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


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 α₁β₃-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 cellular and sarcoma tyrosine-protein kinase activation. On α₁β₃ ligation, JAM-A was shown to be dephosphorylated, which could be prevented by protein tyrosine phosphatase nonreceptor type 1 inhibition. Mice with or without platelet-specific (tr)JAM-A-deficiency in an apolipoprotein e (apoe⁻/⁻) background were fed a high-fat diet. After ≤12 weeks of diet, trJAM-A⁻/⁻ apoe⁺/⁺ mice showed increased aortic plaque formation when 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 CC chemokine ligand 5 and CXC-chemokine ligand 4 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 proinflammatory effects of JAM-A-deficient platelets could be abolished by the inhibition of α₁β₃ 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. (Circ Res. 2015;116:587-599. DOI: 10.1161/CIRCRESAHA.116.304035.)

Key Words: atherosclerosis ■ blood platelets ■ cell adhesion molecules ■ inflammation ■ phosphoprotein phosphatases

Blood platelets not only have an essential function in hemostasis but also play an important role in immune and inflammatory processes. Recent studies have highlighted platelets as key effectors in, eg, sepsis, rheumatoid arthritis, experimental autoimmune encephalomyelitis, and host defense during bacterial infection. In addition, platelets spark vascular inflammatory processes and adhere to atherosclerotic predilection sites preceding plaque formation. Platelets may also form a bridge between leukocytes and the injured or inflamed vessel wall, promoting the extravasation of monocytes and neutrophils and the response to vascular injury. Accordingly, infusion of activated platelets into hyperlipidemic mice accelerated atherosclerosis in a P-selectin- and cluster of differentiation (CD) 40 ligand (CD40L)–dependent manner.

Editorial, see p 557

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interaction with blood cells.16 Recently, we demonstrated that JAM-A translocated from the intercellular contacts and is ex-ported. Recent work, however, identified JAM-A as an endog-enous inhibitor of platelet function by attenuating cellular and sarcoma tyrosine-protein kinase (c-Src)–dependent outside-in
fluence on platelet function remained poorly characterized.17 Stages of atherosclerotic lesions were determined by evaluation of Elastica van Gieson–stained aortic roots.24

**Platelet Isolation and Activity Measurement**
Platelets were isolated and washed by centrifugation from platelet-deficient background (C57Bl/6)) backcrossed for ≥10 generations. These mice were crossed with JAM-A<sup>Alox11</sup> apo<sup>e<sup>−/−</sup></sup> mice<sup>17</sup> to obtain platelet-specific (tr)JAM-A<sup>−/−</sup> apo<sup>e<sup>−/−</sup></sup> mice. Littermates not containing the PF4<sup>−/−</sup> cre transgene were used as (tr)JAM-A<sup>−/−</sup> apo<sup>e<sup>−/−</sup></sup> controls. All human and animal experiments were approved by local authorities (Regierung von Oberbayern, Munich, Germany).

**Platelet Isolation and Activity Measurement**
Platelets were isolated and washed by centrifugation from platelet-poor plasma as described.<sup>12</sup> Platelet aggregation in response to 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 or ADP after pretreatment of the platelets with α<sub>i</sub>β<sub>i</sub> integrin antagonist tirofiban (Aggrastat; MSD, 1 μg/mL) or the Src kinase inhibitors PP2 (4-amino-5-(4-chlorophenyl)-7-(4-butylypyrazolo[3,4-d]pyrimidine), 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.<sup>13,25</sup>

**Immunoprecipitation, Western Blotting, and Quantitative Polymerase Chain Reaction**
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.<sup>26,27</sup> In some experiments, the platelets were incubated with protein tyrosine phosphatase (PTP) 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 (diluted methyl sulf-oxide) during adhesion. The platelets were subsequently lysed, and JAM-A was immunoprecipitated using specific antibodies and protein G-linked magnetic beads (Life Technologies) and analyzed by SDS-PAGE and Western blotting. For immunoblotting or quantitative polymerase chain reaction, isolated platelets and harvested arteries after whole body perfusion with ice-cold phosphate-buffered saline solution (PBS; Sigma Aldrich) were homogenized and analyzed as described.<sup>28</sup>

