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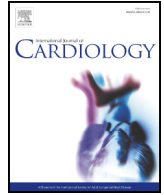
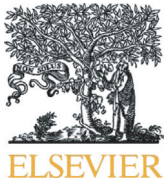
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## Reg3 $\beta$ is associated with cardiac inflammation and provides prognostic information in patients with acute coronary syndrome

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### ABSTRACT

**Background:** Regenerating islet-derived protein 3 beta (Reg3 $\beta$ ) is a cardiomyocyte-derived chemokine for macrophages that is upregulated after myocardial infarction (MI) in mice. Here, we hypothesized that monitoring Reg3 $\beta$  expression might provide specific information on the degree of cardiac inflammation, which is a key determinant in disease progression and prognosis of patients with acute coronary syndrome (ACS).

**Methods and results:** The expression of Reg3 $\beta$  and other inflammatory markers including C-reactive protein (CRP) and myeloperoxidase (MPO) was measured by immunoblotting at serial time points in the hearts and serum of mice with acute MI. We identified a rapid increase of Reg3 $\beta$ , CRP and MPO expression in cardiac tissue and serum within the first 24 h after MI. The expression of Reg3 $\beta$  peaked at day 4 and thereby paralleled the kinetic profile of the early immune-inflammatory response at sites of cardiac injury, which has been characterized by multicolor flow cytometry.

In a retrospective analysis including 322 ACS patients and 117 apparently healthy individuals, we detected increased Reg3 $\beta$  serum concentrations in ACS patients on admission by ELISA. Multiple regression analysis revealed significant relationships between Reg3 $\beta$  and hs-CRP, age, diabetes and NT-proBNP in ACS. Moreover, elevated Reg3 $\beta$  levels on admission were associated with an increased risk of death independent of cardiovascular risk factors and hs-CRP. **Conclusions:** Reg3 $\beta$  is a prognostic biomarker for ACS and is strongly associated with the intensity of cardiac inflammation. Accordingly, Reg3 $\beta$  may complement established strategies of acute risk assessment in the management of ACS.

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### 1. Introduction

The infiltration and local expansion of immune cells contribute to the pathogenesis, progression and complications of acute myocardial infarction (MI) [1]. Among different classes of leukocytes, macrophages constitute one major immune cell population that accumulates at the site of myocardial damage in great numbers, and that is required for successful tissue repair after MI [2–4]. Insufficient, excessive or prolonged recruitment of macrophages, however, has been associated with adverse cardiac remodeling and transition to post-infarction heart failure [4–7].

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<sup>2</sup> This author takes responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

The recruitment of macrophages and other leukocyte subsets is orchestrated by chemokines that direct cellular trafficking and positioning within the injured tissue [8]. In a murine model of MI, we recently identified regenerating-islet derived protein 3 $\beta$  (Reg3 $\beta$ ), also known as pancreatitis-associated protein, as a cardiomyocyte-derived chemokine that attracts macrophages to the site of injury [9]. Using mutant mice with a constitutive knockout of Reg3 $\beta$ , we demonstrated that the local secretion of Reg3 $\beta$  is instrumental for a sustained accumulation of macrophages in the infarct, and subsequent tissue repair and survival after MI.

Given their importance for cardiac repair and remodeling, secreted inflammatory mediators may serve as biomarkers and provide insight in the pathophysiology and prognosis of patients with acute coronary syndrome (ACS) [2,10–12]. Established inflammatory biomarkers such as C-reactive protein (CRP), however, are rather unspecific as they do not provide information about the origin or site of injury and do not reflect distinct dynamics of inflammatory responses in the heart [13].

Based on its local production by cardiomyocytes and relevance for directed macrophage recruitment to the damaged heart, we hypothesized that monitoring Reg3 $\beta$  expression may provide information on the degree of cardiac inflammation [9]. Accordingly, we first studied and compared the expression kinetics of Reg3 $\beta$  to other inflammatory markers and leukocyte subpopulations within the infarcted heart of mice. To assess the potential of Reg3 $\beta$  as a biomarker in ACS, we quantified Reg3 $\beta$  expression in serum samples from infarcted mice and patients with ACS and apparently healthy individuals.

## 2. Materials and methods

### 2.1. Mice and surgical procedures

Ten to 12 week-old male C57BL/6J mice were subjected to permanent ligation of the left anterior descending coronary artery (LAD) as previously described [9]. All animal experiments were performed in accordance with German animal protection laws and were approved by the local governmental animal protection committee Regierungspräsidium Darmstadt.

### 2.2. Immunoblotting of Reg3 $\beta$ , CRP and MPO in infarcted hearts and serum of mice

At indicated time points, left ventricles of infarcted mice were dissected into the infarcted zone (IZ) that is composed of the border zone and apical granulation tissue. The non-infarcted remote zone (RZ) was also harvested and homogenized in lysis buffer as previously reported [9]. In parallel, serum samples were collected in serum collection tubes (Kabe Labortechnik) and diluted 1:50 in the same lysis buffer. Subsequently, SDS-PAGE was performed on Gradient NuPAGE 4–12% Bis-Tris gels (Invitrogen), followed by protein transfer on nitrocellulose membranes (Invitrogen). Equal loading of samples was controlled by membrane staining with RedAlert Western blot stain (Merck). For immunoblotting, the following antibodies were used: sheep anti-mouse Reg3 $\beta$  (R&D Systems), goat anti-mouse CRP (R&D Systems) and goat anti-mouse MPO (R&D Systems). Secondary antibodies conjugated with horseradish peroxidase were ordered from R&D Systems. Visualization and semi-quantitative analysis of immunoreactive proteins was performed by using the ChemiDoc MP Imaging System (Bio-Rad) and Image Lab 5.0 software (Bio-Rad). All proteins were quantified by the mean volume pixel density of the corresponding bands. In addition, relative expression ratios between the IZ and RZ were calculated in order to compare spatial accumulation over time.

