BMP Antagonist Gremlin 2 Limits Inflammation After Myocardial Infarction

Lehanna N. Sanders, John A. Schoenhard, Mohamed A. Saleh, Amrita Mukherjee, Sergey Ryzhov, William G. McMaster Jr, Kristof Nolan, Richard J. Guminia, Thomas B. Thompson, Mark A. Magnuson, David G. Harrison, Antonis K. Hatzopoulos

Rationale: We have recently shown that the bone morphogenetic protein (BMP) antagonist Gremlin 2 (Grem2) is required for early cardiac development and cardiomyocyte differentiation. Our initial studies discovered that Grem2 is strongly induced in the adult heart after experimental myocardial infarction (MI). However, the function of Grem2 and BMP-signaling inhibitors after cardiac injury is currently unknown.

Objective: To investigate the role of Grem2 during cardiac repair and assess its potential to improve ventricular function after injury.

Methods and Results: Our data show that Grem2 is transiently induced after MI in peri-infarct area cardiomyocytes during the inflammatory phase of cardiac tissue repair. By engineering loss- (Grem2−/−) and gain- (TGgrem2) of-Grem2-function mice, we discovered that Grem2 controls the magnitude of the inflammatory response and limits infiltration of inflammatory cells in peri-infarct ventricular tissue, improving cardiac function. Excessive inflammation in Grem2−/− mice after MI was because of overactivation of canonical BMP signaling, as proven by the rescue of the inflammatory phenotype through administration of the canonical BMP inhibitor, DMH1. Furthermore, intraperitoneal administration of Grem2 protein in wild-type mice was sufficient to reduce inflammation after MI. Cellular analyses showed that BMP2 acts with TNFα to induce expression of proinflammatory proteins in endothelial cells and promote adhesion of leukocytes, whereas Grem2 specifically inhibits the BMP2 effect.

Conclusions: Our results indicate that Grem2 provides a molecular barrier that controls the magnitude and extent of inflammatory cell infiltration by suppressing canonical BMP signaling, thereby providing a novel mechanism for limiting the adverse effects of excessive inflammation after MI. (Circ Res. 2016;119:434-449. DOI: 10.1161/CIRCRESAHA.116.308700.)

Key Words: bone morphogenetic protein 2 ■ endothelial cells ■ Grem2 protein, mouse ■ inflammation ■ myocardial infarction

Coronary heart disease resulting in myocardial infarction (MI) is the major cause of death in men and women. Each year about 735,000 people in the United States have an MI and most suffer irreversible tissue damage, leading to ventricular remodeling, hypertrophy, dilatation, and eventually heart failure. After MI, the adult heart undergoes a sequence of molecular and cellular events that delineate the different stages of tissue repair. Initially, cardiomyocytes within infarcted myocardium begin to die within minutes after coronary artery occlusion. Toxic products and signals released from dying cells induce endothelial cell adhesion proteins, as well as cytokines and chemokines, to recruit inflammatory cells that remove tissue debris, and activate proteases to degrade extracellular matrix. After debris is cleared, usually within 2 to 3 days after MI, the gap is filled with granulation tissue that is composed of proliferating cells, mainly endothelial cells that form new capillaries, and myofibroblasts that secrete collagen and other matrix proteins. Two to 3 weeks after the original

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Correspondence to Antonis K. Hatzopoulos, PhD, Division of Cardiovascular Medicine, Department of Medicine, Vanderbilt University Medical Center, Nashville, TN 37232. E-mail antonis.hatzopoulos@vanderbilt.edu

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434
MI, the infarct begins to mature into a dense scar. We have recently demonstrated that canonical Wnt signaling activation after MI attenuates fibrosis and promotes arteriole formation and cardiogenesis, suggesting that developmental pathways, critical for embryonic cardiac development, are reactivated after injury in the adult heart to regulate tissue repair.

Bone morphogenetic proteins (BMPs) play important roles during various stages of cardiac development, including early cardiogenic differentiation of mesoderm, cardiac tube assembly, looping and jogging, cardiac chamber identity, cardiomyocyte differentiation, and cardiac cushion formation. BMP signaling is also induced after ischemic injury in the adult mouse heart and implicated in cardiomyocyte apoptosis during ischemia/reperfusion. Histological analyses showed strong induction of Bmp2 in peri-infarct cardiomyocytes; however, the exact role of BMP signaling in cardiac tissue repair, or how BMP signaling is regulated after cardiac injury, is not well understood.

BMPs belong to the transforming growth factor β (TGFβ) superfamily of secreted ligands and bind to type I and type II receptors as dimers, leading to phosphorylation and activation of the type I receptor by its type II partner. Activated type I receptors then phosphorylate Smad1/5/8 intracellular proteins, which complex with Smad4 and translocate to the nucleus, where they regulate expression of target genes such as the Id family of transcriptional repressors. BMP signaling is modulated in the extracellular space by a large number of secreted, structurally diverse antagonists, such as Chordin, Noggin and members of the DAN family, that bind to BMP ligands and thereby hinder binding to the corresponding receptors.

Gremlin 2 (Grem2), also called protein related to Dan and Cerberus, belongs to the DAN family of BMP antagonists together with its close paralog Gremlin 1, Dan, Dante (or Coco), Cerberus-like 1, Uterine sensitization-associated gene-1, and Sclerostin. Gremlin 2 was first discovered 15 years ago, but its biological function and mechanism of BMP inhibition have remained largely obscure. Gremlin 2 expression has been detected in the developing spinal cord and lung mesenchyme, and Gremlin 2 has been implicated in follicle, neuronal, and bone development. Gremlin 2 in vitro inhibits Bmp2 and Bmp4 but not Tgfβ1 or activin. Although several DAN-family members, such as Dante and Gremlin 1, have been linked to pulmonary arterial hypertension, chronic kidney disease, and cancer, little is known about the role of Gremlin 2 in disease.

We recently established that during embryonic development in zebrafish, grem2 first appears in the pharyngeal mesoderm next to the forming heart tube. Loss- and gain-of-function approaches demonstrated that Gremlin 2 is necessary for cardiac tube jogging and looping, cardiac laterality, and cardiomyocyte differentiation by suppression of Smad1/5/8 phosphorylation. Moreover, we found that Gremlin 2 promotes differentiation of pluripotent mouse embryonic stem cells to atrial-like cardiomyocytes. Here, we show that Gremlin 2 is not essential for mouse embryonic development. In the adult heart, we discovered that Gremlin 2 is highly induced in peri-infarct cardiomyocytes at the end of the inflammatory phase after MI. Using genetic gain- and loss-of-Gremlin-2-function models and chemical compounds that inhibit BMPs, we present evidence that Gremlin 2 is necessary and sufficient to modulate the inflammatory response and keep inflammation in check through suppression of canonical BMP signaling.

**Methods**

A complete Methods section is available in the Online Data Supplement.

**Results**

**Gremlin 2 Is Transiently Induced After MI After the Initial Inflammatory Response**

To place BMP-signaling components within the context of the MI repair process, we analyzed whole mouse heart RNA samples prepared at distinct time points after left anterior descending artery ligation, namely at days 0 (baseline, prior to injury), 1, 2, 3, 5, 7, and 21 after MI. Using typical inflammatory gene markers, such as Il-1β and E-selectin, and markers of granulation tissue formation and fibrosis, such as Tgfb1 and α-Smooth muscle actin (αSma), we determined that proinflammatory genes are induced early and peak at days 1 to 2 after MI, whereas fibrosis genes are induced at day 5, as expected. Gene induction of inflammatory genes returned to baseline levels between days 3 and 5, whereas Tgfb1 expression returned to baseline at day 21. αSma levels declined but were still detectable at day 21, reflecting the presence of myofibroblasts during the scar maturation phase (Figure 1A).

Investigation of BMP ligands after MI showed that Bmp2 is the earliest induced ligand of the BMP family at day 1 with its expression peaking at day 3, a pattern that corresponds to the inflammatory phase of cardiac repair. Previous work documented that Bmp2 protein induction takes place primarily in peri-infarct area cardiomyocytes and not in recruited immune cells. Bmp2 is then downregulated to preinjury levels by day 7 after MI. Bmp2 suppression coincides with upregulation of Bmp4, Bmp6, and Bmp10, the expression of which starts around day 5, and persists during fibrosis and scar formation (Figure 1B). Consistent with the induction of distinct BMP ligands during different stages after MI, there was an overlapping expression of the BMP-signaling target gene Id2 throughout the repair process (Online Figure IA). In contrast, analysis of BMP-signaling antagonists showed minimal changes in their expression levels after MI with the notable exception of Gremlin 2 that is induced around day 2, peaks at day 5 after MI, and returns to preinjury levels at day 21 (Figure 1C). The Gremlin 2 induction pattern follows the pattern of Bmp2 with 1-day delay. We did not detect a
signal for its close paralog Grem1 at baseline or at the tested time points after MI (Online Figure IB). Although Grem2 is the most prominently induced BMP antagonist after MI, absolute expression values indicate that during homeostasis, the heart maintains expression of Dan, Sost, Twsg1, and Chordin, which, however, are at least 40 (Chordin) to 400 (Dan and Twsg1) times less potent BMP inhibitors than Grem2 or do not bind directly to BMP ligands (Sost) (Online Figure IB).36,37

Consistent with the Bmp2 induction pattern after MI, immunofluorescence analysis at day 2 post MI, using antibodies recognizing the phosphorylated, that is, activated form of Smad1/5/8, demonstrated the activation of canonical BMP signaling in endothelial cells in the peri-infarct area and cardiomyocytes at the border zone of the infarcted tissue. In contrast, we did not detect active Smad1/5/8 within ventricular tissue prior to injury (Figure 1D).

