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

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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 $\alpha_{IIb}\beta_3$-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 $\alpha_{IIb}\beta_3$ 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 $\alpha_{IIb}\beta_3$ 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.[1,2] Recent studies have highlighted platelets as key effectors in, eg, sepsis,[3] rheumatoid arthritis,[4] experimental autoimmune encephalomyelitis,[5] and host defense during bacterial infection.[6] In addition, platelets spark vascular inflammatory processes and adhere to atherosclerotic predilection sites preceding plaque formation.[7] Platelets may also form a bridge between leukocytes and the injured or inflamed vessel wall,[8] promoting the extravasation of monocytes and neutrophils[9] and the response to vascular injury.[10] Accordingly, infusion of activated platelets into hyperlipidemic mice accelerated atherosclerosis in a P-selectin– and cluster of differentiation (CD) 40 ligand (CD40L)–dependent manner.[11,12]

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Nonstandard Abbreviations and Acronyms

Apoe	apolipoprotein E
c-Src	cellular and sarcoma tyrosine-protein kinase
CCL5	CC chemokine ligand 5
CD	cluster of differentiation
CSK	c-terminal Src kinase
CVD	cardiovascular disease
CXCL4	CXC-chemokine ligand 4
HFD	high-fat diet
JAM-A	F11R, junctional adhesion molecule A, F11 Receptor
PF4	platelet factor 4
PTPN1	protein tyrosine phosphatase nonreceptor type 1

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.[13] On leukocytes, it mediates cell migration by regulating integrin de-adhesion.[14] On epi- and endothelial cells, JAM-A is a component of the tight junctions and regulates cell layer permeability through homophilic interactions.[15] On 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.[16] Recently, we demonstrated that JAM-A has a cell-type–specific effect on atherosclerotic plaque formation.[17] Whereas JAM-A on leukocytes protected against atherosclerosis, endothelial JAM-A promoted plaque formation by enhanced luminal availability under proatherosclerotic conditions, thus guiding monocytes to sites of plaque development.[17]

Although JAM-A was first identified in platelets,[18] its influence on platelet function remained poorly characterized. Earlier studies demonstrated phosphorylation of JAM-A on platelet activation[19] and its association with $\alpha_{IIb}\beta_3$ integrin,[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 cellular and sarcoma tyrosine-protein kinase (c-Src)–dependent outside-in signal transduction of $\alpha_{IIb}\beta_3$ integrin, through the recruitment of the Src inhibitory enzyme c-terminal Src kinase (CSK).[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.[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, proinflammatory phenotype, and thus to an acceleration of early phase plaque formation.

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 Hospital Basel,[23] and were backcrossed in an apolipoprotein e (*apoe*)–deficient background (C57Bl/6) backcrossed for ≥10 generations. These mice were crossed with *JAM-A*$^{flox/flox}$ *apoe*$^{-/-}$ mice[17] 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 a 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.[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-poor plasma as described.[12] 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 $\alpha_{IIb}\beta_3$ integrin antagonist tirofiban (Aggrastat; MSD, 1 µg/mL) or the Src kinase inhibitors PP2 (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[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.[12,25]

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.[26,27] 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 (dimethyl sulfoxide) 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.[28]

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-conjugated (AbDSerotec) and anti-CD41 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-CD41–FITC (BD), anti-CD45–eFluor

450, anti-CD115–phycoerythrin-Cy7, and anti–lymphocyte antigen 6G –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 version 10 software (Tree Star Inc).

Platelet and Leukocyte Recruitment Assays

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 1×10^6 leukocytes/mL and 1×10^8 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 $CaCl_2$ 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 minutes, followed by leukocytes for 5 minutes and additional washing of nonadherent cells (all at 0.15 N/m^2).[29] 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*$^{+/+}$ *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 to 1.1×10^4 Pa. Previous studies have demonstrated that the vessel, including the endothelial and smooth muscle cells, remains intact.[17,30] Whole blood was diluted with isotonic citrate buffer to adjust the platelet count to 1×10^8 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.[17]

