Hyperreactivity of Junctional Adhesion Molecule A–Deficient Platelets Accelerates Atherosclerosis in Hyperlipidemic Mice

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ABSTRACT

Rationale: Besides their essential role in hemostasis, platelets also have functions in inflammation. In platelets, junctional adhesion molecule (JAM)-A was previously identified as an inhibitor of integrin αIIbβ3-mediated outside-in signaling and its genetic knockdown resulted in hyperreactivity.

Objective: This gain-of-function was specifically exploited to investigate the role of platelet hyperreactivity in plaque development.

Methods and Results: JAM-A-deficient platelets showed increased aggregation and c-Src activation. Upon αIIbβ3 ligation, JAM-A was shown to be dephosphorylated, which could be prevented by PTPN1 inhibition. Mice with or without platelet-specific (tr)JAM-A-deficiency in an apolipoprotein e (apoE−/−) background were fed a high-fat diet. After up to 12 weeks of diet, trJAM-A−/− apoE−/− mice showed increased aortic plaque formation compared with trJAM-A+/− apoE−/− controls and these differences were most evident at early time points. At 2 weeks, the plaques of the trJAM-A−/− apoE−/− animals revealed increased macrophage-, T cell- and smooth muscle cell content. Interestingly, plasma levels of chemokines CCL5 and CXCL4 were increased in the trJAM-A−/− apoE−/− mice and JAM-A−deficient platelets showed increased binding to monocytes and neutrophils. Whole-blood perfusion experiments and intravital microscopy revealed increased recruitment of platelets and monocytes to the inflamed endothelium in blood of trJAM-A−/− apoE−/− mice. Notably, these pro-inflammatory effects of JAM-A−deficient platelets could be abolished by inhibition of αIIbβ3 signaling in vitro.

Conclusions: Deletion of JAM-A causes a gain-of-function in platelets, with lower activation thresholds and increased inflammatory activities. This leads to an increase of plaque formation, particularly in early stages of the disease.

Keywords: Atherosclerosis, platelets, cell adhesion molecules, protein phosphatases, inflammation
Nonstandard Abbreviations and Acronyms:

<table>
<thead>
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<th>Abbreviation</th>
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<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
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<td>Apoe</td>
<td>apolipoprotein E</td>
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<td>c-Src</td>
<td>cellular and sarcoma tyrosine-protein kinase</td>
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<td>CD</td>
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<td>CEACAM</td>
<td>CD66a, carcinoembryonic antigen-related cell adhesion molecule 1</td>
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<tr>
<td>CSK</td>
<td>c-terminal Src kinase</td>
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<tr>
<td>CVD</td>
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<tr>
<td>C(X)CL</td>
<td>C(X)C chemokine ligand</td>
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<td>CXC chemokine receptor 3</td>
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<tr>
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<td>ESAM</td>
<td>endothelial cell-specific adhesion molecule</td>
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<tr>
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<td>elastica van Gieson</td>
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<tr>
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<td>fibrous cap atheroma</td>
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<tr>
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<td>fibrinogen</td>
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<td>high-fat diet</td>
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<td>JAM-A</td>
<td>F11R, Junctional Adhesion Molecule A F11 Receptor</td>
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<tr>
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INTRODUCTION

Blood platelets have an essential function in hemostasis, but also play an important role in immune and inflammatory processes\textsuperscript{1,2}. Recent studies have highlighted platelets as key effectors in e.g. sepsis\textsuperscript{3}, rheumatoid arthritis\textsuperscript{4}, experimental autoimmune encephalomyelitis\textsuperscript{5} and host defense during bacterial infection\textsuperscript{6}. In addition, platelets spark vascular inflammatory processes and adhere to atherosclerotic predilection sites preceding plaque formation\textsuperscript{7}. Platelets may also form a bridge between leukocytes and the injured or inflamed vessel wall\textsuperscript{8}, promoting the extravasation of monocytes and neutrophils\textsuperscript{9} and the response to vascular injury\textsuperscript{10}. Accordingly, infusion of activated platelets into hyperlipidemic mice accelerated atherosclerosis in a P-selectin- and CD40 ligand (CD40L)-dependent manner\textsuperscript{11,12}.

Junctional adhesion molecule A (JAM-A, F11R) is a member of the immunoglobulin superfamily adhesion molecules and expressed on a large variety of cell types, including platelets, leukocytes and endothelial cells\textsuperscript{13}. On leukocytes, it mediates cell migration by regulating integrin de-adhesion\textsuperscript{14}. On epithelial and endothelial cells, JAM-A is a component of the tight junctions and regulates cell layer permeability through homophilic interactions\textsuperscript{15}. Upon inflammatory stimulation of endothelial cells, JAM-A translocated from the intercellular contacts and is exposed on the apical surface, thereby becoming available for the interaction with blood cells\textsuperscript{16}. Recently, we demonstrated that JAM-A has a cell type-specific impact on atherosclerotic plaque formation\textsuperscript{17}. Whereas JAM-A on leukocytes protected against atherosclerosis, endothelial JAM-A promoted plaque formation by enhanced luminal availability under pro-atherosclerotic conditions, thus guiding monocytes to sites of plaque development\textsuperscript{17}.

Although JAM-A was first identified in platelets\textsuperscript{18}, its influence on platelet function remained poorly characterized. Earlier studies demonstrated phosphorylation of JAM-A upon platelet activation\textsuperscript{19} and its association with $\alpha_{IIb}\beta_{3}$ integrin\textsuperscript{20}, yet no functional consequences for platelet function were reported. Recent work however identified JAM-A as an endogenous inhibitor of platelet function by attenuating c-Src–dependent "outside-in" signal transduction of $\alpha_{IIb}\beta_{3}$ integrin, through the recruitment of the Src inhibitory kinase CSK\textsuperscript{21,22}. Deficiency of JAM-A in platelets resulted in increased aggregation in response to some platelet agonists and led to a prothrombotic phenotype in mice\textsuperscript{21}.

The importance of platelets in the pathogenesis of atherosclerosis and the gain-of-function phenotype of JAM-A–deficient platelets prompted us to investigate the role of platelet hyperreactivity in the progression of atherosclerosis. Using mice with a platelet-specific deletion of the JAM-A gene, we demonstrate that loss of JAM-A on platelets leads to hyperreactivity, pro-inflammatory phenotype and thus to an acceleration of early-phase plaque formation.
METHODS

Detailed experimental procedures can be found in the online supplement.

Mice.
Mice carrying cre-recombinase under the control of the platelet factor 4 (PF4)-promoter were a kind gift from Dr. R.C. Skoda, University Hospital Basel and were backcrossed in an apolipoprotein e (apo)e-deficient background (C57Bl/6) backcrossed for at least ten generations. These mice were crossed with JAM-A^{lox/lox} apoe^{−/−} mice to obtain platelet-specific (tr)JAM-A^{−/−} apoe^{−/−} mice. Littermates not containing the PF4-cre transgene were used as (tr)JAM-A^{+/+} apoe^{−/−} controls. All human and animal experiments were approved by local authorities (Regierung von Oberbayern, Munich, Germany).

Plaque formation, quantification and histological analysis of atherosclerosis.
Plaque formation was induced in 7–week old male and female mice (n=7-14) by feeding a high-fat diet (HFD, 21% fat, 19.5 % casein, 0.15% cholesterol, ssniff) for 2, 6 and 12 weeks and the extent of atherosclerosis and plaque-histology was determined as described. Stages of atherosclerotic lesions were determined by evaluation of Elastica van Gieson (EVG)-stained aortic roots.

Platelet isolation and activity measurement.
Platelets were isolated and washed by centrifugation from platelet poor plasma (PPP) as described. Platelet aggregation in response to adenosine diphosphate (ADP) and thrombin was assessed in mouse whole blood within 2 hours after isolation by multiple electrode aggregometry technology using a Multiplate® platelet analyzer according to manufacturer's instructions (Roche Diagnostics). In some experiments, platelet aggregation was initiated using thrombin and ADP after pretreatment of the platelets with αIIbβ3 integrin antagonist tirofiban (Aggrastat®, MSD, 1 µg/mL) or the Src kinase inhibitors PP2, SU6656 and KB Src 4 (Merck Millipore or Tocris, 20, 2.5 and 0.09 µmol/L respectively).

Thrombus formation on collagen under flow.
Platelet adhesion onto fibrillar collagen under shear flow was performed essentially as described.

Immunoprecipitation, western blotting and QPCR.
Isolated washed platelets from humans and mice were incubated on immobilized heat-inactivated BSA or fibrinogen for 60 or 90 minutes at 37°C respectively as described. In some experiments, the platelets were incubated with protein tyrosine phosphatase inhibitors: IV (20 µmol/L), XXXI (30 µmol/L), NSC-87877 (0.7 or 10 µmol/L) and PTP1B inhibitor (8 µmol/L), or vehicle (DMSO) during adhesion. The platelets were subsequently lysed and JAM-A was immunoprecipitated using specific antibodies and protein G-coupled magnetic beads (Life Technologies) and analyzed by SDS page and western blotting. For immunoblotting or qPCR, isolated platelets and/or harvested arteries after whole body perfusion with ice-cold phosphate-buffered saline solution (PBS, Sigma Aldrich) were homogenized and analyzed as described.