**Plasma Lipid and Chemokine Determination**
Concentrations of chemokines CXC-chemokine ligand 4 (CXCL4) and CC chemokine ligand 5 (CCL5) were measured in platelet-poor plasma from mice fed a HFD for 2, 6, and 12 weeks and without HFD using 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 Alexa Fluor 488-conju-gated (AbDSerotec) and anti-C4D1 phycoerythrin-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 minutes at 37°C. Finally, cells were stained with anti-C4D1–FITC (BD), anti-C4D5–eFluor Hospital Basel,<sup>23</sup> and were backcrossed in an apolipoprotein e (apo<sup>e</sup>)–deficient background (C57Bl/6) backcrossed for ≥10 generations. These mice were crossed with JAM-A<sup>Alox11</sup> apo<sup>e<sup>−/−</sup></sup> mice<sup>17</sup> to obtain platelet-specific (tr)JAM-A<sup>−/−</sup> apo<sup>e<sup>−/−</sup></sup> mice. Littermates not containing the PF4<sup>−/−</sup> cre transgene were used as (tr)JAM-A<sup>−/−</sup> apo<sup>e<sup>−/−</sup></sup> controls. All human and animal experiments were approved by local authorities (Regierung von Oberbayern, Munich, Germany).

**Methods**
Detailed experimental procedures are available in the Online Data 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

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**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Apoe</td>
<td>apolipoprotein E</td>
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<tr>
<td>c-Src</td>
<td>cellular and sarcoma tyrosine-protein kinase</td>
</tr>
<tr>
<td>CCL5</td>
<td>CC chemokine ligand 5</td>
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<tr>
<td>CD</td>
<td>cluster of differentiation</td>
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<tr>
<td>CSK</td>
<td>c-terminal Src kinase</td>
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<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
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<tr>
<td>CXCL4</td>
<td>CXC-chemokine ligand 4</td>
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<tr>
<td>HFD</td>
<td>high-fat diet</td>
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<tr>
<td>JAM-A</td>
<td>F11R, junctional adhesion molecule A, F11 Receptor</td>
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<tr>
<td>PF4</td>
<td>platelet factor 4</td>
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<td>PTPN1</td>
<td>protein tyrosine phosphatase nonreceptor type 1</td>
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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.<sup>11</sup> On leukocytes, it mediates cell migration by regulating integrin α<sub>i</sub>β<sub>i</sub>-adhesion.<sup>14</sup> On epi- and endothelial cells, JAM-A is a component of the tight junctions and regulates cell layer permeability through homophilic inter- actions.<sup>15</sup> On inflammatory stimulation of endothelial cells, JAM-A translocated from the intercellular contacts and is expressed on the apical surface, thereby becoming available for the interaction with blood cells.<sup>16</sup> Recently, we demonstrated that JAM-A has a cell-type–specific effect on atherosclerotic plaque formation.<sup>17</sup> Whereas JAM-A on leukocytes protected against atherosclerosis, endothelial JAM-A promoted plaque formation by enhanced luminal availability under proatherosclerotic condi-tions, thus guiding monocytes to sites of plaque development.<sup>17</sup>

Although JAM-A was first identified in platelets,<sup>18</sup> its influence on platelet function remained poorly characterized. Earlier studies demonstrated phosphorylation of JAM-A on platelet activation<sup>19</sup> and its association with α<sub>i</sub>β<sub>i</sub> integrin,<sup>20</sup> yet no functional consequences for platelet function were reported. Recent work, however, identified JAM-A as an endoge-nous inhibitor of platelet function by attenuating cellular and sarcoma tyrosine-protein kinase (c-Src)–dependent outside-in signal transduction of α<sub>i</sub>β<sub>i</sub> integrin, through the recruitment of the Src inhibitory enzyme c-terminal Src kinase (CSK).<sup>21,22</sup>

Deficiency of JAM-A in platelets resulted in increased aggregation in response to some platelet agonists and led to a prothrombotic phenotype in mice.<sup>21</sup>

The importance of platelets in the pathogenesis of ath-erosclerosis 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, proinflam-matory phenotype, and thus to an acceleration of early phase plaque formation.
Karshovska et al. Elevated Atherosclerosis in Platelet-JAM-A KO-Mice

In Vitro Adhesion Assay
SV-40-large T antigen-immortalized mouse endothelial cell monolayers were challenged with tumor necrosis factor-α (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–Alexa Fluor 488 (Bio-Rad) and anti-CD41–phycoerythrin (BD). Immediately before 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 minutes, followed by leukocytes for 5 minutes and additional washing of nonadherent cells (all at 0.15 N/m²). Cell adhesion was expressed as percentage surface coverage of platelets or leukocytes over multiple microscopic fields using ImageJ software (National Institutes of Health).

Ex Vivo Adhesion Assay
Carotid arteries of trJAM-A⁺/⁺ and trJAM-A⁻/⁻ mice fed a HFD for 2 weeks were carefully explanted, mounted in a customized perfusion chamber, and pressurized at physiological pressure of 0.8 to 1.1×10⁴ Pa. Whole blood was diluted with isotonic citrate buffer to adjust the platelet count to 1×10⁸ platelets/mL blood. Before perfusion, the endothelium was labeled with anti-CD31–eFluor 450 antibody for 30 minutes. Platelets were stained with anti-CD41–fluorescein isothiocyanate 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) before perfusion. Adherent platelets were visualized using 2-photon laser scanning microscopy as described.

