Platelets Play an Essential Role in Separating the Blood and Lymphatic Vasculatures During Embryonic Angiogenesis

Laura Carramolino, Joana Fuentes, Clara García-Andrés, Valeria Azcoitia, Dieter Riethmacher, Miguel Torres

Rationale: Several mutations that impair the development of blood lineages in the mouse also impair the formation of the lymphatic vasculature and its separation from the blood vasculature. However, the basis for these defects has remained unknown because the mutations characterized affect more than one blood lineage.

Objective: We tested the hypothesis that megakaryocytes/platelets are required for the formation of the lymphatic vasculature and its separation from the blood vascular system.

Methods and Results: We characterized the vascular patterning defects of mice deficient for the homeodomain transcription factor Meis1 (myeloid ecotropic viral integration site 1), which completely lack megakaryocyte/platelets. Meis1 null embryos fail to separate the blood and lymphatic vasculature, showing blood-filled primary lymphatic sacs and superficial lymphatic vessels. To test the involvement of megakaryocytes/platelets in this phenotype, we generated megakaryocyte/platelet-specific deficient mice by targeted lineage ablation, without affecting other blood lineages. This model reproduces the lymphatic/blood vasculature separation defects observed in Meis1 mutants. A similar phenotype was induced by antibody-mediated ablation of circulating platelets in wild type mice. Strong association of platelets with vascular endothelium at regions of contact between lymphatic sacs and veins confirmed a direct role of platelets in the separation of the 2 vasculatures.

Conclusions: In addition to their known protective function in the response accidental vascular injury, platelets are also required during embryonic lymphangiogenesis for the separation of the nascent lymphatic vasculature from blood vessels. (Circ Res. 2010;106:1197-1201.)

Key Words: megakaryocytes • platelets • lymphatics • Meis1 • angiogenesis • lymphangiogenesis

The mammalian lymphatic vasculature develops at midgestation from lymphendothelial precursors produced from embryonic veins.1 Endothelial cells in different regions of the cardinal veins start to express the lymphatic growth factor Proxl at embryonic development day (E)10.5.2 This activates expression of lymphatic-specific molecules and the formation of lymphatic sacs from the vein-derived lymphendothelial precursors, stimulated by vascular endothelial growth factor-C/vascular endothelial growth factor receptor 3.3,4 The mature postnatal lymphatic vasculature is completely independent of the blood vascular system, except for the connections of the thoracic and right ducts with the subclavian veins, through which lymph drains into the blood circulation. At these junctions, specialized valves prevent blood reflux into the lymphatic vessels.5 However, during development, connections between the venous system and the forming lymphatic vasculature lack valves and therefore additional mechanisms must ensure their separation. Mutations in Syk and Slp-76,6,7 and Runx1,8 all affecting the development of blood lineages, block separation of the blood and lymphatic vasculature; however, the nature of the cell lineages involved and the mechanisms by which they control blood/lymphatic vessel separation remains unclear, because all mutations studied affect more than one blood lineage. Meis1 (myeloid ecotropic viral integration site 1) encodes a homeodomain transcription factor important for definitive hematopoietic stem cell development in the...
embryo and essential for megakaryocyte lineage development. Here, through characterization of Meis1-deficient mice and targeted ablation of megakaryocytes/platelets, we demonstrate an essential role for platelets in the separation of the blood and lymphatic vasculature.

**Methods**

Immunohistochemistry and immunofluorescence were performed as previously described. Primary antibodies were as follows: anti-CD31 (553370) and anti-CD41 (553847) (BD Biosciences-Pharminagen); anti-CD61 (NB100–79980CC, Novus Biologicals); anti-Ly-1 (103-PA505 ReliaTech); and anti-Meis1. Secondary antibodies were as follows: biotinylated goat antirat (Ab7096, Abcam) and goat anti-rabbit biotin (111-066-003, Jackson Immunoresearch), followed by Streptavidin-Alexa 488 (s-11223, Invitrogen) or -Cy3 (016-160-084, Jackson Immunoresearch). For immunohistochemistry, we used Vectastain ABC (Vector Laboratories) with alkaline phosphatase (AK-5000) and developed with FastRed (11496549001, Roche Diagnostic).