Plasma lipid and chemokine determination.
Concentrations of chemokines CXCL4 and CCL5 were measured in platelet poor plasma (PPP) from mice fed a HFD for 2, 6 and 12 weeks and w/o HFD using Enzyme-linked immunosorbent assays (ELISA) kits (both R&D Systems).

Src kinase phosphorylation assay.
Mouse platelets were incubated on BSA or fibrinogen, detached, fixed and permeabilized. Phosphorylated c-Src was subsequently detected using an eFluor®660-conjugated mouse monoclonal antibody, specific for mouse/human Src phosphorylation at tyrosine 418 residue (Y418) (eBioscience), by flow cytometry.
Flow cytometry.
Platelets were labeled with anti-JAM-A AF488-conjugated (AbDSerotec) and anti-CD41 PE-conjugated (BD Pharmingen) antibodies. For platelet-leukocyte interactions, isolated platelets were activated with 0.5 U/mL thrombin and added to isolated erythrocyte-free leukocytes for 20 min at 37°C. Finally, cells were stained with anti-CD41–FITC (BD), anti-CD45–eFluor®450, anti-CD115–PE-Cy7 and anti-Ly6G–PerCP-Cy5.5 antibodies (all eBioscience). In some experiments, platelet-leukocyte interactions were analyzed after pretreatment of the platelets with tirofiban (1 µg/mL). Samples were measured by flow cytometry (FACSCantoII, BD) and analyzed by FlowJo v.10 software (Tree Star Inc.).

Platelet and leukocyte recruitment assays.

In vitro adhesion assay.
SV-40-large T antigen-immortalized mouse endothelial cell (mECs) monolayers were challenged with tumor necrosis factor-α (TNFα, 10 ng/mL) for 4 hours. Freshly isolated leukocytes and platelets were suspended in HBSS containing 5 mg/mL human albumin and 10 mmol/L HEPES at 1×10⁶ leukocytes/mL and 1×10⁸ platelets/mL. In some experiments, isolated platelets were pretreated with tirofiban (1 µg/mL). The blood cells were then labeled with anti-CD45–AF488 (Bio-Rad) and anti-CD41–PE (BD). Immediately prior to perfusion, 0.5 U/mL thrombin and 1 mmol/L CaCl₂ and MgCl₂ were added to the platelets and leukocytes at 37°C. The endothelial cells were assembled in a flow chamber and platelets were perfused for 20 min, followed by leukocytes for 5 min and additional washing of non-adherent cells (all at 0.15 N/m²)²⁹. Cell adhesion was expressed as percentage surface coverage of platelets or leukocytes over multiple microscopic fields using ImageJ software (NIH).

Ex vivo adhesion assay.
Carotid arteries of trJAM-A⁺⁺ apoe⁻⁻ and trJAM-A⁻⁻ apoe⁻⁻ mice fed a HFD for 2 weeks were carefully explanted, mounted in a customized perfusion chamber and pressurized at physiological pressure of 0.8-1.1×10⁴ Pa. Previous studies have demonstrated that the vessel, including the endothelial and smooth muscle cells, remains intact¹⁷,³⁰. Whole blood was diluted with isotonic citrate buffer to adjust the platelet count to 1×10⁸ platelets/mL blood. Prior to perfusion, the endothelium was labeled with anti-CD31–eFluor®450 antibody for 30 minutes. Platelets were stained with anti-CD41–FITC antibody and the whole blood was perfused through the mounted and pressurized vessel for 10 minutes at 0.5 mL/min. In some experiments, diluted blood was pretreated with tirofiban (1 µg/mL) prior to perfusion. Adherent platelets were visualized using two-photon laser scanning microscopy as described¹⁷.

In vivo adhesion assay.
Intravital microscopy was performed in the carotid artery of mice that were fed a HFD for 4 weeks to visualize leukocyte-endothelium interactions along the atherosclerotic carotid artery as described³¹. Leukocytes were considered adherent when no rolling was observed for more than 30 sec.

Statistical analysis.
Statistical analysis was performed using Prism 6.0 (GraphPad Software). Means were compared between 2 groups by 2-tailed, unpaired Student’s t-test, without or with Welch correction or among more than 2 groups by 1- or 2-way ANOVA with Tukey’s or Bonferroni’s post-test or Kruskal-Wallis test with Dunn’s post-test, as indicated. Differences with P<0.05 were considered as statistically significant. Each experiment was independently repeated at least 3 times.
RESULTS

Deficiency in platelet JAM-A results in hyperreactivity.

Previous studies have shown that genetic deletion of JAM-A results in platelet hyperreactivity\textsuperscript{21,22}, yet the functional consequences for the progression of atherosclerosis have not been investigated. For this, we implemented platelet-specific JAM-A knock out (trJAM-A\textsuperscript{−/−}) mice and compared them to their control littermates (trJAM-A\textsuperscript{+/+}). Specific genetic deletion of JAM-A in platelets from trJAM-A\textsuperscript{−/−} mice was shown by western blotting and immunocytochemistry (Fig. 1A, Online Fig. I), whereas JAM-A expression on leukocytes, endothelial cells, smooth muscle cells (SMCs) and in homogenates from aortae and carotid arteries was not affected (Online Fig. I and Online Fig. II). In addition, JAM-A deficiency did neither affect the platelet count, nor the volume or the counts of other blood cell populations and the expression of the related adhesion molecules such as JAM-C and ESAM on platelets was not altered in the absence of JAM-A (Online Table I, and Online Fig. III).

To recapitulate previous observations by Naik and coworkers\textsuperscript{21,22}, we examined in vitro platelet aggregation upon activation. Specific platelet JAM-A-deletion resulted in enhanced aggregation in response to different agonists such as ADP, thrombin and collagen (Fig. 1B-C and not shown), supporting the role of JAM-A as an endogenous platelet function inhibitor. Since integrin $\alpha_{\text{IIb}\beta_3}$ outside-in signaling is the reported target of JAM-A\textsuperscript{21,22}, the $\alpha_{\text{IIb}\beta_3}$ antagonist tirofiban was added to modulate this pathway, by preventing binding to fibrinogen. The presence of tirofiban normalized the aggregation of JAM-A-deficient platelets to the level of JAM-A\textsuperscript{+/+} platelets (Fig. 1B,C).

Outside-in signaling by $\alpha_{\text{IIb}\beta_3}$ is controlled by c-Src kinase\textsuperscript{22,26}. To investigate the influence of JAM-A-deficiency in our PF4-Cre–based mouse model, platelets from trJAM-A\textsuperscript{+/+} and trJAM-A\textsuperscript{−/−} mice were adhered to BSA or fibrinogen and c-Src activation was assessed by measuring Src phosphorylation at Y418 residue by flow cytometry. Absence of JAM-A in platelets resulted in a significant increase in Y418 phosphorylation of c-Src after adhesion to fibrinogen, compared to control conditions (Fig. 1D,E). Blockade of Src activation (and thus $\alpha_{\text{IIb}\beta_3}$ outside-in signaling) with the inhibitors PP2, SU6656 or KB Src 4 abrogated the increased agonist-induced aggregation of JAM-A-deficient platelets (Fig. 1F,G and not shown), albeit that baseline aggregation was increased, possibly by the solvent DMSO.

Tyrosine phosphorylated JAM-A recruits the c-Src–inhibiting kinase CSK to integrin $\alpha_{\text{IIb}\beta_3}$. Upon ligation of $\alpha_{\text{IIb}\beta_3}$, JAM-A is dephosphorylated allowing the dissociation of CSK from the JAM-A/c-Src/integrin $\alpha_{\text{IIb}\beta_3}$complex\textsuperscript{22}. In order to investigate the mechanism responsible for the dephosphorylation of JAM-A, we performed immunoprecipitation experiments using human and mouse platelets. After adhesion to fibrinogen, a reduction of tyrosine phosphorylation of platelet JAM-A was observed, compared to platelets incubated on BSA (Fig. 1H,I). Dephosphorylation of tyrosine residues is carried out by protein tyrosine phosphatases (PTPs). To date, 20 PTPs are known in platelets and regulate signaling events during platelet activation\textsuperscript{32}. Addition of broad-spectrum PTP inhibitors (IV and XXXI) to fibrinogen-adhered platelets resulted in a decreased dephosphorylation of JAM-A (Fig. 1H,I). A previous study described an association of PTPN1 (PTP1B) with the c-Src/integrin $\alpha_{\text{IIb}\beta_3}$ complex and identified PTPN1 as a positive regulator of platelet outside-in signaling\textsuperscript{27}. Thus, we investigated a possible role of PTPN1 in the dephosphorylation of JAM-A. Probing immunoprecipitated JAM-A with antibodies against PTPN1 revealed a co-precipitation of PTPN1 with JAM-A both in resting and outside-in activated human platelets (Fig. 1H), suggesting a constitutive physical association of PTPN1 with JAM-A. Addition of a specific inhibitor against PTPN1 resulted in an increase of tyrosine-phosphorylated JAM-A in fibrinogen-adhered platelets, compared to vehicle (Fig. 1I). Interestingly NSC-87877, an inhibitor of PTPN6 and -11 (SHP1 and -2), did not increase JAM-A phosphorylation at a concentration (0.7 µmol/L) that specifically affected PTPN6 and -11, indicating that these PTPs do not mediate JAM-A dephosphorylation (Fig. 1I). However,
NSC-87877 also blocks PTPN1 when used at a tenfold higher concentration. Addition of NSC-87877 at 10 µmol/L indeed resulted in a reduced tyrosine dephosphorylation of JAM-A (Fig. 1I). These findings indicate that the dephosphorylation of JAM-A in platelets is mediated by PTPN1.