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.
leukocytes along the atherosclerotic carotid artery as described. Leukocytes were considered adherent when no rolling was observed for >30 s.

Statistical Analysis
Statistical analysis was performed using Prism 6.0 (GraphPad Software). Means were compared between 2 groups by 2-tailed, unpaired Student t test, without or with Welch correction or among >2 groups by 1- or 2-way ANOVA with Tukey or Bonferroni post-test or Kruskal–Wallis test with Dunn post-test, as indicated. Differences with P<0.05 were considered as statistically significant. Each experiment was independently repeated ≥3x.

Results
Deficiency in Platelet JAM-A Results in Hyperreactivity
Previous studies have shown that genetic deletion of JAM-A results in platelet hyperreactivity, yet the functional consequences for the progression of atherosclerosis have not been investigated. For this, we implemented platelet-specific JAM-A knockout (trJAM-A–/–) mice and compared them with their control littermates (trJAM-A+/+). Specific genetic deletion of JAM-A in platelets from trJAM-A–/– mice was shown by Western blotting and immunocytochemistry (Figure 1A; Online Figure I), whereas JAM-A expression on leukocytes, endothelial cells, smooth muscle cells, and in homogenates from aortae and carotid arteries was not affected (Online Figures I and II). In addition, JAM-A deficiency did affect neither 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 endothelial cell–specific adhesion molecule on platelets, was not altered in the absence of JAM-A (Online Table I; Online Figure III).

To recapitulate previous observations by Naik et al., we examined in vitro platelet aggregation on activation. Specific platelet JAM-A–deletion resulted in enhanced aggregation in response to different agonists, such as ADP, thrombin, and collagen (Figure 1B and 1C and not shown), supporting the role of JAM-A as an endogenous platelet function inhibitor. Because integrin αIIbβ3 outside-in signaling is the reported target of JAM-A, the αIIbβ3 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+/+ platelets (Figure 1B and 1C).

Outside-in signaling by αIIbβ3 is controlled by c-Src kinase. To investigate the influence of JAM-A deficiency in our PF4-Cre–based mouse model, platelets from trJAM-A–/– and trJAM-A+/– mice were adhered to BSA or fibrinogen, and c-Src activation was assessed by measuring Src phosphorylation at Y418 residue by flow cytometry. The absence of JAM-A in platelets resulted in a significant increase in Y418 phosphorylation of c-Src after adhesion to fibrinogen when compared with control conditions (Figure 1D and 1E). Blockade of Src activation (and thus αIIbβ3 outside-in signaling) with the inhibitors PP2, SU6656, or KB Sre 4 abrogated the increased agonist-induced aggregation of JAM-A–deficient platelets (Figure 1F and 1G and not shown), albeit that baseline aggregation was increased, possibly by the solvent dimethyl sulfoxide.

Tyrosine-phosphorylated JAM-A recruits the c-Src–inhibiting kinase CSK to integrin αIIbβ3. On ligation of αIIbβ3, JAM-A is dephosphorylated allowing the dissociation of CSK from the JAM-A/c-Src/integrin αIIbβ3 complex. 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 when compared with platelets incubated on BSA (Figure 1H and II). Dephosphorylation of tyrosine residues is performed by PTPs. To date, 20 PTPs are known in platelets and regulate signaling events during platelet activation. Addition of broad-spectrum PTP inhibitors (IV and XXXI) to fibrinogen-adhered platelets resulted in a decreased dephosphorylation of JAM-A (Figure 1II and I). A previous study described an association of PTP nonreceptor type 1 (PTP11 [PTP11β]) with the c-Src/integrin αIIbβ3 complex and identified PTP11 as a positive regulator of platelet outside-in signaling. Thus, we investigated a possible role of PTP11 in the dephosphorylation of JAM-A. Probing immunoprecipitated JAM-A with antibodies against PTP11 revealed a coprecipitation of PTP11 with JAM-A both in resting and outside-in activated human platelets (Figure 1H), suggesting a constitutive physical association of PTP11 with JAM-A. Addition of a specific inhibitor against PTP11 resulted in an increase of tyrosine-phosphorylated JAM-A in fibrinogen-adhered platelets when compared with vehicle (Figure II). Interestingly, NSC-87877, an inhibitor of PTPN6 and PTPN11 (Src homology region 2 domain-containing phosphatase-1/2), did not increase JAM-A phosphorylation at a concentration (0.7 µmol/L) that specifically affected PTPN6 and PTPN11, indicating that these PTPs do not mediate JAM-A dephosphorylation (Figure II). However, NSC-87877 also blocks PTPN1 when used at a 10-fold higher concentration. Addition of NSC-87877 at 10 µmol/L indeed resulted in a reduced tyrosine dephosphorylation of JAM-A (Figure II). 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 αIIbβ3 complex and a negative regulator of platelet αIIbβ3 outside-in signaling, and that absence of JAM-A results in hyperreactivity.