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

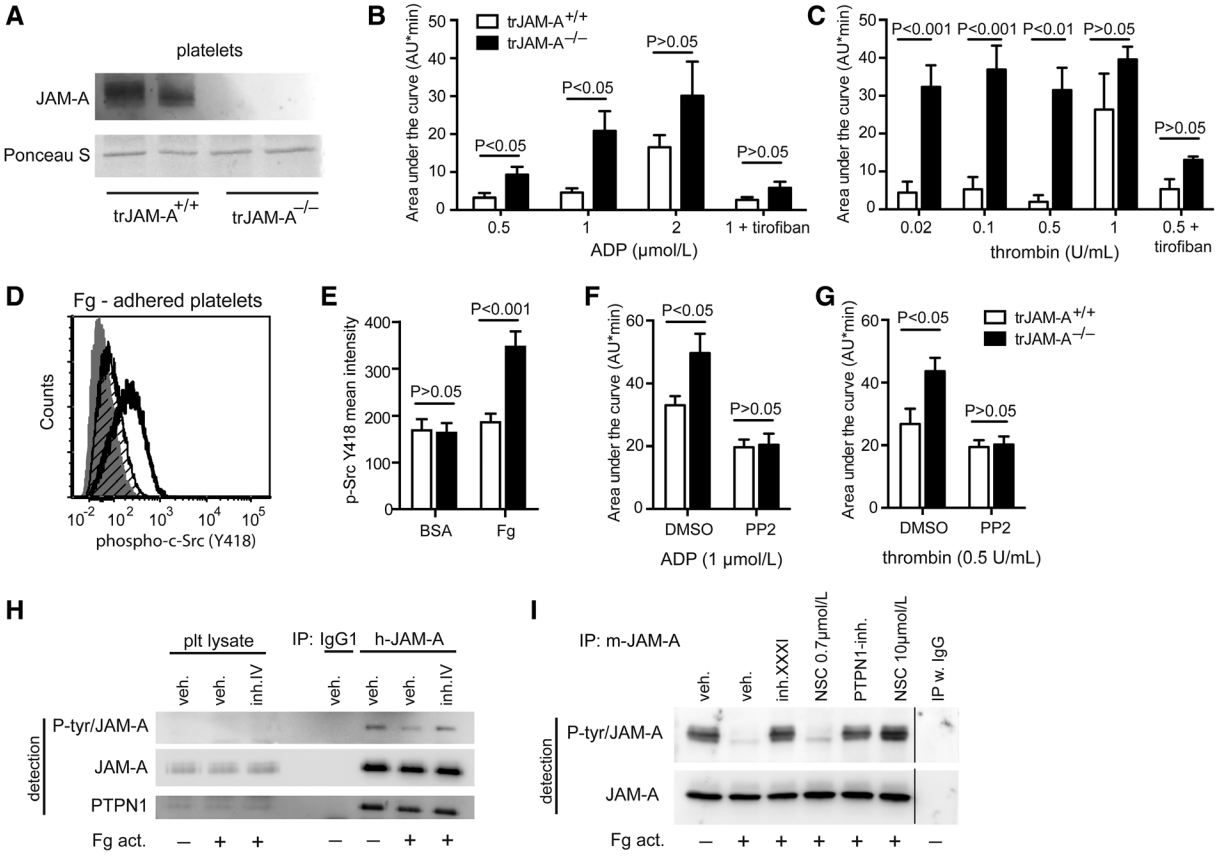

Figure 1. Hyperreactivity of platelets in platelet-specific junctional adhesion molecule A (*trJAM-A*)$^{-/-}$ mice. Absence of JAM-A on platelets was confirmed by Western blotting. Ponceau S served as a loading control (**A**). Aggregation of *trJAM-A*$^{+/+}$ (open bars) and *trJAM-A*$^{-/-}$ (solid 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 post-test. Platelets were adhered to fibrinogen (Fg) or to heat-treated BSA for 90 minutes and stained for phosphorylated cellular and sarcoma tyrosine-protein kinase (c-Src) at tyrosine 418. (**D**) Representative flow cytometry histogram of phospho-c-Src staining in Fg-adhered platelets (gray 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 is shown (**E**). Quantified aggregation of *trJAM*$^{+/+}$ and *trJAM-A*$^{-/-}$ platelets treated with dimethyl sulfoxide (DMSO) or the Src inhibitor PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; 20 µmol/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 post-test. Representative immunoblots of immunoprecipitated JAM-A from human (**H**) or mouse (**I**) platelets (plt) adhered to BSA or Fg in the absence or presence of protein tyrosine phosphatase (PTP) inhibitors or vehicle, detected using antiphosphotyrosine (P-tyr; **H** and **I**, **top**), anti-JAM-A (**H**, **middle**; **I**, **bottom**), or anti-PTP nonreceptor type 1 (PTPN1; **H**, **bottom**) antibodies.

interactions along the atherosclerotic carotid artery as described.[31] 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 ≥3×.

Results

Deficiency in Platelet JAM-A Results in Hyperreactivity

Previous studies have shown that genetic deletion of *JAM-A* results in platelet hyperreactivity,[21,22] 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,[21,22] 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 $\alpha_{IIb}\beta_3$ outside-in signaling is the reported target of JAM-A,[21,22] the $\alpha_{IIb}\beta_3$ 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*$^{+/+}$ platelets (Figure 1B and 1C).