In conclusion, our data indicate a biphasic pattern of BMP ligand induction after MI, that is, early upregulation of Bmp2 expression during the inflammatory phase, followed by a second phase during granulation tissue and scar formation that is dominated by several ligands, including Bmp4, Bmp6, and Bmp10. Conversely, Grem2 is the prominent BMP antagonist induced after MI with its expression starting at day 2, peaking during the transition from inflammation to granulation tissue formation, and returning to baseline levels during scar formation. Induction of Bmp2 coincided with activation of canonical/p-Smad BMP signaling in endothelial cells and cardiomyocytes in the peri-infarct area.
Loss of Grem2 Leads to an Increase of Endothelial Proinflammatory Markers After MI

To determine whether Grem2 has a role in cardiac repair, we generated a loss of function (Grem2−/−) mouse model by deleting most of exon 2 via homologous recombination (Figure 2A; Online Figure II; Methods section in Online Data Supplement). This approach deleted the entire coding sequence and most of the 3′ untranslated region of the Grem2 gene, leading to complete loss of Grem2 protein. Grem2−/− mice are viable without major structural or physiological defects or apparent differences in heart size, cardiac tissue morphology, and cardiac function when compared with wild-type (WT) siblings, with the exception of a small increase in heart rate (Online Table I; Online Figure II). Thus, although the Grem2 expression pattern has been conserved in zebrafish and mouse embryos, where Grem2 is first expressed in the area of the secondary heart field,34,35 Grem2 seems to be dispensable for mouse development.

Antibody staining revealed that the robust induction of Grem2 protein after MI takes place primarily in peri-infarct cardiomyocytes (Figure 2B). There was no detected Grem2 in distal areas away from the infarct (data not shown). There was also a complete absence of Grem2 protein in Grem2−/− mice, further corroborating their null phenotype (Figure 2B).

To test whether the Grem2 induction after the upregulation of Bmp2 expression during the inflammatory phase plays a role in inflammation after acute injury, we challenged Grem2−/− and WT sibling mice with experimental MI by permanent ligation of the left anterior descending coronary artery. We then isolated whole-heart RNA at days 0, 2, 7, and 21 after MI and analyzed the expression of proinflammatory genes. Our data show that induction of genes encoding endothelial cell membrane proteins implicated in the rolling and adhesion of circulating immune cells to the vascular wall are higher in Grem2−/− hearts compared with WT mice. Specifically, RNA analysis showed that expression of E-selectin, Vcam1, and Icam1 is further upregulated compared with WT controls at days 2 and 7 after MI; however, their expression decreases at day 21 to levels comparable with WT hearts (Figure 2C).

Consistent with the gene induction results, immunofluorescence analysis of cardiac tissue sections at day 7 after MI revealed that endothelial cells within the infarct and peri-infarct areas stain positively for E-selectin protein in Grem2−/− mice, whereas, at this stage, E-selectin is almost undetectable in WT controls (Figure 2D). Comparison of chemokine expression, such as Ccl2, Il-8, and Il-1β, showed that although chemokines are induced after MI as expected, relative expression levels were comparable between Grem2−/− and WT mice, except a further 1.7-fold increase of Ccl2 expression in Grem2−/− mice compared with WT (Online Figure IIIA). Together, our data show that Grem2 protein induction takes place in the peri-infarct zone and lack of Grem2 enhances the expression of proinflammatory genes in endothelial cells in and around the injury site after MI.

Loss of Grem2 Leads to an Increase in the Magnitude of Inflammation

To test whether increased expression of proinflammatory makers after MI augments infiltration of immune cells, we isolated hearts at day 5 after MI and analyzed histological sections with an antibody recognizing the leukocyte marker, CD45. The results showed that infiltration of CD45+ cells after MI appeared more abundant in Grem2−/− hearts compared with WT (Figure 3A). To quantify the increase in inflammatory infiltrate and better characterize the corresponding cells, we prepared single-cell suspensions of noncardiomyocyte cells and conducted flow cytometry using antibodies recognizing various immune cell markers, such as CD45, F4/80, Ly6C, Ccr2, Ly6G, and CD3.38-40 As shown in Figure 3B, there was a 3-fold increase in infiltrating inflammatory cells, identified as CD45+ cells, in Grem2−/− hearts after MI when compared with WTs. Within the CD45+ population, there was a 2–3-fold increase in Ly6C+ cells that represent mostly monocytes (but may also include neutrophils and T cells that express intermediate levels of Ly6C), neutrophils (Ly6G+), T cells (CD3+), and macrophages (F4/80+). There was a similar increase in Grem2−/− hearts compared with WT mice of proinflammatory F4/80+ macrophages expressing high levels of Ly6C (F4/80+/Ly6C+), the monocyte chemoattractant protein-1 (or Ccl2) receptor Ccr2 (F4/80+/Ccr2+), or both (F4/80+/Ly6C+/Ccr2+). The gating strategy and representative flow cytometry plots are shown in the Online Figure IV.

Analysis of the initial necrosis area using triphenyl tetrazolium chloride staining 1 day after MI showed that infarct sizes are comparable between WT and Grem2−/− hearts, suggesting that the observed differences in the inflammatory response are not because of more severe infarcts in Grem2−/− mice (Online Figure V). Furthermore, flow cytometry analysis on blood CD45+ cells isolated from Grem2−/− and WT mice at baseline and 5 days after MI revealed that leukocyte numbers in the blood were not significantly different at baseline. After MI, the number of circulating leukocytes increased as expected,41 although numbers were 2× higher in Grem2−/− mice, likely because circulating leukocyte numbers correlate with the magnitude of the inflammatory response (Online Figure VI).42

Finally, we investigated whether the loss of Grem2, besides increased inflammatory cell infiltration, also leads to prolonged inflammation. To this end, we quantified inflammatory cells by flow cytometry at day 14 after MI, a time point when inflammatory cell numbers return close to baseline levels in WT mice. Our results showed a dramatic drop in inflammatory cells in Grem2−/− mice to levels comparable with WT controls (Figure 3C), consistent with the eventual downregulation of cell adhesion molecules in Grem2−/− hearts after MI (Figure 2B). Furthermore, molecular analysis indicated an increase in the induction levels of genes encoding proteins involved in the resolution of inflammation, such as Tgfβ1 and Il-10 in Grem2−/− mice compared with WT animals, which may account for the clearing of excessive inflammatory cells in Grem2−/− cardiac tissue (Figure 3D). Taken together, our results indicate that Grem2 is necessary to regulate the magnitude but not the duration of the inflammatory cell infiltration after MI.

Grem2 Overexpression Attenuates the Inflammatory Response After MI

To explore the possibility that Grem2 controls the extent of inflammation after MI, we generated a transgenic mouse...
line where Grem2 is postnatally overexpressed in adult cardiomyocytes under the control of regulatory elements from the α-Myosin heavy chain (Myh 6) promoter that are active in the adult heart (TG\textsuperscript{Grem2}; Figure 4A; Methods section in Online Data Supplement). TG\textsuperscript{Grem2} mice do not exhibit differences in cardiac morphology and function when compared with WT counterparts (Online Table II; Online Figure VII).

