Table 4) and in their LV internal diameter in systole (LVPWs, 1.7 ± 0.3 vs. 1.2 ± 0.1 mm in sham-operated controls, P = 0.006, Table 4) shown by echocardiography. In contrast, WT mice that were reconstituted with Jinx marrow displayed a less robust increase in cardiac mass at 5 wk after TAC. Although the absolute HW:BW ratio was higher in Jinx→WT mice that underwent TAC surgery than in sham-operated controls, the difference did not reach statistical significance (6.7 ± 1.0 vs. 5.6 ± 0.6, P = 0.32, Table 4). The same was observed with LVPWd (0.9 ± 0.3 vs. 0.8 ± 0.1 mm, P = 0.685, Table 4). Masson’s trichrome staining of heart sections confirmed smaller LV size in WT mice with Jinx marrow cells compared with WT mice that received WT marrow (Fig. 5A). These results suggest that the absence of Munc13-4 in hematopoietic cells protects from cardiac hypertrophy at late times after pressure overload.

To determine whether the lower heart mass at 5 wk after TAC in Jinx→B6 mice was due to a lack of cardiomyocyte hypertrophy, histomorphometric analysis was performed on LV sections stained with PAS. Compared with cardiomyocyte size in hearts from B6→B6 mice after TAC, the cardiomyocytes in Jinx→WT mice were threefold smaller after TAC (Fig. 5, B and C). As was observed at 7 days after TAC, upregulation of Nppa, Nppb, Myh7, and Acta1 occurred in both WT→WT and Jinx→WT mice at 5 wk after TAC (Fig. 5D) despite the lower cardiac mass in Jinx→WT mice. Perivascular fibrosis (Fig. 6, A and B) and interstitial fibrosis (data not shown) were similar in the two groups, as was expression of the fibrosis markers Mmp9 and Col1a (Fig. 6C).

Platelets and platelet releasate partially restores cardiac hypertrophy after TAC in mice lacking Munc13-4 in hematopoietic cells. Lack of Munc13-4 impairs degranulation of natural killer cells, cytotoxic T lymphocytes, and platelets. Platelets, by storing and releasing granule content with activation, may be an important source for proteins and small molecules, such as TGF-β1 found in α-granules and S1P, which influence cardiac hypertrophy. Jinx platelets do not release contents from dense granules and have severely impaired release of α-granules (27). To test whether the phenotype observed in irradiated WT mice that had been reconstituted with Jinx marrow was due to a defect in platelet secretion, WT platelets or releasate from thrombin-stimulated WT platelets were administered to Jinx→WT chimeras. The administration of WT platelets to Jinx→WT mice increased the HW:BW ratio after TAC to 7.7 ± 1.7 vs. 6.8 ± 0.8 mg/g observed in control Jinx→WT mice. The HW:BW ratios observed in Jinx→WT mice treated with platelets were similar to those observed in WT→WT mice after TAC (7.6 ± 2.3 mg/g). The effect was not solely dependent on intact platelets because cardiac hypertrophy following TAC was slightly although not significantly increased by administering platelet releasate to Jinx→WT chimeras. HW:BW ratio was 7.4 ± 0.4 mg/g in Jinx→WT mice that received releasate from thrombin-stimulated platelets and 6.8 ± 0.8 mg/g in animals that received buffer control (containing thrombin and hirudin). Cardiomyocyte size was consistently and significantly increased in Jinx→WT mice with WT platelet releasate treatment (Fig. 7). These results support a role for Munc13-4-mediated platelet secretion in pressure-induced cardiac hypertrophy.

TGF-β1, which is secreted by a variety of cells, has been implicated in the development of cardiac hypertrophy and

Table 3. Complete blood counts at 5 wk after surgery

<table>
<thead>
<tr>
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<th>WT→WT</th>
<th>Jinx→WT</th>
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<tbody>
<tr>
<td></td>
<td>Sham (n = 3)</td>
<td>TAC (n = 3)</td>
</tr>
<tr>
<td>WBC, K/µl</td>
<td>14.2 ± 2.8</td>
<td>7.9 ± 2.6</td>
</tr>
<tr>
<td>HCT, %</td>
<td>40.7 ± 1.6</td>
<td>41.6 ± 1.2</td>
</tr>
<tr>
<td>PLT, K/µl</td>
<td>792 ± 73</td>
<td>846 ± 121</td>
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</table>

Statistical significance was determined by two-way ANOVA.

