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***FAST*TRACK – CLINICAL RESEARCH**
Vascular biology and medicine

Malondialdehyde-specific natural IgM inhibit NETosis triggered by culprit site–derived extracellular vesicles from myocardial infarction patients

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Abstract

Background and Aims Neutrophil extracellular traps (NETs) trigger atherothrombosis during acute myocardial infarction (AMI), but mechanisms of induction remain unclear. Levels of extracellular vesicles (EV) carrying oxidation-specific epitopes (OSE), which are targeted by specific natural immunoglobulin M (IgM), are increased at the culprit site in AMI. This study investigated EV as inducers of NETosis and assessed the inhibitory effect of natural anti-OSE–IgM in this process.

Methods Blood from the culprit and peripheral site of ST-segment elevation myocardial infarction (STEMI) patients ($n = 28$) was collected, and myocardial function assessed by cardiac magnetic resonance imaging (cMRI) 4 ± 2 days and 195 ± 15 days post-AMI. Extracellular vesicles were isolated from patient plasma and cell culture supernatants for neutrophil stimulation *in vitro* and *in vivo*, in the presence of a malondialdehyde (MDA)-specific IgM or an isotype control. NETosis and neutrophil functions were assessed via enzyme-linked immunosorbent assay and fluorescence microscopy. Pharmacological inhibitors were used to map signalling pathways. Neutrophil extracellular trap markers and anti-OSE–IgM were measured by ELISA.

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Results CD45+ MDA+ EV and NET markers were elevated at the culprit site. Extracellular vesicles induced neutrophil activation and NET formation via TLR4 and PAD4, and mice injected with EV showed increased NETosis. Malondialdehyde-specific IgM levels were inversely associated with citH3 in STEMI patient blood. An MDA-specific IgM inhibited EV-induced NET release *in vitro* and *in vivo*. CD45+ MDA+ EV concentrations inversely correlated with left ventricular ejection fraction post-AMI.

Conclusions Culprit site–derived EV induce NETosis, while MDA-specific natural IgM inhibit this effect, potentially impacting outcome after AMI.

Structured Graphical Abstract

Key Question

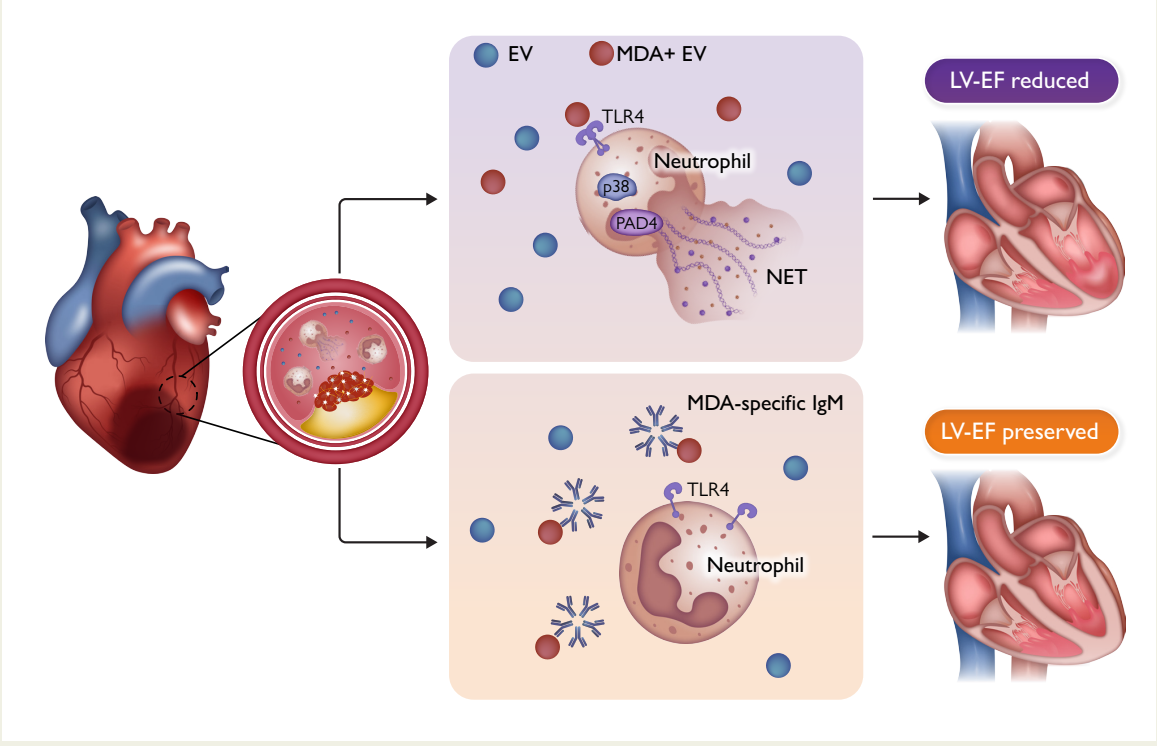
What induces and modulates neutrophil extracellular traps (NET) formation during ST-elevation acute myocardial infarction (STEMI)?

Key Finding

Culprit site-derived extracellular vesicles of STEMI patients triggered NET formation in vitro and in mice. Toll-like receptor 4 (TLR4) and PAD4 were identified as key mediators of culprit site-derived EV-induced NETosis. This effect was inhibited by natural IgM recognizing oxidation-specific epitopes. The level of extracellular vesicles carrying these epitopes was inversely associated with left ventricular ejection fraction in STEMI.

Take Home Message

Extracellular vesicles and natural IgM are endogenous modulators of NETosis in AMI.



Different subsets of extracellular vesicles (EV) are released at the culprit site of ST-segment elevation myocardial infarction (STEMI), and a high percentage of leukocyte-derived EV carry oxidation-specific epitopes (OSE) such as malondialdehyde (MDA) epitopes (red). Culprit site EV from STEMI patients (MI-EV) can activate neutrophils and trigger peptidyl-arginine deiminase 4 (PAD4)–dependent formation of neutrophil extracellular traps (NETs) via toll-like receptor 4 (TLR4) signalling. The presence of the MDA-specific IgM antibody LR04 reduces MI–EV-induced NETosis. ST-segment elevation myocardial infarction patients with high levels of MDA-specific IgM, which have the capacity to balance activity of pro-inflammatory NETogenic EV, present with preserved left ventricular ejection fraction (LVEF).

Keywords Neutrophil extracellular traps • Natural IgM • Oxidation-specific epitopes • Extracellular vesicles • Acute myocardial infarction

Introduction

Cardiovascular diseases (CVDs) remain the leading cause of death worldwide despite increasing efforts in prevention.[1,2] Acute myocardial infarction (AMI) is a manifestation of atherosclerosis and is promoted by inflammation.[3] Plaque rupture and erosion are the culprits of acute atherothrombosis. We have previously demonstrated an accumulation of neutrophils at the culprit site,[4] which was predictive of long-term mortality after AMI.[5] The functional role of neutrophils in AMI has been attributed to their ability to form neutrophil extracellular traps (NETs).[6] Subsequently, NETs were implicated in plaque destabilization and increased vulnerability,[7] but also in thrombosis.[8] Thus, NETs are involved in both chronic and acute stages of atherosclerotic CVD. These expulsed webs of chromatin interspersed with granule proteins and neutrophil proteases have the capacity to trap invading pathogens,[9] but have also shown to effectively entangle platelets, erythrocytes, other immune cells, and coagulation factors.[10,11] We have identified NETs as an integral part of coronary thrombi,[10] and NET markers are specifically elevated in blood aspirated from the culprit site.[10,12–14] In addition to coronary occlusion, we could associate NET burden with microvascular obstruction and decreased left ventricular ejection fraction (LVEF) after AMI.[13] Reduction of NET formation in murine ischaemia–reperfusion models decreased myocardial damage suggesting promise for NETosis blockers as well as DNase treatment in AMI.[15] Therefore, understanding mediators involved in induction and inhibition of NETosis in the context of atherothrombosis may become important for the treatment of AMI. The exact triggers of NETosis during AMI have been elusive.

