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Natural IgM antibodies and microvesicles in coagulation and thrombosis

Doctoral thesis at the Medical University of Vienna
for obtaining the academic degree

Doctor of Philosophy

Submitted by

Dr.med.univ. Georg Obermayer

Supervisor: Univ. Prof. DDr. Christoph J Binder
Klinisches Institut für Labormedizin
Lazarettgasse 14 BT 25.2
Medizinische Universität Wien

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Declaration

This thesis includes a shared first author publication. I performed and designed all experiments and data analysis included in the manuscript (unless otherwise specified below) at the Medical University of Vienna (Department of Laboratory Medicine) and CeMM (Research Center for Molecular Medicine). I was supervised by Univ. Prof. Christoph J Binder, MD PhD. Taras Afonyushkin, PhD, who is currently a Postdoctoral Fellow at the Medical University of Vienna (Department of Laboratory Medicine), is a shared first author on the publication and did not or will not use any content of this manuscript for the purpose of a dissertation.

The results section consists of the paper published in Blood (DOI:10.1182/blood.2020007155) with the title „Natural IgM antibodies inhibit microvesicle-driven coagulation and thrombosis“. The material and methods sections consists of the online material / supplemental material, published in Blood (DOI: 10.1182/blood.2020007155), that I wrote for the manuscript with the title „Natural IgM antibodies inhibit microvesicle-driven coagulation and thrombosis“.

Individual and shared contributions of the original research publication are as follows (which is also included in the publication): Georg Obermayer, Taras Afonyushkin, and Waltraud Schrottmaier conducted experiments and performed data analysis; Georg Obermayer, Taras Afonyushkin, and Christoph J Binder designed experiments and wrote the article; Georg Obermayer and Taras Afonyushkin handled all in vivo experiments; Laura Göderle and Taras Afonyushkin established and performed histologic treatment and analysis of mouse lungs; Soreen Taqi performed histologic analysis of mouse lungs; Florian Puhm designed, conducted, and supervised the flow cytometry experiments; Michael Schwameis performed and supervised the rotational thromboelastometry experiments; Waltraud Schrottmaier conducted thrombocyte aggregation experiments and analysis; Florian Puhm, Cihan Ay, Ingrid Pabinger, Bernd Jilma, Alice Assinger, and Nigel Mackman contributed to writing and critical evaluation of the article; and Taras Afonyushkin, and Christoph J Binder supervised and coordinated the effort.

The contributors to the publication are as follows (superscript numbers indicate affiliations): Georg Obermayer[1,2],* Taras Afonyushkin[1,2],* Laura Göderle[1], Florian Puhm[1,2], Waltraud Schrottmaier[3], Soreen Taqi[1], Michael Schwameis[4,5], Cihan Ay[6,7], Ingrid Pabinger[6], Bernd Jilma[4], Alice Assinger[3], Nigel Mackman[8], and Christoph J. Binder[1,2]

1 Department of Laboratory Medicine, Medical University of Vienna, Vienna, Austria;
2 Research Center for Molecular Medicine of the Austrian Academy of Sciences,
Vienna, Austria;
3 Centre of Physiology and Pharmacology,
4 Department of Clinical Pharmacology,
5 Department of Emergency Medicine, and
6 Clinical Division of Hematology and Hemostaseology, Department of Medicine I,
Medical University of Vienna, Vienna, Austria;
7 I.M. Sechenov First Moscow State Medical University (Sechenov University), Moscow, Russia;
8 Department of Medicine, University of North Carolina at Chapel Hill School of Medicine, Chapel Hill, NC

* authors contributed equally

Table of Contents

List of Tables

List of Figures

Abstract

Thrombosis, manifesting as myocardial infarction, stroke and pulmonary embolism, is the leading cause of morbidity and mortality in the world. Insights into its pathophysiology and how to prevent and treat it therefore have the potential to greatly impact public health. This thesis explores the interaction of coagulation, microvesicles and natural IgM antibodies in cardiovascular disease. Furthermore, the interactions between inflammation, oxidized lipoproteins and venous thrombosis is discussed in depth in the attached review. The paper titled "Natural IgM antibodies inhibit microvesicle-driven coagulation and thrombosis" constitutes the main work behind this thesis. In this study I demonstrate that natural IgM antibodies, which have previously been shown to mediate beneficial effects in inflammation, also possess protective properties with respect to coagulation and thrombosis. Our group has previously shown that circulating microvesicles, which are mediators of coagulation, are bound by the same natural antibodies that also bind oxidatively modified lipoproteins. In this study I show that the presence of natural IgM antibodies that bind oxidation-specific epitopes inhibits the procoagulatory properties of microvesicles. This effect can be seen in the inverse association of free and MV-bound IgM with the propagation of thrombin generation. Furthermore, the presence of IgM antibodies that bind oxidation-specific epitopes inhibits MV-driven plasmatic coagulation, as well as coagulation of whole blood, without affecting thrombocyte aggregation. I prove these anticoagulatory effects by co-injecting these antibodies and microvesicles in mouse models of thrombosis and hemostasis. While the IgM has no effect in a tail bleeding model or a platelet-driven pulmonary thrombosis model, it reduces thrombus burden and protects mice from death in an MV-driven pulmonary embolism model, demonstrating the powerful anticoagulatory effects of IgM antibodies that bind oxidation-specific epitopes in a setting where MVs are the prothrombotic trigger.

Finally, in the discussion, our findings are put into the context of the interplay between immunity and coagulation and their potential impacts are briefly explored.

Zusammenfassung

Thrombosen, welche die Ursache hinter Herzinfarkten, Schlaganfällen und Pulmonalembolien darstellen, sind weltweit die führende Todesursache. Erkenntnisse die sich mit der Pathophysiologie, der Prävention, und der Therapie von thrombotischen Ereignissen beschäftigen, können daher das Gesundheitswesen enorm beeinflussen. Diese Dissertation beschäftigt sich mit den Grundlagen von Blutgerinnung und Thrombosen, und den Rollen von Mikrovesikeln und natürlichen IgM Antikörpern in kardiovaskulären Erkrankungen. Weiters werden die Zusammenhänge von Entzündung, oxidierten Lipoproteinen und venösen Thrombosen im beiliegenden Review besprochen. Abschließend bildet die Publikation "Natural IgM antibodies inhibit microvesicle-driven coagulation and thrombosis" das zentrale Resultat, welches dieser Dissertation zugrunde liegt. In dieser Arbeit beschreiben wir, dass natürliche IgM Antikörper, deren positive Effekte wir in entzündlichen Vorgängen bereits aufzeigen konnten, protektive Eigenschaften in Blutgerinnung und Thrombosen besitzen. Unsere Forschungsgruppe beschrieb zuvor, wie zirkulierende Mikrovesikel, welche blutgerinnungsfördernde Mediatoren sind, von jenen natürlichen IgM Antikörpern gebunden werden, welche auch oxidierte Lipoproteine erkennen.

Wir zeigen in dieser Studie auf, dass natürliche IgM Antikörper, welche oxidationsspezifische Epitope binden, die blutgerinnungsfördernden Eigenschaften von Mikrovesikeln hemmen. Dieser Effekt ist durch die inverse Assoziation von freien und MV-gebundenen IgM. Weiters konnten wir zeigen, dass IgM Antikörper welche oxidationsspezifische Epitope binden, MV-induzierte plasmatische und Vollblut-gerinnung inhibieren, ohne die Plättchenaggregation zu beeinflussen. Diese in vitro Effekte konnten wir auch in in vivo Versuchen aufzeigen. Interessanterweise beeinflusste die Injektion weder Hämostase, noch Thrombosen in einem kollageninduzierten Pulmonalthrombosenmodell in Mäusen. In einem MV-induzierten Pulmonalthrombosenmodell konnten wir jedoch die protektiven Eigenschaften durch eine dramatische Reduktion der Thrombenanzahl und Todesrate demonstrieren. Diese Ergebnisse demonstrieren die wirksamen Effekte von natürlichen IgM Antikörpern in Mikrovesikel-induzierter Blutgerinnung und Thrombose.

Abschließend werden die Ergebnisse unserer Arbeit im Kontext des Zusammenhangs zwischen Immunsystem und Blutgerinnung besprochen und potentielle weiterführende Resultate unserer Forschungsergebnisse diskutiert.

Publications arising from this thesis

Review:

Oxidized low-density lipoprotein in inflammation-driven thrombosis.

Journal of Thrombosis and Haemostasis, 2018 Jan 24;16(3):418–28, DOI: 10.1111/jth.13925

Georg Obermayer, Taras Afonyushkin and Christoph J. Binder

Main Manuscript:

Natural IgM antibodies inhibit microvesicle-driven coagulation and thrombosis

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Georg Obermayer,* Taras Afonyushkin,* Laura Göderle, Florian Puhm, Waltraud Schrottmaier, Soreen Taqi, Michael Schwameis, Cihan Ay, Ingrid Pabinger, Bernd Jilma, Alice Assinger, Nigel Mackman, and Christoph J. Binder

**authors contributed equally*

Abbreviations

BCR	B cell receptor
CAT	cancer-associated thrombosis
CVD	cardiovascular disease
DOAC	direct oral anticoagulant
DVT	deep vein thrombosis
EV	extracellular vesicle
HIT	heparin-induced thrombocytopenia
IVIG	intravenous immunoglobulin
LDL	low density lipoprotein
miRNA	micro ribonucleic acid
mRNA	messenger ribonucleic acid
MV	microvesicle
OSE	oxidation-specific epitopes
PE	pulmonary embolism
PS	phosphatidylserine
RBC	red blood cell
TF	tissue factor
VTE	venous thromboembolism

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I want to thank Christoph Binder and Taras Afonyushkin, who taught me how to be and think like a scientist. Furthermore, I want to express my gratitude to everyone who supported me during my PhD, who are too numerous to list here. Finally, I also want to acknowledge the institutions which have made this possible, Medical University of Vienna, KILM, CeMM and the SFB-54 special research program.

"I love deadlines. I love the whooshing noise they make as they go by."

- Douglas Adams

1. Introduction

1.1 Coagulation and thrombosis

1.1.1 Basics of coagulation

Blood coagulation is a highly conserved process throughout evolution (Jiang and Doolittle, 2003) (Davidson et al., 2003), consistent with the need to preserve and quickly repair the integrity of the circulatory systems of complex organisms. In its most simplified form, blood coagulation relies on the activation of vascular and circulating cells and a complex network of humoral coagulation factors and their regulators. Upon the disruption of blood vessel integrity, a platelet plug is quickly formed (primary hemostasis) and reinforced by the formation of a fibrin mesh (secondary hemostasis). In combination with other processes, such as blood vessel constriction, these mechanisms prevent significant blood loss after injury.

The initial models of plasmatic coagulation were conceptualized as a waterfall, with activation of one clotting factor leading to the activation of the next, ending with the formation of fibrin fibers. This model was further developed into the simplified version of plasmatic coagulation with its intrinsic, extrinsic, and common arms that is still used today. While this model still serves to explain the basics of plasmatic coagulation, more complex depictions are a better way to describe the multiple feedback and inhibitory loops, and the central role of thrombin in those pathways (Figure 1).

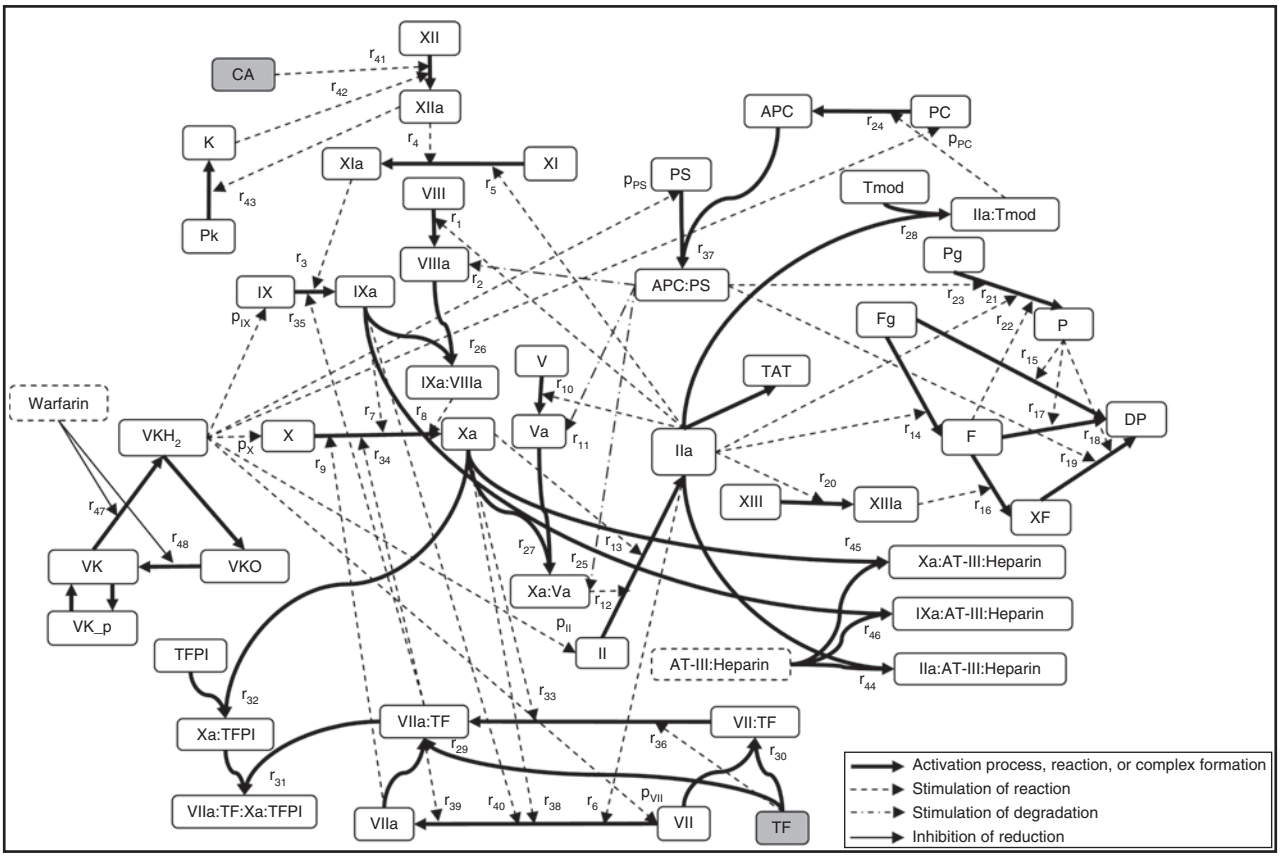


Figure 1. Scheme showing activation, stimulation and inhibition networks of the coagulation cascade (Wajima et al., 2009), *reproduced with permission from the publisher.*

In addition to plasmatic coagulation, several cell types are crucial components of physiological and pathological blood clotting. Platelets serve a central role in focussing the coagulation reactions to sites of injury by forming the initial platelet plug as well as providing the phospholipid surface necessary by exposing phosphatidylserine (PS) and releasing microvesicles (MVs) upon activation. A variety of agonists can induce platelet shape change and activation. Importantly, thrombin, which is one of the central coagulation proteases, is also one of the most potent platelet activators (Colman, 1991) and therefore one of the main links between the cellular and plasmatic components of blood clotting. Activated platelets express glycoprotein receptors which bind connective tissue proteins such as collagen, fibronectin and laminin, which is the primary mode of platelet adhesion in low shear conditions, as opposed to high shear conditions, in which platelet - von Willebrand factor interactions initiate platelet adhesion (Broos et al., 2011).

Immune cells have also taken the stage in coagulation and thrombosis, which led to the term of immunothrombosis, describing host-defense pathways leading to thrombus formation. Both monocytes and neutrophil granulocytes promote coagulation by expressing tissue factor (TF) on their surface as well as releasing TF+ MVs upon activation. Neutrophils in particular have gained much attention after the discovery that neutrophil extracellular traps, which are antimicrobial chromatin webs released by activated neutrophilic granulocytes (Brinkmann et al., 2004), play a major role in promoting thrombosis (Fuchs et al., 2010).

Healthy endothelial cells on the other hand express the coagulation regulators antithrombin and thrombomodulin and therefore play a significant role in keeping coagulatory processes confined to sites of endothelial injury. Furthermore, endothelial cells also help to keep platelets quiescent by releasing nitric oxide (NO) as well as prostacycline (PGI2) (van Hinsbergh, 2012).

Aberrant coagulation can present itself as either hyper- or hypocoagulability.

Hypocoagulability can be caused by defects or deficiencies of plasmatic coagulation factors or platelets, as seen in genetic disorders such as hemophilia (Mannucci and Tuddenham, 2001), von Willebrands disease (Swami and Kaur, 2017), and gray platelet syndrome (Nurden and Nurden, 2007). Furthermore, hypocoagulability can be caused by a multitude of pathological conditions, such as chronic liver disease (Tripodi and Mannucci, 2011), severe trauma (Moore et al., 2021), or vitamin K deficiency as seen in neonates (Pichler and Pichler, 2008).

Unwanted coagulation manifesting itself as thrombosis, on the other hand, has been the leading cause of morbidity and mortality worldwide for the past decades (World Health Organization, 2018). Thrombosis can be classified as arterial and venous thrombosis, which share many pathways, while being distinct in their pathogenesis, which will be discussed in the following chapters.

1.1.2 Arterial thrombosis

The pathogenesis of arterial thrombosis is - with rare exceptions such as spontaneous artery dissections and coronary embolisms - closely tied to the development of atherosclerotic plaques and in these cases termed atherothrombosis (Davì and Patrono, 2007). Even though atherosclerosis is not the primary topic of this thesis, a short overview shall outline the main mechanisms in the disease underlying the formation of most arterial thrombi. While initially thought to be a purely lipid-driven disease, studies investigating the importance of inflammation in atherosclerosis have rapidly increased in recent years, with the recent CANTOS study demonstrating that limiting inflammation by blocking interleukin-1β reduces the number of recurrent cardiovascular events independently of lipid levels (Ridker et al., 2017). The accumulation of low-density lipoprotein (LDL) in the subendothelial space of the arterial wall initiates and propagates the development of atherosclerotic lesions (Borén et al., 2020). After LDL deposition in the arterial wall, its retention is mediated via the interaction of apoB100 with arterial wall proteoglycans (Skålén et al., 2002). Subsequently, oxidative modification of accumulated LDL, which can occur via enzymatic and non-enzymatic processes leads to the formation of oxidized LDL (Binder et al., 2016). The uncontrolled, scavenger receptor-driven uptake of this oxidatively modified LDL by macrophages is the main driver behind the transformation to so-called foam cells which ultimately leads to the formation of an atherosclerotic plaque (Bäck et al., 2019). The rupture or erosion of these plaques leads to the exposure of the procoagulant plaque contents to the circulation, inducing the formation of platelet-rich thrombi, leading to ischaemic strokes (Campbell et al., 2019) and myocardial infarctions (Morel et al., 2009) (Figure 2).

Exposure of collagen and von Willebrand factor (VWF) leads to the rapid adhesion, activation and aggregation of platelets. Platelet activation also leads to the direct and indirect (via the release of MVs) exposure of phosphatidylserine, which is a prerequisite for the propagation of the common pathway of the coagulation cascade. Tissue factor, which is found in particular high concentrations in the lipid-rich necrotic core - more specifically on the MVs shed from apoptotic cells (Tedgui and Mallat, 2001), activates the coagulation cascade via the extrinsic pathway leading to the generation of thrombin. Thrombin, one of the central proteases of the common pathway, in turn further activates platelets mainly via protease activated receptors (PARs) (De Candia, 2012). Interestingly, tissue factor pathway inhibitor (TFPI) which is the main inhibitor of TF-induced coagulation colocalizes with TF in atherosclerotic plaques (Winckers et al., 2013). This could be considered a compensation mechanism, which has also been proposed for the presence of increased circulating TFPI found in coronary artery disease (CAD) patients (Winckers et al., 2011).