Whole mount in situ hybridization was performed using standard procedures. Riboprobes were obtained by SP6 polymerase transcription from cDNA PCR-amplified fragments. Meis1 mutant mice were backcrossed to C57BL/6J mice for more than 20 generations. Platelet factor (PF)4-Cre and R26: lacZbpAfoxDTA were backcrossed to the C57BL/6J background for more than 10 generations.

To transiently deplete platelets during gestation, pregnant C57BL/6J females received IP injections twice, at E11.5 postcoitum and 9 hours later, with 125 μg (100 μL) of rabbit anti-mouse thrombocyto antibody (AIA31440 Accurate Chemical). Animals were housed in accordance with Spanish bioethical regulations for laboratory animals.

**Results and Discussion**

Meis1-deficient embryos accumulate blood at ectopic foci, resulting eventually in generalized edema with internal hemorrhage. The distribution of ectopic blood foci matches the pattern of nascent lymphatic vessels at E12.5 and E13.5 (Figure 1a through 1d), and analysis of endothelial and lymphoendothelial markers identified the ectopic blood foci as the recently formed lymphatic sacs, which were filled with erythrocytes (Figure 1e through 1g and 1i through 1k). Meis1 is not expressed in blood vascular endothelial cells or lymphatic endothelium (Figure 1h and 1l), suggesting a nonautonomous involvement of nonvascular cells. We therefore explored the involvement of Meis1 blood lineage defects in this phenotype. At the stage when the first lymphatic defects are observed in Meis1 mutant embryos the only blood cells severely affected are the megakaryocytes. Expression of the megakaryocyte-specific marker cxcl7 is first detected in WT embryos at E9.5, in cells distributed throughout the embryo, but not in the yolk sac (Figure 2a). From E10.5, the yolk sac and the
megakaryocyte-depleted fetuses survive to E15.5 and do not show liver hypoplasia (Figure 3h through 3k; Figure 1b and 1d). Blood-filled peripheral lymphatic vessels are still evident at these later stages (Figure 3i through 3k, 3m through 3o, and 3q through 3s).

To test whether platelets were involved in the observed defects, we injected pregnant females twice, at 11.5 and 12 days postcoitum, with anti-thrombocyte antibody. Treated fetuses examined at E12.5 showed ectopic blood foci similar to those observed in Meis1-null and PF4-deleted embryos (Figure 4a through 4c; N=6/8), and histological analysis confirmed correspondence of these foci to the nascent lymphatic sacs (Figure 4d through 4g). Consistently, circulating platelets, which are abundant in untreated E12.5 WT embryos, were undetectable in Meis1-null and PF4-deleted embryos (Figure 4h through 4m). CD41 staining of anti-platelet–treated embryos confirmed that the megakaryocyte cell population was unaffected (Figure 4o), but that circulating platelets were agglutinated in large aggregates. Analysis of the junctions between primary lymphatic sacs and cardinal veins detected platelets adhering specifically to the lymphatic and venous endothelia at the sites where the 2 vasculatures meet (Figure 4r, 4s, and 4u), but not to endothelia outside this region (Figure 4r, 4h, and 4i).