Interestingly, extracellular vesicles (EV) have been suggested as potential triggers of NETosis in different settings,[16–18] but their involvement in NET formation in AMI has not been studied. Extracellular vesicle is an umbrella term for phospholipid bilayer vesicles released by resting and activated cells. Although there is no clear consensus on EV terminology, EV can be divided into two major classes according to their biogenesis, either in the intracellular endosomal system or by budding from the outer cell membrane. Although larger EV (~.1–1 μm), previously called microvesicles, seem to be predominantly released by blebbing from the cytoplasmic membrane, there is limited evidence that the mode of release shows clear size restrictions,[19–22] as arrestin domain containing protein 1 (ARRDC1)–mediated budding has been described for smaller EV.[23,24] Extracellular vesicles are loaded with different classes of biologically active molecules such as lipids, metabolites, or regulatory RNAs; thus, their content may reflect the activation status of their parental cells,[25] making them suitable as biomarkers.[26–30] Functionally, they act as mediators of intercellular communication, and the properties of cells targeted by EV may be influenced by both uptake of their content and sensing of surface moieties.[31] Moreover, exposure of pro-coagulant phospholipids and tissue factor on EV harbours a direct pro-thrombotic potential.[26,32–35] Thus, characterization of the biological activities of EV is crucial for the understanding of their role in atherosclerotic CVD.

The circulating levels of EV were found to be elevated in AMI,[36,37] and we have identified a subset of EV that are recognized by immunoglobulin M (IgM) antibodies with specificity for oxidation-specific epitopes (OSE).[38] Oxidation-specific epitopes are adducts formed by the modification of different biomolecules with lipid peroxidation products and usually accumulate at sites of inflammation with increased generation of reactive oxygen species (ROS)[39] such as atherosclerotic plaques. Oxidation-specific epitopes exhibit robust pro-inflammatory activities and represent a distinct class of danger-associated molecular patterns (DAMP),[39–41] which are present on oxidized low-density lipoproteins (OxLDL), dying cells, and a subset of EV.[38,42,43] OSE are generated in different pathologies associated with increased oxidative stress.[44,45] Importantly, we found OSE+ EV to be increased at the culprit site of coronary occlusion in AMI,[38] though their functional role in this has not been addressed.

Different components of innate immunity recognize OSE and modulate their biological activities.[39] Neutralization of specific OSE is protective in mouse models of atherosclerosis.[46,47] For example, the pro-atherogenic role of OSE has been documented in a murine model of atherosclerosis by the beneficial effects of ectopic expression of the single-chain variable fragment of the natural IgM antibody E06 that binds and neutralizes oxidized phospholipids.[47] Natural IgM are germline-encoded antibodies that represent important effectors of innate immunity,[48] and we have previously found that a large part of natural IgM has specificity for OSE.[48] Moreover, several OSE-specific IgM have been cloned, and their protective effects were demonstrated *in vitro* and in animal models of different pathologies.[38,49,50]

We and others have shown that circulating levels of OSE-specific natural IgM, including malondialdehyde (MDA)-specific IgM, are inversely associated with cardiovascular risk.[51–53] The MDA-specific IgM antibody LR04 can inhibit pro-inflammatory and pro-coagulant effects of EV,[38,50] which may provide a mechanistic explanation for these epidemiological observations. Importantly, we have recently demonstrated that low titres of IgM antibodies recognizing a unique peptide mimotope of MDA, which was identified by peptide phage display using LR04, are associated with increased AMI incidence in the Pakistan Risk of Myocardial Infarction Study.[54] Several protective mechanisms for MDA-specific IgM have been proposed with respect to plaque development and progression, but the mechanism by which they mediate protection in the acute event has not been addressed. Considering the accumulating evidence on a key role for NETosis in AMI, we hypothesized that OSE+ EV trigger NET formation during AMI and that binding of IgM to OSE+ EV inhibits this process. Therefore, we investigated the effect of culprit site EV on neutrophils, specifically examining NET formation *in vitro* and *in vivo* and assessed the inhibitory role of OSE-specific natural IgM antibodies in this process.

Methods

All methodological procedures are described in detail in the Supplementary Data.

Patient study protocol (STATIM trial)

This patient population was a subset of the 'Strategic targeted temperature management' study (STATIM, NCT01777750).[55] Patients were recruited at the time of diagnosis of ST-segment elevation myocardial infarction (STEMI). Inclusion criteria were anterior or inferior infarction with ST-segment elevations > .2 mV in two contiguous leads and a maximum duration of 6 h between symptom onset and presentation to emergency medical service. All patients received 250 mg of intravenous acetylsalicylic acid, oral prasugrel or ticagrelor, and intravenous unfractionated heparin to achieve an activated coagulation time > 300 s (4000–10 000 IE). Only patients who had originally been randomized to the control arm and had been eligible for coronary aspiration of thrombus material ($n = 28$) were included in the present investigation. Detailed study exclusion criteria, criteria for thrombectomy, and the processing protocols for blood samples are provided in the Supplementary Data. Myocardial function was assessed by cardiac magnetic resonance imaging (cMRI) 4 ± 2 days (for simplification, a 72-h time point is used) and 195 ± 15 days (for simplification, a 6-month

time point is used) after primary percutaneous coronary intervention (pPCI) as previously described.[55]

Healthy subjects for neutrophil isolation were recruited during routine healthcare check-ups, were above 18 years, and did not have documented CVD. The study protocols were approved by the Ethics Committee of the Medical University of Vienna (approval numbers 1497/2012 and 1947/2014) and were carried out according to the Declaration of Helsinki and its amendments. All patients gave written informed consent.

Statistical analysis

Gaussian distribution was assessed by Shapiro–Wilk tests and histograms. Two independent groups were analysed with Student's *t*-test or Mann–Whitney *U* test, while two paired groups were compared by paired *t*-tests or Wilcoxon matched-pairs signed-rank test. More than two groups were subjected to one-way analysis of variance (ANOVA). A mixed model was utilized to assess the effect of the sampling site and cellular origin on the presence of MDA on EV. In our model, EV subset, site, and the interaction between EV subset and site were treated as fixed effects. Subject, interaction between subject and EV subset, and interaction between subject and site were considered random effects. Subset and site were matched between patients. Tukey's multiple comparisons test was applied to adjust the *P*-values. The specific tests that were used are stated in the respective figure legends; data were only transformed for statistical comparison if specifically stated in the figure legend, even if data are plotted on a log scale for presentation. To test the predictive value of EV levels for reduced LVEF (<40%), the area under the receiver operating characteristic (ROC) curve was calculated. The confidence intervals were calculated using the method by Wilson/Brown. A *P*-value of <.05 was considered statistically significant. Statistical analysis was done with IBM SPSS version 29 and Graph Pad Prism version 9.5.1.

Results

Extracellular vesicles at the culprit site

In order to investigate a potential link between NET formation and OSE–IgM in STEMI, we measured levels of natural IgM antibodies recognizing MDA epitopes in plasma of STEMI patients, in whom we had correlated outcome with NET surrogate markers.[13] We selected patients undergoing pPCI subsequent to thrombus aspiration from the culprit site ($n = 28$). Patients were predominantly male and relatively young, with an average age of 55 ± 10 years, and a typical cardiovascular risk factor profile (see Supplementary data online, *Table S1*).

Levels of MDA-specific IgM correlated inversely with the degree of NET formation as measured by concentrations of citH3 in plasma. This effect was observed in peripheral blood ($r_s = -.535$, $P = .005$, $n = 27$, *Figure 1A*) and in coronary blood ($r_s = -.393$, $P = .043$, $n = 27$, *Figure 1B*). In general, neutrophil activation and NET surrogate markers were significantly higher at the culprit site as compared to the peripheral site (see Supplementary data online, *Figure S1*). Based on these observations, we hypothesized that MDA-specific IgM might interfere with NET formation in STEMI.