Lastly, lipid modification, which plays a central role in the development of atherosclerosis (Libby et al., 2019), can also directly affect atherothrombosis by driving plasmatic coagulation and

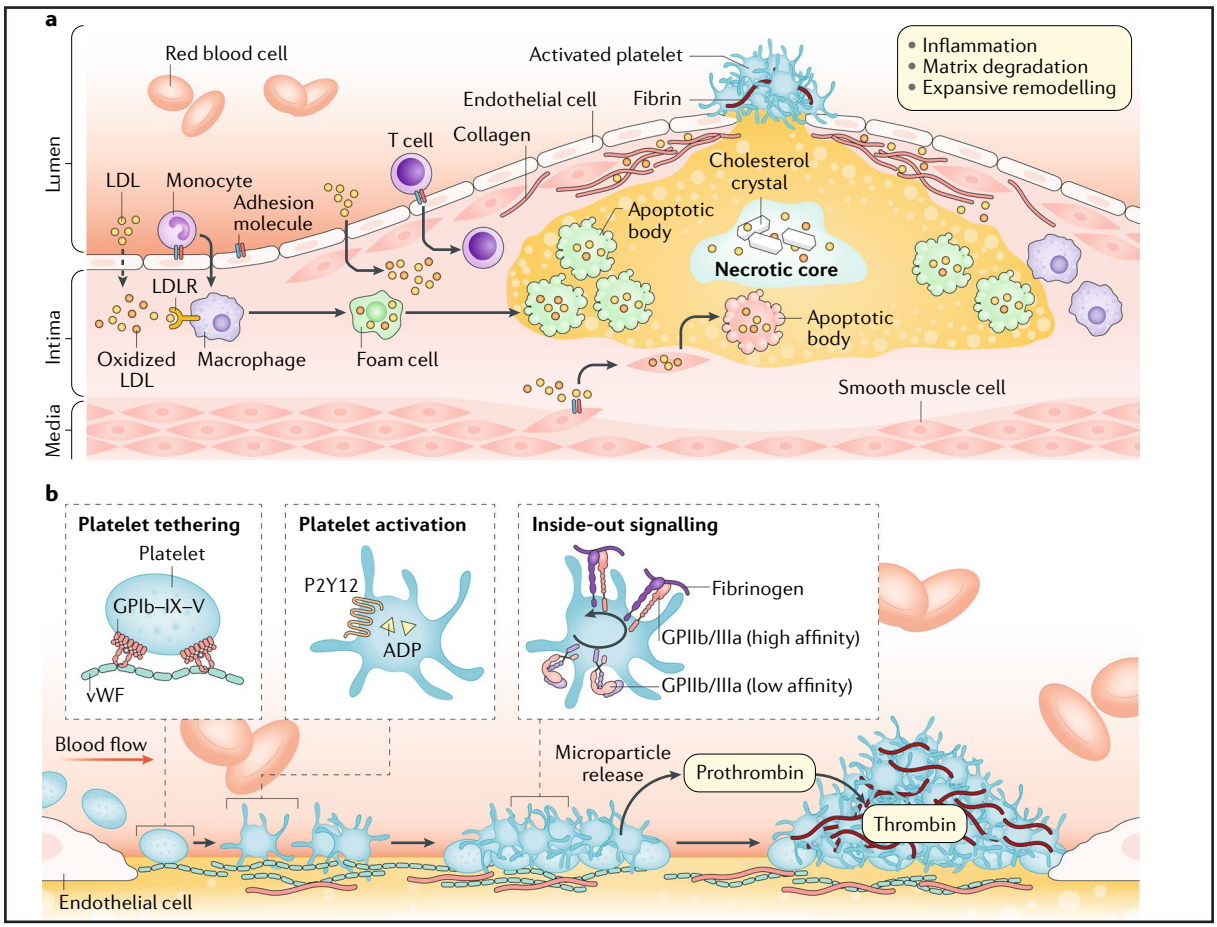


Figure 2. Mechanisms of plaque formation and arterial thrombus formation upon plaque rupture (Vogel et al., 2019), *reproduced with permission from the publisher.*

thrombocyte activation, as discussed later in the attached review which is part of this thesis.

As mentioned before, arterial thrombosis can also occur in the absence of atherosclerotic plaques. Arterial embolisms can be the cause for coronary artery occlusions, most commonly triggered by either infective endocarditis, atrial fibrillation and artificial heart valves (Lacey et al., 2020). Another cause for arterial thrombosis in the absence of plaques are arterial dissections, which most commonly affect cerebral arteries and subsequently cause ischemic strokes (Montalvan et al., 2020).

Furthermore, thrombosis can occur in arteries which are affected by vasculitis but unaffected by atherosclerosis. Giant cell arteritis, which is characterized by the inflammation of cranial and large vessels in people of advanced age, can cause arterial thrombi leading to strokes, tongue infarctions and scalp necrosis (Dejaco et al., 2017). Similarly, Takayasu's arteritis, a vasculitis that mainly affects the aortic arch and its descending arteries, can be the cause of thrombotic occlusions in a variety of locations (Vaideeswar and Deshpande, 2013).

Lastly, platelet-driven arterial thrombosis can be seen in the absence of vessel inflammation, as seen in myeloproliferative neoplasms such as essential thrombocythemia and polycythemia vera (Hultcrantz et al., 2018) or can be drug-induced, as seen in heparin-induced thrombocytopenia (HIT) (Jang and Hursting, 2005).

1.1.3 Venous thrombosis

The most common manifestation of thrombotic venous events are venous thromboembolisms (VTE), which includes deep vein thrombosis (DVT) and pulmonary embolism (PE). VTE is a major cause of mortality in the world, ranking third directly after coronary heart disease and ischemic stroke (Raskob et al., 2014). Among caucasians, a rate of 1-2 VTE events per 1000 person years has been found in several studies (Blom et al., 2006; Cushman et al., 2004; Heit, 2005; Silverstein et al., 1998; Spencer et al., 2006).

While research and practice often focuses on VTE due to its immense global disease burden, venous thrombosis can also occur in other sites, such as the hepatic veins, cerebral veins, retinal veins or renal veins, albeit more rarely (Shatzel et al., 2019).

Venous thrombosis shares certain risk factors with arterial thrombosis, such as advanced age (Figure 3), obesity, diabetes and smoking (Wolberg et al., 2015). Some factors however, play a major role mainly in the pathogenesis of venous thrombi, including major surgery, trauma, prolonged immobilization and cancer (Anderson and Spencer, 2003) (Table 1).

Strong risk factors	Moderate risk factors	Weak risk factors
Fracture (hip or leg)	Arthroscopic knee surgery	Bed rest >3 days
Major general surgery	Central venous lines	Immobility due to sitting
Major trauma	Chemotherapy	Age
Spinal cord injury	Congestive heart or respiratory failure	Laparoscopic surgery
	Hormone replacement therapy	Obesity
	Malignancy	Pregnancy (antepartum)
	Oral contraceptive therapy	Varicose veins
	Paralytic stroke	
	Pregnancy (postpartum)	
	Previous venous thromboembolism	
	Thrombophilia	

Table 1. Risk factors for the development of venous thrombosis. *Adapted from (Anderson and Spencer, 2003)*

Certain types of cancer coincide with a particularly high risk of developing VTE, such as those of ovarian, bone, brain and pancreatic origin (Blom et al., 2006). The pathophysiology of cancer-related VTE may differ between the different types, such as increased podoplanin expression leading to enhanced platelet activation in brain tumors (Riedl et al., 2017). Microvesicle-associated TF activity in particular has been suggested to play an important role in the development of cancer-associated thrombosis (CAT) (Ay et al., 2017) and has been the subject of an increasing number of studies in recent years.

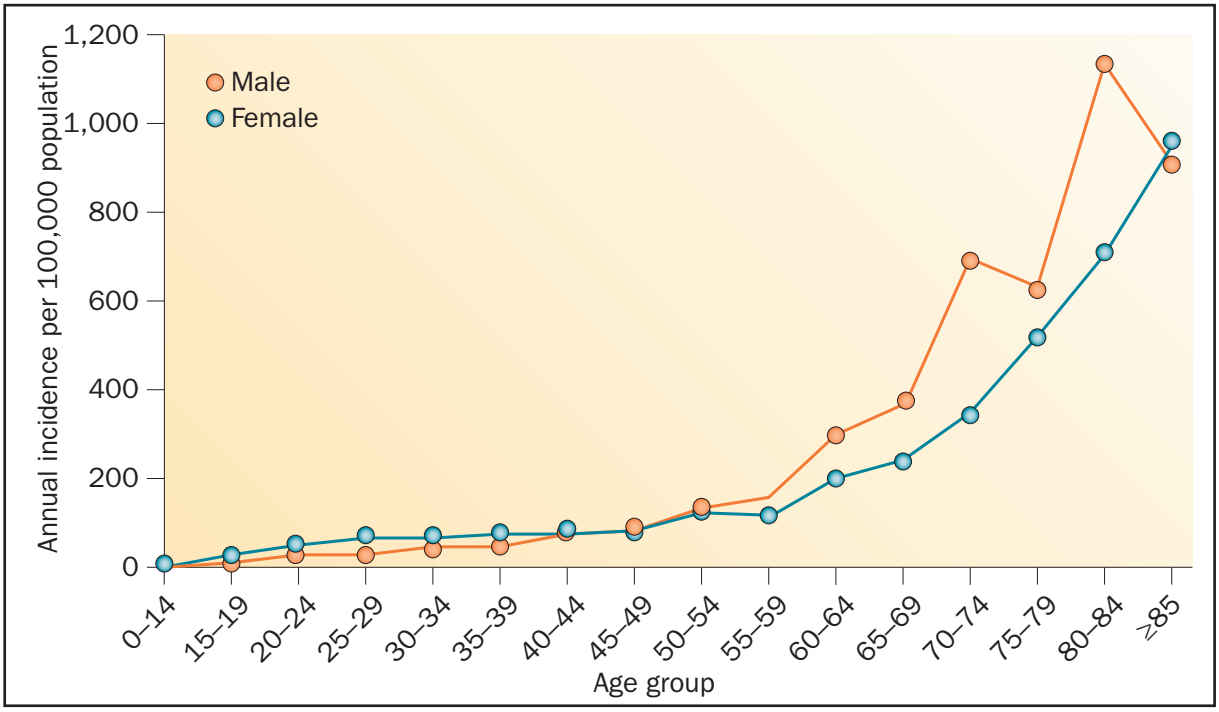


Figure 3. Age distribution of VTE cases in a US cohort (Heit, 2015), *reproduced with permission from the publisher.*

Due to their high content of red blood cells and fibrin, fresh venous thrombi appear mostly red in color and are therefore often called “red thrombi” (though older venous thrombi or those derived from pulmonary emoblisms may have a more heterogenous appearance) as opposed to arterial platelet rich “white thrombi”, which are predominantly made up of platelets and fibrin. While the mortality of acute VTEs is quite high, with pulmonary embolisms posing an 18-fold increased risk of early death compared to patients with only DVTs (Heit et al., 1999), long-term complications after the occurrence of a venous thrombotic event also pose a clinical challenge. Recurrence after initial VTE within 10 years is estimated to approximately 30%, with rates as high as 15-25% within the first three months, if unmedicated (Heit, 2015). A common complication after resolution of DVT is the so-called post-thrombotic syndrome (Prandoni and Kahn, 2009), characterized by pain, swelling, tingling or cramps, usually occurring while standing or walking. A serious complication after the occurrence of pulmonary embolism is chronic thromboembolic pulmonary hypertension (CTEPH), in which intraluminal thrombus organization leads to pulmonary vascular remodeling and severe pulmonary hypertension (Hoeper et al., 2006). These long term complications pose a challenge in the therapy after the occurrence of a venous thrombotic event, especially with respect to the type and duration of anticoagulatory therapy outlined below.

1.1.4 Pharmacological inhibition of blood coagulation

Approved anticoagulatory drugs either directly or indirectly target proteins of the common pathway of the coagulation cascade, or inhibit platelet function (Mackman et al., 2020), (Figure 4). Heparin and coumarins were the first anticoagulatory substances to be discovered in the early 20th century and subsequently the first to be investigated in a randomized controlled trial for the treatment of acute pulmonary embolism in the late 1950s (Barritt and Jordan, 1960). Similarly, acetylsalicylic acid, which was discovered to inhibit platelet aggregation in 1967 (Weiss and Aledort, 1967), with its benefit in suspected myocardial infarction patients being demonstrated in 1988 (ISIS-2 Collaborative Group, 1988). Since then, several novel thromboprotective substances specifically targeting coagulation factor Xa or thrombin (Levy et al., 2014) or platelet activation pathway proteins such as P2Y12 or glycoprotein IIb/IIIa (McFadyen et al., 2018) have been introduced.

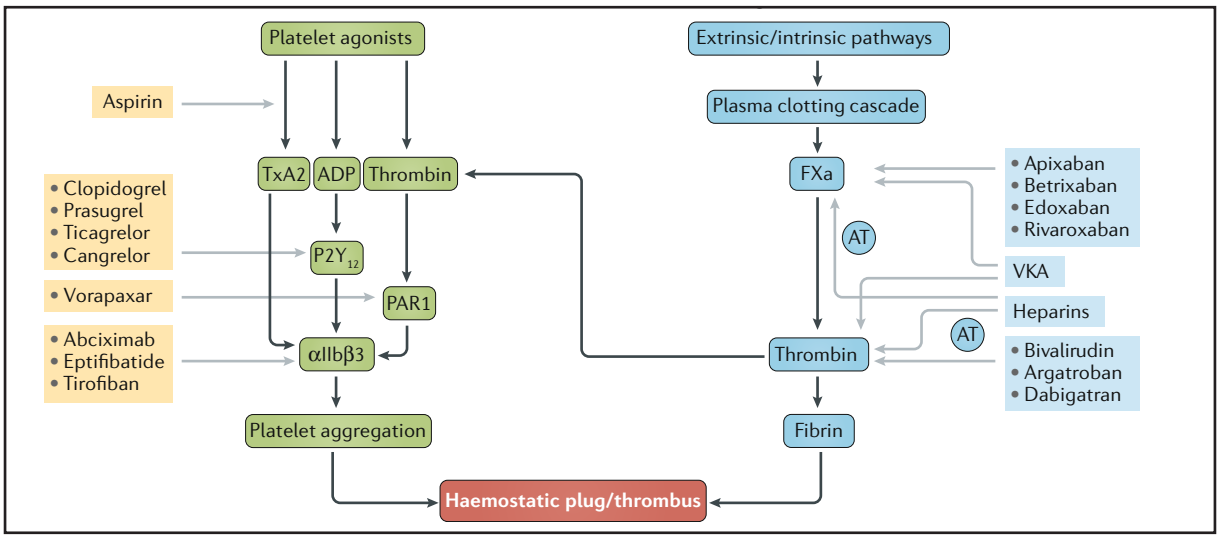


Figure 4. Overview of current anticoagulatory/antiplatelet drugs and their targets (Mackman et al., 2020), *reproduced with permission from the publisher.*

With respect to anticoagulatory therapy, low molecular weight heparin (LMWH) has continued to be the gold standard in the initial treatment of uncomplicated DVT. It causes a conformational change after binding to antithrombin, leading to a 1000-fold increase in its ability to deactivate FXa and thrombin.

LMWH does not require anticoagulant monitoring, but has to be given subcutaneously, which is why it is usually not ideal for long term anticoagulant treatment. Instead, vitamin K antagonists such as warfarin have been used in the past decades. The advantage compared to LMWH is the oral route of administration, with the downside of mandatory anticoagulatory monitoring. They work by inhibiting the enzyme vitamin K epoxide reductase that recycles vitamin K, which is needed for the postranslational carboxylation of glutamic acid residues needed for the

function of the following zymogens: prothrombin, factor VII, factor IX, factor X and protein C. Since protein C, an inhibitor of coagulation, has a shorter half-life than the coagulation factors, bridging with heparin has to be performed for 4-5 days when initiating therapy with vitamin K inhibitors. Furthermore, as just mentioned, anticoagulatory monitoring has to be maintained over the course of treatment, to ensure the international normalized ratio (INR) stays in the effective therapeutic window of 2-3.

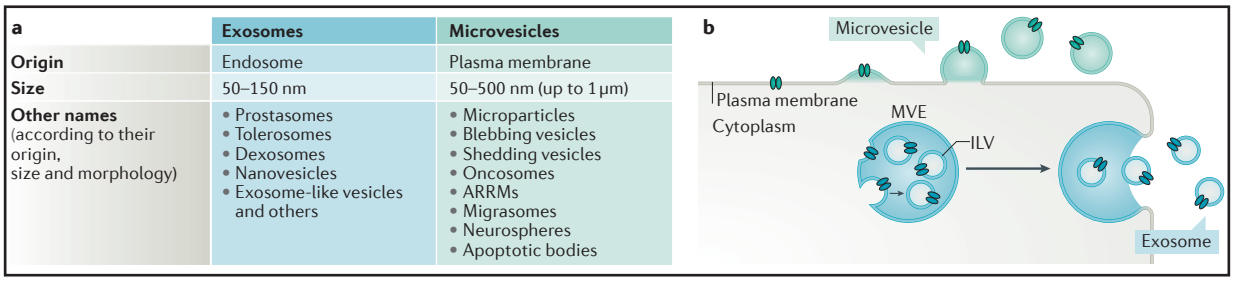
In recent years, direct oral anticoagulants (DOACs), which inhibit factor Xa or thrombin, have become safer and more convenient alternatives of vitamin K antagonists with similar or superior efficacy in long term anticoagulation of different patient populations (van Es et al., 2014; Male et al., 2020; Posch et al., 2015). The initial major downside of DOACs was the lack of reversal agents, which have entered the market only recently (Pollack et al., 2015; Siegal et al., 2015), with the rivaroxaban reversal agent (andexanet alfa) having been approved by the European Medicine Agency only in 2019.

As all current drugs are limited by their propensity to also increase bleeding risk, considerable efforts have pursued novel ways to inhibit coagulation without affecting hemostasis. One of these potential avenues is the inhibition of the contact activation system (Fredenburgh et al., 2017). Out of all the candidates, the inhibition of coagulation factors XI and XII has shown to be effective in different preclinical and clinical trials. FXI and FXII antibodies (Koch et al., 2019; Larsson et al., 2014; Weitz et al., 2020) an FXI antisense oligonucleotide (Büller et al., 2015) or FXI small molecule inhibitors (clinicaltrials.gov: NCT03891524) are all under ongoing investigation and may prove to exhibit similar thromboprotective effects with a better safety profile than current anticoagulatory drugs.

1.2 Microvesicles

1.2.1 Introduction to extracellular vesicles

Extracellular vesicles (EVs) are membrane blebs released by all pro- and eukaryotic cells (Deatherage and Cookson, 2012). Depending on their origin within the cell, they can be classified into two groups: exosomes, which originate from multivesicular endosomes, and microvesicles (MVs), sometimes also called microparticles, which are formed upon the budding of the cell membrane itself (van Niel et al., 2018) and are part of the main focus of this work (Figure 5). Heterogeneous in nature, their size range is most commonly set as 0.1-1µm, and they are defined by the exposure of phosphatidylserine (PS) (though PS-negative MVs are a heavily debated topic), on their surface, as opposed to exosomes, which do not expose PS.



a	Exosomes	Microvesicles
Origin	Endosome	Plasma membrane
Size	50–150 nm	50–500 nm (up to 1 µm)
Other names (according to their origin, size and morphology)	• Prostasomes • Tolerosomes • Dexosomes • Nanovesicles • Exosome-like vesicles and others	• Microparticles • Blebbing vesicles • Shedding vesicles • Oncosomes • ARRMs • Migrasomes • Neurospheres • Apoptotic bodies

Figure 5: Overview of the classification and formation of extracellular vesicles. (van Niel et al., 2018), *reproduced with permission from the publisher.*

The main constituents of extracellular vesicles are proteins, nucleic acids and (phospho)lipids (Figure 6). Mass spectrometry-based proteomic analyses have been used to analyze EV-associated proteins to obtain insights about EV-biology. Broadly speaking, EV-associated proteins can be classified into two different groups. The first group are ubiquitous EV-proteins, such as cytoskeletal proteins, metabolic enzymes or adhesion proteins. These proteins can be found in almost every proteomic analysis and are often related with EV biogenesis. The second group is more specific to the respective cell releasing EVs and therefore often defines the biological effects of the vesicle (e.g. tissue factor present on MVs released by activated macrophages (Del Conde et al., 2005) or cancer cells (Davila et al., 2008) The lists of all proteins that have been found to associate with extracellular vesicles, identified by a large body of different proteomic studies, e.g. (Choi et al., 2012; Duijvesz et al., 2013; de Menezes-Neto et al., 2015) are publicly accessible in the Vesiclepedia database (http://www.microvesicles.org/). EVs can also carry different types of nucleic acids, though most research in this field focuses on exosomes rather than microvesicles. A hallmark study in the EV field demonstrated that exosomes mediate transfer of mRNA and miRNA, and that those mRNAs could be translated into proteins by the recipient cells (Valadi et al., 2007). More recently, another group demonstrated that tumor-derived exosomes contain double-stranded DNA (dsDNA), reflecting

the mutational status of their parental cells, highlighting their potential use as an oncologic biomarker (Thakur et al., 2014).

Considering the fact that phosphatidylserine is used as a major classifier for MVs, it is surprising that lipids remain the least well understood component of extracellular vesicles, perhaps owing to the relative difficulty in performing lipidomic analyses. Similar to the analyses of nucleic acids in EVs, quantitative analyses have mostly been performed on exosomes (Laulagnier et al., 2004; Llorente et al., 2013; Phuyal et al., 2015; Trajkovic et al., 2008), with most studies showing enrichment of cholesterol, sphingomyelin, glycosphingolipids and phosphatidylserine in vesicles compared to the parental cells.

