Cre/lox Studies Identify Resident Macrophages as the Major Source of Circulating Coagulation Factor XIII-A

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Objective—To establish the cellular source of plasma factor (F)XIII-A.

Approach and Results—A novel mouse floxed for the F13a1 gene, FXIII-A\textsuperscript{Flox/Flox} (Flox), was crossed with myeloid- and platelet-cre–expressing mice, and cellular FXIII-A mRNA expression and plasma and platelet FXIII-A levels were measured. The platelet factor 4-cre.Flox cross abolished platelet FXIII-A and reduced plasma FXIII-A to 23±3% (P<0.001). However, the effect of platelet factor 4-cre on plasma FXIII-A was exerted outside of the megakaryocyte lineage because plasma FXIII-A was not reduced in the Mpl\textsuperscript{−/−} mouse, despite marked thrombocytopenia. In support of this, platelet factor 4-cre depleted FXIII-A mRNA in brain, aorta, and heart of floxed mice, where FXIII-A\textsuperscript{pos} cells were identified as macrophages as they costained with CD163. In the integrin αM-cre.Flox and the double copy lysozyme 2-cre.cre.Flox crosses, plasma FXIII-A was reduced to, respectively, 75±5% (P=0.003) and 30±7% (P<0.001), with no change in FXIII-A content per platelet, further consistent with a macrophage origin of plasma FXIII-A. The change in plasma FXIII-A levels across the various mouse genotypes mirrored the change in FXIII-A mRNA expression in aorta. Bone marrow transplantation of FXIII-A\textsuperscript{−/−} bone marrow into FXIII-A\textsuperscript{−/−} mice both restored plasma FXIII-A to normal levels and replaced aortic and cardiac FXIII-A mRNA, while its transplantation into FXIII-A\textsuperscript{−/−} mice did not increase plasma FXIII-A levels, suggesting that a limited population of niches exists that support FXIII-A-releasing cells.

Conclusions—This work suggests that resident macrophages maintain plasma FXIII-A and exclude the platelet lineage as a major contributor.

Visual Overview—An online visual overview is available for this article. (Arterioscler Thromb Vasc Biol. 2017;37:1494-1502. DOI: 10.1161/ATVBAHA.117.309271.)

Key Words: animal models of human disease ■ bone marrow ■ platelets ■ macrophages ■ transplantation

Factor (F)XIII-A is a moderately abundant plasma coagulation protein with a half-life of 5 to 10 days\textsuperscript{1}; therefore, the cell type that releases FXIII-A into the plasma must be reasonably numerous. In mammals, FXIII-A is expressed in megakaryocytes, monocytes, osteocytes, chondrocytes, dendritic cells, and bone marrow (BM)–derived macrophages, which have polarized to the M2 phenotype.\textsuperscript{2} FXIII-A is expressed in resident macrophages in organs, including heart, aorta, skin, and placenta.\textsuperscript{3,4} but not in liver, spleen, and kidney.\textsuperscript{5} FXIII-A lacks a classical signal peptide,\textsuperscript{6} and its mechanism of release remains uncharacterized. As a consequence, plasma FXIII-A may not be released from every cell type in which it is expressed. The outcomes of human BM transplant (BMT) studies have variously implicated platelets, macrophages, and unidentified extra-hematopoietic cells as possible sources of plasma FXIII-A.\textsuperscript{5-11} Our previous studies suggested that the platelet is not the source of plasma FXIII-A because in 2 severely thrombocytopenic mouse lines, B6.129P2(\textit{F}cgr\textit{IIa})\textsuperscript{−/−} and Mpl\textsuperscript{−/−}, plasma FXIII-A levels were normal.\textsuperscript{3} However, a possibility that we did not fully address is that thrombocytopenia per se might induce upregulation of platelet proteins, including FXIII-A, either within the megakaryocyte lineage or within a rescuing cell type. Therefore, in the current study, we have measured FXIII-A in platelets from the Mpl\textsuperscript{−/−} mouse and have investigated the source of plasma FXIII-A by crossing a novel FXIII-A floxed mouse with mice that individually express platelet factor (Pf)4-cre, integrin αM (CD11b)-cre, or lysozyme 2 (LysM)-cre. CD11b is highly expressed on monocytes, inflammatory macrophages, and osteoclasts and is low or absent in certain populations of yolk sac (YS)–derived macrophages,\textsuperscript{12,13} while LysM is expressed on numerous macrophage subpopulations.\textsuperscript{14} The Pf4-cre was designed to delete...
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Resident Macrophages Maintain Plasma FXIII-A

In the platelet lineage, but a recent reporter mouse study has also shown Pf4-cre expression within resident macrophages.

In this study, we relate the extent of plasma FXIII-A depletion to the loss of FXIII-A mRNA levels in our cre/lox crosses and deduce that a macrophage population maintains plasma FXIII-A.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Plasma FXIII-A Levels Are Consistent With Either a Platelet or a Myeloid Origin in cre/lox FXIII-A-Deficient Mice

The presence of the flippase recombinase target or LoxP sites within the F13a1 gene of the floxed mouse (Figure I in the online-only Data Supplement) slightly decreased plasma FXIII-A activity to 85±5% of C57BL/6 wild-type (WT) mice (Figure 1A). Consequently, results are presented either relative to WT mice (Figure 1A) or, where more appropriate, relative to FXIII-A.Flox mice (Figure 1B). Plasma FXIII-A activity was absent in the novel FXIII-A−/− mouse, while plasma FXIII-A activity in heterozygous mice was 61±3% (P<0.001; Figure 1A), in agreement with previously described FXIII-A−/− mice. FXIII-A activity measurements were corroborated by immunoblotting, with generally good agreement between methods (Figure 1).

FXIII-A.Flox mice were crossed with mice expressing cre recombinase in megakaryocytes and certain macrophage populations (Pf4-cre15,16) or exclusively in myeloid cells (LysM-cre14 and CD11b-cre13). In Pf4-cre.Flox mice, plasma FXIII-A activity was reduced to 23±3% (P<0.001; Figure 1B). To determine whether the residual 23% was explained by suboptimal Pf4-cre expression, we bred mice harboring 2 copies of this transgene, using quantitative polymerase chain reaction of genomic (g)DNA to assess copy number (1 copy normalized to 1.00±0.038, n=12 and 2 copy to 1.83±0.060, n=6).

