**Treatment with anti-RANKL antibody reduces infarct size and attenuates
dysfunction impacting on neutrophil-mediated injury**

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Short running title: RANKL and myocardial ischemia.

Abstract

Selective pharmacological treatments targeting reperfusion injury produced modest protective effects and might be associated with immunosuppression. In order to identify novel and better-tolerated approaches, we focused on the neutralization of receptor activator of nuclear factor kappa-B ligand [RANKL], a cytokine recently shown to activate inflammatory cells (i.e. neutrophils) orchestrating post-infarction injury and repair. Myocardial ischemia (60 minutes) and reperfusion injury was surgically induced in C57Bl/6 mice. In hearts and serum, RANKL was early upregulated during reperfusion. A “one-shot” injection with neutralizing anti-RANKL IgG during ischemia ameliorated myocardial infarct size and function, but not adverse remodelling (determined by Magnetic Resonance Imaging [MRI]) as compared to Vehicle or control IgG. These beneficial effects were accompanied *in vivo* by reduction in cardiac neutrophil infiltration, reactive oxygen species (ROS) and MMP-9 release. Anti-RANKL IgG treatment suppressed sudden peak of neutrophil granule products in mouse serum early after reperfusion onset. *In vitro*, RANK mRNA expression was detected in isolated mouse neutrophils. Co-incubation with neutralizing anti-RANKL IgG abrogated RANKL-induced mouse neutrophil degranulation and migration, suggesting a critical role of RANKL in neutrophil-mediated injury. Conversely, anti-RANKL IgG did not affect salvage pathways in cardiac cells (i.e. ERK p42/p44, Akt and STAT-3) or macrophage cardiac infiltration. Finally, treatment with anti-RANKL IgG showed no effect on B and T lymphocyte polarization (in serum, spleen and infarcted myocardium) and circulating chemokines as compared with Vehicle or control IgG. In conclusion, acute treatment with anti-RANKL IgG improved cardiac infarct size and function by potentially impacting on neutrophil-mediated injury and repair.

Key words: myocardial infarction, inflammation, neutrophils.

1. Introduction

Mechanic or pharmacological reperfusion after an ST-elevation myocardial infarction (STEMI) has been recommended as a pivotal approach to improve survival and preserve cardiac function [1]. However, reperfusion itself was demonstrated to induce myocardial inflammation and injury [2, 3]. Differently from mononuclear leukocytes that were shown to play a pivotal role in the healing of experimental infarction in mice [4, 5], early recruitment and activation of neutrophil subsets (i.e. N1, N2) within the cardiac necrosis were strongly associated with post-infarction myocardial injury [2, 6]. In particular, the abrogation of neutrophil recruitment in the early reperfusion time (within the first 24 hours) was associated with reduced cardiac accumulation of toxic products, such as reactive oxygen species (ROS) and proteases that increased cardiac necrosis and post-ischemic adverse remodelling [7-9]. Several anti-inflammatory treatments were assessed in experimental models of cardiac ischemia in order to selectively counteract neutrophil detrimental effects. Despite controversial and sometimes weak results [10], the more promising strategies were anti-chemokine treatments (such neutralizing antibodies, chemokine binding proteins and chemokine analogues) anti-integrin and anti-complement approaches that were able to abrogate neutrophil infiltration and improve cardiac infarct size and partially function at 24-hours follow up [7-9, 11-13]. In addition, those treatments were not selective for neutrophils, since they abrogated also macrophages and several systemic inflammatory molecules, raising major concerns on the potential induction of immunosuppression that delayed the clinical tests in human beings [7-9]. These studies suggested us to investigate some other inflammatory molecules that should be altered during reperfusion, activate neutrophils and possibly not reduce other cell subsets, thus limiting the risk of systemic immunosuppression.

We focused on the receptor activator of nuclear factor kappa-B ligand (RANKL)/Osteoprotegerin (OPG) system, since the cytokine RANKL via the binding to its transmembrane receptor RANK was recently demonstrated to potently activate human neutrophil degranulation [14]. In addition, the clinical use in osteoporosis of the specific antibody that binds to and inhibits RANKL (i.e. Denosumab) was not clearly associated with a relevant increase in the infection rate [15]. Evidence from both basic and clinical research studies highlighted RANKL as a cytokine playing a critical role in bone resorption in several diseases (including cancer metastasis and myeloma) [16, 17], while high serum levels of OPG (the soluble decoy receptor of RANKL) were also associated with an increased cardiovascular risk [18]. In previously published animal models of liver and brain ischemia, RANKL was also shown to be protective against post-ischemic inflammation and modulates cytokines [19, 20]. On this basis, we tested the potential role of pharmacologic RANKL inhibition with a neutralizing anti-mouse RANKL IgG *in vivo* in the early phase of reperfusion on myocardial infarct size and function, inflammatory cell infiltration (i.e. neutrophils), reactive oxygen species (ROS) production and systemic inflammation. *In vitro*, direct inhibition by anti-RANKL IgG co-incubation on RANKL-induced neutrophil degranulation and migration were assessed.

2. Methods

2.1 In vivo I/R protocol

Male C57Bl/6 mice (8-12 weeks of age) were obtained from the University Medical Centre (CMU) animal facility, Faculty of Medicine, University of Geneva. The investigation conforms to the guidelines from Directive 2010/63/EU, from the National Institutes of Health (updated 2011) and has been approved by the local and