Outside-in signaling by $\alpha_{IIb}\beta_3$ is controlled by c-Src kinase.[22,26] 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 $\alpha_{IIb}\beta_3$ outside-in signaling) with the inhibitors PP2, SU6656, or KB Src 4 abrogated the increased agonist-induced aggregation of JAM-A–deficient platelets (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 $\alpha_{IIb}\beta_3$. On ligation of $\alpha_{IIb}\beta_3$, JAM-A is dephosphorylated allowing the dissociation of CSK from the JAM-A/c-Src/integrin $\alpha_{IIb}\beta_3$complex.[22] 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 1I). Dephosphorylation of tyrosine residues is performed by PTPs. To date, 20 PTPs are known in platelets and regulate signaling events during platelet activation.[32] Addition of broad-spectrum PTP inhibitors (IV and XXXI) to fibrinogen-adhered platelets resulted in a decreased dephosphorylation of JAM-A (Figure 1H and 1I). A previous study described an association of PTP nonreceptor type 1 (PTPN1 [PTP1B]) with the c-Src/integrin $\alpha_{IIb}\beta_3$ complex and identified PTPN1 as a positive regulator of platelet outside-in signaling.[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 coprecipitation of PTPN1 with JAM-A both in resting and outside-in activated human platelets (Figure 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 when compared with vehicle (Figure 1I). 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 1I). 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 1I). These findings indicate that the dephosphorylation of JAM-A in platelets is mediated by PTPN1.

Taken together, our observations and those of others[21,22] indicate that JAM-A is an integral member of the CSK/c-Src/integrin $\alpha_{IIb}\beta_3$ complex and a negative regulator of platelet $\alpha_{IIb}\beta_3$ 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,[21] 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 (Figure 2A, 2D, and 2G). In addition, poststaining with the JON/A antibody, specific for the activated conformation of $\alpha_{IIb}\beta_3$ 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

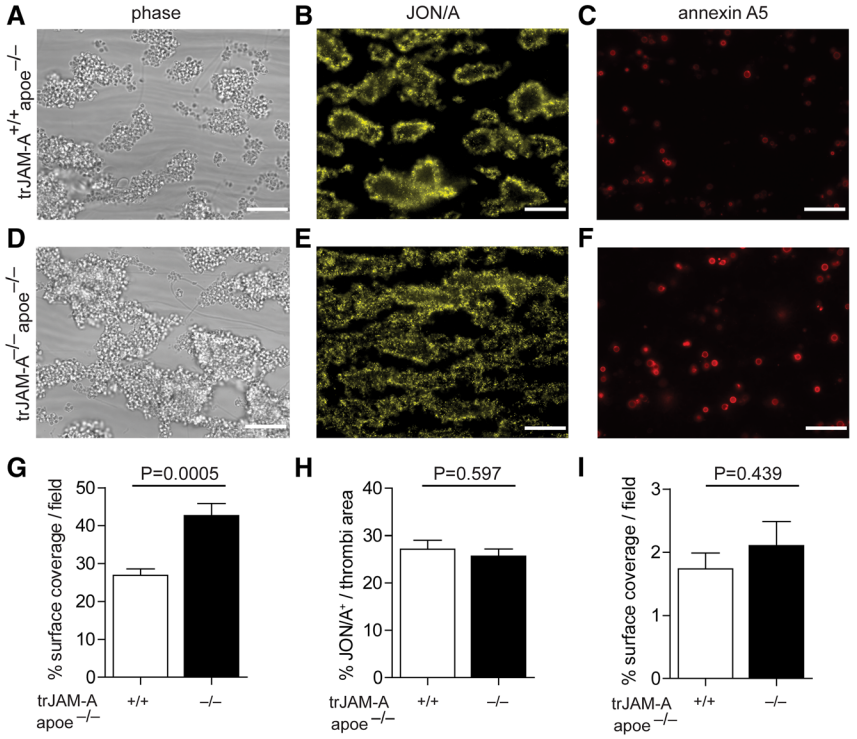

Figure 2. Platelet aggregation on collagen under flow conditions. Whole blood from platelet-specific junctional adhesion molecule A (*trJAM-A*)$^{+/+}$ apolipoprotein e (*apoe*)$^{-/-}$ (**A**–**C**) and *trJAM-A*$^{-/-}$ *apoe*$^{-/-}$ (**D**–**F**) mice was perfused over collagen type I and platelet 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**). The expression of activated $\alpha_{IIb}\beta_3$ 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.

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.