Pf4-cre.cre.Flox mice did not show a statistically significant further decrease in plasma FXIII-A activity (17±1%, P=0.48; Figure 1B), suggesting that incomplete depletion is not explained by suboptimal expression of Pf4-cre.

In CD11b-cre.Flox and LysM-cre.Flox mice, plasma FXIII-A was reduced to 75±5% (P=0.003; Figure 1B) and 57±5% (P<0.001; Figure 1B), respectively. The LysM-cre knock-in undergoes silencing, and possibly on account of this, we observed a significant further reduction in plasma activity to 57±5% of C57BL/6 wild-type (WT) mice (Figure 1B).

Nonstandard Abbreviations and Acronyms

BM bone marrow
BMT bone marrow transplantation
CD11b integrin αM
Flox FXIII-Alox/lox
FXIII-A factor XIII-A
LysM lysozyme 2
Pf4 platelet factor 4
YS yolk sac

Figure 1. Plasma factor (F)XIII-A activity was measured by biotin-pentylamine incorporation (red bars). Activity measurements were confirmed by immunoblotting FXIII-A protein normalized to α1-antitrypsin (blue bars). A, Relates FXIII-A activity in wild-type (WT) mice (100%) to mice in which FXIII-A expression originates from the WT allele and also to the FXIII-A.Flox mouse. B, Relates FXIII-A activity in FXIII-A.Flox mice (100%) to crosses between the FXIII-A.Flox and –cre expressing mice. Numbers of separate mouse samples are shown in parentheses. Results are displayed as mean±SEM. Pf4-cre.cre and LysM-cre.cre are homozygous for the respective transgenes. CD11b indicates integrin αM; Flox, FXIII-Afloxflox; LysM, lysozyme 2; and Pf4, platelet factor 4.
FXIII-A (to 30±7%) in 2-copy LysM-cre.cre.Flox mice relative to LysM-cre.cre.Flox mice (P<0.001; Figure 1B).

The sum of the plasma FXIII-A reductions achieved individually with Pf4-cre.Flox (77%) and either CD11b-cre.Flox (25%) or LysM-cre.cre.Flox (70%), respectively, equals or exceeds 100%. In contrast, while dual-expressing CD11b-cre.Pf4-cre.Flox mice showed a further reduction in plasma FXIII-A activity (11±2%; Figure 1B) over the Pf4-cre.Flox mice (P<0.001), plasma FXIII-A was not completely eliminated. These results suggest that the plasma FXIII-A-releasing cell expresses both Pf4 and myeloid markers.

Platelet FXIII-A Concentrations in cre/lox FXIII-A-Deficient and Thrombocytopenic Mpl−/− Mice Discount the Platelet and Support a Myeloid Origin for Plasma FXIII-A

Although our previous studies suggested that the platelet was not the source of plasma FXIII-A,1 the substantial deletion of plasma FXIII-A in Pf4-cre.Flox mice might be
interpreted as indicating that the megakaryocyte–platelet lineage makes a significant contribution to plasma FXIII-A. To further exclude the platelet lineage as the major source of plasma FXIII-A, we demonstrated that Mpl−/− mice have normal plasma FXIII-A activity (95±4%, P=0.49; Figure 1A), despite platelet counts in whole blood being 6.4±5% of WT (P<0.0001). In contrast, platelet counts did not differ between WT mice and the various cre/lox mice (Figure 2A). We established that the size distribution of Mpl−/− platelets was equivalent to that of WT (Figure 2B) and that FXIII-A activity and protein per platelet were normal (92±6%; Figure 2C). In addition, because thrombocytopenia has been suggested to induce hepatic FXIII-A expression,9 we determined by reverse transcriptase polymerase chain reaction that hepatic FXIII-A mRNA was undetectable in either the Mpl−/− or WT mice (C≥40). Together, these results both exclude compensatory FXIII-A upregulation in platelets or hepatic cells as a mechanism whereby plasma FXIII-A is maintained and further discount the platelet as the source of plasma FXIII-A.

In the platelet, FXIII-A activity was abolished in Pf4-cre. Flox mice (Figure 2D), showing that single-copy Pf4-cre mice underwent efficient deletion. Moreover, platelet FXIII-A was unaffected in the CD11b-cre. Flox (83±10%), LysM-cre. Flox (111±3%), and LysM-cre.cre. Flox (94±10%) mice (Figure 2D).

Figure 3. A, Genomic F13a1 recombination in the tissues indicated was determined in Pf4-cre. Flox (dark bars) and CD11b-cre. Flox (white bars) mice. Results are expressed as mean±SEM (n≥3). B and C, Immunofluorescent staining detects factor (FXIII-A (red) cells in wild-type (WT) but not FXIII-A−/− heart sections. Nuclei are shown in blue. B, A proportion of FXIII-A-labeled cells adopt a spindle-shaped morphology characteristic of cardiac macrophages (inset). Scale bar represents 50 μm. C, Some of these FXIII-A+ cells costain for CD163 (green). Scale bar is 5 μm. D–F, mRNA was isolated from indicated tissues and cDNA subjected to quantitative polymerase chain reaction (qPCR) using housekeeping (HK) transcripts ribosomal protein L32 and β-actin. All results are displayed as mean with 95% confidence interval (CI). D, FXIII-A mRNA expression was measured in WT aorta (light yellow bars) and heart (dark yellow bars) (n=4) were measured by reverse transcriptase (RT)-PCR, and reaction products were resolved on agarose gels (inset). Transcript levels were enriched in WT-cultured BM-derived macrophages (BMDM, n=4) at levels similar to endogenous CD11b transcripts (dark pink bars), as well as in a FXIII-A-enriched cardiac cell fraction (FEF, n=4). F, FXIII-A mRNA expression was measured in brains (n=3) of young mice of the genotypes indicated. CD11b indicates integrin αM; Flox, FXIII-Afloxflox; LysM, lysozyme 2; and Pf4, platelet factor 4.
confirming that these myeloid-cre mice exert their effects on plasma FXIII-A through the myeloid rather than the megakaryocyte lineage. Together, these results suggest that Pf4-cre depletes plasma FXIII-A by acting within myeloid cells, and that these cells are the major site from which plasma FXIII-A is released.

