

# Hematopoietic stem cell transplantation alters susceptibility to pulmonary hypertension in *Bmpr2*-deficient mice

Alexi Crosby<sup>1,\*</sup>, Mark R. Toshner<sup>1,\*</sup>, Mark R. Southwood<sup>2</sup>, Elaine Soon<sup>1</sup>, Benjamin J. Dunmore<sup>1</sup>, Emily Groves<sup>1</sup>, Stephen Moore<sup>1</sup>, Penny Wright<sup>3</sup>, Katrin Ottersbach<sup>4</sup>, Cavan Bennett<sup>4,5</sup>, Jose Guerrero<sup>4,5</sup>, Cedric Ghevaert<sup>4,5</sup> and Nicholas W. Morrell<sup>1</sup>

<sup>1</sup>Department of Medicine, University of Cambridge School of Clinical Medicine, Addenbrooke's Hospital, Cambridge, UK; <sup>2</sup>Department of Pathology, Papworth Hospital, Cambridge, UK; <sup>3</sup>Addenbrooke's Hospital, Cambridge, UK; <sup>4</sup>Department of Haematology, University of Cambridge, Cambridge Biomedical Campus, Cambridge, UK; <sup>5</sup>National Health Service Blood and Transplant, Cambridge Biomedical Campus, Cambridge, UK

## Abstract

Increasing evidence suggests that patients with pulmonary arterial hypertension (PAH) demonstrate abnormalities in the bone marrow (BM) and hematopoietic progenitor cells. In addition, PAH is associated with myeloproliferative diseases. We have previously demonstrated that low-dose lipopolysaccharide (LPS) is a potent stimulus for the development of PAH in the context of a genetic PAH mouse model of *BMPR2* dysfunction. We hypothesized that the hematopoietic progenitor cells might be driving disease in this model. To test this hypothesis, we performed adoptive transfer of BM between wild-type (Ctrl) and heterozygous *Bmpr2* null (Mut) mice. Sixteen weeks after BM reconstitution, mice were exposed to low-dose chronic LPS (0.5 mg/kg three times a week for six weeks). Mice underwent right heart catheterization and tissues were removed for histology. After chronic LPS dosing, Ctrl mice in receipt of Mut BM developed PAH, whereas Mut mice receiving Ctrl BM were protected from PAH. BM histology demonstrated an increase in megakaryocytes and there was an increase in circulating platelets in Ctrl mice receiving Mut BM. These findings demonstrate that the hematopoietic stem cell compartment is involved in the susceptibility to PAH in the Mut mouse. The results raise the possibility that hematopoietic stem cell transplantation might be a potential treatment strategy in genetic forms of PAH.

## Keywords

bone marrow, LPS, megakaryocytes

Date received: 25 June 2018; accepted: 27 August 2018

Pulmonary Circulation 2018; 8(4) 1–9

DOI: 10.1177/2045894018801642

Bone marrow (BM) disorders are recognized as a cause of pulmonary hypertension (PH), currently classified in group 5 PH.<sup>1</sup> This can be mediated through associated changes in thrombotic risk or by alternative mechanisms not well understood.<sup>2</sup> In patients with myelofibrosis it has been reported that up to 36% of patients have evidence of pulmonary arterial hypertension (PAH).<sup>3</sup> In the idiopathic form of PAH, Farha et al.<sup>4</sup> identified a subclinical myeloproliferative process and, unexpectedly, in non-affected family members similar myeloid abnormalities were described.

A number of studies have investigated the role of BM-derived cells in PAH. Patients with PAH have higher circulating numbers of CD133+ cells and CD34+ cells compared with healthy controls.<sup>4,5</sup> Studies of the peripheral circulating CD133+ fraction from PAH patients suggest

\*Equal contributors.

Corresponding author:

Nicholas W. Morrell, Department of Medicine, Box 157, Addenbrooke's Hospital, Hills Road, Cambridge CB2 0QQ, UK.

Email: nwm23@cam.ac.uk



Creative Commons Non Commercial CC BY-NC: This article is distributed under the terms of the Creative Commons Attribution-NonCommercial 4.0 License (<http://www.creativecommons.org/licenses/by-nc/4.0/>) which permits non-commercial use, reproduction and distribution of the work without further permission provided the original work is attributed as specified on the SAGE and Open Access pages (<https://us.sagepub.com/en-us/nam/open-access-at-sage>).

© The Author(s) 2018.

Article reuse guidelines:  
[sagepub.com/journals-permissions](https://sagepub.com/journals-permissions)  
[journals.sagepub.com/home/pul](https://journals.sagepub.com/home/pul)



there are more multipotent progenitors which showed a greater myeloid commitment.<sup>6</sup> Furthermore, it was previously shown that mice infused with CD133+ cells from PAH patients developed pulmonary vascular remodeling, thromboses, and right ventricular hypertrophy.<sup>6</sup> The potential importance of hematopoietic progenitors in the pathobiology of PAH has been suggested in the *Bmpr2* mutant mouse overexpressing the human mutation, R889x.<sup>7</sup> In this severe loss of function model of *BMPR2*, transplantation of R889x *Bmpr2* BM cells was sufficient to induce PAH.

