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Genetic deletion of platelet Glycoprotein Ib alpha but not its extracellular domain protects from atherosclerosis

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Summary

The pathogenesis of atherosclerosis involves the interplay of hematopoietic, stromal and endothelial cells. Platelet interactions with endothelium and leukocytes are pivotal for atherosclerosis promotion. Glycoprotein (GP) Iba is the ligand-binding subunit of the platelet GPIb-IX-V receptor complex; its deficiency causes the Bernard-Soulier syndrome (BSS), characterized by absent platelet GPIb-IX-V, macrothrombocytopenia and bleeding. We designed this study to determine the role of platelet GPIba in the pathogenesis of atherosclerosis using two unique knockout models. *Ldlr*^{-/-} mice were reconstituted with wild type (wt), *GPIba*^{-/-} (lacks GPIba) or chimeric IL-4R/*GPIba*-Tg (lacks GPIba extracellular domain) bone marrow and assayed for atherosclerosis development after feeding with pro-atherogenic “western diet”.

Here, we report that *Ldlr*^{-/-} mice reconstituted with *GPIba*^{-/-} bone marrow developed less atherosclerosis compared to wt controls; accompanied by augmented accumulation of pro-inflammatory CD11b⁺ and CD11c⁺ myeloid cells, reduced oxLDL uptake and decreased TNF and IL12p35 gene expression in the aortas. Flow cytometry and live cell imaging in whole blood-perfused microfluidic chambers revealed reduced platelet-monocyte aggregates in *GPIba*^{-/-} mice, which resulted in decreased monocyte activation. Interestingly, *Ldlr*^{-/-} mice reconstituted with IL-4R/*GPIba*-Tg bone marrow, producing less abnormal platelets, showed atherosclerotic lesions similar to wt mice. Platelet interaction with blood monocytes and accumulation of myeloid cells in

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Disclosures

The authors have no conflicting financial interests.

the aortas was also essentially unaltered. Moreover, only complete *GPIb α* ablation altered platelet microparticles and CCL5 chemokine production.

Thus, atherosclerosis reduction in mice lacking GPIb α may not result from the defective GPIb α -ligand binding, but more likely is a consequence of functional defects of GPIb $\alpha^{-/-}$ platelets and reduced blood platelet counts.

Keywords

aorta; atherosclerosis; inflammation; myeloid cells; platelets

Introduction

The pathogenesis of atherosclerosis involves deregulated lipid metabolism and multiple immune and stromal cell interactions in developing plaques. Platelets contribute to atherosclerosis development(1-4) and have been shown to adhere to endothelium at sites of future plaque formation(5). Platelets are also a key determinant of thrombo-embolic complications when erosion and rupture of advanced lesions promote platelet activation, aggregation and thrombus formation(6). Aberrantly activated platelets have been found in the blood of atherosclerotic mice(2) and patients(7).

Platelets may contribute to atherosclerosis development via production of cytokines and chemokines(1, 3). During adhesion, activated platelets release IL-1 β , CCL5 (RANTES) and CXCL4 (PF4), and up-regulate surface expression of the TNF superfamily member CD40L(8, 9). Platelet-derived CCL5 and CXCL4 mediate monocyte activation(2), and CCL5-CXCL4 heterodimers deposited on endothelial cells promote monocyte arrest(1). Binding of activated platelets to monocytes and neutrophils in the blood may induce activation of latter and their recruitment to the inflamed aortas. Platelet adhesion triggers NF- κ B activation and increases inflammatory gene and adhesion molecule (such as VCAM-1) expression also in endothelial cells; events that precede atherosclerotic plaque invasion by leukocytes(10-13). In addition, endothelial cell-platelet interactions trigger secretion of CCL2 (MCP1), a key monocyte chemoattractant. Altogether, these platelet-mediated events promote leukocyte recruitment and attachment, events that are crucial for atherosclerosis development (11-13).

Platelet interactions with endothelial cells and leukocytes involve several cell surface adhesive receptors and counter-ligands. Among them, P-selectin and its binding partner, PSGL-1, promote platelet adhesion to endothelial cells in atherosclerosis (14-19). The platelet glycoprotein (GP) Ib, a two-subunit (α and β chains) component of the GPIb-IX-V receptor complex (20, 21), binds P-selectin(22) and also contributes to the interaction of platelets with myeloid cells expressing CD11b/CD18 (α M β 2, Mac-1) (23-25). In a vascular injury model, blocking GPIb α -Mac1 interaction prevents myeloid cell adhesion and, therefore, leukocyte accumulation in the vessel wall (24). The concurrent expression of three distinct gene products [GPIb α (CD42b), GPIb β (CD42c) and GPIX (CD42a)] is required for the presence of GPIb-IX-V on platelet membrane (26-29). GPIb-IX-V deficiency causes a bleeding disorder known as Bernard-Soulier syndrome (BSS), characterized by decreased

number of dysfunctional “giant” platelets in blood (26). Relevant to the thrombotic complications of atherosclerosis, GPIb is also the receptor for von Willebrand factor (vWF), necessary to initiate platelet adhesion and aggregation in high shear arterial flow (23-25).

At present, the role that GPIba may play in atherosclerosis remains incompletely understood. Studies with an anti-GPIba antibody showed a significant reduction of plaque size and marked decrease of CD11b and CD14 mRNA expression in the aortas(5). However, injection of anti-GPIba antibodies causes severe thrombocytopenia(30, 31), which is atheroprotective by itself, and therefore complicates the discrimination between GPIba–dependent and independent platelet contribution to atherosclerosis.