Figure 2. Loss of Gremlin 2 (Grem2) increases expression of genes encoding endothelial cell membrane proteins that mediate inflammatory cell recruitment after myocardial infarction (MI). A, Schematic diagram of the Grem2 gene knockout strategy before (top) and after homologous recombination (bottom). The entire coding region of Grem2 in exon 2 was replaced by the pu(Δ)TK (fusion of puromycin and truncated thymidine kinase genes)/EM7neo (kanamycin resistance gene under the synthetic bacterial EM7 promoter) selection cassette as described in the Methods section in Online Data Supplement. The location of fragments A to D used to modify the corresponding BAC (bacterial artificial chromosome) clone, the extent of homology arms, and the position of DNA probes used for screening putative Grem2\textsuperscript{−/−} embryonic stem cell clones is indicated. B, Immunofluorescence analysis with antibodies recognizing Grem2 (green) and CD31 (red) shows that Grem2 protein is induced in the peri-infarct area. Grem2 expression appears primarily in cardiomyocytes but not in CD31+ endothelial cells. Grem2 protein expression is missing in Grem2\textsuperscript{−/−} mice. DAPI marks cellular nuclei. Scale bar, 100 μm. C, Quantitative polymerase chain reaction analysis of whole-heart RNA samples isolated from wild-type (WT) and Grem2\textsuperscript{−/−} mice at days 0, 2, 7, and 21 post MI. Induction levels of genes encoding endothelial cell–specific adhesion membrane proteins E-selectin, Vascular cell adhesion molecule-1 (Vcam1), and Intercellular cell adhesion molecule-1 (Icam1) are significantly higher in Grem2\textsuperscript{−/−} hearts compared with WTs. *P<0.05 and ****P<0.0001. Two-way ANOVA with Bonferroni multiple comparisons test. n=3 for all time points. All data are presented as means±SEM. D, Immunofluorescence analysis of cardiac tissue sections at day 7 post MI shows that E-selectin protein expression (red) persists in endothelial cells (Tie1, green) in the infarct (INF) and peri-infarct border zone (BZ) areas in Grem2\textsuperscript{−/−} hearts compared with WTs. DAPI marks cellular nuclei. Scale bar, 100 μm.
TGGrem2 and WT siblings underwent permanent left anterior descending coronary artery ligation, and whole-heart RNA was isolated at days 0, 2, and 7 after MI. Quantitative polymerase chain reaction analysis showed that gain-of-Grem2-function reduced the induction levels of inflammatory gene markers, such as E-selectin and Vcam1, after MI.
with no major changes in the induction of proinflammatory cytokines (Figure 4B; Figure IIIB). Flow cytometry of cardiac cells 5 days after MI, excluding cardiomyocytes, confirmed that reduced expression of proinflammatory markers leads to a significant decrease in the number of CD45+, Ly6C+ (intermediate and high expression levels), and F4/80+ cells within cardiac tissue (Figure 4C). Attenuation of inflammation correlates with reduced induction of genes encoding the anti-inflammatory cytokines Il-10 and Tgfβ1 (Figure 4D). These data indicate that Grem2 overexpression reduces the magnitude of the inflammatory response after MI.

Grem2 Promotes Functional Recovery After MI

The phenotypic analysis of Grem2−/− and TGGrem2 mice indicates that Grem2 levels are inversely related to the magnitude of inflammation after MI. Excessive inflammation has been linked to poor prognosis after MI both in animal models and human patients. To determine the effects of Grem2 gain- and loss-of-function on cardiac recovery, we compared cardiac functional parameters among WT controls and TGGrem2 and Grem2−/− mice by M-mode echocardiography at various time points after MI (Figure 5; Online Figure VIII).

Our data show that TGGrem2 and Grem2−/− mice have comparable ventricular dimensions and functional values to the corresponding WT control mice at baseline. After left anterior descending coronary artery ligation, TGGrem2 mice have better preserved cardiac function compared with WT littermate controls of C57BL/6 background, as evidenced by higher fractional shortening and ejection fraction values 21 days after MI.

Figure 4. Gremlin 2 (Grem2) overexpression attenuates inflammation after myocardial infarction (MI). A, Schematic diagram of the DNA construct used to generate the TGGrem2 transgenic mice. The Grem2 cDNA coding part was cloned behind a fragment of the αMHC (Myh6) gene promoter that specifically directs expression in adult cardiomyocytes. The construct includes the polyadenylation sequences of the human growth hormone gene (hGH PA). B, Quantitative polymerase chain reaction analysis of whole-heart RNA samples isolated from wild-type (WT) and TGGrem2 mice at days 0, 2, and 7 post MI. Induction of endothelial cell–specific membrane proteins E-selectin and Vcam1 is significantly attenuated in TGGrem2 hearts compared with WT. ***P<0.001 and ****P<0.0001 (2-way ANOVA with Bonferroni multiple comparisons test). n=3 per group for all time points. All data are presented as mean±SEM. C, Flow cytometry analysis of single-cell suspensions of noncardiomyocyte cells isolated from whole hearts 5 days post MI shows decreased number of CD45+, Ly6C+, and F4/80+ cells in TGGrem2 hearts compared with WT. *P<0.05 (Student 2-tailed unpaired t test). WT, n=6; TGGrem2, n=8. Bars represent mean±SEM. D, Quantitative polymerase chain reaction analysis of whole-heart RNA samples isolated from WT and TGGrem2 mice at days 0, 2, and 7 post MI shows lower fold induction of Tgfβ1 and Il-10 in TGGrem2 hearts compared with WT. **P<0.01 and ***P<0.001 (2-way ANOVA with Bonferroni multiple comparisons test). n=3 per group for all time points. All data are presented as mean±SEM.
Figure 5. Gremlin 2 (Grem2) improves cardiac function after myocardial infarction (MI). Wild-type (WT) and TGGrem2 mice (both in C57BL/6 background), as well as WTmix and Grem2−/− mice (both in mixed C57BL/6 and 129/Sv background) underwent permanent ligation of the left anterior descending artery, and cardiac dimensions were determined by echocardiography at several time points post MI. A. Fractional shortening (FS) and ejection fraction (EF) measurements at days 0 (baseline), 7, and 21 post MI indicate cardiac function is improved in TGGrem2 mice compared with their WT siblings, whereas Grem2−/− mice exhibit worse cardiac function compared with WTmix beginning at day 7 post MI. B. Improvement of cardiac function in TGGrem2 mice is because of preservation of both diastolic and systolic left ventricular internal diameter (LVIDd and LVIDs) 21 days post MI. Conversely, worsened function in Grem2−/− mice is mainly because of higher LVIDs parameters when compared with WTmix controls. *P<0.05 and **P<0.01 (Student 2-tailed unpaired t test). WT, n=6; TGGrem2, n=10; WTmix, n=9; and Grem2−/−, n=8. Bars represent mean±SEM. ns indicates not significant.
(Figure 5A). In contrast, Grem2<sup>−/−</sup> mice have worse cardiac function compared with their corresponding WT siblings of mixed C57BL/6 and 129/Sv background (WT<sup>+/−</sup>).

Functional recovery in TG<sup>Grem2</sup> mice was because of preservation of both systolic and diastolic dimensions with lower overall values compared with WT control mice at day 21 after MI (Figure 5B). Conversely, only systolic diameters are higher in Grem2<sup>−/−</sup> mice compared with WT<sup>+/−</sup> controls. These results are consistent with the Grem2<sup>−/−</sup> mice defects because both the magnitude and spread of inflammation have been linked to systolic dysfunction (Figure 5B).<sup>4</sup> In brief, echocardiography data indicate that Grem2 levels directly correlate with functional recovery after acute MI.

**Grem2 Regulates Canonical BMP Signaling in Cardiomyocytes Next to Ischemic Areas**

Grem2 is known to inhibit the canonical BMP-signaling pathway by preventing BMP ligand-mediated phosphorylation of Smad1/5/8. <sup>35</sup> To test whether Grem2 regulates canonical BMP signaling in the heart, we analyzed cardiac tissue sections from WT, Grem2<sup>−/−</sup>, and TG<sup>Grem2</sup> mice at day 7 after MI, shorty after the peak of Grem2 expression. Immunofluorescence staining with antibodies recognizing the phosphorylated, that is, the active form of Smad1/5/8, showed that canonical BMP signaling is active in peri-infarct cardiomyocytes of WT mice, in agreement with previous reports showing that BMP ligands are expressed in this region (Figure 6A).<sup>37</sup> The intensity of p-Smad1/5/8 was increased in Grem2<sup>−/−</sup> mice and decreased in TG<sup>Grem2</sup> hearts when compared with WT mice (Figure 6A and 6B). However, unlike the early stages after MI (Figure 1), we did not detect p-Smad1/5/8 in endothelial cells.

The p-Smad changes overlap with the Grem2 expression domain (Figure 2B) in peri-infarct area cardiomyocytes, suggesting that Grem2 acts as a barrier to limit the infiltration of inflammatory cells into neighboring, relatively healthy cardiac tissue. In agreement with this notion, we observed inflammatory cells in the peri-infarct tissue past the infarct border zone in Grem2<sup>−/−</sup> hearts, whereas inflammatory cells were confined within the infarct area in WT controls (Figure 6C).

To test whether the increased inflammatory cell infiltration is because of p-Smad1/5/8 mediated signaling, we injected Grem2<sup>−/−</sup> mice with the canonical BMP-signaling chemical inhibitor DMH1<sup>46</sup> (4-(6-(4-isopropoxyphenyl)pyrazolo[1,5-a]pyrimidin-3-yl)quinolone) and vehicle control (DMSO) on days 2, 3, and 4 after MI, which correspond to the peak days of inflammation. We used DMSH1 because it is highly specific to canonical BMP signaling without known off-target effects, as tested in various mouse disease models.<sup>46-48</sup> We found that DMH1 treatment rescues the pro-inflammatory phenotype in Grem2<sup>−/−</sup> mice. Flow cytometry analysis at day 5 after MI showed that treated Grem2<sup>−/−</sup> hearts have a dramatic decrease in infiltrated CD45<sup>+</sup>, Ly6C<sup>+</sup>, and F4/80<sup>+</sup> cells when compared with vehicle-injected controls (Figure 6D).