Swiss authorities. The study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. As previously described [21], mice were initially anaesthetized with 4% isoflurane and intubated. After starting mechanical ventilation (tidal volume of 150 μ l, 120 breaths/min) by supplementation with 100% oxygen, anesthesia was maintained with 2% isoflurane. A thoracotomy was performed in the left third intercostal space and the pericardium was removed. Ligature of the left anterior coronary artery at the inferior edge of the left atrium was performed using an 8-0 Prolene suture. A small piece of polyethylene tube was used to secure the ligature without damaging the artery. After 5 minutes of ischemia normal goat IgG control ([CTL IgG], 200 μ g/mouse by R&D Systems, Minneapolis, MN, USA), goat anti-mouse RANKL IgG (neutralizing RANKL bioactivity, at 200 μ g/mouse, R&D Systems) or Control Vehicle (PBS) were administered by intravenous injection. After 60 min of ischemia, the occlusion of the left anterior coronary artery occlusion was released and reperfusion occurred. Reperfusion was confirmed by visible restoration of color to the ischemic tissue. Then, chest was closed and the ventilator removed to restore normal respiration. Adequacy of anesthesia was monitored by careful visual and tactile control of mouse consciousness (changes in breathing rate and volume, heart rate and tearing). Analgesia was administered 30 minutes before surgery and was extended with subcutaneous injection of 0.05 mg/kg buprenorphine HCl every 12 hours in the first 24 hours after surgery. Sham-operated animals were submitted to the same surgical protocol as described but without arterial occlusion. In selective preliminary experiments (time course of RANKL/OPG expression in infarcted hearts and serum), mice were submitted to the same I/R protocol without any treatment injection. At different reperfusion time points, animals were sacrificed for infarct size

determination, Magnetic Resonance Imaging (MRI) and immunohistochemical analysis. Animals were sacrificed by exsanguination after intraperitoneal injection of ketamine-xylazine [4mg/0.2%]).

2.2 Area at risk (AAR) and infarct size (I) assessment

To assess area at risk (AAR) and infarct size (I) in the in vivo I/R protocol, mice were sacrificed after 24h of reperfusion, as previously described [21]. The left anterior coronary artery was re-occluded and Evan's blue dye (2%; Sigma) was injected within the cardiac left ventricle to delineate the in vivo AAR. The heart was rapidly excised and rinsed in NaCl 0.9%. Then, hearts from both assays were frozen and sectioned into 2-mm transverse sections from the apex to the base (5-6 slices/heart). The sections were, incubated at 37°C with 1% triphenyltetrazolium chloride (TTC) in phosphate buffer (pH 7.4) for 15 min, fixed in 10% formaldehyde solution and photographed with a digital camera (Nikon Coolpix) to distinguish continuously perfused tissue (blue), stained ischemic viable tissue (red) and unstained necrotic tissue (white). The different zones were determined using MetaMorph software (version 6.0, Universal Imaging Corporation). AAR and left ventricular infarct zone (I) were expressed as percentage of ventricle surface (AAR/V and I/V), respectively.

2.3 In vivo three-dimensional magnetic resonance imaging

Cardiac Magnetic Resonance Imaging was performed 24 hours after reperfusion onset on a clinical 3T MR scanner (Magnetom PRISMA, Siemens Medical Solutions, Erlangen, Germany) with a dedicated 2-channel mouse heart receiver coil (Rapid biomedical GmbH, Rimpar, Germany). At 24h from reperfusion onset after 60 min ischemia, Vehicle-treated (n=8), CTL IgG-treated (n=6) and anti-RANKL IgG-treated

(n=7) mice were re-anesthetized with isoflurane 1-2% for doing the exam by MRI. Respiratory and ECG monitoring and triggering was controlled by an external trigger set-up (S.A. Inc., Stony Brook, US). Gadolinium (Dotarem; Guerbet, Zurich, Switzerland) at a dose of 0.1 ml of solution diluted to 0.1 mmol/ml was injected intraperitoneally before imaging to provide blood to myocardium contrast and also provide enhancement of the infarct region. The triggered 3D gradient echo cine sequence was used to assess myocardial function. This protocol had an isotropic resolution of 344 μ m, flip angle 30°, TE/TR 2.9/7.8ms. Scan time was approximately 20-30 minutes depending on respiration and ECG rate. Ejection fraction (EF) calculation was performed with the Osirix software (Open source <http://www.osirix-viewer.com/>). The endocardial contour was delineated by hand for systolic and diastolic phases, for each slice of the myocardium (not including papillary muscles. EF (%) was defined as $(EDV-ESV)/EDV \times 100$, where EDV is the end-diastolic volume and ESV the end-systolic volume. The EF measurement was done by the same observer for all mice blinded to the condition/treatment. The septum and lateral wall thickness calculated via the full width half height of a profile through the 4-chamber view at the level of the papillary muscle in both systole and diastole. After MRI protocol, animals were sacrificed by exsanguination after intraperitoneal injection of ketamine-xylazine [4mg/0.2%]) and hearts were collected for immunohistology and nitrobluetrazolium chloride ([NBT], to identify metabolically inactive tissues) staining. Infarct size (IS) was determined as the NBT-negative area [7].

2.4 Immunostaining

Hearts from animals sacrificed after different times of reperfusion (up to 72 hours) were frozen in OCT and cut serially from the occlusion locus to the apex in 7 μm sections. Immunostainings for mouse RANKL, OPG, MMP-9 (all from R&D Systems), neutrophils (anti-mouse Ly-6G antibody, from BD Biosciences, San Jose, CA, USA) and macrophages (anti-mouse CD68, from ABD Serotec, Kidlington, UK) were performed on 5 midventricular cardiac sections per animal. Sections were counterstained with Mayer's hemalun and rinsed in distilled water. Quantification performed with MetaMorph software. Results were expressed as percentages of stained area on total heart surface area (RANKL, OPG, MMP-9) and as number of infiltrating cells on mm^2 of total heart surface area (neutrophils and macrophages).

2.5 Oxidative stress determination

The production of ROS was assessed by staining of the highly toxic product of lipid membrane peroxidation 4-hydroxy-2-nonenal (mouse anti-4-HNE monoclonal antibody, Oxis International Inc, Foster City, CA) and of 3,5-dibromotyrosine (mouse anti-Di bromo tyrosine monoclonal antibody, AMS biotechnology, LTD, Abingdon, UK). Staining within the hearts of these oxidative mediators was performed as previously described [21]. To avoid any potential cross-reactivity with mouse heart antigens and to increase the specificity of the primary antibodies, the VECTOR M.O.M Immunodetection kit and the VECTOR VIP substrate kit for peroxidase (Vector Laboratories, Inc. Burlingame, CA) were used, following the manufacturer's instructions. Quantification was performed with MetaMorph software. Results were expressed as percentages of stained area on total heart surface area.