In this study, we used an established bone marrow transfer approach(32, 33) to transplant atherosclerosis-prone *Ldlr*^{−/−} mice with either *GPIba*^{−/−} or IL4R/GPIba transgenic (Tg) bone marrow. In the latter case, platelets express a normal number of GPIb-IX-V complexes where endogenous GPIba extracytoplasmic ligand-binding domain is replaced by human IL-4 receptor α -chain (34). *IL4R/GPIba*-Tg $\rightarrow$ *Ldlr*^{−/−} mice still retained the BSS bleeding phenotype, but the blood platelet count and platelet volume was closer to normal (34). Atherosclerotic lesion sizes, number of myeloid cells and their activation in the aortic wall were markedly reduced in *Ldlr*^{−/−} mice with *GPIba* platelets, but similar to control in *Ldlr*^{−/−} mice with *IL4R/GPIba*-Tg platelets. We conclude that GPIba ligand-binding functions are not directly involved in proatherogenic effects of platelets. Rather, altered platelets functions in the absence of GPIba cytoplasmic domain and overall decreased blood platelet counts likely curb myeloid cell recruitment and activation in the aorta and reduce inflammation.

Materials and methods

Mice

C57Bl/6J (B6), DsRed, and *Ldlr*^{−/−} mice were obtained from Jackson Labs (Bar Harbor, ME) and bred in an SPF-barrier AAALAC-approved facility at the La Jolla Institute for Allergy and Immunology (LIAI). *GPIba*^{−/−} and *IL4R/GPIba*-Tg mice have been previously described(27, 34). All mice were on C57Bl/6J background. For bone marrow transfer experiments, 6-8 wks old *Ldlr*^{−/−} mice were irradiated twice in a day for a total whole body dose of 1100 rad. Reconstitution efficiency assessed by GPIba and CD41 (α IIB β 3) expression on platelets was 97 $\pm$ 2%. Four wks after reconstitution, female and male littermate *Ldlr*^{−/−} mice were fed western diet (WD) (Harlan) for 16 wks. Animal experiments were approved by the Animal Care Committee at LIAI. All mice were genotyped using standard PCR protocols.

Quantification of atherosclerosis, histological analysis and immunostaining

Quantification of atherosclerosis lesion sizes in whole aortas and aortic roots as well as immunostaining of cut frozen sections were performed as previously described (33).

Aortic single cell preparations for flow cytometry

Staining of cells isolated from aortas was done as described before(35). Cell suspensions were stained with CD41-FITC (MW Reg30) (BD Pharmingen), Ly6G-AF647 (Biolegend), Ly6C-PerCP-Cy5.5 (HK1.4) (eBiosciences) and previously described antibodies (35).

Gene expression

For gene expression analysis, aortas were isolated and snap frozen in liquid nitrogen. Platelets for residual mRNA analysis were isolated from whole blood obtained from the retro-orbital plexus using heparin as anticoagulant, followed by addition of Sodium Citrate (ACD) (1 part for 5 parts of blood). Blood was diluted with equal amount of Tyrode Buffer (135 mM sodium chloride, 2.9 mM potassium chloride, 4.2 mM sodium phosphate monobasic, 11.9 mM sodium hydrogen carbonate, 10 mM Hepes, 5.5 mM dextrose) pH 6.5, and platelet-rich plasma (PRP) was prepared by centrifugation at 1000 rpm for 10 min. Obtained PRP was separated from the remaining blood, which was additionally treated with ACD and Tyrode's buffer following by centrifugation. A platelet pellet was obtained by centrifuging PRP at 2800 rpm for 8 min. Aortas and platelet pellets were homogenized in RLT buffer (Qiagen, Germantown, MD) and RNA was isolated using the RNeasy-Plus kit (Qiagen) followed by cDNA synthesis using the Superscript II with random priming (Invitrogen). Gene expression was analyzed by SybrGreen real-time PCR using primers for ribosomal L32, β -actin, TNF, IL12p35, Arg1, CCL5, CXCL4, CXCL7, CXCL12, IL1 β and TGF β . Sequences of the primers were obtained from NIH QPrimerDepot (<http://mouseprimerdepot.nci.nih.gov>). Reactions were performed in triplicate for each gene and gene expression was normalized to L32 or β -actin expression.

LDL uptake and foam cell formation *in vivo*

To assay oxidized LDL (oxLDL) uptake by myeloid cells mice were injected with 50 μ g of Dil-oxLDL intravenously (i.v.). After 24 hrs, mice were sacrificed and Dil-oxLDL uptake was analyzed in the aortas and blood monocytes by flow cytometry.

Epifluorescence microscopy of platelet-myeloid cell interactions

Mixed bone marrow chimeric *Ldlr*^{-/-} mice reconstituted with DsRed and *GPIIb*^{-/-} bone marrow at 50 to 50 percent ratio were used for the experiment after feeding with WD for 16 wks. Experiments were done using the auto-perfused microfluidic flow-chamber assay as described previously (36, 37). The interaction of platelets with rolling monocytes was recorded using dual-color simultaneous epifluorescence microscopy (38). The trans-illumination bright field lamp was kept on at a low power setting in all experiments to visualize non-fluorescent *GPIIb*^{-/-} platelets. The leukocyte population that rolls on P-selectin both *in vitro* and *in vivo* is mostly composed of monocytes and neutrophils (39, 40). Since integrin α 4 β 1 (CD49d/CD29 (VLA-4)) is expressed abundantly on monocytes (41) but minimally on mouse neutrophils(42), 30 μ g of DyLight488-conjugated-anti-mouse CD49d (α 4) (PS/2) was injected via the catheter connected to the right carotid artery to stain all monocytes yellow (DyLight 488(green)+DsRed (red)) while unstained neutrophils remained red (DsRed) (Fig. S4). Number of rolling neutrophils or monocytes interacting

with platelets was plotted as a fraction of the total number of rolling neutrophils or monocytes over 5-10 field of views.