**FXIII-A** Cardiac Cells Are Pf4-Expressing Macrophages

We assayed the extent of *F13a1* genomic deletion using quantitative polymerase chain reaction of gDNA, with reference to a standard curve (Figure II in the online-only Data Supplement). Genomic *F13a1* recombination was essentially as previously described in the organs of CD11b-cre. Flox mice.13 In Pf4-cre.Flox mice, genomic deletion totaled 3.5% in heart and 3.4% in aorta and was lower in other organs examined (Figure 3A), excluding the possibility that Pf4-cre expression was promiscuous in Pf4-cre.Flox mice.

We examined the expression of FXIII-A mRNA in heart and aorta, where functional roles of FXIII-A have been described.19,20 Immunofluorescence studies revealed FXIII-A**m** cells in WT, but not knockout heart (Figure 3B). Some of these FXIII-A**m** cells appeared as spindles (inset Figure 3B), in agreement with their previous identification as macrophages.21 Moreover, coimmuno-fluorescence for CD163 and FXIII-A (Figure 3C) and immunohistochemistry of consecutive WT heart sections (Figure III in the online-only Data Supplement) confirmed that ∼80% of the FXIII-A**m** cells are CD163**m** macrophages (Table 1).

FXIII-A mRNA was abolished in heart and depleted in aorta in Pf4-cre.Flox mice (Figure 3D). The complete depletion of FXIII-A mRNA in the heart is broadly consistent with the extent of gDNA recombination (3.5%) and the frequency of FXIII-A**m** cells (5%; Table 1), provided that Pf4-cre expression is restricted to FXIII-A**m** cells. Importantly, in LysM-cre.Flox mice, FXIII-A mRNA was substantially depleted from these tissues, while it was conserved in the Mpl−/− mouse, confirming that FXIII-A**m** cells are myeloid rather than platelet/megakaryocyte in origin. As expected, the proportional deletion of the FXIII-A mRNA reflected the loss of FXIII-A**m** cells across the various genotypes (Table 1).

Authentic Pf4 transcripts were detected in WT hearts and aorta (insert, Figure 3E), as well as in Mpl−/− hearts (data not shown). Pf4 transcripts were 40-fold enriched in cultured BM-derived macrophages over whole hearts, while the ratio of Pf4 mRNA to CD11b mRNA was comparable between BM-derived macrophages and whole heart (Figure 3E). This is consistent with Pf4 and CD11b mRNA being present at a similar concentration within cardiac macrophages, which represent a minor proportion of the tissue.

Finally, we analyzed cell fractions from collagenase-digested hearts obtained with the Miltenyi neonatal cardiac fibroblast isolation kit. In a fraction that was ∼8-fold enriched in FXIII-A mRNA (FEF) and which represented ∼3% of total heart cell mass, Pf4 and CD11b mRNA were, respectively, 20-fold and 14-fold enriched (Figure 3E). Together, these data indicate that the FXIII-A**m** cardiac cells are Pf4-expressing macrophages.

**Plasma FXIII-A Originates From Cells Resembling Aortic Macrophages**

In the brain, where because of the blood–brain barrier, macrophages derive exclusively from primitive YS hematopoiesis,22,23 we observed that FXIII-A**m** cells are present at similar levels of FXIII-A mRNA in heart and aorta. Depletion of plasma FXIII-A mRNA in the Pf4-cre.Flox mouse greatly exceeds depletion of the plasma pool, making cardiac tissue an unlikely source of plasma FXIII-A. Depletion of FXIII-A mRNA in the aorta, however, generally reflects the depletion of plasma FXIII-A. CD11B indicates integrin αM; Flox, FXIII-Aflox/flox; FXIII-A, factor XIII-A; LysM, lysozyme 2; and Pf4, platelet factor 4.

### Table 2. Comparison of Plasma and Platelet FXIII-A Enzyme Activities With FXIII-A mRNA Levels in Heart and Aorta

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Plasma Activity</th>
<th>Platelet Activity</th>
<th>Cardiac mRNA</th>
<th>Aortic mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mpl−/−</td>
<td>0.95</td>
<td>0.06*</td>
<td>0.93</td>
<td>1.70</td>
</tr>
<tr>
<td>FXIII-A.Flox</td>
<td>0.83</td>
<td>0.87</td>
<td>0.76</td>
<td>0.85</td>
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<tr>
<td>Pf4-cre.Flox</td>
<td>0.19</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.14</td>
</tr>
<tr>
<td>CD11b-cre.Flox</td>
<td>0.62</td>
<td>0.83</td>
<td>0.93</td>
<td>0.60</td>
</tr>
<tr>
<td>LysM-cre.Flox</td>
<td>0.30</td>
<td>0.94</td>
<td>0.20</td>
<td>0.41</td>
</tr>
</tbody>
</table>

Values are shown relative to WT mice represented as 1.0. Depletion of cardiac FXIII-A mRNA in the Pf4-cre.Flox mouse greatly exceeds depletion of the plasma pool, making cardiac tissue an unlikely source of plasma FXIII-A. Depletion of FXIII-A mRNA in the aorta, however, generally reflects the depletion of plasma FXIII-A. CD11B indicates integrin αM; Flox, FXIII-Aflox/flox; FXIII-A, factor XIII-A; LysM, lysozyme 2; and Pf4, platelet factor 4.

*Platelet FXIII-A in Mpl−/− mice is shown as the platelet count (6%) multiplied by the FXIII-A content per platelet (100%).
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in WT and Mpl−/− mice (Figure 3F). This shows that, as in heart and aorta, the FXIII-A+ cells in the brain do not arise from the platelet lineage. Moreover, FXIII-A mRNA was depleted from the brain of the Pf4-cre.Flox mouse (Figure 3F), suggesting that YS-derived macrophages, as BM-derived macrophages, coexpress Pf4 and FXIII-A.