Mutations in the gene encoding the bone morphogenetic protein type 2 receptor (*BMPR2*) are the commonest genetic cause of PAH. More than 70% of patients with familial PAH and up to 25% of patients with sporadic cases of idiopathic PAH carry a mutation in *BMPR2*. Dysfunction of *BMPR2* signaling has been shown by our group<sup>8,9</sup> and others<sup>10,11</sup> to contribute to non-genetic forms of PH in experimental animal models<sup>12,13</sup> and in man.<sup>8</sup> The *BMPR2* pathway plays an important role in the inhibition of cellular proliferation in pulmonary arterial smooth muscle cells and fibroblasts.<sup>14,15</sup> Several studies have shown that mutations in *BMPR2* promotes pulmonary artery endothelial cell apoptosis, proliferation, and dysfunction.<sup>5,16,17</sup>

Intriguingly, *Bmpr2* heterozygous null mice do not spontaneously develop PAH. In human heritable PAH, the penetrance of PAH in patients carrying heterozygous *BMPR2* mutations is reduced (on average 20–30%) and the concept that a “second hit” is needed has arisen. Inflammation has been posited as one possible trigger for disease.<sup>15,18</sup> Consistent with a role for inflammation driving the development of PAH, *Bmpr2* heterozygous null mice developed marked PAH when exposed chronically to low dose lipopolysaccharide (LPS) for six weeks, whereas wild-type mice did.<sup>19</sup>

Since inflammation is known to mobilize hematopoietic cells from the BM, we questioned whether transplantation of hematopoietic stem cells would influence susceptibility to PAH in *Bmpr2* heterozygous mice exposed to chronic low-dose LPS.

## Methods

### Description of mouse genetic models

Mice heterozygous for a null allele in *Bmpr2* (Mut) generated on a C57BL/6J strain were kindly provided by H. Beppu.<sup>20</sup> Hereafter, these mice will be referred to as Mut mice. The B6-tg CAG-EGFP mouse (Jax, USA) was used in order to be able to identify the transplanted BM cells.

### Hematopoietic stem cell transplantation

*Bmpr2*<sup>+/+</sup> C57/BL6 mice (Ctrl) (n = 22) were lethally BM irradiated using two split doses of 5 Gy<sup>21</sup> and transplanted with BM from GFP-expressing Ctrl mice (1 × 10<sup>6</sup> cells, n = 11) or BM from GFP-expressing *Bmpr2*<sup>+/-</sup> mice (Mut) (n = 11) via a tail-vein injection.<sup>7</sup> Mut mice (n = 8) were

lethally BM irradiated using two split doses of 5 Gy and transplanted with whole BM from GFP-expressing Mut mice or Ctrl mice (1 × 10<sup>6</sup> cells) via a tail-vein injection.<sup>22</sup> In order to prepare the BM cells for transplantation, the femurs were flushed and the BM cells were put through a 40-um strainer to remove debris. Cells were incubated with penicillin streptomycin, spun down at 100 rpm for 5 min at room temperature, and suspended in the desired volume of phosphate-buffered saline (PBS). Both male and female mice were used. All protocols and surgical procedures were approved by the local animal care committee.

### Assessment of bone marrow reconstitution

At four and 16 weeks post irradiation, the degree of BM reconstitution was determined by flow cytometry. All mice had a fully reconstituted BM determined by percentage GFP expression in the BM, by 16 weeks post irradiation (Suppl. Fig. 1). To assess the degree of BM reconstitution, peripheral blood was collected in EDTA-coated tubes (Sarstedt, Germany), red blood cells were lysed in Pharmlyse (BD Biosciences, USA), and samples were run on a flow cytometer (FACSCantoII, BD Biosciences, USA). To determine full blood counts, peripheral blood was collected in EDTA-coated tubes and analyzed on a Wooley ABC (mouse) analyzer (Bolton, UK).

### LPS administration

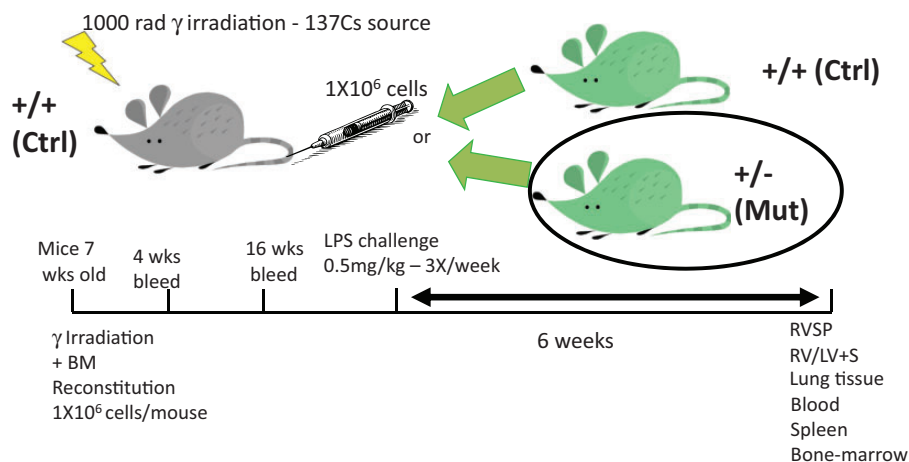
Mice were exposed to LPS derived from *Escherichia coli* O111:B4 (Sigma-Aldrich, St. Louis, MO, USA). Mice were injected intraperitoneally three times per week with LPS (0.5 mg/kg) and humanely killed after six weeks, as previously described.<sup>23</sup> In addition, control animals were injected with PBS. In all figures, mice were treated with LPS, except if explicitly stated.

### Measurement of indices of pulmonary hypertension

At the end of six weeks exposure to LPS, mice were anesthetized using fentanyl and medazolam before hemodynamic assessment. Body weight was recorded and right heart catheterization was performed to measure right ventricular systolic pressure (RVSP), as previously described.<sup>18</sup> The degree of right ventricular hypertrophy was determined from the RV/(LV+S) ratio, as previously described.<sup>18</sup>

### Lung tissue preparation

Following hemodynamic assessment, the left lung was fixed in situ in the distended state by infusion of 4% paraformaldehyde (PFA):OCT (Sakura, Japan) into the trachea, and then placed into 4% PFA for 2 h before embedding in OCT by freezing on dry ice. Samples were subsequently embedded in paraffin. The remaining lung lobes were frozen in liquid nitrogen.



