Neutrophil stunning by metoprolol reduces infarct size

Jaime García-Prieto1,2, Rocío Villena-Gutiérrez1, Mónica Gómez1, Esther Bernardo3, Andrés Pun-García1, Inés García-Lunar1,2,4,5, Georgiana Crainiciuc1, Rodrigo Fernández-Jiménez1,2,3, Vinatha Sreeramkumar1,4, Rafael Bourio-Martínez1,6, José M. García-Ruiz1,2,7, Alfonso Serrano del Valle1, David Sanz-Rosa1,2,4, Gonzalo Pizarro1,2,4,8, Antonio Fernández-Ortiz1,2,3, Andrés Hidalgo1,9, Valentin Fuster1,10 & Borja Ibanez1,2,11

The β1-adrenergic-receptor (ADRB1) antagonist metoprolol reduces infarct size in acute myocardial infarction (AMI) patients. The prevailing view has been that metoprolol acts mainly on cardiomyocytes. Here, we demonstrate that metoprolol reduces reperfusion injury by targeting the haematopoietic compartment. Metoprolol inhibits neutrophil migration in an ADRB1-dependent manner. Metoprolol acts during early phases of neutrophil recruitment by impairing structural and functional rearrangements needed for productive engagement of circulating platelets, resulting in erratic intravascular dynamics and blunted inflammation. Depletion of neutrophils, ablation of Adrb1 in haematopoietic cells, or blockade of PSGL-1, the receptor involved in neutrophil–platelet interactions, fully abrogated metoprolol’s infarct-limiting effects. The association between neutrophil count and microvascular obstruction is abolished in metoprolol-treated AMI patients. Metoprolol inhibits neutrophil–platelet interactions in AMI patients by targeting neutrophils. Identification of the relevant role of ADRB1 in haematopoietic cells during acute injury and the protective role upon its modulation offers potential for developing new therapeutic strategies.

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Heart attack (acute myocardial infarction, AMI) is one of the principal manifestations of cardiovascular disease and a chief contributor to mortality and morbidity worldwide. The main determinant of poor outcome post AMI is the extent of irreversible injury (infarct size). The mainstay of AMI treatment is rapid reperfusion to restore blood flow, which reduces complications and improves survival. However, reperfusion itself accelerates and exacerbates the inflammatory response associated with myocardial injury. Thus, the injury inflicted on the myocardium during AMI is the result of ischaemia and reperfusion processes, and is known as ischaemia/reperfusion (IR) injury. The development of effective therapies to reduce myocardial IR injury is an unmet clinical need.

The injured myocardium is infiltrated by circulating neutrophils, and these cells are critically involved in myocardial IR injury. In an inflammatory milieu, neutrophils bind to platelets and red blood cells, forming plugs. Upon reperfusion, these plugs are dispersed into the microcirculation, where they form embolisms, precluding tissue perfusion despite blood flow restoration in the large coronary arteries. This phenomenon, known as microvascular obstruction (MVO), is a major contributor to IR injury and infarct size. Moreover, neutrophil infiltration into acutely damaged organs is dependent on their interaction with platelets, and these interactions are critical to the formation of harmful co-aggregates and the initiation of inflammatory-like responses before tissue infiltration. Overall, neutrophil dynamics (including neutrophil–platelet interactions) are an attractive therapeutic target for the prevention of IR injury.

The intravenous (i.v.) administration of the selective β1-adrenergic receptor (ADRB1) antagonist metoprolol has been shown to reduce infarct size and improve long-term cardiac function after AMI in the recent METOCARD-CNIC trial. The mechanism underlying metoprolol's cardioprotective effect remains unclear. Identifying this mechanism could have significant implications for the understanding of IR and the development of novel infarct-limiting therapies. The adrenergic system is critically involved in inflammatory reactions. In particular, the inflammatory response of neutrophils involves the de novo production and release by these cells of catecholamines. Induced catecholamine stress (as during ischaemia) alters neutrophil trafficking and promotes formation of neutrophil–platelet co-aggregates.

We hypothesized that pre-reperfusion i.v. metoprolol administration alters neutrophil dynamics, resulting in a dampened inflammatory response, less severe reperfusion injury and smaller infarcts. Here we show that pre-reperfusion administration of i.v. metoprolol to AMI patients significantly reduces the incidence of MVO, and moreover that metoprolol inhibits deleterious neutrophil inflammatory responses both in patients and in animal models of IR. The infarct-limiting effect of metoprolol is abolished in neutrophil-depleted mice and when neutrophils are prevented from interacting with platelets. The beneficial effects of metoprolol are also abolished by genetic ablation of Adrb1 and are rescued by restoration of Adrb1 expression only in haematopoietic cells. These results identify the neutrophil dynamics as the target of the cardioprotective effect of metoprolol against myocardial IR injury.

Results

Intravenous metoprolol reduces MVO in AMI patients. The METOCARD-CNIC trial recruited patients with an ongoing acute ST-segment elevation AMI and randomized them to receive i.v. metoprolol (15 mg) or control before reperfusion. A total of 220 AMI patients underwent a cardiac magnetic resonance (CMR) imaging exam 1 week after AMI. To study the potential mechanisms underlying the infarct-limiting effect of metoprolol, we analysed the 1-week CMR data to evaluate the extent of MVO. MVO was defined as the absence of contrast wash-in inside the delayed gadolinium-enhanced area, and was quantified as grams of left ventricle (LV) (Fig. 1a,b and Supplementary Fig. 1a,b). Patients treated with metoprolol during ongoing AMI had a 40% lower extent of MVO (Fig. 1c). This significant effect was maintained after adjusting for factors potentially affecting MVO by performing linear multiple regression analysis and including sex, age, ischaemia duration, diabetes, and use of thrombectomy or glycoprotein IIb/IIIa inhibitors as covariates. To exclude the possibility that this effect simply reflected the reduction in total infarct size, MVO was further assessed as a percentage of the infarcted area (total late gadolinium enhanced area). Metoprolol-treated patients had 24% less infarct-normalized MVO than control patients (Fig. 1d). As expected, the extent of MVO was significantly associated with poor long-term outcome, evaluated as chronic ventricular performance (Supplementary Fig. 1c). These data suggest that MVO reduction might be involved in the cardioprotective effect of metoprolol administration in patients during ongoing AMI.

Intravenous metoprolol dampens neutrophil–MVO association in AMI. White blood cell (WBC) and neutrophil counts during an AMI are known to be associated with larger infarct sizes and extensive MVO. We explored these associations in AMI patients from the METOCARD-CNIC trial. We found a significant positive correlation between absolute leukocyte count on admission and the extent of MVO on CMR: the higher the leukocyte count, the larger the extent of MVO (Fig. 2a). We further studied the association of the different WBC subpopulations and MVO. As expected, neutrophil count was significantly correlated with the extent of MVO (Fig. 2b). Conversely, there was no sign of association between other WBC subpopulations and MVO: lymphocyte, monocyte, eosinophil or platelet counts did not correlate with the extent of MVO (Fig. 2e). Next, we studied the effect of metoprolol on WBCs and on the association between these and MVO. Metoprolol treatment was not associated with any different in WBC count nor in any WBC subpopulation (Supplementary Table 1). Of note, we found a significant interaction between metoprolol treatment and the correlation between leukocyte count and MVO: the significant positive correlation between neutrophil count and the extent of MVO was only present in control patients (that is, not receiving metoprolol); in patients receiving i.v. metoprolol before reperfusion there was no sign of association between total leukocyte or neutrophil counts and the extent of MVO (Fig. 2c,d). These results suggest that the administration of i.v. metoprolol during ongoing AMI do not affect the circulating levels of WBCs but modulates the impact of neutrophils on MVO.