### 2.3. Flow cytometry-based analysis of leukocytes within the infarcted hearts of mice

The accumulation of leukocytes and their subpopulations including neutrophil granulocytes, tissue macrophages and dendritic cells, T lymphocytes and B lymphocytes in the IZ was analyzed by flow cytometry as described recently [9]. The following antibodies were used in two multicolor FACS panels: rat anti-mouse CD45-PE-Cy5.5 monoclonal antibody (Clone: 30-F11, eBioscience), rat anti-mouse CD45-FITC monoclonal antibody (Clone: 30-F11, eBioscience), rat anti-mouse CD11b-PE-Cy5 monoclonal antibody (Clone: M1/70, eBioscience), rat anti-mouse CD11b-APC-eFluor780 monoclonal antibody (Clone: M1/70, eBioscience), rat anti-mouse Ly6G-PE-eFluor610 monoclonal antibody (Clone: RB6-8C5, eBioscience), rat anti-mouse CD64-AlexaFluor700 monoclonal antibody (Clone: 290,322, R&D systems), rat anti-mouse MerTK-PE-Cy7 (Clone: DS5MMER, eBioscience), rat anti-mouse MHC class II-APC-eFluor780 monoclonal antibody (Clone: M5/114.15.2, eBioscience), rat anti-mouse CD11c-FITC monoclonal antibody (Clone: N418, eBioscience), rat anti-mouse CD115-APC monoclonal antibody (Clone: AFS98, eBioscience), rat anti-mouse CD103-eFluor450 monoclonal antibody (Clone: 2E7, eBioscience), rat anti-mouse CD3e-PerCP-Cyanine5.5 monoclonal antibody (Clone: 145-2C11), rat anti-mouse CD19-PE-eFluor610 monoclonal antibody (Clone: eBio1D3, eBioscience) and rat anti-mouse

IgM-eFluor450 monoclonal antibody (Clone: IL41, eBioscience). Within the population of CD45<sup>hi</sup> leukocytes, neutrophils were defined as CD45<sup>hi</sup>/CD11b<sup>hi</sup>/Ly6G<sup>hi</sup>/CD115<sup>lo</sup>, tissue macrophages as CD45<sup>hi</sup>/CD11b<sup>hi</sup>/Ly6G<sup>lo</sup>/(MerTK/CD64)<sup>hi</sup>/CD115<sup>lo</sup>, and myeloid dendritic cells as CD45<sup>hi</sup>/CD11b<sup>hi</sup>/Ly6G<sup>lo</sup>/(MerTK/CD64)<sup>lo</sup>/(CD11c/MHC-II/CD103)<sup>hi</sup>. Referring to lymphocytes, B lymphocytes were defined as CD45<sup>hi</sup>/CD11b<sup>lo</sup>/(CD19/IgM)<sup>hi</sup> and T lymphocytes as CD45<sup>hi</sup>/CD11b<sup>lo</sup>/(CD19/IgM)<sup>lo</sup>/CD3<sup>hi</sup>. Flow cytometry was performed with the BD FACS Aria III cell sorter. Data were analyzed using the BD FACS Diva v6 Software. For calculation of the total cell number of leukocytes in the infarcted area, samples were normalized to the weight of infarct areas.

### 2.4. ACS patients

Between October 2007 and October 2011, we recruited 322 patients with a final diagnosis of ACS [unstable angina, non-ST-elevation MI (NSTEMI), or ST-elevation MI (STEMI)] who were admitted to Hannover Medical School. ACS was diagnosed according to the criteria of the Joint ESC/AACF/AHA/WHF Task Force and guidelines of the European Society of Cardiology [14]. In brief, NSTEMI was diagnosed in patients showing a rise and/or fall of cardiac troponin above the diagnostic reference threshold for MI during serial testing for at least 12 h. Patients were diagnosed with unstable angina if they had cardiac troponin T (cTnT) concentrations consistently below the diagnostic threshold or persistent cTnT elevations. Furthermore we also required that these patients had signs of myocardial ischemia on echocardiography (ECG), a history of coronary artery disease, or at least 1 stenosis of >50% in a major coronary artery on angiography [15,16]. Cardiac magnetic resonance imaging was not included in the patient's examination. We measured Reg3 $\beta$  in serum samples obtained on admission median (25th–75th percentiles) 265 (130–771) minutes after the onset of symptoms. ECG was performed during the initial hospital stay. There was no pre-specified point in time since all treatment and management decisions were left to the discretion of the attending cardiologist. Coronary angiography was performed in 303 (94%) patients. The number of vessels showing at least a 50% luminal stenosis was documented. Left main coronary artery stenosis was classified as 2-vessel disease. Left ventricular (LV) systolic function was assessed by angiography or echocardiography in 284 (88%) patients. Treatment decisions were left to the discretion of the attending cardiologist. Follow-up for cardiovascular mortality was accomplished by telephone contact or questionnaire at least 6 months after discharge. The study was approved by the ethics committee of Hannover Medical School. All patients provided written informed consent [16–18].

