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Calpastatin overexpression impairs postinfarct scar healing in mice by compromising reparative immune cell recruitment and activation

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Wan F, Letavernier E, Le Saux CJ, Houssaini A, Abid S, Czibik G, Sawaki D, Marcos E, Dubois-Rande JL, Baud L, Adnot S, Derumeaux G, Gellen B. Calpastatin overexpression impairs postinfarct scar healing in mice by compromising reparative immune cell recruitment and activation. Am J Physiol Heart Circ Physiol 309: H1883–H1893, 2015. First published October 9, 2015; doi:10.1152/ajpheart.00594.2015.—The activation of the calpain system is involved in the repair process following myocardial infarction (MI). However, the impact of the inhibition of calpain by calpastatin, its natural inhibitor, on scar healing and left ventricular (LV) remodeling is elusive. Male mice ubiquitously overexpressing calpastatin (TG) and wild-type (WT) controls were subjected to an anterior coronary artery ligation. Mortality at 6 wk was higher in TG mice (24% in WT vs. 44% in TG, P < 0.05) driven by a significantly higher incidence of cardiac rupture during the first post-MI week, despite comparable infarct size and LV dysfunction and dilatation. Calpain activation post-MI was blunted in TG myocardium. In TG mice, inflammatory cell infiltration and activation were reduced in the infarct zone (IZ), particularly affecting M2 macrophages and CD4+ T cells, which are crucial for scar healing. To elucidate the role of calpastatin overexpression in macrophages, we stimulated peritoneal macrophages obtained from WT and TG mice in vitro with IL-4, yielding an abrogated M2 polarization in TG but not in WT cells. Lymphopenic Rag1−/− mice receiving TG splenocytes before MI demonstrated decreased T-cell recruitment and M2 macrophage activation in the IZ day 5 after MI compared with those receiving WT splenocytes. Calpastatin overexpression prevented the activation of the calpain system after MI. It also impaired scar healing, promoted LV rupture, and increased mortality. Defective scar formation was associated with blunted CD4+ T-cell and M2-macrophage recruitment.

myocardial infarction; calpain; calpastatin; inflammation; scar healing; cardiac rupture; lymphocytes; macrophages

NEW & NOTEWORTHY

Ubiquitous calpastatin overexpression is associated with increased mortality in mice after myocardial infarction (MI) by impairing wound healing and thereby favoring cardiac rupture. Thus compromising the reparative immune cell recruitment and activation by systemic calpain inhibition seems not to be a promising approach to improve outcome after large MI.

MYOCARDIAL INFARCTION (MI) is the leading cause of heart failure in developed countries despite improvement in revascularization and pharmacological treatment (45). A complex tissue remodeling occurs at the acute phase in association with systemic and local inflammatory responses (18, 51, 52), involving cardiomyocyte necrosis, granulation tissue formation, and scar maturation (3, 8). Alteration of the inflammatory responses following MI impacts the healing process, in particular the production of fibrous tissue, which is critical for stable scar formation (11). Reduced inflammation results in a non-adaptive fibrosis that may increase the risk of cardiac rupture. In mice, cardiac rupture occurs mostly at 3–6 days post-MI and depends on both infarct size and degree of inflammatory and neurohormonal responses (14). When excessive, the inflammatory response counteracts appropriate wound healing, favoring adverse left ventricle (LV) remodeling and promoting heart failure (7, 8, 14, 19, 35, 37). Macrophages are key players in this post-MI inflammatory and reparative process. Recent studies have identified two distinct phenotypes: the proinflammatory M1 and the anti-inflammatory M2 populations, with deleterious and protective roles in post-MI LV remodeling, respectively (30, 40, 41). Promoting the reparative M2-macrophage polarization may be pivotal for adequate post-MI wound healing.