Taken together, our observations and those of others indicate that JAM-A is an integral member of the CSK/c-Src/integrin αmβ3 complex and a negative regulator of platelet αmβ3 outside-in signaling, and that absence of JAM-A results in hyperreactivity.

Absence of JAM-A promotes thrombus formation.

As JAM-A–deficient mice showed a prothrombotic phenotype, blood from trJAM-A+/– apoE–/– and trJAM-A+/+ apoE–/– mice was compared to determine the specific role of JAM-A on platelet in flow-dependent thrombus formation on a thrombogenic surface. Perfusion at high shear rates over fibrillar collagen induced the formation of platelet aggregates, which was markedly enhanced for trJAM-A+/– apoE–/– platelets (Fig. 2A,D,G). In addition, post-staining with the JON/A antibody, specific for the activated conformation of αmβ3 integrin, showed no difference in the JON/A+ stained thrombi area from trJAM-A+/– apoE–/– mice, indicating that inside-out signaling is not affected by the absence of JAM-A (Fig. 2B,E,H). Under the same conditions, little procoagulant surface (annexin A5 binding) was observed and no differences were present between platelets from trJAM-A+/– apoE–/– mice and controls (Fig. 2C,F,I). Taken together, these results suggest that genetic ablation of JAM-A on platelets results in increased platelet reactivity associated with enhanced thrombus formation.

Platelet-specific JAM-A–deficiency accelerates early-stage atherosclerosis.

To investigate the role of JAM-A on platelets during the progression of atherosclerosis, trJAM-A+-/+- apoE–/– and trJAM-A+/+ apoE–/– mice were fed a HFD for 2, 6 and 12 weeks. Of note, in very early stages of atherosclerosis (2 weeks HFD) platelet JAM-A deficiency resulted in a significantly increased lesion area in the aortic root and in the aortic arch (Fig. 3A,C,E,G). At an intermediate time point (6 weeks HFD), whole-aortic plaque area was still significantly increased in trJAM-A+/– apoE–/– mice compared with controls (Fig. 3G), whereas an increased plaque area was no longer observed in the aortic roots (Fig. 3C). Similar results were obtained after 12 weeks HFD, where a lack of JAM-A on platelets led to an increased lesional area only in the whole aorta (Fig. 3F,G), but not in the aortic root (Fig. 3B,C). An evaluation of the plaque phenotype in aortic roots revealed that JAM-A deficiency on platelets resulted in more advanced plaque phenotypes, compared to controls, expressed as a percentage of the total number of plaques that appeared as intimal xanthoma (IX), representing early stages, pathological intimal thickening (PIT) and fibrous cap atheroma (FCA), the latter representing more advanced atherosclerosis stages in plaques of the aortic roots (Fig. 3D). No differences in plaque formation were found in apoE–/– mice carrying only the PF4-Cre transgene, compared with apoE–/– mice (data not shown) and no differences were detected in the platelet counts, the size, or in other blood cell populations at any time point during HFD in the trJAM-A+/+ apoE–/– and trJAM-A+/– apoE–/– groups (Online Table I). In addition, no significant changes were detected in plasma levels of cholesterol or triglycerides in absence of JAM-A on platelets during atherosclerosis development (Online Table II). These observations strongly suggest an atheroprotective role of platelet JAM-A, notably in early stages of atherosclerosis. Of note, the vascular expression of JAM-A was unaffected at this time point (Online Fig. II).

Absence of JAM-A on platelets accelerates lesional infiltration of mononuclear cells.

Quantification of plaque composition in the aortic root revealed that the lesional MAC-2+ macrophage content in very early stages (2 weeks HFD) was significantly increased in trJAM-A+/– apoE–/– mice, compared with control littermates (Fig. 4A). In contrast, at intermediate (6 weeks HFD) (data not shown) or advanced time points (12 weeks HFD), the macrophage content did not differ in trJAM-A+/–...
apoe<sup>−/−</sup> mice versus trJAM-A<sup>++</sup> apoe<sup>−/−</sup> controls (Fig. 5A). Of note, absence of platelet JAM-A markedly augmented infiltration of the CD3<sup>+</sup> T-cells in very early stages (Fig. 4B), whereas trJAM-A−deletion did not influence T cell content at intermediate (data not shown) and in late time points (Fig. 5B). Moreover, as compared to control mice, platelet JAM-A−deficiency strongly increased α-SMA<sup>+</sup> smooth muscle cell (SMC) content at the early time point (Fig. 4C) and neither had effect on SMC at intermediate (data not shown) nor at late time points (Fig. 5C).

In addition, investigation of gene expression in atherosclerotic aortae in mice fed for 2 and 12 weeks HFD showed that deletion of JAM-A on platelets significantly increased the aortic expression of chemokine receptor CXCR3 (Fig. 5D) and of the inflammatory cytokines interferon gamma (IFN<sub>γ</sub>) (Fig. 5E) and TNFα (Fig. 5F), after 2 weeks but not after 12 weeks of HFD. Thus, platelets may differentially modulate the inflammatory lesion phenotype and the atherosclerotic plaque stage.

**Deficiency of JAM-A leads to increased release of chemokines from platelets.**

Platelets store many inflammatory proteins in their α-granules and they can release chemokines, e.g. platelet factor 4 (PF4, CXCL4) and regulated on activation, normal T cell expressed and secreted (RANTES, CCL5) upon activation, leading to increased recruitment of mononuclear cells to the inflamed vessel wall<sup>33,34</sup>. Therefore, we investigated whether the deletion of platelet JAM-A might have an influence on the secretion of chemokines during the course of atherosclerosis. Interestingly, CXCL4 and CCL5 immunofluorescence intensities were reduced in platelets isolated from trJAM-A<sup>−/−</sup> apoe<sup>−/−</sup> mice compared with those from trJAM-A<sup>++</sup> apoe<sup>−/−</sup> mice after 12 weeks and 2 weeks HFD, respectively (Fig. 6A,B). In addition, absence of JAM-A on platelets incrementally increased the levels of circulating CXCL4 after 2, 6 and 12 weeks of HFD (Fig. 6C). Similarly, CCL5 levels were markedly enhanced in plasma from trJAM-A<sup>−/−</sup> apoe<sup>−/−</sup> mice compared to controls in the early and intermediate time points of the HFD (Fig.6D), whereas there was no difference on the baseline chemokine levels without HFD (data not shown) between both groups. These data are indicative for an increased chemokine release by JAM-A-deficient platelets.

**JAM-A−deficient platelets show an increased interaction with leukocytes.**

Activated platelets have been described to form complexes with leukocytes<sup>11,29</sup>, which might be relevant during acute coronary events<sup>35,36</sup>. To investigate whether JAM-A−deficiency leads to increased interactions with leukocytes, activated platelets isolated from trJAM-A<sup>−/−</sup> apoe<sup>−/−</sup> mice were incubated with leukocytes. Indeed, JAM-A−deficient platelets showed significantly more platelet-monocyte (Fig.6E,F) and platelet-neutrophil (Fig. 6G,H) aggregation than platelets from trJAM-A<sup>++</sup> apoe<sup>−/−</sup> mice, suggesting that JAM-A−deficient platelets might more effectively augment monocyte recruitment to sites of endothelial inflammation. Interestingly, blockade of integrin α<sub>IIbβ3</sub> reverted the increased interaction of JAM-A−deficient platelets with monocytes to that observed for JAM-A−positive platelets (Fig. 6E,F) and also significantly reduced complex formation between platelets and neutrophils, albeit to a lesser extent (Fig. 6G,H).

**Leukocyte recruitment is enhanced in the absence of JAM-A on platelets.**

Platelets are able to form a bridge between monocytes and endothelial cells during inflammation<sup>8</sup>. To assess the contribution of JAM-A to platelet and leukocyte adhesion to endothelial cells, we performed in vitro, ex vivo and in vivo assays. Thrombin-activated platelets from trJAM-A<sup>++</sup> apoe<sup>−/−</sup> and trJAM-A<sup>−/−</sup> apoe<sup>−/−</sup> mice were perfused over mouse endothelial monolayers challenged with TNFα, followed by perfusion of activated leukocytes from the same mouse genotype. A significantly increased endothelial cell surface coverage with platelets (Fig. 7A,B) or leukocytes adherent on the platelets (Fig. 7A,B) was observed in trJAM-A<sup>−/−</sup> apoe<sup>−/−</sup> mice compared to the control group. Treatment of the platelets with tirofiban prior to
perfusion abolished the observed differences in platelet and leukocyte adhesion between trJAM-A+/+ apoε−/− and trJAM-A−/− apoε−/− mice (Fig. 7A,B).