2.6 Serum inflammatory molecule and cardiac troponin I level detection

Colorimetric enzyme-linked immunosorbent assay (ELISA) kits to measure serum RANKL levels (from RayBiotech, Inc., Norcross, GA), serum OPG, CXCL1, CCL2, pro-matrix metalloproteinase (MMP)-9, myeloperoxidase (MPO) and Tissue inhibitor of metalloproteinase-1 (TIMP-1) levels (all from R&D Systems), serum MMP-8 levels (Uscn Life Science Inc., Hubei, PRC) and serum cardiac troponin I ([cTnI], Life Diagnostics Inc., West Chester, PA) were used following manufacturer's instructions. The limit of detection was 2.74 pg/ml for RANKL, 31.3 pg/ml for OPG, 15.6 pg/ml for CXCL1, 3.9 pg/ml for CCL2, 31.25 pg/ml for pro-MMP-9, 250 pg/ml for MPO, 37.5 pg/ml for TIMP-1, 312.5 pg/ml for MMP-8, 156 pg/ml for cTnI. Mean intra- and inter-assay coefficients of variation (CV) were below 6%.

2.7 Western Blot analysis

After 60 minutes of *in vivo* ischaemia and 15 minutes of reperfusion, hearts from mice treated with Vehicle, CTL IgG and anti-RANKL IgG (n=5 per group) were excised and proteins were extracted in lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1% NP40, 0.25% sodium deoxycholate, 1 mM EDTA and complete protease inhibitor cocktail (Thermo Scientific, Waltham, MA, USA). Proteins (50 µg per lane) were electrophoresed through polyacrylamide/SDS gels and transferred by electroblotting onto PVDF membranes. Membranes were blocked for 1h in 5% (w/v) nonfat milk before O/N incubation with appropriate dilutions of primary phospho-specific anti-mouse ERK p42/p44 (R&D Systems, Minneapolis, MN, USA), anti-mouse Akt (p62) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-mouse tyrosine 705 STAT-3 antibodies (both from Cell Signaling Technology, Danvers, MA) as well as corresponding secondary antibodies. The blots were developed using the ECL system (Immobilion™ Western, Millipore, Billerica, MA, USA). To verify

equal loading of the total proteins, membranes were stripped (for 15 min at 50 C in Tris-HCL pH 6.7, 2% SDS and 0.1 M β -mercaptoethanol), reblocked and reprobed to detect total ERK p42/p44 (R&D Systems), Akt p56 (Santa Cruz Biotechnology) or STAT-3 (Cell Signaling Technology). Relative intensities were calculated by dividing the phosphorylated protein through the total protein.

2.8 Mouse peritoneal neutrophil isolation and migration assay

Mouse neutrophils were obtained after intraperitoneal injection of 4% thioglycollate solution, as previously described [21]. Analgesia was administered 30 minutes before thioglycollate injection with a single subcutaneous injection of 0.05 mg/kg buprenorphine HCl. Normal male C57Bl/6 mice without surgery (8-12 weeks of age) were euthanized by neck dislocation after deep anesthesia induced by 4% Isoflurane. The peritoneal lavage fluids of mice (n=4) was used. Then, neutrophils were resuspended at a density of 10^6 cells/ml in HBSS containing 1 mM CaCl_2 , 2 mM MgCl_2 (ICN Biomed) and 0.2% BSA (Sigma Chimica) (mouse chemotaxis medium). Mouse neutrophil chemotaxis was assessed in a 48-well microchemotaxis chamber using a 5 μm pore size, 5- μm -thick polyvinylpyrrolidone-free filter (Neuro Probe). 4×10^5 neutrophils were seeded in the upper well while control medium, recombinant mouse CXCL2 (200 ng/ml, R&D Systems) or recombinant mouse RANKL (0.01-10 ng/ml, R&D Systems) were added to the lower wells. To assess in vitro the potential direct effects of mouse serum (at 24h of reperfusion) from CTL IgG-treated (n=4) or anti-RANKL IgG-treated mice (n=4) on neutrophil migration, cells were also resuspended in different dilutions (1:1, 1:2 or 1:4) of mouse serum that was added with cells in the upper wells. To assess the direct effect of CTL IgG and anti-RANKL IgG on neutrophil migration, 10 ng/ml recombinant mouse RANKL was pre-

incubated 1h before the test in the presence or absence of the IgGs (at 6 µg/ml for CTL IgG and 0.06-6 µg/ml for anti-RANKL IgG). Then, without washing, neutrophil migration towards control medium (CTL) and mouse RANKL was assessed. The concentrations of recombinant CXCL2 and RANKL were selected on the basis of previous studies and the present investigation.¹ After incubation (60 min, 37°C), the filters were removed from the chambers, washed and stained with Diff-Quick (Baxter, Rome, Italy). The cells of five random oil-immersion fields were counted and the chemotaxis index (C.I.) was calculated by dividing the number of cells migrated towards chemoattractants through the number of cells migrated to chemotaxis medium alone.

2.9 Mouse neutrophil degranulation assay

Mouse neutrophils were obtained after intraperitoneal injection of 4% thioglycollate solution, as previously described [21]. Normal male C57Bl/6 mice without surgery (8-12 weeks of age) were used. The peritoneal lavage fluids of mice (n=13) was used. Neutrophils were resuspended in culture medium (serum-free RPMI 1640 medium containing 25 mmol/l Hepes) and cultured at 5×10^5 cells per well in the presence or absence of control medium, 10 ng/ml phorbol-12-myristate-13-acetate (PMA, positive control from Sigma-Aldrich, Buchs, Switzerland), or different doses (up to 10 ng/ml) of recombinant mouse RANKL (R&D Systems) [14]. In selective experiments, to assess the direct effect of CTL IgG and anti-RANKL IgG on neutrophil degranulation, 10 ng/ml recombinant mouse RANKL was pre-incubated 1h before the test in the presence or absence of the IgGs (at 6 µg/ml for CTL IgG and 0.006-0.6 µg/ml for anti-RANKL IgG). After an incubation of 30 min at 37°C in a humidified atmosphere 5% CO₂ in polystyrene plates, supernatants were collected for being

tested for degranulation of pro-MMP-9. Levels of pro-MMP-9 and TIMP-1 in neutrophil supernatants were assessed by the same ELISA kits from R&D systems that were used to detect this molecule in mouse serum.