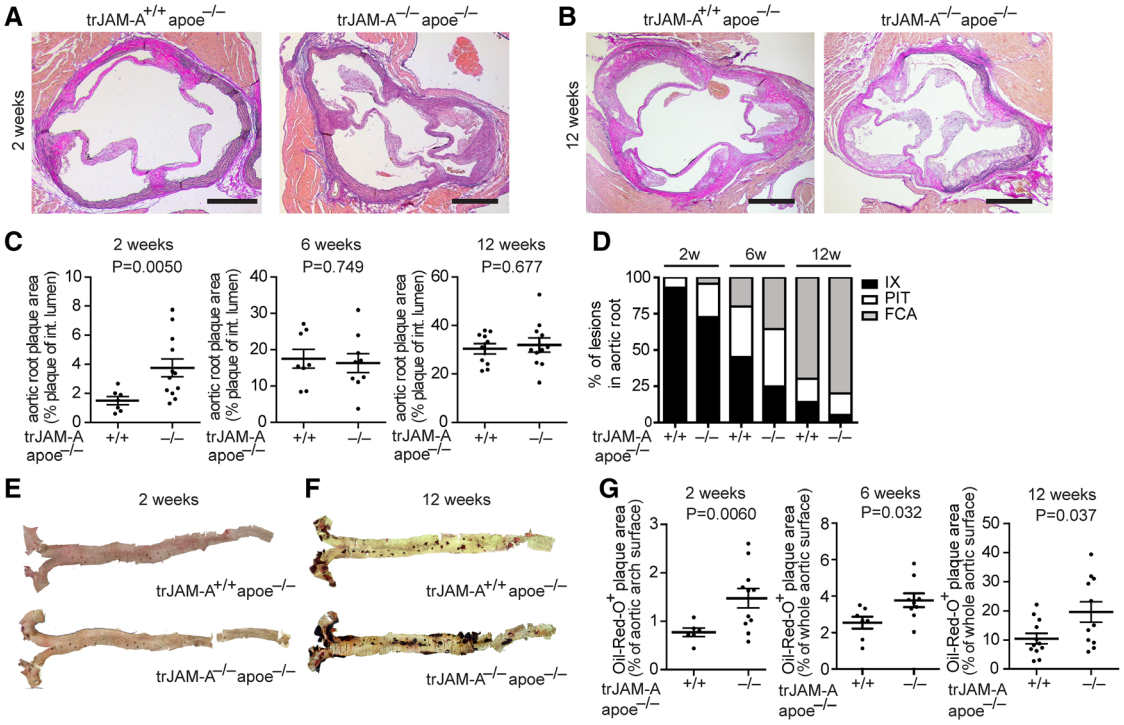

Figure 3. Specific role of junctional adhesion molecule A **(JAM-A) on platelets during atherogenesis.** Platelet-specific JAM-A (*trJAM-A*)$^{+/+}$ apolipoprotein e (*apoe*)$^{-/-}$ and *trJAM-A*$^{-/-}$ *apoe*$^{-/-}$ mice were fed a high-fat diet (HFD) for 2 weeks (**A**, **C**, **E**, and **G**), 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). At an intermediate time point (6-week HFD), whole-aortic plaque area was still significantly increased in *trJAM-A*$^{-/-}$ *apoe*$^{-/-}$ mice when compared with controls (Figure 3G), whereas an increased plaque area was no longer observed in the aortic roots (Figure 3C). Similar results were obtained after 12-week HFD, where a lack of JAM-A on platelets led to an increased lesional area only in the whole aorta (Figure 3F and 3G) but not in the aortic root (Figure 3B and 3C). 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 as intimal xanthoma, representing early stages, pathological intimal thickening, and fibrous cap atheroma, the latter representing more advanced atherosclerosis stages in plaques of the aortic roots[24] (Figure 3D). No differences in plaque formation were found in *apoe*$^{-/-}$ mice carrying only the PF4-Cre transgene when 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 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).