To investigate the interaction of platelets with the atherosclerotic vessel wall in a more physiologic approach, carotid arteries were explanted from trJAM-A+/+ apoε−/− and trJAM-A−/− apoε−/− mice, fed a HFD for 2 weeks and subsequently perfused with whole blood taken from the same mice. Compared with JAM-A–positive blood, a pronounced increase in flow-resistant platelet adhesion onto the vessel wall was observed when platelet JAM-A–deficient blood was perfused through the artery. Platelets mainly adhered as single cells and absolute counts were modest for both trJAM-A+/+ apoε−/− and trJAM-A−/− apoε−/− genotypes in this experimental setup (4.00±1.87 vs. 32.4±10.1, respectively), indicating that the endothelial cells were intact and not damaged by handling. The addition of tirofiban abrogated the difference in adhesion between JAM-A–deficient and control platelets (Fig. 7C,D). Moreover, the adhesion of leukocytes to atherosclerosis-prone sites was assessed using intravital microscopy of the carotid artery. Flow-resistant CD11b+ monocyte–endothelium interactions in trJAM-A−/− apoε−/− mice were significantly more pronounced than in trJAM-A+/+ apoε−/− mice (Fig. 7E,F), whereas neutrophil adhesion in trJAM-A−/− mice showed a non-significant tendency to an increase (Fig. 7E,G). Taken together, these results indicate that platelet hyperreactivity due to JAM-A–deficiency is driven by a lack of negative integrin αIIbβ3 regulation, which leads to increased platelet-leukocyte interactions that in turn may facilitate leukocyte recruitment to the vessel wall during atherogenesis.

DISCUSSION

It is well established that platelets play a decisive role in the clinical precipitation and recurrence of cardiovascular disease (CVD), and antiplatelet drugs are widely used in the after-care and prevention of individuals who suffered, or are at risk of, a cardiovascular event. However, it is less well known that platelets can also initiate and propagate vascular inflammation, which may ultimately lead to the development of atherosclerotic plaques. In both cases, platelet hyperreactivity might contribute to an increased risk of CVD, by initiating endothelial dysfunction and propagating vascular inflammation, and through lower aggregation thresholds, aiding to the occurrence or recurrence of ischemic events due to vascular occlusion. Increased platelet reactivity accompanying resistance to antiplatelet therapy is known to pose an enhanced risk for CVD in patients with e.g. diabetes or hypertension. In this respect, an increase of both the inflammatory and the hemostatic functions of platelets might play an underlying role, but experimental evidence for this notion is scarce.

Deficiency of JAM-A was shown to increase the response of platelets to various agonists in recent studies by Naik and coworkers. Ablation of JAM-A led to shortened bleeding times and increased platelet aggregation in response to collagen, ADP and PAR-4 agonists, without leading to increased activation of αIIbβ3 integrin. Our findings support these observations and we have also demonstrated increased adhesiveness and aggregation of JAM-A–deficient platelets to immobilized collagen and to endothelial cells of the vessel wall under flow. In addition, unlike negatively charged phospholipids, active αIIbβ3 integrin (i.e. expression of the JON/A epitope) was abundantly expressed on these shear-resistant platelet aggregates from both trJAM-A−/− apoε−/− and trJAM-A+/+ apoε−/− mice, yet no differences between genotypes were observed. This appears to be in concordance with the previously postulated notion that JAM-A–deficiency does not influence “inside-out” αIIbβ3 integrin activation.

In literature, several other adhesion molecules were shown to down-regulate platelet function. For example, a direct relative of JAM-A, endothelial cell-selective adhesion molecule (ESAM), is expressed on platelets and localized to platelet-platelet contacts in platelet aggregates. Deficiency of ESAM was
associated with increased platelet aggregation and thrombus formation in laser-damaged arterioles. Similar observations were made for CD66a (Carcinoembryonic antigen-related cell adhesion molecule 1), which was identified as a negative regulator of platelet-collagen interactions. In addition, CD31 (platelet endothelial cell adhesion molecule) was shown to be involved in regulating platelet aggregation as its crosslinking reduced agonist-induced platelet aggregation and genetic deletion of CD31 in platelets, but not endothelial cells increased thrombus formation in vivo.

A mechanistic model of how JAM-A can regulate integrin outside-in signaling was initially described by Naik et al., who found that JAM-A is tyrosine-phosphorylated in resting platelets. Phosphorylation of JAM-A was also observed in other studies and was hypothesized to be involved in regulating interaction of JAM-A with its intracellular binding partners. In quiescent platelets, a complex exists between the endogenous c-Src inhibitor CSK and the integrin–c-Src complex. Naik et al. identified tyrosine-phosphorylated JAM-A as an additional component of this complex and found that JAM-A recruits CSK, thereby maintaining c-Src (and integrin) in an inactive state. Upon engagement of integrin with its ligand fibrinogen, JAM-A is dephosphorylated causing CSK and JAM-A to dissociate from integrin. This leads to activation of c-Src by phosphorylation at Y418, propagating integrin outside-in signaling. In the present study we show that JAM-A dephosphorylation is blocked by inhibiting the tyrosine phosphatase PTPN1, but not PTPN6 and -11, thereby adding a novel aspect to this model. In addition, both in resting and outside-in activated platelets, JAM-A appeared to be associated with PTPN1 and this phosphatase was previously shown to interact with integrin. Given the close proximity of JAM-A to the CSK/c-Src/integrin complex and their association with PTPN1, it appears feasible that PTPN1 is the phosphatase responsible for the dephosphorylation of the substrates in this complex upon outside-in signaling.

The absence of JAM-A may lead to a reduced recruitment of CSK and thus to a lower activation threshold caused by increased integrin signaling. Our experiments support this notion, since an increased c-Src activation was observed in JAM-A–deficient platelets after incubation on immobilized fibrinogen. We and others have also observed increased aggregation particularly at low agonist concentrations in JAM-A–deficient platelets, compared to JAM-A–wild type platelets. It is known that outside-in signaling of integrin serves as a feedback loop that amplifies initial platelet activation stimuli such as ADP or thrombin, particularly in whole blood (i.e. in the presence of fibrinogen). Absence of JAM-A leads to an increased outside-in signaling and thus to an increased amplification of inside-out signaling, hence the lower activation thresholds for ADP and thrombin in inducing platelet aggregation. Interfering with outside-in signaling by preventing fibrinogen binding to integrin through the antagonist tirofiban, or blocking c-Src activity by specific inhibitors eliminated this increased aggregation of JAM-A–deficient platelets. This indicates that interfering with integrin outside-in signaling leads to a reversal of the platelet hyperreactivity associated with deficiency of JAM-A.

In trJAM-A–/– apoe–/– mice, we found decreased granular staining of the chemokines CCL5 and CXCL4 in platelets and increased plasma levels of these chemokines during the course of a high-fat diet. Thus, we postulate that the decreased activation threshold of JAM-A–deficient platelets results in a continuous low-level release of α-granule contents, leading to increased circulating chemokine concentrations. These chemokines might also be more effectively deposited onto the vessel walls of the trJAM-A–/– apoe–/– mice, resulting in increased recruitment of mononuclear cells to developing plaques. In addition, activated platelets avidly bind to leukocytes and uncontrolled platelet activation might lead to increased levels of circulating platelet–leukocyte complexes. Since platelets have been shown to actively recruit mononuclear cells to the vessel wall, it is conceivable that increased platelet-leukocyte aggregation in trJAM-A–/– apoe–/– mice might also contribute to plaque development. This might explain the increased content of T cells, macrophages and the elevated pro-inflammatory cytokine expression levels, particularly in the lesions of trJAM-A–/– apoe–/– mice at very
early time-points. Previous studies have implicated the role of platelets and their secretion products particularly in early phases of plaque development\textsuperscript{7,12,48}. Platelets might initiate endothelial inflammation through the action of cytokines such as CD40L\textsuperscript{2,12} and increased adhesion of platelets was found at atherosclerotic predilection sites, such as the bifurcation of the carotid artery, where plaques developed at later stages\textsuperscript{7}. Yet there is also evidence that platelets play a role at advanced phases of atherosclerotic lesion formation. For example, inhibition of glycoprotein Ib by a blocking antibody in apo-e\textsuperscript{−/−} mice resulted in a pronounced reduction of plaque formation after 18 weeks\textsuperscript{7}. In addition, repeated infusion of activated platelets was shown to also exacerbate atherosclerosis after plaques had already been formed\textsuperscript{12} and mice lacking the α\textsubscript{IIb} subunit of integrin α\textsubscript{IIb}β\textsubscript{3} showed a significant reduction in lesion burden particularly after 12 weeks of HFD\textsuperscript{49}. In this respect, it is interesting that the effects of platelet-specific JAM-A–deficiency declined at later time points during the high-fat diet, particularly in the aortic root. We therefore hypothesize that platelet hyperreactivity, though present throughout the entire course of our study, might have the most pronounced effects on initial events of atherosclerosis, such as the induction of endothelial dysfunction and the propagation of leukocyte recruitment by chemokine release or platelet-leukocyte aggregation. In later stages, these platelet-related activities might become secondary and other cell types, not affected by our model of platelet-specific deletion, might gain prominence. Similar may apply for neutrophils, which were found to preferentially invade early-stage lesions, whereas their influence declined at advanced phases of plaque development\textsuperscript{48}.