Calpains are calcium-dependent nonlysosomal ubiquitously expressed cysteine proteases, localized in the cytosol as inactive proenzymes, and activated by an increase in intracellular Ca2+ concentrations. The two major members of the calpain family, calpain 1 (μ-calpain) and calpain 2 (m-calpain), control a variety of cellular processes, such as motility, proliferation, intracellular signaling, plasma membrane repair, apoptosis (15), and regulation of inflammation (28, 32, 59). Their
activity is tightly regulated by their specific endogenous inhibitor, calpastatin (23). Interestingly, calpain inhibition impairs reparative mechanisms in response to a variety of injuries in different organs (27, 38). In the heart, calpain expression and activity are upregulated after MI (25, 48). Pharmacological calpain inhibition has been suggested to prevent LV remodeling and dysfunction in the acute phase (36, 46). Thus specific deletion of calpain and calpastatin overexpression have also been shown to be protective after MI long term (35, 58). Therefore, an apparent conundrum exists emerging from studies using inhibitors of calpain or genetically modified mouse strains. Whether calpain inhibition results in beneficial or deleterious effects may depend on the experimental animal models and on the time point when the effects are observed. Cardiac-specific calpain deletion is associated with more severe LV dilation and dysfunction after transverse aortic constriction or sustained catecholamine stress (54). On the other hand, calpain inhibition prevents cardiac hypertrophy in an angiotensin II-induced hypertension model (29). Furthermore, uncontrolled calpain activation in calpastatin-deficient mice in the first week post-MI results in more severe LV dilation and increased mortality (25). The aim of this work was to elucidate the impact of ubiquitous calpastatin overexpression in mice during the inflammatory response after MI and its subsequent consequences on LV remodeling at long term.

MATERIALS AND METHODS

Experiments were conducted according to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and were approved by the Institutional Animal Care and Use Committee of the French National Institute of Health and Medical Research (INSERM)-Unit 955, Créteil, France. All animals were housed in a constant temperature room with 12-h light-dark cycle and fed ad libitum.

Mouse strains and procedures. Male 12-wk-old C57BL/6 (WT; Janvier; Le Genest-Saint-Isle, France) or transgenic (TG) mice constitutively overexpressing rabbit calpastatin under the control of a CMV promoter (courtesy of L. Baud; Refs. 29, 44), backcrossed to C57BL/6 genetic background for more than 10 generations, were obtained from Janvier; Le Genest-Saint-Isle, France) or transgenic (TG) mice constitutively overexpressing rabbit calpastatin under the control of a CMV promoter (courtesy of L. Baud; Refs. 29, 44), backcrossed to C57BL/6 genetic background for more than 10 generations, were subjected to permanent coronary artery ligation under mechanical ventilation and anesthesia with 3–4% mixture of isoflurane/room air (47). Sham-operated mice underwent the same procedure except left anterior descending artery ligation. Operated mice were monitored daily and thoracotomy was performed in all deceased animals to check for the presence of hemothorax, as evidence for pericardial effusion. Transmural necrosis circumference were traced manually at the pericardial and the endocardial border on digital photographs of each section, using the ImageJ software. For each section, infarct size was expressed as percentage of necrotic vs. total circumference, using the arithmetic mean of the pericardial and endocardial values. The final MI size was expressed as the mean of the nine transverse sections.

Transhilar echocardiography. Transhilar echocardiography (TTE) was performed by placing mice on a homeothermic operating table, under light isoflurane anaesthesia, using a Vivid 7 apparatus (GE Ultrasound) with a 13-MHz probe (1.3L). LV end-diastolic posterior wall thickness, LV end-systolic diameter (LVEDd), LV end-diastolic diameter (LVEDd), LV end-systolic volume (LVESv), LV end-diastolic volume (LVEDV) were calculated, and the fractional shortening was calculated as [(LVEDd – LVESd)/LVEDd] × 100.

Western blot analysis. Infarct zone (IZ) myocardium was homogenized in ice-cold RIPA buffer containing protease and phosphatase inhibitor (Sigma-Aldrich). Total protein (60 μg) was loaded to 10% SDS-PAGE. Separated proteins were transferred to nitrocellulose membranes, blocked for nonspecific protein binding using TBS-Tween 0.1 and 5% milk, and probed for selected proteins with a primary antibody at 4°C overnight. Primary antibodies used were raised against calpastatin, α-calpain and m-calpain (1:1,000; Cell Signaling), spectrin (1:8,000; Millipore), α-smooth muscle actin (α-SMA; 1:400; Sigma-Aldrich), collagen I (1:4,000; Abcam), matrix metalloproteinase-9 (MMP-9; 1:400; R&D Systems), and GAPDH (1:5,000; Cell Signaling), followed by incubation with the corresponding horseradish peroxidase-conjugated secondary antibody (1:1,000; GE Healthcare). Densitometric quantification was normalized to GAPDH in each sample (Gene Tools; Ozyme).