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

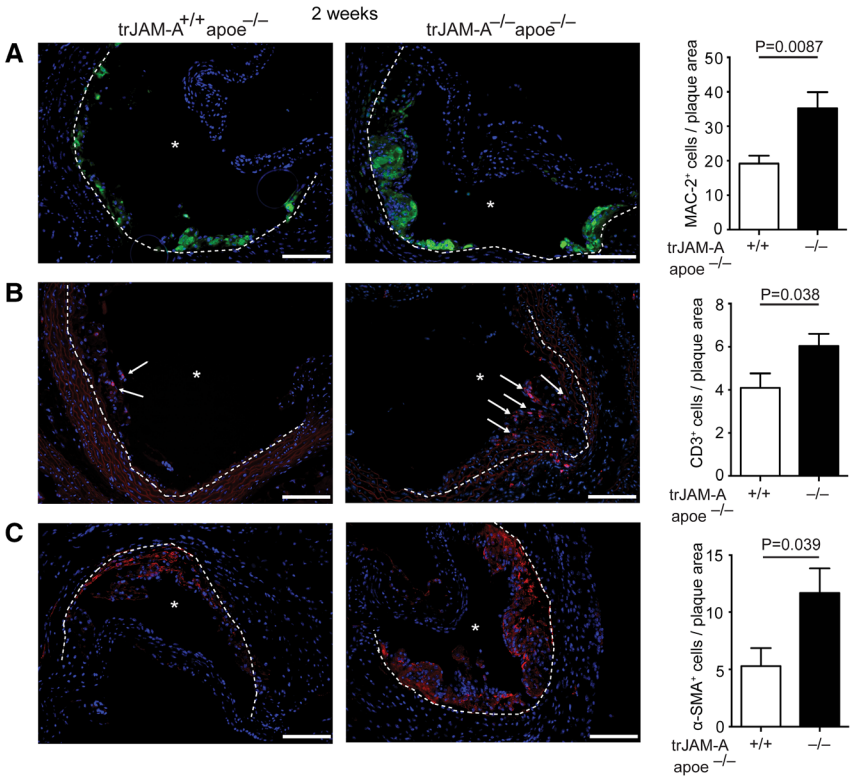

Figure 4. Atherosclerotic lesion phenotype in platelet-specific junctional adhesion molecule A *(trJAM-A)*$^{-/-}$ apolipoprotein e *(apoe)*$^{-/-}$ mice at an early stage of atherosclerosis. Representative pictures and quantifications of macrophage antigen (MAC)-2 (**A**), cluster of differentiation (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.