These results demonstrate a morphogenetic role for platelets during the process that separates the blood and lymphatic vasculatures. Adhesion of platelets to the vascular wall suggests that they are activated by contact with the endothelium at the lymphatic/venous interface. Platelets are not components of lymph and therefore might be activated on contact with lymphendothelial-specific sur-

megakaryocyte development is abrogated in Meis1 mutants. Whole mount in situ hybridization detection of cxcl7 (pro-platelet basic protein) (a through c; f through h) and cxcl4 (PF4) (d and i) in WT (a through d) and Meis1-deficient embryos (f through i). The presence of positive cells in peripheral tissues of WT embryos is evident from E9.5 (see high-magnification in inset in a) and in the liver from E10.5 (b through d). Expression is abolished in Meis1-deficient embryos (f through i). Arrowheads mark the liver primordium. e and j, Immunofluorescent detection of CD41 (red) in sections of WT (e) and Meis1-deficient E12.5 livers (j). YS indicates yolk sac.
face molecules, thus preventing connections between the lymphatic and venous vasculatures by forming a structural barrier. This model is strongly supported by evidence that platelets are activated by contact with the lymphendothelial molecule podoplanin and by the occurrence of blood–lymphatic separation defects in podoplanin-deficient mice, but additional local signaling roles of platelets cannot be discarded. We suggest that the known involvement of Syk and SLP-76 in platelet activation and the defective megakaryocyte differentiation in Runx1 mutants contribute to the blood/lymphatics separation defects in these mutants. These results indicate that platelets, in addition to their role in repairing accidental vascular injuries, play a morphogenetic role during angiogenesis that allows the proper separation of blood and lymphatic circulation.

Acknowledgments
We thank Radek Skoda (Basel) for the PF4-Cre mice; Tamara Córdoba and Virginia García for mouse care; Silvia Vela for mouse genotyping; Rosin Doohan for histology; José Manuel Ligos, Mariano Vitoñ and Raquel Nieto for FACS; and Simon Bartlett for text editing.

Sources of Funding
The Centro Nacional de Investigaciones Cardiovasculares is supported by the Spanish Ministry of Science and Innovation and the Pro-CNIC Foundation. This work was supported by Spanish Ministry of Science and Innovation (RD06/0010/0008 and BFU2009-08331/BMC) and the EU COST program (COST-BM0805).

Disclosures
None.

References

Figure 4. Platelets are required for the separation of the lymphatic and blood vasculatures. *a* through *c*, Ectopic blood foci (arrowheads) in the primary lymphatic sacs of E12.5 fetuses after anti-Platelet treatment. *d* through *g*, Immunohistochemical detection of Lyve-1 and DAPI staining confirms ectopic blood in lymphatic sacs of anti-platelet–treated fetuses. *h* through *m*, Immunohistochemical detection of CD41 in large vessels of E12.5 fetuses shows abundant platelets in the circulation of WT specimens (*h* and *i*) and their absence in Meis1-deficient specimens (*j* and *k*) and PF4-Cre; Rosa26R-LacZbp-DTA specimens (*l* and *m*). *o* and *p*, CD41 staining in E12.5 anti-platelet–treated fetuses demonstrates preservation of the liver megakaryocyte population (*o*) and aggregation of circulating platelets (*p*). *q* through *s*, Immunofluorescence detection of PECAM, Lyve-1, and CD41 and DAPI staining at the venous–lymphatic junction indicates that the CD41 signal corresponds to platelets (arrowheads) and not to nucleated CD41-expressing cells.


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noreceptor signaling. *Arterioscler Thromb Vasc Biol*. 2006;26:


### Novelty and Significance

**What Is Known?**

- The lymphatic vasculature emerges during mammalian embryonic development from preexisting blood vessels through a centripetal sprouting process.
- The separation of the blood and lymphatic circulations is linked to the differentiation of the blood lineages, but until now, it was unknown which lineage controls blood/lymphatic vessel separation.

**What New Information Does This Article Contribute?**

- Using 3 independent methods to eliminate the megakaryocyte/platelet lineage in mice, we show that platelets are required for the separation of the blood and lymphatic vasculatures.
- This novel action of platelets involves their specific activation and adhesion with the endothelium at the junctions between blood and lymphatic vasculatures.
- These findings identify a previously unknown morphogenetic role for platelets and hint at a general role in vascular morphogenesis and remodeling.

