B lymphocyte-deficiency in mice promotes venous thrombosis

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ABSTRACT

Cells of the innate immune system, including monocytes and neutrophils, are key players in the process of venous thrombosis. T lymphocytes have recently been implicated in venous thrombus resolution but the role of B lymphocytes in thrombosis is unknown. The present study was conducted to address this question using a mouse model of partial ligation of the inferior vena cava. Although only a very low number of B cells was found in the venous thrombi of wild-type mice, B cell-deficient JHT mutant mice developed larger venous thrombi than the wild-type controls. Consistent with enhanced thrombogenesis, increased neutrophil counts were found in the circulating blood and in the thrombi of B cell-deficient mice. One of the mechanisms by which neutrophils contribute to venous thrombosis is the formation of neutrophil extracellular traps (NETs). In agreement, higher quantities of NETs were observed in the thrombi of B cell-deficient mice. In vitro assays showed no difference in the NET building capacity of the isolated neutrophils between B cell-deficient and wild-type mice, indicating that the enhanced NET formation in the thrombi of B cell-deficient mice is attributable to the increased number of circulating neutrophils in these animals. Furthermore, increased concentration of the clot-stabilizing macromolecule fibrinogen was detected in the plasma of B cell-deficient mice. In conclusion, B cell-deficiency in mice indirectly promotes venous thrombosis by increasing neutrophil numbers and elevating fibrinogen levels.

1. Introduction

The major cause of death has shifted globally from infectious to non-communicable diseases, with cardiovascular disease being a leading contributor [1]. Venous thromboembolism (VTE), which includes deep-vein thrombosis (DVT) and pulmonary embolism (PE) as its complication, is the third most common cause of cardiovascular mortality, ranking only after ischemic heart disease and stroke [2]. The common pathology underlying all these three major cardiovascular disorders is thrombosis [1]. In an incidence-based epidemiology model, 684019 DVT events, 434723 PE events, and a total of 543454 VTE-related deaths have been estimated across the European Union in 2004 with a population of 454.4 million [3].

Both in human DVT and in murine DVT models, leukocytes and platelets have been identified as the major cellular components [2]. Leukocyte recruitment to activated endothelial cells expressing adhesion molecules is a rapid response. Adhesion of monocytes and neutrophils to endothelial cells can be detected within hours of flow reduction [4]. While the contribution of cells from the innate immune system, such as monocytes and neutrophils, to the pathophysiology of DVT has been well established [2, 4], the role of the adaptive immune system in DVT remains elusive.

As an integral part of the adaptive immune system, effector memory T cells were identified to participate in venous thrombosis [5]. Sterile vascular inflammation leads to activation of memory T cells in a non-antigen-dependent manner. Through release of interferon-γ and...
recruitment of monocytes and neutrophils, infiltrated effector memory T cells delay the resolution of venous clots [5]. In contrast, a specialized population of regulatory T cells has been found to be crucial for blood clot resorption [6]. Of note, a recent study demonstrated that depletion of CD4- and CD8-positive T cells impairs venous thrombus resolution in mice [7]. Therefore, it is likely that the adaptive immune system has a regulatory role in venous thrombogenesis.

However, so far, the role of B lymphocytes in thrombosis is unresolved. Interestingly, there are first indications for a role of B cells from a cross-sectional study from Egypt, which could show that patients diagnosed with Behçet Disease have significantly lower proportions of B cells in the peripheral blood [8]. Because Behçet Disease is a multi-organ vasculitis characterized by widespread thrombosis, this observation suggests a link between B cell deficiency and thrombosis. This idea is supported by our recent finding that B cell-deficiency in mice leads to neutrophilia and endothelial dysfunction, two major factors promoting venous thrombosis [9]. To causally address whether the lack of B cells impacts on venous thrombus formation, we analyzed a genetic mouse model of B cell-deficiency combined with a standardized model of deep vein thrombosis (DVT). Here, we provide first experimental evidence that B cell-deficiency is linked to venous thrombus growth.