Table 4. WT mice with Jinx marrow are partially protected from LVH at 5 wk after TAC

<table>
<thead>
<tr>
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<th>WT→WT</th>
<th>Jinx→WT</th>
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<tr>
<td></td>
<td>Sham (n = 3)</td>
<td>TAC (n = 3)</td>
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<tr>
<td>BW, g</td>
<td>28.3 ± 1.2</td>
<td>28.6 ± 2.7</td>
</tr>
<tr>
<td>HW/BW, mg/g</td>
<td>5.3 ± 0.6</td>
<td>7.6 ± 2.3</td>
</tr>
<tr>
<td>LVIDss, mm</td>
<td>2.6 ± 0.2</td>
<td>2.0 ± 0.7</td>
</tr>
<tr>
<td>LVPWs, mm</td>
<td>1.2 ± 0.1</td>
<td>1.7 ± 0.3*</td>
</tr>
<tr>
<td>LVIDd, mm</td>
<td>3.9 ± 0.2</td>
<td>3.5 ± 0.6</td>
</tr>
<tr>
<td>LVPWd, mm</td>
<td>0.8 ± 0.1</td>
<td>1.3 ± 0.2*</td>
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<tr>
<td>EF, %</td>
<td>61 ± 4</td>
<td>74 ± 13</td>
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<td>FS, %</td>
<td>33 ± 3</td>
<td>43 ± 11</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>470 ± 18</td>
<td>470 ± 12</td>
</tr>
</tbody>
</table>

BW:BW, heart weight to body weight ratio; LVH, left ventricular hypertrophy. For HW:BW measurement, sham mice n = 3, TAC mice n = 9. For echocardiography, sham mice n = 3, TAC mice n = 5. *P < 0.05 compared with WT→WT TAC; †P < 0.05 compared with WT→WT sham by two-way ANOVA. Data are presented as mean ± SD.
fibrosis. Platelets store TGF-β1 in α-granules and therefore serve as a major source for TGF-β1. Platelet-derived TGF-β1 has been implicated in LVH (23). We established a role for Munc13-4 in the release of TGF-β1 from platelets by measuring its release from isolated WT and Jinx platelets following thrombin stimulation. Thrombin, but not PGE1, triggered release of TGF-β1 from WT platelets (Fig. 8A). TGF-β1 in releasate from thrombin-stimulated Jinx platelets was fourfold lower than that released from WT platelets and was no different than levels observed after PGE1 treatment (Fig. 8A). No differences in plasma or serum levels of TGF-β1 were noted in control, unoperated WT, or Jinx mice (Fig. 8B). Interestingly, 7 days after TAC, plasma TGF-β1 levels in WT→WT mice were higher (1.73 ± 0.38 ng/ml) than in sham-operated or in Jinx→WT mice (1.26 ± 0.15 ng/ml, Fig. 8C); no difference between WT→WT sham-operated (1.51 ± 0.24 ng/ml) and Jinx→WT sham-operated mice (1.27 ± 0.42 ng/ml) was observed (Fig. 8C). S1P, a known regulator of hypertrophic responses in cardiomyocytes, is also released by platelets. However, no difference in plasma S1P level was observed between Jinx→WT and WT→WT TAC mice (0.88 ± 0.2 vs. 0.79 ± 0.15 pmol/μl).

**DISCUSSION**

We report that mice lacking the Rab27 effector protein Munc13-4 in bone marrow cells (Jinx→WT) were partially protected from the development of LVH at 5 wk after pressure overload due to smaller cardiomyocyte size. Our results imply that there is a role for granule secretion in propagating the hypertrophic response that is initiated by pressure overload. It is surprising that the initial response to pressure overload at 7
days was not detectably different in mice with Jinx bone marrow cells and those with normal bone marrow (WT
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WT); both developed cardiac hypertrophy, inflammatory responses, and an upregulation of cardiac fetal gene expression in response to the mechanical stress of pressure. Given the known role of Munc13-4 in secretory vesicle priming and fusion in lymphocytes, macrophages, platelets, and neutrophils (5, 7, 11, 17, 25–27), Munc13-4 therefore does not appear to be required for the bulk of inflammatory cell recruitment. It is possible that we failed to detect small differences in cell numbers, changes in subpopulations of inflammatory cells, or secreted mediators. Alternate pathways may promote cell recruitment. For example, in the absence of Munc13-4, the defects in platelet granule secretion can be partially rescued by high agonist concentration, indicating the presence of pathways not reliant on Munc13-4 for platelet secretion (27).