Separation of the blood and lymphatic vasculatures is disrupted by mutations that affect blood lineage differentiation, but the specific lineages and mechanisms involved were unknown. We show that specific elimination of the megakaryocyte/platelet lineage results in blood-filled lymphatic vessels, indicating a failure to separate the blood and lymphatic circulations. Adhesion of platelets at the junctions between blood and lymphatic vasculatures indicates that local activation of platelets is involved in this process. These results identify a previously unknown morphogenetic role for platelets during lymphangio-

genesis and suggest a general role of platelets in vascular morphogenesis and remodelling potentially relevant in vascular disease.
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Circ Res. 2010;106:1197-1201; originally published online March 4, 2010;
doi: 10.1161/CIRCRESAHA.110.218073
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/106/7/1197

Data Supplement (unedited) at:
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Methods

Antibody staining

Staged mouse embryos were washed with PBS and fixed in 4% PFA overnight. Embryos were paraffin embedded or cryopreserved in OCT or sucrose-gelatin mix. Primary antibodies used were rat-anti mouse CD31 (553370) and CD41 (553847) (BD Biosciences-Pharmingen); rabbit anti human CD61 (NB100-79980CC, Novus Biologicals, Littleton, CO); rabbit anti mouse Lyve1 (103-PA50S ReliaTech, Wolfenbüttel, Germany). Rabbit anti-mouse Meis1 was generated in house. Secondary antibodies were biotinylated goat anti-rat (Ab7096, Abcam, Cambridge, UK) and goat anti rabbit biotin (111-066-003, Jackson Immunoresearch) followed by Streptavidin-Alexa 488 (s-11223, invitrogin) or -Cy3 (016-160-084, Jackson Immunoresearch). For immunohistochemistry we used the Vectastain ABC detection system (Vector Laboratories) with alkaline phosphatase (AK-5000), followed by signal development with FastRed (11496549001. Roche Diagnostic GmbH, Manheim, Germany).

Whole-mount in situ hybridization

Whole mount in situ hybridization was carried out manually as described or in an Insitu Pro VS robot (Intavis AG Bioanalytical Instruments, Köln, Germany). In both cases riboprobe was obtained by SP6 polymerase replication of a PCR fragment from E12.5 liver cDNA. Oligonucleotides were as follows: PBPP (CXCL7) 1U, GCC TGC CCA CTT CAT AAC CT; PBPP 1L, ATT TAG GTG ACA CTA TAG AAG CGA AAA CTG CTT GAC TCC; Pf4 1L, AGC CCT AGA CCC ATT TCC TC; PF4.SP6 1R, ATT TAG GTG ACA CTA TAG ATA TAG GGG TGC TTG CCG.

Mice

Animals were housed in accordance with Spanish bioethical regulations for laboratory animals. Meis1 mutant mice were maintained in heterozygosity by crossing with C57BL/6J mice (Harlan, UK). More than 20 backcrosses to the C57BL/6J background were done before analysis. Meis1-deficient and WT control embryos were generated by crossing Meis1-deficient heterozygous mice. Progeny were genotyped as described. PF4-Cre and R26:IacZbpAfloxDTA mice were both in the C57BL/6J background for more than 10 generations. Embryos obtained from these crosses were genotyped for both genotypes with oligonucleotides described in the cited references. To transiently deplete platelets during gestation we used an in vivo antibody treatment as previously described. Pregnant C57BL/6J females were injected i.p. twice with 125 μg of rabbit anti-mouse thrombocyte antibody (AIA31440 Accurate Chemical), at E11.5 p.c. and again nine hours later. Embryos were obtained from CO2 euthanasied females at E12.5 and fixed with 4% PFA overnight. As reported before, antibody treatment induced strong depletion of platelet counts and only minor alterations in other blood cell counts.

References to Methods