2. Material and methods

2.1. Animals

Wild-type C57BL/6J mice were obtained from Janvier Labs (Le Genest-Saint-Ise, France). The B cell-knockout mice, termed JHT mice, were generated by deletion of the exons encoding the joining region and the intron enhancer of the immunoglobulin heavy chain locus [10]. Their genetic background is C57BL/6J. To limit genetic drift, the JHT strain background was refreshed by backcrossing to the C57BL/6J control strain. The last backcross was carried out in 2020. The animal experiment was approved by the responsible regulatory authority (Landesuntersuchungsamt Rheinland-Pfalz; 23 177-07/G 17-1-020) and was conducted in accordance with the German animal protection law, the EU Directive 2010/63/EU for animal experiments and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

2.2. Inferior Vena Cava (IVC) flow restriction thrombosis model

Ten weeks old male mice were anesthetized by intraperitoneal injection of a solution of midazolame (5 mg/kg; Ratiopharm GmbH, Ulm, Germany), medetomidine (0.5 mg/kg body; Pfizer Deutschland GmbH, Berlin, Germany), and fentanyl (0.05 mg/kg; Janssen-Cilag GmbH, Neuss, Germany) after initial inhalable anesthesia in a chamber –– 0.1 L/min 100% O2). The abdomen of the mice abdomen for visualization and optimal image. IVC, stenosis and the electrocardiogram (ECG). Sound transmission gel was applied on the body temperature was measured with a rectal probe and kept at 37°C using a heating system within the handling platform. This platform was also used to monitor the ventilation and the end-tidal CO2. Transmission gel was applied on the mice abdomen for visualization and optimal image. IVC, stenosis and the formed thrombus were visualized in a long axis view. A freeze-frame picture was taken manually in B-mode. With the Vevo LAB software, the cross-sectional area of the clot was traced to receive the thrombus measurements [12].

2.4. Flow cytometry

The thrombi were mechanically disrupted on a 70-μm strainer and washed with 10 ml PBS (Sigma-Aldrich) substituted with 2% FCS (Sigma-Aldrich) and 1 mM EDTA (Thermo Fisher Scientific) (FACS buffer). After centrifugation (300 g, 6 min, 4°C) red blood cells lysis was performed with distilled water for 10 s, 2 ml of FACS buffer were added and centrifugation was repeated. The resulting single-cell suspensions were treated with Fc-block and washed. Cells were stained for 1 h with commercial antibodies in dilutions as recommended by the vendor. The following antibodies were utilized: CD3e-PE-Cy7 (145-2C11), CD11b-BV421 (M1/70), CD45-APC (30-F11), F4/80-PE (T45-2342), NK1.1-BV605 (PK136), Ly6C-PerCP-Cy5.5 (AL21), Ly6G-FITC (1A8) and Puri- fied Rat Anti-Mouse CD16/CD32 (Mouse BD Fc Block™) (2.4G2) were obtained from BD Biosciences. LIVE/DEAD™ Fixable Near-Infrared Dead Cell Stain Kit, for 635 nm or 635 nm excitation, was from Thermo Fisher Scientific. Blood samples were processed after staining. Erythrocyte lysis was performed by adding 1 ml of 1×BD PharmLyse™ lysis Buffer to 150 μl blood for an incubation duration of 8 min at room temperature in the dark. Samples were washed three times with 1 ml FACS buffer (300 g, 6 min and 4°C). Cells were suspended in FACS buffer and directly transferred into FACS tubes. All samples were analyzed using a BD FACS CANTO II flow cytometer (Becton Dickinson) and FACSDiva Software 7.0 (Becton Dickinson), respectively.