Analysis of platelet-derived microparticles

Blood from *GPIIb α ^{-/-} → Ldlr^{-/-}*, *IL4R/GPIIb α -Tg → Ldlr^{-/-}* or wt → *Ldlr^{-/-}* mice, or from *GPIIb α ^{-/-}*, *IL4R/GPIIb α -Tg* or wt healthy mice was collected from the retro-orbital plexus into sodium citrate solution. Whole blood was diluted 1:100 in Tyrode Buffer (pH 7.4) followed by the addition of monoclonal antibodies against mouse α IIb β 3 (anti-mCD41, clone MWReg30, Biolegend, San Diego CA) and mGPIIb α (for C57BL/6J; clone 5A7, raised at TSRI) or hIL4 receptor (for IL-4R; anti hCD124, clone HIL4R-M57). After 15 minutes incubation, blood was further diluted 1:10 and platelets and platelet-derived microparticles (PMP) were counted by flow cytometry. Standard beads with a 1 μ m diameter were used to determine FSC size for microparticles gating. Particles with diameter smaller than 1 μ m were considered microparticles. Microparticles double positive for mouse α 2b β III (all strain) and mGPIIb α (for C57BL/6J chimera) or hIL-4 receptor (for IL4R/GPIIb α -Tg chimera) or positive only to mouse α IIb β 3 (for *GPIIb α ^{-/-}* chimera) were considered platelet-derived.

Statistical analysis

Data were analyzed using Prism software (GraphPad, San Diego, CA). Student's two-tailed t-test, Mann-Whitney U-test and one-way ANOVA test were used to compare conditions. Wilcoxon signed-rank test was used to compare fold induction of gene expression by real-time PCR. Data are expressed as mean $\pm$ SEM. *p < 0.05, **p < 0.001.

Results

Reduced atherosclerosis in mice with Bernard-Soulier syndrome

To elucidate the role of GPIIb α versus its ligand-binding extracellular domain in a mouse model of atherosclerosis, we transplanted bone marrow of *GPIIb α ^{-/-}*, *IL4R/GPIIb α -Tg* or wild type (wt) mice into *Ldlr^{-/-}* mice (*GPIIb α ^{-/-} → Ldlr^{-/-}*, *IL4R/GPIIb α -Tg → Ldlr^{-/-}* and wt → *Ldlr^{-/-}*, respectively). Four wks after bone marrow transplantation, mice were placed on a high fat western diet (WD) for 16 wks. The three groups showed no difference in body weight, total HDL cholesterol, triglycerides (Fig. S1A, B) and blood leukocyte counts (data not shown).

A reduction in plaque burden was evident in the lesser curvature of the aorta, innominate artery and origins of the right carotid and subclavian arteries of *GPIIb α ^{-/-} → Ldlr^{-/-}* (Fig. 1A). Microscopic analysis of atherosclerotic lesions in the aortic roots revealed a reduction of atherosclerosis in *GPIIb α ^{-/-} → Ldlr^{-/-}*, but not in *IL4R/GPIIb α -Tg → Ldlr^{-/-}* mice compared to wt controls (Fig. 1B, C). Both male and female *GPIIb α ^{-/-} → Ldlr^{-/-}*, but not *IL4R/GPIIb α -Tg → Ldlr^{-/-}*, mice displayed decreased lesion sizes when compared to wt → *Ldlr^{-/-}* gender-matched littermate controls (Fig. 1C). In agreement with that, *en face* analysis of aortas stained with Sudan IV revealed a significant decrease of atherosclerotic plaque surface area in aortas of *GPIIb α ^{-/-} → Ldlr^{-/-}* mice compared to wt → *Ldlr^{-/-}* controls (data not shown). Collagen content was unchanged (Fig. S2A, B), while smooth

muscle actin (SMA) expression was enhanced in the aortic arches of *GPIIb α ^{-/-} -> Ldlr^{-/-}* compared to wt -> *Ldlr^{-/-}* mice (Fig. S2C, D), suggesting increased plaque stability.

Genetic ablation of GPIIb α leads to decreased myeloid cell recruitment to mouse aortas in atherosclerosis

Since atherosclerosis is associated with myeloid cell accumulation in the aortic wall, we examined the cellular composition of aortas from *GPIIb α ^{-/-} -> Ldlr^{-/-}*, *IL4R/GPIIb α -Tg-> Ldlr^{-/-}* or wt mice. Flow cytometry analysis revealed a significant reduction of CD45⁺ cells in aortas of *GPIIb α ^{-/-} -> Ldlr^{-/-}*, but not *IL4R/GPIIb α -Tg-> Ldlr^{-/-}* mice compared to wt -> *Ldlr^{-/-}* controls (Fig. 2A, B). Among CD45⁺ cells, a reduction of myeloid cells was particularly strong in the aortic wall of *GPIIb α ^{-/-} -> Ldlr^{-/-}* (Fig. 2C, D) with a marked decrease of all three major myeloid cell populations (CD11b⁺, CD11b⁺CD11c⁺ and CD11c⁺) (Fig. 2C, D) (35). By contrast, the number and composition of myeloid cells in *IL4R/GPIIb α -Tg-> Ldlr^{-/-}* aortas were similar to wt controls, in agreement with the unaltered course of atherosclerosis development (Fig. 2A-D). The percentage of MHCII-expressing cells was also decreased in *GPIIb α ^{-/-} -> Ldlr^{-/-}* mice (Fig. S3).