Metoprolol blunts neutrophil infiltration in experimental IR. To identify the factors underlying the metoprolol-induced reduction in MVO within the reperfused myocardium, we examined a mouse model of in vivo myocardial IR injury (Fig. 3a). Given the observed modulator effect of i.v. metoprolol in the association between neutrophil count and MVO in patients suffering an AMI, we focused our attention into this cell population. We first tested the infarct-limiting effect of metoprolol in wild-type mice by occluding the left anterior descending (LAD) coronary artery for 45 min followed by reperfusion. Mice were randomized to receive a single i.v. bolus (50 μl) of metoprolol (10 mM) or vehicle (saline) 35 min after...
ischaemia onset (10 min before reperfusion). Infarct size was evaluated at 24 h reperfusion by TTC staining and normalized to area at risk (AAR, negative Evans blue staining). Metoprolol-treated mice had significantly smaller infarcts (as % of AAR) than vehicle-treated mice (Fig. 3b–d). Next, we examined mice carrying a GFP reporter in myeloid derived cells (LysM-GFP). LysM-GFP mice underwent the myocardial IR procedure and were randomized to receive i.v. metoprolol or vehicle. Capillary

Figure 1 | Metoprolol administration during ongoing AMI reduces MVO in patients. (a) METOCARD-CNIC trial scheme: patients with ongoing ST-segment elevation myocardial infarction (STEMI) were recruited and randomized to receive metoprolol (15 mg i.v. doses) or control before reperfusion. A total of 220 patients were evaluated for MVO by cardiac magnetic resonance (CMR) imaging 1 week after AMI and 202 patients for an additional CMR at long-term LVEF 6 months after AMI for ventricular function assessment. (b) Representative CMR exams (short-axis covering the entire left ventricle (LV) from base to apex), showing significant differences in 1-week MVO between a control patient (left) and a metoprolol-treated patient (right). Lower panels show detailed views of the boxed images, revealing MVO (blue area, automatic quantification), defined as the absence of contrast wash-in inside the delayed gadolinium-enhanced area (red, semiautomatic quantification). Yellow arrowheads indicate MVO in the LV wall. (c) Quantification of MVO in grams of left ventricle. (d) Quantification of MVO relative to the infarcted area (%). Dots represent values for individual patients: 114 in the control group (grey) versus 106 in the metoprolol group (blue). Data are means ± s.e.m. and compared by unpaired Student's t-test.
obliteration (a histological surrogate for MVO) and leukocyte infiltration were quantified at 6 h post-reperfusion (Fig. 3e–m). Metoprolol administration during ongoing AMI resulted in a significant reduction of capillary obliteration by circulatory cell plugins when evaluated at 6 h post-reperfusion (Fig. 3e,f). Confocal microscopy analyses revealed a significant reduction in the number of myeloid cells plugins (LysM-GFP^+^ particles) and percentage of LysM-GFP^+^ area within the LV sections, indicating rapid inhibition of leukocyte recruitment and protection of lumen vessel patency (Fig. 3g, Supplementary Fig. 2a–d and Supplementary Movie 1). Temporal (6 and 24 h) evaluation of myeloid-derived cells infiltration into injured myocardium showed a persistent abrogation of neutrophil infiltration (Fig. 3h–m and Supplementary Movie 2) and a differential relative proportion of different myeloid cells in hearts from metoprolol and vehicle-treated mice (Fig. 3j and Supplementary Fig. 3). These mouse experiments confirm the clinical findings that pre-reperfusion metoprolol administration during ongoing AMI limits infarct size and reduces MVO, and further show that metoprolol reduces neutrophil infiltration, suggesting neutrophils as a potential target in this cardioprotective effect.

Metoprolol does not protect from AMI in the absence of neutrophils. Catecholamine-stimulation of βARs alters neutrophil function, cytokine release and neutrophil–platelet aggregate formation^17,23,24, processes associated with aggravated injury during AMI (refs 25–27). To decipher the role of neutrophils in the protection afforded by metoprolol during ongoing AMI, we evaluated the effect of the drug in the absence of neutrophils. Neutrophil depletion in mouse peripheral blood was achieved by administration of an anti-Ly6G mAb over 2 days^8,28. After neutrophil depletion, animals were subjected to myocardial IR, and infarct size was evaluated at 24 h post-reperfusion (Fig. 3n). Confirming earlier reports^29, neutrophil-depleted mice had smaller infarcts than controls. Administration of metoprolol to these mice during ongoing AMI did not reduce infarct size (Fig. 3o–q). The abrogation of the cardioprotective effect of metoprolol confirms circulating neutrophils as a target of the beneficial effect associated with this pharmacological therapy.

Metoprolol inhibits neutrophil migration by targeting ADRB1. The effect of metoprolol on primary neutrophils’ function was
evaluated in a chemokine-induced transwell migration assay and by evaluation of the chemotactic FPR activator-peptide, W-peptide30, -induced reactive oxygen species (ROS) production assay31. First, neutrophils were exposed across the transwell filter to the chemoattractant CXCL1 in the presence or absence of metoprolol for 1.5 h, and migration through the transwell membrane was quantified by flow cytometry. Metoprolol inhibited baseline and epinephrine-stimulated neutrophil migration towards CXCL1, reducing migration to the same level in both cases (Fig. 4a). Second, ROS production in vitro was measured using DHR 123 and W-peptide activation. ROS production was tested in metoprolol-treated and untreated neutrophils with and without W-peptide stimulation. Metoprolol-treated neutrophils presented significant decreased oxidative burst compared to the non-treated cells after stimulation (Fig. 4b,c). Metoprolol alone had no effect on ROS endogenous production.

Metoprolol is a selective ADRB1-blocker, and ADRB1 signalling has been shown to mediate some of the pro-inflammatory response of monocytes32. Neutrophils and monocytes are both myeloid derived cells, and we therefore reasoned that ADRB1 might be involved in the anti-migratory effect of metoprolol. Adrb1 mRNA expression in fresh and viable isolated neutrophils was confirmed by PCR in wild-type mice and absence in neutrophils from Adrb1-knockout mice (Adrb1KO) (Supplementary Fig. 4a–c).

In vitro, the -epinephrine-mediated
Migration- and -W-peptide-mediated ROS production-inhibitory effect of metoprolol was lost in Adrb1KO neutrophils (Fig. 4a–c). β2-adrenergic receptor-knockout mice (Adrb2KO) was not involved in the effects observed after metoprolol administration (Supplementary Fig. 5).

We next explored whether metoprolol directly inhibits the capacity of neutrophils to infiltrate tissues in vivo. For this, we first used a model of thioglycolate-induced peritonitis (Fig. 4d). Thioglycolate induces massive leucocyte migration into the peritoneal cavity within the first 16 h (Supplementary Fig. 6a), with the majority of infiltrating cells being neutrophils (Supplementary Fig. 6b–d). Metoprolol i.v. administration sharply inhibited thioglycolate-induced neutrophil infiltration into the peritoneal cavity of wild-type mice (Fig. 4e,i), but the inhibitory effect of metoprolol was lost in Adrb1KO mice (Fig. 4f and Supplementary Fig. 6a,b).

To better define the cell compartment targeted by metoprolol, we lethally irradiated Adrb1KO mice and restored haematopoiesis with bone marrow (BM) transplanted from wild-type donors, generating chimeric mice expressing ADRB1 only in circulating cells. At 4 weeks after irradiation, transplanted animals presented >85% BM engraftment (Supplementary Fig. 7) and were subjected to thioglycolate-induced peritonitis. The replenishment of ADRB1 only in haematopoietic cells was enough to rescue the anti-leucocyte-infiltration effect of metoprolol (Fig. 4g). Together, these data show a direct effect of metoprolol on neutrophil function and demonstrate that the presence of ADRB1 in circulating cells is essential for the ability of metoprolol to reduce neutrophil infiltration into injured tissue.