2.10 Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was extracted from mouse neutrophils, heart and spleen with the Nucleospin kit and on-column DNase digested (Macherey-Nagel, Düren, Germany), following the manufacturer's instructions. The concentration and purity of RNA were determined by spectrophotometry analysis and cDNA was prepared from 200 ng total RNA using the ImProm-II Reverse Transcription System (Promega Corporation, Madison, USA), as suggested by the manufacturer. As a negative control, reverse transcription experiments were performed by omitting the reverse transcriptase enzyme from the reactions. PCR was performed using primer pairs specific for mouse RANK based on the nucleotide sequences available in GenBank. Forward and reverse primers were used for RANK were: Rank forward: 5' GAC CAA CTG CAC CCT CCTT 3'. Rank reverse: 5' CAC CGT CTT CTG GAA CCA TC 3'. The PCR products were separated by electrophoresis in a 1% agarose gel and visualized by UV illumination in the presence of SYBR Safe (Invitrogen Corporation, Carlsbad, USA). The molecular identity was confirmed by specific endonuclease digestion (MspI, from New England Biolabs, Ipswich, USA).

2.11 Real time RT PCR

Total mRNA was isolated with Tri-reagent (MRC, Cincinnati, Ohio) from spleen, cardiac atria, left and right ventricle tissues. The method is described in detail in the online supplementary material.

2.12 Statistical analysis

Statistical analyses were performed with IBM SPSS Statistics for Windows, Version 20.0 (IBM Corp., Armonk, NY, USA). Continuous variables are presented as mean (standard deviation [SD]) or median (interquartile range [IQR]) as appropriate. Two group comparisons were then performed by two-tailed t test (paired and unpaired) or non-parametric Mann-Whitney U test when the normality assumption of variable distribution was violated. Finally, one-way ANOVA with Bonferroni's post-hoc test was used for multiple group comparison. A 2-sided p-value <0.05 was considered statistically significant.

3. Results

3.1 RANKL levels are increased during reperfusion in infarcted hearts and systemic circulation

An investigation of the possible role of RANKL/OPG in myocardial ischemia/reperfusion was performed in sham-operated mice (SHAM) and animals exposed to 60 minutes of ischemia and different times of reperfusion (up to 72 hours). In infarcted left ventricles, RANKL protein expression was significantly increased from 12 to 72 hours of reperfusion as compared to Time 0 (Figure 1A and 1B). In consecutive sections from the same infarcted mice, OPG staining were mainly positive in heart vessels (Figure 1A). OPG protein expression did not change overtime during reperfusion as compared to SHAM (Figure 1C). In mouse serum, RANKL levels significantly increased already 5 minutes after reperfusion onset in mice submitted to 60 minutes of ischemia as compared to Sham-operated animals (Figure 2A). A peak in serum RANKL levels was observed at 12 hours of reperfusion (Figure

2A). Conversely, OPG serum levels were significantly reduced 5 minutes and at 12 hours after reperfusion onset as compared to SHAM (Figure 2B). No other changes in OPG serum levels were observed at later time points (up to 24 hours of reperfusion) as compared to SHAM (Figure 2B). Finally, RANKL/OPG ratio was increased at any reperfusion time points (up to 24 hours) as compared to SHAM (Figure 2C).

3.2 Treatment with anti-RANKL IgG reduces myocardial infarct size and improves left ventricular function

An *in vivo* treatment using an antibody neutralizing mouse RANKL bioactivity (anti-RANKL IgG) was performed and compared to Vehicle and isotype CTL IgG treatments. Based on timing of RANKL up regulation within the infarcted myocardium (12 hours of reperfusion), we assessed the potential effects of cardiac RANKL inhibition on infarct size and cardiac function at 24 hours of reperfusion. The AAR was similar in three treatment groups, indicating that ligation was reproducibly performed at the same level of the left anterior coronary artery (Figure 3A). Treatment with anti-RANKL IgG diminished infarct size as compared to controls (Figures 3B-3D). Accordingly, treatment with anti-RANKL IgG reduced serum cTnI levels as compared to controls at both 16 and 24 hours of reperfusion (Figure 3E). MRI was used to assess post-infarction cardiac function and remodeling. Importantly, both MRI and post-mortem histology confirmed well-detectable infarcted areas in the same regions of the mouse left ventricle (Figure 4). As shown in Table 1, mouse body weight and post-infarction heart weight were similar in the three treatment groups. MRI measurements demonstrated that mice had similar myocardium volume and end-systolic lateral wall and septum thicknesses (Table 1, Figure 4). Conversely, anti-RANKL IgG treatment improved left ventricular ejection fraction as compared to

control groups (Table 1). A weak improvement in right ventricular ejection fraction value was also observed in anti-RANKL IgG-treated mice as compared to CTL IgG control (Table 1).

3.3 Treatment with anti-RANKL IgG reduces neutrophil and MMP-9 contents in infarcted hearts

Despite some controversies, neutrophil infiltration, release and production of proteases has been associated with increased myocardial injury in mice [21-23]. Therefore, we assessed treatments on neutrophil post-infarction cardiac infiltration and on content of a neutrophil granule product (such as MMP-9). Treatment with anti-RANKL IgG significantly reduced neutrophil infiltration at 16h and 24h after reperfusion as compared to the Vehicle-treated group and at 24h of reperfusion as compared to CTL IgG-treated mice (Figure 5A and 5B). Accordingly, a reduction in MMP-9 content was demonstrated in anti-RANKL IgG-treated mice as compared controls at 24h of reperfusion (Figure 5C and 5D). Treatment with anti-RANKL IgG was also associated with a weak reduction in MMP-9 content at 16h of reperfusion as compared to Vehicle (Figure 5C and 5D). Considering the role of neutrophilic inflammation in ROS release during reperfusion [21], we tested the same animals on cardiac levels of 4-HNE and dibromotyrosine, two biomarkers of lipid modification. Among them, dibromotyrosine was particularly associated this neutrophil-mediated oxidative stress [21]. As predicted, at 24h of reperfusion, treatment with anti-RANKL IgG was associated with a significant reduction in 4-HNE and dibromotyrosine levels as compared with both control groups (Supplementary figure 1A-1D). Finally, we assessed the circulating levels of neutrophil granule products and the protease inhibitor TIMP-1 at different time points of reperfusion in infarcted mice. Based on