Given that a significant portion of circulating EV carry MDA epitopes,[38] we hypothesized that this subset of EV could be a potential trigger of neutrophil activation and NET formation in STEMI. Extracellular vesicles were isolated from culprit site plasma (MI–EV) of STEMI patients and thoroughly characterized. Nanoparticle tracking analysis revealed the presence of smaller and larger EV with an average diameter of 150 nm (see Supplementary data online, *Figure S2A*). Flow cytometry demonstrated that MDA+ EV represented a subset with a bigger diameter carrying phosphatidylserine, thereby allowing classification as larger EV (see Supplementary data online, *Figure S2B*). Furthermore, isolated MI–EV and corresponding plasma samples were analysed by mass spectrometry, confirming different protein contents through principal component analysis (see Supplementary data online, *Figure S3A*). Compared with plasma, EV preparations were enriched in proteins typically listed in VesiclePedia[56,57] (see Supplementary data online, *Figure S3B* and *Table S2*). Analysis of minimal information for studies of EV (MISEV) category 1 proteins (transmembrane or GPI-anchored proteins associated with plasma membrane and/or endosomes;[19] Supplementary data online, *Figure S3C and D*) and MISEV category 2 proteins (cytosolic proteins;[19] Supplementary data online, *Figure S3E*) revealed significant enrichment of these EV hallmark proteins in isolated MI–EV compared with plasma. Although certain lipoproteins were present after differential centrifugation (see Supplementary data online, *Figure S3F*), they constituted a smaller proportion of the total protein content in the EV preparation compared to the parental plasma (see Supplementary data online, *Table S3*).

Considering that MDA+ EV constituted a larger diameter subset, we measured the concentrations and frequencies of larger (.24–1.1 µm) MDA+ EV derived from endothelial cells (CD144+), platelets (CD41a+), and leukocytes (CD45+) at the culprit site and in peripheral arterial blood. As expected, concentrations of larger EV and MDA+ EV were significantly higher at the culprit site as compared to the arterial peripheral circulation (see Supplementary data online, *Figure S4A and B*).

Larger CD45+ and CD45+ MDA+ EV exhibited the highest quantitative difference between peripheral and culprit sites (*Figure 1C and D*). Importantly, also the frequency of this CD45+ MDA+ subset was increased at the culprit site, suggesting a site-specific enrichment of MDA-carrying leukocyte-derived EV (*Figure 1E*). The concentration of CD41a+ EV was significantly higher at the culprit site than in the periphery (see Supplementary data online, *Figure S4C*), while those of CD144+ EV were the same at the culprit and peripheral sites (see Supplementary data online, *Figure S4E*), as were the concentrations of CD41a+ MDA+ and CD144+ MDA+ EV (see Supplementary data online, *Figure S4D and F*). The frequency of MDA+ EV derived from leukocytes (CD45+) was significantly higher than of the other two EV subsets at the culprit site (*Figure 1F*), while there was no difference at the peripheral site. These data indicated local leukocyte activation and increased release of leukocyte EV at the site of coronary occlusion. Levels of EV ($r_s = .488$, $P = .010$, $n = 27$), CD45+ EV ($r_s = .468$, $P = .014$, $n = 27$), and CD45+ MDA+ EV ($r_s = .402$, $P = .038$, $n = 27$) correlated with culprit site neutrophil elastase (NE) levels, but not with other NET surrogate markers (see Supplementary data online, *Table S4*).

Myocardial infarction–extracellular vesicles from the culprit site induce NETosis

Next, we tested the effect of EV on neutrophil-like differentiated HL60 (dHL60) cells (see Supplementary data online, *Figure S5A and B*). Culprit site MI–EV induced a prominent release of typical NET structures of chromatin interspersed with MPO. Notably, EV derived from the peripheral venous circulation 72 h after intervention also induced NETosis, but this was less accentuated (see Supplementary data online, *Figure S5C*), indicating a declining pro-inflammatory activity in the recovery phase of STEMI. This observation is in accordance with decreasing concentrations of MDA+ EV (see Supplementary data online, *Figure S5D–F*), leukocyte counts (see Supplementary data online, *Figure S6A and B*), and NET levels (see Supplementary data online, *Figure S6D–F*).

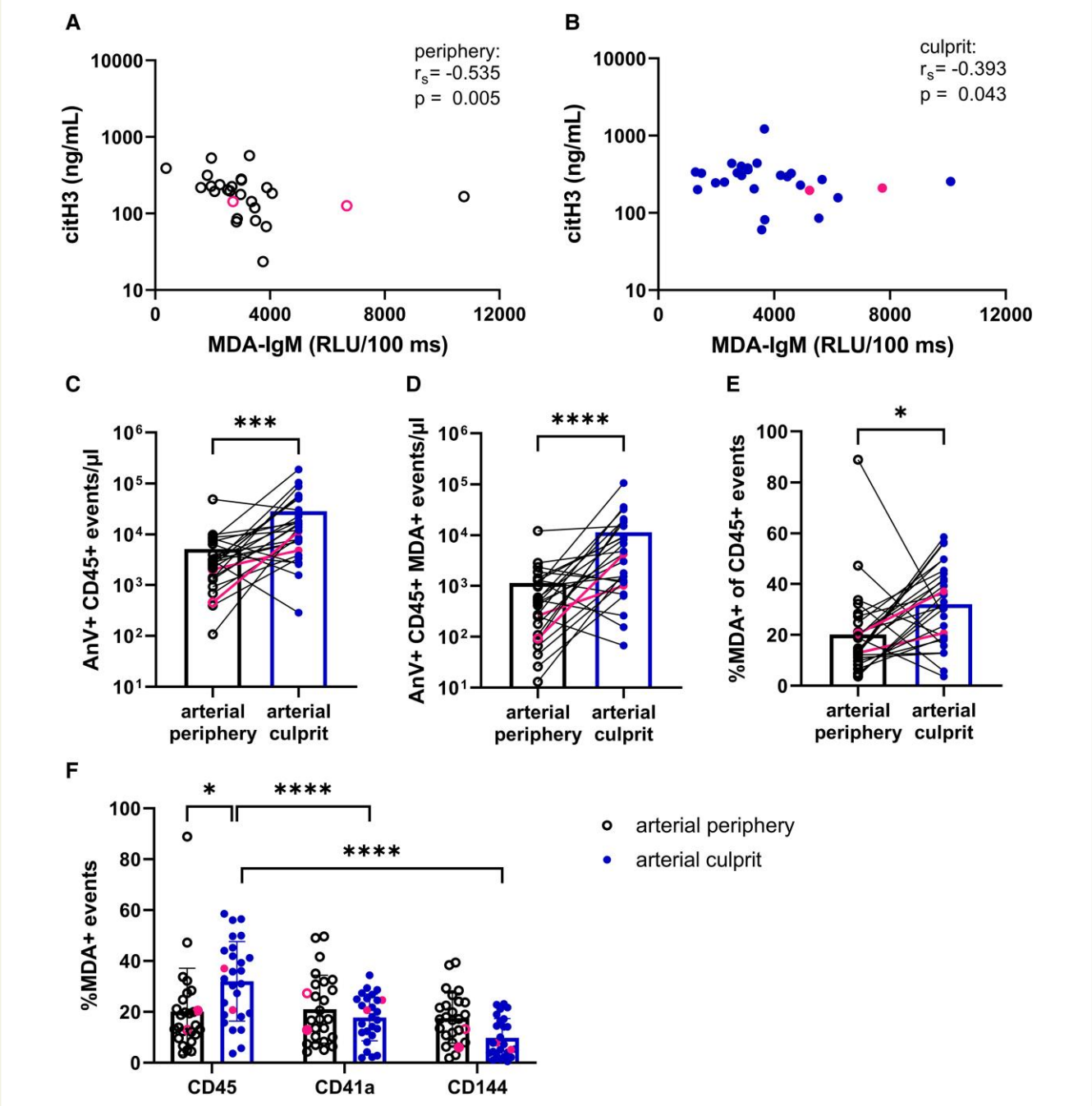


Figure 1 Concentration and percentage of CD45+ extracellular vesicles carrying malondialdehyde epitopes are elevated at the culprit site. Citrullinated histone H3 (citH3), malondialdehyde-specific immunoglobulin M, and extracellular vesicles were measured in culprit site and peripheral site plasma of acute myocardial infarction patients. Annexin V positivity and a size range of .2–1.1 µm were used to define large extracellular vesicles. Anti-CD45 and malondialdehyde-specific LR04 antibodies were used to define leukocyte origin and MDA+ extracellular vesicles. (*A*) Correlation of plasma levels of citH3 with malondialdehyde-specific immunoglobulin M in the periphery and (*B*) at the culprit sites; Spearman signed-rank correlations. Comparison between the periphery and culprit site of (*C*) annexin V–positive CD45+ events/µL, (*D*) Annexin V–positive CD45+ malondialdehyde-positive events/µL, and (*E*) percentage of malondialdehyde-positive extracellular vesicles gated from annexin V–positive CD45+ events; Wilcoxon matched-pairs signed-rank tests. (*F*) Percentages of malondialdehyde-carrying extracellular vesicles derived from leukocytes (CD45+), platelets (CD41a+), and endothelial cells (CD144+) were compared at the culprit site and in the periphery using a mixed model *$P < .05$, **$P < .01$, *** $P < .001$, ****$P < .0001$. Light-shaded dots represent female patients. RLU, relative light units

Since CD45+ EV were the subset with the highest frequency of MDA epitopes at the culprit site as compared to the peripheral circulation (*Figure 1F*), we specifically tested the stimulatory capacity of leukocyte-derived EV on primary human neutrophils. Therefore, we generated EV *in vitro* by activating THP-1 monocytic cells with lipopolysaccharide (LPS) as previously described.[58]

Lipopolysaccharide-stressed cells released a higher number of EV enriched in EV-carrying MDA epitopes (see Supplementary data online, *Figure S7*).