An interesting role in our study is reserved for integrin α\textsubscript{IIb}β\textsubscript{3}, which is the reported target of the (down-) regulation of platelet function by JAM-A. Inhibition of α\textsubscript{IIb}β\textsubscript{3} using the small molecular inhibitor tirofiban, effectively reverted the detrimental effects associated with JAM-A–deficiency in platelets, such as the increased platelet-leukocyte aggregation, the increased platelet adhesion to the vessel wall and the recruitment of platelets and leukocytes to inflamed endothelial cells. Several studies in mice implicate α\textsubscript{IIb}β\textsubscript{3} in inflammatory platelet functions and the development of atherosclerosis\textsuperscript{7,49,50}. For example, the expression of CD40L was shown to be increased after engagement of α\textsubscript{IIb}β\textsubscript{3} by fibrinogen\textsuperscript{50} and flow-resistant adhesion of platelets to the vessel wall in mice was significantly reduced after inhibition of α\textsubscript{IIb}β\textsubscript{3}\textsuperscript{7}. Mice deficient in functional α\textsubscript{IIb}β\textsubscript{3} showed a reduction in atherosclerosis\textsuperscript{49}. However, care should be taken in translating these findings to humans, as individuals with a genetic deficiency in α\textsubscript{IIb}β\textsubscript{3} can still develop atherosclerosis\textsuperscript{51}.

Taken together, JAM-A plays a crucial role in controlling platelet reactivity. Uncontrolled platelet reactivity might lead to continuous low-level activation of circulating platelets, due to a decreased stimulation threshold, which could be reversed by inhibition of α\textsubscript{IIb}β\textsubscript{3}.

Since activated platelets exacerbate atherosclerosis, hyperreactive JAM-A–deficient platelets might both initiate and accelerate early plaque formation. Whereas other studies have addressed the role of platelets in atherosclerosis\textsuperscript{7,11,12,49}, this is to our knowledge the first study linking a genetic gain-of-function phenotype to an accelerated development of atherosclerosis, particularly at early phases of plaque development. This study thus highlights the detrimental role of activated platelets in preclinical phases of CVD and adds another fundamental facet to the role of JAM-A in vascular disease.
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DISCLOSURES
None.

AUTHOR CONTRIBUTIONS
E.K. performed and designed experiments, analyzed data, wrote the manuscript; Z.Z. performed and
analyzed experiments, X.B. performed immune assays and immunoprecipitations, M.M.S. and R.T.A.M
performed imaging, K.B. performed qPCR analysis, O.S. performed intravital microscopy, P.v.H. provided
intellectual and material input, N.J.M. and J.M.E.M.C. performed platelet adhesion experiments, T.A.K.,
A.S., provided necessary resources, T.M.H. provided intellectual input, C.W. raised funding and provided
intellectual input, R.R.K. performed experiments, supervised study, analyzed data, obtained funding and
wrote the manuscript.

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FIGURE LEGENDS

Figure 1. Hyperreactivity of platelets in trJAM-A+/– mice. Absence of JAM-A on platelets was confirmed by western blotting. Ponceau S served as a loading control (A). Aggregation of trJAM-A+/+ (open bars) and trJAM-A–/– (solid bars) platelets by ADP (B) and thrombin (C) in the absence or presence of tirofiban (1 µg/mL), expressed as area under the curve in aggregation units per minute (AU*min). Data represent mean ± SEM (n=3-15) and P values were calculated by 2-way ANOVA with Tukey’s post test. Platelets were adhered to fibrinogen (Fg) or to heat-treated BSA for 90 minutes and stained for phosphorylated c-Src at tyrosine 418. (D) Representative flow cytometry histogram of phospho-c-Src staining in Fg-adhered platelets (grey histogram: control, hatched histogram: trJAM-A+/+, open histogram: trJAM-A–/–) and quantification of phospho-c-src mean intensity of trJAM-A+/+ and trJAM-A–/– platelets adhered to BSA or Fg are shown (E). Quantified aggregation of trJAM-A+/+ and trJAM-A–/– platelets treated with DMSO or the Src inhibitor PP2 (20µmol/L) after activation with ADP (F) or thrombin (G). Data represent mean ± SEM (n=4-7) and P values were calculated by 2-way ANOVA with Tukey’s post test. Representative immunoblots of immunoprecipitated JAM-A from human (H) or mouse (I) platelets (plt) adhered to BSA or fibrinogen (Fg) in the absence or presence of PTP inhibitors or vehicle, detected using anti-phosphotyrosine (P-tyr) (H, I, top image), anti-JAM-A (H middle, I lower image) or anti-PTPN1 (H, lower image) antibodies.

Figure 2. Platelet aggregation on collagen under flow conditions. Whole blood from trJAM-A+/+ apoe–/– (A–C) and trJAM-A–/– apoe–/– (D–F) mice was perfused over collagen type I and platelet aggregation or fluorescence stained area was quantified as % surface area coverage per analyzed visual field (G and I) or as % positively stained thrombi area (H). Expression of activated αIIbβ3 integrin or negatively charged phospholipids was quantified after staining with JON/A (B,E) or anti-annexin A5 antibodies, respectively (C,F). Scale bar= 20 µm. Data represent mean ±SEM (n=6-8) and all P values were calculated by Student’s t-test.

Figure 3. Specific role of JAM-A on platelets during atherogenesis. trJAM-A+/+ apoe–/– and trJAM-A–/– apoe–/– mice were fed a high-fat diet (HFD) for 2 weeks (A,C,E,G), 6 weeks (C, G) and 12 weeks (B, C,F,G), as indicated. Representative pictures display the atherosclerotic areas in aortic roots (A,B) and in whole aortae (E,F) of trJAM-A+/+ apoe–/– and trJAM-A–/– apoe–/– mice after 2 weeks and 12 weeks HFD, as indicated. Scale bar=500µm. Lesional areas were quantified in the aortic roots after EVG-staining (A-C) and the lesions were phenotypically characterized according to stage of atherosclerosis (D). IX: intimal xanthoma, PIT: pathologic intimal thickening, FCA: fibrous cap atheroma. Oil-Red-O-positive plaque surface was determined in the aortic arch after 2 weeks HFD and in the whole aorta 6 and 12 weeks after HFD (E-G). Data represent mean ±SEM (n=7-12) and all P values were calculated by Student’s t-test with (A, C, G) and without (B) Welch correction.

Figure 4. Atherosclerotic lesion phenotype in trJAM-A+/+ apoe–/– mice at an early stage of atherosclerosis. Representative pictures and quantifications of MAC-2 (A), CD3 (B) and αSMA (C) -stained aortic roots from trJAM-A+/+ apoe–/– and trJAM-A–/– apoe–/– mice 2 weeks after HFD. Plaque area was demarcated with dashed lines, CD3-positive cells were marked with arrows and the luminal direction of the aortic valves leaflets was marked with an asterisk. Nuclei were stained with DAPI (blue). Scale bar=100µm. Data represent mean±SEM (n=7-12) and P values were calculated by Student’s t-test with (A, C) or without (B) Welch correction.

Figure 5. Atherosclerotic lesion phenotype in trJAM-A–/– apoe–/– mice at an advanced stage of atherosclerosis. Representative pictures and quantifications of MAC-2 (A), CD3 (B) and αSMA (C) -stained aortic roots from trJAM-A+/+ apoe–/– and trJAM-A–/– apoe–/– mice 12 weeks after HFD. Plaque area was demarcated with dashed lines, CD3-positive cells were marked with arrows and the luminal direction of the aortic valves leaflets was marked with an asterisk. Nuclei were stained with DAPI (blue). Scale
bar=200µm (A,C) and 100 µm (B). Relative mRNA expression of CXCR3 (D), IFNγ (E) and TNFα (F) in whole aortae from trJAM-A+/+ apoe−/− mice and trJAM-A−/− apoe−/− mice after 2 weeks and 12 weeks HFD, expressed as ratio between target gene and 18S RNA expression and normalized to the levels of trJAM-A+/+ apoe−/− mice 2 weeks and 12 weeks HFD, respectively. Data represent mean±SEM (n=7-12) and P values were calculated by Student's t-test (A-C) or 1-way ANOVA with Tukey's post test (D-F).