Taken together, our data indicate that Grem2 generates a molecular barrier at the border zone between infarcted and unaffected tissue that contains both the number and spatial extent of infiltrating inflammatory cells. In the absence of Grem2, increased p-Smad–mediated BMP signaling is responsible for excessive inflammation because canonical BMP inhibition rescues the loss-of-Grem2-phenotype.

**Grem2 Inhibits the Proinflammatory Effect of Bmp2 on Endothelial Cells**

The data described above suggest that secretion of Grem2 protein by peri-infarct cardiomyocytes regulates the extent of inflammation by affecting directly or indirectly the proinflammatory phenotype of cardiac endothelial cells. Further gene expression analysis at various time points after MI showed that Tnfα is induced first, followed by Bmp2 and then Grem2 (Figure 7A). To investigate whether the sequential temporal induction patterns of the 3 genes after MI are linked, we tested the effects of TNFα, BMP2, and Grem2 on the human microvascular endothelial cell line 1 (HMEC-1). We found that TNFα induces expression of E-SELECTIN, as expected; however, it also induces BMP2, suggesting that TNFα contributes to the induction of the Bmp2 gene after MI (Figure 7B). BMP2 in turn induces E-SELECTIN expression in endothelial cells (Figure 7C). BMP2 also stimulates GREM2 expression, suggesting that BMP2 induces a negative regulatory loop to limit its own activity (Figure 7D).

To determine whether (1) BMP2 acts synergistically with TNFα and (2) Grem2 blocks the proinflammatory effect of BMP2 on endothelial cells, we treated human microvascular endothelial cells with TNFα, BMP2, and Grem2 in different combinations (Figure 7E and 7F). When protein factors are added alone, both TNFα and BMP2 induce E-SELECTIN expression. Costimulation with TNFα and BMP2 leads to E-SELECTIN induction levels higher than either factor alone, suggesting that TNFα and BMP2 have a synergistic or additive effect. Grem2 does not affect the TNFα induction of E-SELECTIN but completely inhibits the BMP2 effect. In a similar fashion, coincubation with TNFα, BMP2, and Grem2 reduces E-SELECTIN levels to those induced by TNFα treatment alone (Figure 7E). In accordance with its function as a canonical BMP-signaling antagonist, Grem2 blocks induction of BMP-signaling target ID2, whereas TNFα alone has no effect on ID2 expression, although it reduces the fold induction of ID2 by BMP2 (Online Figure IX). Treating cells with the chemical inhibitor of canonical BMP-signaling DMH1 showed similar effects as Grem2, indicating that BMP2-stimulated induction of E-SELECTIN is because of activation of canonical BMP signaling (Figure 7F).

To test the functional significance of the modulation of proinflammatory gene expression in endothelial cells by Grem2, we performed cell adhesion assays of monocytes to endothelial cells in vitro. These assays showed that preincubation of endothelial cells with BMP2 increases adhesion of monocytes to endothelial cells with BMP2 having a synergistic or additive effect. Grem2 does not affect the TNFα induction of E-SELECTIN but completely inhibits the BMP2 effect. In a similar fashion, co-incubation with TNFα, BMP2, and Grem2 reduces E-SELECTIN levels to those induced by TNFα treatment alone (Figure 7E). In accordance with its function as a canonical BMP-signaling antagonist, Grem2 blocks induction of BMP-signaling target ID2, whereas TNFα alone has no effect on ID2 expression, although it reduces the fold induction of ID2 by BMP2 (Online Figure IX). Treating cells with the chemical inhibitor of canonical BMP-signaling DMH1 showed similar effects as Grem2, indicating that BMP2-stimulated induction of E-SELECTIN is because of activation of canonical BMP signaling (Figure 7F).
These data further support the idea that the primary cellular targets of Grem2 are the endothelial cells. Together, our data suggest a sequential relationship among MI-induced Tnfα, Bmp2, and Grem2, where Tnfα induces Bmp2 and Bmp2 synergizes with Tnfα to further increase the proinflammatory phenotype of endothelial cells. Grem2 induction then inhibits canonical BMP signaling and the positive effect of Bmp2 on inflammatory gene expression.

Systemic Grem2 Administration Attenuates Inflammation After MI

The results obtained in TGGrem2 mice and isolated endothelial cells suggest that Grem2 is sufficient to attenuate cardiac tissue inflammation after ischemic injury. To test whether this activity is confined to a specific time window after MI and does not depend on structural or functional changes caused by permanent Grem2 overexpression in the heart of TGGrem2 mice, we injected WT mice intraperitoneally with Grem2 protein at days 2, 3, and 4 after MI, during the critical time window of the inflammatory phase. We then isolated hearts at day 5 after MI was determined by flow cytometry. DMH1-injected mice have a decreased inflammatory cell infiltration compared with vehicle controls. **P<0.01 (Student 2-tailed unpaired t test). Vehicle, n=5; +DMH1, n=6. Bars represent mean±SEM. BZ indicates border zone; and INF, infarct.

Figure 6. Gremlin 2 (Grem2) regulates canonical/p-Smad1/5/8 BMP signaling in peri-infarct area cardiomyocytes.

A, Immunofluorescence analysis of cardiac tissue sections 7 days post myocardial infarction (MI) using antibodies recognizing p-Smad1/5/8 (green) and myosin heavy chain (MF20, red) shows activation of canonical BMP signaling in cardiomyocytes in the peri-infarct border zone. DAPI marks cellular nuclei. The number of p-Smad1/5/8− cardiomyocytes is increased in Grem2−/− mice and decreased in TGGrem2 mice. Scale bar, 100 μm. B, Quantification of p-Smad1/5/8+ cells in the infarct border zone as percentage of total MF20+ cells per viewing area between wild-type (WT), Grem2−/−, and TGGrem2 mice. **P<0.01 (Student 2-tailed unpaired t test). n=3; all data are presented as mean±SEM. C, Hematoxylin and eosin staining 5 days post MI shows that inflammatory cell infiltration (arrows) beyond the infarct border (dotted line) is greater in the Grem2−/− mice compared with WT counterparts. Scale bar, 10 μm. D, Grem2−/− mice were injected once daily via intraperitoneal administration with either the canonical BMP-signaling inhibitor DMH1 or vehicle (DMSO) 2, 3, and 4 days post MI. The number of total CD45+, Ly6C+, and F4/80+ cells in whole hearts at 5 days post MI was determined by flow cytometry. DMH1-injected mice have a decreased inflammatory cell infiltration compared with vehicle controls. **P<0.01 (Student 2-tailed unpaired t test). Vehicle, n=5; +DMH1, n=6. Bars represent mean±SEM. BZ indicates border zone; and INF, infarct.
In conclusion, our data support the following model of cardiac tissue repair: induction of proinflammatory cytokines such as Tnfα after MI initiates a transient inflammatory response, which is sustained by subsequent induction of Bmp2 by Tnfα. Bmp2 increases the magnitude of inflammation through induction of proinflammatory cell adhesion membrane proteins in endothelial cells. Grem2 is then induced as part of a negative feedback loop to inhibit Bmp2’s proinflammatory activity and act as a barrier of inflammation at the infarct border zone (Figure 8B).

**Discussion**

Here we provide evidence for a new mechanism that regulates the magnitude and extent of the inflammatory response after MI. Specifically, we show that the BMP antagonist Grem2 is robustly and transiently induced after MI during the late