### 2.5. Apparently healthy individuals

We obtained serum samples from 117 apparently healthy individuals who were recruited in Heidelberg, Germany. Individuals taking regular medication, individuals with chronic drug or alcohol abuse, and individuals with established cardiovascular disease or other chronic disease or acute illness were excluded. All participants were required to have non-pathologic serum creatinine, aspartate aminotransferase, alanine aminotransferase, thyroid-stimulating hormone, and hemoglobin concentrations, normal leukocyte and platelet counts, a normal resting 12-lead electrocardiogram, normal physical examination, normal oral glucose tolerance test, and a serum N-terminal pro-B-type natriuretic peptide (NT-proBNP) concentration below 125 ng L<sup>-1</sup>. All individuals had undergone cardiac magnetic resonance imaging with dobutamine or adenosine stress and were found not to have left ventricular (LV) hypertrophy, LV dilation, LV systolic dysfunction, valvular heart disease, or stress-induced LV wall motion abnormalities or perfusion defects. All participants provided written informed consent, and the ethics committee of the University of Heidelberg approved the study [17].

### 2.6. Biomarker assays of Reg3 $\beta$ and other biomarkers in human individuals

Concentration of circulating Reg3 $\beta$  in serum was determined by a commercially available ELISA according to the manufacturer's instructions (PancrePAP, DYNABIO SA, La Gaude, France). According to the manufacturer's information, no cross-reactivity have been observed with other inflammation-related cytokines including IL-2, IL-6, IFN- $\gamma$ , TNF- $\alpha$  and *E. coli*-derived proteins. Cross-reactions with other members of the Reg family, however, have not been documented to date.

NT-proBNP was measured by a sandwich immunoassay, CRP by a high-sensitivity (hs) latex particle-enhanced immunoassay, and cTnT by a hs electrochemiluminescence assay (all from Roche Diagnostics). Estimated glomerular filtration rate (eGFR) was calculated with the Modification of Diet in Renal Disease equation [18].

### 2.7. Statistical analysis

Data are presented as mean with SEM, median with 25th and 75th percentiles, or numbers and percentages. Differences between mouse samples obtained from serum and cardiac tissue at different time points were assessed by two-tailed unpaired t-test. Referring to human samples, we compared continuous variables with the Kruskal-Wallis test. Reg3 $\beta$  was further related to baseline characteristics by use of single and multiple variable linear regression analysis. Age, NT-proBNP, hs-CRP, and hs-cTnT were logarithmically transformed (ln-transformed) before their use in these analyses. Kaplan-Meier survival curves were examined to assess the relationship between Reg3 $\beta$  and all-cause mortality. To determine whether Reg3 $\beta$  was independently associated with outcome, Cox regression models were built that included age and sex (model 1). Diabetes, hypertension, and current smoking were added to create model 2. Finally, hs-CRP was added

to create model 3. A 2-tailed  $P$  value  $<0.05$  was considered to indicate statistical significance. All analyses were performed with SAS 9.1 (SAS Institute), STATA 14.0 (StataCorp) and GraphPad Prism 5.0 (GraphPad Software).

### 3. Results

#### 3.1. Kinetic profile of Reg3 $\beta$ and inflammatory markers in cardiac tissue and serum of mice after myocardial infarction

In view of a direct relationship between local Reg3 $\beta$  production and intensity of inflammation at sites of injury, we first monitored Reg3 $\beta$  expression throughout the process of myocardial healing in cardiac tissue of infarcted mice by immunoblotting (Fig. 1A). We found a rapid increase of Reg3 $\beta$  within the infarction zone (IZ) when compared with the non-infarcted remote zone (RZ) at day 1 post-MI (Fig. 1A, B). This elevation in local Reg3 $\beta$  expression peaked at day 4 post-MI and remained sustained within the following days post-MI at the site of injury (Fig. 1A, B). Intriguingly, we detected a continuous increase of Reg3 $\beta$  expression within the non-infarcted RZ over the period of observation, albeit less pronounced than in the IZ (Fig. 1B).

We further analyzed the kinetic profile of established inflammatory markers including C-reactive protein (CRP) and myeloperoxidase (MPO) [19,20] (Fig. 1A). Similar to Reg3 $\beta$ , CRP and MPO were increased early after the onset of MI (Fig. 1A, B). Thereafter, CRP gradually declined over the period of observation, while MPO remained high until day 4 before its expression dropped from day 7 to non-significant levels at day 14 post-MI (Fig. 1A, B).

The relative expression ratios of Reg3 $\beta$ , CRP and MPO between IZ and RZ were additionally calculated in order to compare their spatial accumulation over time after MI. Here, it became apparent that Reg3 $\beta$ , CRP and MPO particularly accumulate within injured regions during the first 24 h followed by a gradual decline over the next 14 days (Fig. 1C).