Figure 6. Role of JAM-A on platelets in release and platelet-leukocyte interactions. Representative images of CXCL4 (A) and CCL5 (B) staining in platelets after 12 weeks (A) and 2 weeks HFD (B). Scale bar=20 µm. Chemokine levels of CXCL4 (C) and CCL5 (D) in platelet poor plasma (PPP) from trJAM-A+/+ apoe−/− and trJAM-A−/− apoe−/− mice fed a HFD for 2 weeks, 6 weeks and 12 weeks, as indicated. Thrombin-activated platelets and leukocytes were incubated in the absence or presence of tirofiban (1 µg/mL) and platelet-monocyte aggregates (CD41+/CD115+) (E,F) and platelet-neutrophil (CD41+/Ly6G+) (G,H) complexes were quantified by flow cytometry as a percentage of all CD45+ cells. Data represent mean ±SEM (n=7-14) and all P values were calculated by 1-way ANOVA with Bonferroni's post test.

Figure 7. Role of JAM-A on platelets in leukocyte recruitment. Representative images display adherent platelets (plt, green) and leukocytes (leu, red) in vitro (A). Adherent CD41+ platelets (B, left) and CD45+ leukocytes (B, right) in the absence or presence of tirofiban (1 µg/mL) were analyzed on TNFα-activated mECs as % of cell surface coverage per visual field in trJAM-A+/+ apoe−/− and trJAM-A−/− apoe−/− mice. Whole blood from trJAM-A+/+ apoe−/− and trJAM-A−/− apoe−/− mice was perfused through pre-stained (CD31, blue) carotid arteries of the same mice fed a HFD for 2 weeks in the absence or presence of tirofiban (1 µg/mL) (C). Adherent platelets, visualized by CD41 staining (green), were quantified per visual field as described17 (D). Firm arrest of CD11b+ monocytes (E, left) and Ly6G+ neutrophils (E, right) was assessed in atherosclerotic carotid artery in vivo using intravital microscopy and presented as number of adherent cells per visual field. Scale bar=100 µm (A, E) and 20 µm (C). Data represent mean ±SEM (n=5-8) and P values were calculated by 1-way ANOVA with Tukey's post test (B), Kruskal-Wallis test with Dunn's post test (D) or Mann-Whitney test (F,G).
Novelty and Significance

What Is Known?

- Besides their essential role in hemostasis, platelets also act as immune cells.
- Loss of platelet function reduces atherosclerotic plaque formation in hyperlipidemic mice.
- Junctional Adhesion Molecule A (JAM-A, F11R) inhibits platelet outside-in signaling through integrin $\alpha_{IIb}\beta_3$.
- Deletion of JAM-A in platelets increases their reactivity.

What New Information Does This Article Contribute?

- Hyperlipidemic mice with a specific JAM-A–deficiency in platelets showed increased atherosclerosis development.
- Platelets deficient in JAM-A showed increased interactions with leukocytes and with inflamed vascular endothelium.
- Inhibition of integrin $\alpha_{IIb}\beta_3$ using tirofiban normalized platelet reactivity in JAM-A–deficient platelets and reduced their interactions with leukocytes and endothelial cells.

Through the release of pro-inflammatory factors (e.g. bioactive lipids, cyto- and chemokines) or by binding to leukocytes, platelets can initiate and propagate vascular inflammation. For example, repeated injections of activated platelets exacerbate atherosclerosis in hyperlipidemic mice. Although genetic loss-of-platelet-function mutations led to an amelioration of atherosclerosis in mice, less is known about the effects of gain-of-function mutations in platelets on plaque development. Specific deletion of JAM-A in platelets led to an enhanced response to various agonists. Hyperlipidemic mice with platelet-JAM-A–deficiency had accelerated plaque formation, particularly during early phases of atherogenesis. In addition, increased plasma levels of the chemokines CCL5 and CXCL4 were measured in the mice with platelet-specific deficiency of JAM-A. Further, JAM-A–deficient platelets showed increased interactions with leukocytes and with inflamed vascular endothelium. An increased number of monocytes adhered to atherosclerosis-prone areas in the platelet-JAM-A–deficient mice. Blockade of integrin $\alpha_{IIb}\beta_3$ by the small molecular inhibitor tirofiban (Aggrastat®) normalized the increased aggregation of JAM-A–deficient platelets and reversed their increased tendency to interact with leukocytes and with the vessel wall. Taken together, this study demonstrates that platelet hyper-reactivity contributes to plaque development in mice and highlights the detrimental role of activated platelets in preclinical phases of cardiovascular disease.
Figure 5

A

trJAM-A<sup>+/+</sup> apoe<sup>−/−</sup> 12 weeks  trJAM-A<sup>−/−</sup> apoe<sup>−/−</sup>

B

C

D

E

F

P>0.05 P<0.05

P<0.01 P<0.01

P<0.05 P<0.05

CXCR3 expression / control

IFNγ expression / control

TNFα expression / control
Hyperreactivity of Junctional Adhesion Molecule A-Deficient Platelets Accelerates Atherosclerosis in Hyperlipidemic Mice

Ela Karshovska, Zhen Zhao, Xavier Blanchet, Martin M. N. Schmitt, Kiril Bidzhekov, Oliver Soehnlein, Philipp von Hundelshausen, Nadine J Mattheij, Judith M Cosemans, Remco T Megens, Thomas Koeppel, Andreas Schober, Tilman M Hackeng, Christian Weber and Rory R Koenen

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SUPPLEMENTAL MATERIAL

Hyperreactivity of junctional adhesion molecule A-deficient platelets accelerates atherosclerosis in hyperlipidemic mice.

Ela Karshovska; Zhen Zhao; Xavier Blanchet; Martin M. Schmitt; Kiril Bidzhekov; Oliver Soehnlein; Philipp von Hundelshausen; Nadine J. Mattheij; Judith M. E. M. Cosemans; Remco T. A. Megens; Thomas A. Koeppel; Tilman M. Hackeng; Andreas Schober; Christian Weber; and Rory R. Koenen
Detailed Methods

Mouse models

Mice carrying cre-recombinase under the control of the platelet factor 4 (PF4)-promoter were a kind gift from Dr. R.C. Skoda, University Hospital Basel\(^1\) and were backcrossed in an apolipoprotein e (apoE)-deficient background (C57Bl/6) for at least 10 generations. These mice were crossed with JAM-A\(^{-\text{floxed/floxed}}\)apoE\(^{-/-}\)mice\(^2\) to obtain platelet-specific (tr)JAM-A\(^{-/-}\)apoE\(^{-/-}\)mice. Littermates not containing the PF4-cre transgene were used as (tr)JAM-A\(^{+/+}\)apoE\(^{-/-}\)controls. Specific gene deletion of JAM-A was confirmed by flow cytometry (FACS), immunofluorescence and western blot analysis demonstrating nearly complete absence of JAM-A in platelets in trJAM-A\(^{-/-}\)mice compared to controls.

Blood collection and analysis

Mice were anesthetized with ketamine (80 mg/kg) and medetomidine (0.3 mg/kg) and blood was retro-orbitally collected into citrate-, EDTA- or hirudin-coated tubes for flow cytometry-, cell counts and platelet function assessment (see below). Cell counts and mean platelet volume (MPV) were determined using an automated hematology analyzer (sci: Animal Care). Human leukocytes and platelets from platelet poor plasma (PPP) were isolated from whole venous blood as described\(^3\). All human and animal experiments were approved by local authorities (Regierung von Oberbayern, Munich, Germany).

Plaque formation, quantification and histological analysis of atherosclerosis

Plaque formation was induced in 7–week old male and female mice (n=7-14) by feeding a high-fat diet (HFD, 21% fat, 19.5% casein, 0.15% cholesterol, ssniff, Soest, Germany) for 2, 6 and 12 weeks. Mice were anesthetized as above and the heart and the whole aorta were excised after whole body in situ perfusion with 4% buffered formaldehyde (PFA) (Carl Roth, Karlsruhe, Germany). After overnight fixation and adventitia removal, the aorta was longitudinally opened and stained en face with Oil-Red-O to visualize the lipid deposition. The plaque area was calculated as percentage of the Oil-Red-O\(^+\)stained area by dividing the aortic arch- and whole aortic area. After paraffin-embedding and cutting into 4-μm transverse sections, aortic root samples were assessed for atherosclerotic plaque size after staining with Elastic van Gieson (EVG) (Baacklab, Schwerin, Germany). The plaque area was quantified as percentage of plaque area of all three aortic valves divided to the area of the internal lumen. Adjacent sections were used for evaluating plaque cellular content by immunofluorescence staining for macrophages, CD3\(^+\) T cells and smooth muscle cells (SMCs). Briefly, after heat-induced antigen retrieval (Dako, Hamburg, Germany) and blocking of unspecific protein binding (goat- and sheep sera, Sigma Aldrich, St. Louis, MO) aortic root samples were reacted with anti-MAC-2- (Cedarlane Labs, Burlington, Canada), anti-CD3- and anti-αSMA antibodies (both Dako), respectively. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Vector
Laboratories, Burlingame, CA). For the background assessment, samples were stained instead of primary antibodies with the appropriate IgG controls followed by the secondary antibodies. For immunofluorescence staining on platelets, cells were isolated as described⁴, fixed with 4% formaldehyde, permeabilized with saponin (BD Biosciences, Franklin Lakes, NJ) and incubated with anti-CXCL4 and anti-CCL5 antibodies (both R&D Systems, Minneapolis, MN). Finally, the primary antibodies were detected after incubation with DyLight®-488, -550 and -650–conjugated secondary antibodies (all Abcam, Cambridge, UK). Images were recorded with a DM 6000B fluorescence microscope (Leica, Solms, Germany), connected to a monochrome digital camera (DFC 365FX) for the fluorescent images and DFC425C camera for the EVG-stained aortic root pictures. MAC-2-, CD3- and αSMA cells were manually quantified as positive cells per plaque area. Stages of atherosclerotic lesions were determined by evaluation of EVG-stained aortic roots⁵. All animal experiments were approved by local authorities (Regierung von Oberbayern, Munich, Germany) to comply with German animal protection law.