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**Figure 7.** Gremlin 2 (Grem2) inhibits the proinflammatory effect of bone morphogenetic protein-2 (BMP2) on endothelial cells. A, Quantitative polymerase chain reaction (qPCR) analysis of whole-heart RNA samples isolated from wild-type (WT) mice at days 0, 1, 2, 3, 5, and 7 post myocardial infarction (MI) shows sequential induction of Tnfα, Bmp2, and Grem2 during the cardiac repair process. *P<0.05 and **P<0.01 (1-way ANOVA with Dunnett multiple comparisons test). n=3 for all time points. All data are presented as mean±SEM. B, qPCR analysis of RNA samples isolated from human microvascular endothelial cells (HMECs) at 4 (left) and 24 hours (right) after tumor necrotic factor α (TNFα) treatment shows TNFα induces E-SELECTIN and BMP2 expression. *P<0.05 (Student 2-tailed unpaired t test). n=3 per group. All data are presented as mean±SEM. C and D, qPCR analysis shows that BMP2 induces E-SELECTIN and GREM2 in HMECs after 24 hours. *P<0.05 and **P<0.01 (Student 2-tailed unpaired t test). n=3 per group. All data are presented as mean±SEM. E and F, qPCR analysis of RNA sample isolated from HMEC 24 hours after treatment with TNFα, BMP2, Grem2, and DMH1 in different combinations as indicated. E, TNFα and BMP2 together superinduce expression of E-SELECTIN. Grem2 specifically inhibits the BMP2 effect but has no effect on the E-SELECTIN induction by TNFα. F, Canonical BMP-signaling inhibitor DMH1 specifically inhibits the BMP2-induced E-SELECTIN. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 (1-way ANOVA with Dunnett multiple comparison test). n=3 for all treatments. All data are presented as mean±SEM. G, Adhesion of human monocytes (THP-1 cells) to HMECs was measured after HMEC incubation with TNFα, BMP2, or in combination for 24 hours. TNFα and BMP2 induce binding of endothelial cells to monocytes. Grem2 specifically inhibits the BMP2 effect. *P<0.05 and ****P<0.0001 (1-way ANOVA with Bonferroni multiple comparison test). n=36. All data are presented as mean±SEM. ns indicates not significant.
inflammatory phase and the early proliferative phase of granulation tissue formation. Genetic loss and gain of function approaches revealed that Grem2 (1) controls the magnitude of the inflammatory cell infiltration, although not its long-term duration and (2) acts as a molecular barrier to limit infiltration of inflammatory cells in the relatively healthy cardiac tissue adjacent to the infarct border zone. Grem2 administration during the inflammatory phase of cardiac tissue repair after MI decreases the number of inflammatory cells recruited to the infarct site. Our results further demonstrate that the anti-inflammatory effects of Grem2 depend, at least in part, on suppression of canonical BMP signaling through inhibition of Smad1/5/8 phosphorylation within ventricular tissue at the infarct border zone. In agreement with this idea, administration of DMH1, a chemical inhibitor of canonical BMP signaling, rescued the inflammatory phenotype in Grem2−/− mice. The effects of Grem2 in attenuating the inflammatory response correlate with a significant amelioration of ventricular function after MI, as evidenced by improved systolic and diastolic parameters in the TGGrem2 mice that overexpress Grem2 in cardiomyocytes. In contrast, cardiac function after MI further deteriorated over time in Grem2−/− mice when compared with WT controls.

Grem2 seems to be an integral part of an orchestrated sequence of events that regulates the inflammatory response. Our results suggest that this sequence starts with induction of Bmp2 by Tnfα, which is released shortly after cardiac tissue injury. Bmp2 then acts synergistically or additively with Tnfα to further increase and sustain the induction of pro-inflammatory membrane proteins in endothelial cells, as the Tnfα effects decrease. The proinflammatory action of Bmp2 is then blocked by Grem2, which itself is induced by Bmp2,
thus forming a negative regulatory loop. This concept is supported by the sequential induction of Tnfa, Bmp2, and Grem2 after MI, each approximately 24 hours apart. In vitro assays showing that Bmp2 acts on endothelial cells to promote cell adhesion of monocytes, the consistent changes in the number of inflammatory cells across a wide spectrum of various immune cell types, and histological analyses showing minimal changes in BMP signaling in infiltrating leukocytes all suggest that the main cellular targets of Grem2 in the regulation of the inflammatory response in the heart are the endothelial cells. However, at present, we cannot exclude that Grem2 may also affect inflammatory cell differentiation, activation, or mobilization before their recruitment in the infarct area.

Previous studies have linked BMP ligands to stimulation of the proinflammatory phenotype of endothelial cells and adhesion of leukocytes in response to shear stress in vitro. Likewise, BMP signaling has been associated with promotion of inflammation in models of atherosclerosis and with anemia caused by chronic inflammatory conditions. Conversely, BMP antagonists, such as BMPER (BMP-binding endothelial regulator) and Noggin, can inhibit inflammation by reducing the expression of proinflammatory cell adhesion molecules, and administration of chemical inhibitors of BMP signaling, such as LDN-193189 and dorsomorphin, reduced vascular inflammation and atherosclerosis. Our data indicate that BMP signaling also plays an important role in the cardiac repair process after MI. Although it was known that BMP ligands might cause cardiomyocyte apoptosis, to our knowledge, ours is the first study to show that canonical BMP-signaling activation after MI controls the magnitude of the inflammatory response. Importantly, this is the first indication that induction of the BMP antagonist Grem2 in the heart after MI may be a critical mechanism in the suppression of the vascular proinflammatory phenotype, as a way to reduce and eventually stop recruitment of circulating leukocytes.

Grem2 protein is synthesized primarily in peri-infarct cardiomyocytes, a domain that overlaps both with the expression of BMP2 after MI and the area of p-Smad1/5/8, that is, canonical BMP-signaling activation. Histological analysis in Grem2−/− mice suggests that Grem2 inhibits p-Smad activation. Some p-Smad1/5/8 persist in cardiomyocytes, even with Grem2 induction, which may be because of the presence of ligands, such as GDFs (growth differentiation factors) or activins that can induce Smad1/5/8 phosphorylation, but are not inhibited by Grem2. Persistent activity suggests that additional canonical BMP-signaling inhibition may be required after MI to supplement Grem2. Previous reports have linked activation of BMP signaling to cardiomyocyte apoptosis during the early stages of ischemia/reperfusion injury, as well as fibrosis and hypertrophy, so Grem2 may have additional cardioprotective properties.

We have previously shown that Grem2 is necessary for cardiac asymmetry and atrial development in zebrafish. However, it seems that Grem2 is dispensable for mouse development. This result is not without precedent among BMP-signaling components. For example, single Bmp5, Bmp6, and Bmp7 knockout mice are viable, but double knockouts of either Bmp5/7 or Bmp6/7 are embryonic lethal because of cardiac developmental defects. It is likely that, as with Bmp ligands, there is redundancy among BMP antagonists in cardiac development. Such redundancy may also happen in the adult heart. Although Grem2 is the highest induced BMP antagonist after MI, the heart maintains expression of many BMP antagonists such as Chordin, Sost, Twsg1 and Dan. There are also low levels of Noggin expression. Sost does not inhibit Bmp2, and thus, it is unlikely that Sost plays a role in the inflammatory response, which is dominated by Bmp2 induction. Dan, Chordin, and Twsg1 are weaker antagonists than Grem2 and may not compensate for Grem2 in the peri-infarct area, where Grem2 is prominently induced. It will be informative to generate double loss-of-function model by crossing the Grem2−/− mice to mice with conditional inactivation of other BMP antagonists to directly test redundant functions during development or after injury.

Increased numbers of infiltrating immune cells or prolonged inflammation negatively impacts subsequent steps of cardiac repair and functional recovery. Activated immune cells may continue to degrade matrix, impede angiogenesis, or affect myofibroblast proliferation and differentiation. However, clinical trials testing whether blocking inflammation improves outcomes have so far produced mixed results. For example, inhibitors of the complement system, TNFα, or integrins required for immune cell binding showed no significant improvement of infarct size and MI outcomes. Glucocorticoids actually had severe adverse effects, likely because of their interference with functions that are essential for healing. On the other hand, inhibitors of activated T cells, such as cyclosporine and the P-selectin antagonist inclacumab, showed promising results, indicating that moderating inflammation can be beneficial for cardiac repair after MI. However, the benefits of cyclosporine use after MI could not be replicated in a larger clinical trial. The failure or modest success to reduce infarct size in patients with MI by targeting direct players of reparative inflammatory processes underscores the need to better understand the endogenous regulatory mechanisms that control the temporal and spatial extent of inflammation. Even if blocking overactive postinfarction inflammation does not affect the size of the infarct, it might moderate dilative remodeling.

Recent crystallographic evidence revealed that Grem2 folds into a unique tertiary shape that has not been described before. Specifically, Grem2 dimerizes in a head-to-tail manner, unlike the head-to-head pairing of Noggin. This head-to-tail arrangement gives rise to a concave and convex 3-dimensional structure, which precludes Grem2 from wrapping around BMP dimers as Noggin does. We currently test whether this unique structural arrangement is also critical for the function of Grem2 in cardiac repair. Future biochemical analyses may identify critical structural motifs, which could be exploited to design molecules that mimic the biological effects of Grem2. Recognizing these mechanisms may offer novel insights in the cardiac healing process and provide new ways to regulate inflammation in a physiological manner. Moreover, because of the wide interest in regulating BMP signaling in bone fractures, osteoporosis, and cancer, many chemical compounds and peptides, to either promote or hinder BMP signaling, are being developed for clinical use.
Our findings may facilitate future repurposing of these new pharmacological resources for potential treatment of patients with MI to expand current strategies that aim to restore circulation to infarcted areas with thrombolitics and percutaneous interventions.

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Disclosures
None.

References


Novelty and Significance

**What Is Known?**

- Recruitment of inflammatory cells is an early critical step in the repair of heart tissue after a myocardial infarction (MI).
- Although inflammatory cells are necessary to clear debris from dead cells, excessive inflammation after MI is correlated with adverse cardiac remodeling and poor prognosis.
- Bone morphogenetic proteins (BMPs), which are critical for heart development, are induced shortly after MI, but their role in heart repair is largely unknown.