**IR reduction by metoprolol involves haematopoietic cells’ ADRB1.** We next investigated the involvement of ADRB1 blockade in haematopoietic circulating cells in the protective effect of metoprolol in the infarcted myocardium. Adrb1KO mice were subjected to myocardial IR and randomized to receive i.v. metoprolol or vehicle during ongoing AMI (Fig. 4h). Unlike the situation in wild-type mice, metoprolol did not limit infarct size in Adrb1KO animals (Fig. 4i–k), demonstrating the critical role of ADRB1 blockade in the cardioprotective effect. To demonstrate the role of ADRB1 expression in haematopoietic circulating cells, we repeated the myocardial IR protocol in the chimeras described above (Adrb1KO mice transplanted with wild-type BM). The presence of ADRB1 only in haematopoietic circulating cells was sufficient to restore susceptibility to the cardioprotective effect of metoprolol (Fig. 4l–n). Conversely, transplant of Adrb1KO BM into irradiated wild-type mice abrogated the protective phenotype associated with metoprolol administration during IR (Fig. 4o–q). These data confirm the involvement of ADRB1 blockade in haematopoietic cells in the cardioprotection afforded by metoprolol administration during AMI.

**Metoprolol alters neutrophil dynamics in vivo.** During acute injury, neutrophils alter their morphology upon adhering to the activated endothelium. These shape change or polarization, permit intercellular interactions critical for the inflammatory response in several conditions, including myocardial IR (refs 8,33). Polarization of adhered neutrophils involves receptor redistribution and the assembly of a rearward protruding microdomain called the uropod, and is essential for the integration of signals coming from the endothelium and activated platelets before infiltration.8. We were therefore interested in investigating whether metoprolol impaired neutrophil migration and infiltration through an effect on neutrophil dynamics. For this, we used bi-dimensional and 3D intravital microscopy (IVM) to image cremaster muscle vessels of mice treated with tumour necrosis factor-α (TNFα), an inflammatory model in which the vast majority of recruited leukocytes are neutrophils. Neutrophil behaviour was evaluated 3h after administration of metoprolol or vehicle (Fig. 5a). Notably, metoprolol reduced neutrophil migratory velocity, accumulated crawling distance and directional movement (Fig. 5b,c and online Supplementary Movie 3). Independent-neutrophil 3D-reconstructions of live inflamed vessels showed that metoprolol consistently disabled the intravascular behaviour of neutrophils without disrupting polarization within activated vessels, resulting in dramatic changes in cell morphology (Fig. 5d–f) that correlated with their abnormal crawling dynamics. These data show that metoprolol ‘stuns’ neutrophils, resulting in altered dynamics and prevents the morphological changes needed to initiate intercellular interactions and subsequent tissue infiltration.

**Metoprolol blocks PSG11-dependent neutrophil platelet interactions.** Correct neutrophil polarization and organization of an extruding microdomain that captures circulating platelets is required to initiate tissue-damaging inflammation.8. Indeed, plugs of neutrophil–platelet co-aggregates in the microcirculation are a major contributor to MVO in AMI and in other models of injury.8,13. We therefore explored the impact of metoprolol-induced neutrophil stunning on neutrophil–platelet interactions.

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**Figure 3 | Metoprolol reduces neutrophil infiltration and capillary obliteration in murine IR.** (a) Mouse model of myocardial IR. (b, c) Histological evaluation of left ventricle (LV) area at risk (AAR) and infarct size (IS) in mice subjected to IR and randomized to receive metoprolol (blue) or vehicle (white); NS stands for non-significant. n = 8. (d) Representative images of LV slices showing AAR (negative for Evans Blue) in upper panels and extent of necrosis (triphenyltetrazoliumchloride (TTC)-negative area in lower panels). (e) Capillary obliteration quantified in H&E ten random images; n = 5–6. (f) Representative H&E myocardial images at 6 h reperfusion showing disarrayed and abundant obstructed capillaries in the vehicle-treated sample; metoprolol-treated samples show injury and nuclear condensation but no signs of MVO; green scale bars, 50 μm. Detailed amplification of the boxes show obstructed capillaries indicated with black arrows; black scale bars, 10 μm. (g) Confocal images from LV at 6 h after reperfusion onset showing massive vascular neutrophil migration (LysM-GFP, green) and co-aggregates with platelets (CD41, red) vehicle- but not in metoprolol-treated mice; scale bar, 25 μm. Next, amplified boxes indicating regions of capillary obstruction; scale bar, 10 μm. Bottom yellow panels show computed 3D reconstructions. (h,i) Myeloid-derived cell infiltration dynamics showing maintained attenuation in hearts from metoprolol-treated mice. n = 5. (j) Neutrophilic proportions infiltrate dynamics. (k) Representative confocal images of LV sections taken from injured mice after 6 and 24 h reperfusion onset. Myeloid infiltration (LysM-GFP, green) most of which are neutrophil (Ly6G +, red) is evident in vehicle-treated mice and significantly attenuated in those from metoprolol-treated mice; merged images show double positive cells (LysM +/Ly6G +, that is, neutrophils). Scale bar, 50 μm; n = 3–5. (l) Representative confocal images of neutrophil infiltration 24 h after reperfusion onset. Vehicle-treated mice show massive myocardial neutrophil infiltration (LysM-GFP +, green), with dispersed cells attached to the injured cardiac fibre membranes (α-actinin, red; laminin, grey). (m) LysM+ total area in the LV section as a %AAR; scale bar, 20 μm; n = 5–6. (n–q) Effect of metoprolol on limiting-infarct size in neutrophil-depleted mice. (m) Neutrophil depletion model. (o) Myocardial area at risk (AAR). (p) Infarct size. (q) Representative transverse ventricular slices showing AAR and infarct size Data are means ± s.e.m. *P < 0.05; **P < 0.01, determined by the nonparametric Wilcoxon–Mann–Whitney test for each panel.
Using the cremaster IVM model of TNFα-induced local inflammation, we evaluated the acute neutrophil–platelet inhibitory effect of metoprolol in polarized neutrophils (Fig. 5). Metoprolol i.v. administration effectively inhibited interactions with the uropod, but not the leading edge (Fig. 5g,h) and rapidly reduced total neutrophil–platelet interactions (Fig. 5i–k). IVM experiments in Adrb1KO mice revealed no differences between metoprolol-treated and vehicle-treated mice, implicating ADRB1 in the inhibitory effect of metoprolol on neutrophil–platelet interactions (Fig. 5j).

Based on these findings, we hypothesized that inhibition of neutrophil–platelet interactions underlies the inhibitory effect of metoprolol on MVO after myocardial IR. To test this, we first evaluated the effect of metoprolol on neutrophil–platelet interactions underlies the inhibitory effect of metoprolol on MVO after myocardial IR. To test this, we first evaluated the effect of metoprolol on neutrophil–platelet interactions underlies the inhibitory effect of metoprolol on MVO after myocardial IR. To test this, we first evaluated the effect of metoprolol on neutrophil–platelet interactions underlies the inhibitory effect of metoprolol on MVO after myocardial IR. To test this, we first evaluated the effect of metoprolol on neutrophil–platelet interactions underlies the inhibitory effect of metoprolol on MVO after myocardial IR. To test this, we first evaluated the effect of metoprolol on neutrophil–platelet interactions underlies the inhibitory effect of metoprolol on MVO after myocardial IR.
co-aggregate formation in mouse myocardial vessels after experimental IR. Administration of metoprolol to wild-type mice during ongoing AMI significantly reduced the number of neutrophils attached to the vessel wall (Fig. 6a,b) and the average number of interacting platelets per neutrophil (Fig. 6c).