Absence of JAM-A Promotes Thrombus Formation
Because 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 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 (Figure 2A, 2D, and 2G). In addition, poststaining with the JON/A antibody, specific for the activated conformation of αIIbβ3 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 (Figure 2B, 2E, and 2H). 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 (Figure 2C, 2F, and 2I). Taken together, these results suggest that genetic ablation of JAM-A on platelets results in increased platelet reactivity associated with enhanced thrombus formation.

**Figure 2.** Platelet aggregation on collagen under flow conditions. Whole blood from platelet-specific junctional adhesion molecule A (trJAM-A)+/– apolipoprotein e (apoe)+/– (A–G) 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 (N). The expression of activated αIIbβ3 integrin or negatively charged phospholipids was quantified after staining with JON/A (B and E) or anti-annexin A5 antibodies, respectively (C and 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 junctional adhesion molecule A (JAM-A) on platelets during atherogenesis. Platelet-specific JAM-A (trJAM-A)+/– apolipoprotein e (apoe)+/– and trJAM-A–/– apoe–/– mice were fed a high-fat diet (HFD) for 2 weeks (A, C, E, and G), 6 weeks (C and G) and 12 weeks (B, C, F, and G), as indicated. Representative pictures display the atherosclerotic areas in aortic roots (A and B) and in whole aortae (E and F) of trJAM-A+/– apoe–/– and trJAM-A–/– apoe–/– mice after 2 and 12 weeks HFD, as indicated. Scale bar, 500 μm. Lesional areas were quantified in the aortic roots after Elastica van Gieson staining (A–C), and the lesions were phenotypically characterized according to stage of atherosclerosis (D). 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 (C, 2 weeks; G, 2 and 12 weeks) or without Welch correction. FCA indicates fibrous cap atheroma; IX, intimal xanthoma; and PIT, pathological intimal thickening.
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 early stages of atherosclerosis (2-week HFD) platelet JAM-A deficiency resulted in a significantly increased lesion area in the aortic root and in the aortic arch (Figure 3A, 3C, 3E, and 3G). An evaluation of the plaque phenotype in aortic roots revealed that JAM-A deficiency on platelets resulted in more advanced plaque phenotypes when compared with controls, expressed as a percentage of the total number of plaques that appeared at an early stage of atherosclerosis (2-week HFD) platelet JAM-A deficiency resulted in a significantly increased lesion area in trJAM-A−/− apoe−/− mice when compared with that in control littermates (Figure 4A). In contrast, at intermediate (6-week HFD; data not shown) or advanced time points (12-week HFD), the macrophage content did not differ in trJAM-A−/− apoe−/− mice versus trJAM-A+/+ apoe−/− controls (Figure 5A). Of note, the absence of platelet JAM-A markedly augmented infiltration of the CD3+ T-cells in early stages (Figure 4B). Moreover, when compared with control mice, platelet JAM-A deficiency strongly increased α-smooth muscle cell content at the early time point (Figure 4C) and neither had effect on smooth muscle cell at intermediate (data not shown) nor at late time points (Figure 5C).

In addition, investigation of gene expression in atherosclerotic aortae in mice fed for 2- and 12-week HFD showed that deletion of JAM-A on platelets significantly increased the aortic expression of CXC chemokine receptor 3 (Figure 5D), and of the inflammatory cytokines interferon-γ (Figure 5E) and tumor necrosis factor-α (Figure 5F), after 2 weeks but not after 12 weeks of HFD. Thus, platelets may differentially

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 Figure 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 macrophage antigen-2+ macrophage content in early stages (2-week HFD) was significantly increased in trJAM-A−/− apoe−/− mice when compared with that in control littermates (Figure 4A). In contrast, at intermediate (6-week HFD; data not shown) or advanced time points (12-week HFD), the macrophage content did not differ in trJAM-A−/− apoe−/− mice versus trJAM-A+/+ apoe−/− controls (Figure 5A). Of note, the absence of platelet JAM-A markedly augmented infiltration of the CD3+ T-cells in early stages (Figure 4B). Whereas trJAM-A deletion did not influence the T-cell content at intermediate (data not shown) and in late time points (Figure 5B). Moreover, when compared with control mice, platelet JAM-A deficiency strongly increased α-smooth muscle cell content at the early time point (Figure 4C) and neither had effect on smooth muscle cell at intermediate (data not shown) nor at late time points (Figure 5C).