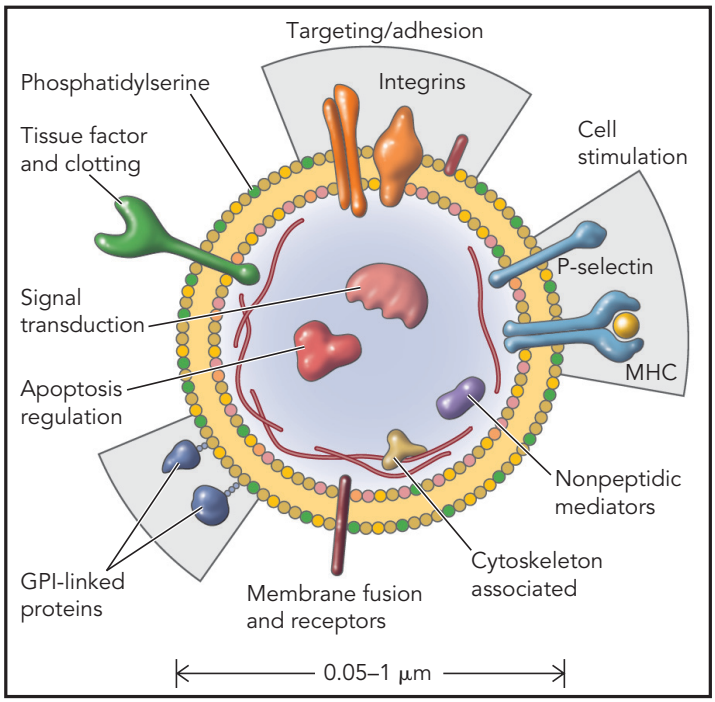


Figure 6: Overview of bioactive molecules that are frequently exposed by or carried within microvesicles (Hugel et al., 2005), *reproduced with permission from the publisher.*

Initially, MVs were isolated from human plasma and described as "platelet dust" (Wolf, 1967), which was observed to possess procoagulatory properties. Following this discovery, much research into EV and MV biology focused on their role in cardiovascular physiology and pathology. Continuous research in EV biology led to a myriad of discoveries about the importance of EVs in many different processes and pathologies, such as inflammation (Buzas et al., 2014), immune response regulation (Robbins and Morelli, 2014), cancer (Xu et al., 2018), as well as metabolic (Martínez and Andriantsitohaina, 2017), neurodegenerative (Thompson et al., 2016) and infectious disease (Raab-Traub and Dittmer, 2017).

1.2.2 Microvesicle generation

All cells - including platelets/megakaryocytes, which are the source of a majority of circulating MVs (Arraud et al., 2014; Flaumenhaft et al., 2009), release MVs both in resting and activated states (Figure 7). Therefore, the amount of measured MVs in circulation at any given time point reflects a steady-state between the generation and clearance of MVs.

Several discoveries about the biogenesis of MVs have been made in recent years. A hallmark feature of MVs is the presence of phosphatidylserine on the outer membrane leaflet. In eukaryotic cells, membrane phospholipids are asymmetrically distributed (Manno et al., 2002), with phosphatidylserine being actively kept on the inner membrane of healthy cells. The main enzymes involved in the distribution of phospholipids between the inner and outer leaflet of the cell membrane, flippase, floppase, scramblase and calpain. A rare disease called Scott syndrome, in which a decreased floppase activity leads to impaired PS exposure and MV generation in platelets, and its bleeding phenotype demonstrates the importance of these pathways in hemostasis (Sims et al., 1989).

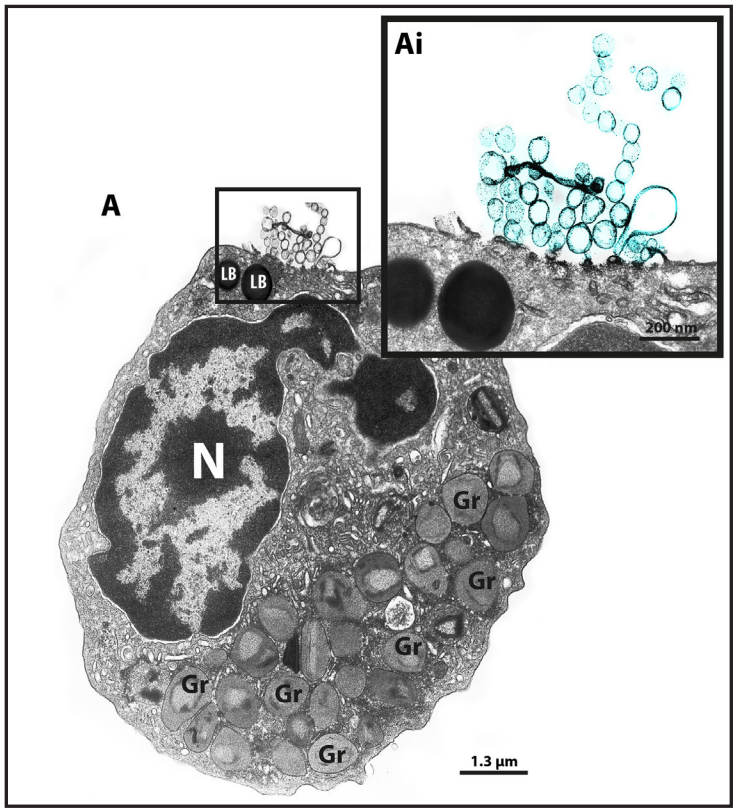


Figure 7: Microvesicles released from an activated neutrophil granulocyte (Akuthota et al., 2016), *reproduced under the terms of the Creative Commons Attribution License (CC BY).*

Cytoskeletal rearrangement is another important factor in the generation of MVs, particular in the fission and subsequent pinching-off of the cellular membrane (van Niel et al., 2018). Since PS binds to cytoskeletal proteins (Comfurius et al., 1989), translocation of PS towards the outer leaflet is considered to allow the membrane deformation necessary for MV formation. Lastly, lipid rafts, which are areas in the cell membrane rich in cholesterol, sphingomyelins and glycosphingolipids, have also been shown to play an important role (Wei et al., 2018), and the depletion of membrane cholesterol has been demonstrated to inhibit the formation of MVs (Del Conde et al., 2005).

1.2.3 Microvesicle clearance

Clearance remains one of the least understood topics of MV biology, owing to a large degree to how hard it is to study these dynamics in vivo. The first study to investigate the clearance of MVs in vivo found that labeled platelet microvesicles injected into rabbits were cleared rapidly within 10 minutes (Rand et al., 2006). This study gave the first insight on the dynamics of MV-homeostasis in the circulation. The difference in the vasculature and therefore hemodynamics between small animals like mice and rabbits and humans renders these results a rough approximation at best. Since the injection of MVs into humans is not possible from an ethical standpoint, other, more creative approaches have been pursued.

Since platelet concentrates contain a significant number of MVs, one can measure their levels after infusion and draw conclusions about the dynamics from their plasma levels. Rank et al, who investigated the clearance of microvesicles in severe thrombocytopenic patients who received platelet transfusions, found the half life of platelet-derived MVs of 5-6 hours (Rank et al., 2011), which is significantly longer than what was found in rabbit experiments.

It has been previously proposed that one of the main clearance mechanisms for MVs in circulation is phosphatidylserine/lactadherin-mediated uptake by splenic macrophages (Dasgupta et al., 2009). Furthermore, it has been shown that phosphatidylserine - developmental endothelial locus-1 (Del-1) mediated binding to endothelial cells can also contribute to the clearance of platelet derived MV from the circulation (Dasgupta et al., 2012). Another interesting mechanism explaining the fast clearance of free MVs from circulation may be the complement mediated adhesion to red blood cells (RBC), as it has been shown that platelet-derived MVs that carry C3b adhere to RBCs via complement receptor 1 in vitro (Cumpelik et al., 2015). One has to be aware that the majority of these studies focus on the uptake of platelet-derived MVs, which is the most abundant type of MV in circulation. Other, more specific mechanisms, which pertain only to certain types of MVs and are dependent on the presence of specific biomolecules on their surface may be discovered in the future. Furthermore, the internalization of MVs can happen through different uptake mechanisms, such as phagocytosis, macropinocytosis, clathrin-mediated endocytosis and plasma or endosomal membrane fusion, which also depends on the MV composition (Mulcahy et al., 2014). The type of pathway taken may influence the effect that MVs exert on the uptaking cells, which in turn may affect e.g. cell-cell communication in cancer and other diseases (Xu et al., 2018).

1.2.4 Isolation and detection of microvesicles

Due to their small size, heterogeneous nature, and overlap with other extracellular vesicles, methods of isolation and detection of MVs have been a controversial and continuously evolving topic within the scientific community. To improve reproducibility between groups, the International Society for Extracellular Vesicles (ISEV) has published a comprehensive compendium titled minimal information guidelines for publication of EV studies (MISEV) (Théry et al., 2018). Commonly used isolation and characterization methods of EVs, as well as their specific advantages and drawbacks are summarized below (Table 1&2).

Isolation method	Scalability	Accessibility	Specificity
High-speed centrifugation	++	+++	-
Density gradient isolation	++	++	+/-
Size-exclusion chromatography	+	+/-	+
Membrane-affinity columns	+	+/-	+
Immuno(affinity)-based isolation	-	+	+++

Table 2. Commonly used methods for the isolation of EVs.

Quantification / characterization method	Scalability	Accessibility	Resolution
Flow cytometry	++	++	+
Nanoparticle tracking analysis	-	+	++
Electron microscopy*	-	-	+++
Total protein / lipid quantification**	++	+++	N/A

Table 3. Commonly used methods for the quantification and/or characterization of EVs. **only applicable for characterization, **only applicable for quantification*

1.2.5 Microvesicles in arterial and venous thrombosis

Following investigations into their procoagulatory activity, several significant discoveries of the role of MVs in cardiovascular physiology and pathology have been made through the past decades. They owe their procoagulatory effects to the exposure of phosphatidylserine and, depending on their origin, also TF on their surface (Owens and Mackman, 2011). As mentioned above, the majority of TF within atherosclerotic plaques is considered to be associated with MVs, making them the central driver of coagulation upon plaque disruption. Additionally, MVs from platelets, endothelial cells and leukocytes can further propagate coagulatory processes after the initial thrombus formation. Investigating the role of MVs in arterial thrombosis, elevated levels of circulating endothelial MVs were found in patients with unstable angina pectoris or myocardial infarction, compared to patients with stable angina or controls (Mallat et al., 2000). Another group corroborated these findings by demonstrating a very high level of TF+ MVs of endothelial and leukocytic origin in the coronary artery of STEMI patients compared to circulation (Morel et al., 2009).

In venous thrombosis, elevated levels of TF+ MVs have been investigated as prognostic biomarkers in cancer-associated thrombosis in particular. Several studies have found that pancreatic cancer patients display increased levels of TF+ MVs prior to developing DVT (van Doormaal et al., 2012; Khorana et al., 2008; Thaler et al., 2012; Zwicker et al., 2009). Of note, most studies investigating TF+ MVs use MV-associated TF-activity, rather than actually measuring TF+ MVs, which lacks the sensitivity to detect MVs with a low amount of TF (Vallier et al., 2019). A particularly high TF-activity per vesicle in pancreatic cell derived MVs may explain why such a high rate of DVT is prevalent in patients suffering from this type of cancer. In line with this, the vesicles used to induce murine pulmonary thrombosis in our study are derived from the pancreatic cancer cell line HPAF-II and displayed an extremely high coagulant potential and were easily detected as TF-positive even in flow cytometry (Obermayer et al., 2021).

1.3 Natural antibodies

Antibodies are a class of glycoproteins which constitute a principal component in the adaptive immune response to foreign pathogens. A subtype of immunoglobulins however are produced without prior exposure to exogenous antigens and have therefore been termed “natural antibodies” and are part of the innate immune response (Holodick et al., 2017). The majority of natural antibodies belong to the immunoglobulin M (IgM) subtype, in addition to IgG3 and IgA (Panda and Ding, 2015). IgM is produced prior to class switching and the first type to be upregulated in the response to infections (Boes, 2000). While adaptive IgM antibodies are produced by B2 cells in a T-cell dependent manner, natural IgM antibodies are produced by B1 cells in a T-cell independent manner.

1.3.1 Natural IgM antibodies in different diseases

Since membrane-bound IgM comprises the B cell receptor (BCR) and therefore plays a crucial role in B-cell survival, most studies investigating the role of IgM antibodies rely on a mouse model of secreted IgM deficiency (sIgM-/-). Using this model, different groups have demonstrated that the absence of secreted natural IgM leads to worse outcomes in a wide variety of different infections (Ehrenstein and Notley, 2010), (Table 4). They perform these functions by direct neutralization (Ochsenbein et al., 1999) but can also form immune complexes together with the complement system. Furthermore, natural IgM assists in the recognition of pathogens for the initiation of adaptive immunity (Atif et al., 2018; Boes et al., 1998).

Viral	Bacterial	Fungal	Parasitic
Influenza virus	Pseudomonas aeruginosa	Cryptococcus neoformans	Toxoplasma gondii
West Nile virus	Ehrlichia muris		Trypanosoma evansi
LCM virus	Nocardia brasiliensis		Trypanosoma brucei
Vesicular stomatitis virus	Borrelia hermsii		Brugia pahangi
	Streptococcus pneumoniae		Plasmodium falciparum
			Plasmodium chabaudi

Table 4: Pathogens which natural IgM has been shown to protect against. *Adapted from (Ehrenstein and Notley, 2010)*

Another large body of evidence for the beneficial effects of natural IgM antibodies can be found in studies investigating their association with atherosclerosis and arterial CVD outcomes. In the early 2000s, an inverse correlation between levels of OxLDL-binding IgM and atherosclerosis levels determined by the intima-media thickness (IMT) of the carotid artery was found even after adjusting for common risk factors such as age, lipid levels and CRP in middle-aged men and women (Karvonen et al., 2003).

Similarly, a different group noted an inverse correlation between the levels of OxLDL-binding IgM and the extent of coronary artery disease in patients undergoing coronary angiography when performing a univariate, but not in a multivariable analysis (Tsimikas et al., 2007). The same group later quantified oxidation-specific biomarkers in subjects of the Bruneck study (Kiechl and Willeit, 2019), noting the same inverse correlation of OxLDL-binding IgM and composite CVD (which includes acute coronary interventions, myocardial infarction, ischemic stroke, new-onset unstable angina, and vascular death), as well as strokes (Tsimikas et al., 2012). Since then, several more studies have corroborated these findings by demonstrating similar inverse associations between CVD and OxLDL-binding IgM (van den Berg et al., 2018; Khamis et al., 2016; Meeuwsen et al., 2017; Prasad et al., 2017).

Interestingly, pneumococcal vaccination has been shown to increase anti-oxLDL antibodies and subsequently lead to reduced atherosclerosis in mice. This can be linked to molecular similarities between the phosphocholine present in the pneumococcal capsule and the phosphocholine group of oxidized phospholipids present on oxidized LDL (Binder et al., 2003). These preclinical results have been corroborated in several observational studies pointing towards a beneficial effect of pneumococcal vaccinations in cardiovascular disease by reducing the risk of acute coronary syndrome and are being further followed up in the Australian randomized controlled AUSPICE trial (Ren et al., 2016).

1.3.2 Natural IgM in coagulation and thrombosis

As outlined in the publication leading to this thesis, the finding that natural IgM antibodies can directly inhibit coagulation is a completely novel one. However, circumstantial evidence leading up to our finding has been published quite a while ago. One of the earliest indirect indications that IgM antibodies could play a role in coagulation was the increased incidence of myocardial infarctions observed in splenectomized WWII veterans (Robinette and Fraumeni, 1977). The authors hypothesized that post-splenectomy thrombocytosis could be the culprit of this increase, even though most ischemia-related deaths occurred more than 15 years after the splenectomy, while the increase in platelets after splenectomy is usually transient. Another large cohort study in over 8000 splenectomized American veterans noted a significant long term increase of venous thrombotic events (Kristinsson et al., 2014).

Another study noted a missing association between postsplenectomy platelet levels and incidence of VTE (Lee et al., 2015), further indicating that thrombocythemia is not the culprit of increased venous thrombotic events.

More direct findings linking natural IgM antibodies and coagulation which are not related to atherosclerosis was published more recently. A recent study found an inverse correlation between the levels of natural IgM antibodies to oxidation-specific epitopes and the risk of developing recurrent VTE (Eichinger et al., 2018). Another study investigating the immune defense against neoehrlichiosis, and IgM antibodies in particular, since splenectomy is a risk factor for severe disease courses. Interestingly, they also noted an inverse relationship between the levels of natural IgM antibodies and the incidence of deep vein thrombosis in a small group of patients (Wennerås et al., 2017).

Considering the correlative aspect of both studies, conclusions about the mechanism behind those findings were not able to be drawn. Taking into account the vast evidence of literature outlining the antiinflammatory aspects of natural IgM antibodies, a reduction in thromboinflammation may have been the obvious explanation for these findings up to now. As will be discussed in this thesis, I believe that the binding and neutralization of procoagulatory microvesicles by natural IgM antibodies is the main driver behind these epidemiological phenomena.

1.4 Immunothrombosis, oxidation-specific epitopes & interlude to review

The terms immunothrombosis (frequently used interchangeably with immunohemostasis and thromboinflammation) describes antimicrobial innate immune responses that lead to the formation of microthrombi as a response to intravascular microorganisms (Engelmann and Massberg, 2013). This physiological process limits their dissemination and tissue invasion, while promoting immune cell recruitment to the site of pathogen invasion. More broadly, immunothrombosis can be used to describe the close interconnectivity between innate immunity and blood coagulation, which have been previously viewed as separate. Examples are the discovery of neutrophil extracellular traps (Brinkmann et al., 2004) and their capacity to promote thrombosis (Fuchs et al., 2010). Vice versa, platelets have been increasingly recognized as important innate immune effectors (Gaertner and Massberg, 2019), and were even discovered to be able to capture and trap bacteria (Gaertner et al., 2017). Furthermore, an increasing amount of studies link the complement system and coagulation.

One powerful mediator of inflammation are oxidation-specific epitopes (OSEs), which are generated during the oxidative modification and degradation of lipids, called lipid peroxidation. These OSEs, which can arise during both enzymatic and non-enzymatic reactions, are recognized by a variety of cellular and soluble pattern recognition receptors (PRRs) which includes natural IgM antibodies.

As we have previously shown, natural IgM antibodies that bind to OSEs present on oxidatively modified lipoproteins also bind to a subset of circulating MVs and can inhibit their proinflammatory effects on cells (Tsiantoulas et al., 2015).

Lipoproteins do not directly promote coagulation, since they do not carry PS (Dashti et al., 2011) or TF. However, OSEs on lipoproteins and MVs are powerful mediators of inflammation (Binder et al., 2016) and therefore can affect the heavily interconnected systems of immunity, inflammation and coagulation. The following review focuses on the effects of oxidatively modified low density lipoprotein and its effect on inflammation-driven thrombosis.

1.5 Review: Oxidized low-density lipoprotein in inflammation-driven thrombosis

Oxidized low-density lipoprotein in inflammation-driven thrombosis.

Journal of Thrombosis and Haemostasis, 2018 Jan 24;16(3):418–28, DOI: 10.1111/jth.13925

Georg Obermayer, Taras Afonyushkin and Christoph J. Binder

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Oxidized low-density lipoprotein in inflammation-driven thrombosis

G. OBERMAYER,*† T. AFONYUSHKIN*† and C. J. BINDER*†
**Department of Laboratory Medicine, Medical University of Vienna; and †Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria*

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Summary. Thrombosis is the defining feature of the most prevalent causes of cardiovascular mortality, such as myocardial infarction, stroke, and pulmonary artery embolism. Although platelet activation and activation of the plasmatic coagulation system are the hallmarks of thrombus formation, inflammatory processes and the cellular responses involved are increasingly being recognized as critical modulators of thrombosis. In the context of many chronic inflammatory diseases that are associated with a high thrombotic risk, oxidized lipoproteins represent a prominent sterile trigger of inflammation. Oxidized low-density lipoprotein and its components play a central role in the initiation and progression of atherosclerotic plaques, but also in other processes that lead to thrombotic events. Moreover, dying cells and microvesicles can be decorated with some of the same oxidized lipid components that are found on oxidized lipoproteins, and thereby similar mechanisms of thromboinflammation may also be active in venous thrombosis. In this review, we summarize the current knowledge on how oxidized lipoproteins and components thereof affect the cells and pathways involved in thrombosis.

Keywords: coagulation; inflammation; microvesicles; oxidized LDL; statins; thrombosis.

Introduction

The hemostatic system involves a tight interplay between plasmatic coagulation and the activation of vascular cells, most prominently platelets. Adherence of platelets to the endothelium initiates a process of activation and aggregation that involves several surface receptors and triggers primary clot formation [1]. Plasmatic coagulation enzymes are activated by charged surfaces and by tissue factor (TF), ultimately leading to the generation of the central coagulation protease thrombin, which generates fibrin. This process is controlled by a myriad of feedback and inhibitory mechanisms [2], and is further enhanced by the generation of microvesicles (MVs), which form an additional surface of anionic phospholipids to further support the assembly of plasmatic clotting factors [3]. Endothelial cells (ECs) not only prevent exposure of blood to procoagulatory smooth muscle cells (SMCs) and the subendothelial matrix, but are also actively involved in coagulation and the prevention thereof. Pathological activation of the hemostatic system can lead to thrombosis and thromboembolism.

Thrombosis is typically classified as arterial or venous, according to the anatomical site of occurrence. Arterial thrombosis is primarily associated with the rupture or erosion of atherosclerotic plaques, exposing prothrombotic material, in particular high local concentrations of TF [4], whereas venous thrombosis typically occurs in low shear stress conditions in the absence of endothelial disruption [5]. Although different pathological changes underlie the development of arterial and venous thrombosis, common mechanistic pathways exist for both. For example, oxidized low-density lipoprotein (OxLDL) and its active lipid components have been primarily demonstrated to contribute to atherothrombosis either directly or by inducing inflammation, which, in turn, has been shown to promote coagulatory processes. Recent findings that the same lipid peroxidation-derived structures are also found on the surfaces of circulating MVs [6] suggest a role for oxidized lipids beyond arterial thrombosis. Therefore, understanding the mechanisms that mediate the effects of OxLDL and its active components in inflammation-driven thrombosis may provide novel insights into thromboembolic diseases. The proinflammatory properties of oxidized lipoproteins and the molecular mechanisms mediating them are reviewed extensively elsewhere [7]. In this review, we present an overview of the effects of oxidized lipoproteins on processes directly and indirectly driving coagulation and thrombosis.