timing of serum RANKL up regulation (5 minutes of reperfusion), we assessed the potential effects of serum RANKL inhibition on neutrophil degranulation already at that time point. At 5 minutes of reperfusion, a peak in the serum levels of pro-MMP-9 (contained in tertiary granules), MMP-8 (secondary granules) and MPO (primary granules) was demonstrated in both Vehicle- and CTL IgG-treated mice as compared to SHAM (Supplementary figure 2A-2C). Treatment with anti-RANKL IgG was associated with a significant reduction in pro-MMP-9 and MMP-8 levels as compared to control groups (Supplementary figure 2A and 2B). No effect of RANKL inhibition was shown on neutrophil product serum levels at any other time point investigated (up to 8h of reperfusion) (Supplementary figure 2A-2C). Interestingly, the sudden peak in serum levels of neutrophil products was not accompanied by any increase in TIMP-1 (Supplementary figure 2D). A delayed peak in TIMP-1 serum concentration (as compared to SHAM) was observed in all infarcted mice independent of treatments (Supplementary figure 2D). These data suggest that neutralization of RANKL bioactivity was associated with a marked reduction of neutrophil systemic activation and recruitment within the post-infarcted myocardium.

3.4 Treatment with anti-RANKL IgG abrogates mouse neutrophil recruitment and degranulation in vitro

The expression of RANK (the transmembrane receptor of RANKL) was recently demonstrated on human neutrophils [14]. Here, we tested the expression of this receptor also on mouse neutrophils. RANK mRNA expression was detected in mouse neutrophils from four non-operated donors (Figure 6). We then tested *in vitro* the effects of RANKL and anti-RANKL IgG treatment on mouse neutrophil recruitment and degranulation. At 24h of reperfusion, the time point at which neutrophils peaked

within infarcted hearts, serum from vehicle-treated mice significantly increased mouse neutrophil migration towards recombinant CXCL2 as compared to anti-RANKL IgG-treated serum or control chemotaxis medium (Figure 7A). When compared to control medium, anti-RANKL IgG-treated serum weakly reduced neutrophil migration towards CXCL2 (Figure 7A), indicating that co-incubation with serum of Vehicle- or anti-RANKL IgG-treated animals might differently favor neutrophil migration towards CXCL2 (a chemokine known to be upregulated within infarcted hearts during reperfusion). We then assessed neutrophil migration in response to different concentrations of recombinant mouse RANKL co-incubated in the presence of different doses of anti-RANKL IgG as well as CTL IgG. In contrast to humans [14], mouse neutrophil locomotion was triggered in the presence of 1 and 10 ng/ml of RANKL (Figure 7B). RANKL-induced migration was significantly reduced in the presence of anti-RANKL IgG, but not CTL IgG (Figure 7B). As expected, incubation of anti-RANKL IgG or CTL IgG did not affect neutrophil migration towards CXCL2, confirming that anti-RANKL IgG-mediated inhibition of neutrophil migration was specific and due to neutralization of RANKL bioactivity (Figure 7C). As regard to mouse neutrophil degranulation *in vitro*, we tested supernatant concentrations of pro-MMP-9 of cells exposed to positive control (PMA) as well as different concentrations of RANKL and anti-RANKL IgG. Similarly to human neutrophils [13], 10 ng/ml recombinant RANKL increased mouse neutrophil degranulation (Figure 8A). Co-incubation of recombinant mouse RANKL with the neutralizing anti-RANKL IgG dose-dependently reduced RANKL-induced degranulation (Figure 8B). As expected, co-incubation with CTL IgG did not induce any effect on RANKL-induced degranulation (Figure 8C). Assessment of the

potential release of TIMPs in mouse neutrophil supernatants, showed that TIMP-1 was below the low range limit of detection (37.5 pg/ml) in all samples.

3.5 Treatment with anti-RANKL IgG does not activate cardiac protective intracellular pathways

An investigation of the potential direct activity of anti-RANKL IgG on the activation of some cardioprotective RISK and SAFE intracellular pathways was performed [24, 25]. When compared to controls (Vehicle and CTL IgG), treatment with anti-RANKL IgG did not affect the phosphorylation of ERK1/2 (Supplementary figure 3A-3C), Akt (Supplementary figure 3D and 3E), STAT-3 tyrosine 705 (Supplementary figure 4A and 4B) within infarcted hearts at 15 minutes of reperfusion. These results suggest that anti-RANKL IgG-mediated neutralization might be not involved on early mechanisms of cardiomyocyte survival.

3.6 Treatment with anti-RANKL IgG does not affect cardiac content of macrophages, B cell and Th cell subsets

An investigation of the potential RANKL-mediated effects on different inflammatory cell subsets within infarcted hearts and spleen during reperfusion was performed. No difference in infiltrating macrophages was detected between treatments at any reperfusion time point investigated (Supplementary figure 5A and 5B). With regard to B and T lymphocytes the expression of genetic cell markers was tested in samples from cardiac atria, right and left ventricles. No difference was shown between groups for the expression of Cd20 mRNA (B lymphocytes) in the cardiac tissues (Supplementary tables 2-4). Considering T_h lymphocytes, no difference was demonstrated between groups in the expression of Cd4 mRNA within hearts

(Supplementary tables 2-4). Considering T_h lymphocyte polarization, no changes in Th1, Th2, Treg and Th17 genetic markers and selective cytokines were demonstrated between groups in cardiac tissues, with the exception of a weak reduction of Th1 in anti-RANKL IgG-treated right ventricles as compared to CTL IgG (Supplementary tables 2-4). Similarly to cardiac samples, no difference on B and T_h lymphocyte mRNA levels was demonstrated between groups in spleen samples at both 6 and 24 hours of reperfusion (Supplementary table 5), indicating that the levels of these cell subsets are not influenced by anti-RANKL IgG treatment.