Histology and immunohistochemistry. Immunofluorescence was performed on LV cross sections using antibodies against CD45 (1:20; AbD Serotec), Ly6G (1:50; AbD Serotec), F4/80 (1:50; AbD Serotec), CD3 (1:20; BioLegend), CD4 (1:20; BioLegend), CD8 (1:20; AbD Serotec), CD19 (1:50; Abcam), collagen I (1:400; Abcam), α-SMA (1:400; Sigma-Aldrich), calpastatin (1:40; Santa Cruz), tropoelin I (1:400; Abcam), and cardiac α-actinin (1:400; Abcam) at 4°C overnight and incubated with the corresponding secondary antibody (1:1,000; Cell Signaling Technology) at room temperature for 1 h. Nuclei were stained with Hoechst 33342 (1 μg/ml; Cell Signaling Technology). Fluorescence was measured using a Zeiss Axioplan 2 Imaging microscope and quantified on digital photographs using the ImageJ software.

Collagen deposition was assessed with Sirius red staining of cross sections. The extent of fibrosis was expressed as percentage of the red colored vs. the total area using the Imagej software (43).

Real-time quantitative RT-PCR. Total RNA was extracted from IZ myocardium and from cultured macrophages using Trizol (Invitrogen) according to the manufacturer’s instructions. First-strand cDNA was generated using SuperScript II reverse transcriptase, SYBR Green RT-PCR was performed using a 7900HT real-time PCR cycler (Applied Biosystems) and analyzed using ABI Prism Sequence Detection Software v.1.7 (Applied Biosystems). Primer sequences are available upon request. All samples were run in duplicate and quantification was performed using the comparative Ct method with GAPDH (tissue) or 18S rRNA (cells) as endogenous control.

Peritoneal macrophage purification and stimulation. Two-month-old male WT and TG mice were anesthetized with 3–4% mixture of isoflurane and room air and injected with 5–10 ml of RPMI 1640 medium (Invitrogen) supplemented with 2% FBS (Invitrogen) into the peritoneal cavity. The abdomen was massaged to mobilize resident peritoneal macrophages, followed by removal of the medium using a 19-gauge needle. Collected cells were spun down, resuspended in RPMI with 10% FBS, counted, and plated 1 × 106 cells per well. Macrophages were stimulated for 24 h with INF-γ (50 ng/ml; PeproTech) + LPS (10 ng/ml; Sigma-Aldrich) to induce polarization into M1 or by IL-4 (10 ng/ml; PetoTech) to induce polarization into M2 phenotype. Total RNA was extracted from cultured macrophages using Trizol (Invitrogen) according to the manufacturer’s instructions.

Gelatin zymography. Zymography was performed with 10% Zymogram (Gelatin) gels (Invitrogen). Tris-glycin SDS sample buffer (Invitrogen) was added to lysed border zone (BZ) myocardium samples and loaded on gels. Electrophoresis was performed in Tris-glycine SDS running buffer (Invitrogen). SDS was removed from the gel by washing with Zymogram renaturing buffer (Invitrogen). Gels were incubated in Zymogram developing buffer (Invitrogen) at 37°C.
overnight and then stained with Coomassie brilliant blue. Gelatino-lytic activity was visualized as clear bands of lysis against a dark background.

Statistical analysis. The data are presented as means ± SE. Mortal-ty rates were compared using log-rank test. For the analysis of mortality, periprocedural deaths occurring within the first 24 h after the operation were excluded. Means were compared using either unpaired two-tailed Student’s t-test (2 groups) or one-way ANOVA with Bonferroni correction (>2 groups). A P < 0.05 was considered significant.

RESULTS

Calpastatin overexpression attenuates calpain activation post-MI. To validate calpastatin-overexpressing TG mice in the context of postinfarct remodeling, we evaluated the expression of calpastatin and related targets in infarcted and sham-operated hearts. Protein levels of calpastatin were mildly, but significantly, higher in TG compared with WT myocardium at baseline and 5 days after MI (Fig. 1A). To estimate the calpain activity after MI, we determined expression of the specific calpain-cleaved fragment of spectrin (145/150 kDa) by immuno-blotting. The level of spectrin proteolysis was low in both sham-operated TG and WT and increased significantly 5 days after MI in WT hearts as expected (P < 0.05). This increase was completely blunted in TG mice (Fig. 1A) suggesting an efficiently suppressed calpain activity by calpastatin overexpression post-MI. The protein levels of calpain 1 and 2 paralleled calpain activity (Fig. 1A).