**Fig. 1.** An example of the BM transplant protocol. Control (Ctrl) mice were irradiated and given a BM transplant from GFP-expressing Ctrl or mutant (Mut) mice. Mut mice were irradiated and given a BM transplant from GFP-expressing Ctrl or Mut mice. Mice were bled for reconstitution check at 4 and 16 weeks post irradiation. Mice were treated with LPS 3 times a week for 6 weeks. Mice then underwent right heart catheterization and tissue was removed.

### Pulmonary vascular morphometry

Lung tissues were stained with anti-smooth muscle  $\alpha$ -actin ( $\alpha$ -SMA) (DakoCytomation, UK) as previously described.<sup>24</sup> To measure the medial thickness of small pulmonary arteries associated with terminal bronchioles, vessels were photographed at 400 $\times$  magnification using MediaCybernetics software (Bethesda, MD, USA). Percentage wall thickness was determined using ImageJ and expressed as the percentage of the average width of the tunica media to the average diameter of the vessel ( $n=10$  arteries per animal), as previously described.<sup>24</sup> Peripheral muscularization was determined by counting the number of non-muscularized, partially muscularized, and fully muscularized vessels in each lung section.<sup>24</sup>

### Bone marrow histology

Femurs were collected and placed in 10% paraformaldehyde for 24 h and then transferred to 10% EDTA to soften and decalcify the bone for histology.<sup>25</sup> The EDTA was changed daily for three weeks. The femurs were then embedded into paraffin blocks and sectioned. Sections were stained with hematoxylin and eosin (H&E). Immunohistochemistry was performed for von Willebrand factor (vWF).

### Spleen histology

The spleens were fixed in 10% paraformaldehyde, embedded into paraffin blocks, and sectioned. Sections were stained with H&E. Photographs were taken at 400 $\times$  magnification with MediaCybernetics software (Bethesda, MD, USA).

### Statistical analysis

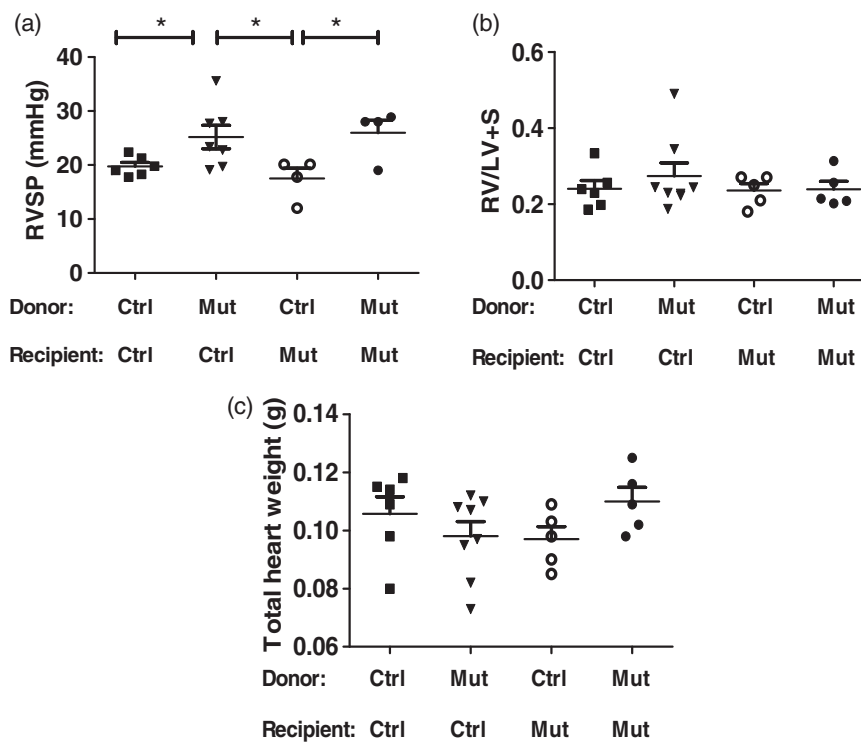
Where appropriate, whether data fitted a normal distribution were tested using the Kolmogorov–Smirnov method

(Prism, La Jolla, CA, USA). Where data did not conform to a normal distribution, a non-parametric analysis was applied. Data are presented as mean  $\pm$  standard error of the mean (SEM) or median, where appropriate. Data were compared using one-way ANOVA and a Tukey's post-hoc test, Student's t-test (parametric), or Mann–Whitney test (non-parametric) as appropriate.

## Results

### The *Bmpr2* status of transplanted hematopoietic stem cells alters susceptibility to PAH

In order to determine whether the *Bmpr2* status of hematopoietic stem cells plays a role in the development of PAH, adoptive transfer of BM was performed. Sixteen weeks after adoptive transfer, mice were injected with low-dose LPS three times per week for six weeks (Fig. 1).<sup>23</sup> Ctrl mice that received Mut BM demonstrated increases in RVSP ( $P < 0.05$ ) compared to their control counterparts (Fig. 2a). Conversely Mut mice that received Ctrl BM were protected from the development of PH during LPS exposure (Fig. 2a), when compared to controls ( $P < 0.05$ ). Ctrl mice transplanted with Ctrl BM, as expected, did not show any increase in RVSP following chronic LPS exposure. There was no difference in the degree of right ventricular hypertrophy (Fig. 2b) or total heart weight (Fig. 2c) between any of the groups. A separate cohort of mice that had undergone adoptive transfer were injected with PBS three times a week for six weeks (Suppl. Fig. 2). The only significant difference between the PBS cohort and the LPS cohort was that Ctrl mice transplanted with Ctrl BM and treated with LPS showed a significant reduction in RVSP ( $P < 0.05$ ). The number of Ctrl mice that received Mut BM and treated with PBS was too low to test for significance.