Table 2 shows that the Pf4-cre-mediated reduction in cardiac FXIII-A mRNA (to <1%) greatly exceeds the depletion of the plasma FXIII-A (to ≈20%), making it unlikely that the majority of cardiac cells contribute to the plasma FXIII-A pool. However, in aorta, we observed a similar profile of depletion of FXIII-A mRNA (to ≈14%) to plasma FXIII-A. We, therefore, conclude that plasma FXIII-A is released from cells, similar or identical to those present in the aorta, and that these cells resemble YS-derived macrophages.

BMT Repopulates Resident Macrophages in Heart and Aorta and Reconstitutes Plasma FXIII-A in FXIII-A−/− Mice

BM was transferred from WT mice into irradiated FXIII-A−/− and WT recipients; survival was 7 of 8 in both groups. At 10 weeks post-BMT, FXIII-A−/− recipients expressed normal levels of plasma and platelet FXIII-A (Figure 4A). Recipient genotype at harvest was confirmed by showing that liver gDNA remained predominantly FXIII-A−/−, while a minor proportion became FXIII-A+/+, consistent with engraftment of donor macrophages (Figure 4B).

FXIII-A mRNA was measured in the hearts, aortas, and brains of WT (light green bars) and FXIII-A−/− (dark green bars) BMT recipients. Results are represented as mean with 95% confidence interval (CI). BMT indicates bone marrow transplant.

Discussion

Previously, we demonstrated that plasma FXIII-A levels are normal in the thrombocytopenic Mpl−/− and BClx Plt20/Plt20 mice. However, we did not assay platelet FXIII-A in the Mpl−/− mouse, in which depletion of ≈94% platelets and their precursors results from knockout of the thrombopoietin receptor.24 Here, we have shown that the amount of FXIII-A per platelet has not increased to compensate for thrombocytopenia. Platelets arise from fragmentation of megakaryocytes; therefore, cytosolic FXIII-A concentrations in megakaryocytes from the Mpl−/− mouse should also be unchanged. This makes it improbable for platelets and megakaryocytes to be major contributors to the plasma FXIII-A pool, although we cannot exclude a minor contribution.

We next compared the effects of recombination by lineage-specific cre mice on plasma FXIII-A and cellular FXIII-A mRNA levels. Plasma FXIII-A, but not platelet FXIII-A, was partially depleted in CD11b-cre.Flox and LysM-cre.cre.Flox mice, suggesting that macrophages maintain plasma FXIII-A and further excluding the megakaryocyte lineage as a major contributor.

In Pf4-cre.Flox mice, platelet FXIII-A was abolished, while plasma FXIII-A was reduced to 23%. This result further supports a macrophage origin for FXIII-A because a stop/lox study has established that Pf4-cre expression occurs within resident tissue macrophages in addition to megakaryocytes.16 Notably, Pf4-cre-mediated recombination within macrophages is mosaic,16 potentially accounting for incomplete depletion of plasma FXIII-A. The FXIII-A-expressing cells in brain, heart, and aorta have been previously identified as macrophages,19,20 and consistent with this, we show (1) that in heart, as previously demonstrated in skin,26 FXIII-A partially co-localizes with the M2 macrophage marker CD163 and (2) that in heart, brain, and aorta, Pf4-cre depleted FXIII-A mRNA.

Figure 4. Factor (F)XIII-A+ bone marrow (BM) was transplanted into wild-type (WT) (WT BMT) and FXIII-A−/− (FXIII-A−/− BMT) recipients. A, Plasma (red bars) and platelet (blue bars) FXIII-A activity was measured by biotin-pannylamine incorporation assay. Average activities in nontransplanted mice are shown for comparison. Results are presented as means±SEM. B, Livers were excised from a single WT (lane +) and 3 representative FXIII-A−/− (lanes 1, 2, and 3) recipients of WT BM. Amplification of gDNA by polymerase chain reaction (PCR) revealed a preponderance of the deleted allele (200 kDa) in the livers of the FXIII-A−/− recipients and partial repopulation with FXIII-A+/+ cells (130 kDa). C, FXIII-A mRNA was measured in the hearts, aortas, and brains of WT (light green bars) and FXIII-A−/− (dark green bars) BMT recipients. Results are represented as mean with 95% confidence interval (CI). BMT indicates bone marrow transplant.
The expression of Pf4-cre within resident macrophages is likely to be faithful because expression of Pf4 protein was previously demonstrated in adherent cultures of human monocytes27,28 and confirmed by us in mouse BM-derived macrophages. In whole heart, as well as in a FXIII-A-enriched cardiac cell fraction, we demonstrated that the ratio of Pf4 to CD11b transcripts was similar to that in BM-derived macrophages, as expected if the Pf4pos cells in the heart are macrophages.

The profile of depletion of plasma FXIII-A in the various cre/lox crosses (Table 2) closely resembles that of aortic FXIII-A mRNA, implicating cells identical or related to aortic resident macrophages as the source of plasma FXIII-A. FXIII-A within aortic macrophages has been implicated in arterial repair and remodeling.19 If local release contributes to this function, this could also suggest that aortic macrophages, or cells similar to them, release FXIII-A into the plasma. Similarly, placental macrophages express FXIII-A and may stabilize this organ during pregnancy.7 Because intravenous FXIII-A prevents spontaneous abortion, 29 this shows that the requirement is extracellular and suggests that placental macrophages can release FXIII-A.