We complementarily monitored Reg3 $\beta$  expression in serum after MI. Basal serum samples were harvested and utilized as a reference to calculate relative changes in Reg3 $\beta$  expression over time. We observed a 2.9-fold increase of circulating Reg3 $\beta$  within the first 24 h after MI (Supplementary Fig. 1A). Afterwards, the levels of Reg3 $\beta$  increased over time and peaked at day 7 with an 8.5-fold increase (Supplementary Fig. 1A). In line with cardiac tissue, we detected a sustained elevation of Reg3 $\beta$  (5.3-fold) at day 14 post-MI (Supplementary Fig. 1A).

By contrast, the expression kinetics of CRP and MPO in serum was characterized by a rapid increase peaking at day 1 (1.8-fold for CRP and 2.9-fold for MPO) (Supplementary Fig. 1B, C). Thereafter, CRP expression dropped but remained moderately elevated during our period of observation, while MPO expression followed a more biphasic course (Supplementary Fig. 1B, C).

#### 3.2. Association of Reg3 $\beta$ expression with immune cell kinetics at the site of ischemic damage

By comparing the kinetic profile of individual leukocyte subsets with the dynamic expression of Reg3 $\beta$  within the infarcted region, we explored whether its expression levels are directly associated with the accumulation of distinct immune cells during myocardial healing. Overall, we observed similar temporal courses of leukocyte numbers and Reg3 $\beta$  expression at the site of cardiac injury within the first 4 days post-MI (Fig. 2A). This association included virtually all major immune cell subsets including neutrophil granulocytes, tissue macrophages, dendritic cells, T lymphocytes as well as B lymphocytes upon day 4 post-MI (Fig. 2B–F). Thereafter, tissue macrophages were sustained at a higher level together with local Reg3 $\beta$  expression at day 7 post-MI, while all other leukocyte subpopulations declined in numbers (Fig. 2B–F). At day 14 post-MI, we finally observed a strong reduction of all major immune cell subsets, whereas Reg3 $\beta$  expression levels were maintained (Fig. 2B–F). Hence, these findings support the initial idea that local expression of Reg3 $\beta$  is

positively associated with the degree of cardiac inflammation within the early phase of myocardial healing. Similar associations between CRP, MPO and immune cell dynamics during the early phase of cardiac repair, however, were not observed (Supplementary Fig. 2).

#### 3.3. Circulating concentrations of Reg3 $\beta$ in healthy individuals and patients with ACS

The association of Reg3 $\beta$  with inflammation in cardiac tissue and its systemic increase in serum upon MI in mice prompted us to analyze Reg3 $\beta$  concentrations in 322 human patients admitted with an ACS. For reference, we measured serum Reg3 $\beta$  concentrations in 117 apparently healthy individuals. Reg3 $\beta$  levels were detected in all 439 samples by using an ELISA that has been previously used in a multicenter study [21].

The median (25th–75th percentiles) Reg3 $\beta$  concentration in apparently healthy individuals was 5.54 (4.57–6.88)  $\mu\text{g L}^{-1}$  (Fig. 3A). Thereof, serum concentrations of 59 male and 58 female individuals with a median (25th–75th percentiles) age of 42 (29–52) years displayed similar Reg3 $\beta$  concentrations [males, 5.32 (3.82–6.58)  $\mu\text{g L}^{-1}$ ; females, 5.94 (4.81–7.24)  $\mu\text{g L}^{-1}$ ]. With increasing age, we observed a slight increase of circulating Reg3 $\beta$  levels ( $R = 0.24$ ;  $P = 0.029$ ; Supplementary Fig. 3).

The median (25th–75th percentiles) concentration of Reg3 $\beta$  in ACS patients on admission was 9.52 (6.42–16.62)  $\mu\text{g L}^{-1}$  and significantly higher than in healthy individuals ( $P < 0.001$ ) (Fig. 3A). The patient cohort included more men and was older with 236 men and 86 women and a median (25th–75th percentiles) age of 67 (56–75) years, respectively ( $P < 0.001$ ). An age- and gender-matched analysis of Reg3 $\beta$  in serum of 49 healthy men and 49 ACS male patients with a median age 52 (48–57) years validated increased levels of Reg3 $\beta$  in ACS patients (Supplementary Table 1).

Baseline characteristics of our patient cohort are summarized in Supplementary Table 2. Overall, Reg3 $\beta$  concentrations were significantly higher in females, in older patients, in current smokers, and in patients with diabetes, a history of myocardial infarction, or with more severe coronary artery disease. Increasing Reg3 $\beta$  concentrations were further related to the concentrations of hs-cTnT, NT-proBNP and hs-CRP. Reg3 $\beta$  was inversely related to eGFR. Referring to the type of ACS, we observed a considerable overlap in Reg3 $\beta$  concentrations between patients with unstable angina [9.76 (6.56–18.20)  $\mu\text{g L}^{-1}$ ], NSTEMI [10.53 (6.88–17.50)  $\mu\text{g L}^{-1}$ ] and STEMI [8.61 (5.99–14.79)  $\mu\text{g L}^{-1}$ ] ( $P = 0.06$ ).