**Fig. 2.** (a) Bar graph showing right ventricular systolic pressure (RVSP), control mice with control BM (Ctrl/Ctrl) ( $n = 6$ ) or with mutant BM (Ctrl/Mut) ( $n = 7$ ), mutant mice with control BM (Mut/Ctrl) ( $n = 4$ ) or with mutant BM (Mut/Mut) ( $n = 4$ ). (b) Right ventricular index (RV/LV+S), Ctrl/Ctrl ( $n = 6$ ), Ctrl/Mut ( $n = 8$ ), Mut/Ctrl ( $n = 5$ ), Mut/Mut ( $n = 5$ ). (c) Total heart weight, Ctrl/Ctrl ( $n = 6$ ), Ctrl/Mut ( $n = 8$ ), Mut/Ctrl ( $n = 5$ ), Mut/Mut ( $n = 5$ ). All mice were treated with LPS. Data are presented as mean  $\pm$  SEM. \* $P < 0.05$ .

### Increased pulmonary vascular remodeling in mice transplanted with Mut bone marrow

Mice that received adoptive transfer of Mut BM and LPS exposure demonstrated an increase in medial thickness of the pulmonary arterioles associated with terminal bronchioles (Fig. 3a) ( $P < 0.05$ ). Representative images are shown in Fig. 3c. There were no differences in wall thickness between the two groups of mice with Mut BM. In addition, mice that received Mut BM exhibited a significant increase in the percentage of fully muscularized peripheral arterioles at the level of the alveolar ducts, compared with mice receiving Ctrl BM (Fig. 3b) ( $P < 0.05$ ).

### Mice with Mut bone marrow exposed to LPS exhibit heavier spleens

There was a significant increase in spleen weight ( $P < 0.05$ ) in Ctrl mice receiving Mut BM and exposed to LPS (Fig. 4a). In contrast, spleen weight did not increase in mice receiving Ctrl BM and exposed to LPS. Since only mice that received Mut BM exhibited an increase in spleen weight, we compared the splenic histology between groups. Spleen from mice transplanted with Mut BM exhibited increased numbers of granulocytes, erythrocytes, and megakaryocytes than mice with wild-type BM. In a quantitative analysis, mice transplanted with Mut BM exhibited more

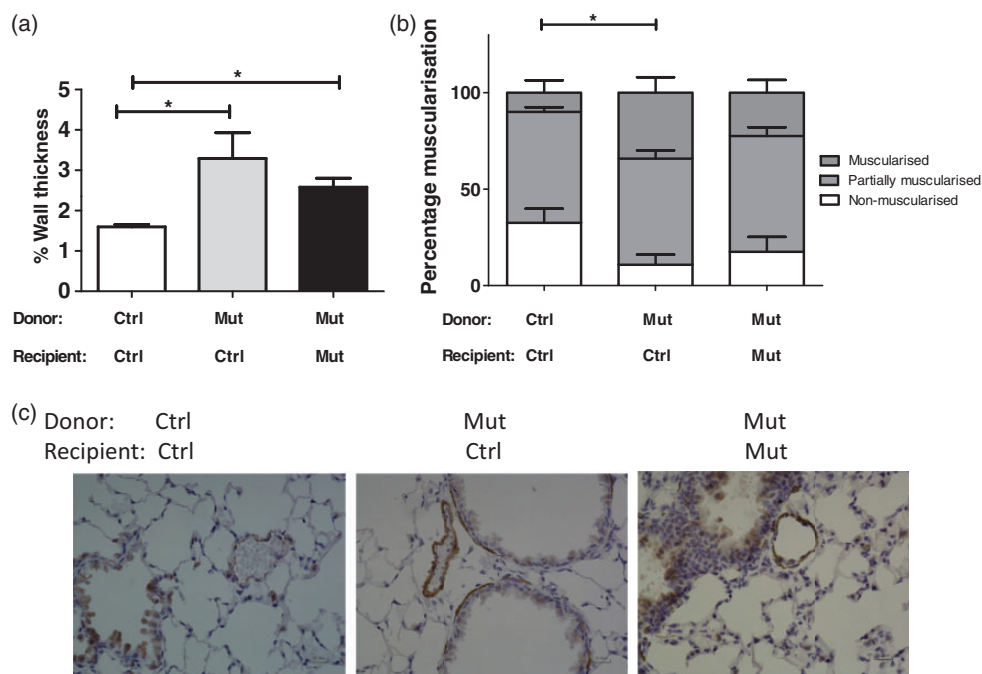
megakaryocytes in the spleen compared with mice with Ctrl BM (Fig. 4b). This was statistically significant in Mut mice with Mut BM ( $P < 0.05$ ) and narrowly failed to reach significance in Ctrl mice with Mut BM ( $P = 0.055$ ). Figure 4c shows representative H&E images of the spleen.