2.5. Quantification of neutrophil extracellular traps (NETs)

The excised IVC samples (including the thrombi) were immediately rinsed with PBS, embedded in OCT, and frozen at –80°C. For NETs quantification, 7 μm sections were cut from the middle part of the thrombi [13]. Specimens were fixed with 4% formalin for 4 min, washed in PBS, and blocked with 4 μg/ml goat IgG and 1% BSA for 30 min. The sections were incubated with primary antibodies (rabbit antibody against histone H3 (citrulline R2 + R8 + R17); rabbit antibody against neutrophil elastase; NE; rat antibody against Ly6G; all from Abcam, Cambridge, UK) for 24 h at 4°C followed by washing in PBS + 0.1% Tween. Next, incubation with secondary antibodies was performed for 24 h at 4°C. DNA was stained with 2 μg/ml Hoechst 33342. Images were acquired using a fluorescence microscope. Included into the quantification of neutrophil extracellular traps (NETs) were structures with triple positive staining: (i) Hoechst 33342-positive for extracellular DNA; (ii) positive for Ly6G as a neutrophil marker; (iii) positive for the marker of neutrophil granule protein NE or for citrullinated histone H3 [4]. NETs quantification was performed at random locations within the images to minimize bias.
Bone marrow was harvested by flushing the femur with HBSS (Hanks’ balanced salt solution) containing 25 mM HEPES and 10% FCS (fetal calf serum) [14]. After flushing through a 70 μm cell strainer, the suspension was centrifuged at 300g for 10 min. The pellet was resuspended in HBSS containing 25 mM HEPES, overlaid on a Histopaque gradient (an equal volume of Histopaque-1077 over Histopaque-1119) and centrifuged for 30 min at 700g without brake. Neutrophils at the interface were carefully collected and washed with 20 ml washing buffer. After a centrifugation at 300g for 10 min, 10 ml of the wash buffer was removed, 10 ml of fresh washing buffer added. The cells were resuspended and centrifuged at 300g for 10 min. Then, the supernatant was removed completely, the cells washed with 20 ml washing buffer and centrifuged at 300g for 10 min. Finally, washed neutrophils were resuspended in 1 ml RPMI 1640 medium and counted [14].

To study the role of B lymphocytes in venous thrombosis, a standardized model of inferior vena cava (IVC) stenosis was applied. In this model, the blood flow is restricted by 80% through partial IVC ligation without endothelial injury [4]. It has been shown that this model could be enhanced following ligation of the IVC. Indeed, more NETs were observed in the thrombi of JHT mice compared to wild-type control mice, as detected by immunofluorescence imaging of citrullinated histone H3 (cH3) and by NE (Figure 3A and B, red signals). Quantitative image analysis revealed significantly elevated counts of cH3-positive and NE-positive NETs in the thrombi of JHT mice relative to B6 WT controls (Figure 3A and B). This suggests that the observed increased thrombus growth in the B cell-deficient JHT mouse model might be due to increased NET formation.

3. Results

3.1. Enhanced venous thrombus formation in B cell-deficient mice

The bodyweight of JHT mice (27.4 ± 3.0 g) was slightly lower than that of the C57BL/6J mice (29.0 ± 1.7 g) at 10 weeks of age before surgery (mean ± SD, p < 0.05). IVC ligation was performed in 35 C57BL/6J and 35 JHT mice. 15 C57BL/6J and 13 JHT mice did not develop venous thrombosis 48 h after IVC ligation. Consistent with the reported 60% proportion of mice developing occlusive thrombi in this model [4], venous thrombosis was found in 20 C57BL/6J mice and 22 JHT mice. Analysis of thrombus weight and thrombus size by in vivo ultrasound imaging of the formed thrombus revealed that IVC thrombus formation was markedly enhanced at 48 h post stenosis in B cell-deficient JHT mice as compared to wild-type control mice (Figure 2A–C). Our results indicate that the lack of circulating B cells promotes venous thrombogenesis.

3.2. Increased number of neutrophils in B cell-deficient mice

Immune cells, especially neutrophils, monocytes and T cells are fundamental players in venous thrombosis [4, 5, 18]. Therefore, we performed FACS to analyze the counts of major immune cell types in the blood and in the thrombus of mice undergoing IVC ligation. At 48 h after IVC ligation, B cell-deficient JHT mice showed overall more immune cells in the blood and in the developed thrombus (Figure 2A and B). Interestingly, significantly elevated counts of T cells and myeloid cells (predominantly neutrophils) were detected in JHT thrombi as well as in JHT blood relative to B6 mice (Figure 2C–F). In contrast, the numbers of monocytes in the thrombi were unchanged between B6 and JHT mice (Figure 2E) but a slight increase in monocytes was observed in JHT blood (Figure 2F). Our results indicate that B cell-deficiency in JHT mice yields in systemically elevated neutrophil counts and T cell counts, both in blood and IVC thrombi.

3.3. Enhanced counts of NETing neutrophils in the thrombi of B cell-deficient mice

One key mechanism by which neutrophils promote venous thrombosis is by NETosis [4]. Intriguingly, a fraction of activated neutrophils, as well as other granulocytes, undergo a specialized form of cell death [19]. Upon histone citrullination, neutrophils decondensate their chromatin and expel DNA fibers, called NETs. Through polar interactions, NETs are decorated with histones (e.g. histone H3) and serine proteases, such as NE, promoting coagulation activation and the damage of endothelial cells [20, 21]. Extracellular DNA traps provide a scaffold for platelet binding and aggregation, thus contributing to venous thrombosis [22].