These EV robustly activated primary human neutrophils shown by a distinct release of the NET marker deoxyribonucleic acid (DNA)–myeloperoxidase (MPO) complexes (*Figure 2A*; Supplementary data online, *Figure S8A*) and NE (*Figure 2B*) into the supernatant. Extracellular vesicles could also trigger a pronounced release of interleukin-8 (IL-8; *Figure 2C*; Supplementary data online, *Figure S8B*) after 3 h, and degranulation of azurophilic (MPO, *Figure 2D*; Supplementary data online, *Figure S8C*) and specific granules [neutrophil gelatinase–associated lipocalin (NGAL), *Figure 2E*; Supplementary data online, *Figure S8D*] after 30 min, but did not increase the production of ROS (*Figure 2F*; Supplementary data online, *Figure S8E* and *F*).

Finally, we confirmed that MI–EV also have the ability to induce NET formation of primary human neutrophils by visualizing formed NETs in fluorescence microscopy (*Figure 2G* and *H*). Taken together, EV can trigger NET formation and other major functions of neutrophils.

Extracellular vesicle–induced NETosis is dependent on TLR4 and PAD4

We used a pharmacological approach to pinpoint the role of two major signalling axes, i.e. PAD4-dependent and PAD4-independent NET formation in EV-induced NETosis by inhibiting TLR4, PAD4, p38 mitogen activated kinase, protein kinase C (PKC), MEK, and NADPH oxidase. Inhibition of TLR4, PAD4, and p38 significantly inhibited EV-induced NETosis as measured by release of DNA–MPO complexes (*Figure 3A–C*), while PKC, MEK, and NADPH oxidase inhibitors only reduced NET formation by PMA or had no effect (*Figure 3D–G*). TLR4 and PAD4 were confirmed as key mediators for NETosis using MI–EV and primary neutrophils as assessed by fluorescence microscopy (*Figure 3H–K*). Notably, the effect of all tested inhibitors was not uniform across other neutrophil effector functions induced by EV, as, for example, neither inhibition of TLR4 nor PKC or MEK influenced the release of IL-8 (see Supplementary data online, *Figure S9A–F*), MPO (see Supplementary data online, *Figure S10A–F*), or NGAL (see Supplementary data online, *Figure S11A–F*). Thus, extracellular vesicle–induced effects on neutrophils exhibited a distinct signalling pattern compared to 'classical' stimuli (see Supplementary data online, *Figure S8*).

LR04 inhibits NETosis induced by extracellular vesicles from acute myocardial infarction patients *in vitro*

To test the ability of the MDA-specific IgM LR04 to interfere with EV-induced NETosis, primary human neutrophils were stimulated with EV in the presence of LR04. LR04, but not an isotype control, significantly attenuated the release of DNA–MPO complexes from primary neutrophils (*Figure 4A*).

Similarly, release of NETs by primary neutrophils and neutrophil-like dHL60 cells stimulated with MI–EV was also inhibited by LR04 as assessed by microscopy (*Figure 4B* and *C*; Supplementary data online, *Figure S12A* and *C*, correspondingly). To rule out EV-independent effects of LR04 leading to an unspecific decrease in NET formation, we also stimulated neutrophil-like dHL60 cells with IL-8, a well-established, pathophysiological NET trigger.[59] While IL-8 induced NETs, neither isotype control IgM nor LR04 significantly reduced NETosis (see Supplementary data online, *Figure S12B* and *D*).

LR04 inhibits NETosis induced by extracellular vesicles from acute myocardial infarction patients in mice *in vivo*

To validate our findings *in vivo*, we tested the NET-inducing potential of MI–EV in a mouse model. Given the conserved nature of OSE in vertebrates,[39] as well as the conserved binding by pattern recognition receptors, we studied the ability of human MI–EV to activate circulating neutrophils in mice. Mice were injected with MI–EV from human culprit site plasma and sacrificed after 3 h to study systemic neutrophil activation in the blood (*Figure 5A*). The percentage of neutrophils positive for citH3 was significantly higher in blood of mice injected with MI–EV compared to sham-injected animals (*Figure 5B–D*). Moreover, the relative area of *ex vivo*–formed NETs detected by immunofluorescence (IF) microscopy (*Figure 5B* and *C*) was significantly larger in blood of mice injected with MI–EV compared to sham-injected animals (*Figure 5E*).

Importantly, the number of circulating EV was not significantly influenced following injection, indicating that exogenous EV did not substantially alter the steady-state levels of EV in the circulation (see Supplementary data online, *Figure S13A*) but rather increased the proportion of EV with a high potential to induce NETosis. While circulating cell-free double-stranded DNA (dsDNA) was significantly increased in mice receiving MI–EV compared to sham-treated animals (see Supplementary data online, *Figure S13C*), citH3 remained unchanged (see Supplementary data online, *Figure S13B*). Altogether, these data indicate that MI–EV can induce NETosis in mice *in vivo*.

Levels of MDA-specific IgM have been shown to decline in acute settings in humans, suggesting increased consumption of these antibodies.[60] In order to test the hypothesis that binding of MDA-specific IgM to exogenously administered EV can reduce antibody levels in the circulation, plasma IgM concentrations were compared before and after injections.

After 3 h, circulating levels of total IgM were significantly decreased in MI–EV-injected mice (see Supplementary data online, *Figure S13D*) consistent with the fact that a large part of natural IgM has specificity for MDA epitopes. To attribute this decline to specific consumption of OSE–IgM, MDA-specific IgM was normalized to total IgM. Even after normalization, MDA-specific IgM levels were significantly lower in MI–EV-injected mice than mice at baseline (see Supplementary data online, *Figure S13E*) indicating a specific consumption of these antibodies.

Based on this finding and on our *in vitro* results, we aimed to confirm the potential inhibitory effect of MDA-specific IgM LR04 on EV-induced neutrophil activation *in vivo*. Mice were co-injected with MI–EV and either an isotype control or LR04. Antibody administration did not significantly increase the levels of total IgM in both groups (see Supplementary data online, *Figure S14A*). Importantly, IgM reactivity with a peptide mimotope (P2) specifically recognized by LR04[54,61] was robustly increased in plasma of animals injected with LR04 but not with isotype control (see Supplementary data online, *Figure S14B*). Thus, administration of LR04 was responsible for the increase of P2 mimotope-reactive IgM. Moreover, co-injection of MI–EV and antibodies did not affect haematocrit and differential blood counts (see Supplementary data online, *Figure S15A–F*).