**What New Information Does This Article Contribute?**

- The BMP-signaling inhibitor Gremlin 2 is a secreted protein that is transiently induced in infarct border cardiomyocytes at the end of the inflammatory phase after MI, at the site of BMP-signaling activation.
- BMP signaling contributes to the induction of cell adhesion membrane proteins in endothelial cells that capture and recruit inflammatory cells after MI.
- Gremlin2 gene inactivation intensifies BMP signaling, leading to a dramatic increase in the number of infiltrating inflammatory cells after MI and further deterioration of heart function, whereas Greml2 overexpression in the heart or injection of Greml2 protein shortly after MI attenuates inflammation and improves recovery.

Although unregulated inflammation has been linked to adverse effects in the heart, previous anti-inflammatory treatments in patients with MI have been mostly unsuccessful. The failure or modest success of clinical trials aiming to reduce infarct size in MI patients, by targeting direct players of the inflammatory processes, underscores the need to better understand the natural mechanisms that control inflammation in the injured heart tissue. In this study, we provide evidence for a new mechanism that determines the magnitude and extent of the inflammatory response after MI. Specifically, our data show that tumor necrosis factor-α induction after MI initiates a transient inflammatory response, which is sustained by subsequent induction of the BMP ligand BMP2. BMP2 increases the magnitude of inflammation through upregulation of proinflammatory cell adhesion membrane proteins in endothelial cells. Greml2 is then induced by BMP2 as part of a negative feedback loop to inhibit the proinflammatory activity of canonical BMP signaling and act as a barrier to inflammation at the infarct border zone. Our findings suggest that BMP antagonists like Greml2 could attenuate inflammation and thereby improve heart recovery MI.
BMP Antagonist Gremlin 2 Limits Inflammation After Myocardial Infarction
Lehanna N. Sanders, John A. Schoenhard, Mohamed A. Saleh, Amrita Mukherjee, Sergey Ryzhov, William G. McMaster, Jr, Kristof Nolan, Richard J. Gumina, Thomas B. Thompson, Mark A. Magnuson, David G. Harrison and Antonis K. Hatzopoulos

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Supplemental Material

Methods

Generation of genetically engineered Grem2 mice

In order to inactivate the Grem2 gene and generate Grem2−/− mice, we first constructed an insertion vector containing two fragments from the Grem2 gene locus (fragments B and C, each 0.5 kb in length) flanking a kanamycin selection gene cassette under the synthetic EM7 prokaryotic promoter (EM7neo) and a fusion puromycin/truncated herpes simplex virus thymidine kinase gene [pu(Δ)TK; Figure 2A]). Fragment B is located within the Grem2 single intron just upstream of exon 2, which contains the entire Grem2 coding sequence and 3’ untranslated area (UTR). Fragment C resides within the 3’ UTR. The truncated thymidine kinase was incorporated to facilitate clone selection in future recombination strategies. Homologous recombination between a BAC containing the WT Grem2 locus and the insertion vector replaced the entire coding sequence and part of the 3’ UTR of the Grem2 gene with the pu(Δ)TK/EM7neo cassette. A vector containing two additional small fragments of the Grem2 gene locus (Fragments A and D), also 0.5 kb in length, were used to retrieve the resultant targeting vector from the modified BAC (Figure 2A).

200 μg of the targeting vector were linearized and double-electroporated into 3.5 x 10⁷ 129/Sv mouse embryonic stem cells at the Vanderbilt Transgenic Mouse/Embryonic Stem Cell Shared Resource (TMESCSR). After puromycin selection at 1.5 μg/ml, 483 colonies were picked and 25 colonies were identified as having properly recombined by Southern blotting using 5’ and 3’ probes outside the targeting vector (the location of the probes are marked in Figure 2A). The targeting efficiency was 5.2%. Six positive clones were subsequently expanded and confirmed by secondary screening. Two selected clones were then injected into C57BL/6 blastocysts and blastocysts were transplanted into pseudopregnant females. Both clones gave germline transmission generating two independent Grem2−/− mouse lines that displayed identical physiological phenotypes and response to myocardial ischemic injury. The Grem2−/− mice and littermate WT controls (WTmix) were kept on a mixed C57BL/6 and 129/Sv background.

The αMHC-Grem2 plasmid was generated by inserting the full-length Grem2 cDNA into the αMHC (Myh6) gene promoter-polyA hGH cloning vector 1 (kindly provided by Dr. J. Robbins).1 The αMHC-Grem2 transgenic (TG<sup>Grem2</sup>) mice were generated by pronuclear microinjection of the construct into fertilized oocytes at the TMESCSR. TG<sup>Grem2</sup> mice and WT
littermate controls were raised in C57BL/6 background.

Histological, molecular and flow cytometric analyses were conducted using male mice at 12-16 weeks of age fed with a normal chow diet.

**Experimental myocardial infarction (MI) and administration of Grem2 protein and DMH1**

Mice underwent open chest surgery, a 10-0 nylon suture was placed through the myocardium into the anterolateral left ventricular wall around the left anterior descending (LAD) artery and the vessel was permanently ligated. Mice were euthanized at defined time points following surgery to obtain cardiac tissue for molecular, histological and flow cytometric analyses.

For injection of Grem2 protein, we synthesized, purified and measured activity as previously described. WT mice were injected with 1 μg Grem2 protein per gram of body weight or vehicle (sterile 1X PBS via intraperitoneal injection (IP) once per day at day 2, 3, and 4 following MI. For injection of DMH1 (Sigma), Grem2/− mice were injected IP with 13 μg DMH1 per gram of body weight or vehicle (DMSO) once per day at 2, 3 and 4 days following MI.

**Echocardiography**

Mice underwent echocardiography measurements in order to assess cardiac function post-surgery. Mice were rested and calmed before echocardiography was performed. All mice were conscious and unsedated during imaging using the VEVO 2100 machine and transducer MS-400 (VisualSonics) to measure and calculate cardiac parameters. The left ventricle was located in B-Mode and was traced over five consecutive beats in M-Mode. Left ventricular internal dimension and volume in diastole and systole (LVIDd, LVIDs, LVvold, LVvols) were measured from M-Mode using the short axis and used to calculate fractional shortening and ejection fraction.

**RNA analysis by Reverse Transcription and quantitative Polymerase Chain Reaction (RT-qPCR)**

Whole hearts were dissected at the indicated time points after MI, perfused to remove blood cells and RNA was obtained using TriZol Reagent according to the manufacturer’s instruction (Life Technologies). RNA was obtained from cells in culture using the RNeasy Mini Kit (Qiagen). Reverse transcription was conducted by incubating 100 ng of oligo(dt)
(Promega) with 3 μg RNA for 5 min at 70°C. 20 mM of dNTPs (GE Healthcare), 200 U/μl of Mo-MLV reverse transcriptase with 5x associated buffer (Promega), 40 U/μl RNasin (Promega) and water were added to the RNA solution and incubated at 40°C for 1 hour, followed by a 5 minute incubation at 95°C in order to inactivate enzyme activity. 1:100 of the final cDNA solution or ~20 ng served as template for quantitative Real Time PCR with GoTaq qPCR Master Mix (Promega) using a C1000 Thermal Cycler (BioRad) as previously described.2,7 0.5 μM of Gapdh primers were included as an internal control and relative gene induction levels were determined using the $2^{(-\Delta\Delta C_t)}$ formula.8,9 Experiments were done in triplicates. The sequences of gene-specific primers have been included in the Online Table III.

**Immunofluorescence and immunohistochemistry analyses**

For IF on cardiac tissue sections, freshly isolated hearts were perfused with 1X Phosphate Buffered Saline (PBS), bisected transversely, embedded in Optimal Cutting Temperature (OCT) compound, frozen on dry ice, cut into 10 μm thick sections and stored at -70°C until use. Before antibody staining, slides were thawed at room temperature, immersed in cold 1:1 acetone:methanol and fixed for 5 minutes on ice. Slides with cardiac tissue sections were washed three times in 1X PBS for 5 minutes each wash, and incubated with blocking buffer containing 1% bovine serum albumin (BSA) and 0.05% saponin in 1X PBS for 1 hour at room temperature. Next, sections were stained with primary antibodies overnight at 4°C in blocking buffer. Afterwards, slides were washed five times in 1X PBS for 5 minutes each, incubated with secondary antibodies and DAPI for 1 hour at room temperature in blocking buffer, washed in 1X PBS three times for 5 minutes each, and mounted with VECTASHIELD fluorescent mounting medium (Vector Laboratories). The Vanderbilt Histology Core performed histological services, including tissue sectioning and hematoxylin and eosin staining. Bright field images were taken on the Zeiss AxioImager Z1.