Calpastatin overexpression promotes LV rupture post-MI. Infarcted TG and WT mice were followed up over a period of 6 wk. Acute postoperative mortality (day 1) was comparable between groups, without evidence of cardiac rupture at autopsy. Thereafter, the incidence of cardiac rupture evidenced by hemo-thorax on autopsy was two times higher in TG compared with WT (data not shown), with a peak 5 days after MI.

This increase in cardiac rupture in TG accounts for the significantly increased post-MI mortality from day 2 to 42 in TG compared with WT mice (Fig. 1B). Of note, infarcted TG and WT mice showed comparable MI size when measured 5 and at 42 days after MI (Fig. 1C). Five days post-MI, infarcted surviving mice of either genotype displayed comparable LV dilation and contractile dysfunction (Fig. 1D). TG mice showed a tendency towards a more important LV hypertrophy in the noninfarcted posterior wall (1.15 ± 0.09 in TG vs. 0.95 ± 0.05 mm in WT; P = 0.09; Fig. 1D). Pulmonary edema as reflected by wet lung weight was significantly increased in TG-MI compared with WT-MI mice (Fig. 1E). The level of expression of calpastatin in WT-MI at day 42 was decreased compared with sham mice (Fig. 1F).

Compromised post-MI scar formation renders calpastatin-overexpressing hearts vulnerable to rupture. Since increased incidence of post-MI cardiac rupture in calpastatin-overexpressing mice could be related to the formation of a compromised scar, we evaluated expression of markers involved in the post-MI repair process. First, a marked upregulation of the myofibroblast marker α-SMA in the IZ was detected, which remained significantly obliviated in calpastatin-overexpressing animals (Fig. 2, A and B). Levels of collagen type I, a major extracellular component of postinfarct remodeling, closely mirrored the α-SMA expression pattern (Fig. 2, A and B). These findings were further supported by the quantification of collagen in the tissue evaluated by picrosirius red staining of the BZ (Fig. 2C).

Infarct-induced scar formation is contingent on degradation of the extracellular matrix. Accordingly, both zymography and Western blot showed induction of MMP-9 activity and expression levels in WT hearts by MI, which was attenuated in the infarcted TG hearts (Fig. 2, D and E). The specificity of the involvement of calpastatin overexpression in the regulation of the activity of MMPs post-MI is supported by the absent effect of calpastatin overexpression on the activity of MMP-2 (Fig. 2D). Taken together, these findings indicate an altered postinfarct repair response by calpastatin overexpression resulting in compromised scar formation and increased susceptibility to rupture.

Calpastatin overexpression reduces CD4+ T-cell and macrophage recruitment to the IZ. It has been shown that calpastatin overexpression impairs inflammatory cell infiltration (5). Thus we hypothesized that calpastatin hampers the post-MI reparative inflammatory process, ultimately promoting vulnerability to cardiac rupture.

Five days after MI, CD45+ leukocyte recruitment was reduced by ~50% in the IZ of TG compared with WT hearts (Fig. 3A). This was not associated with a difference in the accumulation of Ly-6G+ neutrophils (data not shown) but with a significant reduction in CD3+ T lymphocytes and F4/80+ macrophages (Fig. 3, B and C). The difference in CD3+ T lymphocyte was accounted for by a dramatic reduction of CD4+ T lymphocytes in TG mice, while the recruitment of CD8+ T lymphocytes (Fig. 3B) remained unaltered.