Neutrophil–platelet interactions during acute injury are mediated by PSGL1 and signals delivered upon these contacts promote subsequent neutrophil extravasation and injury to the tissue6. Blockade of PSGL1 by pretreatment with PSGL1-mAb (Fig. 6e) significantly reduced infarct size in the myocardial IR model, and administration of metoprolol during ongoing AMI did not yield any further infarct-size reductions (Fig. 6f,g). These data confirm that metoprolol protects the infarcted myocardium by uncoupling neutrophil recruitment and polarization, thereby disrupting neutrophil–platelet interactions and the downstream inflammatory response.

**Metoprolol limits neutrophil–platelet aggregates in patients.**

To investigate whether metoprolol alters neutrophil dynamics and inhibits neutrophil–platelet interactions in humans, whole blood drawn from healthy donors was incubated *ex vivo* with epinephrine (5 μM) and increasing concentrations of metoprolol (0, 2, 5 μM). Samples were then stained, and neutrophil–platelet co-aggregate formation was evaluated by flow cytometry34–37 (morphological parameters, CD14neg, CD45+, CD61+). Metoprolol significantly inhibited epinephrine-stimulated neutrophil–platelet co-aggregate formation (Fig. 7a). The effect of metoprolol in *vivo* was additionally studied in patients undergoing elective coronary angioplasty for acute coronary syndrome (ACS). Samples were collected before and after i.v. administration of metoprolol (15 mg) and circulating neutrophil–platelet co-aggregates were assessed by flow cytometry. Metoprolol administration significantly reduced the number of neutrophil–platelet interactions (Fig. 7b). To elucidate whether metoprolol was acting at the platelet level of action we evaluated effect of metoprolol on platelet function through platelet aggregation using light transmittance aggregometry in platelet-rich plasma (PRP) (Supplementary Fig. 8a) or platelet activation surface markers expression assay using flow cytometry (Supplementary Fig. 8b,c). Metoprolol did not show any effect on platelet aggregation/activation, which together with the aforementioned effects on neutrophil migration and ROS-production suggests that the effect seen on neutrophil–platelet co-aggregates was driven by a direct effect on neutrophils.

**Discussion**

Early metoprolol administration during AMI, given as an adjunct to mechanical reperfusion has been shown to reduce infarct size and ameliorate post-infarction severe cardiac dysfunction11,38. Defining the mechanisms underlying this cardioprotection is therefore of great medical relevance since more efficient and specific protective strategies could be identified.

The ability of ADRB1 selective blockers to reduce infarct size was tested decades ago in several clinical trials, but the results were inconclusive1. However, most of these early studies were performed in the context of non-reperfused AMI. The advent of reperfusion as the treatment of choice for AMI changed the mode of myocardial death: from unrelied ischaemia to a combination of ischaemia and reperfusion processes. Therefore the potential cardioprotective effects of ADRB1 selective blockers needed to be revisited in light of the current evidence of IR injury during AMI (ref. 1). The ability of metoprolol to reduce infarct size in the context of reperfused AMI was recently evaluated in the METOCARD-CNIC trial. In this trial, early i.v. metoprolol administration during ongoing AMI resulted in a significant reduction of infarct size29,39, and also significantly reduced the incidence of severe ventricular dysfunction and heart failure readmissions10. Large animal studies conducted before the METOCARD-CNIC trial showed that metoprolol reduces infarct size only when administered before reperfusion40,41, suggesting that metoprolol might reduce infarct size by inhibiting reperfusion injury. The early studies, testing the infarct-limiting properties of metoprolol in non-reperfused AMI, were undertaken under the hypothesis that ADRB1-blockers would reduce the extent of damage by a direct effect on cardiomyocytes via reducing oxygen consumption. Although our large animal study40 did not investigate the mechanism of metoprolol-mediated protection, it did show that oxygen consumption was similar in metoprolol- and vehicle-treated pigs. In addition, the infarct-size reduction in metoprolol-treated pigs was associated with reduced myeloperoxidase activity in the post-ischaemic myocardium 24 h after reperfusion41. These findings challenged the idea that metoprolol could reduce cardiac damages simply by reducing myocardial oxygen consumption directly in the heart and prompted us to investigate the cellular mechanisms underlying the reperfusion-related injury-limiting effect of metoprolol during IR.

There is ample evidence supporting the critical implication of neutrophils in IR injury4,25,27,29,42,43. Neutrophils have two deleterious effects in this context. First, neutrophils and neutrophil–platelet plugs occlude microvessels, preventing efficient tissue perfusion: MVO. Second, neutrophils adhering to the newly reperfused injured vessels infiltrate the myocardium, prompting deleterious processes associated with reperfusion45,53. Our study confirms these associations in AMI patients: neutrophil count positively correlated with the extent of MVO as evaluated by state-of-the-art MRI 1 week after reperfusion. There exist weak pre-existing evidence linking the action of metoprolol to neutrophils, including reductions in

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**Figure 4 | Metoprolol directly inhibits neutrophil deleterious function through a ADRB1-blockade.** (a) Effect of metoprolol on CXCL1-induced migration of fresh isolated primary neutrophils (Ly6G+) from WT or ADRB1-knockout (β1KO) mice. CXCL1-stimulated cells were incubated with vehicle, epinephrine (10 μM), metoprolol (10 μM) and epinephrine + metoprolol; n = 4 independent experiments. NS, stands for non-significant. (b,c) Inhibitory effect of metoprolol on W-platelet-induced ROS production on fresh isolated primary neutrophils (Ly6G+) from WT or β1KO mice. Mean fluorescent intensity of Rho123 in Ly6G+ neutrophils after W-peptide stimulation. n = 6 independent experiments; flow cytometry plots illustrate reduced expression of Rho123 in metoprolol-treated neutrophils. (d-f) Effect of metoprolol on neutrophil activation surface markers expression (CD115neg; GR1+). (d) Neutrophil–platelet co-aggregate formation was evaluated by flow cytometry34–37 (morphological parameters, CD14neg, CD45+, CD61+). Metoprolol significantly inhibited epinephrine-stimulated neutrophil–platelet co-aggregate formation (Fig. 7a). The effect of metoprolol in *vivo* was additionally studied in patients undergoing elective coronary angioplasty for acute coronary syndrome (ACS). Samples were collected before and after i.v. administration of metoprolol (15 mg) and circulating neutrophil–platelet co-aggregates were assessed by flow cytometry. Metoprolol administration significantly reduced the number of neutrophil–platelet interactions (Fig. 7b). To elucidate whether metoprolol was acting at the platelet level of action we evaluated effect of metoprolol on platelet function through platelet aggregation using light transmittance aggregometry in platelet-rich plasma (PRP) (Supplementary Fig. 8a) or platelet activation surface markers expression assay using flow cytometry (Supplementary Fig. 8b,c). Metoprolol did not show any effect on platelet aggregation/activation, which together with the aforementioned effects on neutrophil migration and ROS-production suggests that the effect seen on neutrophil–platelet co-aggregates was driven by a direct effect on neutrophils.
post-IR myeloperoxidase activity in pig myocardium \(^{41}\) and rat spinal cord \(^{44}\), and inhibition of sepsis-induced inflammation in mice \(^{45}\). The data here presented from patients randomized in a controlled clinical trial are the first human evidence linking metoprolol with altered neutrophil behaviour \(\text{in vivo}\). We show that the strong positive correlation between neutrophil count and MVO was abolished in patients receiving i.v. metoprolol before reperfusion. The fact that metoprolol-treated patients’ high neutrophil count was not associated with the extent of MVO suggests an altered neutrophil dynamics during acute injury.