Immunofluorescence staining of aortic sections for CD11b and CD11c (Fig. 3A) showed significantly lower number of these myeloid cells in aortic roots of *GPIIb α ^{-/-} -> Ldlr^{-/-}* mice compared to wt -> *Ldlr^{-/-}* control (Fig. 3A, B).

Decreased inflammatory gene expression in atherosclerotic aortas of GPIIb α ^{-/-} mice

Some, if not most, monocytes entering atherosclerotic lesions are thought to have been previously in contact with platelets. These interactions between platelets and myeloid cells in the blood could influence the recruitment of myeloid cells to the aortas. Upon atherosclerosis development, monocytes immigrating to the aortic wall can differentiate into macrophages, which may be polarized towards a pro-inflammatory “M1-like” phenotype or a less inflammatory “M2-like” phenotype (43). To elucidate the mechanism of how platelet-myeloid cell interaction can affect accumulation and activation of myeloid cells in atherosclerosis, we analyzed gene expression by real-time PCR analysis on aortas isolated from *GPIIb α ^{-/-} -> Ldlr^{-/-}*, *IL4R/GPIIb α -Tg-> Ldlr^{-/-}* and wt-> *Ldlr^{-/-}* mice. We found a significant reduction of the “M1-like” pro-inflammatory markers TNF and IL12p35 in *GPIIb α ^{-/-} -> Ldlr^{-/-}* mice compared to wt controls (Fig. 3C). The expression of arginase 1 (Arg1), a “M2-like” marker, was upregulated in the aortas of *GPIIb α ^{-/-} -> Ldlr^{-/-}* mice (Fig. 3C). We did not observe significant changes in expression of other “M2” genes including mannose receptor (Mrc1), chitinase 3-like 3 (Chi3l3) or Fizz1 (Retnla) (data not shown). By contrast, we did not find significant changes in gene expression characteristic of macrophage subset differentiation in *IL-4R/GPIIb α -Tg* mice compared to wt controls (Fig. 3C).

To test whether the macrothrombocytopenia of *GPIIb α ^{-/-}* mice influenced *in vivo* foam cell formation, we injected mice with Dil-oxLDL. We found a small decrease of oxLDL uptake by Ly6C⁺ and Ly6C⁻ monocytes in blood and CD11b⁺ myeloid cells in aortas of *GPIIb α ^{-/-} -> Ldlr^{-/-}* mice compared to wt -> *Ldlr^{-/-}* controls (Fig. 3D, E). Thus, macrophage

polarization and functions in atherosclerosis may be modulated by platelet-monocyte interactions that are disrupted by GPIIb α deficiency.

Reduced platelet-derived microparticles and chemokines mRNA level in GPIIb α ^{-/-} platelets

In agreement with previous studies (27, 34) platelets count in GPIIb α ^{-/-} $\rightarrow$ Ldlr^{-/-} and IL4R/GPIIb α -Tg $\rightarrow$ Ldlr^{-/-} mice fed with WD for 16 wks were reduced compared to wt controls (Fig. 4A). Platelets serve as a major source of bioactive molecules, such as cytokines and chemokines as well as platelet-derived microparticles. Platelet-derived microparticles (PMP) have been implicated in the pathogenesis of atherosclerosis (44). Indeed, we observed enhanced PMP release in wt $\rightarrow$ Ldlr^{-/-} atherosclerotic mice compared to wt healthy mice on a regular diet (Fig. 4B), suggesting that the atherosclerotic environment promotes the release of MP by activated platelets. We found a strong reduction of PMP numbers in GPIIb α ^{-/-} $\rightarrow$ Ldlr^{-/-} but only partial decrease in IL4R/GPIIb α -Tg $\rightarrow$ Ldlr^{-/-} mice compared to wt $\rightarrow$ Ldlr^{-/-} controls, which correlated with platelet counts in these mice (Fig. 4B). A similar decrease of PMP was observed in their wt counterparts (Fig. 4B). As different platelet counts (Fig. 4A) may influence PMP number, we calculated the latter as percentage of platelets (Fig. 4C). The value was significantly lower only in GPIIb α ^{-/-} $\rightarrow$ Ldlr^{-/-} compared to wt $\rightarrow$ Ldlr^{-/-} or even IL4R/GPIIb α -Tg $\rightarrow$ Ldlr^{-/-} mice, implying that GPIIb α ^{-/-} platelets but not those lacking the GPIIb α extracytoplasmic domain (IL4R/GPIIb α -Tg) fail to respond to the atherosclerotic environment by increasing PMP release. Therefore, we concluded that decreased PMP numbers might contribute to overall reduced atherosclerosis in GPIIb α ^{-/-} $\rightarrow$ Ldlr^{-/-} mice.

Previous reports demonstrated that platelet-derived cytokines and chemokines are important in regulating atherosclerosis development (1-4). Using real-time RT-PCR analysis, we observed that platelets of GPIIb α ^{-/-} $\rightarrow$ Ldlr^{-/-}, but not of IL4R/GPIIb α -Tg $\rightarrow$ Ldlr^{-/-} mice, had a significant reduction of Ccl5 mRNA compared to wt $\rightarrow$ Ldlr^{-/-} mice after 16 wks of WD (Fig. 4D). No changes were observed for Cxcl4, Cxcl7, Cxcl12 and Il1 β (data not shown). We conclude that altered platelet and/or megakaryocyte functions independent of GPIIb α ligand binding may regulate the abundance of cytokine and chemokine mRNA within platelets.