Despite the level of expression for the chemokines MCP-1 and CCR2 being similar between the strains (Fig. 3D), the number of monocyte/macrophage was reduced at the injury site. Furthermore, the activation of the macrophages appeared altered in TG mice compared with WT as a reduced expression of Cx3cl1 and Cx3cr1 was measured (Fig. 3E). These data suggest that despite comparable recruitment signals in TG and WT myocardium after MI, the...
**A**

5d after MI

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**B**

Kaplan-Meier survival in % after MI

- **WT**
  - 5d: 77, 77, 77, 77, 77, 77, 77
  - 42d: 77, 77, 77, 77, 77, 77, 77

- **TG**
  - 5d: 77, 77, 77, 77, 77, 77, 77
  - 42d: 77, 77, 77, 77, 77, 77, 77

Death from LV rupture
- **WT**
  - 5d: 77, 77, 77, 77, 77, 77, 77
  - 42d: 77, 77, 77, 77, 77, 77, 77

- **TG**
  - 5d: 77, 77, 77, 77, 77, 77, 77
  - 42d: 77, 77, 77, 77, 77, 77, 77

**C**

Day 5

- **WT**
- **TG**

Day 42

- **WT**
- **TG**

**D**

5d after MI

**E**

42d after MI

**F**

42d after MI

- **WT sham**
- **TG sham**
- **WT-MI**
- **TG-MI**

- **Calpastatin**
- **α-SMA**
- **GAPDH**
number and activation of T lymphocytes and macrophages were significantly attenuated.

**Calpastatin overexpression inhibits M2 macrophage activation.** To estimate macrophage function in situ, expression of polarization markers along with polarization-specific secretory products was measured in the BZ 5 days after MI, showing robust induction of both M1 and M2 macrophage markers in WT hearts after MI. However, while M1 polarization (Fig. 4A) was unaltered, polarization to the M2 phenotype was substantially attenuated in the BZ of TG mice (Fig. 4B).

To explore the hypothesis that calpastatin overexpression impacts macrophage polarization, peritoneal macrophages were isolated from WT and TG mice and stimulated with either a combination of IFN-γ and LPS (to polarize to M1) or IL-4 (to polarize to M2). After IFN-γ and LPS stimulation, the levels of expression of the M1 markers IL-1β and TNF-α were comparably upregulated in macrophages of both genotypes (Fig. 4C). Conversely, IL-4 stimulation of TG macrophages completely abolished M2 polarization as demonstrated by the reduction of Arg1, CD206, and TGF-β1 expression levels (Fig. 4D).

These findings suggest that calpastatin overexpression disrupts the regenerative inflammation post-MI at least in part by abrogating M2 macrophage polarization.

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Fig. 2. Calpastatin overexpression is linked to decreased myofibroblast differentiation and collagen deposition 5 days after myocardial infarction. A: representative immunoblot photographs and densitometric quantification of α-SMA and collagen I levels in homogenates of sham and IZ myocardium from WT and TG mice (n = 8–10/group) showing markedly attenuated myofibroblast differentiation and completely blunted collagen deposition in TG-MI compared with WT-MI hearts. B: graphical representation of immunohistological analysis of the number of α-SMA⁺ and collagen I⁺ cells in sham and IZ myocardium from WT and TG mice (n = 5–8/group). C: fibrosis 5 days after MI in the IZ was identified by picrosirius red staining and quantified using the ImageJ software (n = 5–8/group). D: representative gelatin zymographic photographs and densitometric quantification of matrix metalloproteinase (MMP) activities in homogenates of sham and IZ from WT and TG mice showing attenuated MMP-9 and unaltered MMP-2 activity in TG compared with WT mice (n = 5/group). E: representative immunoblot photographs and densitometric quantification of MMP-9, normalized to GAPDH, in homogenates of sham and IZ myocardium from WT and calpastatin overexpressing TG mice 5 days after MI. Data are presented as means ± SE. Statistical significance was obtained using a one-way ANOVA analysis followed by a Bonferroni posttest: *P < 0.05; **P < 0.01; ***P < 0.001.
Calpastatin level in CD4+ T cells is a critical determinant of the post-MI repair process. Given the considerable reduction in CD4+ T-lymphocyte recruitment to the BZ in TG mice and the fact that CD4+ lymphocytes promote macrophage polarization to the M2 phenotype (4, 19), we examined more specifically the consequences of calpastatin overexpression in lymphocytes on the postinfarct inflammatory response. To consolidate the hypothesis that calpastatin overexpression within T cells is a crucial determinant of their impaired recruitment post-MI, we examined T-cell infiltration in the BZ of lymphopenic Rag1−/− mice receiving WT or TG splenocytes (add when they receive the splenocytes compared with the time of MI).