The hypothesis that resident macrophages release plasma FXIII-A is consistent with the outcomes of most human BMT protocols.9–11 In contrast to Wölpl et al,8 Poon et al11 observed that conversion from donor to recipient plasma FXIII-A variant occurred over many months after BMT. Further, Inbal et al30 and Pihusch et al31 observed that BMT caused a much greater decrease in platelet count than plasma FXIII-A, which led Pihusch et al30 to suggest that resident macrophages maintain the plasma pool. Notably, alveolar macrophages persist for ≤80 days after BMT.30,31 Further, the results of these studies seem inconsistent with release occurring from circulating monocytes, because, like platelets, these cells are short-lived.32

BMT of FXIII-Apos cells into FXIII-A−/− mice rescued plasma FXIII-A to normal levels, while transfer into FXIII-A+/− mice caused no further increase in plasma FXIII-A. This may imply that the transplanted cells differentiate within a limited number of niches to become FXIII-A-releasing cells.33,34

Recent studies have shown that arterial macrophages arise variously from (1) YS macrophages, (2) fetal liver monocytes, and (3) a short wave of BM monocytes.35 To determine whether, under normal conditions, monocytes repopulate heart and artery and become FXIII-A-releasing cells, we generated Fms-like tyrosine kinase 3-cre;Flox mice. These mice were generated by breeding Fms-like tyrosine kinase 3-cre mice to Flox mice. These mice were generated by breeding Fms-like tyrosine kinase 3-cre mice to Flox mice. The Fms-like tyrosine kinase 3-cre;Flox mice underwent efficient recombination and reduced plasma FXIII-A to 34% (Figure IV in the online-only Data Supplement). Assuming that this result is representative, it would imply that a proportion of FXIII-A-releasing cells derive from fetal liver or BM-derived cells, presumably monocytes. This would resemble the situation described for arterial macrophages.35 Our model is shown in Figure 5: the aorto-gonad-mesonephros is an early site of hematopoiesis,36 and because it is known that vascular macrophages in the early embryo express FXIII-A,40 it seems probable that they establish here, persist within the developing aorta, and survive postnata! Monocyte-derived macrophages subsequently supplement, or partially displace, YS-derived macrophages and contribute to plasma FXIII-A. Although we have shown that aortic macrophages resemble the plasma FXIII-A-releasing cells, resident macrophages in other tissues may contribute.

The main limitation of the present study is that we have not directly demonstrated that macrophages release FXIII-A. Previously, we detected nonclassical secretion in vitro of interleukin-1β, but not FXIII-A, from IL-4-treated adherent THP-1 cells, which resemble resident macrophages.41 In addition, we have detected externalization of the closely related protein transglutaminase 2,42 but not of FXIII-A, to the surface of mouse macrophages and THP-1 cells (Figure V in the online-only Data Supplement). It may transpire that FXIII-A is released primarily (1) within tissue niches or (2) from Li6Cio resident macrophage precursors,34 and that these cells were sparse in culture. Further studies may address these issues by (1) adoptive transfer of defined macrophage precursors, (2) enriching cultures for particular cell types, or (3) establishing coculture models that mimic niches. In conclusion, our studies...
exclude the platelet and all its precursors as the major source of plasma FXIII-A and instead implicate that resident macrophages maintain plasma FXIII-A.

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Disclosures
None.

References

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Highlights

- Plasma factor XIII-A derives from resident macrophages.
- Plasma factor XIII-A releasing macrophages resemble aortic macrophages.
- Bone marrow–derived monocytes/macrophages can differentiate in situ into plasma factor XIII-A releasing cells.
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Supplemental Materials and Methods

Cre/lox studies identify resident macrophages as the major source of circulating coagulation Factor XIII-A.

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Breeding of mouse lines.
Animal housing, husbandry and procedures were conducted in accordance with guidelines and regulations of the University of Leeds and United Kingdom Home Office. Mice had free access to laboratory chow and water. A C57/129 mixed background mouse with LoxP sites inserted into exon 8 (coding exon 7, which includes the catalytic cysteine residue) of a single Flt3α1 allele was generated by targeted recombination of embryonic stem cells from mouse strain 129 (GenOway, Lille). Following selection of G418 resistant clones and indication that appropriate recombinant ES cells had been obtained, these cells were used to generate chimeric blastocysts with cells from C57BL/6 mice. Chimeric offspring were then mated with C57BL/6 mice expressing flippase (flp) recombinase to generate progeny in which the neomycin phosphotransferase cassette had been excised from the majority of cells to leave a single flp recognition target (FRT) site (supplementary Figure I). Mice mosaic for the flp deletion were backcrossed with C57BL/6 wild-type mice to generate true FXIII-A<sup>+/flox</sup> heterozygotes. Liver gDNA from the FXIII-A<sup>-/flox</sup> mouse was amplified by PCR across the entire FXIII-A floxed locus using primers 5'-TGCTGGTGTTTTTAAACACATTTTAA-3' and 5'-TGGGCCGAGAATGAAATTGGT-3'. The product was sub-cloned into vector pCR4-TOPO (Invitrogen) and subjected to dideoxy sequencing to verify the expected sequences in the WT and floxed alleles (DNA Sequencing & Services, College of Life Sciences, University of Dundee, Scotland).

A FXIII-A<sup>-/-</sup> heterozygous mouse was also derived from the FXIII-A<sup>-/flox</sup> mouse by crossing with C57BL/6 transgenic mice expressing CMV-cre recombinase to generate partially deleted mice and then crossing these mosaic mice with C57BL/6 wild-type mice to obtain true heterozygotes (GenOway).

Both the FXIII-A<sup>-/flox</sup> and the FXIII-A<sup>-/-</sup> mouse lines were backcrossed against C57BL/6 mice to enrich the background to >97.5% C57BL/6 before breeding the floxed or knockout allele to homozygosity. FXIII-A<sup>-/-</sup> mice were in all cases obtained as the offspring of FXIII-A<sup>-/-</sup> females mated with FXIII-A<sup>-/-</sup> males, since as previously reported, 1 female FXIII-A<sup>-/-</sup> mice did not survive pregnancy.