Some preclinical studies have suggested an association between metoprolol exposure and altered neutrophil dynamics \(^{41,44,45}\).
However, the cellular mechanism responsible for this effect has remained unknown. Our results from the in vitro transwell migration assays confirm previous studies showing the ability of metoprolol to inhibit migration of isolated neutrophils. Furthermore, the studies with Adrb1KO mice provide evidence for a critical involvement of the Adrb1 axis in the effects of metoprolol. In the transwell assays, metoprolol had no effect on migration even in the presence of the agonist orciprenaline, while in our study we have used a genetic knockout toll (that is, Adrb1KO mice), which ensures the absence of Adrb1. Finally, we ruled out any involvement of Adrb2 on metoprolol action in an apparently Adrb1-independent manner. In that study authors reported that metoprolol was able to inhibit neutrophil migration even in the presence of the β-adrenergic receptor agonist orciprenaline, while in our study we have used a genetic ablation toll (that is, Adrb1KO mice), which ensures the absence of Adrb1.

Upon acute injury, neutrophils recruited to injured vessels initiate inflammation by scanning for activated platelets present in the circulation, establishing interactions with them through PSGL1 exposed on neutrophil protrusions. The IVM confocal imaging analysis demonstrated that metoprolol prevents the exposure of functional PSGL1 clusters, which are essential for...
interaction with platelets. These events correlated with alterations in neutrophil properties essential for infiltration, including migratory velocity and directionality. In agreement with these data, in the mouse myocardial IR model, metoprolol altered neutrophil 3D structure and reduced the numbers of neutrophil–platelet co-aggregates that occluded myocardial vessels. The inability of metoprolol to provide additional protection after blockade of neutrophil–platelet interactions with the anti-PSGL1 mAB is compelling evidence for interference in this interaction as the mechanism underlying the infarct-limiting action of metoprolol during myocardial IR in mice. The demonstration that metoprolol inhibits neutrophil–platelet interactions in healthy volunteers and ACS patients suggests that this mechanism also operates in humans. There is controversy on the direct effect of metoprolol on platelet aggregation. Some studies suggested that metoprolol was able to inhibit ADP- and epinephrine-mediated platelet aggregation, but recent studies did not show any anti-platelet effect of metoprolol in vitro. In line with the latter evidence, in our study we found that metoprolol had no effect on platelet aggregation or in platelet activation in healthy volunteers. These data strongly suggest that the inhibitory effect of metoprolol on neutrophil–platelet co-aggregates was driven by a direct effect on neutrophils. In our flow cytometry studies we defined a positive neutrophil–platelet interaction as CD14+CD45+, CD61+ particles. It should be noted that there are more specific neutrophil markers and thus our selection is a limitation of these experiments. However, given that all data (human and mouse) point to the same direction, we think this limitation did not have an impact on the conclusions obtained in these experiments.

The role of neutrophils in experimental IR injury is well established; however, the negative results of clinical trials with anti-inflammatory therapies (for example, Csa (refs 50,51), CD-18 (ref. 52)) dampened hopes for this pharmacological strategy, and suggested that parallel mechanisms driving myocardial injury were at play. The demonstration that neutrophil dynamics in general and neutrophil–platelet interactions in particular are the target for the protective actions of metoprolol administered to patients during AMI reinstates neutrophils as a potential target for the therapeutic reduction of infarct size.

Methods

**Human cardiac magnetic resonance imaging.** The METOCARD-CNIC trial (NCT01311700) recruited patients suffering an AMI during hospital transit to undergo mechanical reperfusion by primary angioplasty. Patients were randomized to receive i.v. metoprolol (up to 15 mg) or no drug (control). Patients underwent two CMR studies: 1 week and 6 months after AMI. Images were acquired with a 3.0 Tesla magnet (Achieva Tx, Philips Medical Systems) with vectorcardiographic gating and a dedicated cardiac 32-channel phased-array surface coil. The extent of MVO was measured in the 1-week CMR study; to detect and quantify MVO, a delayed enhancement imaging was performed 10 min after gadolinium contrast injection, using a T1-weighted 2-D Inversion Recovery Turbo Field Echo (2D IR-TFE) sequence. Myocardial necrosis was defined by the extent of abnormal gadolinium enhancement, whereas MVO was defined as black-hypoenhanced areas within the bright-hypoenhanced regions. CMR analysis was undertaken by operators blinded to treatment allocation at the Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC). Myocardial necrosis and MVO were quantified by semi-automated delineation with dedicated software (QMass MR 7.6; Medis, Leiden, the Netherlands). Total MVO was quantified as grams of LV. To correct for infarct size, MVO was also expressed as a percentage of the infarct area.

**Mouse procedures.** Experimental procedures were approved by the CNIC Animal Care and Ethics Committee and regional authorities. IR, thioglycollate-induced peritonitis and IVM experiments were performed in 8–13-week-old wild type male (C57BL/6) mice. β1-adrenergic receptor (Adrb1) knockout (KO; Adrb1KO) mice were in a mixed background. β2-adrenergic receptor (Adrb2) knockout (KO; Adrb2KO) mice were in a C57BL/6 background. For the BM transplant experiments, Adrb1KO mice were backcrossed with mice expressing DsRed under the control of the β-actin promoter to facilitate evaluation of BM engraftment. Male and female mice were used as donors in BM transplant procedures, but only males were used in myocardial IR and thioglycollate-induced peritonitis experiments. All animals were randomized to receive a single i.v. injection (50 μl) of metoprolol tartrate (10 mM) or vehicle (saline). Histological evaluation of injured myocardium in the myocardial IR model was performed in lysozyme M-GFP+ (LyS-GFP) mouse male mice. Intravascular neutrophil and neutrophil–platelet interactions were scored manually in the myocardium. Neutrophils were depleted in C57BL/6 male by i.v. injections of 50 μg anti-mouse Ly6G 24 and 48 h before the myocardial IR procedure5. For in vivo blocking of P-selectin glycoprotein ligand-1 (PSGL1), 50 μg of anti-PSGL1 antibody (clone 4RA19) was administered 15 min after ischemia onset. Mice were maintained under pathogen-free conditions in a temperature-controlled room and a 12-h light–dark cycle at the CNIC animal facilities. Chow and water were available ad libitum.

**Reagents.** Metoprolol tartrate (M5391), Evans blue, triphenyltetrazolium chloride, DAPI (D8417-1MG), Mowiol mounting medium (81381), anti-laminin (19393), anti-α-actin (A7732), dihydroxyrodamine 123 (D1054) were obtained from Sigma-Aldrich. Dylight-650 conjugated anti-1A8 Ly6G (BE0075-1) and anti-PSGL1 antibody (clone 4RA10) from BioXcell. Anti-CD41 (12-04-11-83) and anti-CD11b (12-113283) were obtained from BioXcell. W-peptide (17-5445-02) and Ready-to-go RT-PCR Beads (27-9259-01) from GE Healthcare. CXCL1 was obtained from (453-KC-010) from R&D Systems. W-peptide (WKYMVM, 17998) from Tocris. Thioylcysteine (BD211716) and anti-GP IIb/IIIa from BD Biosciences. Anti-GR1 (ab2557) was obtained from Abcam and AF-647 (A-21472) from Molecular Probes. Anti-CD45-FITC from Miltenyi Biotec, Germany. PC5-conjugated anti-CD14, anti-CD61 PC7 and Versalyse solution were obtained from Beckman Coulter.