Ablation of GPIIb α expression decreases platelet- leukocyte aggregation

Both monocytes(45) and neutrophils(46) play an important role in atherogenesis. Although platelet-monocyte aggregates in the blood of atherosclerotic mice and humans were previously described (24, 47), the role of GPIIb α in platelet interaction with monocytes and subsequent monocyte activation as well as changes of monocytes recruitment to the aortas in atherosclerosis is not clear. We studied the role of GPIIb α ligand-binding function in regulation of platelet-monocyte and platelet-neutrophil aggregates in blood of atherosclerotic GPIIb α ^{-/-} $\rightarrow$ Ldlr^{-/-}, IL4R/GPIIb α -Tg $\rightarrow$ Ldlr^{-/-} and wt $\rightarrow$ Ldlr^{-/-} mice using flow cytometry and live cell microscopy. We found a marked reduction of CD41⁺ platelets interacting with Ly6C⁺ inflammatory monocytes and Ly6G⁺ neutrophils in the blood of GPIIb α ^{-/-} $\rightarrow$ Ldlr^{-/-}, but not IL4R/GPIIb α -Tg $\rightarrow$ Ldlr^{-/-} compared to wt $\rightarrow$ Ldlr^{-/-} controls (Fig. 5A-D). Even though IL4R/GPIIb α -Tg platelets have the same Mac-1 binding defect due to the absence of extracellular part of GPIIb α similar to GPIIb α platelets, yet they form

normal heteroaggregates with leukocytes in blood. Therefore, it seems plausible that other adhesion molecules, but not GPIb α , could mediate interactions of platelets with myeloid cells in the blood *in vivo*.

To directly visualize platelet-leukocyte interactions, we conducted dual-color epifluorescence live imaging of rolling cells in microfluidic flow chamber autoperfused with mouse blood. We used the blood of *Ldlr*^{-/-} mice transplanted with a 1:1 mixture of bone marrow from *GPIb α* ^{-/-} and wt DsRed mice (48) and fed with WD. In these chimera mice, GPIb α expressing platelets are red and *GPIb α* ^{-/-} platelets are non-fluorescent, therefore allowing *in vivo* side-by-side comparison of *GPIb α* ^{-/-} and wt platelets in the same atherosclerotic environment. Injection of DyLight488-labeled CD49d (α 4) mAb (PS/2) into the mice allows visualizing VLA-4 stained DsRed⁺ circulating monocytes (appear in yellow) and unstained DsRed⁺ neutrophils (appear in red) *in vivo* (Fig. S4). In agreement with flow cytometry data, interactions between platelets and neutrophils (Fig. 5E, F) or platelets and monocytes (Fig. 5G, H and Supplementary Movie 1, 2) were significantly reduced in *GPIb α* ^{-/-} mice compared to wt controls.

To gain insights into the functional consequences of defective interactions between platelets and monocytes in blood, we next examined whether interaction with platelets can cause monocyte activation. Mouse monocytes include Ly6C⁺Gr1⁺ “inflammatory” and Ly6C⁻Gr1⁻ “patrolling” subsets(49). We sorted Ly6C⁺ inflammatory and Ly6C⁻ patrolling monocyte from the blood of *GPIb α* ^{-/-} $\rightarrow$ *Ldlr*^{-/-} or wt $\rightarrow$ *Ldlr*^{-/-} mice by FACS sorting. Real-time PCR analysis revealed a reduction of pro-inflammatory TNF gene expression in Ly6C⁺ blood monocytes isolated from *GPIb α* ^{-/-} $\rightarrow$ *Ldlr*^{-/-} compared to wt $\rightarrow$ *Ldlr*^{-/-} controls; in contrast, no changes were found in level of IL12p35 expression (Fig. 5I). These data suggest that during atherosclerosis development, interaction between platelets and monocytes in the blood may modulate monocyte activation as seen by changes in TNF expression. Moreover, since most of myeloid cells are derived from monocytes, the interaction between platelets and monocytes in the blood may curtail monocyte recruitment into aorta and their differentiation to macrophages in atherosclerosis.

Discussion

Atherosclerosis is a chronic inflammatory disease with life-threatening complications. Platelets play a critical role in both initiation and progression of atherosclerosis. Several clinically used drugs target platelets to prevent adverse cardiovascular events triggered by atherosclerosis(50). Inhibition of platelets, however, often increase the risk of hemorrhage, therefore balance of effects (suppression of platelet functions) and side effects (depletion of platelets) is a key for the successful therapy.

A number of previously published studies mainly done using either *in vitro* models or acute models of vascular injury suggest that platelet could interact with leukocytes in the blood and these interactions are mediated by the GPIb α subunit of GPIb-IX-V platelet receptor complex (22-25). Moreover, injections of anti-GPIb α antibodies reduced atherosclerosis(51). These data prompted us to test the role of GPIb α , one of the key

molecules regulating platelet morphology and function via GPIb-IX-V complex expression, versus the function of its ligand-binding extracellular domain in atherosclerosis.