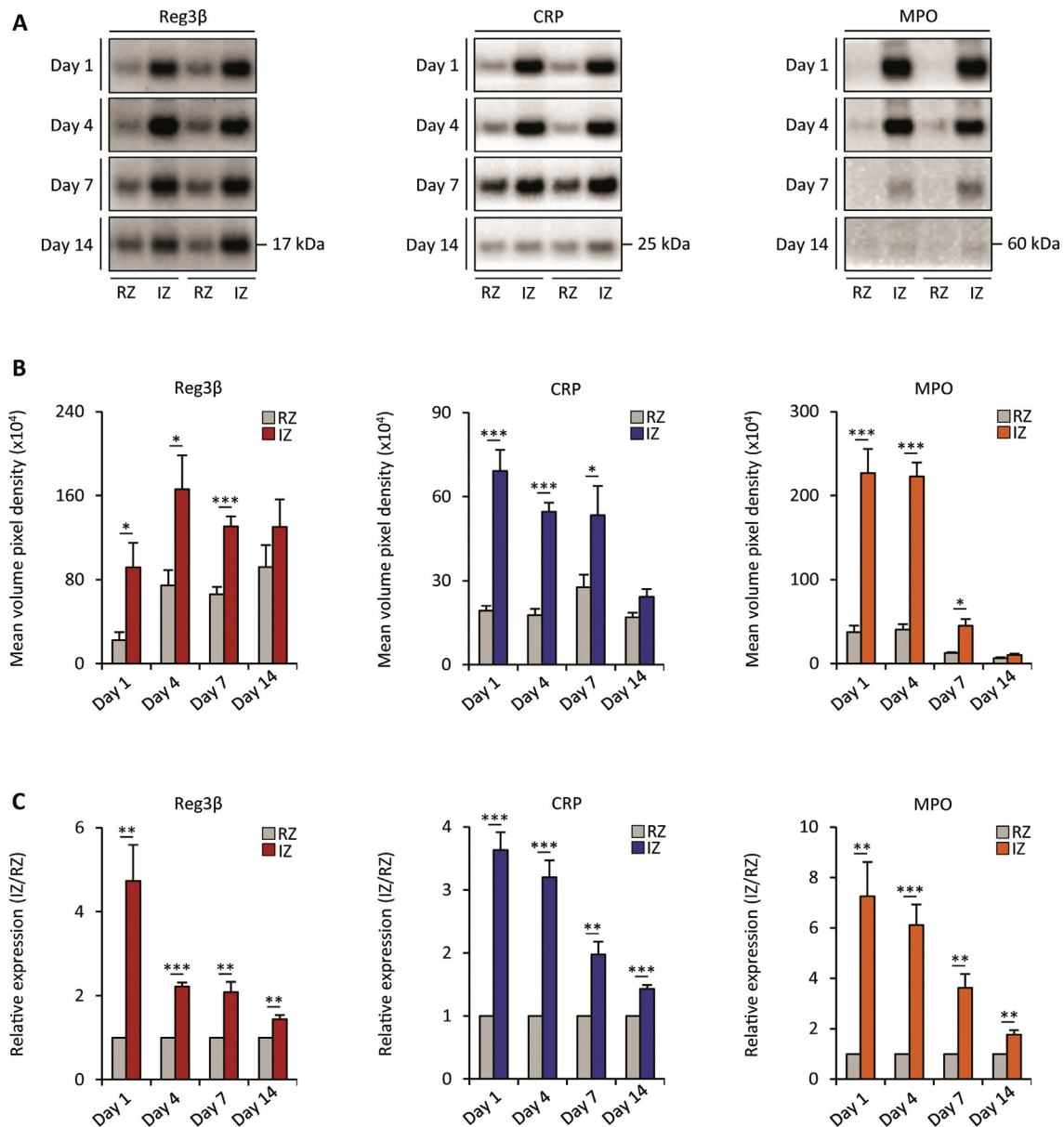
In a multiple regression analysis that used  $\ln(\text{Reg3}\beta)$  as the independent variable, and that included all variables shown in Fig. 3B, age, diabetes, hs-CRP and NT-proBNP were independently associated with Reg3 $\beta$ .

#### 3.4. Association of Reg3 $\beta$ with the risk of acute mortality

During 6 months follow-up 34 patients died. Of note, patients presenting with a Reg3 $\beta$  concentration in the third tertile had the highest mortality (Fig. 4A). Along these lines, patients in the third tertile had a 3.8-fold higher risk of dying within 6 months than patients in the first tertile (95% CI 1.9–4.9;  $P < 0.001$ ). The association of Reg3 $\beta$  serum concentration and mortality remained after adjustment for age and sex (model 1) as well as after including traditional cardiovascular risk factors (model 2) and hs-CRP (Model 3) (Fig. 4B).

### 4. Discussion

We have recently identified Reg3 $\beta$  as a cardiomyocyte-derived chemokine for macrophages, which is increased after MI in mice [9]. Here, we demonstrate that the local expression of Reg3 $\beta$  in the ischemic myocardium of mice parallels the dynamic expression pattern of the inflammatory markers CRP and MPO during the early phase of cardiac healing. The rapid and continuous rise of Reg3 $\beta$  additionally mirrors the kinetic