The LysM-cre mouse<sup>2</sup> (Charles River, UK) is a knock-in of cre recombinase into the macrophage-specific lysozyme 2 gene and so its expression is expected to reflect the endogenous gene. The Pf4-cre mouse<sup>3</sup> (Charles River, UK) is transgenic for a bacterial artificial chromosome construct in which the Pf4 coding sequence has been replaced by cre recombinase, with flanking genes present to promote fidelity of expression. The Flt3-cre mouse<sup>4</sup> is transgenic for a bacterial artificial chromosome construct in which the FMS-like tyrosine kinase (Flt3) coding sequence is replaced with cre recombinase and was supplied by Dr Adam Mead (University of Oxford), with kind permission from Dr Thomas Boehm (University of Freiburg). The CD11b-cre mouse has been previously described and is transgenic for a cre construct under the control of a minimal promoter.<sup>5</sup> All cre-recombinase expressing mice were obtained on a C57BL/6 background. Mpl<sup>-/-</sup> mice<sup>5</sup> were a kind gift of Dr Warren Alexander (Walter and Eliza Hall Institute of Medical Research). The genotypes of all mice listed above were determined by PCR amplification of DNA from ear notches obtained at 4 weeks that were solubilised for 20min at 95°C in 100μl of 25mM NaOH / 0.2mM EDTA prior to neutralisation. Oligonucleotides used for routine mouse genotyping and the sizes of products obtained are shown in supplementary Table I.

In some cases, mice were bred with two copies of Pf4-cre and needed to be distinguished from mice harbouring a single copy. Since the integration site of the transgene has not been reported, the copy number of the Pf4-cre construct was obtained by designing 3 primers, one in the Pf4 promoter sequence common to the authentic Pf4 gene and the transgene, and one each specific to the authentic gene or the transgene. This enabled qPCR of the transgene relative to the endogenous Pf4 gene using 60ng of total gDNA (Supplementary Table I), 10μL of the Roche LightCycler 480 SYBR Green I Master Mix and 500nM of each primer in a total volume of 20μL. Reaction conditions used (95°C for 10min, 45 cycles of 95°C for 20s, 64°C for 20s and 72°C for 45s) were followed by a melting curve from 65°C to 95°C, giving a ΔC<sub>t</sub> which was converted into an amount of gene product by the Livak 2<sup>-ΔΔCt</sup> method.<sup>7</sup>
Blood sampling and organ harvest.
Blood samples from anaesthetised mice (8-12 weeks) were drawn directly into 250µl citrate, theophylline, adenosine, and dipryidamole (CTAD) anticoagulant (Becton Dickinson). Phosphate buffered saline (PBS, Gibco) perfused organs were harvested into liquid nitrogen prior to DNA/RNA analysis.

Bone Marrow Transplantation.
Female C57BL/6 (WT) and FXIII-A<sup>-/-</sup> mice at 7 weeks of age and were placed on water containing enrofloxacin (50µg/ml, Bayer) and subjected to total body irradiation (8.45 Gy) at 8 weeks. BM cells from WT male donors (1.0x10<sup>6</sup>) were transplanted 24h after irradiation. Blood and organs were harvested 10 weeks later.

Platelet counting.
Blood/CTAD mixture was diluted 20-fold in resuspension buffer (50mM Tris, 100mM NaCl, pH7.4) containing 0.1U/ml apyrase (Sigma-Aldrich) and 1µg/ml prostaglandin E-1 (Calbiochem). Platelets stained with 20% vol/vol fluorescein isothiocyanate (FITC)-labelled rat anti-mouse glycoprotein (GP)Ibα (Emfret Analytics, Germany) were counted on a Becton Dickinson Aria II cell sorter. Platelet size in WT and Mpl<sup>-/-</sup> mice was compared by separate labelling with GPIbα-FITC or GPIbα-phycocerythrin (PE).

Platelet harvest.
Platelet rich plasma from ~1.0ml of blood/CTAD was obtained by centrifugation for 5min at 200xg. Platelet pellets, obtained from platelet rich plasma by centrifugation at 1000xg for 10min, were dispersed in resuspension buffer. Platelet pellets, were harvested by cell sorting (5x10<sup>6</sup> aliquot) in initial experiments and in later experiments by centrifugation alone, were washed and stored at -80°C. Platelet depleted plasma was stored at -40°C.

FXIII-A activity assay.
Plasma FXIII-A activity was measured by a modified biotin-pentylamine incorporation assay<sup>8</sup>. To ensure that each individual sample was assayed in the linear range of the assay, it was measured at 3 concentrations and in triplicate at each concentration. The middle value was used for analysis, provided the upper and lower samples were in proportion. FXIII-A activity of detergent-lysed platelets was assayed in duplicate reactions containing 10<sup>5</sup>, 10<sup>6</sup> and 2.5x10<sup>6</sup> cells. In the first instance, flow-sorted platelets were used but subsequently it was confirmed that washed platelets could substitute. A reference sample of human plasma plus a plasma sample from a WT mouse were included throughout. Lactate dehydrogenase was assayed (Cytotox96 kit, Promega) to adjust for variation in platelet yield. The increased volume of the flow sorted platelet samples (100µl for assay) necessitated adjustment of reagent volumes. Plates (96 well, Nunc Maxisorb) were coated overnight with 100µl per well (plasma assay) or 200µl (platelet assay) of fibrinogen (40µg/ml, Calbiochem) in TBS (40mM Tris, 40mM NaCl, pH8.3) and blocked with 1% bovine serum albumin in TBS for 90min at 37°C. Samples were incubated with plasma (150µl) or platelet (100µl) reaction mix for 7.5 min at 25°C; (Plasma/platelet mix: TBS/TBS plus 0.2% Tween-20 containing 110µM/220µM dithiothreitol (Sigma Aldrich), 333µM/1332µM biotin-pentylamine (Pierce), 11mM/22mM CaCl<sub>2</sub>, 1.1U/2.2U.ml<sup>-1</sup> thrombin (Calbiochem)). Reactions were quenched with 200µl of 200mM ethylenediamine tetra-acetic acid (EDTA), incubated for 1h at 37°C with 100µl (plasma assay) or 200µl (platelet assay) of alkaline phosphatase-labelled streptavidin (2µg/ml, Sigma Aldrich) in TBS containing 1% bovine serum albumin and 0.1% Tween-20. Subsequently, 100µl of 2.7mM 4-nitrophenol phosphate (Sigma) in 1M diethanolamine pH9.8 was added and A<sub>405nm</sub> was recorded every min over the linear range at 30°C. Mice of either genders were used, except for Flt3-cre<sup>4</sup> and CD11b-cre<sup>5</sup> mice, which carry the transgene on the Y-chromosome.
FXIII-A antigen assay.
Plasma samples and platelet pellets (10x10⁶ cells) were mixed 1:4 with NuPAGE loading buffer (Life Technologies), resolved on 8% SDS gels and transferred to nitrocellulose membranes (Bio-Rad). Membranes were probed with sheep anti-FXIII-A antibody SAF13A (Enzyme Research Laboratories), mouse anti-β-actin (Sigma Aldrich) or chicken anti-α-1 antitrypsin (Immune systems), washed and incubated with rabbit anti-sheep-, rabbit anti-mouse- (Dako) and goat anti-chicken (Abcam) horseradish peroxidase, respectively. Immunoreactive bands were detected by chemiluminescence (Clarity, Bio-Rad) and analysed using the Fuji Science Imaging System with AIDA Image Analyzer software (Raytest Isotopenmessgeräte). For each assay the FXIII-A/loading control ratios of the cre/lox crosses were compared to the FXIII-A/control ratio of a WT sample which was present on the same gel.