**Mouse model of myocardial IR injury.** Male 8–12-week-old mice were subjected to 45 min occlusion of the LAD coronary artery followed by 6 or 24 h of reperfusion. For infarct size evaluation, reperfusion was maintained for 24 h. For analysis of MVO, neutrophil infiltration and neutrophil–platelet interactions, reperfusion was maintained for 6 or 24 h as indicated. The IR procedure was performed as previously described5. Briefly, fully asleep animals were intubated and temperature controlled throughout the experiment at 36.5 °C to prevent hypothermic cardiac depression. Thoracotomy was then performed and the LAD was ligated with a nylon 8/0 monofilament suture for 45 min. The electrocardiogram was monitored (MP36, Biopac Systems Inc.) to confirm total coronary artery occlusion (ST-segment elevation) throughout the 45 min ischemia. Ten minutes before reperfusion onset, mice were randomized to receive a single i.v. injection (50 μl) of metoprolol tartrate (10 mM) or vehicle (saline) through the femoral vein. Dose of metoprolol was chosen after a dose response study (Supplementary Fig. 9).

At the end of reperfusion, the chest was closed and animals were kept with 100% O2 and analgesized with buprenorphine (S.C., 0.1 mg per kg) until the end of reperfusion.

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**Figure 7** | Metoprolol inhibits neutrophil–platelet interactions in patients. (a) Effect of metoprolol on neutrophil–platelet co-aggregate formation in epinephrine-stimulated whole blood from healthy volunteers (n=20). Whole blood was incubated in vitro with epinephrine 5 μM and metoprolol (Met, concentrations in μM). (b) In vivo effect of metoprolol (up to 15 mg i.v.) on the number of neutrophil i.v.platelet co-aggregates in acute coronary syndrome (ACS) patients scheduled for coronary angioplasty. Blood was drawn before and after metoprolol i.v. administration; n = 6 ACS patients. Pre, before i.v. administration; Post, after i.v. administration. Data are means ± s.e.m. *P < 0.05; **P < 0.01, determined by one-way ANOVA and Holm Sidak’s post-hoc multiple comparisons method.
Mouse infarct size quantification. At the end of follow up, mice were re-anesthetized and re-intubated, and the LAD coronary artery was re-occluded by ligating the suture in the same position as the original infarction. Animals were then killed and rapidly 1% (w/v) Evans Blue dye was infused i.v. to delineate AAR; myocardium lacking blood flow, that is, negative for blue dye staining. The heart was then harvested, LV was isolated, cut into transverse slices (5–7 mm thick slices per LV) and both sides were imaged. Sections post-Evans blue staining present two different areas: one palish negative for Evans blue dye followed by the delineteing AAR, and another blueish (positive Evans blue) area indicating remote tissue. In order to differentiate infarcted from viable tissue, same slices were incubated in triphenyltetrazolium chloride (TTC, 1% (w per v) diluted in PBS) at 37 °C. Image were acquired with an RT-PCR Beads (27-9259-01, GE Healthcare). PCR was performed with 40 cycles of 95 °C for 12 s and 60 °C for 1 min. All PCR reactions were done in triplicates. Primers for Hprt and Adrb1 were as follows: mHprt_fw5-GAGGAGTCTGTAGTGGTGCAG-3, mHprt_rv5-GGCGTT CGCTATAGGCCTATAGTG-3, mAdrb1_fw5-GTGTTGAACTGCTGT TGATG-3, mAdrb1_rv5-GAAGTGGAGCTGTCAGCGGAGA-3. Amplicons generated in the qPCR were loaded on to an agarose gel to confirm PCR products.

Mice for the experiments were randomized into four groups: vehicle, vehicle + Adrb1 KO, metoprolol, and metoprolol + Adrb1 KO. Systemic blood pressure was monitored by tail cuff and the heart rate (HR) was monitored by ECG tracings. After anesthetization, mice were randomized to receive an intraperitoneal injection of vehicle (0.9% saline), metoprolol tartrate (10 mg/kg), or a combination of the two. At 20 min after injection of vehicle or metoprolol, mice were killed and hearts were rapidly harvested and perfused with ice-cold 0.9% saline solution until no visible blood flow was observed. Hearts were then minced and digested with collagenase and incubated with 1 mg/ml dispase and 1% (v/v) trypsin. After the enzymatic digestion, cells were filtered through a 70 mm filter and plated into a 24-well plate at a density of 10^6 cells per well. After 24 h, nonadherent cells were washed away and the remaining adherent cells were treated with 10 μM DHR 123. The fluorescence was measured using a plate reader and percentage of total H^+ influx was calculated. Nonadherent cells were also collected and submitted to qPCR analysis for Adrb1 expression. RT-PCR was performed with 40 cycles of 95 °C for 12 s and 60 °C for 1 min. All PCR reactions were done in triplicates. Primers for Hprt and Adrb1 were as follows: mHprt_fw5-GAGGAGTCTGTAGTGGTGCAG-3, mHprt_rv5-GGCGTT CGCTATAGGCCTATAGTG-3, mAdrb1_fw5-GTGTTGAACTGCTGT TGATG-3, mAdrb1_rv5-GAAGTGGAGCTGTCAGCGGAGA-3. Amplicons generated in the qPCR were loaded on to an agarose gel to confirm PCR products.

Migration transwell assay. The ability of leukocytes to migrate towards chemokine (C-X-C motif) ligand 1 (CXCL1) was assessed using a modification of the method of Villablanca et al. Briefly, wild-type or Adrb1 KO mice were heparinized (30 μl of 0.1% w/v heparin), and residual erythrocytes were lysed with hypotonic buffer. PBS-washed leukocytes of the same genotype were pooled and resuspended in RPMI containing 10% FBS and the appropriate treatment: saline (vehicle control), 10 μM epinephrine (positive control), 10 μM metoprolol-tartrate, or a combination of epinephrine and metoprolol. Transwell inserts (6.5 mm, 5.0 μm pore size, 385 μl) were pre-coated with Costar Coatings. Forty-eight hours after the start of the experiment, 3 × 10^5 cells were applied to each well. After 20 min and placed in 24-well plates before seeding cells (100 μl; 1 × 10^6; > 90% viability). Lower compartments (wells) were filled with 600 μl DMEM medium containing 0.04 ng per μl CXCL1 to induce directional movement. Spontaneous migration was assessed in wells lacking CXCL1. After incubation at 37 °C for 1.5 h, cells in the lower compartment were collected and neutrophils (Ly6G +) were evaluated by flow cytometry. Each independent experiment was conducted with leukocytes pooled from nine animals, and each of the five conditions was run with four replicates. Mean spontaneous migration was subtracted from the migration value of each well, and neutrophil migration was expressed as a percentage of the total number of neutrophils seeded in the upper chamber at the start of the experiment. For comparison between different genotypes and treatments, migration was normalized to the mean control (vehicle) value.

Neutrophil oxidative burst assay. Blood from wild-type or Adrb1 KO mice was collected in heparinized tubes and diluted in 100 μl aliquots, erythrocytes were lysed with hypotonic buffer. After centrifugation, leukocytes were washed and re-suspended in high glucose phenol red free DMEM. Cells were then incubated for 50 min with or without metoprolol tartrate (10 mM). As previously described, dihydrorhodamine 123 (DHR 123) (1799 Tocris). After 20 min and placed in 24-well plates before seeding cells (100 μl; 1 × 10^6; > 90% viability). Lower compartments (wells) were filled with 600 μl DMEM medium containing 0.04 ng per μl CXCL1 to induce directional movement. Spontaneous migration was assessed in wells lacking CXCL1. After incubation at 37 °C for 1.5 h, cells in the lower compartment were collected and neutrophils (Ly6G +) were evaluated by flow cytometry. Each independent experiment was conducted with leukocytes pooled from nine animals, and each of the five conditions was run with four replicates. Mean spontaneous migration was subtracted from the migration value of each well, and neutrophil migration was expressed as a percentage of the total number of neutrophils seeded in the upper chamber at the start of the experiment. For comparison between different genotypes and treatments, migration was normalized to the mean control (vehicle) value.