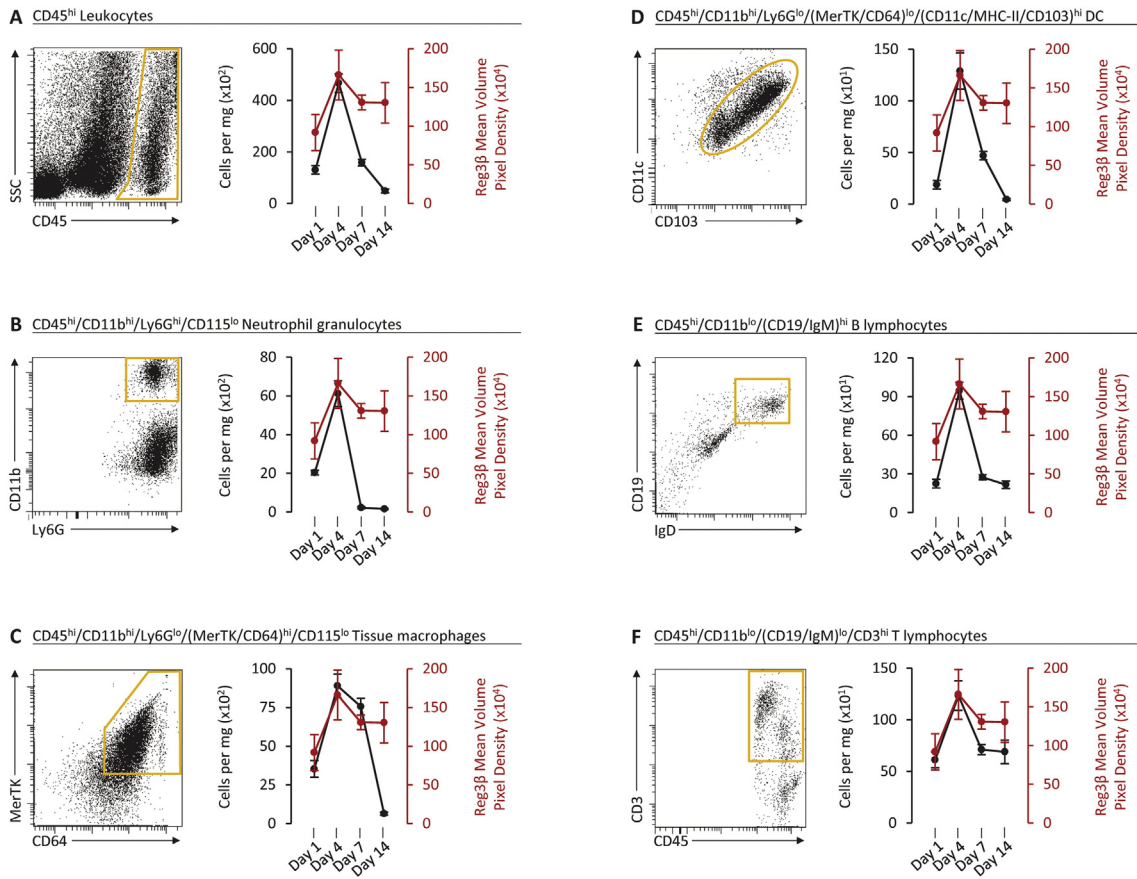


**Fig. 1.** Dynamic expression pattern of Reg3 $\beta$  and inflammatory markers in infarcted hearts of mice. (A) Western blot analysis of Reg3 $\beta$ , CRP and MPO expression in homogenates of infarcted hearts of adult mice fractionated in infarction zone (IZ) and non-infarcted remote zone (RZ). Two representative samples of day-1, day-4, day-7 and day-14 post myocardial infarction (post-MI) are shown. (B) Semi-quantitative analysis of samples described in (A) based on the mean volume pixel density of bands ( $n = 6$  for each time point). (C) Relative expression of Reg3 $\beta$ , CRP and MPO expression within the IZ related to the RZ at indicated time points ( $n = 6$  for each time point). Data are presented as mean  $\pm$  SEM.  $P$  values were determined by two-tailed unpaired t-test. \* $P < 0.05$ , \*\* $P < 0.005$ , \*\*\* $P < 0.0005$ .

profile of virtually all major immune cell subsets within the first 4 days post-MI. Hence, its cardiomyocyte-specific origin, dynamic expression and association with inflammatory markers and leukocyte kinetics imply that Reg3 $\beta$  might be used as a biomarker to monitor the intensity of cardiac inflammation since current tools measuring inflammatory pathways are considered to be imprecise and mostly lack detailed information about the disease site and activity [22].

By means of a multiple regression analysis considering demographic, clinical and biochemical parameters, we similarly identified a strong association between Reg3 $\beta$  and the inflammatory state in serum of ACS patients. In addition to hs-CRP, increasing age and diabetes were found to be independently predictive of Reg3 $\beta$  levels, which have been recently shown to be associated with inflammation [23,24]. Previous data from a rodent model also demonstrated an age-related increase of Reg3 $\beta$  in pancreatic cells [25]. This raises the suspicion that age *per se* may cause increased levels of Reg3 $\beta$  in the older ACS patient

population. In our cohort of apparently healthy individuals, however, we observed only a weak correlation between serum Reg3 $\beta$  concentrations and age, which cannot completely explain the 1.7-fold higher median Reg3 $\beta$  concentration in ACS patients. Of note, such changes in serum concentrations with increasing age have been previously described for different biomarkers including NT-proBNP, heart-type fatty acid binding protein (H-FABP) as well as inflammatory cytokines and chemokines [26–28]. By contrast, we did not detect an independent association of Reg3 $\beta$  concentrations with gender in both the ACS cohort and healthy individuals. Thus, we did not find convincing evidence for a gender-specific effect of Reg3 $\beta$  within this cohort. Nonetheless, estimating age- and gender-adjusted thresholds for Reg3 $\beta$  concentrations in order to determine a reference range within larger studies may conclusively help to enhance accurate data interpretation of ACS patients as it has been demonstrated for other biomarkers used in cardiology [26,29].