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Figure 4. Atherosclerotic lesion phenotype in platelet-specific junctional adhesion molecule A (trJAM-A−/−) apolipoprotein e (apo e−/−) mice at an early stage of atherosclerosis.

Representative pictures and quantifications of macrophage antigen (MAC)-2 (A), cluster of differentiation (CD) 3 (B), and α-smooth muscle cell (SMA; C)-stained aortic roots from trJAM-A−/− apoe−/− and trJAM-A+/− apoe−/− mice 2 weeks after high-fat diet. Plaque area is demarcated with dashed lines, CD3-positive cells are marked with arrows, and the luminal direction of the aortic valves leaflets is marked with an asterisk. Nuclei are stained with DAPI (4’,6-diamidino-2-phenylindole; blue). Scale bar, 100 μm. Data represent mean±SEM (n=7–12), and P values were calculated by Student t test with (A and C) or without (B) Welch correction.
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., PF4 (CXCL4), and regulated on activation, normal T-cell expressed and secreted (RANTES, CCL5) on activation, leading to increased recruitment of mononuclear cells to the inflamed vessel wall. 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−/− apoe−/− mice compared with those from trJAM-A+/+ apoe−/− mice after 12- and 2-week HFD, respectively (Figure 6A and 6B). In addition, the absence of JAM-A on platelets incrementally increased the levels of circulating CXCL4 after 2, 6, and 12 weeks of HFD (Figure 6C). Similarly, CCL5 levels were markedly enhanced in plasma from trJAM-A−/− apoe−/− mice when compared with controls in the early and intermediate time points of the HFD (Figure 6D), whereas there was no difference on the baseline chemokine levels without HFD (data not shown) between both conditions.
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,

Platelets are able to form a bridge between monocytes and endothelial cells during inflammation.

**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.

**Figure 6. Role of Junctional adhesion molecule A (JAM-A) on platelets in release and platelet–leukocyte interactions.**

Representative images of CXC-chemokine ligand 4 (CXCL4; A) and CC chemokine ligand 5 (CCL5; B) staining in platelets after 12 weeks (A) and 2 weeks high-fat diet (HFD; B). Scale bar, 20 μm. Chemokine levels of CXCL4 (C) and CCL5 (D) in platelet-poor plasma from trJAM-A⁺/⁺ apoe⁻/⁻ and trJAM-A⁻/⁻ apoe⁻/⁻ mice fed a HFD for 2, 6, 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 and F) and platelet–neutrophil (CD41⁺/Ly6G⁺; G and 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 post-test.
mouse genotype. A significantly increased endothelial cell surface coverage with platelets (Figure 7A and 7B) or leukocytes adherent on the platelets (Figure 7A and 7B) was observed in $trJAM-A^{-/-} apoe^{-/-}$ mice when compared with the control group. Treatment of the platelets with tirofiban before perfusion abolished the observed differences in platelet and leukocyte adhesion between $trJAM-A^{+/+} apoe^{-/-}$ and $trJAM-A^{-/-} apoe^{-/-}$ mice (Figure 7A and 7B).

To investigate the interaction of platelets with the atherosclerotic vessel wall in a more physiological approach, carotid arteries were explanted from $trJAM-A^{+/+} apoe^{-/-}$ and $trJAM-A^{-/-} apoe^{-/-}$ mice, fed a HFD for 2 weeks and subsequently perfused with whole blood taken from the same mice. When 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 $trJAM-A^{+/+} apoe^{-/-}$ and $trJAM-A^{-/-} apoe^{-/-}$ genotypes in this experimental set up (4.00±1.87 versus 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 (Figure 7C and 7D). 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 $trJAM-A^{-/-} apoe^{-/-}$ mice were significantly more pronounced than in $trJAM-A^{+/+} apoe^{-/-}$ mice (Figure 7E and 7F), whereas neutrophil adhesion in $trJAM-A^{-/-}$ mice showed a nonsignificant tendency to an increase (Figure 7E and 7G). Taken together, these results indicate that platelet hyperreactivity because of JAM-A deficiency is driven by a lack of negative integrin $\alpha_{IIb}\beta_3$ 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, and antiplatelet drugs are widely used in the after-care and prevention of individuals who had, 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 cardiovascular disease, by...
initiating endothelial dysfunction and propagating vascular inflammation, and through lower aggregation thresholds, aiding to the occurrence or recurrence of ischemic events because of vascular occlusion. Increased platelet reactivity accompanying resistance to antiplatelet therapy is known to pose an enhanced risk for cardiovascular disease in patients with, eg, diabetes mellitus or hypertension.\(^{26,39}\) 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 et al.\(^{21,22}\) Ablation of JAM-A led to shortened bleeding times and increased platelet aggregation in response to collagen, ADP, and protease-activated receptor-4 agonists, without leading to increased activation of \(\alpha_{\text{IIb}}\beta_3\) 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 \(\alpha_{\text{IIb}}\beta_3\) integrin (ie, expression of the JON/A epitope) was abundantly expressed on these shear-resistant platelet aggregates from both \(\text{trJAM-A}^-\text{apo}^-\) and \(\text{trJAM-A}^-\text{apo}^+\) mice, yet no differences between genotypes were observed. This seems to be in concordance with the previously postulated notion that JAM-A deficiency does not influence inside-out \(\alpha_{\text{IIb}}\beta_3\) integrin activation.\(^{22}\)