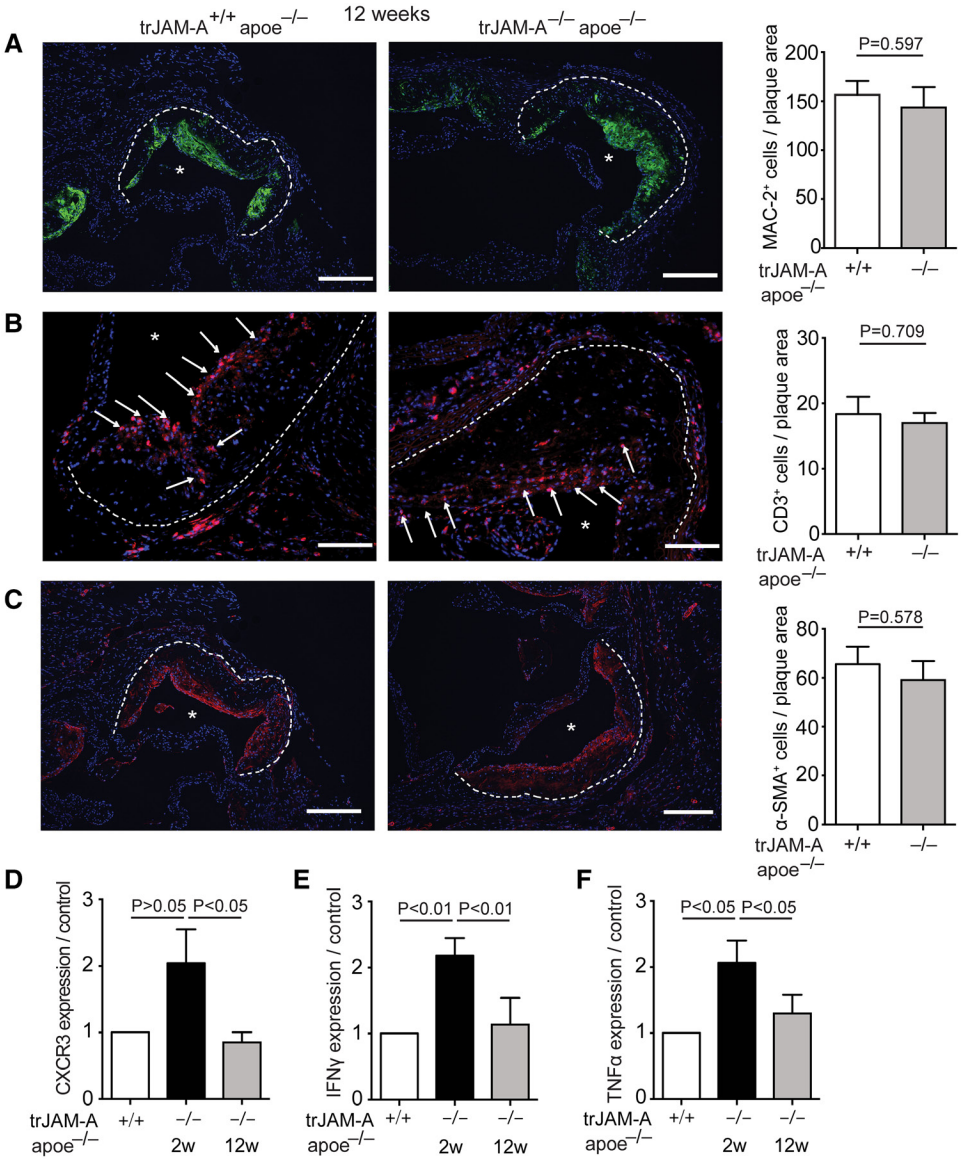

Figure 5. Atherosclerotic lesion phenotype in platelet-specific junctional adhesion molecule A (*trJAM-A*)$^{-/-}$ apolipoprotein e (*apoe*)$^{-/-}$ mice at an advanced stage of atherosclerosis. Representative pictures and quantifications of macrophage antigen (MAC)-2 (**A**), cluster of differentiation (CD) 3 (**B**), and α-smooth muscle cell (SMA; **C**)–stained aortic roots from *trJAM-A*$^{+/+}$ *apoe*$^{-/-}$ and *trJAM-A*$^{-/-}$ *apoe*$^{-/-}$ mice 12 weeks after high-fat diet (HFD). 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, 200 μm (**A** and **C**) and 100 μm (**B**). Relative mRNA expression of CXCR3 (**D**), interferon-γ (IFNγ; **E**) and tumor necrosis factor (TNF)-α (**F**) in whole aortae from *trJAM-A*$^{+/+}$ *apoe*$^{-/-}$ mice and *trJAM-A*$^{-/-}$ *apoe*$^{-/-}$ mice after 2 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 *t* test (**A**–**C**) or 1-way ANOVA with Tukey post-test (**D**–**F**).

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, eg, 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.[33,34] 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

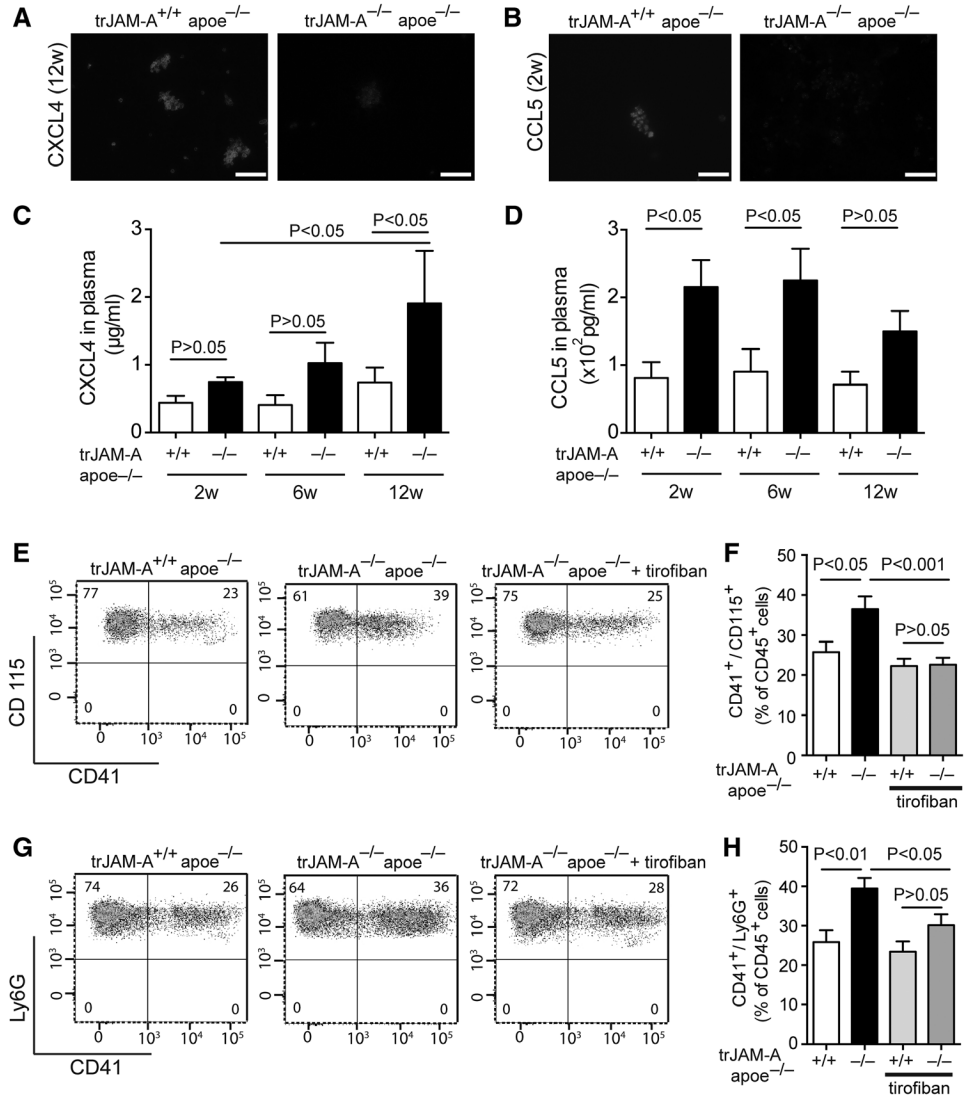

Figure 6. Role of Junctional adhesion molecule A (JAM-A) on platelets in release and platelet–leukocyte interactions. Representative images of CXC-chemokine ligand 4 (CXCL4; **A**) and CC chemokine ligand 5 (CCL5; **B**) staining in platelets after 12 weeks (**A**) and 2 weeks high-fat diet (HFD; **B**). 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.