3.7 Treatment with anti-RANKL IgG does not affect systemic and cardiac chemokine expression

Similarly to previous studies investigating inflammation as a key determinant of myocardial injury during reperfusion [7, 9, 21], we measured serum levels of leukocyte chemoattractants (i.e. CCL2 and CXCL1) at different time points of reperfusion after 60 minutes of ischemia. A significant increase of CCL2 and CXCL1 was demonstrated already at 5 minutes of reperfusion and until 1 hour in infarcted mice as compared to Sham-operated animals (Supplementary figure 6A and 6B). However, treatment with anti-RANKL IgG did not affect either CCL2 or CXCL1 serum levels at any early time points of reperfusion (up to 16 hours) as compared to controls (Supplementary figure 6A and 6B). Anti-RANKL IgG treatment was associated with a reduction of CCL2, but not CXCL1 serum levels at 24 hours of reperfusion as compared to controls (Supplementary figure 6A and 6B). Accordingly, within samples from atria, and right and left ventricles from infarcted hearts no effect was shown on mRNA levels of chemokines and Tnf in anti-RANKL IgG-treated animals as compared to controls (Supplementary table 6). These data suggest that

anti-RANKL IgG treatment did not affect the systemic and cardiac expression of inflammatory molecules triggering leukocyte recruitment as compared to control treatments.

4. Discussion

The present research shows that RANKL is up regulated in the systemic circulation and within the infarcted heart early during reperfusion in mice. On the other hand, serum OPG levels were decreased at 5 minutes of reperfusion and then were similar at later time points (up to 24 hours) as compared to sham-operated animals. Within the infarcted hearts OPG-positive regions were not co-localized with RANKL and showed expression exclusively within the heart vessels. RANKL and OPG expression remains to be demonstrated in human infarcted hearts. Some pilot studies performed in patients with acute myocardial infarction did not confirm the increased serum levels of RANKL observed in mice [26, 27]. This aspect might weaken the clinical relevance of our mouse results. Although the number of patients enrolled in these studies was quite small, an early increase in serum levels of OPG (opposite to our mouse results) was reported. Interestingly, both RANKL and OPG were demonstrated within human and mouse atherosclerotic plaques, indicating these molecules as potential pathophysiological players in plaque progression [27]. Considering these studies, we believe that the potential difference in RANKL/OPG serum levels from mouse and human myocardial infarction desperately requires a pathophysiological confirmation in larger clinical studies with AMI patients. This step is needed to further corroborate mouse data and potentially suggest an anti-RANKL treatment against AMI in humans.

The main finding of our article is represented by the fact that treatment with a neutralizing anti-RANKL IgG improved infarct size and cardiac function at 24 hours of reperfusion. Even under buprenorphine treatment, that can per se induce cardioprotection [28] and potentially attenuate the benefits observed, the improvement of infarct size by anti-RANKL IgG was relevant. These results might be surprising since RANKL was demonstrated as protective against ischemia in other mouse organs, such as the liver and the brain [19, 20]. This apparent discrepancy of opposite activities of a same molecule/treatment in heart and brain ischemia was already found in other mouse studies [29, 30-32]. A potential explanation was based on the fact that certain ischemia-induced reactive oxygen species (ROS) that might be detrimental within the ischemic heart, but can be protective in the brain or other organs. The benefits of RANKL blockade in mouse cardiac infarction were further proven by the development in our Lab of an innovative MRI 3D technology that replaced the 2D MRI used in our previous studies [7, 9]. 3D MRI with injection of paramagnetic contrast was promising to better confirm and potentially identify the infarcted zones within the mouse left ventricle in the treatments groups. While functional MRI data are solid, the 3D MRI measurement of infarct size was not performed in the present study since a validation comparing MRI and histological pictures is still required and ongoing in our Laboratory.

In order to identify the mechanism underlying anti-RANKL IgG-mediated benefits, an investigation of known post-ischemic injury mediators involving inflammatory cells and cardiomyocytes was performed. Although we cannot exclude that the effects on cardiomyocyte survival may per-se account for the reduced neutrophil infiltration following treatment with RANKL antibody, evidence from both *in vivo* and *in vitro* experiments suggested that neutralization of RANKL bioactivity was associated with

the inhibition of neutrophil recruitment and degranulation of proteases both within ischemic hearts and the systemic circulation. These results were also accompanied by a reduction in cardiac ROS in mice treated with anti-RANKL IgG as compared to controls. The inhibition of neutrophil-mediated injury during reperfusion was widely described as a key strategy to potentially improve both cardiac infarct size and function. We already demonstrated similar beneficial effects to those observed in the present study by inhibiting neutrophil chemoattractant bioactivities and levels [7, 21]. However, these treatments were accompanied by a marked reduction of systemic inflammation that was not observed using anti-RANKL IgG. Considering cardiomyocytes, treatment with anti-RANKL IgG was not associated with any activation of cardiomyocyte-related salvage pathways, such as ERK1/2 and Akt (RISK pathway) or Tyr 705 STAT-3 (SAFE pathway) [25, 29, 33]. Considering the low expression of RANK within the infarcted myocardium that we observed (Figure 6), RANKL inhibition might be not a very effective treatment strategy targeting cardiomyocytes.

Interestingly, treatment with anti-RANKL IgG was not associated with immune depression of macrophages or B and T cells both within the ischemic hearts, nor in lymphoid organs (such as spleen). As compared to more established treatments blocking neutrophil post-ischemic infiltration in rodents, such as statins and anti-neutrophil antibodies [34, 35], anti-RANKL approach might be considered to potentially have similar efficacy and acceptable safety in the first 24h from the event. In fact, our mouse model did not allow any conclusion on the relative safety of repeated injections as well as potential long-term disruptive effects of an anti-RANKL treatment on bone homeostasis in subjects with an acute myocardial infarction.

Therefore, caution should be used when analysing our results and before translating them into human beings.