Since JHT mice have increased neutrophil counts in their blood and in the generated IVC thrombi, we assumed that NET formation in these mice could be enhanced following ligation of the IVC. Indeed, more NETs were observed in the thrombi of JHT mice compared to wild-type control mice, as detected by immunofluorescence imaging of citrullinated histone H3 (cH3) and by NE (Figure 3A and B, red signals). Quantitative image analysis revealed significantly elevated counts of cH3-positive and NE-positive NETs in the thrombi of JHT mice relative to B6 WT controls (Figure 3A and B). This suggests that the observed increased thrombus growth in the B cell-deficient JHT mouse model might be due to increased NET formation.

To test whether an enhanced capability of the JHT neutrophils to build NETs could be the causative of enhanced thrombus growth, we performed a functional ex vivo NETosis assay. Therefore, neutrophils were isolated from the bone marrow and stimulated with LPS. Importantly, the extent of LPS-stimulated NET formation was comparable in the isolated bone marrow neutrophils from B6 and JHT mice (Figure 3C). Specificity of the assay was confirmed since the signal could be reduced by DNase I (Figure 3C). Furthermore, LPS-induced NETosis was prevented in bone marrow neutrophils from mice deficient in peptidyl arginine deiminase (PAD4) (Figure 3D), a key enzyme for histone citrullination [23]. Thus, the enhanced NET formation (Figure 3A and B) was not due to a change in the capability of the neutrophils to undergo NETosis (Figure 3C) but could be attributed to the increased circulating neutrophil number detected in JHT mice (Figure 2).
3.4. Increased concentration of fibrinogen in B cell-deficient mice

The interaction of neutrophils with endothelial cells and platelets is facilitated by fibrinogen via binding to Mac-1 (integrin αMβ2) [24, 25, 26]. Hence, we measured the plasma fibrinogen concentration at baseline without IVC ligation. Of note, a higher fibrinogen concentration was detected in the blood of JHT mice (Figure 4A). Consistent with this finding, JHT plasma showed a shortened fibrinogen-dependent clotting time, when stimulated with excess thrombin (Figure 4B). Thus, the elevated capacity of JHT mice to form fibrin polymers may further support the formation of venous thrombi.

4. Discussion

Although the roles of monocytes, neutrophils and T cells in DVT have been clearly demonstrated in previous studies [4, 5, 18], the role of B lymphocytes remained elusive. Our present study demonstrates that B cells are functionally involved in venous thrombosis, although the role of B cells is indirect.

In the partial IVC ligation model without endothelial injury, von Brühl et al. have demonstrated that the reduction of blood flow induces a pro-inflammatory phenotype with activated endothelial cells exposing adhesion molecules on their surfaces, thereby initiating recruitment of innate immune cells, particularly neutrophils and monocytes, with neutrophils being the predominant leukocyte subset within venous thrombi [4]. Blood monocytes and neutrophils crawling along and adhering to the venous endothelium provide the initiating stimulus for DVT development. Recruited leukocytes start fibrin formation via blood cell-derived tissue factor, predominantly originating from monocytes and, to a lesser extent, from neutrophils. This intravascular activation of the extrinsic coagulation pathway is the decisive trigger for fibrin formation [4].

Neutrophils are crucial players in flow restriction-induced venous thrombosis. Thrombus-resident neutrophils are indispensable for DVT propagation by releasing NETs. Although NET formation is not required in the initial phase of fibrin deposition [30], it is essential for the propagation of DVT [4]. NETs act as a procoagulant surface, propagating DVT development through the binding and activation of FXII, the initiator of the intrinsic coagulation pathway. NETs are negatively charged, can bind factor XII (FXII) on their surfaces and provide a scaffold for FXII activation [4]. In addition, NETs act as prothrombotic scaffolds by binding von Willebrand factor (VWF), degrading potent anticoagulants like tissue factor pathway inhibitor (TFPI), and reducing thrombomodulin-dependent protein C activation [4].