Platelet isolation and activity measurement

Platelets were isolated and washed by centrifugation as described⁴. Platelet aggregation in response to adenosine diphosphate (ADP) and thrombin (Sigma Aldrich) or collagen (Loxo GmbH, Dossenheim, Germany) was assessed in mouse whole blood within 2 hours after isolation by multiple electrode aggregometry technology using a Multiplate® platelet analyzer according to manufacturer's instructions (Roche Diagnostics, Mannheim, Germany)⁶. In some experiments, platelet aggregation was initiated using thrombin and ADP after pretreatment of the platelets with α₂β₃ integrin antagonist tirofiban (Aggrastat®, MSD, 1 µg/mL) or the Src kinase inhibitors PP2, SU6656 and KB Src 4 (Merck Millipore or Tocris, 20, 2.5 and 0.09 µmol/L respectively ).

Thrombus formation on collagen under flow

Platelet adhesion onto fibrillar collagen under shear flow was performed essentially as described⁴,⁷. Briefly, mouse blood collected into D-phenylalanyl-L-prolyl-L-arginine chloromethylketone (PPACK) was perfused over collagen type I-coated coverslips in a transparent parallel-plate perfusion chamber at a shear rate of 1000 s⁻¹ for 4 minutes. To assess α₁β₃ integrin activation and phosphatidyl serine binding, thrombi formed on the collagen surface were post-stained with fluorescently labeled anti-JON/A PE-conjugated (Emfret, Würzburg, Germany) and anti-annexin A5 Alexa Fluor® 647-conjugated (Life Technologies, Carlsbad, CA) antibodies, respectively. Phase contrast and fluorescence images were recorded using an EVOS-FL digital microscope (Life Technologies). Finally, surface area coverage of adherent platelets and fluorescence positive area per thrombi area over multiple microscopic fields was analyzed using Metamorph 5.0.0 software (MDS Analytical Technologies).
**Immunoprecipitation**

Isolated washed platelets from mice and humans (1 or 4.5×10^8, respectively) were incubated in petri dishes with immobilized heat-inactivated BSA (5 mg/mL) or fibrinogen (0.1 mg/mL, Enzyme Research Labs, South Bend, IN) for 60-90 minutes as described^8,9. In some experiments, the platelets were incubated with protein tyrosine phosphatase (PTP) inhibitors: broad-spectrum PTP inhibitors IV (CAS 329317-98-8, 20 μmol/L) and XXXI (II-B08, 30 μmol/L) and specific inhibitors SHP1/2 inhibitor NSC-87877 (CAS 56932-43-5, 0.7 and 10 μmol/L) and PTP1B inhibitor (CAS 765317-72-4, 8 μmol/L) or vehicle (DMSO) during adhesion on BSA or fibrinogen (all inhibitors from Merck Millipore). The platelets were subsequently lysed in 20 mmol/L tris pH7.4, 150 mmol/L NaCl, 2 mmol/L CaCl_2, 1 mmol/L Na_3VO_4, 10 mg/mL triton X-100, 5 mmol/L of AEBSF and JAM-A was immunoprecipitated using specific antibodies (clone M.Ab.F11 and H2O2-106 for human or mouse, respectively) and protein G-coupled magnetic beads (Life Technologies) and analyzed by SDS page and western blotting. Antigens were detected with anti-human JAM-A goat polyclonal (R&D systems), anti-mouse JAM-A rat monoclonal (clone H2O2-106, AbD Serotec, Düsseldorf, Germany), anti-phosphotyrosine mouse monoclonal (clone 4G10, Merck Millipore), and anti-PTPN1 goat polyclonal antibodies (N-19, Santa Cruz Biotech, Santa Cruz, CA) as described below.

**Western blotting**

For immunoblotting, isolated platelets and harvested aortae and carotid arteries after whole body perfusion with ice-cold phosphate-buffered saline solution (PBS, Sigma Aldrich) were homogenized as described^10. Briefly, protein extracts were obtained from homogenates after incubation in lysis buffer containing protease inhibitors and detergents^10, following centrifugation at 8,000g for 1 min. Supernatants were analyzed for immunoblotting. Protein concentrations were determined (Bio-Rad protein assay, Bio-Rad). Equal amounts of protein (10 to 30 μg) were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Ponceau S staining served as a loading control. The membranes were reacted with designated antibodies followed by incubation with peroxidase-conjugated secondary antibodies (Santa Cruz Biotech) and detected with chemiluminescence reagent (Thermo Fischer Scientific, Waltham, MA).

**Plasma lipid and chemokine determination**

Concentrations of chemokines CXCL4 and CCL5 were measured in platelet poor plasma (PPP) from mice fed a HFD for 2, 6 and 12 weeks using Enzyme-linked immunosorbent assays (ELISA) kits (both R&D Systems, Minneapolis). Total cholesterol and triglycerides concentrations were analyzed using the enzymatic colorimetric method (Cholesterol-CHOD-PAP kit and Triglycerides-GPO-PAP kit, both Roche Diagnostics) according to the manufacturer’s instructions.
Flow cytometry

For assessment of JAM-A expression, platelets were labeled with anti-JAM-A AF488-conjugated (AbD Serotec) and anti-CD41 PE-conjugated (BD Pharmingen) antibodies. For platelet-leukocyte interactions, isolated platelets were activated with 0.5U/mL thrombin and added to isolated erythrocyte-free leukocytes for 20 min at 37°C. Finally, cells were stained with anti-CD41 FITC-conjugated (BD Pharmingen), anti-CD45 eFluor®450-conjugated (eBioscience), anti-CD115 PE-Cyanine 7-conjugated (eBioscience) and anti-Ly6G PerCP-Cyanine 5.5-conjugated (eBioscience) antibodies. In some experiments, platelet-leukocyte interactions were analyzed after pretreatment of the platelets with α(III)β3 integrin antagonist tirofiban (Aggrastat®, MSD, 1 µg/mL). For the adhesion molecules expression, isolated platelets were stained with anti-JAM-A AF488-conjugated (AbD Serotec), anti-JAM-C PE-conjugated and anti-endothelial cell-selective adhesion molecule (ESAM) (both R&D systems), incubated with DyLight®-650-conjugated secondary antibody (Abcam, Cambridge, UK). Samples were measured by flow cytometry (FACSCantoII, BD Biosciences) after appropriate compensation settings and analyzed by FlowJo v.10 software (Tree Star Inc.).

Src kinase phosphorylation assay

Mouse platelets were isolated as above and incubated on immobilized heat-inactivated BSA or fibrinogen for 90 min at 37°C as described, detached, fixed and permeabilized. Phosphorylated c-Src was subsequently detected using an eFluor660-conjugated mouse monoclonal antibody, specific for mouse/human Src phosphorylation at tyrosine 418 residue (Y418) (eBioscience), by flow cytometry (FACSCantoII, BD Biosciences) and analyzed by FlowJo v.10 software (Tree Star Inc.).

Quantitative real-time PCR

For RNA analysis, total RNA was isolated from mouse aortae after 2 and 12 weeks after HFD and reverse transcribed into cDNA using Mo-MLV RT (Life Technologies). q-PCR was performed using TaqMan Gene Expression Master Mix and real time specific primers (Life Technologies). All reactions were run on a 7900HT thermocycler (Life Technologies). The expression levels of the target CXCR3 (Mm99999054_s1); IFNγ (Mm99999071_m1); TNFα (Mm00443260_g1) were quantified by the ratio to 18S RNA (4319413E) levels. The controls represented trJAM-A+/+ apoE−/− mice, after 2 weeks and 12 weeks HFD, respectively.