Correspondence: Christoph J. Binder, Department of Laboratory Medicine, Medical University of Vienna, Lazarettgasse 14, AKH BT 25.2, Ebene 6, Vienna, Austria
Tel.: +43 1 40400 73755
E-mail: christoph.binder@meduniwien.ac.at

Inflammation and thrombosis

The crucial contribution of inflammation to thrombosis has been a subject of investigation for many decades [8]. Indeed, atherosclerosis, which is the prerequisite for most arterial thrombotic events, is nowadays considered to be a chronic inflammatory disease of arterial blood vessels [9]. The recent conclusion of the Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS) showing a significant benefit of the anti-interleukin (IL)-1β antibody canakinumab in the secondary prevention of atherothrombotic events has clearly demonstrated the causal involvement of inflammation in this disease process [10]. On the other hand, only a modest association between C-reactive protein (CRP) levels – as markers of inflammation – and venous thromboembolism (VTE) risk has been found, as demonstrated in a recent meta-analysis [11]. Nevertheless, increasing clinical evidence shows a close link between inflammation and venous thrombosis. For example, diseases that are associated with profound inflammation, such as inflammatory bowel disease, rheumatoid arthritis, and Behçet's disease, are associated with an increased risk of venous thrombotic events [12]. Thus, both arterial and venous thrombotic events are influenced by inflammatory processes.

In turn, components of both plasmatic and cellular coagulation are now recognized as promoters of inflammatory processes, and growing evidence supports an important role for platelets in inflammation in addition to their central role in thrombus formation [13]. Moreover, various members of the coagulation cascade possess biological activities that go beyond the formation of fibrin. Thrombin in particular is a central effector of cell activation, and induces inflammatory pathways via the protease-activated receptor family [14]. Thus, it is not surprising that several coagulation factors upstream of thrombin, such as TF [15], activated factor X (FXa) [16], FXI [17], and FXII [18], have been found to also propagate inflammation. Indeed, rivaroxaban, an FXa inhibitor, was recently shown to attenuate atheroscerotic plaque progression in mice, in part through inhibiting the proinflammatory activation of macrophages [19]. Furthermore, cells that are considered to be classical mediators of inflammation have been found to directly (via modulating procoagulatory and anticoagulatory pathways) and indirectly (via promoting vascular inflammation and atherosclerosis) influence thrombotic processes. This led to the development, in recent years, of the concept of immunothrombosis, which describes the role of innate immune responses, in particular by neutrophils and monocytes, in triggering prothrombotic effects [20].

Oxidized lipoproteins and inflammation

Oxidized lipoproteins and damage-associated molecular patterns derived from them have been studied extensively in the context of inflammation and atherosclerosis in particular [21]. LDL is a complex lipoprotein containing different lipid species, including triglycerides, phospholipids, and free and esterified cholesterol. Both enzymatic and non-enzymatic mechanisms have been described that result in the oxidation of both the phospholipid and cholesterol moieties of LDL, resulting in the generation of myriads of different lipid oxidation products [22]. Oxidized cholesterol and phospholipids can in turn be hydrolyzed and release free oxidized fatty acids that decompose to malondialdehyde (MDA) and 4-hydroxy-2-nonenal, and modify LDL proteins and phospholipids. For example, when polyunsaturated fatty acids of phosphatidylcholine undergo oxidation, highly reactive decomposition products, such as MDA and 4-hydroxynonenal, are generated that form adducts with ε-amino groups of lysines or amino groups of other phospholipids. In addition, different species of oxidized phospholipids (OxPLs) that are derived from the truncated phospholipids are formed [23]. The contents of different lipid oxidation species are is highly dependent on the mechanisms of OxLDL generation *in vitro*. Nevertheless, many of the same lipid-derived structures have been described in atherosclerotic plaques, in plasma, as on the surfaces of dying cells and MVs shed by activated vascular cells (see below). Many of these are bound by both cellular and soluble receptors of innate immunity that sense and recognize certain products of lipid peroxidation such as oxidation-specific epitopes (OSEs) [21]. These include soluble pattern recognition proteins, such as CRP and certain complement factors, as well as naturally occurring IgM antibodies that mediate the clearance and removal of cellular debris and oxidized lipoproteins. Furthermore, scavenger receptors, such as CD36 and MSR-1, are critical in mediating the uptake of OxLDL, and cooperate with toll-like receptors (TLRs) to transmit proinflammatory responses in cells (Fig. 1). Thus, various lipid peroxidation products are found in OxLDL, dying cells, and MVs, and many of them possess robust biological activities that can promote vascular pathologies.

Oxidized lipoproteins and plasmatic coagulation

The data on direct prothrombotic effects of OxLDL on plasmatic coagulation are scarce. However, it has been shown that OxLDL delays plasmatic coagulation *in vitro* [24], mediated through inhibition of FVIII, FIX and FXI activity [25]. Another group, however, described increased prothrombinase activity and thrombin generation caused by OxLDL [26]. Yet another group reported that OxPLs led to increased prothrombinase activity [27]. Whether these observations are *in vitro* artefacts or are relevant *in vivo* remains to be determined. Potential inhibition of plasmatic coagulation by oxidized lipoproteins *in vivo* may be overshadowed by their potent activating effects on vascular cells. As discussed in depth below, the activation of various vascular cells can lead to dramatic cellular changes that can directly and indirectly modulate thrombotic processes (Fig. 2).

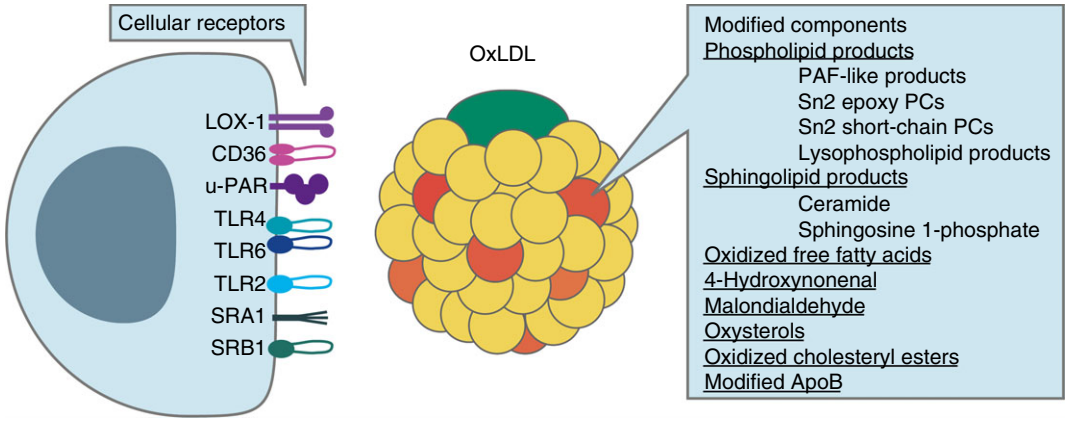

Fig. 1. Components of oxidized LDL (OxLDL) and the cellular receptors involved in their recognition by different cell types. Detailed information on the depicted receptors mediating the thromboinflammatory effects of OxLDL is given in the following references: lectin-like OxLDL receptor-1 (LOX-1) [38,39,47,83], CD36 [29,56,58,59,64,84], urokinase plasminogen activator recdeptor (u-PAR) [56], toll-like receptor 4/6 (TLR4/6) [56,59,73], toll-like receptor 2 (TLR2) [73], and steroid receptor RNA activator 1 (SRA1) [58]. scavenger receptor class B type 1 (SRB1) has also been characterized as a receptor for OxLDL and oxidized phospholipids (OxPLs) [99]. ApoB, apolipoprotein B; Platelet-activating factor (PAF); Phosphatidylcholines (PCs). [Color figure can be viewed at wileyonlinelibrary.com]

Oxidized lipoproteins and platelets

Beyond their central role in thrombus formation, platelets have been recognized to play important roles in acute and chronic inflammatory pathologies that are associated with an elevated thrombotic risk [13].

Activation of platelets upon exposure to OxLDL or OxPLs has been described [28], and has been attributed to recognition by CD36 [29]. Genetic deletion of CD36 significantly attenuated dyslipidemia-associated increased platelet reactivity and thrombosis susceptibility in mice *in vivo*. Therefore, recognition of OxPLs by platelet CD36 has been proposed as a link between prothrombotic risk and dyslipidemia associated with increased oxidative stress [29]. In turn, platelets themselves can – in particular upon activation – contribute to the oxidation of lipoproteins [30], and may thereby further propagate OxLDL-induced thromboinflammation. Platelets treated with OxLDL activate ECs and inhibit endothelial regeneration even more potently than OxLDL alone [31]. OxLDL also induces the formation of platelet–monocyte aggregates, and OxLDL-stimulated platelets have been shown to

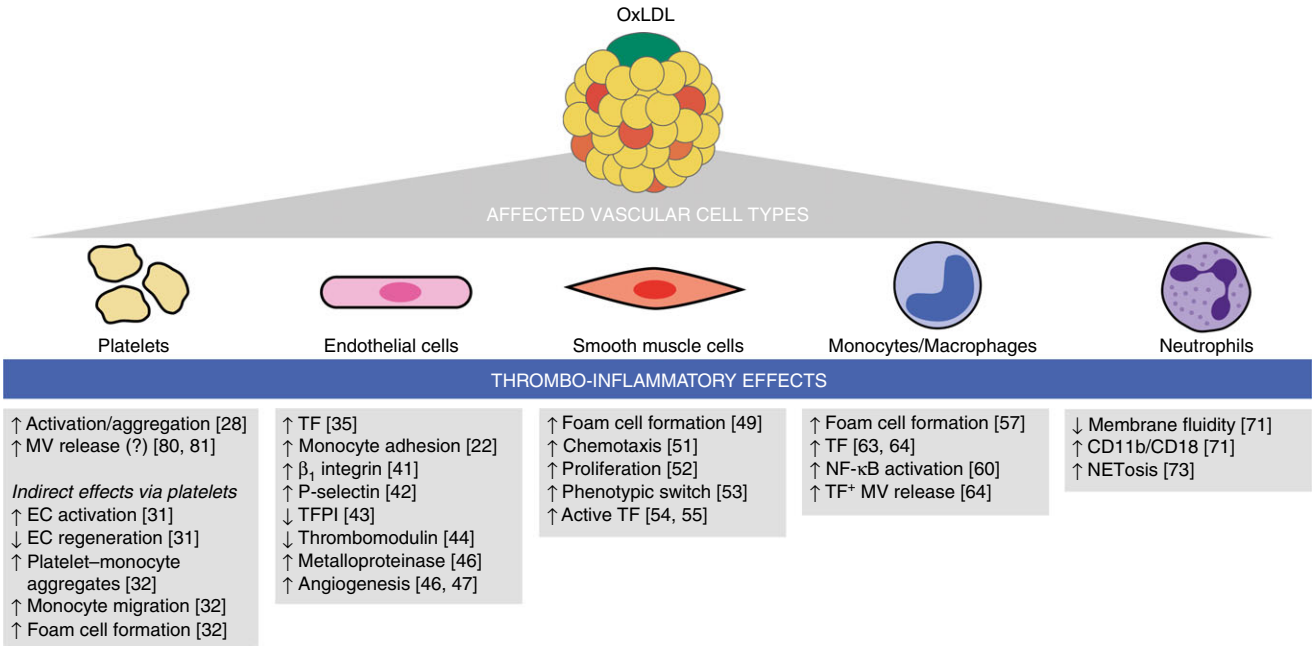

Fig. 2. Effects of oxidized LDL (OxLDL) on vascular cell types in the context of thromboinflammation. OxLDL has been shown to mediate proinflammatory and procoagulatory effects on several vascular cells. EC, endothelial cell; MV, microvesicle; NET, neutrophil extracellular trap; NF-κB, nuclear factor-κB; TF, tissue factor; TFPI, tissue factor pathway inhibitor. [Color figure can be viewed at wileyonlinelibrary.com]

enhance both transendothelial monocyte migration and foam cell formation *in vitro* [32]. Thus, platelets are potent proinflammatory and prothrombotic mediators of the effects of OxLDL.

OxLDL and ECs

Resting ECs are powerful inhibitors of coagulation by displaying tissue factor pathway inhibitor (TFPI), heparan sulfate proteoglycans and thrombomodulin on their surfaces. Furthermore, ECs are important modulators of fibrinolysis by expressing tissue-type plasminogen activator (t-PA) [33]. Proinflammatory activation of ECs leads to a shift towards a prothrombotic state by downregulation of those factors, as well as by the induction of several procoagulant pathways, including the release of stored von Willebrand factor and the expression of adhesion molecules and TF [34]. Furthermore, activated ECs express adhesion molecules on their surfaces, thus recruiting leukocytes and platelets, which can initiate further thromboinflammatory pathways.

Studies in the early 1990s demonstrated that incubation of ECs with OxLDL results in changes in the expression profile of proteins involved in coagulatory processes, including TF expression [35]. Although OxPLs and OxLDL have been demonstrated to induce TF expression on ECs [36], the functional role that this effect has in thrombosis is unclear, as EC-derived TF (in contrast to TF expressed by SMCs and hematopoietic cells) is not considered to play a major role in pathological coagulation [37]. Nevertheless, TF expressed by ECs could contribute indirectly by enhancing inflammatory processes. Lectin-like oxidized LDL receptor-1 (LOX-1) has been identified as a receptor for OxLDL [38]. Interestingly, EC-specific LOX-1 transgenic mice showed attenuated carotid artery thrombosis, accompanied by activation of the Oct-1–SIRT1 pathway in the vasculature. However, direct injection of OxLDL or a Western-type diet (which presumably increases OxLDL levels) led to activation of the Ras–Raf-1–extracellular signal-related kinase 1/2 pathway in the vasculature and increased thrombosis in the transgenic animals [39]. Thus, OxLDL mediates prothrombotic effects via ECs in a LOX-1-dependent manner. Further insights into the proinflammatory and prothrombotic functions of OxLDL came from detailed characterization of its biologically active components, most prominently OxPLs, that were isolated from minimally oxidized LDL [40]. Interestingly, OxPLs induce the recruitment and adhesion of monocytes but not neutrophils to ECs *in vitro* and *in vivo* [23]. This is in part mediated by OxPLs-dependent expression of β_1 integrins [41] and P-selectin [42] on the surfaces of ECs. Moreover, a number of chemokines and cytokines, such as IL-8, monocyte chemoattractant protein-1 (MCP-1), and vascular endothelial growth factor A, have been shown to be expressed upon stimulation of ECs with OxPLs [23], which may further promote the migration of leukocytes and vascular inflammation. Moreover, OxPLs have also been found to bind and dramatically reduce the activity of TFPI [43] and to downregulate thrombomodulin expression in ECs [44]. Therefore, OxPLs are robust inducers of a proinflammatory and procoagulant EC phenotype. Additionally, oxidized cholesteryl esters, which are known to be present in OxLDL, induce adhesion of monocytes to ECs, and thereby promote atherogenesis and directly contribute to procoagulatory processes [45].

Apart from the direct effects that OxLDL and its active lipid components exert on ECs in causing them to switch from an anticoagulatory to a procoagulatory phenotype, they may also influence the clinical complications of plaque development by promoting plaque destabilization, rupture, and erosion, ultimately leading to atherothrombotic events. Indeed, OxPLs have been shown to induce, in human umbilical vein endothelial cells, the expression of ADAMTS-1 metalloproteinase, which can degrade plaque matrix components and therefore promote atherosclerotic plaque instability [46]. Moreover, OxLDL [47] and OxPLs [46] have been reported to promote angiogenic responses *in vitro* and *in vivo*, and these, in turn, also contribute to plaque instability and atherothrombotic complications.

Altogether, these data identify potent activities of OxLDL and its active lipid components in promoting vascular inflammation and altering ECs to be potentially more procoagulant, in addition to recruiting other cells that support thromboinflammatory pathways.

Oxidized lipoproteins and vascular SMCs (VSMCs)

VSMCs are not exposed to blood flow under physiological conditions, but have an essential role in hemostasis by exposing TF upon vessel injury. They promote atherosclerotic plaque stability by producing and excreting extracellular matrix proteins [48]. Inflammatory stimuli lead to VSMC activation and the secretion of matrix metalloproteinases. Furthermore, similarly to macrophages, SMCs can transform into foam cells [49].

OxLDL has been shown to induce SMC-derived foam cells [50], and has other biological activities, including chemotactic [51] and proliferative effects [52] on VSMCs. OxPLs have also been found to induce a phenotypic switch and the expression of proinflammatory genes, such as those encoding MCP-1, monocyte chemoattractant protein-3, and cytolysin, in VSMCs [53]. Interestingly, native LDL was found to induce increased inactive TF expression in SMCs [54], whereas incubation with OxLDL leads to increased expression of its active form [55]. This suggests that oxidative modification of LDL is a prerequisite for induction of functional TF expression as opposed to increased inactive TF expression. More recently, a study revealed that OxLDL triggers the association of urokinase plasminogen activator receptor (u-PAR) with CD36 and TLR4, forming a cluster of pattern

recognition receptors (PRRs) that drive phenotypic changes and granulocyte colony-stimulating factor and granulocyte–macrophage colony-stimulating factor secretion by VSMCs [56]. The authors proposed that this might, in turn, affect macrophage activation in atherosclerotic lesions. Whether these findings also affect the plasminogen activation system, which u-PAR drives by binding urokinase, remains to be elucidated.

Therefore, oxidized lipoproteins may also contribute to atherothrombosis by affecting VSMCs, which are known to play an important role in plaque development and stability – critical determinants of the atherothrombotic risk.

Oxidized lipoproteins and leukocytes

Monocyte-derived macrophages are considered to be hallmark cells of atherosclerotic lesions, and thereby major contributors to arterial thrombosis, as reviewed in detail by Moore and Tabas [57]. Following activation by OxLDL, ECs express chemokines and adhesion molecules that mediate the recruitment of monocytes to the artery wall, where they differentiate into macrophages that further promote the oxidation of LDL by generating reactive oxygen species (ROS) as well as by enzymatic processes. OxLDL is taken up by macrophages via mechanisms that involve scavenger receptors such as CD36 and SR-A [58]. At the same time, OxLDL and OxPLs engage a CD36–TLR4–TLR6 heterotrimer to induce the expression of proinflammatory, mostly nuclear factor-κB-dependent, genes, including chemokines, cytokines, and NACHT, LRR and PYD domain-containing protein 3 (NLRP3) [59]. Similar proinflammatory properties have been shown for MDA adducts, although different signaling mechanisms seem to be involved [60]. Following uptake of OxLDL, intracellular cholesterol crystals are formed, and these act as ligands for the NLRP3 inflammasome, leading to its activation and the production of IL-1b and IL-18 [61]. In analogy to what has been found for both VSMCs and ECs, OxLDL has been demonstrated to induce the expression of matrix metalloproteinases in macrophages [62]. OxLDL has also been shown to both induce TF expression on its own and enhance the lipopolysaccharide-induced expression of TF in monocytes [63]. This is supported by the observation that hypercholesterolemic animals have elevated levels of circulating OxLDL, increased activation of coagulation, and higher TF expression on leukocytes [64]. The authors further demonstrated that OxLDL induces TF expression in THP-1 monocytes in a TLR4-dependent manner *in vitro*, and that the hypercholesterolemia-induced procoagulatory phenotype was dramatically reduced in $TLR4^{-/-}$ and $TLR6^{-/-}$ mice. Thus, OxLDL and its components propagate thromboinflammatory processes via monocytes/macrophages in atherosclerosis.

In contrast, so far, only a few studies have investigated the role of monocytes in venous thrombosis. Monocytes are present in venous thrombi, where they support thrombus formation by expressing TF upon activation [65]. On the other hand, they are critical mediators of venous thrombus resolution, particularly through the expression of urokinase plasminogen activator and t-PA [66,67].

In recent years, the role of neutrophils in cardiovascular disease (CVD) has gained increasing attention. Although they are not as prominently present in atherosclerotic lesions as monocyte/macrophages, they have been shown to contribute to lesion development [68]. Because of their oxidative burst capacity, neutrophils themselves may also mediate the oxidation of LDL [69]. OxLDL has been shown to induce superoxide generation in neutrophils [70], which propagates further lipid peroxidation. Furthermore, OxLDL decreases neutrophil membrane fluidity while upregulating expression of the CD11b–CD18 adhesion receptor complex, which is a core inflammatory effector mediating adhesion and migration of leukocytes [71].

In addition to their role in vascular inflammation, neutrophils have gained much attention in thrombosis research in recent years with the discovery of neutrophil extracellular traps (NETs) and the subsequent finding that NETs promote coagulation and thrombosis [72]. Recently, it has been shown that OxLDL, and two of its components, lysophosphatidylcholine and oxidized 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine in particular, are potent inducers of NETosis [73], and can therefore promote vascular inflammation and thrombosis via NET formation. Thereby, OxLDL exerts multiple effects on neutrophils that may contribute to thrombosis by promoting further amplification of lipoprotein oxidation, activation of vascular cells, and the release of NETs.