In the literature, several other adhesion molecules were shown to downregulate platelet function. For example, a direct relative of JAM-A, endothelial cell-selective adhesion molecule, is expressed on platelets and localized to platelet–platelet contacts in platelet aggregates. Deficiency of endothelial cell–specific adhesion molecule was associated with increased platelet aggregation and thrombus formation in laser-damaged arterioles.\(^{40}\) Similar observations were made for CD66a (carcinoembryonic antigen-related cell adhesion molecule 1), which was identified as a negative regulator of platelet–collagen interactions.\(^{41}\) In addition, CD31 (platelet endothelial cell adhesion molecule) was shown to be involved in regulating platelet aggregation because its crosslinking reduced agonist-induced platelet aggregation\(^ {42}\) and genetic deletion of CD31 in platelets, but not endothelial cells increased thrombus formation in vivo.\(^ {43}\)

A mechanistic model of how JAM-A can regulate integrin outside-in signaling was initially described by Naik et al,\(^ {21}\) who found that JAM-A is tyrosine-phosphorylated in resting platelets. Phosphorylation of JAM-A was also observed in other studies\(^ {9,14}\) and was hypothesized to be involved in regulating interaction of JAM-A with its intracellular binding partners.\(^ {41}\) In quiescent platelets, a complex exists between the endogenous c-Src inhibitor CSK and the \(\alpha_{\text{IIb}}\beta_3\) integrin–c-Src complex.\(^ {26}\) 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 \(\alpha_{\text{IIb}}\beta_3\)) in an inactive state. On engagement of \(\alpha_{\text{IIb}}\beta_3\) with its ligand fibrinogen, JAM-A is dephosphorylated causing CSK and JAM-A to dissociate from \(\alpha_{\text{IIb}}\beta_3\). This leads to the activation of c-Src by phosphorylation at Y418, propagating of \(\alpha_{\text{IIb}}\beta_3\) integrin outside-in signaling. In the present study, we show that JAM-A dephosphorylation is blocked by inhibiting the tyrosine phosphatase PTPN1, but not the PTPN6 and PTPN11, thereby adding a novel aspect to this model. In addition, both in resting and outside-in activated platelets, JAM-A seemed to be associated with PTPN1 and this phosphatase was previously shown to interact with integrin \(\alpha_{\text{IIb}}\beta_3.\(^ {27}\) Given the close proximity of JAM-A to the CSK/c-Src/integrin \(\alpha_{\text{IIb}}\beta_3\) complex and their association with PTPN1, it seems feasible that PTPN1 is the phosphatase responsible for the dephosphorylation of the substrates in this complex on 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 an increased \(\alpha_{\text{IIb}}\beta_3\) integrin signaling. Our experiments support this notion because 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 when compared with JAM-A wild-type platelets. It is known that outside-in signaling of \(\alpha_{\text{IIb}}\beta_3\) integrin serves as a feedback loop that amplifies initial platelet activation stimuli, such as ADP or thrombin, particularly in whole blood (ie, in the presence of fibrinogen).\(^ {45}\) The 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 \(\alpha_{\text{IIb}}\beta_3\) 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 \(\alpha_{\text{IIb}}\beta_3\) outside-in signaling leads to a reversal of the platelet hyperreactivity associated with deficiency of JAM-A.