We evaluated disease development in a mouse model where either the entire GPIb-IX-V complex (*GPIba*^{-/-}) or only the GPIba extracytoplasmic domain (*IL4/GPIba*^{-/-}-Tg) was removed from the platelet membrane. Complete GPIba deficiency results in a multifaceted platelet abnormality and macrothrombocytopenia(26), while removal of the GPIba extracytoplasmic domain only causes impaired platelet interaction with several ligands, including vWF, P-selectin, Mac-1 and α -thrombin, and develops milder thrombocytopenia compared to *GPIba*^{-/-} animals. We show here that *Ldlr*^{-/-} mice transplanted with *GPIba*^{-/-} bone marrow (thus, with macrothrombocytopenia), exhibit reduced atherosclerosis progression with smaller lesions and decreased myeloid cells accumulation and activation in the aortic wall. In contrast, mice transplanted with bone marrow expressing a chimeric IL4R/GPIba subunit within GPIb-IX-V complex (with defective GPIba ligand-binding function, but less abnormal platelet morphology and counts) developed atherosclerotic lesions similar to control mice.

Our results indicate that GPIba binding sites for vWF(52), P-selectin(22), Mac-1(23) and α -thrombin(53) might be dispensable for the development of atherosclerosis *in vivo*, since mice expressing the IL4R/GPIba Tg within GPIb-IX-V and therefore lacking these interactions, are not protected from the disease. This was unexpected because all these ligands are thought to play a role in the atherosclerosis, as shown in vWF (54) and P-selectin (16) deficient mice. Moreover, previous studies have highlighted the role of Mac-1 interaction with GPIba in leukocyte recruitment at sites of vascular injury and inflammation (24, 25); in addition, GPIba is the most abundant site for platelet binding of α -thrombin (53, 55), which may be involved in atherogenesis (56). For all these ligands, however, alternative mechanisms exist that can mediate platelet interactions at site of developing atherosclerosis - such as integrins α IIb β 3(57) and α v β 3 (58) for vWF, PSGL-1 for P-selectin (15), CD40L for Mac-1 (59, 60) and protease activated receptors for α -thrombin (61); these appear sufficient for mediating disease even in the absence of GPIba binding sites. Another possible explanation is that GPIba ligand binding is important for adhesion under high, but not modest shear stress. High shear stresses are reached in severed blood vessels during arterial bleeding, in small arterioles, and in stenosed arteries. By contrast, atherosclerosis develops in large arteries with modest shear stress (shear rates typically 1000 s⁻¹ or less), where GPIIb/IIIa is sufficient for platelet adhesion and aggregation. It is possible that the ligand binding function of GPIba might be involved in stenosis development, but rather play a minor role in chronic inflammation in atherosclerosis.

This notwithstanding, our results support a role for platelets in atherosclerosis development that is independent of GPIba ligand binding function but influenced by membrane expression of the GPIb-IX-V complex, affecting the number and functions of platelets. To explain this apparent paradox, it is important to note that loss of GPIb-IX-V linkage to the membrane skeleton greatly affects thrombocytopoiesis. Indeed, substitution of two GPIba cytoplasmic domain residues required for anchoring to filamin-A is sufficient to recreate the phenotype caused by ablation of the entire GPIb-IX-V complex(62). The GPIba cytoplasmic domain is normal in the *IL4R/GPIba* transgene, explaining why its expression

can correct, but not completely normalize, the abnormal platelet size and count of *GPIb α ^{-/-}* mice (34). As shown here, one consequence of GPIb-IX-V absence is the reduction of platelet heteroaggregates with myeloid cells in blood, which limits the accumulation of all three myeloid subsets (CD11b⁺, CD11c⁺ and CD11b⁺CD11c⁺) in atherosclerotic lesions. However, the phenotype is reversed in mice expressing the *IL4R/GPIb α -Tg*. Because of the demonstrated role of CD11b⁺CD11c⁺ antigen presenting cells in sustaining inflammation and T cell immune response in atherosclerotic aortas (35), the disruption of platelet-myeloid cell interactions in BSS mice may influence the atherosclerosis development by decreasing the “autoimmune component” of the process via curbing pro-inflammatory cell accumulation to the aortas. Decreased platelet-leukocyte interactions may also affect blood monocyte activation and /or reduce the presence of pro-atherogenic platelet components, such as CXCL4 and CCL5 in atherosclerotic lesions (63). Such a reduction can shift the polarization of macrophages from the pro-inflammatory “M1-like” to the less inflammatory “M2-like” phenotype, influencing disease progression. We found a decrease of “M1-like” (pro-inflammatory TNF and IL12p35) and increased “M2-like” (anti-inflammatory arginase 1) macrophage markers in the aortas of *GPIb α ^{-/-}->Ldlr^{-/-}*, but not *IL4R/GPIb α -Tg->Ldlr^{-/-}* mice compared to wt controls, which is in agreement with these concepts. The decreased content of myeloid cells and increased smooth muscle α -actin are both compatible with a more stable plaque phenotype in *GPIb α ^{-/-}->Ldlr^{-/-}* mice.

Another notable abnormality of *GPIb α ^{-/-}->Ldlr^{-/-}* mice was the marked decrease of circulating platelet-derived microparticles (PMP), which are known to contribute to the procoagulant activity of platelets (64) and may have pro-inflammatory and proatherosclerotic activity(65). Even though the reduction of endogenous PMP numbers correlated with decreased platelet counts, only *GPIb α ^{-/-}->Ldlr^{-/-}* mice had significantly reduced PMP production when it was normalized to platelet numbers. It also seems that platelets from *GPIb α ^{-/-}->Ldlr^{-/-}* atherosclerotic mice were less responsive to the pro-inflammatory atherosclerosis environment compared to wt->*Ldlr^{-/-}* or even *IL4R/GPIb α -Tg->Ldlr^{-/-}* as well as healthy non-atherosclerotic mice on a regular diet as seen by PMP production. Overall, this suggests possible intrinsic defect of *GPIb α ^{-/-}* platelets in their ability to produce PMPs.