### Increased circulating platelets and bone marrow megakaryocytes in mice receiving Mut bone marrow

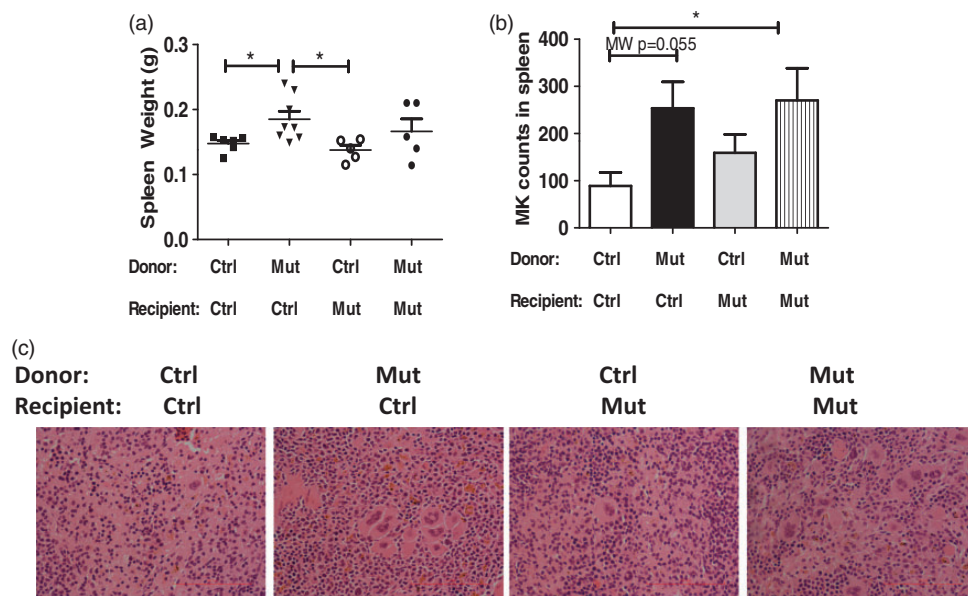
There were no differences in the numbers of peripheral circulating blood cells of all groups, except in the levels of platelets (Fig. 5e). We consistently observed increases in circulating platelet numbers ( $P < 0.05$ ) in mice receiving Mut BM exposed to LPS. Histological examination of the femurs showed an increase in megakaryocyte numbers in the femurs of Mut mice compared with Ctrl mice, and Ctrl mice receiving Mut BM (Fig. 6a). In animals that received LPS, there was a profound alteration of the BM, with reduced hematopoietic cells and a replacement of BM by expansion of adipocytes (Fig. 6b). This was seen in mice with both Ctrl and Mut BM (Suppl. Fig. 3) and is therefore predominantly an effect of LPS treatment.

## Discussion

We demonstrate that susceptibility to PAH can be conferred onto a wild-type (Ctrl) animal by transplantation



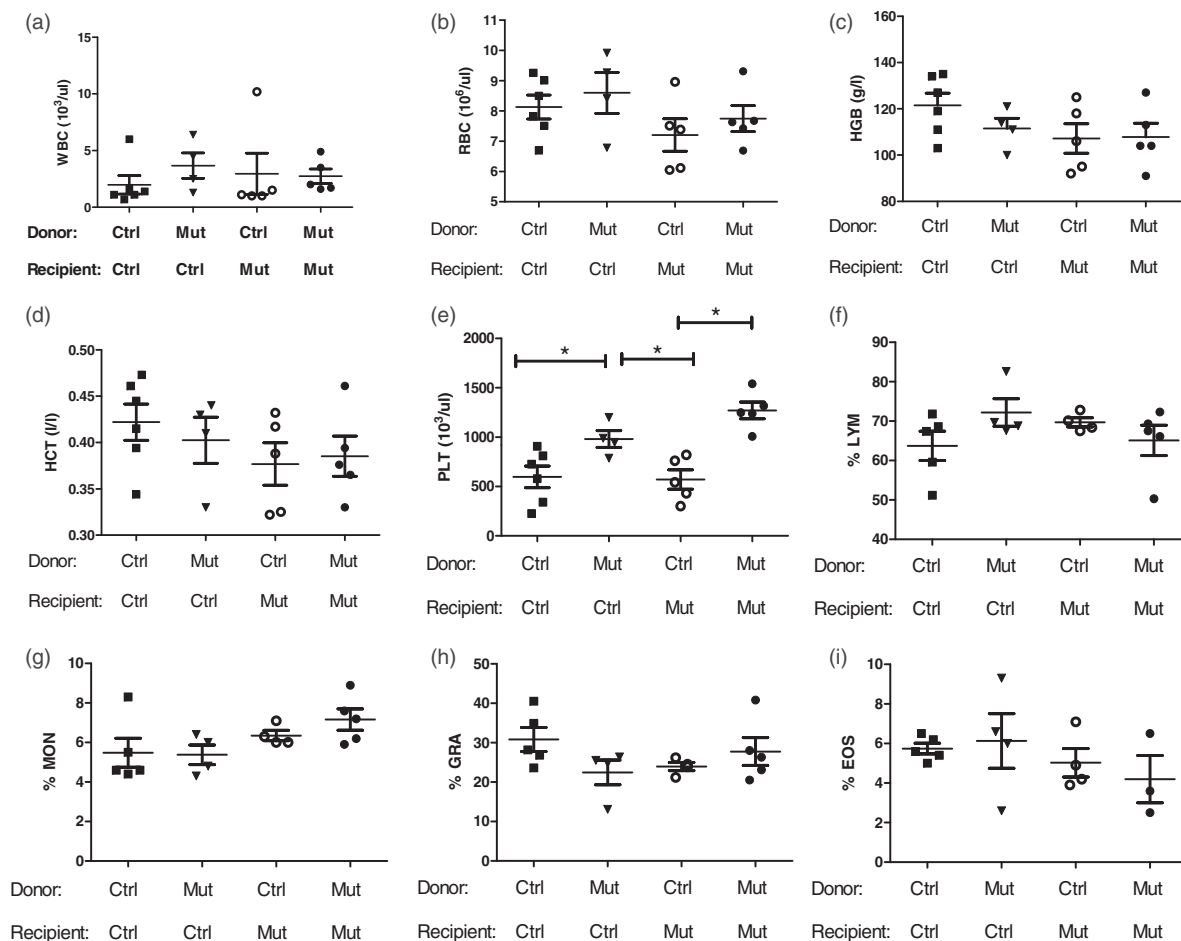
**Fig. 3.** (a) Bar graph showing percentage wall thickness of pulmonary arterioles at the level of the terminal bronchiole in control mice with control BM (Ctrl/Ctrl) (n = 3) or with mutant BM (Ctrl/Mut) (n = 3), or with mutant BM (Mut/Mut) (n = 4). \*P < 0.05 compared with Ctrl/Ctrl. (b) Bar graph showing the percentage muscularization in small peripheral vessels in Ctrl/Ctrl (n = 3), Ctrl/Mut (n = 3), Mut/Mut (n = 4). \*P < 0.05 compared with Ctrl/Ctrl. (c) Representative photomicrographs 400 × magnification of lung sections immunostained with α-smooth muscle actin (α-SMA). Scale bars 100 μm.