**Fig. 2.** Comparative analysis of immune cell kinetics with local Reg3β expression at sites of cardiac damage after myocardial infarction. Single cell suspensions obtained from infarcted regions of mice were analyzed flow cytometry to identify and quantify major leukocyte subsets at indicated time points (n = 6 at day-1, n = 7 at day-4, n = 7 at day-7 and n = 6 at day-14). Representative FACS dot plots and surface markers defining leukocytes, neutrophil granulocytes, tissue macrophages, myeloid dendritic cells (DC), B lymphocytes and T lymphocytes are presented in (A–F). Their individual kinetic profile is plotted and compared with local Reg3β expression (marked in red) within the IZ (compare Fig. 1). Data are presented as mean ± SEM.

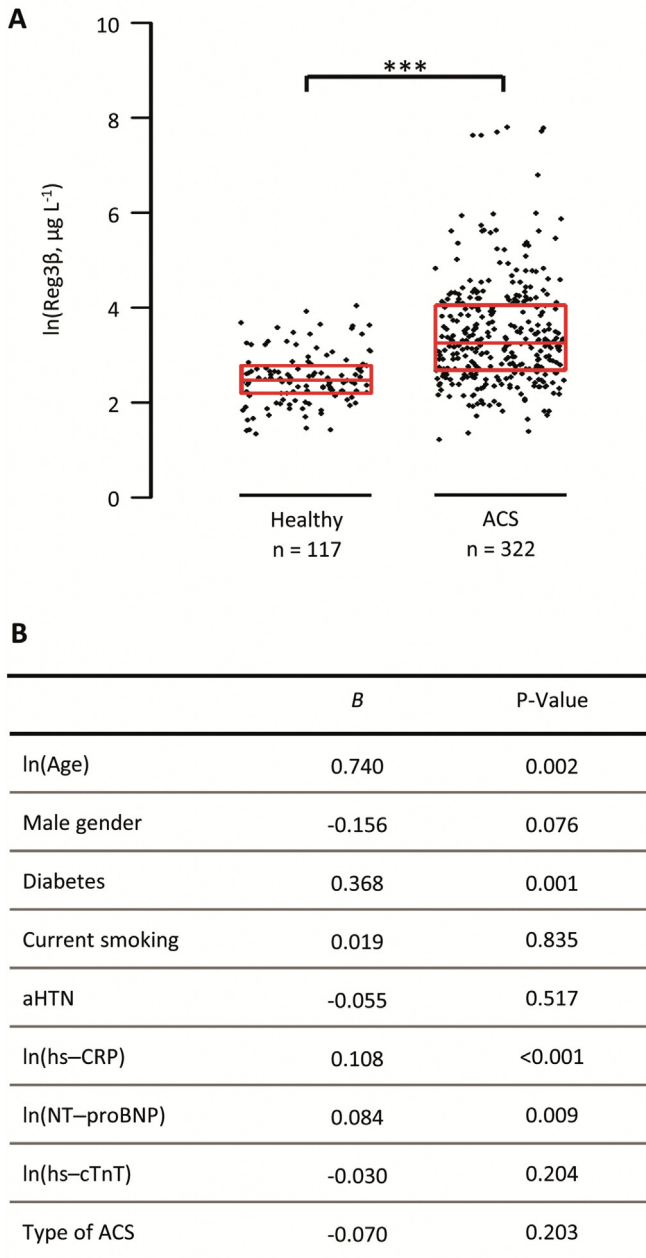
We also observed an independent association of elevated Reg3β concentrations with NT-proBNP in ACS patients. Preclinical and clinical studies have indicated that the expression of this prominent biomarker of acute heart failure may not exclusively depend on cardiac wall stress but on the inflammatory milieu as well [30–33]. Given the association between Reg3β and NT-proBNP, it would be of clinical interest to analyze if prediction models combining both parameters might improve their prognostic power as it has been hypothesized previously [34]. On the other hand, we found an inverse relationship between Reg3β, the type of ACS and hs-cTnT in this study, which suggests that Reg3β levels do not simply reflect the extent of myocardial necrosis at this early stage of ACS [35]. Instead, we hypothesize that immune-inflammatory processes associated with the development and progression of acute cardiac diseases initiate increased synthesis of Reg3β in surviving cardiomyocytes [36]. Along these lines, previous work by our group have demonstrated that an enhanced release of the IL-6 type cytokine Oncostatin M by infiltrating neutrophils and macrophages induces increased Reg3β expression in cardiomyocytes after the onset of myocardial infarction in mice [9]. Of course, we cannot rule out that inflammatory-related preconditions including non-obstructive coronary atherosclerosis and plaque instability may have an impact on systemic Reg3β levels in human individuals. Further studies are needed to clarify the issue if elevated systemic levels of Reg3β are solely confined to the acute inflammatory response after the onset of myocardial ischemia or rather reflect a combined consequence of related preconditions contributing to ACS.