Five days after MI, the time point that corresponds to the peak incidence of cardiac rupture, Rag1−/− mice receiving TG or WT splenocytes showed comparable LV dilatation and contractile dysfunction (Fig. 5A) and infarct size (Fig. 5B). At the cellular level, the recruitment of TG splenocytes to the infarcted site lagged behind that observed in mice receiving WT splenocytes (Fig. 5C). This result was not attributable to differential recruitment of CD19+ B lymphocytes and was not associated with altered expression of M1 markers (Fig. 5D) but with reduced levels of M2 markers in the myocardium of mice receiving TG splenocytes (Fig. 5E). Finally, post-MI collagen deposition in the BZ was attenuated in the myocardium of mice receiving TG splenocytes (Fig. 5F).

Taken together, these findings suggest that increased calpastatin levels in CD4+ T cells might exert a detrimental effect on the post-MI scar formation.

DISCUSSION

Our study indicates that limiting the activation of calpains by ubiquitous overexpression of calpastatin increases mortality by LV rupture during the repair phase in mice with large transmural MI. This impaired healing process is characterized by an inadequate recruitment/activation of CD4+ T lymphocytes and M2 macrophages and by a decreased deposition of collagen in the BZ.

The post-MI inflammatory response serves to remove tissue debris, supply growth factors, and promote granulation tissue and scar formation (9). A well-proportioned, sufficient, but nonexcessive inflammatory response seems necessary to promote adequate scar healing and prevent adverse remodeling. Both reduced and excessive inflammation results in nonadaptive fibrosis leading to cardiac dysfunction instead of the adaptive scar formation (9, 33). An insufficient or impaired inflammation can promote cardiac rupture after MI (19, 35, 37). The macrophage activation immediately following MI has emerged as one of the key features of the post-MI inflammatory reaction. Simplistically, there are two major subsets of macrophages: classically activated M1 that secrete large amounts of proinflammatory mediators and alternatively activated M2 macrophages that promote tissue repair (20, 26). Early depletion of macrophages increased mortality while excess of M2 macrophages leads to adverse remodeling and congestive heart failure (26, 56). In our study, inhibition of calpain activity significantly impaired the scar formation and promotes cardiac rupture most likely by limiting the presence of CD4+ cells and the subsequent activation of the M2 macrophages. Moreover, we have shown that inhibition of calpain could also directly reduce M2 activation.

Our data are in contrast with those of pharmacological calpain inhibition. Indeed, in this model, myocardial structure and function at short-term (4 days) post-MI were preserved (36). At long term (>4 wk) post-MI, both calpain deletion (34) and calpastatin overexpression (58) limit infarct expansion and myocardial remodeling/dysfunction. In ischemia/reperfusion models, calpain activation during reperfusion contributes to myocardial dysfunction and cell death (21). Results derived from different calpain inhibition strategies after myocardial ischemia in mice should be interpreted with caution, since fundamental differences may exist in the experimental design.
and data appearing contradictory at first sight might be reconciled. These differences concern the tissue selectivity of calpain inhibition (cardiac specific vs. ubiquitous), the method used to reduce calpain activity (pharmacological, calpastatin-mediated, calpain knockout), the model of myocardial ischemia (large MI, small-moderate MI, ischemia/reperfusion), and the duration of follow-up (short-term addressing the acute phase vs. long-term addressing chronic heart failure).

The infarct size constitutes a crucial determinant of cardiac rupture and LV remodeling. C57BL/6J mice do not present cardiac rupture when the infarct size is ≤30% of the LV myocardium, and the rate of cardiac rupture increases significantly with more extensive necrosis (13). Moreover, larger MI provokes a more important local and systemic inflammatory reaction and constitutes a model of heart failure on top of myocardial ischemia. Similar calpain inhibition strategies could yield divergent results with regard to mortality and LV remodeling/function if the initial MI size is substantially different. Indeed, the beneficial effect of ubiquitous calpastatin overexpression on LV remodeling/function found recently by Ye et al. (58) in a model of limited MI size (27% of the LV myocardium) is not necessarily contradictory to our results suggesting deleterious effects of ubiquitous calpastatin overexpression after large MI. Of note is that in the present study the mortality rate in the control group was comparable with that observed in other murine MI studies (22, 24, 50).