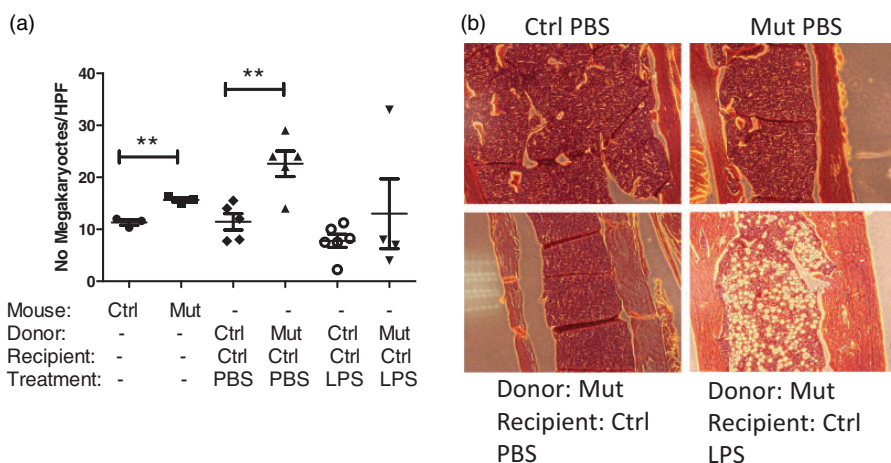
**Fig. 4.** (a) Spleen weight of control mice with control BM (Ctrl/Ctrl) (n = 6) or with mutant BM (Ctrl/Mut) (n = 8), mutant mice with control BM (Mut/Ctrl) (n = 5) or with mutant BM (Mut/Mut) (n = 5). (b) The number of megakaryocytes (MK) in sections of spleen from Ctrl/Ctrl (n = 5), Ctrl/Mut (n = 5), Mut/Ctrl (n = 2), Mut/Mut (n = 4). (c) Representative spleen images stained with H&E from all groups of mice. Images taken using 400 × magnification. Data are presented as mean ± SEM. \*P < 0.05.

of *Bmpr2*-deficient BM. We also demonstrate that transplanting Ctrl BM into Mut mice prevents the development of PAH. An increase in pulmonary vascular remodeling, enlarged spleens and an increase in circulating platelets

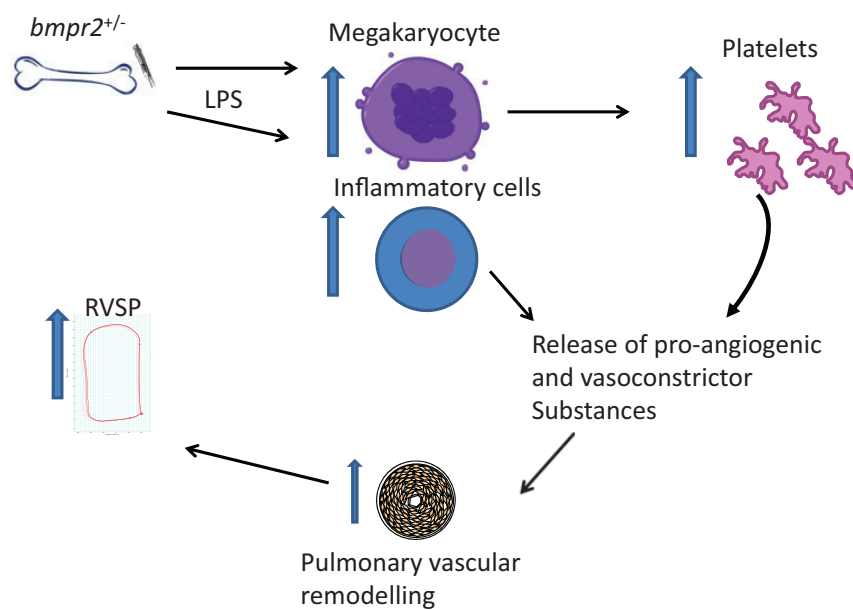
and an increase in megakaryocytes in the femurs were observed in all animals with a reduction of *Bmpr2* in the BM. This demonstrates the importance of the BM in the development of PAH.



**Fig. 5.** Bar graphs showing full blood count parameters from all groups of mice; control mice with control BM (Ctrl/Ctrl) (n = 6) or with mutant BM (Ctrl/Mut) (n = 4), mutant mice with control BM (Mut/Ctrl) (n = 5) or with mutant BM (Mut/Mut) (n = 5). (a) White blood count (WBC); (b) red blood cell count (RBC); (c) hemoglobin (HGB); (d) hematocrit (HCT); (e) platelet count (PLT); (f) percentage of lymphocytes (LYM); (g) percentage of monocytes (MON); (h) percentage of granulocytes (GRA); and (i) percentage of eosinophils (EOS). Data are presented as mean ± SEM. \*P < 0.05.



**Fig. 6.** (a) Plots showing the number of megakaryocytes per high power field (HPF) in baseline control (Ctrl) (n = 3) and mutant mice (Mut) (n = 3), control mice with control BM (Ctrl/Ctrl) treated with PBS (n = 5), control mice with mutant BM (Ctrl/Mut) treated with PBS (n = 4), (Ctrl/Ctrl) treated with LPS (n = 5), (Ctrl/Mut) treated with LPS (n = 6). (b) Histological sections of the BM with H&E stain in Ctrl mice with PBS, Mut mice with PBS, Ctrl/Mut treated with PBS, Mut/Ctrl with LPS. Data are presented as mean ± SEM. \*\*P < 0.01.