Platelet-derived chemokines and cytokines are known players in atherosclerosis. IL-1 β , CCL5 (RANTES) and CXCL4 (PF4) released by activated platelets modulate monocyte activation and recruitment to the inflamed aortas. In our study, we found decreased CCL5 mRNA in platelets from *GPIb α ->Ldlr^{-/-}*, but not *IL4R/GPIb α -Tg->Ldlr^{-/-}* mice compared to wt controls at 16 wks of WD feeding. Therefore, it appears that the absence of GPIb-IX-V, but not lack of the extracytoplasmic GPIb α domain with its ligand binding function, can reduce the susceptibility of platelets to the activating effect of a pro-atherosclerotic environment as reflected by PMP numbers and pro-inflammatory chemokine mRNA levels. It is noteworthy that a previous study of *GPIb β ^{-/-}* mice crossed to *Apoe^{-/-}* background found no difference in atherosclerosis development as compared to *Apoe^{-/-}* controls (66). These mice, unlike in our study, were kept on a normal diet and had only a moderate increase of plasma cholesterol and triglycerides. GPIb α and GPIb β deficiencies result in similar thrombocytopenia; however, platelet counts were 10-fold lower in our

transplanted *GPIIb α ^{-/-}->Ldlr^{-/-}* as compared to *wt->Ldlr^{-/-}*, and only 7-fold lower in the reported *GPIIb β ^{-/-}/Apoe^{-/-}* as compared to *Apoe^{-/-}* controls. Thus, the distinctive results obtained from these two studies can be explained by the combination of lower platelet counts and more challenging atherosclerotic environment in our model with higher blood lipid levels and heightened pro-inflammatory stimuli. Indeed, the conditions we used were more similar to those of a previous study, based on a 12-week treatment with anti-GPIIb α antibody and 0.25% cholesterol diet in *Apoe^{-/-}*, which concluded for a critical role of platelet adhesion in the initiation of atherosclerosis (5). Of note, the antibody used in that study (5) causes severe thrombocytopenia, so it does not just disrupt or neutralize the functionality of GPIIb α but also simply depletes platelets; therefore eliminating their pro-atherogenic impact (30, 31). We conclude that the atheroprotective effect seen in previous and current studies is caused by the reduced blood platelet counts and abnormal platelets function maybe associated with cytoskeleton abnormalities.

In summary, we demonstrate that the ligand-binding function of the GPIIb α extracytoplasmic domain, which supports platelet interactions with endothelial cells and leukocytes, likely does not play an important role in the pathogenesis of atherosclerosis. In contrast, we show the GPIIb α cytoplasmic domain of the GPIIb-IX-V complex, which is essential for normal platelets production, also influences the release of platelet-derived microparticles and chemokines in a pro-inflammatory environment. Along with the markedly reduced platelet count, these findings can explain why GPIIb-IX-V complex deletion reduces the development of atherosclerotic lesions. Importantly, this pathogenic role of GPIIb-IX-V seems to depend on a threshold-effect, since platelet count, PMP release and chemokine gene expression are not completely normalized by expression of the IL4R/GPIIb α -Tg chimeric subunit within GPIIb-IX-V and, yet, disease development is similar to that of control mice. Overall, our data obtained in an *in vivo* model of atherosclerosis suggest that platelet GPIIb α ligand-binding domain itself plays a minor role in atherosclerosis and therefore platelet-derived factors may represent better targets for atherosclerosis prevention and therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1. Decreased atherosclerotic plaque area in *GPIIb*^{-/-} bone marrow transplanted mice *Ldlr*^{-/-} mice were lethally irradiated and reconstituted with 5×10^6 unfractionated bone marrow cells from C57BL/6 (wt), *GPIIb*^{-/-} or *IL4R/GPIIb-Tg* mice. After 4 wks of reconstitution, mice were fed WD for 16 wks. **(A)** Images of aortic arches of *Ldlr*^{-/-} mice receiving C57BL/6 (wt) control, *GPIIb*^{-/-} or *IL4R/GPIIb-Tg* bone marrow. **(B)** Representative images of aortic root cross sections of *Ldlr*^{-/-} mice receiving C57BL/6 (wt), *GPIIb*^{-/-} or *IL4R/GPIIb-Tg* bone marrow. **(C)** Aortic root lesions were quantified on frozen sections stained with Oil-red-O within 500 μ m of the aortic valve [wt (n=5-8 males or females), *GPIIb*^{-/-} (n=5-8 males or females) and *IL4R/GPIIb-Tg* (n=4-6 males or females)]. *p<0.05

Fig 2. Reduced number of leukocytes in aortas of *GPIIb/IIIa*^{-/-}→*Ldlr*^{-/-} mice

(A) Aortas from bone marrow transplanted mice were made into single cell suspensions by enzymatic digestion and stained. Live CD45⁺ leukocytes were counted in aortas of 16 wks WD-fed *Ldlr*^{-/-} mice transplanted with *GPIIb/IIIa*^{-/-} (n=8), *IL4R/GPIIb/IIIa-Tg* (n=5) or C57BL/6 (wt) (n=8) bone marrow. Representative dot plots; percentages represent gate as percent of all cells. (B) Absolute numbers of CD45⁺ cells per aorta in *Ldlr*^{-/-} mice transplanted with *GPIIb/IIIa*^{-/-}, *IL4R/GPIIb/IIIa-Tg* or wt. (C) Live CD45⁺ cells from aortas were stained for CD11b and CD11c. Numbers indicate percentage in each gate as percent of all CD45⁺ cells. (D) Absolute number of live CD45⁺, CD11b⁺CD11c⁻, CD11b⁺CD11c⁺, CD11b⁻CD11c⁺ cells in aortas from *Ldlr*^{-/-} mice transplanted with *GPIIb/IIIa*^{-/-}, *IL4R/GPIIb/IIIa-Tg* or wt bone marrow (n=6-8) each. Data represented as mean ±SEM. *p<0.05.