Co-injection of LR04 with MI–EV resulted in reduced neutrophil activation *in vivo* as documented by a considerably lower NET area in IF microscopy of cytospins of peripheral blood and a marginally

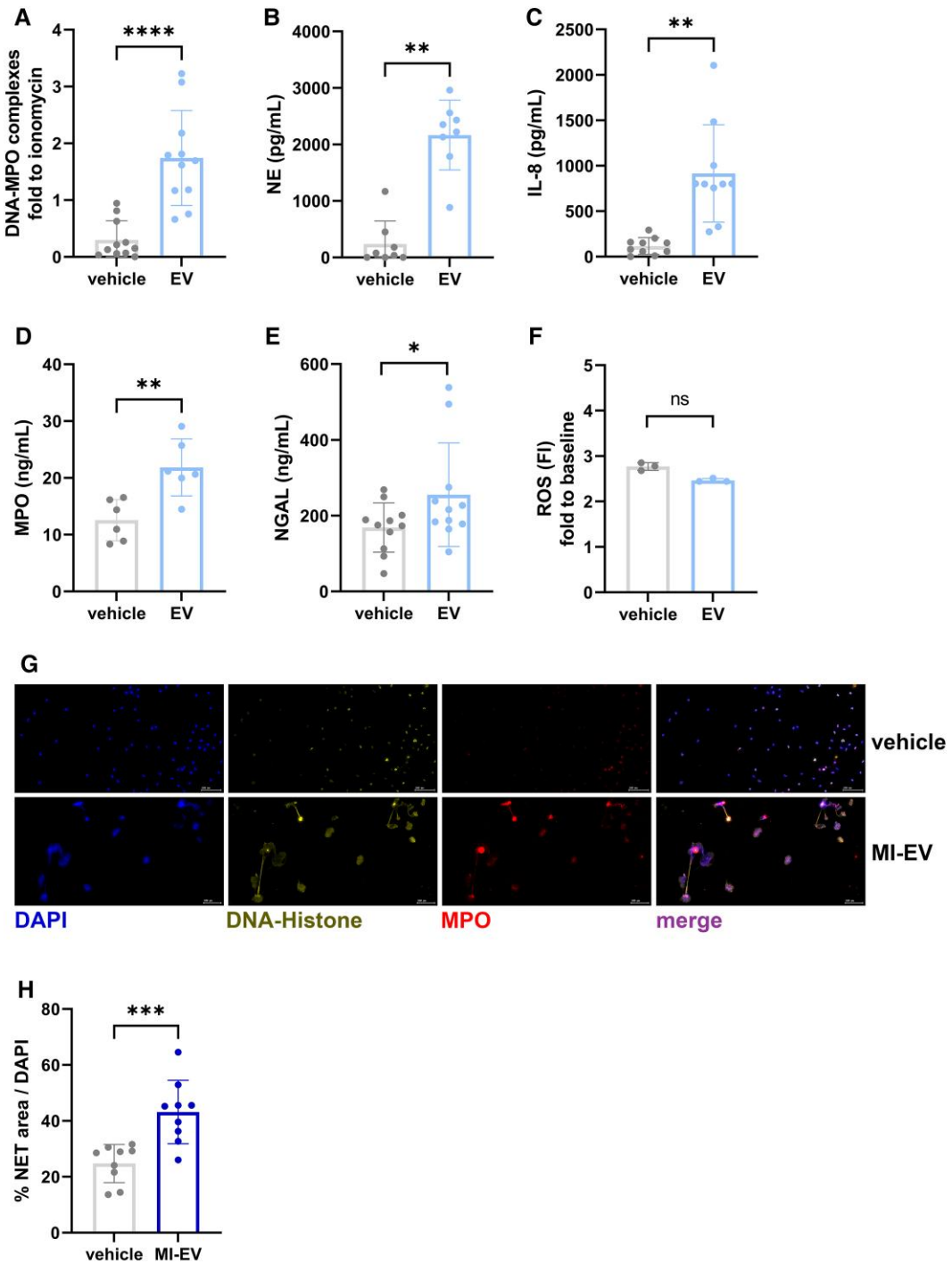


Figure 2 Extracellular vesicles induce neutrophil activation and neutrophil extracellular trap formation. (*A*–*F*) Neutrophils of healthy donors were stimulated either with extracellular vesicles or vehicle control, and supernatants were screened for the release of (*A*) deoxyribonucleic acid–myeloperoxidase complexes as neutrophil extracellular trap surrogate markers after 3 h, (*B*) neutrophil elastase after 3 h, (*C*) interleukin-8 after 3 h, (*D*) degranulation of myeloperoxidase after 30 min, (*E*) degranulation of neutrophil gelatinase–associated lipocalin after 30 min, and (*F*) reactive oxygen species production after 15 min. (*G* and *H*) Neutrophils of healthy donors were stimulated with myocardial infarction–extracellular vesicles, and formed neutrophil extracellular traps were stained with anti-deoxyribonucleic acid–histone (yellow) and anti-myeloperoxidase (red) antibodies. DAPI staining (blue) was used to visualize DNA/cell nuclei. (*G*) Representative pictures of neutrophil extracellular traps formed upon stimulation with vehicle (*top*) and culprit site myocardial infarction–extracellular vesicles (*bottom*), which were (*H*) quantified by normalizing the merged area to total DAPI area; paired *t*-tests, (*E*) data were log-transformed before statistical comparison, **P* < .05, ***P* < .01, ****P* < .001, *****P* < .0001. MPO, myeloperoxidase; NE, neutrophil elastase; NGAL, neutrophil gelatinase–associated lipocalin; ROS, reactive oxygen species