Our recent study has found that B cell-deficiency in mice leads to neutrophilia [9]. Increased number of neutrophils in the circulating blood was observed in the JHT mice (Figure 2). The increased neutrophil number in JHT mice also resulted in the enhanced accumulation of neutrophils in the thrombi (Figure 2). Given the fundamental role of neutrophils in venous thrombosis, it is reasonable to postulate that the neutrophilia is likely to be the major molecular mechanism underlying the enhanced thrombogenesis observed in B cell-deficient JHT mice, involving a quantitative more frequent occurring formation of NETs (Figure 3). It has been shown in several models that elevated levels of circulating neutrophils can enhance the production of NETs and therefore increase venous thrombus size [31, 32, 33, 34]. Consistent with these previous findings, our data indicate that the enhanced NETs formation in the JHT thrombi is not a result of a changed NET-building capacity of the neutrophils but solely attributable to the increased number of circulating neutrophils.

Notwithstanding, dysfunctional vascular endothelium could also contribute to the increased thrombus growth in IVC-stenosed JHT mice. We have shown previously that JHT mice have endothelial dysfunction and increased circulating levels of tumor necrosis factor-α (TNF-α) [9].

Figure 1. Enhanced venous thrombus formation in B cell-deficient mice. Venous thrombosis was induced by partial ligation of the inferior vena cava (IVC) in wild-type B6 and B lymphocyte-deficient JHT mice, respectively. Thrombus weight (A) and size (B) were determined 48 h after IVC ligation. Panel C shows representative transversal ultrasound images with thrombus outlined (turquoise) for size determination. The boxes represent the interquartile range which contains data between the 25th and 75th percentiles. The horizontal lines within the boxes are the medians. The whiskers represent the minimum and the maximum value, respectively. **P < 0.01, unpaired t test.
Although endothelial function is usually tested in arteries, the observed impairment of endothelial function may also apply to venous vessels. TNF-α is known to reduce the expression of endothelial nitric oxide synthase resulting in a decrease of nitric oxide (NO) production in endothelial cells [27, 28]. Because endothelial NO plays a crucial role in mediating vasodilation and in suppressing the expression of adhesion molecules [29], the reduced NO production by TNF-α is an explanation for the endothelial dysfunction but may also contribute to the enhanced leukocyte recruitment in JHT mice. Therefore, the endothelial inflammation triggered by TNF-α is likely to be a contributing mechanism to the enhanced venous thrombogenesis observed in JHT mice.

In the setting of thrombosis, conditions of endothelial dysfunction and oxidative stress can lead to elevated plasma levels of von Willebrand factor (VWF) and coagulation factor VIII; both factors play important roles in venous thrombosis [11, 13]. Noteworthy, no significant difference of VWF was found in JHT and B6 thrombi and baseline factor VIII functional activity (FVIII-dependent clotting time) was not different between JHT and B6 mice (data not shown). Thus, changes in VWF and factor VIII are most likely not involved in the enhanced venous thrombosis observed in the JHT mice.

An interesting question is how B cell-deficiency causes neutrophilia, although it is beyond the scope of the present study. Neutrophil hematopoiesis is known to be regulated by granulocyte colony stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) [35, 36]. However, we found no difference in the serum levels of G-CSF and GM-CSF between JHT and wild-type mice [9]. Orexin has been recently identified as a factor limiting neutrophil hematopoiesis and a decrease in orexin levels leads to neutrophilia [37]. Indeed, JHT mice have reduced serum levels of orexin compared to wild-type mice [9] but its contribution to neutrophilia in the JHT mice remains to be determined. Very recently, an interesting study has provided compelling evidence on the importance of B cells in regulating hematopoiesis [38]. Cholinergic signaling has been found to inhibit myelopoiesis with B cells as the primary source of acetylcholine in the bone marrow. Disruption of acetylcholine production selectively in B cells increases the levels of circulating myeloid cells, including neutrophils [38]. Thus, the lack of B
Figure 3. Enhanced NET formation in B cell-deficient JHT mouse thrombi. NETs in the IVC were immunohistochemically visualized 48 h after induction of DVT by staining for the neutrophil marker Ly6G (green) and for DNA (blue) with the Hoechst 33342 dye. Additional staining was performed for citrullinated histone h3 (red; panel A) or for the neutrophil granule protein neutrophil elastase (NE, red, panel B). Arrows indicate triple positive structures that were assessed as NETs. The boxes represent the interquartile range which contains data between the 25th and 75th percentiles. The horizontal lines within the boxes are the medians. The whiskers represent the minimum and the maximum value, respectively. *P < 0.05; **P < 0.01; unpaired t-test. Panels C and D: Neutrophils were isolated from the bone marrow of wild-type B6 mice, B lymphocyte-deficient JHT mice, and PAD4−/− mice, respectively. NET formation was stimulated ex vivo with LPS and extracellular DNA stained with SYTOX Orange. DNase I was added to digest the extracellular DNA. Columns represent mean ± SEM; n = 8. ***P < 0.001; one-way ANOVA (C); *P < 0.05, Mann-Whitney test (D).