Fig 3. Reduction of myeloid cells in mice receiving *GPIIb α ^{-/-}* bone marrow

(A) Localization of CD11b⁺CD11c⁻ (green), CD11b⁺CD11c⁺ (yellow), CD11b⁻CD11c⁺ (red) cells in aortic roots of *Ldlr*^{-/-} mice transplanted with *GPIIb α ^{-/-}* or wt bone marrow as seen by immunofluorescence. Nuclei stained by DAPI (blue). Representative images from one of four independent experiments. (B) Mean $\pm$ SEM number of CD11b⁺CD11c⁻, CD11b⁺CD11c⁺ and CD11b⁻CD11c⁺ cells per section in aortic roots of *Ldlr*^{-/-} mice transplanted with C57BL/6 (wt) (n=5) and *GPIIb α ^{-/-}* (n=5) bone marrow as shown on (A). (C) Relative cytokine gene expression in *GPIIb α ^{-/-}* $\rightarrow$ *Ldlr*^{-/-} (n=4) and *IL4R/GPIIb α -Tg* (n=4) normalized to β -actin and then normalized to gene expression in wt $\rightarrow$ *Ldlr*^{-/-} (n=4) (=1.0) mice in aortas after 16 wks of WD. (D) Oxidized LDL (oxLDL) uptake was measured in Ly6C⁺ and Ly6C⁻ blood monocytes and aortas at 24 hrs after i.v. DiI-oxLDL injection. oxLDL uptake was decreased in *GPIIb α ^{-/-}* $\rightarrow$ *Ldlr*^{-/-} mice (blue line) compared to wt $\rightarrow$ *Ldlr*^{-/-} (black line) in aorta and blood. Negative control (no DiI-oxLDL) is represented by dotted line. Mean $\pm$ SEM. *p<0.05.

Fig 4. Reduced platelet-derived microparticles and chemokine gene expression in *GPIbα*^{-/-} platelets

(A) Platelet counts were obtained from flow cytometry data of *GPIbα*^{-/-} → *Ldlr*^{-/-} (n=5), *IL4R/GPIbα-Tg* → *Ldlr* (n=5) or wt → *Ldlr*^{-/-} (n=5) or healthy control mice, based on the flow rate and time to acquire 10,000 events in the platelet gate. Absolute PMP number (B) and PMP as percentage of platelet count (C) in blood of wt → *Ldlr*^{-/-}, *GPIbα*^{-/-} → *Ldlr*^{-/-}, and *IL4R/GPIbα-Tg* → *Ldlr*^{-/-} mice fed with WD for 16 wks. (D) Relative chemokine gene expression in platelets of *GPIbα*^{-/-} → *Ldlr*^{-/-} (n=5) and *IL4R/GPIbα-Tg* → *Ldlr*^{-/-} (n=5) normalized to β-actin and then normalized to gene expression in wt → *Ldlr*^{-/-} (=1.0) mice after 16 wks of WD. Mean ± SEM. *p<0.05; **p<0.01.

Fig 5. Reduced interaction of *GPIIbα^{-/-}* platelets with myeloid cells in the blood
 (A-D) Blood isolated from 16 wks WD-fed *Ldlr^{-/-}* mice transplanted with *GPIIbα^{-/-}* (n=5), *IL4R/GPIIbα-Tg* (n=6) or C57BL/6 (wt) (n=5) bone marrow was analyzed for presence of heterotypic aggregates. Live cells were stained for CD11b, Ly6C, Ly6G and GPIIb/CD41. (A) Percentage of aggregates between Ly6C⁺ monocytes and GPIIb/CD41⁺platelets among live CD11b⁺ blood cells. (B) Mean ±SEM platelet-monocytes aggregates as % of live CD11b⁺ cells in the blood. (C) Percentage of aggregates between Ly6G⁺ neutrophils and GPIIb/CD41⁺platelets among live CD11b⁺ cells. Numbers indicate percentage in each gate. Representative FACS plots from at least 4 independent experiments. (D) Platelet-neutrophils aggregates in the blood as % of live CD11b⁺ cells in the blood, mean ± SEM. Representative image of a platelet interacting with a neutrophils (E) or monocyte (G) in the blood of mixed bone marrow transplanted (DsRed and *GPIIbα^{-/-}* in 50/50% ratio) mice rolling on a P-selectin-coated cover slip in a microfluidic flow chamber assay. Monocytes are labeled by Alexa-Fluor 488-VLA-4 Ab. Monocyte-yellow. Neutrophil-red, Platelet-red. Wall shear stress 6 dyn/cm². P-selectin 20 molecules/μm². Percentage of rolling neutrophils (F) or monocytes (H) interacting with platelets under flow in whole blood. (I) Relative cytokine gene expression in *GPIIbα^{-/-}* → *Ldlr^{-/-}* (n=4) normalized to β-actin and then normalized to gene expression in wt → *Ldlr^{-/-}* (n=4) (=1.0) mice in Ly6C⁺ blood monocytes after 16 wks of WD. Data represented as mean ±SEM. *p<0.05.