Calpain inhibition strategies limited to cardiac myocytes cannot be directly compared with a systemic calpain inhibition approach such as the ubiquitous calpastatin overexpression used in the present work or pharmacological inhibition strategies used by others. Because post-MI wound healing implies a complex interplay among cardiac myocytes, inflammatory cells, fibroblasts, and endothelial cells, etc., divergent results between cardiac-specific compared with ubiquitous calpain inhibition models are to be expected. In cardiac myocyte-specific calpain inhibition models (12, 34), calpain expression and activity are unaltered in nonmyocardial cells. Thus inflammatory cell recruitment, activation, and differentiation following MI, as well as the differentiation of fibroblasts into myofibroblasts, are not primarily affected in these myocardium-specific calpain inhibition models. Therefore, the beneficial effects of reduced calpain activity post-MI on LV remodeling and mortality found by Ma et al. (34) are compatible with the deleterious effects observed in the present study, since calpain activity is preserved in all other cell types except in cardiac myocytes in the study of Ma et al., including lymphocytes.

Fig. 4. Calpastatin overexpression is associated with blunted M2 macrophage activation. A and B: qRT-PCR analysis of M1 activation markers (IL-1β, IL-6, TNF-α, iNOS) and M2 macrophage activation markers (CD206, Arg-1, TGF-β1, IL-10) in homogenates of sham and IZ myocardium 5 days after MI, normalized to GAPDH as internal control (n = 6/group). C and D: qRT-PCR analysis of M1 macrophage activation markers (IL-1β, TNF-α) and M2 macrophage activation markers (CD206, Arg-1, TGF-β1) in peritoneal macrophages from WT and TG mice after stimulation with 50 ng/ml IFN-γ + 10 ng/ml LPS for M1 macrophage activation or with 10 ng/ml of IL-4 for M2 macrophage activation for 24 h or left untreated. Values are presented relative to 18S ribosomal RNA as internal control (n = 6/group). Data are presented as means ± SE. Statistical significance was obtained using a one-way ANOVA analysis followed by a Bonferroni posttest: *P < 0.05; **P < 0.01; ***P < 0.001. IL, interleukin; TNF-α, tumor necrosis factor-α; iNOS, inducible nitric oxide synthase; Arg-1, arginase 1; TGF-β, transforming growth factor-β; IFN-γ, interferon-γ; LPS, lipopolysaccharides.
macrophages, and fibroblasts, and might therefore not provoke defective scar healing. The calpain system has been shown to be essential for scar healing by promoting the infiltration of inflammatory cells (42, 49). Our data strongly argue for a role of calpain in both inflammatory cells recruitment and activation.

CD4+ T lymphocytes and macrophages are key players of this inflammatory process during the first week post-MI. They infiltrate the injured myocardium in the inflammatory phase and remain until the end of wound healing (51, 57). Macrophages in the IZ polarize into two distinct phenotypes with different functions: the CCR2-dependent proinflammatory M1, recruited during the initial phase (days 1–4), and the CX3XR1-dependent reparative M2 phenotype, with recruitment culminating at days 4–7 post-MI (8, 39, 40, 51, 55). M2 macrophages facilitate scar healing and regeneration by promoting myofibroblast activation and collagen deposition. Macrophage depletion prevents these processes and thereby impairs myocardial wound healing post-MI (1, 10, 41, 56). Recruited CD4+ T lymphocytes favor the differentiation of macrophages into

Fig. 5. Adoptive transfer of calpastatin overexpressing splenocytes in lymphopenic Rag1−/− mice is associated with reduced T-lymphocyte recruitment and blunted M2 macrophage activation 5 days after myocardial infarction. A: graphical echocardiographic representations of left ventricular dimensions and contractile function 5 days after MI of Rag1−/− mice receiving splenocytes either from WT or TG mice (n = 8–9/group). B: quantification of infarct size using Masson’s trichrome-stained sections (n = 5–8/group). C: Graphical representation of immunohistological analysis of the infiltration of CD3+ T lymphocytes and CD19+ B lymphocytes in the IZ (n = 5–8/group). D and E: qRT-PCR analysis of M1 (IL-1β, IL-6, TNF-α, iNOS) and M2 macrophage activation markers (CD206, Arg-1, TGF-β1) in homogenates of sham and IZ myocardium. Values are represented relative to GAPDH as internal control (n = 5–8/group). F: collagen deposition detected by picrosirius red staining in the IZ, quantified using the ImageJ software (n = 5–8/group). Data are presented as means ± SE. Statistical significance was obtained using a one-way ANOVA analysis followed by a Bonferroni posttest: *P < 0.05.
the reparative M2 phenotype (16, 31). In mice lacking CD4+ T cells, a defect in collagen synthesis and angiogenesis is observed within the IZ, leading to increased LV dilation and more cardiac rupture (19). In the present study, the markedly decreased infiltration of CD4+ T lymphocytes found in the IZ in TG mice 5 days after MI corroborates previous results indicating that calpain inhibition in T lymphocytes hampers their recruitment (2, 28).