Regulators of cardiac hypertrophy. Granules in hematopoietic cells contain a variety of cytokines with many functions

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**Fig. 6. Cardiac fibrosis 5 wk after TAC. A:** representative images of Masson’s trichrome (MT) staining (×20, bar = 145 μm); **B:** quantification of perivascular fibrosis in mice 5 wk after surgery. WT→WT sham-operated mice n = 3/group, WT→WT TAC n = 9/group, Jinx→WT sham-operated mice n = 3/group, Jinx→WT TAC n = 9/group. Data are presented as means (bar) ± SE (error bar). **C:** real-time quantitative PCR of MMP9 (matrix metallopeptidase 9) and Col Ia (collagen Iα). RQ, relative quantification. Values are reported normalized to those observed in WT→WT sham-operated mice (set at 1); n = 9 mice/group for TAC, n = 3 mice/group for sham-operated. No significant difference was observed by two-way ANOVA.

**Fig. 7. WT platelet releasate partially restored cardiomyocyte size in Jinx→WT mice 5 wk after TAC. A:** no difference was detected between buffer and WT platelet-injected groups. **B:** WT platelet releasate significantly increased cardiomyocyte size after TAC compared with the control. *P < 0.05 by Student’s t-test.
More recently, Wang et al. (34) showed that derived TGF-β/71 platelet releasates are consistent with a contribution of platelet-phenotype observed in chimeric Jinx mice and the fact that the \( \text{TGF-β/H9252} \) to the activation of TGF-β/71 released from platelets. In vitro (23), the high shear produced by TAC may contribute regulators remains to be determined. Cardiomyocytes inhibits the expression of cardiac endogenous regulator. Whether interaction between inflammatory cells and \( \text{TGF-β/H9251} \) has been shown that Trx1 is upregulated in pressure overload groups, such as thioredoxin (Trx1), have also been reported. It has been shown that Trx1 is upregulated in pressure overload (36). It is possible that a component of hematopoietic cell releasate influences cardiomyocyte expression of a group II regulator. Whether interaction between inflammatory cells and cardiomyocytes inhibits the expression of cardiac endogenous regulators remains to be determined.

Platelet-derived mediators of cardiac hypertrophy. Platelets, and to a lesser extent platelet releasate, were able to promote cardiac hypertrophy in mice lacking the Rab27 effector protein Munc13-4 in their bone marrow cells. This finding indicates that factors present in platelet granules and secreted in a Munc13-4-dependent manner may influence cardiomyocyte hypertrophy. The loss of Munc13-4 results in defects in platelet α-granules, dense granules, and lysosomes. Possible candidate mediators that are released from platelets and may promote cardiac hypertrophy include TGF-β1 (23), S1P (4, 28), and serotonin (24). In fact, a recent study demonstrated that platelets contribute to plasma TGF-β1 levels and that mice lacking TGF-β1 in their megakaryocytes/platelets are partially protected from developing cardiac hypertrophy after TAC surgery (23). Because shear force has been shown to activate TGF-β1 in vitro (23), the high shear produced by TAC may contribute to the activation of TGF-β1 released from platelets. The phenotype observed in chimeric Jinx mice and the fact that the phenotype is partially reversed by administration of platelets or platelet releasates is consistent with a contribution of platelet-derived TGF-β1. More recently, Wang et al. (34) showed that in both a surgically induced high shear ascending aortic constriction model and a hyperlipidemia-induced spontaneous aortic stenosis model that shear force correlated with TGF-β1 release and activation. In our studies we demonstrated that Munc13-4 is important for thrombin-mediated release of TGF-β1 from platelets and for the increase in plasma TGF-β1 that occurs after TAC. Although previous reports indicate that platelet TGF-β1 release correlates with cardiac fibrosis in the setting of LVH (23) and aortic valve stenosis in Reversa mice (34), we did not detect an effect of Munc13-4 on markers of fibrosis. Instead, Munc13-4 was required for cardiomyocyte hypertrophy. Thus it is possible that additional factors, released in a Munc13-4-dependent manner, also contribute to cardiac remodeling. Finally, although our studies implicate platelets as regulators of cardiac hypertrophy, the phenotype observed in mice lacking Munc13-4 could be due to a lack of cargo release from other hematopoietic cells, which influence cardiomyocytes.

In summary, factors secreted from marrow-derived cells in a Munc13-4-dependent manner appear to be important for maintaining the hypertrophic response of cardiomyocytes to pressure overload. Platelet cargo release accounts for at least some of the effect. Thus inhibition of platelet granule secretion might represent an effective way to reduce cardiac hypertrophy.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

S.S.S. prepared figures; F.Y. and S.S.S. drafted manuscript; F.Y., J.A., and S.S.S. edited and revised manuscript; S.S.S. approved final version of manuscript.

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