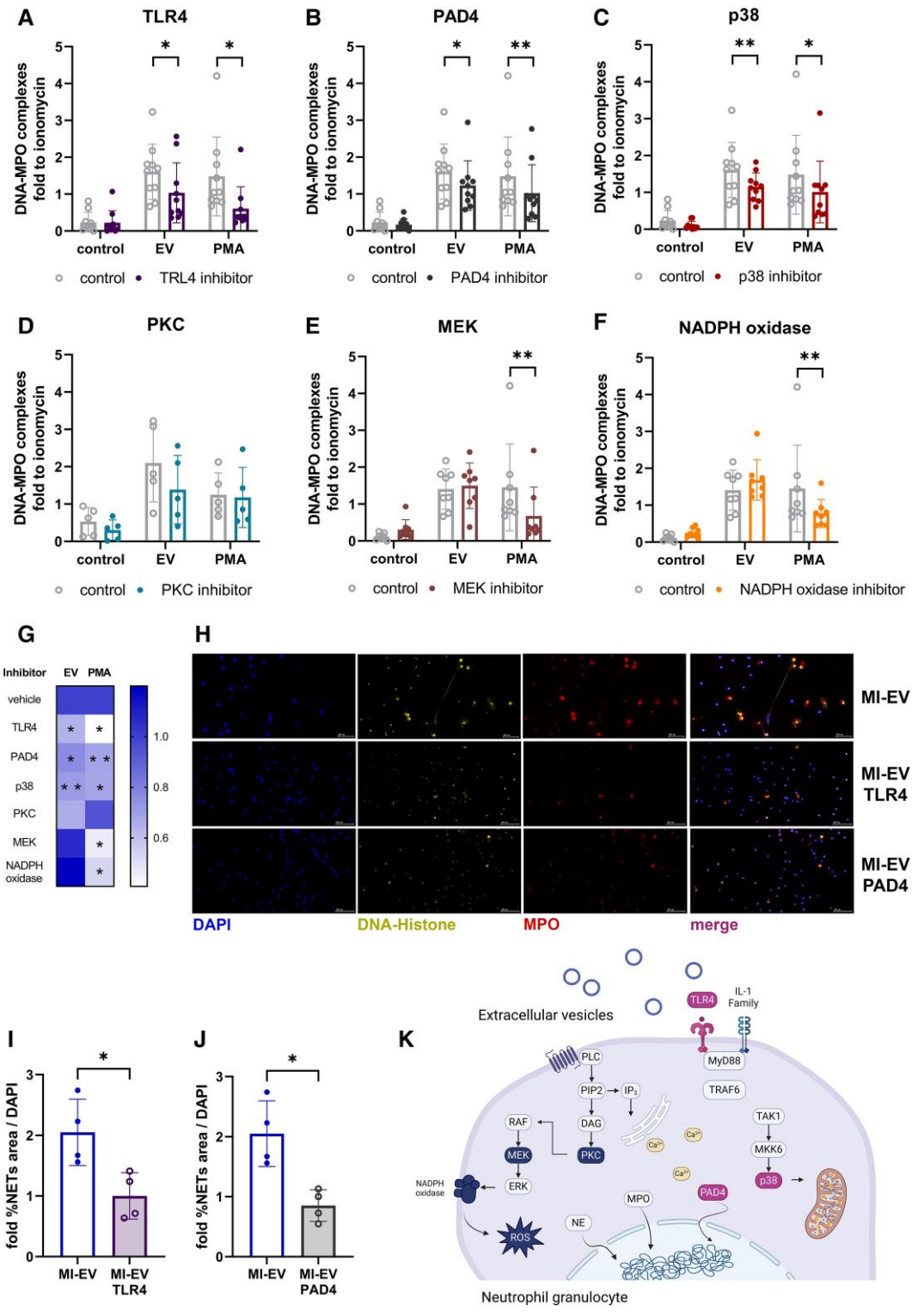


Figure 3 Extracellular vesicle–induced neutrophil extracellular trap formation is dependent on TLR4, p38, and PAD4. Neutrophils of healthy donors were pre-treated with inhibitors of (*A*) TLR4 (TAK-242, 10 μM), (*B*) PAD4 (GSK484, 10 μM), (*C*) p38 (SB203580, 10 μM), (*D*) PKC (Go6979, 2 μM), (*E*) MEK (PD98059, 40 μM), and (*F*) NADPH oxidase (diphenyleniodonium chloride, 20 μM) or vehicle control for 20 min before stimulation with extracellular vesicles, PMA (125 nM), and ionomycin (4 μM) for 3 h to assess neutrophil extracellular trap formation by deoxyribonucleic acid–myeloperoxidase complexes; (*G*) matrix summarizing A–F, Wilcoxon matched-pairs signed-rank tests. (*H–J*) The involvement of TLR4 and PAD4 was confirmed using coronary myocardial infarction–extracellular vesicles on primary neutrophils. (*H*) Neutrophil extracellular traps were visualized by immunofluorescence microscopy and were defined as merge of chromatin and myeloperoxidase. Neutrophil extracellular trap area was normalized to DAPI area and presented as fold to the respective vehicle control for (*I*) TLR4 inhibition and (*J*) PAD4 inhibition; paired *t*-tests. (*K*) Potential signalling pathway of extracellular vesicle–induced neutrophil extracellular trap formation. *$P < .05$, **$P < .01$

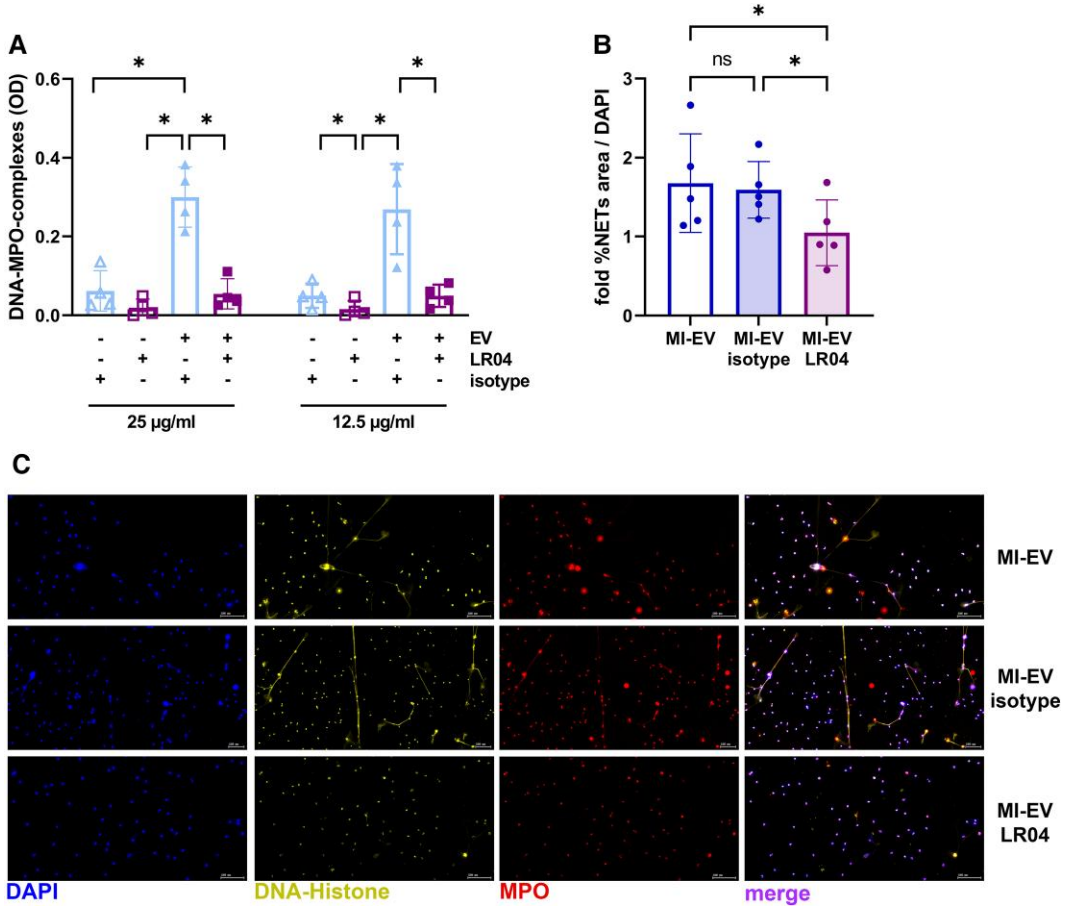


Figure 4 Malondialdehyde-specific immunoglobulin M LR04 attenuate extracellular vesicle–induced neutrophil extracellular trap formation. (*A*) Neutrophils isolated from healthy donors were stimulated with extracellular vesicles released by lipopolysaccharide-activated THP-1 monocytic cells, either in the presence of malondialdehyde-specific immunoglobulin M LR04 or isotype control (25 µg/mL and 12.5 µg/mL). Deoxyribonucleic acid–myeloperoxidase complexes in the supernatant were measured as indicator of neutrophil extracellular trap formation. Repeated measures analysis of variance. (*B* and *C*) Neutrophils isolated from healthy donors were stimulated with myocardial infarction–extracellular vesicles pooled from culprit site plasma of six patients, either in the presence of LR04 or an isotype control (25 µg/mL). Neutrophil extracellular traps were visualized by immunofluorescence microscopy and were defined as merge of chromatin and myeloperoxidase. Neutrophil extracellular trap area was normalized to DAPI area and presented as fold of the respective vehicle control; repeated measures analysis of variance. *$P < .05$

reduced percentage of citH3+ neutrophils (*Figure 5F–I*). Plasma citH3 levels were significantly lower in LR04-injected mice compared to isotype control-injected mice (see Supplementary data online, *Figure S14C*), while dsDNA levels were not different (see Supplementary data online, *Figure S14D*).

Because the monoclonal antibody LR04 represents a naturally occurring IgM antibody, we tested the hypothesis that pre-injection levels of endogenous IgM recognizing the P2 peptide mimotope may be associated with reduced EV-induced NETosis. Indeed, we observed an inverse association of endogenous P2-reactive IgM levels and the percentage of citH3+ neutrophils in mice co-injected with MI–EV and an isotype control IgM antibody ($r = -.743$, $P = .014$, $n = 10$; Supplementary data online, *Figure S14E*). In contrast, this was not observed in mice receiving MI–EV and LR04 due to the significant increase of exogenously added MDA-specific IgM ($r = -.370$, $P = .292$, $n = 10$; Supplementary data online, *Figure S14F*). These data demonstrate that higher levels of endogenous MDA-specific IgM have the capacity to reduce EV-induced NETosis *in vivo*.

Leukocyte-derived malondialdehyde-positive extracellular vesicles predict reduced left ventricular ejection fraction

Finally, we analysed whether the levels of deleterious EV in the acute setting of STEMI may mark the extent of tissue damage and the future outcome of cardiac function. Therefore, we correlated cMRI-derived LVEF 72 h and 6 months after STEMI (follow-up) with EV subsets. Concentrations of MDA+ EV measured at the culprit site were strongly and inversely associated with LVEF at 72 h ($r_s = -.505$, $P = .006$, $n = 28$, *Figure 6A*) and follow-up ($r_s = -.427$, $P = .033$, $n = 25$; Supplementary data online, *Figure S16A*). This effect was specific to MDA+ EV as no association was found with levels of Annexin V (AnV)+ EV.