Macrophage culture.
Epiphyses were removed from bones rinsed in 70% ethanol and PBS and BM cells harvested in RPMI medium containing 100U/ml penicillin, 0.1mg/ml streptomycin and 0.25µg/ml amphotericin B. Homogenised extracts were passed through a 70µm filter, pelleted and resuspended in RPMI containing 20% foetal calf serum, 2nmol/ml L-glutamine and 50ng/ml macrophage colony stimulating factor-1. Cells were plated at a density of 4x10⁶.ml⁻¹. At day 4, 10ml complete RPMI was added. Washed cells were snap frozen at day 7, in preparation for RNA analysis.

Quantitative PCR.
Nucleic acids were released into TRlzol (ThermoFisher) using a TissueLyser II (Qiagen) for organ samples or by gentle mixing to disperse cultured cells. Nucleic acids were precipitated with 100% ethanol and contaminating DNA removed with the DNA-free kit (ThermoFisher). RNA was transcribed to cDNA using the High Capacity Reverse Transcription Kit (ThermoFisher). qPCR was carried out in duplicate with a commercial SyBr Green PCR mix (Vectorlab) and used as negative controls for the FXIII-βHis PCR. Amplification cycles (95°C for 10 min, 45 cycles of 95°C for 10 sec, 60°C for 1 min) were followed by a melting curve to ensure a single product was amplified. β-actin and ribosomal protein L32 (RPL32) were used as housekeeping controls.

Immunofluorescent detection of FXIII-A and CD163 on heart sections.
Mouse hearts were either perfused with 4% paraformaldehyde in PBS, excised and processed for paraffin embedding or were frozen in OCT compound and stored at -80 degrees prior to processing. FXIII-A detection was performed on 4µm cross sections using sheep anti FXIII-A (Enzyme Research Laboratories) followed by donkey anti sheep conjugated with Alexa Fluor 555 (ThermoFisher) while CD163 detection was performed with rabbit anti CD163 (M-96, Santa Cruz) with donkey anti rabbit conjugated with Alexa Fluor 488 (ThermoFisher). Slides were sealed using Vectashield containing DAPI (Vector Laboratories). Fluorescence imaging microscopy was performed using an Olympus BX61WI inverted microscope with an XC10-IR camera under the control of CellSens software (Olympus).

Immunohistochemical detection of FXIII-A and CD163 on consecutive heart sections.
WT mouse hearts and livers and FXIII-A KO hearts were perfused with 4% paraformaldehyde in PBS, excised and processed for paraffin embedding. Consecutive cross sections (4µm) were deparaffinised, rehydrated and stained for either FXIII-A or CD163. FXIII-A KO hearts sections were used as a negative staining control. Heart sections of FXIII-A KO mice were used as negative controls for the FXIII-A staining. WT liver sections were used as the recommended positive control for CD163 staining, while sections without primary antibody were used as negative controls. For FXIII-A detection peroxidase activity was blocked using 30% H₂O₂ in methanol and incubated in rabbit serum from the Vectastain ABC-HRP kit (Vectorlab PK-4006). Sections were incubating with sheep anti FXIII-A (Enzyme Research Laboratories) and an Avidin/HRP biotinylated rabbit anti sheep antibody. Diaminobenzidene (DAB) substrate (Vectorlab SK-4100) was used for visualisation. For CD163 staining antigen
retrieval was performed by pressure cooking for 2 min in 100mM Tris, 1 mM EDTA, pH 9. Sections were blocked in goat serum from the Vectastain ABC-AP kit (Vectorlab AK-5001). An additional Avidin B blocking was performed and sections were incubated with rabbit anti CD163 (M-96) (Santa Cruz 33560) and an Avidin/alkaline phosphatase biotinylated goat anti rabbit antibody. Alkaline phosphatase substrate 3-amino-9-ethylcarbazol (red, Vectorlab SK-5001) was used for visualisation. Slides were counterstained with haematoxylin, dehydrated and mounted. Brightfield microscopy was performed using an Olympus BX61WI inverted microscope with an XC10-IR camera under the control of CellSens software (Olympus). The proportion of FXII-Apos to FXIII-Aneg heart cells was counted in 3 representative images.

Immunofluorescent detection of FXIII-A and TG2 in human macrophages.
Peripheral blood mononuclear cells were isolated from human blood using Ficoll Paque (GE Healthcare) and monocytes were subsequently purified using anti CD14 MACS beads (Miltenyi Biotec) following manufacturers' protocols. Cells were cultured for 6 days on glass coverslips in macrophage serum-free medium (Gibco/Thermo) containing 20ng/ml recombinant macrophage colony stimulating factor (M-CSF, eBioscience). THP-1 cells (acquired from ECACC) were cultured in RPMI 1640 medium supplemented with 10% foetal calf serum (PAA labs), glutamine (2mM) and antibiotic/antibiotic mix (Sigma). Prior to analysis, cells were incubated with phorbol myristate acetate (PMA) at 500ng/ml for 3h, washed three times in Dulbecco’s modified phosphate-buffered saline (DPBS), seeded onto glass coverslips then cultured for 3 days.