**Fig. 7.** Summary slide showing the potential mechanism of how a reduction in BMPR2 in the BM can lead to an increase in right ventricular systolic pressure (RVSP) through an increase in inflammatory cytokines, driving an increase in pulmonary vascular remodeling.

We note that the hemodynamic responses to BM transplant alone are more modest than in our original description of the *Bmpr2<sup>+/-</sup>* LPS model.<sup>23</sup> This is consistent with a role for the immune system in development of PAH but highlights that there is likely an additive effect on top of loss of BMPR2 in the pulmonary vasculature. In addition, these mice underwent a BM transplant and a different lot number of LPS was used in the current study. To some degree this could also help explain the differences in pressure between the studies.

The increase in spleen weight in Mut mice exposed to LPS is particularly interesting. There have been previous reports of splenomegaly in idiopathic PAH (IPAH).<sup>26</sup> The spleen plays an important role not only in the degradation and storage of red blood cells but also plays a central role in the mononuclear phagocytic system.<sup>27</sup> The red pulp of the spleen has been shown to be a reservoir that contains > 50% of the body's monocytes.<sup>27</sup> It may be that extramedullary hematopoiesis is occurring in the spleens of these mice, contributing to the increase in circulating platelets observed.

In mice with Mut BM, not only was there an increase in circulating platelets but also an increase in megakaryocytes in the femurs and in the spleen. Megakaryocytes are hematopoietic cells that account for approximately 0.05–0.1% of all nucleated BM cells.<sup>28</sup> Megakaryocytes produce platelets, which bud off the pseudopodia in the BM sinusoids. Each megakaryocyte can produce up to 1000–3000 platelets. It is possible that altering BMPR2 expression in the BM may affect megakaryocyte development or function.

We view it as surprising that the major difference in the response of mice with Ctrl BM and Mut BM to LPS were in the megakaryocytes and platelets. Platelets have not been extensively studied in PAH; however, they are well described

as having a role in regulation of vascular homeostasis. They are known to be a major regulator of angiogenesis releasing a number of bioactive substances such as PDGF, serotonin, and nitric oxide, which regulate vasoconstriction and thrombosis. In experimental models, platelets have been shown to play a role in the development of PAH.<sup>29</sup> It has recently been shown that platelets from patients with IPAH have a reduction in endothelial nitric oxide synthetase (eNOS) compared with platelets from controls.<sup>30</sup>

An interesting question as to whether GFP+ BM cells were incorporated into the lung tissue remains. Unfortunately, due to technical issues, we were unable to demonstrate this. This information would lead to a further mechanism for the development of PAH in these mice.

A working hypothesis is presented in Fig. 7. We have clarified that the BM-mobilized response is critical for the chronic LSP model of disease; however, we have not yet delineated how this is mediated. We do note an unexpected change in megakaryocytes and platelets with splenomegaly and future work will focus on whether this is pathogenetic.

The importance of the BM in *Bmpr2* mouse models has previously been established in a mice overexpressing mutant *Bmpr2<sup>7</sup>* and now we present data in the setting of *Bmpr2* deficiency combined with a second hit. One obvious question is whether donor-matched BM or even autologous *BMPR2* correction and transplantation can be a therapeutic avenue in PAH. Genetic rescue of autologous hematopoietic stem cells could be considered, reducing the need for immunosuppression after transplantation. A note of caution is appropriate given the example of scleroderma where BM transplantation is being pioneered and PH is known to be a risk factor for short-term complications.<sup>31</sup> Any proposed studies in PAH would have to carefully consider this. In

addition, it should be noted that this is a prevention study and not a reversal of existing disease.

In summary, the *Bmpr2*<sup>+/-</sup> LPS mouse model of PAH is partly driven by the BM-mobilized response and can be abrogated by transplantation of normal BM. This opens up potentially exciting questions about pathogenesis of the disease and novel treatment avenues.

### Conflict of interest

The author(s) declare that there is no conflict of interest.

### Funding

This work was supported by the MRC MDU Mouse Biochemistry Laboratory (MRC\_MC\_UU\_12012/5).