Model of thioglycolate-induced peritonitis. To assess the ability of metoprolol to inhibit neutrophil recruitment, we used a well-established thioglycolate-induced peritonitis model (see Fig. 3). Wild-type mice were intraperitoneally injected with 1 ml of thioglycolate and immediately randomized to receive a single 50 μl i.v. injection of vehicle or metoprolol tartrate (10 mM). After 20 min of injection, neutrophils of each genotype were evaluated by flow cytometry at the same time point. Neutrophils were collected from the peritoneal cavity using EDTA tubes for later haematological analysis in a haematocytometer (Pentra 80). Next, animals were killed, 2 ml PBS was injected intraperitoneally and distributed manually for 30 s to detach infiltrated circulatory cells. Next, another 8 ml PBS was injected to facilitate collection of 6 ml peritoneal exudate. Exudates were gently centrifuged, and cells were washed twice with PBS and incubated for 1 h with anti-G1 (1:200) and PE-conjugated anti-CD11b (1:200). After washing with PBS, cells were incubated for 30 min with anti-rat 647 to detect GR1. Cell nuclei were stained with DAPI. All samples were analysed by flow cytometry for exactly 30 s of constant flow. Neutrophil recruitment efficiency was presented as neutrophils per ml of exudate for each independent animal.

To evaluate the role of ADRB1 in different compartments, we performed the same experiments in Adrb1 KO mice and generated cohorts of chimeric mice by transplantation with BM cells from wild-type and Adrb1 KO donors. Four weeks after transplantation, chimeric mice were evaluated by flow cytometry as the percentage of donated cells. Animals with chimerism below 85% were discarded; those with chimerism above 85% underwent the thioglycolate-induced peritonitis
protocol followed by randomization to receive either i.v. vehicle or metoprolol (10 mM). To compare between the different chimeric groups, the mean value for each metoprolol-treated group was normalized to the mean for the vehicle-treated group.

Bone marrow transplant. BM transplants protocols were adapted from Casanova et al. Recipient mice from Adb1KO or wild-type genotypes (DnRed+ or DsRed- as appropriate) were lethally irradiated (13 Gy in two doses) before bone marrow (BM) donor BM. Donor BM was collected from the appropriate genotype by flushing both tibiae and femurs into PBS containing 2 mM EDTA (Pef-buffer). Contaminating erythrocytes were lysed with hypotonic buffer. Engraftment in recipient animals was assessed by flow cytometry 3–4 weeks after transplantation. Animals bled for engraftment evaluations were rested for 1 week before any other procedure.

Flow cytometry. Neutrophil purity for in vitro migration assay and both neutrophil and platelet mRNA expression analysis were purified by light and bright-field microscopy with Dylight anti-IAb, Ly6G and with DAPI to assess viability. Mouse primary blood leukocytes from peritoneum experiments were incubated with anti-Gr1 conjugated with AF647 and with PE-conjugated anti-CD115 and DAPI. Neutrophils were gated on the basis of Gr1+ positive and CD115-negative staining in a FACS Canto-3L flow cytometer equipped with DIVA software (BD Biosciences). Doublet discrimination and viability (negative to DAPI) was assessed for every sample. Data were analysed with FlowJo (Ashland) software by blinded observer. All experiments were conducted at the CNIC-Cellomics Unit.

Intravital microscopy. IVM of the cremaster muscle was performed after intrascrotal injection of TNF± (0.5 mg R&D Systems), followed immediately by injection of a single i.v. bolus of metoprolol (10 mM) or vehicle, and neutrophil behavior was evaluated 3 h after stimulus. In some experiments metoprolol was injected 3 h after treatment with TNF± and images immediately acquired for analysis (see Fig. 4). The IVM system was built by 3i (Intelligent Imaging Innovations, Denver, CO, USA) on an Axio Examiner Z.1 workstation (Zeiss, Oberkochen, Germany) mounted on a 3-Dimensional Motorized Stage (Sutter Instrument, Novato, CA, USA). This set up allows precise computer-controlled lateral movement between XY positions and a Z focusing drive for confocal acquisition. The microscope is equipped with a CoollED pW widefield fluorescent light source system (CoollED Ltd. UK) and a quad pass filter cube with a Semrock Dm01-R405/488/561/635 dichroic and a FF01-446/523/600/700 excitation and emission for CD45, CD41, Ly6G and PE and a 446/488/561/610 excitation and emission for annexin V, CD64, CD61 and CD115. Images were acquired with a CoolSnap HQ2 camera (6.45 × 6.45 µm pixels, 1,392 × 1,040 pixel format; Photometrics, Tucson, AZ, USA). For confocal high-speed IVM, we used laser stacks for 488, 561 and 640 nm beams coupled with a confocal scanner (Yokogawa CSU-X1; Yokogawa, Japan); images were acquired at 0.5 µm Z-intervals. Image acquisition was coordinated and offline data analysis facilitated with SlideBook software (Intelligent Imaging Innovations), run on a Dell Precision T7500 computer (Dell Inc., Round Rock, TX, USA). For three-dimensional analysis we used the 3D surface view function to determine the position of the CD62L+ clusters relative to the cell body and the nucleus. To six to ten venules per mouse were analysed 210 to 300 min after TNF± treatment by acquisition of fluorescence (Cy3/561 channels for phycocerythrin, FITC/488 channels for FITC and Cy5/640 channels for allophycocyanin) and bright-field images with 2 × 2 for 2 min. For double staining with PE- and FITC-conjugated antibodies, acquisition was facilitated in single (FITC) and quads (PE) filters in order to obtain the necessary range of potential blue cell bodies from the three display areas (xy top view, xz front view, and zy lateral view) of the cell. For all experiments, covered by the cell. Directionality measures how straight the cell track is, and is obtained as the ratio of euclidean distance to accumulated distance.

Analysis of 3D features of intravascular neutrophils. We used the 3D features of intravascular neutrophils using Imaris Software (Bitplane, Oxford, UK). From the parameters provided by the ImarisCell module, we selected prolate ellipticity by obtaining the lengths of the three semi-axes, which correspond with the spherical coordinates, whereas the accumulated distance is the total length of the path covered by the cell. Directionality measures how straight the cell track is, and is calculated as the ratio of euclidean distance to accumulated distance.

Analysis of 3D reconstructions of polarized neutrophils. We measured the 3D features of intravascular neutrophils using Imaris Software (Bitplane, Oxford, UK). From the parameters provided by the ImarisCell module, we selected prolate ellipticity by obtaining the lengths of the three semi-axes, which correspond with the spherical coordinates, whereas the accumulated distance is the total length of the path covered by the cell. Directionality measures how straight the cell track is, and is calculated as the ratio of euclidean distance to accumulated distance.

Human neutrophil-platelet interactions evaluation. Human citrated blood was diluted 1:5 in HEPES-Tyrode’s+ (5 mM hydroxyethylpiperazineethane-sulfonic acid (HEPES), 137 mM NaCl, 2.7 mM NaHCO3, 0.36 mM NaH2PO4, 2 mM NaHPO4, 2 mM CaCl2, 5 mM glucose, bovine albumin 0.2%, pH=7.4) and incubated with 0.2 or 5 µM metoprolol for 10 min. Then, 5 µl of diluted blood was incubated with 5 µM epinephrine for 10 min. Unstimulated and epinephrine-stimulated samples were stained with PCS-conjugated anti-CD14 anti CD45-FITC and anti-CD61 PC7 for 20 min at room temperature in the dark. Erythrocytes were lysed for 10 min using Versalyse solution. Appropriate mouse isotype controls were used for each antibody. Flow cytometry analysis was performed with a Gallios cytometer (Beckman Coulter, Miami, FL, USA). Leukocytes were by CD45-FITC staining. Neutrophils identification was adapted from refs 34,35 and performed by morphological parameters (side scatter) and negative staining for CD14-PC5 but positive staining for CD45-FITC. Neutrophil–platelet conjugates were identified as CD61-PE and positive to CD14-PC5 and CD61-PC7 (refs 36,37). The acquisition process was stopped after collection of 5,000 monocytes. Data are expressed as the percentage of neutrophil–platelet aggregates.