For cell surface labelling, cells were placed on ice, washed 3 times with ice-cold DPBS and blocked for 30 min with DPBS containing 2% w/v bovine serum albumin (BSA) and a 1:10 dilution of Fc Receptor blocker solution (Miltenyi Biotec). Cells were rinsed in DPBS/BSA and incubated for 1h with primary antibodies diluted in DPBS/BSA (anti FXIII-A: SAF13A-AP 2μg/ml (Enzyme Research Laboratories), anti TG2 mixed monoclonal antibodies CUB7402 and TG100 1μg/ml (Thermo Fisher), anti giantin rabbit polyclonal antiserum 1:1000, ab24586 (Abcam)). After 3 further washes in DPBS/BSA, cells were incubated for 45 minutes with 0.5μg/ml multiple labelling grade secondary antibodies (DyLight 488 donkey anti rabbit, DyLight 549 donkey anti sheep and DyLight 649 donkey anti mouse (Jackson Immunoresearch)), washed 3 times with DPBS/BSA and twice with DPBS before fixation with warmed 3% paraformaldehyde in DBPS (pH7.4). Subsequently samples were washed 3 times in DBPS, quenched for 10 minutes in 50mM NH₄Cl in DBPS and washed a further 3 times in DPBS. Coverslips were mounted using ProLong Gold antifade with DAPI (Invitrogen) and single 1μm thick confocal sections through the centre of the cell were imaged on a Zeiss LSM510 inverted laser-scanning confocal microscope using a 63x oil 1.4NA objective at 1024x1024 resolution.

Statistics.
For all assays performed n was ≥3 unless otherwise stated. Data are reported as mean ± SEM unless otherwise indicated. ANOVA tests were followed by Student t tests. P values below 0.05 were considered significant.
Reference List


Beckers et al Supplemental Figures

Cre/lox studies identify resident macrophages as the major source of circulating coagulation Factor XIII-A.

Cora M.L. Beckers1*, Kingsley R. Simpson1*, Kathryn J. Griffin1*, Jane M. Brown1, Lih T. Cheah1, Kerrie A. Smith1, Jean Vacher2, Paul A. Cordell1, Mark T. Kearney1, Peter J. Grant1# and Richard J. Pease1#

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*CMLB., KRS. and KJG. These authors contributed equally to this article.
#PJG. and RJP. These authors share senior authorship.

Running title: Resident macrophages maintain plasma FXIII-A

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Supplementary Figure I. Lox P sites were inserted into the mouse $F13a1$ gene to span coding exon 7 (genomic exon 8), at the sites numbered according to the NCBI reference genome for the C57BL/6 mouse strain, NC000079.6 (chromosome 13). A FRT site remains after flp recombinase-mediated excision of the neomycin phosphotransferase cassette that was used for selection.

Supplementary Figure II. Genomic DNA, isolated from the livers of FXIII-A$^{\text{Flox/Flox}}$ mice and from FXIII-A$^{-/-}$ mice (CMV-cre recombed FXIII-A$^{\text{Flox/Flox}}$ mice), was mixed in known proportions and subjected to quantitative PCR to establish a calibration curve, using primer pairs shown in Supplementary Table 1.
Supplementary Figure III. **A**) FXIII-A staining on FXIII-A−/− heart section is shown as a negative control (no antigen) for the DAB staining protocol. **B**) Negative (no primary antibody) and positive controls for CD163 alkaline phosphatase staining (red) on liver sections. **C**) Negative (no primary antibody) and positive controls for CD163 staining on heart sections. **D**) FXIII-A and **E**) CD163 staining of consecutive WT heart sections. FXIII-A positive cells (brown) that are also clearly CD163 positive (pink) are indicated in the enlargements of D and E by green arrows. In the case of other FXIII-A positive cells co-staining with CD163 cannot be determined (yellow arrows). This suggests that a proportion of the FXIII-A positive cells in the heart are also CD163 positive. Scale bars represent 50 μm.
Supplementary Figure IV. A) The gDNA from the ear, liver and bone marrow of Flt3-cre.Flox mice was analysed by PCR, together with gDNA from mice of the genotypes shown. In a single Flt3-cre.Flox mouse (#1, which showed no recombination in ear notch gDNA) efficient recombination of the F13a1 gene occurred in bone marrow, while in Flt3-cre.Flox mice #2,3,4 minimal or no recombination occurred. In mouse #1, ~50% recombination occurred in liver, in line with reports that a high proportion of adult liver cells are Flt3-dependent. The 196 base pair (bp) band represents the KO allele, the 177bp the floxed allele and the 126bp band the WT allele. B) Plasma and platelet FXIII-A activity (red bars) and protein (blue bars) levels were decreased in the efficiently recombined Flt3-cre.Flox mouse #1, but not in the other, non-recombined mice of the same genotype. Results are displayed as mean±SEM.
Supplementary Figure V. Cultured adherent human monocyte-derived macrophages (MDM) or human monocytic leukaemic cells (THP-1) were treated with antibodies to TG2 (green) or FXIII-A (red), under conditions where cell integrity was preserved (as confirmed by the absence of giantin staining, not shown). Cell nuclei detected with DAPI are shown in blue. Intact cells showed a ring of TG2 staining on the outer face of the plasma membrane. Very faint staining with the anti-FXIII-A antibody did not exceed the isotype control, and did not reveal cell-surface labelling. As previously observed,² cytosolic FXIII-A staining was apparent in permeabilised cells, while permeabilisation also resulted in staining of the Golgi apparatus with giantin (not shown). Using acquisition settings optimised for experimental samples, no labelling of cells was detected when primary antibodies were omitted from the staining procedure. Scale bar represent 20μm.
## Supplementary Table I

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### References