Platelet and leukocyte recruitment assays

In vitro adhesion assay - SV-40-large T antigen-immortalized mouse endothelial cell (mECs) monolayers were challenged with TNFα (10 ng/mL) for 4 hours. Freshly isolated leukocytes and platelets were suspended in HBSS containing 5 mg/mL human albumin and 10 mmol/L HEPES at 1×10⁶ leukocytes/mL and 1×10⁸ platelets/mL. In some experiments, isolated platelets were pretreated with α(III)β3 integrin antagonist tirofiban (Aggrastat®, MSD, 1 µg/mL). The blood cells were then labeled with CD45 AF488-conjugated (Bio-Rad) and CD41 PE-conjugated (BD Pharmingen).
Immediately prior to perfusion, 0.5 U/mL thrombin and 1 mmol/L CaCl$_2$ and MgCl$_2$ were added to the platelets and leukocytes at 37°C. The endothelial cells were assembled in a flow chamber and platelets were perfused for 20 min, followed by leukocytes for 5 min and additional washing of non-adherent cells (all at 0.15 N/m$^2$). Images were recorded with a fluorescence microscope (DM6000, Leica), connected to a digital camera (DFC365FX, Leica) and quantified as percentage surface coverage of platelets or leukocytes over multiple microscopic fields using ImageJ software (NIH).

**Ex vivo adhesion assay** – Ex vivo perfusion assay was performed in carotid arteries of trJAM-A$^{+/+}$ apoe$^{-/-}$ and trJAM-A$^{-/-}$ apoe$^{-/-}$ mice fed a HFD for 2 weeks. Carotid arteries were carefully explanted, mounted in a customized perfusion chamber and pressurized at physiological pressure of $0.8-1.1\times10^4$ Pa. Whole blood was retro-orbitally taken from the mice and diluted with isotonic citrate buffer to adjust the platelet count to $1\times10^8$ platelets/mL. Prior to perfusion, the endothelium was labeled with anti-CD31 eFluor®450-conjugated antibody (eBioscience) for 30 minutes. Platelets were stained with anti-CD41 FITC-conjugated antibody (BD Pharmingen) and the blood was perfused along the mounted and pressurized vessel for 10 minutes at 0.5 mL/min. In some experiments, diluted blood was pretreated with $\alpha$IIb$\beta$3 integrin antagonist tirofiban (Aggrastat®, MSD, 1 $\mu$g/mL) prior to perfusion. The first 500$\mu$m close to the mounting pipettes were excluded due to possible handling damage. Adherent platelets were recorded by two-photon laser scanning microscopy (TPLSM) and manually counted per field of view of Z-stack maximum projections (FOV=240$\mu$m$^2$; voxel size: 0.23 x 0.23 x 1$\mu$m$^3$). Z-stacks were acquired at 0.1 Hz including two-fold frame averaging$^2$.

**In vivo adhesion assay**

Intravital microscopy was performed to visualize leukocyte-endothelium interactions along the atherosclerotic carotid artery. Mice were fed a HFD for 4 weeks, anesthetized as described above and the right jugular vein was cannulated with polyethylene tubing for intravenous administration of anti-CD11b eFluor®650NC-conjugated and anti-Ly6G PE-conjugated antibodies (both eBioscience). The left carotid artery was exposed and the firm arrest of the labeled leukocytes was captured by epifluorescence microscopy (Olympus BX51 10x saline-immersion objective) and recorded using a digital camera (Hamamatsu EM-CCD, C9100) as described$^{12}$. Olympus cellR software (Olympus, Shinjuku, Japan) was used for image acquisition and quantification. Leukocytes were considered adherent when no rolling was observed for more than 30 sec.

**Two-photon laser scanning microscopy**

For two-photon laser scanning microscopy (TPLSM) mice were fed a HFD for 2 weeks and carotid arteries were carefully harvested, mounted in custom-made perfusion chambers and incubated at physiological pressure of $0.8-1.1\times10^4$ Pa. Previous studies have demonstrated that the vessel, including the endothelial and smooth muscle cells, remains intact$^2$.$^{13}$. To detect endothelial JAM-A (eJAM-A), vessels were intraluminally incubated with anti-JAM-A AlexaFluor®488-conjugated
(AbSerotec) and anti-CD31 eFluor®450 (eBioscience) antibodies for 1 hour. TPLSM imaging was performed using a Leica SP5MP system with a pulsed Ti:Sapphire laser (Spectra Physics MaiTai DeepSee) tuned at 800 nm and a 20×NA1.00 water immersion objectives. Emitted fluorescent signals were detected by three internal Hybrid detectors tuned for the corresponding wavelengths using an acousto-optical beam splitter: 400-440 nm for second-harmonic generation (SHG); 460-490 nm (eFluor® 450) and 510-560 nm (Alexa Fluor®488 or FITC). Z-stacks were acquired at 0.1 Hz over time including two-fold line averaging; FOV = 740 μm² (for JAM-A images) or 240μm² (for platelet images). All pictures were processed using LAS software (Leica) and Image Pro Analyzer v 7.0 software (Media Cybernetics, Rockville, MD). All pictures were processed using LAS software (Leica) and Image Pro Analyzer v7.0 software (Media Cybernetics, Rockville, MD).

**Statistical Analysis**

Statistical analysis was performed using Prism 6.0 (GraphPad Software). Means were compared between 2 groups by 2-tailed, unpaired Student’s t-test, without or with Welch correction or among more than 2 groups by 1- or 2-way ANOVA with Tukey's or Bonferroni's post-test or Kruskal-Wallis test with Dunn's post-test, as indicated. Differences with P<0.05 were considered as statistically significant. Each experiment was independently repeated at least 3 times.
Supplemental Figures and Figure legends

**Online Figure I.** JAM-A deletion on platelets and cell-specific JAM-A expression in trJAM-A<sup>+/−</sup> apoe<sup>−/−</sup> mice. JAM-A was stained on isolated platelets (A, JAM-A: green, CD41: red). JAM-A expression on platelets (B) or leukocytes (D) was measured by flow cytometry. Upper right quadrants depict the percentage of platelet– (B) and leukocyte– (D) JAM-A expression in trJAM-A<sup>+/−</sup> apoe<sup>−/−</sup> and trJAM-A<sup>−/−</sup> apoe<sup>−/−</sup> mice, as indicated. Immunocytochemical images of the carotid arteries from trJAM-A<sup>−/−</sup> apoe<sup>−/−</sup> and trJAM-A<sup>+/−</sup> apoe<sup>−/−</sup> mice, endothelial cells (C, JAM-A: green, von Willebrand Factor, vWF: red and nuclei: blue) or SMC (E, JAM-A: green, αSMA: red and nuclei: blue). Scale bar=20µm (A, C, E). Western blot experiments in homogenates from aortae and carotid arteries from trJAM-A<sup>−/−</sup> apoe<sup>−/−</sup> and trJAM-A<sup>+/−</sup> mice apoe<sup>−/−</sup> are shown (F). Ponceau S staining served as a loading control. Representative picture from 3 independent experiments.
Online Figure II. Endothelial JAM-A expression in platelet-deficient JAM-A mice. Endothelial JAM-A (A,B, merged, right) was stained in carotid arteries from trJAM-A+/+ apoE−/− (A) and trJAM-A−/− apoE−/− (B) mice fed a HFD for 2 weeks. JAM-A: green (A, B left), CD31: red (A,B, middle), collagen: blue (second harmonics generation). Autofluorescence signal derived from the arterial wall was detectable in all channels. Scale bar= 20 µm.
Online Figure III. JAM-C and ESAM expression on platelets from trJAM-A⁺/⁺ apoe⁻/⁻ and trJAM-A⁻/⁻ apoe⁻/⁻ mice. JAM-C (A) and ESAM (B) were stained on isolated platelets from trJAM-A⁺/⁺ apoe⁻/⁻ (blue histogram) and trJAM-A⁻/⁻ apoe⁻/⁻ mice (black histogram), compared with controls (grey histogram) and measured by flow cytometry. Fluorescence was quantified and represented as mean±SEM (n=3), P values were calculated by Student's t-test (n=3).
## Supplemental Tables

<table>
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<tr>
<th>genotype</th>
<th>platelets</th>
<th>MPV</th>
<th>WBC</th>
<th>lymphocytes</th>
<th>monocytes</th>
<th>neutrophils</th>
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**Online table I:** Platelet counts, mean platelet volume (MPV) and white blood cell counts (WBC) in trJAM-A−/− apoe−/− and trJAM-A+/+ apoe−/− mice fed a HFD for 2 weeks (2w), 6 weeks (6w), 12 weeks (12 w) and without (w/o) HFD. Values are shown as 10⁹ cells/L blood and MPV as femtoliter. Data represent mean ±SEM (n=7-14) and all P values were calculated by Student's t-test.

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<th>triglycerides (mmol/L)</th>
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**Online table II:** Plasma cholesterol and triglyceride levels in trJAM-A−/− apoe−/− and trJAM-A+/+ apoe−/− mice fed a HFD for 2 weeks (2w), 6 weeks (6w) and 12 weeks (12 w) HFD. Values are expressed as mg/dl. Data represent mean ±SEM (n=7-13) and all P values were calculated by Student's t-test.
Supplemental References