We could finally show that ACS patients in the third tertile of serum Reg3β concentration had the highest risk of overall death during 6

month follow-up. Reg3β remained independently associated with outcome in the context of demographic and traditional cardiovascular risk factors. Even after adding hs-CRP to the model, Reg3β kept its predictive value, indicating that Reg3β provides information not reflected by this traditional inflammatory biomarker.

Though having shown that several inflammatory factors were independently associated with Reg3β levels, the R<sup>2</sup> value of our multiple regression analysis indicates that only one quarter of serum Reg3β concentration can be explained by demographic and biochemical parameters being included in our retrospective study. Other, yet unknown disease conditions and pathophysiological pathways may contribute to the elevated levels of Reg3β in patients presenting with ACS. Furthermore, the restricted number of samples obtained from two heart centers limits the strength and significance of our findings. Larger studies will be needed to specify the predictive value of Reg3β as a biomarker for distinct events after ACS such as recurrent MI and acute heart failure. Along these lines, serial monitoring of Reg3β upon admission might yield additional information on disease progression. Since elevated Reg3β levels are strongly associated with the inflammatory state of ACS patients at the onset of symptoms, it is intriguing to evaluate in further studies if Reg3β is sustained and corresponds with a prolonged and or excessive inflammatory state over time. In addition, serial evaluations of Reg3β levels might be complementarily used in future to assess the specificity and efficiency of therapeutic strategies targeting the immune-inflammatory response within the diseased heart.

In conclusion, our study identifies Reg3β as a prognostic biomarker for ACS. The local production of Reg3β at sites of cardiac damage, its increase in serum and association with inflammatory pathways suggest

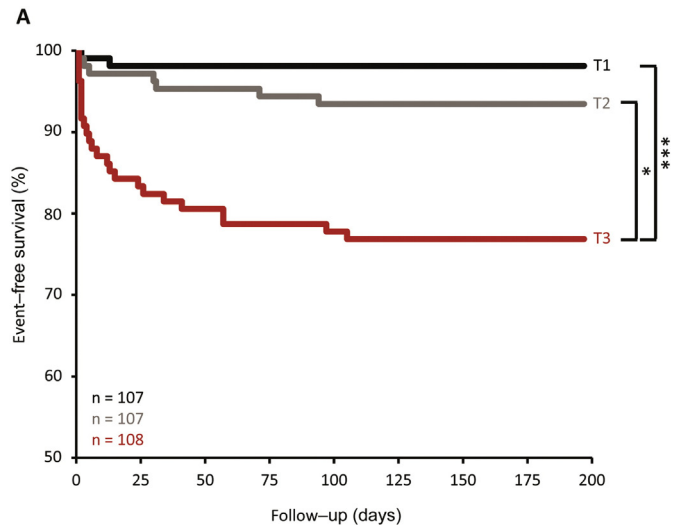


**Fig. 3.** Concentration of Reg3 $\beta$  and its association to baseline variables in serum of patients with acute coronary syndrome. (A) Box plots of ln-transformed concentrations of Reg3 $\beta$  in serum of healthy individuals and patients with acute coronary syndrome (ACS). Each box indicates the interquartile range with a median cross line for both groups. \*\*\* $P < 0.001$  was determined by Kruskal–Wallis test. (B) Multiple regression analysis that used ln(Reg3 $\beta$ ) as the independent variable and that included all variables shown in Supplementary Table 1. The  $R^2$  value of this model was 0.26. aHTN indicates arterial hypertension; hs-CRP, high-sensitivity C-reactive protein; NT-proBNP, N-terminal prohormone of brain natriuretic peptide; hs-cTnT, high-sensitivity cardiac troponin T.

that Reg3 $\beta$  may serve as a novel biomarker to assess the intensity of the inflammatory response during ischemia, which is known to constitute an independent predictor for disease progression and prognosis in ACS patients [9,37,38].

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**B**

Model	Hazard Ratio (95% CI) <sup>a</sup>	P-Value
Unadjusted	3.79 (2.12 – 6.80)	<0.001
Adjusted for		
Model 1	2.60 (1.42 – 4.78)	0.002
Model 2	2.60 (1.41 – 4.80)	0.002
Model 3	2.28 (1.23 – 4.20)	0.009

<sup>a</sup>Hazard ratios (95% CI) associated with tertiles of Reg3 $\beta$  on admission in the context of Model 1, 2 or 3 variables. Model 1 included information on age and sex. Information on diabetes, hypertension and current smoking was added to model 1 to create model 2. Circulating levels of hsCRP were added to model 2 to create model 3.

**Fig. 4.** Cumulative survival and mortality hazard ratio depending on Reg3 $\beta$  concentration in patients with acute coronary syndrome. (A) Kaplan–Meier analysis for event-free survival of ACS patients according to tertiles (T) of Reg3 $\beta$  concentration in serum.  $P$  values were determined by log-rank test. \* $P < 0.05$ , \*\*\* $P < 0.001$ . (B) Hazard ratios (95% CI) associated with tertiles of Reg3 $\beta$  on admission in the context of Model 1, 2 or 3. Model 1 included information on age and sex. Information on diabetes, hypertension and current smoking was added to model 1 to create model 2. Circulating levels of hs-CRP were added to model 2 to create model 3.

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#### Conflict of interest

None declared.

#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijcard.2018.01.043>.

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