To gain further insight into the respective roles of M2 macrophage differentiation and CD4+ T-lymphocyte recruitment post-MI independent of the effects of calpastatin expression in other cell types, we performed adoptive splenocyte transfer from WT and from TG mice into lymphopenic Rag1−/− mice. Since in lymphopenic Rag1−/− mice T-cell recruitment in the IZ was only inhibited in those receiving TG splenocytes, calpastatin overexpression within T cells rather than that in cardiac myocytes seems the determinant factor of impaired T-cell recruitment.

The reduced infiltration of macrophages in the IZ in TG mice 5 days post-MI is in accordance with data from the literature (6, 29, 59). Decreased recruitment of CD4+ T lymphocytes in TG mice might have contributed to the limited differentiation of macrophages into the M2 phenotype and thereby to the impaired reparative response. Interestingly, calpastatin overexpression did not alter the number of M1 macrophages and the expression of MCP-1. The defective scar healing found in TG-MI mice is consistent with results found in CX3CR1−/− mice (17, 41) showing reduced recruitment of reparative M2 macrophages. Decreased recruitment of CD4+ T lymphocytes in TG mice might have contributed to the limited differentiation of macrophages into the M2 phenotype and thereby to the impaired reparative response.

To examine if calpain inhibition in macrophages impacts on their polarization independently of external stimuli such as T lymphocytes, we provoked macrophage polarization in vitro to the M1 and the M2 phenotype. The fact that polarization of TG macrophages into M2 was completely abolished in this in vitro setting indicates that limitation of calpain activity within these cells exerts an inhibitory effect on differentiation into the reparative phenotype and might therefore contribute to defective scar formation post-MI.

Conclusion and perspectives. After large transmural MI, ubiquitous calpain inhibition is detrimental by favoring death from cardiac rupture within a week after MI and adverse LV remodeling in survivors. Our results support the hypothesis that calpain activation post-MI is essential for a well-balanced inflammatory response, which is a prerequisite of an adequate wound healing. Limited calpain activation by calpastatin overexpression inhibits essentially the reparative component of the post-MI inflammatory response, exacerbating the risk of defective scar healing. Considering that cardiomyocyte-specific calpain inhibition was previously found to be protective post-MI, the deleterious effects observed here might be explained at least in part by inhibition of the calpain activity in inflammatory cells.

Taken together, systemic calpain inhibition seems not to be a promising approach to improve outcome after large MI. Future strategies aimed at limiting calpain activation might be more promising if they selectively target calpains in cardiac myocytes, without affecting calpain activity in other cell types such as inflammatory cells.

Limitations of the study. Despite a number of arguments in favor of an impact of calpastatin overexpression in the inflammatory cells on the observed phenotype, in the absence of reciprocal bone marrow transplantation experiments we cannot exclude that calpastatin overexpression in other cell types might also have contributed to the observed effects, such as endothelial cells that play an important role in the recruitment of inflammatory cells. Moreover, the causal relationship between the reduced recruitment of the reparative inflammatory cell phenotypes (M2 macrophages and T lymphocytes) and the defective scar repair in the IZ was not proven and remains therefore speculative. This assumption seems plausible since the profibrotic role of these cell types is established in the literature, as well as the link between impaired fibrosis and increased risk of cardiac rupture.

Finally, the MI model by permanent coronary ligation that was used here is fundamentally different from ischemia/reperfusion. Therefore, the presented results might not be extrapolated on ischemia/reperfusion, especially data concerning inflammatory cell recruitment collected at day 5 after myocardial injury.

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

AUTHOR CONTRIBUTIONS

REFERENCES