Human blood sampling. Functional tests were performed in blood samples from 20 volunteers (36 ± 6 years, 15 men). Exclusion criteria were as follows: any platelet, anticoagulant or anti-inflammatory drug taken within the 2 previous weeks; abnormal platelet or leucocyte count; or any history of abnormal bleeding, thrombosis, or active inflammatory disease. Written consent was obtained from all volunteers. Blood samples were collected into polystyrene tubes containing sodium citrate from an antecubital vein with a 21 gauge needle, discarding the first 2 ml to avoid platelet activation. Blood was collected between 8:00 and 10:00 after overnight fasting. Samples were processed immediately. ACS patients were recruited at our cath-lab (both genders, age < 80) from among those referred for coronary angiogram and subsequent percutaneous coronary intervention. Exclusion criteria were: active treatment with anti-inflammatory or other concomitant therapy; severe obstructive lung disease; heart failure or valvular heart disease; atrial fibrillation requiring antiarrhythmic therapy; renal failure with creatinine ≥ 2 mg per ml; liver disease with bilirubin ≥ 2 mg per ml; acute illness or any malignancy; pregnancy; nursing; body mass index ≥ 27 kg per m². Doublet discrimination and viability (negative to DAPI) was assessed for every sample. Data were analysed with FlowJo (Ashland) software by blinded observer. All experiments were conducted at the CNIC-Cellomics Unit.

Analysis of blood cell interactions. Platelets in the inflamed cremaster muscle were visualized as CD41-labelled cells and quantified as reported. Briefly, we defined the uropod of adherent neutrophils as the domain staining positive for CD62L and the leading edge as the CD62L-negative pole forming multiple protrusions and showing guided movement. Six to ten venules per mouse were recorded, and platelet interactions with neutrophils were counted and analysed manually at the two distinct domains of the polarized neutrophil with the help of Slidebook software.

Analysis of tracking of crawling neutrophils. Time-lapse movies of crawling neutrophils were analysed with ImageJ, which includes the Manual Tracking and the Chemotaxis and Migration Tool plugins. For each movie we first adjusted channel intensities and converted them into RGB format. Movies were rotated so that the vessels and the blood flow were positioned horizontally and oriented left-right. When necessary, the Background subtraction and Image stabilization pre-established plugins were applied to eliminate noise and reduce tissue twitching, respectively. Both plugins were set up with xy calibration values, which depend on the camera and microscope parameters, to convert pixels into linear measures, as well as the time interval value between movie frames (3s). Each polarized neutrophil (identified by a clear polarized morphology or uropod staining) was tracked individually for 1 min using automated tracking. Tracks were then used to generate a data set with the respective xy track coordinates. We then used the Chemotaxis and Migration Tool to plot and the velocity (µm per s), accumulated distance (µm), euclidean distance (µm) and directionality of the tracks obtained. The Euclidean distance is the length of the straight-line segment connecting the initial and finishing points, whereas the accumulated distance is the total length of the length of the path covered by the cell. Directionality measures how straight the cell track is, and is calculated as the ratio of euclidean distance to accumulated distance.
All experiments were conducted at the Hospital Universitario Clínico San Carlos, Madrid.

**Human platelet function evaluation.** Platelet aggregation was assessed using light transmittance aggregometry in PRP by the turbidimetric method in a four-channel aggregometer (Chrono-Log 490 Model, Chrono-Log Corp., Havertown, PA, USA) according to standard protocols. The PRP was obtained from citrated blood at centrifugation (800 g r.p.m.) for 10 min and platelet-poor plasma was obtained after a second centrifugation (2,500 g r.p.m.) for 10 min. PRP will be adjusted to 250,000 per μl with autologous plasma. PRP was incubated with metoprolol 2 and 5 μM or saline buffer for 15 min and then stimulated using epinephrine (5 μM). Light transmission was adjusted to 0% with PRP and to 100% with platelet-poor plasma for each measurement. Curves were recorded during 5 min and platelet aggregation was determined as the maximal percent change in light transmittance.

Platelet function was determined by assessing platelet activation as surface expression of activated GP Ib/IIa (Becton Dickinson) and P-selectin using flow cytometry. Whole blood from healthy donors were drawn into trisodium citrate tubes diluted with Hanks-tyrodes-buffer (0.2% BSA) to a final volume of 1:8:1 (blood: Hepes-tyrodes:citrate). Diluted blood was incubated with metoprolol 2 and 5 μM or saline for 15 min. Following activation with epinephrine (5 μM) samples were incubated for 20 min with polyclonal PAC1-FITC conjugated or PE-conjugated anti-Cd62p. Appropriate isotype controls were used in each case. Platelet aggregation was expressed as the percentage of platelets positive for antibody binding. Platelets were gated on the basis of light scatter and CDE1 antibody expression. Activated platelets were defined as the percentage of expressing the activated confirmation of PAC1 binding and P-selectin (CD62p). Data were expressed as the percentage of platelets positive for antibody binding. All experiments were conducted at the Hospital Universitario Clínico San Carlos, Madrid.

**Statistics.** Data are represented as mean ± s.e. of the mean (s.e.m.), and analysed using Prism software (Graph pad, Inc.) and Stata 12.0: StataCorp LP, College Station, TX, USA. Comparisons between two groups were performed by using the unpaired two-tailed Student’s t-test or the nonparametric Wilcoxon–Mann–Whitney test as appropriate. Comparisons between more than two groups were performed by using the one-way ANOVA. The P-value was adjusted with the Holm Sidak’s multiple comparison test. Multiple linear regression analysis was used to study the influence of metoprolol on MVO, adjusted for factors potentially affecting MVO such as sex, age, ischaemia duration, diabetes, use of thrombectomy or glycoprotein Ib/IIa inhibitors. Test for linear trend after one-way ANOVA was used to study the relationship between LVEF at 6 months and MVO quartiles at 1 week. Power calculations were applied to obtain statistically significant at P values below 0.05 significant. *P<0.05, **P<0.01, ***P<0.001.

**Study approval.** All studies in patients and volunteers were approved by the ethics committee of Hospital Clínico San Carlos, Madrid. Written informed consent was received from all participants before inclusion in the study.

**Data availability.** The data that support the conclusions of this study are available from the corresponding author on reasonable request.

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Author contributions

B.I. is responsible for the design of the entire study, assisted by J.G.-P. Experimental myocardial IR, perfusion experiments, neutrophil migration assays and bone marrow transplants were done by J.G.-P., M.G., R.V.-G. and A.P.-G. Interpreted by B.I. Genotyping and transcripts expression evaluation were done by M.G., R.V.-G. and D.S. R. Histological processing and evaluations were done by M.G., R.V.-G., R.B.-M. and J.G.-P. MVO evaluation in MRI studies from the METOCARD-CNIC trial were analysed by J.G.-L., R.F.-J., J.M.G.-R. and G.P. METOCARD-CNIC trial Pls: B.I and V.F. Correlation studies of WBC and MVO were done by J.M.G.-R. and J.G.-P. Interpreted by B.I. Intravital microscopy experiments and analysis were done by G.C., V.S. and A.S.V. Interpreted by A.H. Human neutrophil-platelet interaction and platelet function experiments were done by E.B. and analysed by J.G.-P. Interpreted by A.F.-O and B.I. Statistical analyses were done by J.G.-P., J.M.G.-R. and R.F.-J. Manuscript was drafted by J.G.-P. and critically revised by A.H., V.F. and B.I. B.I. and J.G.-P. are responsible for the final version of the manuscript, which was approved by all authors.

Additional information

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