Figure 4. Increased concentration of fibrinogen in B cell-deficient mice. Concentration of fibrinogen was measured in the plasma of wild-type B6 mice and B lymphocyte-deficient JHT mice using an ELISA kit (A). Fibrinogen functional activity was determined with a KC4 Delta Amelung Coagulometer (B). Shorter coagulation time indicates higher activity. The boxes represent the interquartile range which contains data between the 25th and 75th percentiles. The horizontal lines within the boxes are the medians. The whiskers represent the minimum and the maximum value, respectively. *P < 0.05; ***P < 0.001; unpaired t-test.
cells and B cell-derived acetylcholine is likely to be an underlying mechanism explaining the increased neutrophil count also in the JHT mice.

Although lymphocytes were not found in high number in earlier DVT studies [4], a recent research has shown that T cells rapidly infiltrate the thrombotic vein wall and become integral constituents of the developing thrombus [5]. Moreover, effector-memory T cells were found to play an important role by orchestrating the inflammatory response in this IVC ligation model [5]. These cells delay thrombus resolution through antigen-independent production of interferon-γ and activation of myeloid cells [5]. In addition, a special subset of regulatory T cells has been found in venous thrombi, characterized by expression of "secreted protein acidic and rich in cysteines" (SPARC). In contrast to effector-memory T cells, the clot regulatory T cells boost monocyte production of matrix metalloproteinases and thereby facilitate clot resolution [6]. Global CD4+/CD8+ depletion impairs thrombus clearance [7]. Because different subtypes of T cells may play different or even opposite roles in thrombus formation and resolution, the contribution of T cells to the enhanced thrombosis observed in the JHT mice in our model remains elusive.

An additional mechanism underlying the observation of an enhanced venous thrombosis in JHT may be the increased concentration of fibrinogen (Figure 4). Fibrinogen can bind to neutrophilic Mac-1 and to platelet integrin αIIbβ3, and thereby facilitate the formation of neutrophil-platelet complex [24]. Furthermore, fibrinogen and fibrin (ogen)-degradation products promote adhesion of leukocytes (including neutrophils) to the endothelium [25]. Both are important for the pro-thrombotic properties of neutrophils.

Our study has some limitations. First, we used only male mice in our study. Although the overall risk of developing a first acute venous thromboembolism (VTE) is similar between men and women, there exist considerable sex differences in the VTE presentation and prognosis [39, 40, 41]. Therefore, in future studies, it will be interesting to study whether the impact of B cell deficiency on VTE is influenced via sex-specific mechanisms. Second, we used only one model of B cell-deficiency in this study. The JHT mice are a chronic model with permanent B cell depletion. It would be interesting to address whether acute B cell depletion in wild-type mice has the same effect on thrombogenesis as identified in JHT mice.

In conclusion, our study provides first evidence that B cell-deficiency in mice promotes venous thrombosis. The low number of B cells in the thrombi (Figure 2) argues for an indirect effect of B cells on venous thrombosis. The enhanced venous thrombosis caused by B cell-deficiency, shown in the IVC stenosis model, is most likely due to the increased number of circulating neutrophils, leading to a higher abundance of NETs in the thrombus, and due to the upregulated production of fibrinogen.

Declarations

Author contribution statement

Solveig Hasselwander, Ning Xia: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data. Maximilian Mimmller, Stefanie Ascher, Tanja Knopp, Gisela Reifenberg: Performed the experiments; Analyzed and interpreted the data. Susanne Karbach, Wolfram Ruf: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data. Christoph Reinhardt, Huige Li: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest’s statement

The authors declare no conflict of interest. S.K. received consultancy honoraria from Almiral not associated with this work.

Additional information

No additional information is available for this paper.

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