Primary antibodies used for IF analysis were as follows: rabbit polyclonal anti-human Tie1 (Santa Cruz Biotechnology, 1:100, Cat. No. sc-342 (C-18), rat monoclonal anti-mouse CD31/PECAM1 (BD Pharmingen; 1:100, Cat. No. 553370), rabbit monoclonal anti-mouse p-Smad1/5/8 (Cell Signaling; 1:50, Cat. No. 9511), rat monoclonal anti-mouse CD45 (BD Pharmingen; 1:100, Cat. No. 550539), mouse monoclonal anti-mouse MF20 (Developmental Studies Hybridoma Bank; 1:5, Cat. No. MF 20, RRID:AB_2147781), rabbit polyclonal anti-human Grem2 (GeneTex; 1:100, Cat. No. GTX108414), rat monoclonal anti-mouse CD62E/E-selectin (BD Pharmingen; 1:100, Cat. No. 550290), and mouse monoclonal α-Actinin (Sigma;
Secondary antibodies used for IF were: goat anti-mouse Cy3-conjugated (Cat. No. A7811), goat anti-rat Cy3 (Jackson ImmunoResearch, Cat. No. 712-165-150), and goat anti-rabbit Alexa-Fluor-488-conjugated (Life Technologies, Cat. No. A21206). Cy3 antibodies were used at a 1:200 dilution and Alexa-Fluor-488 was used at a 1:400 dilution. Cardiac tissue sections were stained with the fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI, 1:5000 dilution; Invitrogen) to mark cellular nuclei. Control images using isotype controls and minus primary antibody controls are shown in Online Figure XII. Images were taken on the Olympus FV-1000 inverted confocal microscope and processed using the FV10-ASW 1.6 Viewer software (Olympus).

p-Smad1/5/8+ cardiomyocytes were quantified using ImageJ 1.46r (NIH) color thresholding, as a percentage of cells double positive for MF20 and p-Smad1/5/8 amongst all DAPI positive cells in the viewing field; at least 4 viewing fields were used for calculations. N=3 mice for each group.

**Flow Cytometry**

We prepared single cell suspensions of cardiac cells depleted of cardiomyocytes from freshly isolated whole hearts perfused with 1X PBS to remove blood cells. Next, hearts were digested with Collagenase D (2 mg/ml; Roche) and DNase I (100 μg/ml) in a solution of RPMI 1640 (Gibco) containing 10% FBS using an AUTOMacs Dissociator (Miltenyi Biotech), and then incubated at 37°C for 30 minutes in an orbital shaker to prepare single cell suspensions. The digested tissue was then passed through a 70-micron cell strainer and centrifuged at 500 g for 10 minutes. The cell pellet was suspended in 2 ml 1X PBS and centrifuged at 300 g for 5 minutes. Two more centrifugation/wash steps followed, and the pellet was suspended in 100 μl of FACS buffer (1% BSA, 0.5% NaN₃ in 1X PBS). We then added 2 μl of Fc blocker (eBioscience) and cells were incubated for 10 minutes at 4°C to prevent non-specific antibody binding, then washed with 1 ml of FACS buffer and centrifuged at 300 g for 5 minutes. The cells were resuspended in 100 μl of FACS buffer and antibodies were added at 1 μl or 0.25 μg per 1 million cells and incubated for 30 minutes at 4°C. The antibodies used were Brilliant Violet 510–conjugated (BV510) anti-CD45 antibody (Biolegend, Cat. No. 103107), Alexa Fluor 488–conjugated anti-F4/80 antibody (Biolegend, Cat. No. 123119), PE-Cy conjugated anti-Ly6C (eBioscience, Cat. No. 25-5932-80), Brilliant Violet 421-conjugated anti-Ccr2 (Biolegend, Cat. No. 150605), PE-conjugated anti-Ly6G (Biolegend, Cat. No. 127607), and APC-Cy7-conjugated anti-CD3e (BD Pharmingen, Cat. No. 557596).
After incubation, cells were centrifuged at 300 g for 5 minutes and washed twice with 1 ml FACS buffer. 5 μl of 7-AAD (eBioscience) was added to 100 μl of the solution and incubated for 10 minutes at room temperature for live/dead staining. After 300 μl of FACS staining buffer was added, cell samples were analyzed by flow cytometry using the BD FACSCanto II cytometer. Total cell number was determined by adding 50 μl of counting beads (~49500-52000 beads per μl; Life Technologies). Flow-minus-one was used for gating. Low voltage gating was conducted in order to capture the counting bead population. All leukocyte populations were quantified within the CD45+ gate, and pro-inflammatory monocyte cell numbers were quantified within the F4/80+ gate. Data acquisition was completed using FloView.

Regarding flow cytometry of blood leukocytes, 100 μl of fresh heparinized blood was directly stained with 1.5 μl of each of the previously mentioned antibodies for 30 minutes at 4°C. The samples were then washed with FACS buffer. 2 ml of red blood cell lysis buffer was applied per 100 μl of blood for 4 minutes at room temperature. Two additional rounds of washing and centrifugation at 1500g for 3 minutes followed. 7-AAD staining and incubation with primary and secondary antibodies was conducted as described above.

**TTC Staining**

Whole mouse hearts were isolated 24 hours post-MI, flash frozen and cut into 1 mm sections. The sections were incubated in 1.5% TTC at 37°C for 30 minutes and then fixed in 10% formalin overnight, and finally imaged. ImageJ 1.46r (NIH) was used to outline infarct (white) tissue. Infarct size is reported as a percentage of the total left ventricular (LV) area.

**Cell culture**

Human Microvascular Endothelial cells (HMECs) were kindly provided by Dr. Sergey Ryzhov. Cells used for experiments were between the third and fourth passages and cultured in 199 media (Gibco 11150) containing 15% FBS, 10 U/ml Heparin (Sigma), and 30 μg/ml endothelial cell growth supplement (Biomedical Technologies). Cells were grown in full growth serum and then seeded in 12-well plates. Prior to growth factor addition, cells were incubated with serum starvation media (same as normal media with 1% FBS) over night. Cells were subsequently treated with rhTNFα (R&D; 10 ng/ml), rhBMP2 (R&D; 100 ng/ml-200 ng/ml), Grem2 (100 ng/ml), and DMH1 (10μM) or the equivalent volume of vehicle solution (PBS or
DMSO). After 4 hours or 24 hours of treatment, cells were lysed for RNA extraction. Data are representative of at least two independent experiments.

For binding assays, HMEC cells were grown for three days until they reached monolayer confluency (3 x 10^4 cells/well within a 96-well plate). Calcein AM labeled (1 μM, 30 min) human monocytes (THP-1 cells) were added to the HMEC monolayer at a concentration of 10 x 10^4 cells per well (HMEC:THP-1 ratio 1:3) and incubated together for 30 minutes. After incubation, non-adherent THP-1 cells were aspirated off and the remaining cells were washed with PBS 5 times. The fluorescence intensity was measured using the Modulus microplate multimode reader. The number of adherent THP-1 cells was calculated from a calibration curve prepared using increasing concentrations (ranging from 0.1 to 100 x 10^3 cells) of THP-1 cells. 300 ng/ml of BMP2, 100 ng/ml of Grem2 and 100ng/ml of TNFα were used alone or combination for 24 hours to treat either HMEC or THP-1 cells.

**Statistical Analysis**

Statistical analysis was performed using GraphPad Prism software. Data are represented as the mean ± SEM. Student’s two-tailed unpaired t-test was used for comparison between two groups, one-way ANOVA was used to compare multiple groups, and two-way ANOVA was used to compare gene induction in each mouse model over time. Dunnett’s and Bonferroni’s multiple comparisons test was used post-hoc. *P<0.05, **P<0.01, ***P< 0.001, ****P<0.0001 were considered significant.