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,[11,29] which might be relevant during acute coronary events.[35,36] To investigate whether JAM-A deficiency leads to increased interactions with leukocytes, activated platelets isolated from *trJAM-A*$^{-/-}$ *apoe*$^{-/-}$ mice were incubated with leukocytes. Indeed, JAM-A–deficient platelets showed significantly more platelet–monocyte (Figure 6E and 6F) and platelet–neutrophil (Figure 6G and 6H) aggregation than platelets from *trJAM-A*$^{+/+}$ *apoe*$^{-/-}$ mice, suggesting that JAM-A–deficient platelets might more effectively augment monocyte recruitment to sites of endothelial inflammation. Interestingly, blockade of integrin $\alpha_{IIb}\beta_3$ reverted the increased interaction of JAM-A–deficient platelets with monocytes to that observed for JAM-A–positive platelets (Figure 6E and 6F) and also significantly reduced complex formation between platelets and neutrophils, albeit to a lesser extent (Figure 6G and 6H).

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.[8] 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*$^{+/+}$ *apoe*$^{-/-}$ and *trJAM-A*$^{-/-}$ *apoe*$^{-/-}$ mice were perfused over mouse endothelial monolayers challenged with tumor necrosis factor-α, followed by perfusion of activated leukocytes from the same

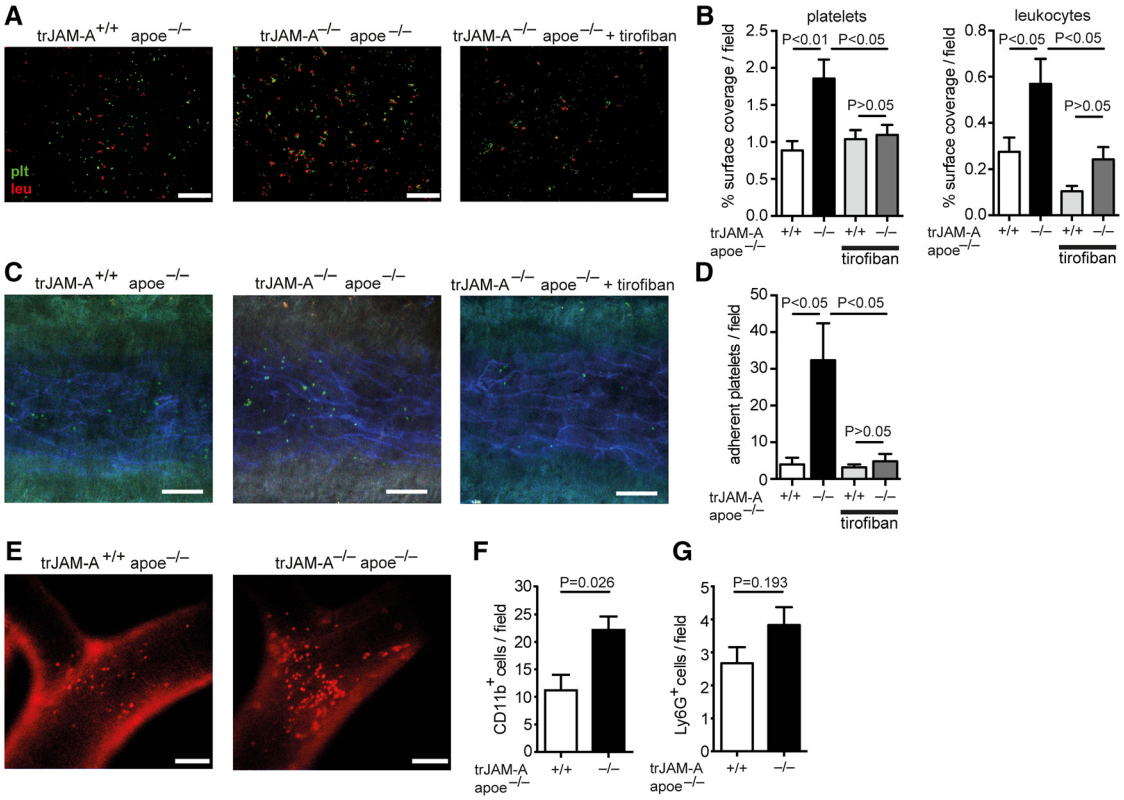

Figure 7. Role of Junctional adhesion molecule A (JAM-A) on platelets in leukocyte recruitment. Representative images display adherent platelets (plt, green) and leukocytes (leu, red) in vitro (**A**). Adherent CD41+ platelets (**B**, **left**) and CD45+ leukocytes (**B**, **right**) in the absence or presence of tirofiban (1 μg/mL) were analyzed on tumor necrosis factor (TNF)-α–activated mECs (mouse endothelial cells) as % of cell surface coverage per visual field in platelet-specific JAM-A (*trJAM-A*)$^{+/+}$ apolipoprotein e (*apoe*)$^{-/-}$ and *trJAM-A*$^{-/-}$ *apoe*$^{-/-}$ mice. Whole blood from *trJAM-A*$^{+/+}$ *apoe*$^{-/-}$ and *trJAM-A*$^{-/-}$ *apoe*$^{-/-}$ mice was perfused through prestained (CD31, blue) carotid arteries of the same mice fed a high-fat diet 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 described[17] (**D**). Representative images and quantification of firm arrest of CD11b+ monocytes (**E**, **F**), and Ly6G+ neutrophils (**G**) 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 post-test (**B**), Kruskal–Wallis test with Dunn post-test (**D**) or Mann–Whitney test (**F** and **G**).

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.[37] 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.[1,2] 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.[38,39] 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 $\alpha_{IIb}\beta_3$ 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 $\alpha_{IIb}\beta_3$ integrin (ie, expression of the JON/A epitope) was abundantly expressed on these shear-resistant platelet aggregates from both *trJAM-A*$^{-/-}$ *apoe*$^{-/-}$ and *trJAM-A*$^{+/+}$ *apoe*$^{-/-}$ 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 $\alpha_{IIb}\beta_3$ 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,[22] who found that JAM-A is tyrosine-phosphorylated in resting platelets. Phosphorylation of JAM-A was also observed in other studies[19,44] and was hypothesized to be involved in regulating interaction of JAM-A with its intracellular binding partners.