In addition, systemic chemokine levels were also comparable in the mouse treatment groups at different times of reperfusion. The acute anti-RANKL IgG treatment that we performed in our study was well tolerated in mice and the mortality rate at 24h-follow up was similar in the treatment groups and around 10%. Also in humans anti-RANKL IgG treatment was not suggested to be associated with clinical manifestations of immune suppression [15]. Therefore, we observed that the acute treatment with anti-RANKL IgG was safe in a mouse model of myocardial infarction and 24h reperfusion.

The brief follow up period represents a main limitation of our study. A longer follow up is required to confirm the early benefits on cardiac function and promising results on safety. In addition, considering recent evidence for neutrophils in activating protective macrophages during cardiac repair [36], we cannot exclude that the apparent discrepancy of RANKL blockade (that reduces infarct size without affecting early adverse remodeling following infarction) might result in later detrimental consequences during post-infarction recovery. Also, we studied the potential impact of RANKL inhibition on macrophages, B cells, and Th cells up to 24 hours. This short time point might not be good as these cells infiltrate later than neutrophils, and peak after day 3 post-infarction [37]. On the other hand, the mouse model used in this study (characterized by the surgical occlusion of the coronary that allows a transient myocardial ischemia, followed by reperfusion) might be not the most appropriate to investigate clinical outcomes at a prolonged follow up. In fact, other mouse models (such permanent coronary occlusion) are associated with more relevant adverse cardiac remodeling and heart failure [7, 9]. Considering pathophysiological

differences between the ischemia/reperfusion and permanent ischemia models, we preferred to not mix these experimental approaches in the same project. Another methodological limitation is represented by the fact that we used the entire left ventricle for various assays instead of specifically the area at risk. As performed in previous publications [7, 9, 21, 29], this approach was performed to obtain the sufficient amount of sample for analysis. However, this strategy might dilute out the effects of the end points by introducing a type II error of missing effects due to the sample mix of ischemic and non-ischemic tissues. In addition, a relative low number of mice (n=155) included in the global study. Despite appropriately statistically powered on primary (infarct size) and secondary (left ventricle ejection fraction and neutrophil infiltration) outcomes, to be conformed with 3R (Replace, Refine, Reduce) principles, we limited this first study targeting RANKL inhibition to early benefits without exploring survival and cardiac scar evolution or adverse remodeling. Finally, we were unable to determine which cell subsets was responsible for RANKL upregulation in the systemic circulation and within the ischemic hearts. This point deserves additional pathophysiological studies.

In conclusion, we demonstrated that a “one-shot” treatment during cardiac ischemia and before reperfusion with an anti-RANKL IgG improved infarct size and post-infarction left ventricular function in mice. These beneficial effects were associated with a selective and potent inhibition of neutrophil recruitment and degranulation in response to RANKL both *in vivo* and *in vitro*. Finally, anti-RANKL IgG treatment was safe and did not induce any alteration on the early inflammatory response during reperfusion in the systemic circulation, infarcted myocardium and spleen. Although we cannot exclude that RANKL inhibition might target also other cell subsets, these

data indicate that the pharmacologic neutralization of RANKL bioactivities might impact on neutrophil-mediated injury in a mouse model of ischemia and reperfusion.

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Author contributions

Conceived the study: FMontecucco, FMach, JPV. Designed the experiments: FMontecucco, FC. Performed the *in vivo* experiments, immunohistochemistry, PCR: FMontecucco, LC, AR, FB, SL, VB, KJB, JPV. Performed the *in vitro* experiments: FMontecucco, FC, LC, AR, FB, SL, VB, KJB, AQ. Analysed the data: FMontecucco, LC, FC. Wrote the paper: FMontecucco, FMach, FC, LC, with critical input from all co-authors. All authors have read and approved the final version of the manuscript.

Conflict of interest

Authors declare no conflicts of interest.

Acknowledgments

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Figure legends

Figure 1. RANKL is upregulated within infarcted hearts during reperfusion. Left ventricle ischemia was performed for 60 minutes in male untreated C57Bl/6 mice. Intracardiac levels of RANKL and OPG were assessed at different time points of reperfusion in untreated and sham-operated mice (SHAM), sacrificed at 24h. Data are expressed as median (interquartile range). **A.** Representative images of RANKL- and OPG-stained consecutive middle heart sections of infarcted and sham-operated hearts. **B.** Quantification of RANKL stained area within the infarcted myocardium (n=5 per time point). **C.** Quantification of OPG stained area within the infarcted myocardium (n=5 per time point).

Figure 2. RANKL serum levels are increased in infarcted mice during reperfusion. Serum RANKL (**A**), OPG (**B**), and RANKL/OPG ratio (**C**) in sham-operated animals (SHAM) and in mice submitted to 60 minutes ischemia a different times of reperfusion. Results are expressed as median (interquartile range) (Sham: n=9; 5 minutes: n=5; 1h: n=5; 8h: n=13; 12h: n=23; 24h: n=10).

Figure 3. Treatment with anti-RANKL IgG reduces infarct size *in vivo* after 24h of reperfusion. Vehicle, CTL IgG (200 µg/mouse) or anti-RANKL IgG (200 µg/mouse) were administered in one shot at 5 min after ischemia onset. Data are expressed as median (interquartile range). **A.** Quantification of area at risk (AAR) per ventricle surface (V) (n=10 per group). **B.** Quantification of infarct size (I) per AAR (n=10 per group). **C.** Quantification of infarct size (I) per ventricle surface (V) (n=10 per group). **D.** Representative images of TTC stained middle heart sections of Vehicle- CTL IgG- or anti-RANKL IgG-treated mice. The AAR was demarcated with

the red line. **E.** Serum cardiac troponin I (cTnI) levels of Vehicle- CTL IgG- or anti-RANKL IgG-treated mice after 16h and 24h of reperfusion (n=7 per group at 16 hours, n=10 per group at 24 hours).

Figure 4. Treatment with anti-RANKL IgG improves left ventricular function at 24h of reperfusion. **A, C, E.** Representative magnetic resonance images (MRI) (among n=8 in Vehicle-treated, n=6 in CTL IgG-treated and n=7 in anti-RANKL IgG-treated mice) of 2-chamber, 4-chamber long axis and short axis at mid papillary muscle level of end-systole and end-diastole of Vehicle- (**A**), CTL IgG- (**C**) and anti-RANKL IgG-treated (**E**) mice at 24 hours of reperfusion. Arrows indicate the infarcted zones. **B, D, F.** Representative microphotographs (among n=8 in Vehicle-treated, n=6 in CTL IgG-treated and n=7 in anti-RANKL IgG-treated mice) at mid papillary muscle level of the same infarcted hearts tested with MRI showing Nitrobluetetrazolium chloride staining (arrows indicate white colored regions that are metabolically inactive).