Although very little is known about the role of lymphocytes in thromboinflammation, they may mediate indirect effects. OxLDL-specific T and B cells have been described, and their role in atherosclerosis is discussed extensively elsewhere [74]. These cellular responses may also modulate processes of thrombosis more directly, as effector memory T cells were recently demonstrated to affect venous thrombus resolution by recruiting monocytes and neutrophils into post-thrombotic vein walls [75]. Furthermore, certain T-cell clones derived from atherosclerotic lesions have been shown to recognize OxLDL [76]. Whether OxLDL-induced T-cell activation plays a significant role in directly affecting thrombotic processes remains to be elucidated. B cells, on the other hand, may modulate thrombosis via the secretion of antibodies. Natural IgM antibodies with specificity for oxidation-specific epitopes protect against OxLDL-induced inflammation and atherosclerosis [77]. A number of epidemiological studies have also demonstrated an inverse association between OxLDL-specific IgM antibody titers and cardiovascular events. This protective effect of B cells is mediated by the subset of B1a cells, which produce natural IgM antibodies, a large percentage of which are

reactive to OxLDL [78]. Furthermore, a recent study found an inverse association between IgM antibodies reactive to OxLDL and phosphatidylcholine (PC) and recurrent venous thrombosis in patients with unprovoked VTE [79]. This finding suggests a potential value of certain IgM antibodies in predicting and lowering the venous thrombotic risk.

MVs and oxidized lipoproteins

MVs constitute a subclass of extracellular vesicles (100–1000 nm in diameter), and are released by blebbing of the cytoplasmic membranes of resting and activated cells. Circulating MVs can originate from different parental cells, including platelets, red blood cells, leukocytes, and ECs [3]. Because of the exposure of phosphatidylserine and, in some cases, TF on their surfaces, MVs are potent inducers of coagulation [3]. Similarly to OxLDL, MVs have been reported to accumulate in atherosclerotic plaques and proposed to promote their progression. Moreover, cell activation by different stimuli (including OxLDL) has been shown to induce the release of procoagulatory and proinflammatory MVs [3]. Thus, similarly to OxLDL, MVs can indirectly elevate the thrombotic risk by inducing diverse inflammatory responses, but also directly activate coagulation.

Activation of platelets upon exposure to OxLDL or OxPLs has been described [28]. However, studies investigating whether stimulation with OxLDL leads to platelet MV release *in vitro* are contradictory [80,81]. In either case, the effects of OxLDL on the release of MVs by platelets *in vivo* remain to be elucidated. Stimulation of ECs with OxLDL has also been found to trigger the release of MVs, which, in turn, induce the expression of ICAM-1 and promote monocyte adhesion to ECs [82]. However, a direct procoagulatory role for OxLDL-induced EC-derived MVs still needs to be established.

OxLDL has also been demonstrated to induce the release of of highly procoagulant TF-expressing MVs by monocytes *in vitro* [64]. The authors also found elevated numbers of circulating TF-expressing MVs in animal models of hypercholesterolemia as well as in patients with familial hypercholesterolemia [64].

Recently, we showed that a subset of *in vitro*-generated and circulating MVs of different cellular origin is characterized by the presence of the same OSEs that are found in OxLDL [6]. These products can be either inherited from parental cells or newly formed upon enzymatic and non-enzymatic modification after MV release analogous to the modification of LDL. Consistent with this, cholesterol loading of macrophages has been shown to promote the release of MVs that carry MDA epitopes [83]. OSEs present on MVs may also contribute to prothrombotic and proinflammatory processes by interacting with the same PRRs and inducing similar functional effects as active OxLDL components. For example, MVs released by cholesterol-loaded macrophages were found to induce endothelial activation and leukocyte adhesion to postcapillary venules in mice *in vivo* [83]. This effect was significantly reduced in LOX-1-deficient mice or upon treatment with anti-LOX-1 antibodies, suggesting that these effects are mediated by OSEs [83]. Moreover, similarly to OxLDL, a critical role for CD36 on platelets in enhancing ADP-dependent platelet activation and aggregation by EC-derived MVs has been demonstrated [84]. These effects are also probably dependent on the exposure of OSEs on EC-derived MVs. On the other hand, the same components of innate immunity that are known to protect against OxLDL-mediated effects may also reduce thromboinflammatory responses induced by MVs. Notably, we have shown that 50% of circulating MVs are recognized by natural IgM that also bind OxLDL [6]. The ability of MDA-positive MVs to induce IL-8 secretion in monocytes was significantly reduced in the presence of a monoclonal IgM antibody with specificity for MDA adducts, which are also present on OxLDL [6]. A similar effect has also been described for PC-specific IgM antibodies, which neutralized the capacity of apoptotic blebs to mediate activation of and subsequent monocyte adhesion to ECs [45]. Whether OxLDL-specific IgM antibodies could also directly inhibit the procoagulatory effects of OSE-carrying MVs remains to be demonstrated.

Thus, OxLDL induces the cellular release of MVs, which can directly promote coagulation by the exposure of active TF, and indirectly promote it by propagating inflammatory responses. The latter may be mediated by the presence of the same OSEs that are present on OxLDL and recognized by the same PRRs. Although MVs have been shown to inherit the surface molecules of their parental cells and presumably PRRs such as CD36, possible interactions of OxLDL with MVs and the functional consequences thereof are unknown. One may speculate that recognition of OxLDL by PRRs present on MVs may compete with cellular recognition of OxLDL and therefore serve as a mechanism limiting overt thromboinflammation. The evaluation of OSE-carrying MVs may have prognostic value in CVD and associated thrombotic events, as has been shown for OxLDL.

Oxidized lipoproteins as a risk factor for thrombosis in clinical studies

Many studies have presented clinical evidence for a relationship between plasma OxLDL levels and arterial CVD. Measurements of OxLDL were shown to be highly sensitive and specific in discriminating stable and unstable coronary artery disease (CAD) [85]. Furthermore, the amount of OxPLs bound to apoB-100 and, more specifically, Lp(a) lipoprotein levels have been identified as strong predictors of the presence and extent of CAD [86]. Thus, OxPLs have been suggested to constitute a

predictive biomarker of arterial cardiovascular events. A major limitation of these studies is the heterogeneity and the lack of robustness of the OxLDL measurements employed. Only reproducible assays that determine the levels of specific components of OxLDL, such as OxPLs, independently of total LDL levels will provide reliable insights. Nevertheless, OxLDL (or its components) may be a useful biomarker in the assessment and prediction of atherothrombotic risk.

In contrast to the many studies investigating OxLDL in arterial thrombosis, only a single study has investigated the relationship between OxLDL and venous thrombosis. Slightly elevated levels of OxLDL were found in a small cohort of patients suffering from antiphospholipid syndrome (APS) who had developed venous thrombosis [87]. However, these findings might be confounded by the presence of autoantibodies recognizing OxLDL–β_2GPI complexes in APS patients. Clearly, there is a need for more studies investigating OxLDL levels with reliable assays in different and larger cohorts at risk for venous thrombosis to determine whether it can serve as a biomarker for VTE. Considering the similarities between OxLDL and MVs, quantifying the levels of the latter, in particular the subset carrying OSE, may also provide important insights.

Targeting lipoproteins as a preventive measure in thrombosis

The classical anticoagulatory drugs used to prevent arterial and venous thrombosis also increase the risk of bleeding complications. Therefore, targeting other risk factors of thrombosis might provide an alternative approach to reduce thrombotic risk in certain situations.

Statins are widely used to lower LDL cholesterol in the primary and secondary prevention of CVD caused by atherosclerosis [88]. Additionally, statins have been found to lower the risk of venous thrombosis, as first reported in a large-scale retrospective study by Ray *et al*. [89], an effect that was not observed for non-statin lipid-lowering agents. More recently, Glynn *et al*. confirmed the protective effects of statins in venous thrombosis in a prospective randomized trial [90]. In this subanalysis of the JUPITER trial, apparently healthy individuals with normal lipid profiles treated with rosuvastatin developed significantly fewer VTEs. However, a population-based cohort study found no association between overall lipid levels and VTE [91]. Additional benefits of statins that are independent of LDL cholesterol lowering have been described and termed 'pleiotropic effects'. Several pleiotropic effects might directly and indirectly affect thrombotic processes, such as altering the expression of endothelial nitric oxide synthase, atherosclerotic plaque stabilization, lowering platelet reactivity, and the production of proinflammatory cytokines and ROS [92]. Interestingly, a study investigating the levels of OxLDL and the severity of CAD found that patients undergoing statin treatment had significantly lower levels of OxLDL than untreated patients [93]. Furthermore, a prospective study investigating the effect of atorvastatin treatment on biomarkers of inflammation and oxidative stress in subjects with metabolic syndrome found a dose–response relationship for atorvastatin in its capacity to lower OxLDL levels [94]. Another group comparing atorvastatin treatment with atorvastatin/ezetimibe treatment showed that the addition of ezetimibe, while lowering total LDL levels, did not affect OxLDL levels [95]. Notably, Owens *et al*. demonstrated that simvastatin treatment reduced the prothrombotic phenotype in experimental animal models of hypercholesterolemia [64]. Interestingly, simvastatin treatment resulted in lower OxLDL levels without changing total LDL levels.

Thus, total LDL lowering in itself, although of great benefit in the prevention of arterial thrombosis, does not seem to affect the risk of developing venous thrombosis. However, in addition to their capacity to affect total LDL levels, statins also lower VTE risk, which has not been observed with non-statin lipid-lowering drugs. The positive effect of statin treatment on VTE risk might be related to their capacity to reduce oxidative stress, and possibly to lower OxLDL. The potential changes in OxLDL levels caused by statin treatment might be explained by their general anti-inflammatory effects. However, CRP and OxLDL levels have shown no correlation with each other in a majority of studies [96], suggesting other mechanisms. Moreover, these data should be interpreted with caution, because OxLDL measurements are not standardized, and are variable in their quality.

Another – more direct – approach to consider in modulating thromboinflammation induced by OxLDL could be based on specific antibodies. Anti-OxLDL IgM antibodies protect against atherosclerosis, and several studies have shown a correlation between low levels of anti-OxLDL IgM antibodies and an increased risk of CAD [21]. Thus, the protective capacity of IgM antibodies with specificity for oxidation-specific epitopes could be exploited for therapeutic purposes. Indeed, beyond their neutralizing activity on OxLDL, these antibodies also target MVs, and therefore may have indications beyond atherosclerotic CVD. Interestingly, epidemiological data suggest a connection between low levels of total IgM antibodies and long-term venous thrombotic risk after splenectomy [97]. Additionally, an inverse correlation between OxLDL-specific IgM levels and recurrence of venous thrombosis has recently been demonstrated [79], as discussed above. Strategies to boost OxLDL-specific antibody production that are being investigated may provide potential tools with which to prevent inflammation and thrombosis. These could include vaccination approaches, passive infusions with purified IgM antibodies, or the targeting of negative regulators of IgM production on B cells. In

murine models, these intervention strategies have demonstrated profound effects on atherosclerotic lesion formation and inflammation in general [60,77,98]. Translating these approaches into use in humans will be a major challenge in the future.

Conclusion

Various ligands of microbial and sterile origin have been shown to promote thrombosis either directly or by driving inflammatory responses. OxLDL represents a prominent driver of vascular inflammation and atherothrombotic events. The underlying mechanisms by which OxLDL promotes atherosclerosis initiation and progression and subsequently arterial thrombosis (e.g. foam cell formation and plaque matrix degradation) are well recognized. Although these effects are not relevant in the pathophysiology of venous thrombosis, OxLDL and its biologically active components have nevertheless been shown to directly and indirectly promote coagulation and thrombosis. Notably, many of the same biologically active components that are found on OxLDL are present on a subset of circulating MVs, which are considered to be critical mediators of arterial and venous thrombosis. Investigating the contribution of oxidized lipoproteins in the context of these findings will provide novel insights and identify new pathways of thromboinflammation. Understanding the function and targeting the generation and activities of active components of OxLDL and OSE-carrying MVs may provide novel therapeutic approaches to prevent thromboinflammatory diseases.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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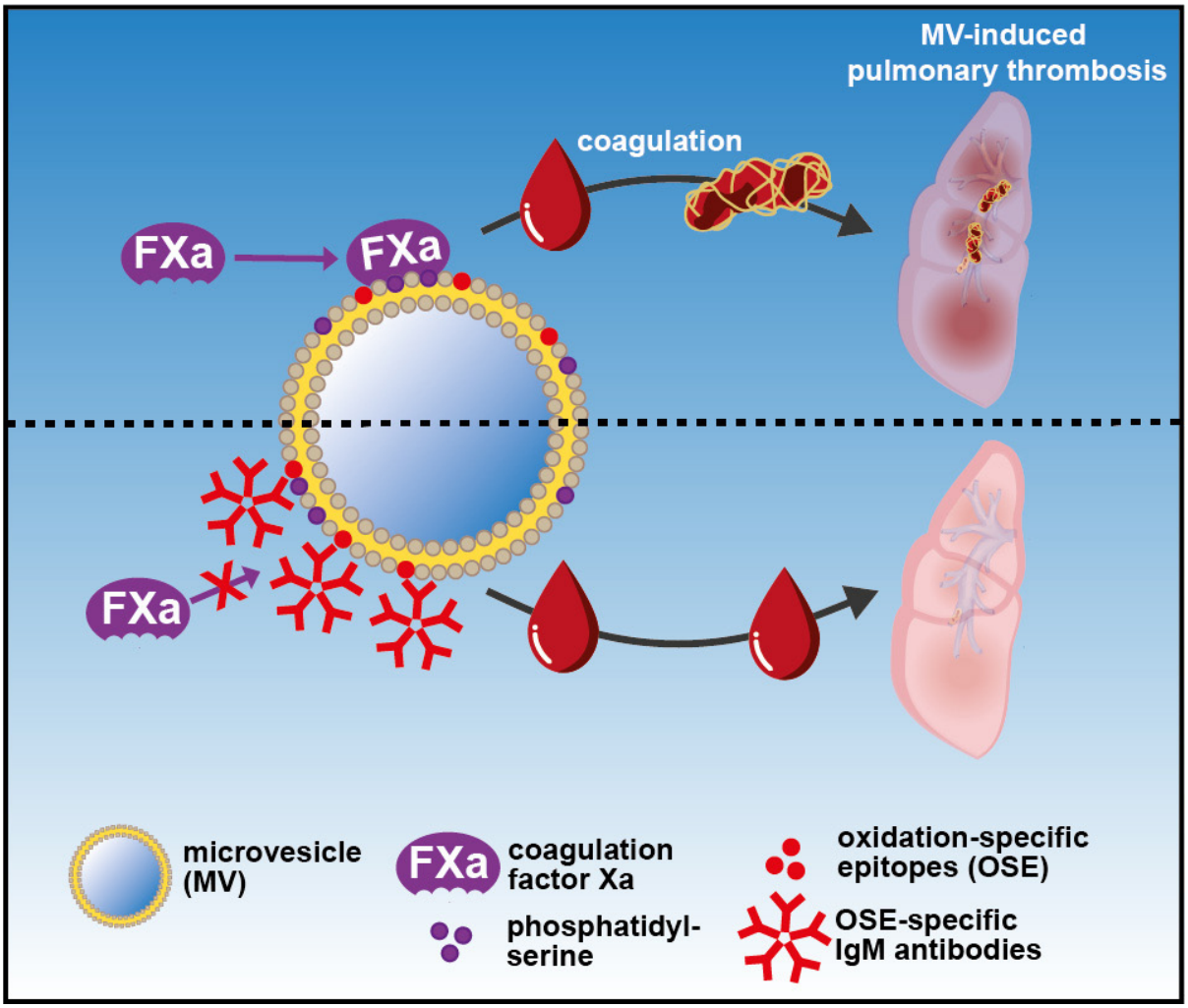
1.6 Interlude to Manuscript

As discussed in the review above, inflammatory processes are also significant drivers of coagulation and thrombosis. Therefore, natural IgM antibodies can confer beneficial effects in cardiovascular disease by limiting inflammation, affecting not just atherosclerosis and subsequently arterial thrombosis, but also venous thrombosis. Previous to our findings, the obvious explanation for the inverse associations that have been found between natural IgM levels and arterial (Tsimikas et al., 2012) and venous (Eichinger et al., 2018) thrombosis would have been this connection between inflammation and thrombosis. These indirect positive effects of natural IgM antibodies most likely still play a significant role by affecting the cellular components of blood coagulation. For example, OSE-binding IgM has been demonstrated to neutralize the proinflammatory effect of MVs on monocytes (Tsiantoulas et al., 2015). However, in the following manuscript, I demonstrate that natural IgM can affect coagulation and thrombosis in a direct manner by inhibiting the procoagulant properties of MVs.

In the publication, I outline an inverse association of free and MV-bound endogenous IgM antibodies and plasmatic coagulation. Furthermore, I demonstrate that the addition of OSE-binding IgM antibodies inhibits MV-dependent coagulation in plasma independently of extrinsic and intrinsic activation. Factor X/Xa binding to the surface is inhibited by the presence of these antibodies, suggesting that sterical hindrance of the prothrombinase complex is the mechanism behind this anticoagulatory effect. I further confirm the ex vivo effects of these antibodies in whole blood coagulation and exclude that platelet aggregation is affected by their presence. Finally, I translate these results to murine in vivo models of thrombosis and hemostasis. I show that coinjecting the antibodies does not affect hemostasis in a tail clipping model, or in a collagen-induced pulmonary thrombosis model. However, coinjection of the antibodies rescues mice from death in an MV-induced pulmonary embolism model. I further confirm these results by demonstrating a lower amount of pulmonary thrombi and visualize the presence of MVs in the pulmonary thrombi via immunofluorescence histology.

2. Results

2.1 Graphical abstract



2.2 Manuscript: Natural IgM antibodies inhibit microvesicle-driven coagulation and thrombosis

Natural IgM antibodies inhibit microvesicle-driven coagulation and thrombosis

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Georg Obermayer,* Taras Afonyushkin,* Laura Göderle, Florian Puhm, Waltraud Schrottmaier, Soreen Taqi, Michael Schwameis, Cihan Ay, Ingrid Pabinger, Bernd Jilma, Alice Assinger, Nigel Mackman, and Christoph J. Binder

**authors contributed equally*

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THROMBOSIS AND HEMOSTASIS

Natural IgM antibodies inhibit microvesicle-driven coagulation and thrombosis

Georg Obermayer,[1,2,*] Taras Afonyushkin,[1,2,*] Laura Göderle,[1] Florian Puhm,[1,2] Waltraud Schrottmaier,[3] Soreen Taqi,[1] Michael Schwameis,[4,5] Cihan Ay,[6,7] Ingrid Pabinger,[6] Bernd Jilma,[4] Alice Assinger,[3] Nigel Mackman,[8] and Christoph J. Binder[1,2]

[1]Department of Laboratory Medicine, Medical University of Vienna, Vienna, Austria; [2]Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria; [3]Centre of Physiology and Pharmacology, [4]Department of Clinical Pharmacology, [5]Department of Emergency Medicine, and [6]Clinical Division of Hematology and Hemostaseology, Department of Medicine I, Medical University of Vienna, Vienna, Austria; [7]I.M. Sechenov First Moscow State Medical University (Sechenov University), Moscow, Russia; and [8]Department of Medicine, University of North Carolina at Chapel Hill School of Medicine, Chapel Hill, NC

KEY POINTS

- **Natural IgM antibodies in plasma bind to circulating MVs and modulate coagulation.**
- **A monoclonal natural IgM antibody specific for malondialdehyde epitopes inhibits MV-induced coagulation and thrombosis in mice in vivo.**

Thrombosis and its associated complications are a major cause of morbidity and mortality worldwide. Microvesicles (MVs), a class of extracellular vesicles, are increasingly recognized as mediators of coagulation and biomarkers of thrombotic risk. Thus, identifying factors targeting MV-driven coagulation may help in the development of novel antithrombotic treatments. We have previously identified a subset of circulating MVs that is characterized by the presence of oxidation-specific epitopes and bound by natural immunoglobulin M (IgM) antibodies targeting these structures. This study investigated whether natural IgM antibodies, which are known to have important anti-inflammatory housekeeping functions, inhibit the procoagulatory properties of MVs. We found that the extent of plasma coagulation is inversely associated with the levels of both free and MV-bound endogenous IgM. Moreover, the oxidation epitope-specific natural IgM antibody LR04, which recognizes malondialdehyde adducts, reduced MV-dependent plasmatic coagulation and whole blood clotting without affecting thrombocyte aggregation. Intravenous injection of LR04 protected mice from MV-induced pulmonary thrombosis. Of note, LR04 competed the binding of coagulation factor X/Xa to MVs, providing a mechanistic explanation for its anticoagulatory effect. Thus, our data identify natural IgM antibodies as hitherto unknown modulators of MV-induced coagulation in vitro and in vivo and their prognostic and therapeutic potential in the management of thrombosis. (*Blood*. 2021;137(10):1406-1415)

Introduction

Cardiovascular diseases remain the major cause of morbidity and mortality worldwide.[1] Most cardiovascular events such as stroke, myocardial infarction, and pulmonary embolism are caused by thrombotic vessel occlusion.[2,3] Moreover, several chronic conditions, such as diabetes[4] and certain autoimmune[5] and autoinflammatory[6] disorders, as well as many types of cancer,[7] are associated with a high thrombotic risk. Current strategies for the prevention and treatment of thrombosis are based on targeting the coagulation cascade and/or platelet reactivity and therefore increase the risk of bleeding.[8-12] Further insights into the pathophysiology of thrombosis and the development of novel antithrombotic treatments are thus needed.