In \(\text{trJAM-A}^-\text{apo}^-\) 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 HFD. Thus, we postulate that the decreased activation threshold of JAM-A–deficient platelets results in a continuous low-level release of \(\alpha\)-granule contents, leading to increased circulating chemokine concentrations. These chemokines might also be more effectively deposited onto the vessel walls of the \(\text{trJAM-A}^-\text{apo}^-\) mice, resulting in an 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, eg, during acute cardiovascular events.\(^ {35,36}\) Because platelets have been shown to recruit mononuclear cells to the vessel wall actively,\(^ {3,46,47}\) it is conceivable that increased platelet–leukocyte aggregation in \(\text{trJAM-A}^-\text{apo}^-\) mice might also contribute to plaque development. This might explain the increased content of T cells, macrophages, and the elevated proinflammatory cytokine expression levels, particularly in the lesions of \(\text{trJAM-A}^-\text{apo}^-\) mice at early time points. Previous studies have implicated the role of platelets and their secretion products particularly in early phases of plaque development.\(^ {3,12,48}\) Platelets might initiate endothelial inflammation through the action of cytokines, such as CD40L,\(^ {2,12}\) 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. 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 apoE−/− mice resulted in a pronounced reduction of plaque formation after 18 weeks. In addition, repeated infusion of activated platelets was shown to also exacerbate atherosclerosis after plaques had already been formed, and mice lacking the αIbβ2 subunit of integrin αmβ3 showed a significant reduction in lesion burden particularly after 12 weeks of HFD. In this respect, it is interesting that the effects of platelet-specific JAM-A deficiency declined at later time points during the HFD, particularly in the aortic root. Therefore, we hypothesize that platelet hyperreactivity, although 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 invade early stage lesions preferentially, whereas their influence declined at advanced phases of plaque development.

An interesting role in our study is reserved for integrin αIbβ2, which is the reported target of the (down-) regulation of platelet function by JAM-A. Inhibition of αIbβ2 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 αmβ3 in inflammatory platelet functions and the development of atherosclerosis. For example, the expression of CD40L was shown to be increased after engagement of αmβ3 by fibrinogen, and flow-resistant adhesion of platelets to the vessel wall in mice was significantly reduced after inhibition of αmβ3. Mice deficient in functional αmβ3 showed a reduction in atherosclerosis. However, care should be taken in translating these findings to humans because individuals with a genetic deficiency in αmβ3 can still develop atherosclerosis.

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 because of a decreased stimulation threshold, which could be reversed by the inhibition of αIbβ2.

Because activated platelets exacerbate atherosclerosis, hyperreactivity JAM-A-deficient platelets might both initiate and accelerate early plaque formation. Whereas other studies have addressed the role of platelets in atherosclerosis, 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 cardiovascular disease and adds another fundamental facet to the role of JAM-A in vascular disease.

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Disclosures

None.

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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, F11 receptor) 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 proinflammatory factors (e.g., bioactive lipids, cytokines, 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 CC chemokine ligand 5 and CXC chemokine ligand 4 were measured in the mice with platelet-specific deficiency of JAM-A. Furthermore, 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 molecule 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 hyperreactivity contributes to plaque development in mice and highlights the detrimental role of activated platelets in preclinical phases of cardiovascular disease.
Hyperreactivity of Junctional Adhesion Molecule A-Deficient Platelets Accelerates Atherosclerosis in Hyperlipidemic Mice


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SUPPLEMENTAL MATERIAL

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

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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 and were backcrossed in an apolipoprotein e (apoE)-deficient background (C57Bl/6) for at least 10 generations. These mice were crossed with JAM-A^floxflox^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. 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 (scil Animal Care). Human leukocytes and platelets from platelet poor plasma (PPP) were isolated from whole venous blood as described.

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 described\(^4\), 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 roots\(^5\). 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 described\(^4\). 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 $\alpha_{IIb}\beta_3$ 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\(^4,7\). 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 $\alpha_{IIb}\beta_3$ 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 CaCl2, 1 mmol/L Na3VO4, 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 αIIbβ3 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 described11, 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 1×10^6 leukocytes/mL and 1×10^8 platelets/mL. In some experiments, isolated platelets were pretreated with αIIbβ3 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 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). 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 0.8-1.1×10⁴ Pa. Whole blood was retro-orbitally taken from the mice and diluted with isotonic citrate buffer to adjust the platelet count to 1×10⁸ 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 α₁β₃ integrin antagonist tirofiban (Aggrastat®, MSD, 1 µg/mL) prior to perfusion. The first 500µm 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=240µm²; voxel size: 0.23 x 0.23 x 1µm³). Z-stacks were acquired at 0.1 Hz including two-fold frame averaging.

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. 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 0.8-1.1×10⁴ Pa. Previous studies have demonstrated that the vessel, including the endothelial and smooth muscle cells, remains intact. 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 v 7.0 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.
Online Figure I. JAM-A deletion on platelets and cell-specific JAM-A expression in trJAM-A−/− apoe−/− 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+/+ apoe−/− and trJAM-A−/− apoe−/− mice, as indicated. Immunocytochemical images of the carotid arteries from trJAM-A−/− apoe−/− and trJAM-A+/+ apoe−/− 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−/− apoe−/− and trJAM-A+/+ mice apoe−/− 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).
### 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 $10^9$ 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.