Figure 5. Treatment with anti-RANKL IgG reduces neutrophil and MMP-9 content in infarcted hearts *in vivo*. **A.** Quantification of infiltrated neutrophils (Ly6G⁺ cells) per area in frozen sections of infarcted hearts at different reperfusion time points (1h, 16h, and 24h). Data are expressed as median (interquartile range). (n=4 per group at 1h, n=7 per group at 16h, n=9 per group at 24h). **B.** Representative images of neutrophil infiltration at 16 and 24h reperfusion are shown. **C.** Quantification of MMP-9 stained areas in frozen sections of infarcted hearts at different reperfusion time points (1h, 16h, and 24h). Data are expressed as median (interquartile range). (n=4 per group at 1h, n=7 per group at 16h, n=9 per group at

24h). **D.** Representative images of MMP-9 stained heart sections at 16 and 24h of reperfusion are shown.

Figure 6. RANK is expressed in mouse primary neutrophils. Representative agarose gel of PCR products from mouse neutrophils (isolated from four different mouse donors M_1-M_4) showing mRNA expression of RANKL receptor RANK. On the left the molecular weights of the markers are shown.

Figure 7. *In vitro* incubation of anti-RANKL IgG with recombinant RANKL inhibits mouse neutrophil migration towards this cytokine. **A.** Mouse neutrophil migration towards 200 ng/ml CXCL2 or control medium in the presence or absence of control medium (NIL) or of different dilutions of serum from infarcted mice treated with CTL IgG or anti-RANKL IgG at 24h of reperfusion. Data are expressed mean $\pm$ SD, n=4. **B.** Mouse neutrophil migration towards different doses (0.01-10 ng/ml) of RANKL or control medium pre-incubated with CTL IgG (6 μ g/ml) or different concentrations (0.06-6 μ g/ml) of anti-RANKL IgG. Data are expressed mean $\pm$ SD, n=4 per group. **C.** Mouse neutrophil migration towards 200 ng/ml CXCL2 or control medium pre-incubated in the presence or absence of 6 μ g/ml CTL IgG or 6 μ g/ml of anti-RANKL IgG. Data are expressed mean $\pm$ SD, n=4 per group.

Figure 8. *In vitro* incubation of anti-RANKL IgG with recombinant RANKL inhibits mouse neutrophil degranulation in response to this cytokine. **A.** Pro-MMP-9 levels in mouse neutrophil supernatants in the presence or absence of control medium (CTL), 10 ng/ml phorbol-12-myristate-13-acetate (PMA, positive control) or different concentrations of mouse recombinant RANKL. Data are expressed as

median (interquartile range), n=13 per group. **B.** Pro-MMP-9 levels in mouse neutrophil supernatants of in the presence or absence of control medium (CTL), 10 ng/ml PMA or 10 ng/ml RANKL pre-incubated with different concentration of anti-RANKL IgG. Data are expressed as median (interquartile range), n=13 per group. **C.** Pro-MMP-9 levels in mouse neutrophil supernatants of in the presence or absence of control medium (CTL), 10 ng/ml PMA or 10 ng/ml RANKL and pre-incubated with 6 µg/ml CTL IgG. Data are expressed as median (interquartile range), n=13 per group.

Table 1. Magnetic resonance imaging (MRI) assessment of left ventricular dimension and wall thickness at mid papillary muscle level.

Heart measurements at 24 hours from reperfusion onset					
Measurements	Vehicle-treated mice (n=8)	CTL IgG-treated mice (n=6)	anti-RANKL IgG-treated mice (n=7)	<i>p</i> -value (anti-RANKL IgG vs. Vehicle)	<i>p</i> -value (anti-RANKL IgG vs. CTL IgG)
Heart weight, mg	143.2±27.5	147.6±27.8	134.1±30.4	0.554	0.421
Body weight, g	25.5±2.0	26.4±1.8	25.7±3.0	0.885	0.593
MRI measurements at 24 hours from reperfusion onset					
Measurements	Vehicle-treated mice (n=8)	CTL IgG-treated mice (n=6)	anti-RANKL IgG-treated mice (n=7)	<i>p</i> -value (anti-RANKL IgG vs. Vehicle)	<i>p</i> -value (anti-RANKL IgG vs. CTL IgG)
Myocardium volume, mm ³	85.5± 4.9	83.1±19.0	75.9±19.0	0.413	0.514
End-systolic lateral wall thickness, mm	1.16±0.15	1.22±2.27	1.30±0.14	0.090	0.473
End-systolic septum thickness, mm	1.03±0.14	1.24±0.16	1.12±0.10	0.213	0.148
End-systolic septum-to-lateral wall thickness ratio	0.90±0.16	1.05±0.28	0.86±0.07	0.552	0.163
Left ventricle end-systolic volume (μl)	43.1±9.0	36.3±10.4	28.6±5.0	0.002	0.140
Left ventricle end-diastolic volume (μl)	63.7±15.1	56.5±12.4	53.8±7.0	0.125	0.647
Left ventricle ejection fraction, %	32.0±6.3	36.6±5.7	46.6±7.6	0.002	0.021
Right ventricle end-systolic volume (μl)	25.2±8.3	28.7±6.5	21.3±4.0	0.258	0.042
Right ventricle end-diastolic volume (μl)	44.7±12.4	51.0±9.7	46.9±7.5	0.681	0.422
Right ventricle ejection fraction, %	43.3±12.3	44.1±2.8	53.9±9.4	0.090	0.033

Data are expressed as mean±SD.

Figure 1

Figure 2

Figure 3

Figure 4
Vehicle

Figure 5

A

Neutrophils
(cells/mm²)

B

C

D

Figure 6

Figure 7

Figure 8

A

B

C

In vivo

In vitro