Inflammation and the cellular responses involved in it are increasingly recognized as important modulators of thrombosis, mainly by activating the procoagulant pathway.[13-15] Increased oxidative stress and the production of reactive oxygen species are a hallmark of inflammatory processes. In turn, oxidative damage of membrane lipids give rise to the formation of oxidation-specific epitopes (OSEs)[16] such as malondialdehyde (MDA) and oxidized phosphatidylcholine. OSEs are considered a class of danger-associated molecular patterns that trigger inflammatory responses in both acute and chronic settings.[17] For example, oxidized low-density lipoprotein (LDL), which carries different OSEs, represents a key trigger of vascular inflammation, in particular in the context of atherosclerosis.[18] Moreover, oxidized LDL has been shown to induce tissue factor (TF) expression in monocytes,[19,20] neutrophil extracellular cellular trap formation,[21] and a prothrombotic phenotype in endothelial cells.[22-24] Thus, innate immune receptors that recognize OSEs can mediate procoagulant and prothrombotic responses.[18] However, whether recognition of OSEs could also directly dampen coagulation has not yet been investigated, to the best of our knowledge.

We and others have previously shown that low levels of immunoglobulin M (IgM) antibodies binding OSEs are inversely associated with the risk of atherosclerotic cardiovascular events and venous thrombosis.[25-28] Most, if not all, of these

immunoglobulins are germline encoded (so-called natural antibodies), produced without prior exposure to pathogens.[29] We have previously shown that a large part of IgM antibodies recognize OSEs.[16,30] The protective effects of these OSE IgM antibodies have been largely attributed to their ability to neutralize the proinflammatory effects of oxidized lipids and mediate the clearance of apoptotic cells carrying OSEs.[30-34]

We recently reported that OSE IgM antibodies recognize a subset of circulating microvesicles (MVs),[35] a class of extracellular vesicles (EVs) that can be found in all biological fluids.[36] Cellular stress, in particular inflammatory activation, increases MV production.[37] Accordingly, the levels of circulating MVs are elevated in many pathologic settings and have therefore been suggested as mediators and biomarkers in a variety of diseases,[38] including metabolic[39] and cardiovascular disease,[40] cancer,[41] and infections.[42] Initially, MVs were discovered as mediators of coagulation and have been studied for their contribution to thrombosis[43-45] by presenting negatively charged phospholipids, which enable the assembly of the prothrombinase complex,[46] and initiating the extrinsic coagulation pathway by exposing TF.[47] Furthermore, MVs may also indirectly contribute to a prothrombotic state by triggering inflammatory responses in vascular cells. MVs are therefore considered important mediators of arterial[48] and venous[49] thrombosis. The current study investigated whether natural IgM antibodies and OSE IgM antibodies in particular could directly modulate the procoagulatory potential of MVs and thereby limit thrombotic processes.

Methods

IgM antibodies recognizing OSEs

Monoclonal IgM antibodies with specificity for MDA adducts (LR04 and NA17; kind gifts of J.L. Witztum, University of California, San Diego) and for phosphocholine (E06; Avanti Polar Lipids) were used in this study. The cloning and characterization of these antibodies have been described before.[30,50,51] A mouse IgM isotype (clone number MM30, 401604; BioLegend) was used as a control. All antibodies were tested to be free of endotoxin contamination.

Thrombin generation assay

Thrombin generation (TG) was performed by using a Ceveron alpha analyzer (Technoclone). Isolated MVs were used to initiate clotting as described in the figure legends. Plasma deficient in factors V (5134004), VII (5144015), VIII (5154004), IX (5164003), XI (5184004), and XII (5194008) and a control plasma (5020020) (all from Technoclone) were used. OSE-specific antibodies (LR04, NA17, and E06) or isotype control were used to inhibit coagulation at concentrations of 25 and 50 μg/mL. For blocking of TF, an anti-CD142 monoclonal antibody (clone number HTF-1, 16-1429-82; eBioscience) was used (10 μg/mL). Antibodies were mixed with MVs in phosphate-buffered saline (125 μL) and incubated for 20 minutes at room temperature before adding to 125 μL of plasma.

Pulmonary thrombosis model

A modified mouse model of pulmonary thrombosis was used.[52] Eight- to 12-week-old C57BL6/J mice were anesthetized by intraperitoneal injection of ketamine (AniMedica) (100 μg/g body weight [bw]) and xylazine (Bayer) (10 μg/g bw), diluted in sterile saline (injection volume, 100 μL). The mixture containing epinephrine (60 ng/g bw), HPAF-II–derived MVs (0.1 μg/g bw), or rat tail collagen (Corning) (3 μg/g bw), and LR04 or isotype control IgM (25 μg/mL of estimated blood volume; corresponding to 2.25 μg/g bw) in sterile phosphate-buffered saline (total volume, 120 μL), was injected into the retro-orbital plexus using 29 gauge insulin syringes. The antibody concentrations were chosen according to the effective concentrations (25 μg/mL) observed in plasma (TG) and whole blood (rotational thromboelastometry) coagulation assays. Terminal breath was counted as time of death. Surviving animals were killed by cervical dislocation after 30 minutes. Experiments were performed in a blinded manner.

A detailed description of the methods is provided in the supplemental Methods (available on the *Blood* Web site).

Statistics

GraphPad Prism 8.3 for Windows (GraphPad Software) software was used for statistical analyses. The Mann-Whitney *U* test was used for comparing data from 2 unpaired groups, and the Wilcoxon matched-pairs signed-rank test was used for data from 2 paired groups. The log-rank (Mantel-Cox) test was used for the comparison of survival curves. One-way analysis of variance test with subsequent Bonferroni's multiple comparison tests were used for multiple group data analysis. Data are presented as mean ± SEM.

Ethics

Human blood and plasma samples were collected under the approval by the Ethics Committee of the Medical University of Vienna (EK2051/2013 and EK1845/2015). All in vivo experiments were conducted in accordance with the approval of the animal ethics committee of the Medical University of Vienna (BMBWF-66.009/0017-V/3b/2018).

Results

Endogenous IgM antibodies affect MV-dependent TG

To investigate whether IgM antibodies can influence MV-induced plasma coagulation, we selectively depleted circulating IgM antibodies (Figure 1A; supplemental Figure 1A) from pooled, EV-depleted (supplemental Figure 1B) human plasma. Blood drawing leads to plasma preactivation via the contact pathway[53] and therefore does not require TF as a trigger. Gradual depletion of endogenous IgM antibodies led to a concurrent increase in propagation of TG triggered by platelet-derived MVs (TF^-), reflected by a peak height increase (Figure 1B; supplemental Figure 1C) and by TF^+ MVs (supplemental Figure 1D). Activated partial thromboplastin time (aPTT), which in contrast to TG[54] is not sensitive to the presence of MVs,[55] was not affected by IgM depletion (supplemental Figure 1E).

To investigate whether endogenous IgM antibodies bound to MVs affect their coagulatory potential, we assessed the presence of IgM on circulating annexin V–positive MVs of healthy volunteers (n = 22) using flow cytometry (supplemental Figure 1F). MVs were isolated and divided into IgM^{low} and IgM^{high} based on the mean percentage of IgM-positive MVs (Figure 1C). MVs were used to induce TG in the equivalent volume of pooled MV-free plasma. Although the total amount of MVs did not significantly differ between the 2 groups (supplemental Figure 1G), IgM^{high} MVs induced TG to a significantly

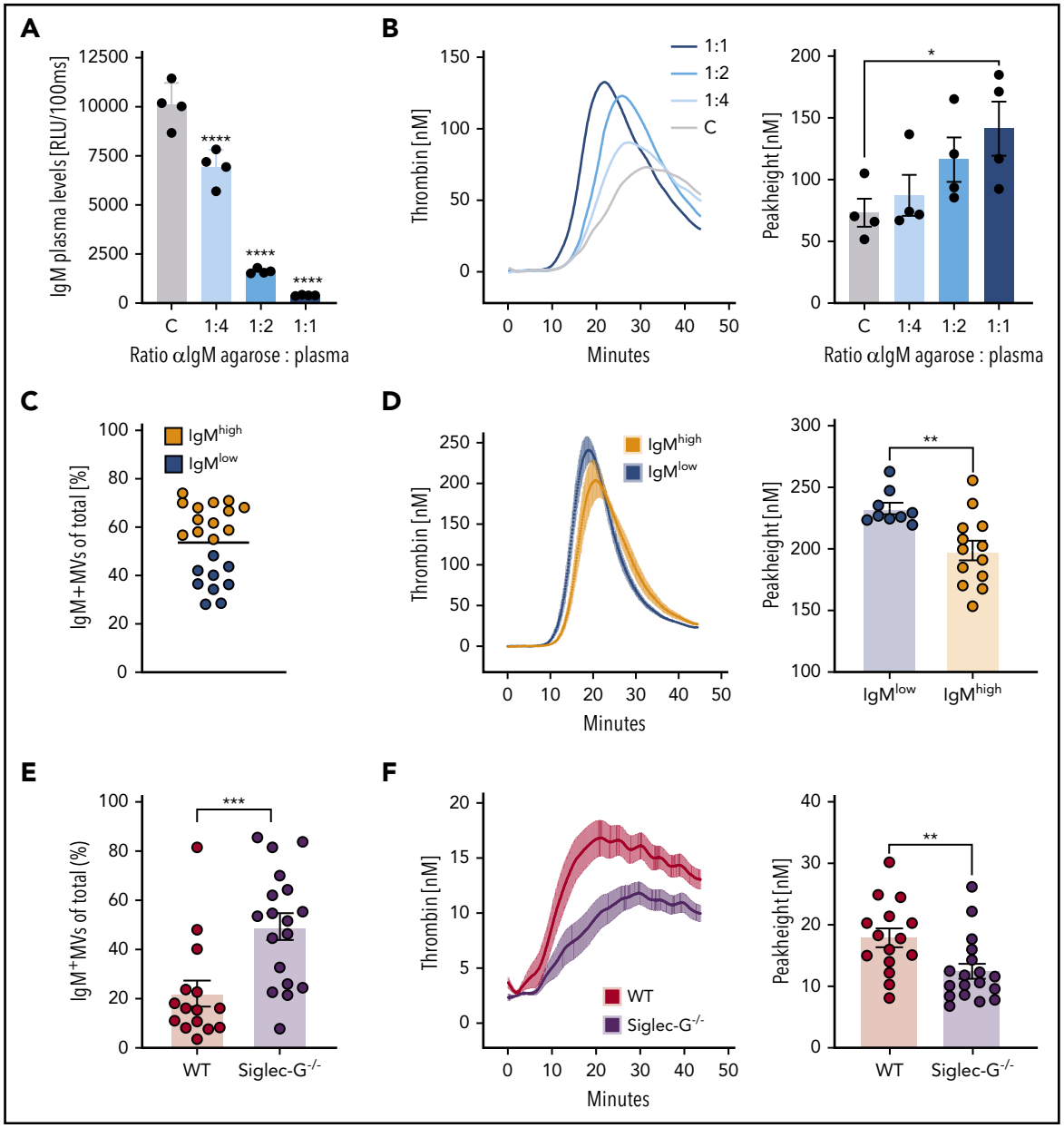

Figure 1. Endogenous IgM antibodies decrease MV-dependent TG. (A) Chemiluminescent enzyme-linked immunosorbent assay of plasma IgM levels after depletion of free IgM from pooled EV-free human plasma with increasing amounts of anti–human IgM/agarose beads. (B) TG curves and peak heights of IgM-depleted MV-free plasma triggered by addition of platelet-derived MVs. Plots depict representative experiment of n = 4. Bars represent mean ± SEM of each group. $*P < .05$, $****P < .001$, two-way analysis of variance with Bonferroni's multiple comparisons test. (C) Percentages of IgM^+ MVs within annexin V–positive events isolated from plasma of healthy volunteers (n = 22) measured by using flow cytometry. Groups were divided into MV IgM^{low} and MV IgM^{high} based on the mean percentage (horizontal bar). (D) Comparison of the procoagulatory potential of IgM^{low} and IgM^{high} MV average TG curves and peak heights of pooled MV-depleted plasma reconstituted with isolated IgM^{low} (n = 9) and IgM^{high} (n = 13) MVs. Columns and error bars or solid lines and light-colored areas represent the mean ± SEM of each group. (E) Percentages of IgM^+ MVs of WT (n = 15) and Siglec-$G^{-/-}$ (n = 18) mice within annexin V–positive events measured by flow cytometry. Bars represent mean ± SEM. (F) Spontaneous TG of platelet-poor plasma from WT and Siglec-$G^{-/-}$ mice. Average TG curves and peak heights of pooled platelet-poor plasma of WT vs Siglec-$G^{-/-}$ mice. Columns and error bars or solid lines and light-colored areas represent the mean ± SEM of each group. (D-F) $**P < .01$, $***P < .005$, Mann-Whitney *U* test. RLU, relative light units.

lower degree compared with IgM^{low} MVs (Figure 1D; supplemental Figure 1H). Similar results were obtained when MVs were dichotomized into IgM^{high} and IgM^{low} MVs within the subset of $CD41a^+$ platelet MVs that are considered to represent the majority of circulating MVs (supplemental Figure 1I).

To further characterize the capacity of endogenous IgM antibodies to modulate coagulation, we characterized circulating MVs from mice lacking sialic acid-binding immunoglobulin-like lectin G (Siglec$G^{-/-}$), which possess elevated levels of total and OSE-specific IgM.[31] Siglec$G^{-/-}$ mice exhibited a significantly higher percentage of IgM^+ MVs compared with wild-type (WT) mice (Figure 1E), whereas the total number of MVs was not different between the 2 groups of mice (supplemental Figure 1J). Platelet-poor plasma of Siglec$G^{-/-}$ mice still containing endogenous MVs exhibited significantly reduced TG compared with plasma of WT mice (Figure 1F; supplemental Figure 1K).

OSE-specific natural IgM LR04 inhibits MV-sensitive plasma coagulation and factor X binding to MVs

We have previously shown that the majority of endogenous IgM antibodies bound to circulating MVs have specificity for OSEs; OSE-specific natural IgM antibodies, in particular with specificity for MDA epitopes, bind circulating MVs.[35]

To address whether OSE-specific natural IgM antibodies modulate coagulation similarly to endogenous IgM, we investigated the effects of LR04, a previously characterized monoclonal MDA-specific IgM antibody[50,56] on coagulation. LR04 has been shown to bind circulating and in vitro generated MVs,[35] which we confirmed for the platelet-derived MVs (PMV) used (supplemental Figure 2A). To test the effect of LR04 on TG, PMVs were preincubated with increasing concentrations of either LR04 or an isotype control antibody and added to MV-free plasma. LR04 significantly inhibited initiation (lag time) and propagation (peak height) of PMV-triggered TG (Figure 2A; supplemental Figure 2B).

Preincubation with a previously described peptide mimotope (P2) of MDA that is specifically bound by LR04[50] led to a significant inhibition of LR04 binding to PMVs (Figure 2B). Consistent with that action, preincubation with increasing concentrations of P2 led to a dose-dependent inhibition of the anticoagulatory effects of LR04 in PMV-triggered TG (Figure 2C; supplemental Figure 2C). Similarly to LR04, different monoclonal IgM antibodies with specificity for MDA (NA17) or the phosphocholine headgroup of oxidized phospholipids (E06) also inhibited TG (Figure 2D; supplemental Figure 2D). This finding is consistent with the previously reported presence of both types of OSEs on MVs.

To test whether LR04 inhibits TG in the presence of exogenous MV-associated TF, we used TF^+ MVs generated from activated THP-1 cells, which are also bound by LR04 (supplemental Figure 2E), as a trigger. LR04 resulted in a dose-dependent reduction of THP1-MV triggered peak TG without affecting initiation (lag time), whereas a TF-blocking antibody (HTF-1) significantly delayed lag time (Figure 2E; supplemental Figure 2F), indicating that MDA-specific IgM antibodies limit the propagation of MV-dependent coagulation, irrespective of the presence of TF.

We next investigated whether LR04 also has an effect on clotting tests that is not dependent on the presence of MVs.[55] We used aPTT and prothrombin time (PT) tests, which rely on the addition of exogenous phospholipids. Preincubation of pooled platelet-poor plasma with LR04 had no effect on aPTT or PT (Figure 2F). This outcome suggests that LR04 exerts its anticoagulatory effects by interacting with MVs, rather than by directly inhibiting the activity of certain coagulation factors. To further test this hypothesis, we evaluated the effect of LR04 on THP-1/MV–induced coagulation in plasmas with selective deficiency of individual coagulation factors of the extrinsic or intrinsic pathway (FV, FVII, FVIII, FIX, FXI, and FXII). A reduction of TG (peak height) by LR04 was observed in all deficient plasmas tested (Figure 2G), suggesting that LR04 does not selectively affect either the intrinsic or extrinsic arm of the coagulation cascade.

To investigate whether the anticoagulatory effect is caused by promoting aggregation of MVs, and thereby reducing the procoagulant surface, we assessed the size profile of MVs after incubation with LR04 or concanavalin A, which has been previously described as causing MV aggregation.[57] Although concanavalin A caused a significant shift in the size profile of MVs, no measurable shift occurred after incubation with LR04 (Figure 2H; supplemental Figure 2G). We next tested whether LR04 affects the interaction between MVs and components of the common coagulation pathway. Because the binding of factor X/Xa to phospholipid surfaces (eg, MVs) is a crucial step in the formation of the prothrombinase complex, we tested whether LR04 could directly inhibit factor Xa binding to the phospholipids of MVs. To address this theory, we established an enzyme-linked immunosorbent assay for the detection of factor Xa binding to immobilized MVs. Purified factor Xa bound to immobilized MVs in a concentration-dependent manner. In contrast to LR04,[35] no binding of factor Xa to MDA-modified bovine serum albumin was observed (supplemental Figure 2H). Furthermore, we reported the binding of LR04 to immobilized MVs (supplemental Figure 2I), which could be inhibited with the MDA peptide mimotope P2 (supplemental Figure 2J). To assess whether LR04 can compete with plasma-derived factor X/Xa binding to MVs, we established a competition enzyme-linked immunosorbent assay in which plasma was added to immobilized MVs (supplemental Figure 2K). Preincubation of immobilized MVs with LR04 but not an isotype control antibody significantly reduced binding of plasma-derived factor X/Xa to MVs in a concentration-dependent manner (Figure 2I).

LR04 delays initiation and propagation in whole blood clotting without affecting platelet aggregation

Activation of blood cells during coagulation results in the production of MVs, which in turn contributes to the propagation of blood clotting. Indeed, we observed dramatically higher numbers of MVs in serum compared with plasma of the same donor (supplemental Figure 3A). Using rotational thromboelastometry, we therefore tested the effect of LR04 on whole blood coagulation during which MVs are continuously released. Addition of LR04 but not an isotype control antibody significantly increased clotting time, clot formation time, and time of maximum clot firmness while significantly decreasing maximum clot firmness (Figure 3A-D). The strongest effect was observed in clot formation time, a measure of clotting propagation, which is consistent with the effects seen in TG. This effect could depend on the circulating MVs present, as well as affecting MVs that are generated during the clotting process.

Thrombocyte aggregation assays were performed to test the effect of LR04 on platelet function. Neither LR04 nor an isotype control antibody affected the aggregation of platelets isolated from healthy volunteers (Figure 3E-G; supplemental Figure 3B-C).