Concentrations of CD45+ MDA+ EV showed a strong negative association with LVEF at 72 h ($r_s = -.489$, $P = .008$, $n = 28$, *Figure 6B*) and follow-up ($r_s = -.442$, $P = .027$, $n = 25$; Supplementary data online, *Figure S16B*), highlighting the impact of leukocyte-driven

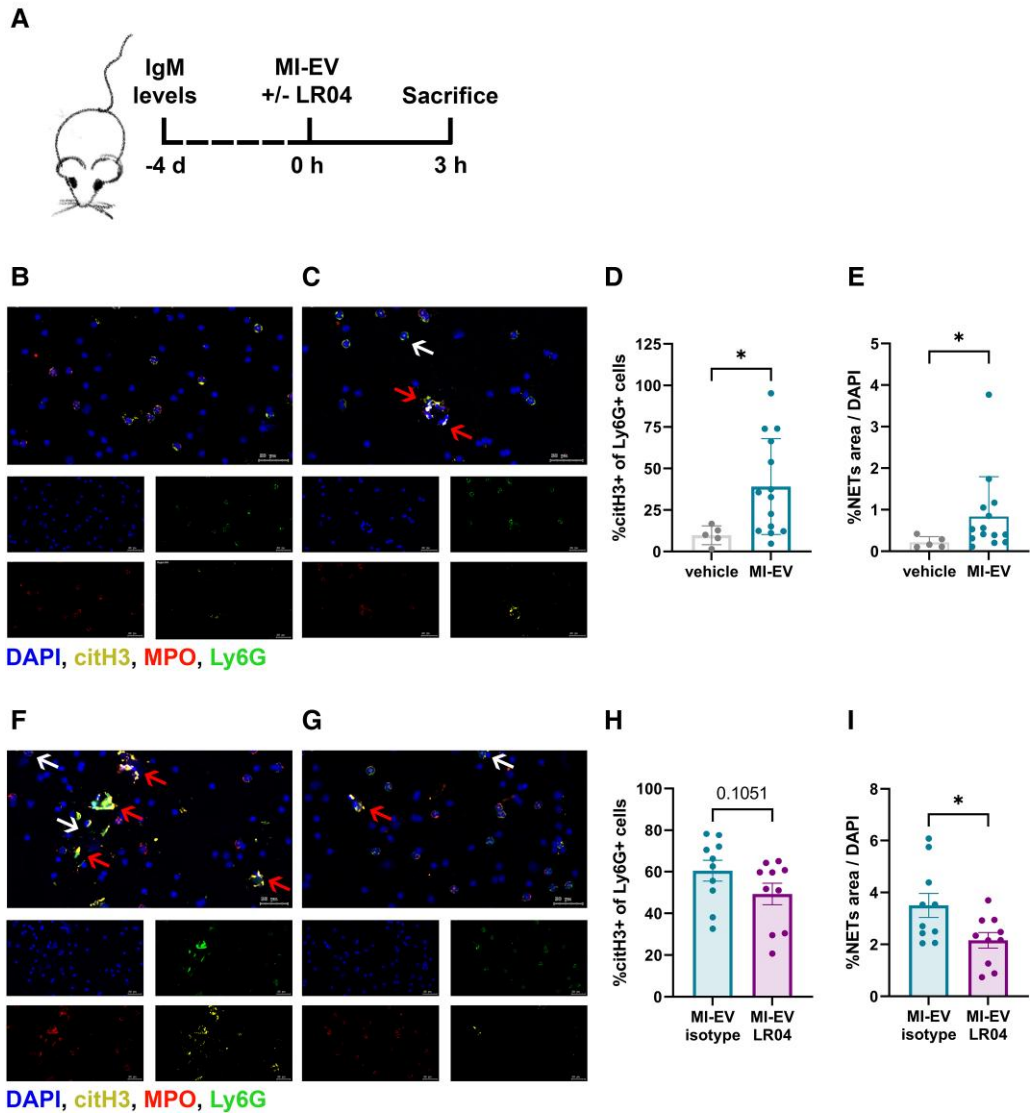


Figure 5 Malondialdehyde-specific immunoglobulin M LR04 attenuates myocardial infarction–extracellular vesicle–induced neutrophil extracellular trap formation *in vivo*. (*A*) Mouse treatment protocol. Baseline blood was drawn to determine malondialdehyde-specific immunoglobulin M levels 4 days prior to any injections. Upon sacrifice 3 h after injections, cytospins of haemolysed whole blood were prepared and analysed by immunofluorescence microscopy. (*B* and *C*) Representative pictures of cytospins of a mouse injected with (*B*) vehicle or (*C*) myocardial infarction–extracellular vesicles, respectively. (*D* and *H*) Ly6G+ (green) nuclei were identified, and the percentage of citH3+ (yellow) neutrophils was quantified. (*E* and *I*) An overlay of DNA and citH3 was quantified as NETs. (*F* and *G*) Representative cytospin pictures of a mouse treated with (*F*) myocardial infarction–extracellular vesicles and isotype or (*G*) myocardial infarction–extracellular vesicles and LR04. Red arrows indicate NETs, white arrows citH3+ neutrophils. Mann–Whitney *U* test in (*D*, *E*, and *H*), unpaired *t*-test in (*I*); * $P < .05$.

inflammation on patient outcome. In contrast, neither the levels of AnV+ MDA (see Supplementary data online, *Figure S17A*) nor CD45+ MDA+ EV (see Supplementary data online, *Figure S17B*) in the peripheral circulation displayed an association. To test the predictive value of larger CD45+ MDA+ EV levels, we conducted ROC analysis stratifying patients based on LVEF into a group with LVEF > 40% (IQR 49%–60%) and a group with LVEF < 40% (IQR 31%–38%). We identified the levels of culprit site but not peripheral CD45+ MDA+ EV as strong predictors of reduced LVEF 72 h after STEMI (A = .789, $P = .001$, *Figure 6C*). To integrate the potential beneficial effect of MDA-specific IgM, we calculated the ratio between CD45+ MDA+ EV and IgM for each patient. This individual ratio displayed an even stronger negative association with LVEF at 72 h (*Figure 6D*) and follow-up (see Supplementary data online, *Figure S16C*). These data emphasize the importance of the local balance of pro- (EV) and anti-NETogenic (IgM) factors at the culprit site.

Discussion

Neutrophils are significantly elevated during AMI, particularly at the culprit site.[5,6,62] They are considered important drivers of immunothrombosis in CVD[10,11,13] especially due to their ability to form NETs.

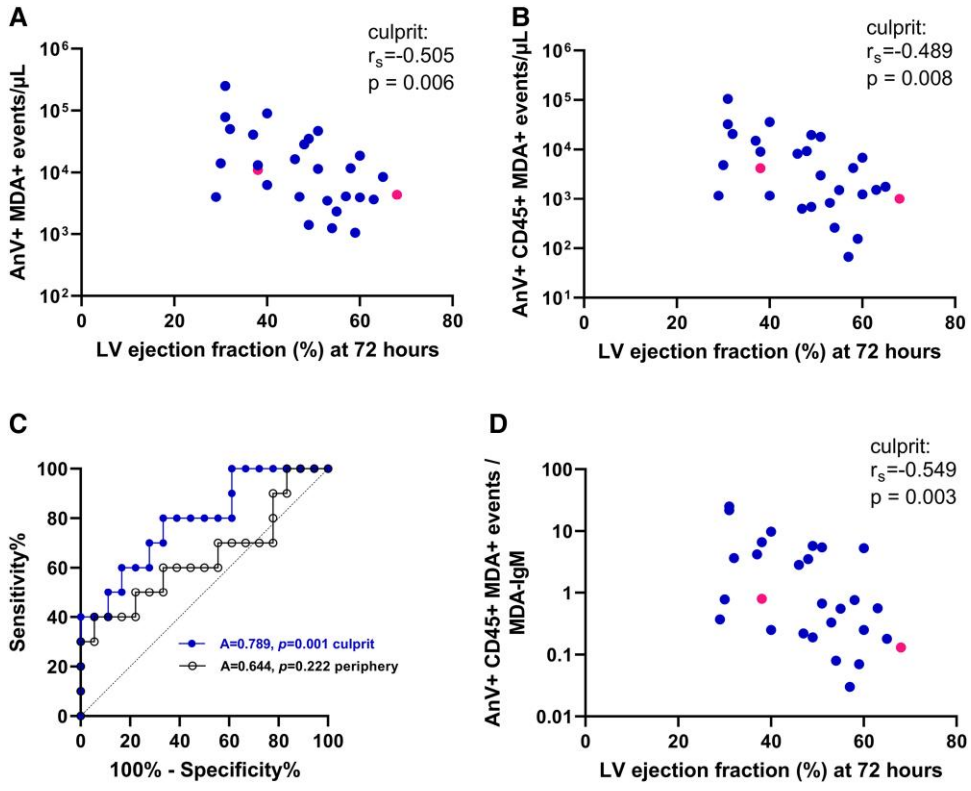


Figure 6 Association of 72-h left ventricular ejection fraction with malondialdehyde-positive extracellular vesicles. Spearman signed-rank correlations of cardiac magnetic resonance imaging–derived left ventricular ejection fraction (%) at 72 h with culprit site (*A*) malondialdehyde-positive extracellular vesicles and (*B*) CD45+ malondialdehyde-positive extracellular vesicles. (*C*) Receiver operating characteristic analysis of CD45+ malondialdehyde-positive extracellular vesicles from the culprit site (dots) and the periphery (empty circles) predicting reduced left ventricular ejection fraction (<40%). (*D*) Spearman signed-rank correlation of cardiac magnetic resonance imaging–derived left ventricular ejection fraction (%) at 72 h with the ratio of culprit site CD45+ malondialdehyde-positive extracellular vesicles and malondialdehyde–immunoglobulin M. Light-shaded dots represent female patients. A, area