[44] In quiescent platelets, a complex exists between the endogenous c-Src inhibitor CSK and the $\alpha_{IIb}\beta_3$ 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 $\alpha_{IIb}\beta_3$) in an inactive state. On engagement of $\alpha_{IIb}\beta_3$ with its ligand fibrinogen, JAM-A is dephosphorylated causing CSK and JAM-A to dissociate from $\alpha_{IIb}\beta_3$. This leads to the activation of c-Src by phosphorylation at Y418, propagating $\alpha_{IIb}\beta_3$ 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 $\alpha_{IIb}\beta_3$.[27] Given the close proximity of JAM-A to the CSK/c-Src/integrin $\alpha_{IIb}\beta_3$ 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 $\alpha_{IIb}\beta_3$ 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 $\alpha_{IIb}\beta_3$ 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 $\alpha_{IIb}\beta_3$ 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 $\alpha_{IIb}\beta_3$ 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 HFD. 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 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,[8,46,47] 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 proinflammatory cytokine expression levels, particularly in the lesions of *trJAM-A*$^{-/-}$ *apoe*$^{-/-}$ mice at early time points. Previous studies have implicated the role of platelets and their secretion products particularly in early phases of plaque development.[7,12,48] 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.[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 *apoe*$^{-/-}$ mice resulted in a pronounced reduction of plaque formation after 18 weeks.[7] In addition, repeated infusion of activated platelets was shown to also exacerbate atherosclerosis after plaques had already been formed[12], and mice lacking the α_{IIb} subunit of integrin $\alpha_{IIb}\beta_3$ showed a significant reduction in lesion burden particularly after 12 weeks of HFD.[49] 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.[48]

An interesting role in our study is reserved for integrin $\alpha_{IIb}\beta_3$, which is the reported target of the (down-) regulation of platelet function by JAM-A. Inhibition of $\alpha_{IIb}\beta_3$ 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 $\alpha_{IIb}\beta_3$ in inflammatory platelet functions and the development of atherosclerosis.[7,49,50] For example, the expression of CD40L was shown to be increased after engagement of $\alpha_{IIb}\beta_3$ by fibrinogen[50], and flow-resistant adhesion of platelets to the vessel wall in mice was significantly reduced after inhibition of $\alpha_{IIb}\beta_3$.[7] Mice deficient in functional $\alpha_{IIb}\beta_3$ showed a reduction in atherosclerosis.[49] However, care should be taken in translating these findings to humans because individuals with a genetic deficiency in $\alpha_{IIb}\beta_3$ can still develop atherosclerosis.[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 because of a decreased stimulation threshold, which could be reversed by the inhibition of $\alpha_{IIb}\beta_3$.

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,[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 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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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, 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 (eg, 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 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 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 hyperreactivity contributes to plaque development in mice and highlights the detrimental role of activated platelets in preclinical phases of cardiovascular disease.