LR04 protects mice from MV-induced pulmonary thrombosis but does not affect hemostasis

We next tested whether LR04 could modulate hemostasis and MV-driven thrombosis in vivo. To assess the potential influence of LR04 on hemostasis, a murine tail bleeding assay was performed.[58] After anesthesia, mice received injections with either LR04 or an isotype control antibody, and bleeding time was

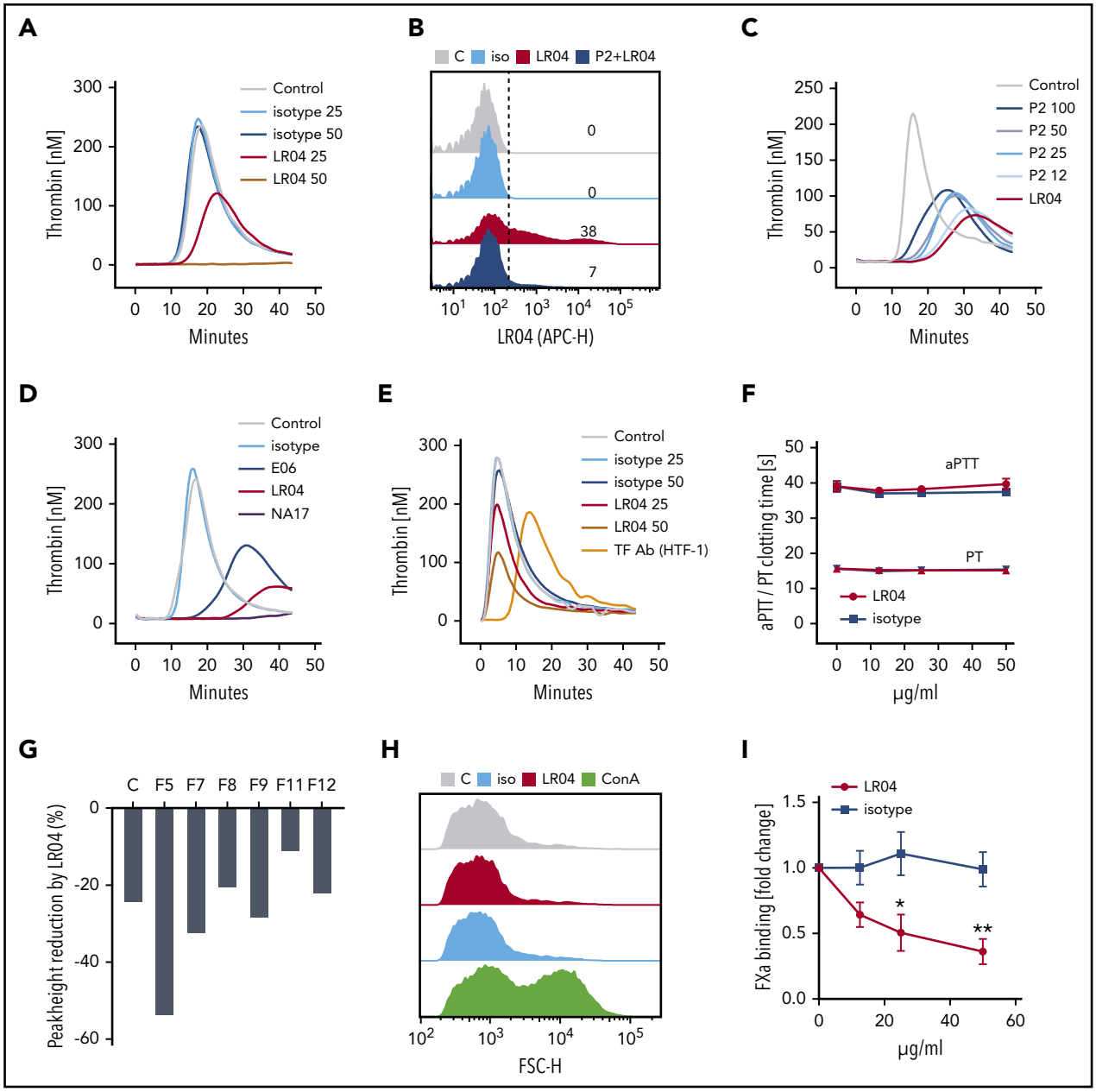

Figure 2. Natural IgM antibodies inhibit MV-sensitive plasma coagulation and factor Xa (FXa) binding to MVs. (A) Effect of the MDA-specific natural IgM antibody (ab) LR04 on TG. TG curves of MV-depleted plasma, triggered by platelet-derived MVs (2 µg/mL), which were preincubated with 2 concentrations of LR04 or an isotype control ab (0, 25, or 50 µg/mL). (B-C) Inhibitory effect of the LR04-specific peptide mimotope P2. Flow cytometry histograms depict binding of LR04 to platelet-derived MVs in the presence or absence of P2 (100 µg/mL) (B); and TG curves of MV-depleted plasma triggered by platelet-derived MVs (2 µg/mL) incubated with LR04 (25 µg/mL) in the presence of increasing concentrations of P2 (0, 12, 25, 50, or 100 µg/mL) (C). (D) TG curves of MV-depleted plasma triggered by platelet-derived MVs (2 µg/mL), which were preincubated with 25 µg/mL of the MDA-specific natural IgM LR04 or NA17, the phosphocholine-specific natural IgM E06, or an isotype control ab. (E) TG curves of MV-depleted plasma triggered by THP-1 cell-derived MVs (2 µg/mL). MVs were preincubated with LR04 or an isotype control ab (25 or 50 µg/mL) or a TF-blocking ab (HTF-1, 10 µg/mL). (F) Activated partial thromboplastin time (aPTT) and prothrombin time (PT) of platelet-poor plasma that was preincubated with increasing amounts of LR04 or an isotype control ab (0, 12, 25, or 50 µg/mL). (G) Effect of LR04 on THP-1/MV–triggered (2 µg/mL) TG of MV-depleted plasma deficient for single coagulation factors (FVII, FVIII, FIX, FXI, and FXII). Shown is the relative reduction of peak height after preincubation of MVs with LR04 (25 µg/mL) compared with the peak height of each respective plasma. (H) Flow cytometry–based size profiles of platelet-derived MVs after incubation (30 minutes, 37°C) with LR04 (25 µg/mL), isotype control ab (25 µg/mL), or concanavalin A (ConA) (5 µg/mL). (I) Inhibition of the binding of plasma-derived factor X/Xa to MVs by increasing concentrations of LR04. Symbols represent the mean ± SEM of 4 independent experiments. $^{*}P < .05$, $^{**}P < .01$, two-way analysis of variance, Bonferroni's multiple comparisons test. In panels A through G, plots depict representative experiments of least 4 experiments. APC-H, allophycocyanin; FSC-H, forward scatter.

assessed after tail tip amputation. We observed no differences in bleeding time (Figure 4A) between the groups injected with either LR04 or the isotype. However, hemostasis is mediated largely by platelet aggregation and extravascular TF, whereas the contribution of circulating MVs is debated.[44]

To assess the effect on MV-driven coagulatory processes in vivo, we adapted an established model of MV-induced murine pulmonary thrombosis,[52] using epinephrine together with MVs released by the pancreatic cancer cell line HPAF-II as a trigger, which possess a strong procoagulatory activity.[59] The rapid

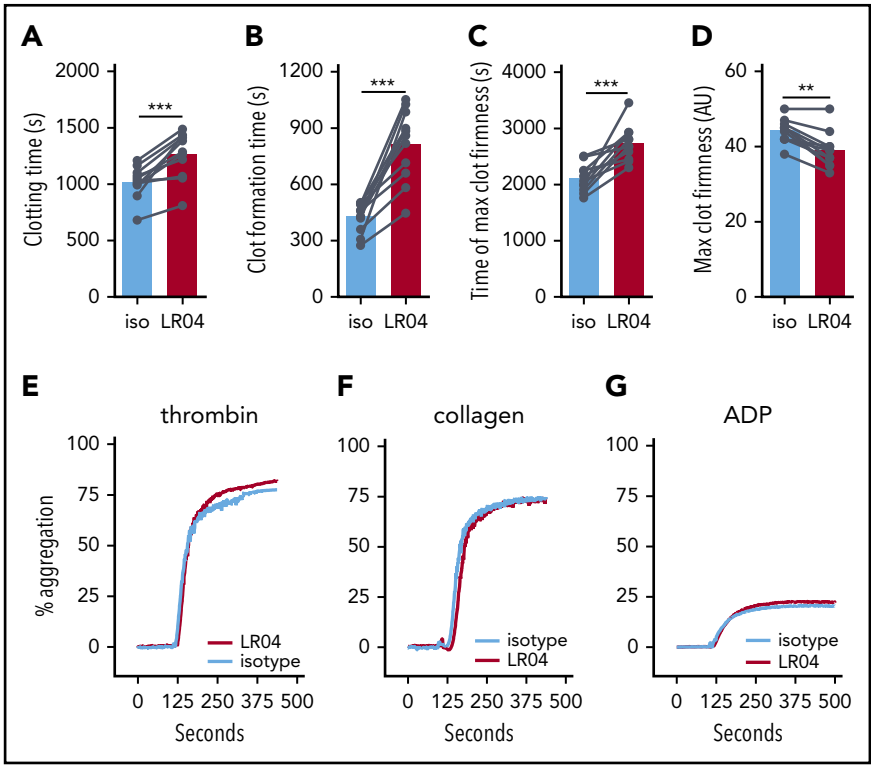

Figure 3. LR04 delays whole blood clotting without affecting platelet aggregation. (A-D) Effect of LR04 on whole blood clotting using rotational thromboelastometry. Freshly drawn blood of healthy individuals (n = 11) was incubated for 20 minutes with 25 μg/mL of either LR04 or an isotype (iso) control antibody before initiating rotational thromboelastometry in the non-activated method mode. (A) Clotting time, (B) clot formation time, (C) time of maximum clot firmness, and (D) maximum clot firmness. Bars represent the mean of each group. $^{**}P < .01$, $^{***}P < .005$, Wilcoxon matched-pairs signed-rank test. (E-G) Effect of LR04 on platelet aggregation. Washed platelets isolated from healthy volunteers (n = 11-15) were preincubated for 20 minutes with LR04 or an IgM control antibody (25 μg/mL) before activation with thrombin (E), collagen (F), or adenosine 5′-diphosphate (ADP) (G). Shown are representative aggregation curves. au, arbitrary units.

occurrence of thrombotic events in this model allows assessment of the direct anticoagulatory properties of IgM antibodies, independent of their known anti-inflammatory effects. The isolated HPAF-MVs displayed high TF positivity (supplemental Figure 4A) as well as LR04 binding (supplemental Figure 4B), and induced TG in MV-free plasma already after the addition of low concentrations. Preincubation with LR04 led to a strong inhibition of TG peak height (supplemental Figure 4C).

We next tested the prothrombotic effect of HPAF-MVs in vivo. Coinjection of HPAF-MVs and epinephrine, but not epinephrine alone, led to death within 10 minutes (supplemental Figure 4D). Microscopic evaluation of the lungs of these animals revealed the presence of thrombi, as indicated by deposition of fibrin (supplemental Figure 4E) and platelets (supplemental Figure 4F-G). Importantly, we observed the presence of injected fluorescently labeled HPAF-MVs within thrombi (Figure 4B). Coinjection of HPAF-MVs with LR04 but not an isotype control significantly rescued mice from death due to pulmonary thrombosis (Figure 4C). However, LR04 had no effect when collagen was used as a prothrombotic trigger (Figure 4D), further illustrating the MV-dependent mode of action of these antibodies. Importantly, the number of thrombi was significantly lower in lung sections of animals that were coinjected with MVs and LR04 compared with MVs and isotype control (Figure 4E-F).

Discussion

Circulating MVs are considered to be critical mediators of coagulation, and their increased levels and procoagulatory potential have been reported in cardiovascular disease and other pathologic states associated with increased risk for thrombotic events.[38,45,48] The current study identified natural IgM antibodies, and in particular IgM antibodies that bind to OSEs, as important modulators of the procoagulatory properties of MVs (Figure 4G). We found that depletion of IgM antibodies from plasma increases its coagulatory capacity and that the presence of IgM on circulating MVs is associated with a lower coagulatory potential. These findings identify IgM antibodies as a protective factor with a direct anticoagulatory function.

Interestingly, splenectomy has been shown to lead to an elevated long-term risk of venous thrombosis[60] and an increased rate of myocardial infarctions.[61] However, the mechanisms behind these observations have been elusive. Notably, splenectomy results in decreased IgM levels,[62] which might explain the prothrombotic state of these patients. In line with this, we and others have recently shown that low levels of OSE IgM antibodies are associated with an increased risk of venous thrombosis.[27,63] Thus, our findings offer an explanation for the high thrombotic risk associated with low IgM levels. Moreover, elevated numbers of circulating MVs in splenectomized individuals have been suggested to contribute to a prothrombotic state.[64]

High levels of IgM antibodies, and IgM antibodies with a specificity for OSEs in particular, have also been shown to be inversely associated with atherosclerotic cardiovascular events.[25,26,28] The protective effect of OSE-specific IgM antibodies in atherosclerotic cardiovascular disease has largely been attributed to their capacity to inhibit atherogenesis by neutralizing the proinflammatory effects of oxidized phospholipids, blocking the scavenger receptor–mediated uptake of oxidized LDL, and promoting the clearance of apoptotic cells.[16,29,65] Of note, we have shown that MDA-specific IgM antibodies inhibit the ability of MVs to induce interleukin-8 production by monocytes.[35] The anticoagulatory effect of OSE-specific IgM antibodies that we describe here identifies a novel function that is not directly linked to the antibodies' ability to neutralize the

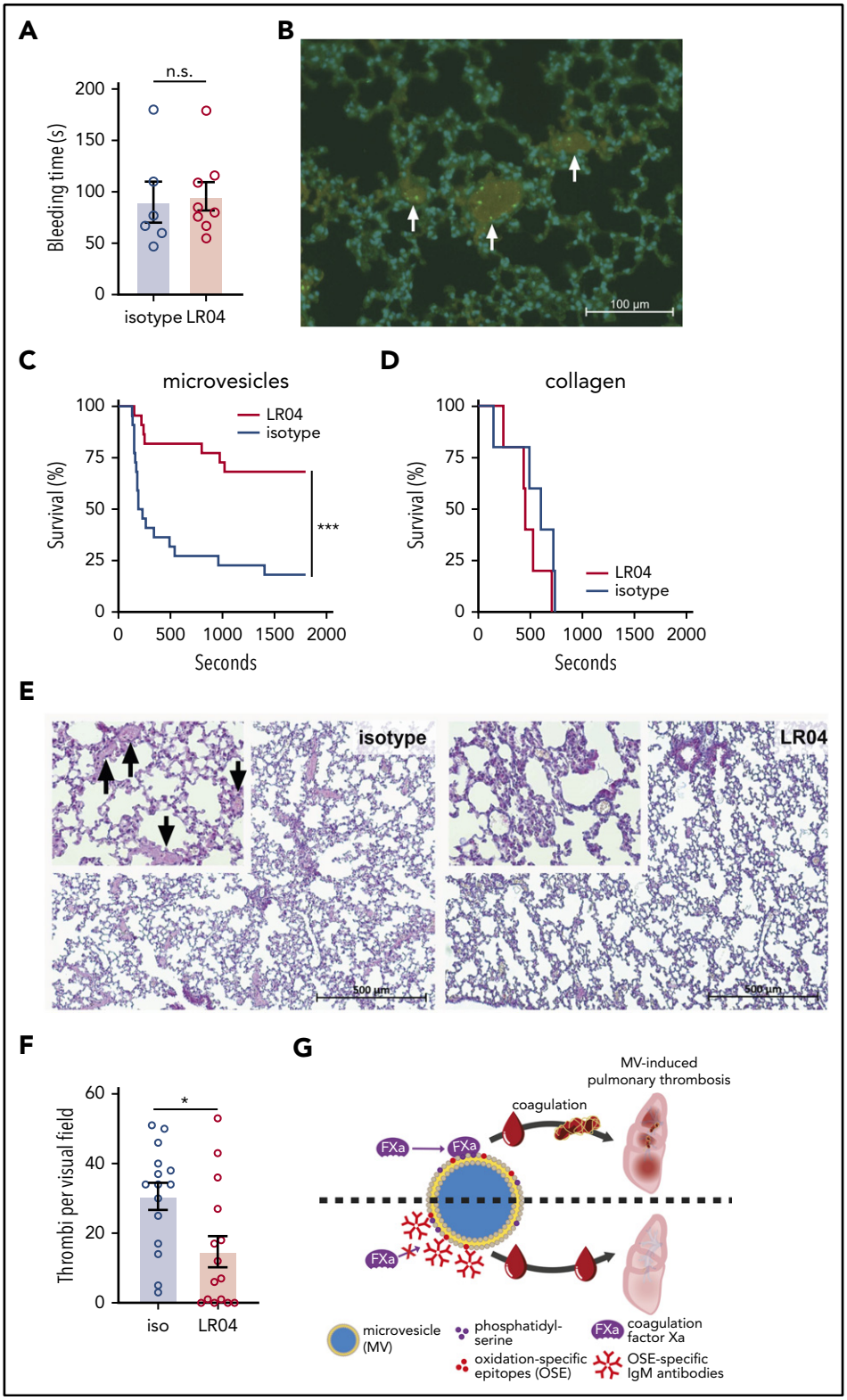

Figure 4. LR04 does not affect tail bleeding time but protects mice in an MV-induced pulmonary thrombosis model. (A) Tail bleeding times of mice injected with LR04 (n = 8) or an isotype control antibody (n = 6), 25 µg/mL of estimated total blood volume. Columns represent mean ± SEM. (B) Representative microscopic fluorescent image of lung sections of mice injected with a mixture containing epinephrine (60 ng/g bw) and fluorescently labeled HPAF-MVs (0.1 µg/g bw). Nuclei were stained with 4′,6-diamidino-2-phenylindole (blue) and platelets with an anti-CD41 antibody (red), injected labeled MVs (green) (20× magnification). Arrows indicate thrombi (fibrin = bright pink). Kaplan-Meier survival curves of mice intravenously injected with a mixture containing epinephrine (60 ng/g bw) and HPAF-MVs (0.1 µg/g bw) (n = 22 per group) (C) or epinephrine (60 ng/g bw) and collagen (3 µg/g bw) (n = 5 per group) (D). The mixture was preincubated with LR04 or an isotype control antibody, 25 µg/mL of estimated total blood volume. Mice were observed for a maximum duration of 30 minutes. ***$P < .005$, log-rank (Mantel-Cox) test. (E) Representative microscopic images of picro-Mallory–stained lung sections of mice, which were injected with a mixture of epinephrine (60 ng/g bw) and HPAF-MVs (0.1 µg/g bw).The mixture was preincubated either with 25 µg/mL estimated blood volume (2.25 µg/g bw) of LR04 or isotype control antibody. 5× magnification and 20× magnification (top left), arrows indicate fibrin deposits. (F) Numbers of thrombi per visual field, counted at 10× magnification (n = 15 per group). Columns represent mean ± SEM. *$P < .05$, Mann-Whitney U test. (G) Summary cartoon (details are provided in the text). n.s., not significant.

proinflammatory effects of lipid peroxidation products. Oxidized phospholipids, such as phosphatidylethanolamine and phosphatidylcholine, have been shown to increase the phosphatidylserine accessibility of membranes to coagulation factors by changing the size and curvature of liposomes.[66] Similar effects may occur on the surface of MVs, which are a major source of procoagulant phospholipids in vivo.[38] Therefore, the recognition of lipid oxidation products present on the surface of MVs may modulate their procoagulant potential. Indeed, we found that MV-dependent TG is delayed by the addition of different OSE-specific IgM antibodies binding either MDA (LR04 and NA17) or the phosphocholine headgroup of oxidized phospholipids (E06) but not a control IgM antibody. Notably, we previously showed that one-third of the natural IgM in plasma has specificity for OSE

and that most MV-associated IgM binds to MDA- or copper-oxidized LDL, the cognate antigens of NA17, LR04, or E06, respectively.[30,35] Because IgM antibodies with specificity for different OSEs mediate a similar anticoagulatory function, their combined effects may be even more potent.

Importantly, LR04 delayed TG (in particular, its propagation) triggered by both TF^+ and TF^- MV, showing that the effect occurs after initiation by both the extrinsic and intrinsic pathways. This scenario indicates that LR04 interferes with MVs interacting with the common pathway of the coagulation cascade. A key event in the common pathway is factor X binding to negatively charged phospholipids as found on MVs, leading to the assembly of the prothrombinase complex.[67] This explanation is supported by our finding that LR04 competes binding of factor X/Xa to immobilized MVs, whereas it does not cause MV aggregation. The fact that factor Xa itself does not directly bind to MDA epitopes, which are recognized by LR04, indicates that binding of LR04 to MVs indirectly interferes with the phosphatidylserine accessibility for factor Xa on MV surfaces and therefore decreases their procoagulatory capacity.

LR04 also delayed clot formation, most prominently clot formation time (ie, propagation), in the dynamic situation of constant MV production that occurs during whole blood clotting, which depends on both plasmatic and cellular components of coagulation. Of note, LR04 did not affect aggregation of isolated platelets, providing further support for the notion that the effect of LR04 relies on the interaction between MVs and plasmatic coagulation components. Importantly, the antithrombotic effect of LR04 was also observed in vivo, when MVs were used to induce pulmonary thrombosis in mice but not when collagen was used. These data further illustrate the specificity of the anticoagulatory effect of LR04 for MV-driven coagulation in contrast to collagen-induced clot formation that primarily depends on platelets.

Notably, OSE-specific IgM antibodies had no effect on clotting assays that depend on the addition of exogenous phospholipids and are not sensitive to MVs (aPTT). This finding is in contrast to antiphospholipid syndrome antibodies that recognize the phospholipid cardiolipin and/or β_2-glycoprotein 1, and are associated with an increased risk of thrombosis but prolong phospholipid-dependent clotting times in vitro.[68] Furthermore, epidemiologically, OSE-specific IgM antibodies are associated with lower risk of arterial[25,26,28] and venous[27,63] thrombotic events. Thus, these natural IgM antibodies represent a different group of antibodies that confer MV-dependent anticoagulatory effects both in vitro and in vivo.

Our findings have several clinical implications. First, assessing OSE-specific IgM levels could improve patient stratification within at-risk populations to decide on the necessity or intensity of anticoagulatory therapies. This might be particularly important in thrombotic pathologies in which MVs are considered to contribute to the thrombotic burden, such as cancer-associated thrombosis[49] and acute coronary syndrome.[69] Second, the therapeutic potential of OSE-specific IgM antibodies could be explored by both developing therapeutic monoclonal antibodies and identifying factors that stimulate production of OSE-specific natural IgM antibodies.