We and others have described endogenous triggers of NETosis, such as monocyte chemo-attractant protein 1,[12] IL-1β,[63] activated platelets,[64] and OxLDL.[65] Here, we report that EV isolated from STEMI patients have the capacity to activate neutrophils *in vitro* and *in vivo*. An abundance of EV in acute CVD renders them potent sterile triggers of NET formation. The observation that culprit site MI–EV could enhance the NETing of circulating neutrophils in mice, which typically have low neutrophil numbers,[66] underscores the robust NETogenic potential of these EV. Furthermore, we identified the TLR4–PAD4 axis as key signalling pathway in MI-EV-induced NETosis. Notably, pharmacological inhibitor studies revealed differences between EV-induced NETosis compared to NETosis induced by 'classical' triggers, such as PMA or ionomycin. This points to a distinct signal transduction triggered by EV and emphasizes the limitations of the use of 'artificial' NET inducers to study pathophysiological processes.

Extracellular vesicles are recognized for their functional effects and biomarker potential in chronic and acute settings of CVD. For example, EV can induce expression of tissue factor, cytokines, and adhesion molecules in vascular cells promoting a pro-coagulatory phenotype and thereby thrombosis. In addition, EV-associated phospholipids also directly contribute to thrombus formation.[67] We now show that EV have the capacity to trigger NETosis and other functions of neutrophils relevant to CVD, such as release of IL-8, NE, and MPO. Interestingly, these effector mechanisms seem to be differently regulated as they did not follow the same inhibitory pattern observed for EV-induced NETosis. The lack of correlation between EV subsets and certain NET surrogate markers in patient samples suggests a complex interplay that warrants further investigation. Their association with NE plasma levels likely indicates a prodromal phase of general neutrophil activation, increasing the NETing capacity. Moreover, due to degradation of NET chromatin filaments by circulating deoxyribonucleases,[10] NE plasma levels might better capture the ongoing cycle of neutrophil activation, of which NETs are only a part.

Extracellular vesicles have been proposed to play both beneficial and deleterious roles in AMI,[68] and the identification of EV subsets with different biological activities is important. Despite the robust pro-inflammatory and pro-thrombotic potential of EV, their local contribution at the site of coronary occlusion is still inadequately investigated. Here, we show that leukocyte-derived EV contained the highest percentage of MDA+ EV at the culprit site when compared to platelet- and endothelial-derived EV. This is consistent with the high potential of ROS production by activated leukocytes, suggesting that MDA+ EV may be shed in the process of NET formation, thus providing a positive feedback loop for more NETosis. Consistently, THP-1 cells stimulated with LPS *in vitro* shed more EV, with a significantly higher proportion of MDA+ EV compared with resting cells. The generation of OSE does not only reflect increased oxidative stress, but OSE also have the capacity to induce inflammatory responses.[39,69]

A recent study found that oxidized phospholipids induced NET formation in lymphocyte adapter protein (LNK)–deficient mice which is a model for NETosis.[70]

Oxidation-specific epitope–specific IgM antibodies represent a large part of naturally occurring IgM,[48] and their plasma levels are inversely associated with cardiovascular events.[54,71] A recent study highlighted that high levels of OSE-specific IgM, including IgM with specificity for a peptide mimotope of MDA that is specifically bound by LR04, are associated with reduced AMI risk. Thus, in our experimental studies, we investigated the effect of an IgM antibody with the exact same specificity as the endogenous IgM titres showing an inverse association with clinical outcomes.[54] The protective mechanisms of these IgM have been attributed to their capacity to neutralize the pro-inflammatory activities of OxLDL and aid the clearance of dying cells. However, the potential effect of these IgM in the acute manifestation of atherosclerosis, i.e. AMI, has been unknown. In our study, we discovered an inverse correlation between the levels of NET markers, such as citH3, and MDA-specific IgM in the circulation of AMI patients, suggesting that targeting of MDA epitopes can reduce the deleterious potential of EV (*Structured Graphical Abstract*). Nevertheless, natural IgM may exert additional protective effects by targeting OSE on the surface of a range of molecules exposed at the culprit site.

The notable finding of a strong, negative correlation between leukocyte-derived MDA+ EV at the culprit site and LVEF measured by the gold standard method of cMRI underscores the invaluable insights that can be gained from analysing local blood samples. While our stratification based on LVEF may have limited implications for clinical diagnostics, it serves as a compelling testament to the importance of investigating the local milieu through in-depth analysis of culprit site blood, offering a perspective that goes beyond peripheral blood assessments. Importantly, we have found that the ratio between levels of MDA+ EV inducing NETosis and MDA-specific IgM show an even more robust inverse association with LVEF 72 h after intervention and 6 months after AMI. These data support the notion that the balance of deleterious players in AMI, such as MDA+ EV, and protective factors, such as OSE–IgM, need to be considered. Despite the fact that we only show a robust association in culprit site samples with clinical outcomes, future studies in larger cohorts should investigate the potential of a combined assessment of these factors in peripheral blood samples. Furthermore, future research should address potential gender differences, which were not discernible in our study due to the predominantly male study cohort.

In summary, EV from AMI patients are potent inducers of NETosis *in vitro* and *in vivo* triggering distinct intracellular signalling pathways in neutrophils. The demonstration that IgM targeting OSE on EV can modulate NET formation *in vitro* and in mice *in vivo* and the association of the ratio between CD45+ MDA+ EV and MDA-specific IgM with LVEF provide novel mechanistic insights into the protective effects of OSE–IgM in AMI.

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Supplementary data

Supplementary data are available at *European Heart Journal* online.

Declarations

Disclosure of Interest

A.S.O. received support for attending meetings and travel from Janssen Austria, AOP Health, and MSD. T.M.H. received payment or honoraria for lectures, presentations, etc. from Sanofi Aventis. M.H. received payment or honoraria for lectures, presentations, etc. from Szabo Scandic (Vienna, Austria), and Beckman Coulter Life Sciences (Austria, Vienna). D.B. received payment or honoraria for lectures, presentations, etc. from Siemens Healthineers and GE Healthcare. I.M.L. received grants from Neutrolis; consulting fees from AOP Health, MSD, Janssen, Pulnovo, and MSD; payment or honoraria for lectures, presentations, etc. from AOP Health, MSD, Janssen, Pulnovo, and MSD; and support for attending meetings from Medtronic. C.J.B. has received grants from Oxitope Pharma and Biotest AG; consulting fees from SOBI GmbH, Novartis, and Daiichi Sankyo; payment or honoraria for lectures, presentations, etc. from Novartis, Amgen, Sanofi, and Daiichi Sankyo; he is Vice President of the European Atherosclerosis Society and a board member at Technoclone Ges.m.b.H.

Data Availability

The data underlying this article will be shared on reasonable request to the corresponding authors.

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Ethical Approval

Patient study protocols were approved by the Ethics Committee of the Medical University of Vienna and carried out under approval numbers 1497/2012 and 1947/2014. Animal studies were approved by the Animal Ethics Committee of the Medical University of Vienna and conducted according to the protocol BMWFW-66.009/0188-WF/V/3b/2015.

Pre-registered Clinical Trial Number

The pre-registered clinical trial number is NCT01777750.

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