**Online Methods References**


Online Figure I. Induction of canonical BMP signaling target gene *ld2*, as well as relative expression of BMP antagonists after MI. (A) qPCR analysis of whole heart RNA samples isolated from WT mice at days 0, 1, 2, 3, 5, 7 and 21 post-MI shows *ld2* gene induction throughout the cardiac tissue repair process. ** *P < 0.01* compared to day 0. One-way ANOVA with Dunnett’s multiple comparisons test. N=3 for all time points. All data are means ± SEM. *ld2*: Inhibitor of DNA binding 2. (B) Absolute expression of BMP antagonist genes following normalization to *Gapdh*. delta Ct= Ct$_{\text{gene}}$ - Ct$_{\text{Gapdh}}$. All data points (N=3) are means ± SEM.
Online Figure II. *Grem2* gene inactivation by homologous recombination. (A) qPCR analysis of whole heart RNA samples isolated from WT, *Grem2*+/− and *Grem2*−/− mice shows that *Grem2* baseline gene expression levels are approximately halved in *Grem2*+/− mice and completely absent in *Grem2*−/− mice. * P < 0.05. Student’s two-tailed unpaired t-test. N=3 for all groups. All data are means ± SEM. (B) Schematic drawing to mark the location of the primer pairs used to genotype WT, *Grem2*+/− and *Grem2*−/− mice. Primer pairs 1,2 and 4,6 are specific to the endogenous WT locus, whereas 1,3 and 5,6 amplify DNA fragments generated after homologous recombination. (C) Example of conventional PCR results using genomic DNA isolated by mouse tail tip clipping. Mice a,b are WT, c,d are *Grem2*−/−. The expected size of the amplicons is indicated below. *Gapdh* primers served as controls. L: DNA ladder marker. (D, E) *Grem2*−/− hearts appear morphologically normal. (D) Whole mount images of 12-week old WT and *Grem2*−/− hearts show no differences in morphology and size between the two genotypes. (E) Hematoxylin & Eosin stained cardiac sections from the left ventricle of WT and *Grem2*−/− show no apparent cellular and tissue abnormalities in *Grem2*−/− hearts. Scale bar, 10 μm.
Online Figure III. Chemokine expression levels after MI are comparable among WT and Grem2 mouse lines. qPCR analysis of whole heart RNA samples isolated from WT, Grem2−/−, and TG<sup>Grem2</sup> mice at days 0, 2, and 7 post-MI. Relative expression levels of chemokines in Grem2−/− (A) and TG<sup>Grem2</sup> (B) mice before and after MI are comparable to WT with the exception of a moderate increase in Ccl2 in Grem2−/− mice. Student’s two-tailed unpaired t-test between genotypes at various time points. N=3 per group for all time points. All data are means ± SEM.
Online Figure IV. Flow cytometry gating strategy. (A) Representative graphs of the flow cytometry analysis of non-cardiomyocyte cells isolated from whole hearts showing the gating strategy, where we gated out debris first, followed by gating for live cells and then singlets. SSC=side scatter, FSC= forward side scatter, 7AAD= 7-Aminoactinomycin D. (B) Representative graphs of the flow cytometry analysis of non-cardiomyocyte single cells from a WT mouse. CD45+ cells, which represent all leukocytes, were then gated for antibodies used to identify various inflammatory cell subpopulations: Ly6C\(^+\) that marks primarily monocytes, Ly6G for neutrophils, CD3 for T-cell lymphocytes, and F4/80 for macrophages. Inflammatory macrophages within the F4/80 population were further characterized as Ly6C\(^+\) and/or Ccr2\(^+\).

Online Figure V. Infarct sizes are comparable in WT and Grem2 mice. (A) TTC staining on WT and Grem2−/− mouse hearts at day 1 post-MI illustrates no significant differences in initial infarct sizes. N=6-7 per group. All data are means ± SEM. (B) Echocardiography measurements conducted demonstrate comparable drop in %FS at 1 day post-MI, further supporting that the initial injury in these mice are comparable. N=6-8 per group. All data are means ± SEM.
Online Figure VI. Flow cytometry of circulating leukocytes. CD45$^+$ cells in blood samples from WT and Grem2$^-$ mice demonstrate that there are no significant differences in circulating leukocytes before injury. There is a ~2-fold increase in the level of leukocytes in Grem2$^-$ mice compared to WT at day 5 post-MI. * $P < 0.05$. Student’s two-tailed unpaired t-test. No injury N=3 per group, post-MI N=5-6. All data are means ± SEM.
Online Figure VII. **TG^{Grem2} hearts appear morphologically normal.** (A) Whole mount images of 12-week old WT and TG^{Grem2} hearts show no differences in morphology and size between the two genotypes. (B) Hematoxylin & Eosin stained cardiac sections from the left ventricle of WT and TG^{Grem2} hearts show no apparent cellular and tissue abnormalities. Scale bar, 10 μm. (C) qPCR analysis of whole heart RNA samples isolated from WT and TG^{Grem2} mice shows a significant increase in Grem2 expression in TG^{Grem2} compared to WT. ***P < 0.001. Student's two-tailed unpaired t-test. N=3 for all groups. All data are means ± SEM.
Online Figure VIII. Representative M-mode images from WT, TG<sup>Grem2</sup>, WT<sup>mix</sup> and Grems<sup>-/-</sup> mice 21 days post-MI.
Online Figure IX. Grem2 inhibits canonical BMP signaling in endothelial cells. *ID2* induction in endothelial cells is completely inhibited by Grem2. TNFα has a moderate effect on BMP2-mediated *ID2* induction. **** *P* < 0.0001. One-way ANOVA with Dunnett’s multiple comparisons test. N=3 for all treatments. All data are means ± SEM.
Online Figure X. **Grem2 acts specifically on endothelial cells.** Incubation of human monocytes (THP-1 cells) with BMP2, Grem2, or in combination did not alter binding to endothelial cells (HMEC). N=12. All data are means ± SEM.
Online Figure XI. Canonical BMP signaling is not active in infiltrating leukocytes. IF analysis of cardiac tissue sections 5 days post-MI using antibodies recognizing p-Smad1/5/8 (green) and CD45, (red) shows that BMP signaling is not active in infiltrating inflammatory cells of WT and Grem2<sup>-/-</sup> hearts. DAPI marks cellular nuclei. Scale bars, 100 μm. BZ=infarct border zone; INF=infarct.
Online Figure XII. Control antibody analyses for immunofluorescence staining. (A) IF using non-immune immunoglobulins (isotype controls) of same species and same concentration as the corresponding primary antibodies used on cardiac tissue sections post-MI. Rat and mouse isotypes controls are shown in combination (left) and the rabbit isotype control is shown independently (right). (B) IF staining with respective secondary antibody-only of the sections in the absence of primary antibodies.
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<th>HR (bpm)</th>
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**Online Table I. Physiological and cardiac functional parameters of Grem2−/− mice and WT siblings.**
Body weight, left ventricle (LV) mass, heart rates, LV wall and cavity dimensions, including calculated functional parameters (measured by echocardiography), are comparable in WT_mix and Grem2−/− adult mice at baseline, with the exception of heart rate. HR=heart rate; IVS=interventricular septum; LVPW=Left Ventricle Posterior Wall; LVID=Left Ventricle Internal Dimension; vol=volume, EF=ejection fraction, FS=fractional shortening; SV=stroke volume; CO=cardiac output; d=dystole; s=systole. ns=not significant. Student’s two-tailed unpaired t-test. WT N=9, Grem2−/− N=8. All data represent means ± S.D.
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<td>TG\textsuperscript{Grem2}</td>
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<th>FS (%)</th>
<th>SV (µl)</th>
<th>CO (ml/min)</th>
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<td>WT</td>
<td>0.8 ± 0.01</td>
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**Online Table II. Physiological and cardiac functional parameters of TG\textsuperscript{Grem2} mice and WT siblings.**

Body weight, left ventricle (LV) mass, heart rates, LV wall and cavity dimensions, including calculated functional parameters (measured by echocardiography), are comparable in WT and TG\textsuperscript{Grem2} adult mice at baseline. HR=heart rate; IVS=interventricular septum; LVPW=Left Ventricle Posterior Wall; LVID=Left Ventricle Internal Dimension; vol=volume, EF=ejection fraction, FS=fractional shortening; SV=stroke volume; CO=cardiac output; d=dystole; s=systole. ns=not significant. Student’s two-tailed unpaired t-test. Body weight, LV mass, HR, IVS\textsubscript{d}, IVS\textsubscript{s}, LBPW\textsubscript{d}, LVPW\textsubscript{d}, LVPW\textsubscript{s}, SV, CO N=4 per group; LVID\textsubscript{d}, LVID\textsubscript{s}, EF, FS WT N=6, TG\textsuperscript{Grem2} N=10. All data represent means ± S.D.
### Online Table III. Primer Sequences used in qPCR analyses and *Grem2<sup>-/-</sup>* mouse genotyping

**Mouse primers qPCR:**

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| 3' CACATACGACACAGTCGC  
Vcam1 | 5' AGAGAAACCATTATTTGTTGACATCTCCC  
| 3' CAAGTGGCCCACTCATTTTAAATTACTGG

**Human primers qPCR:**

**BMP2**
| 5' ACCCGCTGTCTTCTAGCGT  
| 3' TTTCAAGCCGAACATGCTGAG  

**E-SELECTIN**
| 5' GCTGGACTCTCCCTCCTGACATTAGC  
| 3' CATAAAGGCATCTGGCATAGTAGGCAAG  

**GAPDH**
| 5' AAGGTGAAGGTGACGGGATGCAAC  
| 3' GGGTCATTGATGGCAACAATA  

**GREM2**
| 5' ATCCCCCTCGCTTTACAAGGA  
| 3' TCTTGACACCAGTCACCTTTGA  

**ID2**
| 5' GCATCCCCAGAACAAGGAGGTGAG  
| 3' CGTTATTCAAGCCACAGTGCTTGA

**Genotyping Primers:**

**Primer 1**
| 5' CTGTGCAGCAGAAAGAAGCTG  
**Primer 2**
| 3' TGGCAATGTACCTCATCTCA  
**Primer 3**
| 3' CTGCCATCTGACGAGACT  
**Primer 4**
| 5' TCTGGTACCACGAGGACAAGC  
**Primer 5**
| 5' GTCTGAGTAGGTGTCAATTCTA  
**Primer 6**
| 3' CACAGATCACTGAGCTCT