<table>
<thead>
<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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<tbody>
<tr>
<td>trJAM+/+ w/o HFD</td>
<td>386±148</td>
<td>6.57±0.35</td>
<td>5.08±0.85</td>
<td>5.25±0.60</td>
<td>0.225±0.03</td>
<td>1.63±0.23</td>
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<tr>
<td>trJAM–/– w/o HFD</td>
<td>499±157</td>
<td>6.00±0.17</td>
<td>5.50±0.15</td>
<td>5.73±0.07</td>
<td>0.303±0.03</td>
<td>1.47±0.15</td>
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<tr>
<td>p-value</td>
<td>0.63</td>
<td>0.23</td>
<td>0.22</td>
<td>0.38</td>
<td>0.09</td>
<td>0.62</td>
</tr>
<tr>
<td>trJAM+/+, 2w HFD</td>
<td>719±36.9</td>
<td>6.00±0.05</td>
<td>6.23±0.48</td>
<td>4.56±0.39</td>
<td>0.231±0.03</td>
<td>1.44±0.09</td>
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<tr>
<td>trJAM–/–, 2w HFD</td>
<td>766±64.9</td>
<td>5.97±0.06</td>
<td>6.18±0.38</td>
<td>4.47±0.25</td>
<td>0.241±0.03</td>
<td>1.48±0.19</td>
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<tr>
<td>p-value</td>
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<td>0.66</td>
<td>0.94</td>
<td>0.83</td>
<td>0.81</td>
<td>0.40</td>
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<td>trJAM+/+, 6w HFD</td>
<td>765±130</td>
<td>6.67±0.31</td>
<td>4.75±0.67</td>
<td>3.18±0.25</td>
<td>0.167±0.07</td>
<td>1.40±0.43</td>
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<td>trJAM–/–, 6w HFD</td>
<td>839±125</td>
<td>6.66±0.41</td>
<td>5.24±0.38</td>
<td>3.66±0.26</td>
<td>0.211±0.03</td>
<td>1.38±0.14</td>
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<td>p-value</td>
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<td>0.71</td>
<td>0.24</td>
<td>0.26</td>
<td>0.21</td>
<td>0.26</td>
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<tr>
<td>trJAM+/+, 12w HFD</td>
<td>947±124</td>
<td>6.76±0.17</td>
<td>4.84±0.47</td>
<td>3.03±0.33</td>
<td>0.193±0.03</td>
<td>1.62±0.02</td>
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<tr>
<td>trJAM–/–, 12w HFD</td>
<td>1286±146</td>
<td>6.45±0.13</td>
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<td>2.77±0.28</td>
<td>0.164±0.03</td>
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<tr>
<td>p-value</td>
<td>0.09</td>
<td>0.18</td>
<td>0.37</td>
<td>0.58</td>
<td>0.47</td>
<td>0.57</td>
</tr>
</tbody>
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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.

<table>
<thead>
<tr>
<th>genotype</th>
<th>cholesterol (mmol/L)</th>
<th>triglycerides (mmol/L)</th>
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</thead>
<tbody>
<tr>
<td>trJAM+/+ apoe–/–, 2w HFD</td>
<td>25.4 ± 3.41</td>
<td>1.85 ± 0.462</td>
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<tr>
<td>trJAM+/+ apoe–/–, 2w HFD</td>
<td>28.6 ± 3.26</td>
<td>1.59 ± 0.231</td>
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<tr>
<td>p-value</td>
<td>0.51</td>
<td>0.62</td>
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<tr>
<td>trJAM–/– apoe–/–, 6w HFD</td>
<td>23.7 ± 3.41</td>
<td>1.86 ± 0.356</td>
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<tr>
<td>trJAM–/– apoe–/–, 6w HFD</td>
<td>24.6 ± 4.16</td>
<td>2.08 ± 0.551</td>
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<tr>
<td>p-value</td>
<td>0.88</td>
<td>0.96</td>
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<tr>
<td>trJAM+/+ apoe–/–, 12w HFD</td>
<td>31.6 ± 3.13</td>
<td>2.01 ± 0.299</td>
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<tr>
<td>trJAM–/– apoe–/–, 12w HFD</td>
<td>34.9 ± 3.78</td>
<td>2.51 ± 0.344</td>
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<tr>
<td>p-value</td>
<td>0.51</td>
<td>0.29</td>
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</table>
Supplemental References