### References

- Galie N, Humbert M, Vachiery JL, et al. 2015 ESC/ERS Guidelines for the diagnosis and treatment of pulmonary hypertension: The Joint Task Force for the Diagnosis and Treatment of Pulmonary Hypertension of the European Society of Cardiology (ESC) and the European Respiratory Society (ERS); Endorsed by: Association for European Paediatric and Congenital Cardiology (AEPC), International Society for Heart and Lung Transplantation (ISHLT). *Eur Respir J* 2015; 46(4): 903–975.
- Guilpain P, Montani D, Damaj G, et al. Pulmonary hypertension associated with myeloproliferative disorders: a retrospective study of ten cases. *Respiration* 2008; 76(3): 295–302.
- Cortelezzi A, Gritti G, Del Papa N, et al. Pulmonary arterial hypertension in primary myelofibrosis is common and associated with an altered angiogenic status. *Leukemia* 2008; 22(3): 646–649.
- Farha S, Asosingh K, Xu W, et al. Hypoxia-inducible factors in human pulmonary arterial hypertension: a link to the intrinsic myeloid abnormalities. *Blood* 2011; 117(13): 3485–3493.
- Toshner M, Voswinkel R, Southwood M, et al. Evidence of dysfunction of endothelial progenitors in pulmonary arterial hypertension. *Am J Respir Crit Care Med* 2009; 180(8): 780–787.
- Asosingh K, Farha S, Lichtin A, et al. Pulmonary vascular disease in mice xenografted with human BM progenitors from patients with pulmonary arterial hypertension. *Blood* 2012; 120(6): 1218–1227.
- Yan L, Chen X, Talati M, et al. Bone marrow-derived cells contribute to the pathogenesis of pulmonary arterial hypertension. *Am J Respir Crit Care Med* 2016; 193(8): 898–909.
- Atkinson C, Stewart S, Upton PD, et al. Primary pulmonary hypertension is associated with reduced pulmonary vascular expression of type II bone morphogenetic protein receptor. *Circulation* 2002; 105(14): 1672–1678.
- Morrell NW, Yang X, Upton PD, et al. Altered growth responses of pulmonary artery smooth muscle cells from patients with primary pulmonary hypertension to transforming growth factor-beta(1) and bone morphogenetic proteins. *Circulation* 2001; 104(7): 790–795.
- Machado RD, Pauciulo MW, Thomson JR, et al. *BMPR2* haploinsufficiency as the inherited molecular mechanism for primary pulmonary hypertension. *Am J Hum Genet* 2001; 68(1): 92–102.
- Thomson JR, Machado RD, Pauciulo MW, et al. Sporadic primary pulmonary hypertension is associated with germline mutations of the gene encoding *BMPR-II*, a receptor member of the TGF-beta family. *J Med Genet* 2000; 37(10): 741–745.
- Long L, Crosby A, Yang X, et al. Altered bone morphogenetic protein and transforming growth factor-beta signaling in rat models of pulmonary hypertension: potential for activin receptor-like kinase-5 inhibition in prevention and progression of disease. *Circulation* 2009; 119(4): 566–576.
- Morty RE, Nejman B, Kwapiszewska G, et al. Dysregulated bone morphogenetic protein signaling in monocrotaline-induced pulmonary arterial hypertension. *Arterioscler Thromb Vasc Biol* 2007; 27(5): 1072–1078.
- Yang J, Davies RJ, Southwood M, et al. Mutations in bone morphogenetic protein type II receptor cause dysregulation of *Id* gene expression in pulmonary artery smooth muscle cells: implications for familial pulmonary arterial hypertension. *Circ Res* 2008; 102(10): 1212–1221.
- Yang X, Long L, Southwood M, et al. Dysfunctional Smad signaling contributes to abnormal smooth muscle cell proliferation in familial pulmonary arterial hypertension. *Circ Res* 2005; 96(10): 1053–1063.
- Davies RJ, Holmes AM, Deighton J, et al. *BMP* type II receptor deficiency confers resistance to growth inhibition by TGF-beta in pulmonary artery smooth muscle cells: role of proinflammatory cytokines. *Am J Physiol Lung Cell Mol Physiol* 2012; 302(6): L604–615.
- Yang X, Long L, Reynolds PN, et al. Expression of mutant *BMPR-II* in pulmonary endothelial cells promotes apoptosis and a release of factors that stimulate proliferation of pulmonary artery smooth muscle cells. *Pulm Circ* 2011; 1(1): 103–110.
- Long L, MacLean MR, Jeffery TK, et al. Serotonin increases susceptibility to pulmonary hypertension in *BMPR2*-deficient mice. *Circ Res* 2006; 98(6): 818–827.
- Soon E, Crosby A, Southwood M, et al. Bone morphogenetic protein receptor type II deficiency and increased inflammatory cytokine production. A gateway to pulmonary arterial hypertension. *Am J Respir Crit Care Med* 2015; 192(7): 859–872.
- Beppu H, Kawabata M, Hamamoto T, et al. *BMP* type II receptor is required for gastrulation and early development of mouse embryos. *Dev Biol* 2000; 221(1): 249–258.
- West J, Harral J, Lane K, et al. Mice expressing *BMPR2R899X* transgene in smooth muscle develop pulmonary vascular lesions. *Am J Physiol Lung Cell Mol Physiol* 2008; 295(5): L744–L755.
- Li J, Spensberger D, Ahn JS, et al. *JAK2 V617F* impairs hematopoietic stem cell function in a conditional knock-in mouse model of *JAK2 V617F*-positive essential thrombocythemia. *Blood* 2010; 116(9): 1528–1538.
- Soon E, Crosby A, Southwood M, et al. *BMPR-II* deficiency promotes pulmonary hypertension via increased inflammatory cytokine production. *Am J Respir Crit Care Med* 2015; 192(7): 859–872.
- Crosby A, Jones FM, Southwood M, et al. Pulmonary vascular remodeling correlates with lung eggs and cytokines in murine schistosomiasis. *Am J Respir Crit Care Med* 2010; 181(3): 279–288.
- Liu H, Zhu R, Liu C, et al. Evaluation of decalcification techniques for rat femurs using HE and immunohistochemical staining. *Biomed Res Int* 2017; 2017: 9050754.



26. Tonelli AR, Yadav R, Gupta A, et al. Spleen size in idiopathic and heritable pulmonary arterial hypertension. *Respiration* 2013; 85(5): 391–399.
27. Swirski FK, Nahrendorf M, Etzrodt M, et al. Identification of splenic reservoir monocytes and their deployment to inflammatory sites. *Science* 2009; 325(5940): 612–616.
28. Pang L, Weiss MJ and Poncz M. Megakaryocyte biology and related disorders. *J Clin Invest* 2005; 115(12): 3332–3338.
29. Bauer EM, Chanthaphavong RS, Sodhi CP, et al. Genetic deletion of toll-like receptor 4 on platelets attenuates experimental pulmonary hypertension. *Circ Res* 2014; 114(10): 1596–1600.
30. AYTEKIN M, AULAK KS, HASERODT S, et al. Abnormal platelet aggregation in idiopathic pulmonary arterial hypertension: role of nitric oxide. *Am J Physiol Lung Cell Mol Physiol* 2012; 302(6): L512–520.
31. Burt RK, Traynor AE, Craig R, et al. The promise of hematopoietic stem cell transplantation for autoimmune diseases. *Bone Marrow Transplant* 2003; 31(7): 521–524.