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Authorship

Contribution: G.O., T.A., and W.S. conducted experiments and performed data analysis; G.O., T.A., and C.J.B. designed experiments and wrote the article; G.O. and T.A. handled all in vivo experiments; L.G. and T.A. established and performed histologic treatment and analysis of mouse lungs; S.T. performed histologic analysis of mouse lungs; F.P. designed, conducted, and supervised the flow cytometry experiments; M.S. performed and supervised the rotational thromboelastometry experiments; W.S. conducted thrombocyte aggregation experiments and analysis; F.P., C.A., I.P., B.J., A.A., and N.M. contributed to writing and critical evaluation of the article; and C.J.B. and T.A. supervised and coordinated the effort.

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ORCID profiles: G.O., 0000-0003-4930-6628; L.G., 0000-0003-1037-3137; W.S., 0000-0003-0550-4120; M.S., 0000-0003-2741-6877; C.A., 0000-0003-2607-9717; B.J., 0000-0001-5652-7977; A.A., 0000-0002-5670-5910; N.M., 0000-0002-9170-7700; C.J.B., 0000-0001-8313-7050.

Correspondence: Christoph J. Binder, Medical University of Vienna, Department of Laboratory Medicine, Lazarettgasse 14, AKH BT25/2, 1090 Vienna, Austria; e-mail: christoph.binder@meduniwien.ac.at.

Footnotes

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*G.O. and T.A. contributed equally to this study.

Requests for original data may be submitted to the corresponding author (Christoph J. Binder; e-mail: christoph.binder@meduniwien.ac.at).

The online version of this article contains a data supplement.

There is a *Blood* Commentary on this article in this issue.

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2.3 Manuscript: Supplemental Methods

Supplemental Methods:

Blood collection, plasma handling, MV-isolation and analysis.

Human blood was collected from fasted healthy volunteers via venipuncture into 9 ml sodium citrate (3.8%) containing collection tubes. For platelet-poor plasma (PPP), samples were centrifuged twice for 10 min at 2,000 *g* at room temperature using an Allegra X-13 R (SX4750A swingbucket rotor) centrifuge.

For microvesicle-poor plasma (MVPP), additional centrifugation for 30 min at 18,000 *g* at 4°C was performed, using a microfuge 22R (F241,5P fixed-angle rotor) centrifuge. The pellet was washed once in DPBS. For extracellular vesicle poor plasma (EVPP), additional centrifugation at 100,000 *g* for 60 min at 4°C was performed using a Sorvall WX Ultra 100 (TFT-80.4 fixed-angle rotor) centrifuge. For murine plasma, 1 ml syringes, preloaded with 80 µl of 3.8% sodium citrate were used to collect blood from the inferior vena cava after euthanasia by CO_2. To obtain mouse PPP, blood samples were centrifuged at 2,000 *g* for 10 min at room temperature, using an Eppendorf centrifuge 5424 (FA-54-24-11 fixed-angle rotor).

Microvesicle generation and isolation

Platelet-derived microvesicles were generated from platelet concentrates obtained from the Department of Transfusion Medicine of the Medical University of Vienna. Two washing steps with DPBS after centrifugation (900 *g*, at room temperature, 10 min, using an Allegra X-13 R (SX4750A swingbucket rotor) centrifuge) were performed, after which platelets were stimulated with ionomycin (10 µM) and $CaCl_2$ (1mM) in PBS for 30 min at 37°C, stimulation was stopped by adding EDTA (5 µM). Platelets were removed using two centrifugation steps (2,000 *g* / 3,900 *g*, at room temperature, 10 min, using an Allegra X-13 R (SX4750A swing bucket rotor) centrifuge), MVs were twice pelleted (18,000 *g*, 4°C, 30 min, using a microfuge 22R (F241,5P fixed-angle rotor) centrifuge) and washed in PBS. HPAF-II derived MVs were isolated from HPAF-II cells (ATCC CRL-1997), cultured in MEM (Gibco) containing 10% FBS, every 24 h for 5 days after cells had reached 80% confluency. For monocytic MVs, THP-1

cells (ATCC TIB-202) (1*10^6 cells/ml) were stimulated with 0.5 µg/ml LPS for 16 h in RPMI 1640 containing 10% FBS (10270106, ThermoFisher). A potential contribution of FBS-derived EVs was excluded in separate experiments. Conditioned media from both THP-1 and HPAF-II cells was centrifuged at 400 *g* for 5 min and 1,500 *g* for 10 min to remove cells and cellular debris. The supernatant was centrifuged twice at 18,000 *g* for 30 min at 4°C. using a Sorvall RC 6+ (F14-6x250y fixed-angle rotor) for the first centrifugation and a microfuge 22R (F241,5P fixed-angle rotor) centrifuge for the second centrifugation.

Factor X/Xa ELISA

To measure the ability of LR04 to compete with Factor X/Xa binding to MVs, U-bottom 96 well plates were coated with platelet-derived MVs (15 µg/ml in 20 µl of PBS) and incubated at room temperature for 1 h. Thereafter, plates were washed, blocked for 1 h with 120 µl per well with 0.5% FA-free BSA in PBS and washed again. Then, 25 µl diluted of LR04/ or isotype at 25 µg/ml in TBS $CaCl_2$ 5mM were added, incubated for 30 min at RT and washed. Thereafter, 25 µl plasma 1:1000 in TBS per well was added, incubated 15 min and washed. Anti FX/Xa antibody (Abcam, ab79929) diluted 1:2K in TBS-BSA (1%) 5 mM $CaCl_2$ was added, incubated 1 h at room temperature and washed. 50 µl of anti-rabbit AP IgG (A-3687, Sigma-Aldrich) 1:20000 in TBS-BSA (1%) was added and incubated for 1 h at room temperature and washed. 25 µl of Lumiphos plus per well diluted 1:3 in A.dest. per well was added and incubated for 30 min at room temperature protected from light, after which luminescence was detected using a Synergy 2 plate reader (Biotek). TBS+$CaCl_2$ (5 mM) was used as a washing buffer except for the last washing step where TBS was used. Human Factor Xa Native Protein (Thermo Fisher RP-43114) was biotinylated using EZ-Link™ Sulfo-NHS-LC-1 Biotinylation Kit (Thermo Fisher) according to the manufacturer's protocol, and used in 0.5 µg/ml, diluted in TBS-BSA (1%) 5 mM $CaCl_2$.

IgM depletion

IgM depletion of human plasma was performed using anti-human IgM beads (A9935, Sigma-Aldrich). The amount of anti-human IgM beads was chosen based on their IgM binding capacity (2-3 mg/mL of IgM) according to the manufacturer´s information, in order to achieve gradual depletion of plasma IgM, which is typically 0,4-2,3 mg/ml in the plasma of healthy adults. Beads were washed twice with PBS and excess liquid was discarded after centrifuging at 300 *g* for 2 min at 4°C. 100 µl of pooled citrated EVPP was incubated with beads for 60 min at 4°C. Beads were removed by centrifugation at 300 *g* for 2 min at 4°C. After depletion, total IgM and IgG levels were measured by a chemiluminescent-based ELISA as described previously [1]

Flow cytometry

MV characterization was performed with a BD LSRFortessa II flow cytometer. Megamix-Plus SSC beads and latex beads (1.1 µm) were used for size calibration. The detection gate was set between 0.24 µm and 1.1 µm size beads (Stago). Acquisition was performed for 30 s (12 µl/min), keeping the event rate below 5,000 events/s. Annexin V positivity was used as a marker for MVs. In order to control staining specificity, MV preparations were stained with PE-conjugated Annexin V (Biolegend) in the presence or absence of either 0.05% Triton X-100 or 50 µM EDTA for 30 min according to the MISEV recommendations by Théry et.al.[2] (Supplemental Figure 5). To identify MVs carrying IgM antibodies on their surface, MV preparations were stained with an APC-conjugated anti-human IgM antibody **(**1:100; II/41,17-5790-82, ThermoFisher) together with either PE-conjugated Annexin V or a Brilliant Violet 421-conjugated anti-human CD41a antibody (1:100; HIP8, 740074 , BD Biosciences) in 50 µl of Annexin V buffer for 30 min at RT, followed by addition of 200 µl of the same buffer for subsequent analysis. Binding of LR04 or a mouse IgM control was assessed by incubating MVs with 10 µg/ml of either IgM antibody in 50 µl of Annexin V buffer for 30 min at RT, followed by addition of 25 µl of a secondary APC-conjugated anti-mouse IgM (1:200 final dilution, RMM-1 APC Biolegend,) and incubation for 30 min at RT. Thereafter, the sample was diluted by adding 125 µl of Annexin V buffer and analyzed. The same procedure

was used to assess the presence of IgM antibodies on the surface of mouse MVs (except the use of primary antibodies). MV-associated TF was detected by addition of an APC-conjugated CD142 monoclonal antibody (5 µg/ml final concentration, HTF-1, eBioscience, 16-1429-82) for 30 min at RT.

Analysis of Extracellular Vesicles by Nanosight tracking analysis (NTA)

Nanoparticle Tracking Analysis (NTA) (LM10; Nanosight) was used to measure concentration and size distribution of particles in plasma. 200 nm diameter Microspheres-Nanospheres (Cold Spring) were used for calibration. Samples were diluted 20-100 fold in PBS. Each sample was measured for 5x1 min.

Tail bleeding assay

To assess effect of IgM on tail bleeding, 8-12 week old C57BL6/J mice were anesthetized as described above and injected intravenously with LR04 or a control IgM antibody (50 µg/ml of estimated blood volume, corresponding to 4.5 µg/g bw) in sterile PBS (volume 120 µl). After 10 min, a 5 mm piece of the tail was amputated and the tail was immersed into 37° C prewarmed PBS. Time of bleeding cessation was recorded as the disappearance of visible bleeding.

Rotational Thromboelastometry

Rotational Thromboelastometry was performed using a ROTEM device (Pentapharm). Blood of 11 healthy volunteers was drawn as described above. 300 µl were incubated for 20 min with either 25 µg/ml LR04 or an IgM isotype control antibody at room temperature. ROTEM as was performed in Non-Activated Rotational Thromboelastometry mode (NATEM) mode according to the manufacturer´s instructions.

Immunohistology and histochemistry

Mouse lungs were excised immediately after death and suspended overnight in PBS containing 3.6% formaldehyde. Then, tissues were placed for at least 16 hours in PBS containing 0.1% formaldehyde before dehydration and subsequent embedding in paraffine for sectioning. Tissue sections (4 µm) were placed on glass slides, deparaffinized and processed for histochemical and immunofluorescent stainings.

Platelets in lung sections were visualized by immunofluorescence microscopy using a rabbit anti-mouse CD41 antibody (ab134131, Abcam) and rabbit IgG antibody (ab27478, Abcam) as isotype control. First, tissue sections were blocked with 10% donkey serum in PBS, washed 3 times with PBS and incubated with a 1:2000 dilution of respective antibodies overnight at 4° C. slides were washed again 3 times with PBS and sections were incubated with an AlexFluor546-conjugated donkey anti rabbit antibody (1:200 dilution, A10040, ThermoFisher) for 1 hour at RT in the dark. Thereafter, sections were washed again and incubated with DAPI to visualize nuclei (1:1000 dilution, Company) for 10 min in the dark. Sections were washed with PBS, mounted under coverslips using a Fluoromount™ Aqueous Mounting Medium (Sigma) and examined with a Axio Imager.Z2 microscope and photographed using Zen Blue software (both Carl Zeiss Inc., Jena, Germany).

To visualize the presence of injected MVs within thrombi fluorescently labeled HPAF II MVs were prepared. In brief, MVs were generated and isolated as decribed above, and incubated with 1 µM of CellTrace™ CFSE (ThermoFisher Sientific) in PBS for 30 min at RT. Labeling was quenched by addition of an equal volume of 10% BSA–PBS, and excess CFSE was removed by centrifugation at 18000 g for s30 min at 4° C. MV pellets were resuspended in PBS, successful labelling was confirmed by flow cytometry, and labeled MVs were used for injections as described above.

To visualize pulmonary thrombi, sections of each animal (n=15 per group) were stained with a modified Lendrum's Picro-Mallory staining, using Bouin's fluid, Weigert's hematoxylin, Martius yellow, 1% acid Fuchsin, 1% phosphomolybdic acid and 2.5% aqueous methylblue.

Sections were mounted and eamined as described above. The presence and numbers of thrombi per visual field at 10X magnification was assessed by two independent investigators, blinded to the treatment and survival outcome.

Platelet isolation and aggregation

Blood was drawn from healthy donors as described above. Following centrifugation (20 min, 120 *g*) supernatant platelet-rich plasma was taken and re-centrifuged for 90 s at 3,000 *g* in the presence of 0.1 µg/ml prostacyclin. Platelets were resuspended in Tyrode's *HEPES* buffer at a density of 250,000 cells/µl. Platelet aggregation was assayed by light transmission aggregometry (PAP-8 Profiler, möLab). Platelets were pre-incubated with 50 µg/ml LR04 or control IgM for 30 min prior to stimulation with 110 mU/ml thrombin (Technoclone), 16 µg/ml collagen (möLab) or 60 µM adenosine diphosphate (Sigma-Aldrich).

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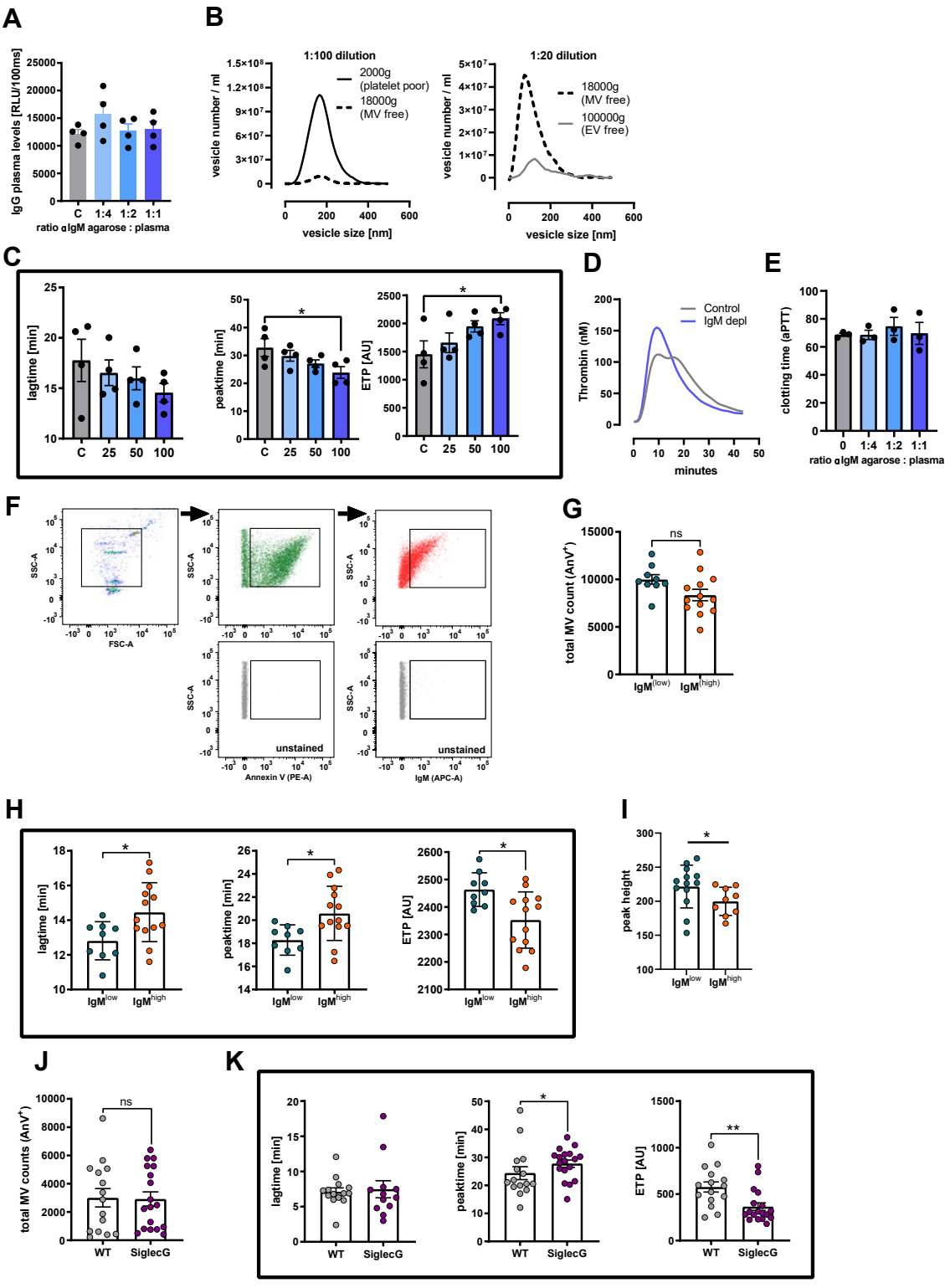
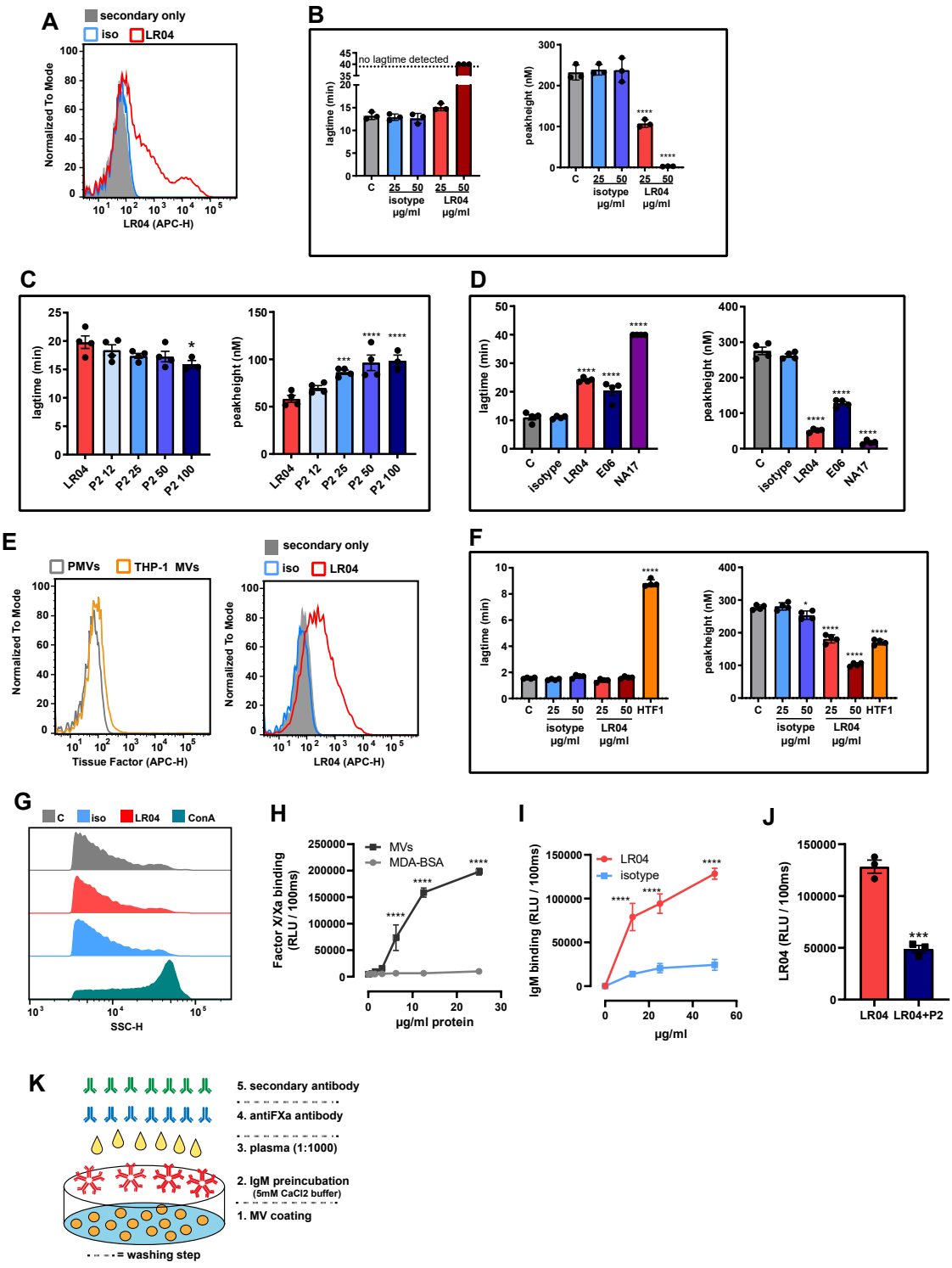
2.4 Manuscript: Supplemental Figures



Supplemental Figure 1

Characterization of plasma and circulating MVs from healthy volunteers and mice

(A) Plasma IgG levels after depletion of IgM antibodies with increasing amounts of Anti-Human IgM (µ-chain specific)−Agarose antibody. IgM depletion, measured by ELISA. Before IgM-depletion, EVs were removed via sequential centrifugation steps (2000g, 10 min x2 -> 18000 g, 30 min -> 100000 g, 1 h) **(B)** Size distribution and concentration of EVs in plasma after depletion by sequential centrifugation steps (2000 g, 10 min x2 = platelet poor / 18000 g, 30 min = MV f*ree / 100000 g, 1 h* = EV-free), as measured by nanoparticle tracking analysis (NTA). **(C)** Lag time, peak time, and ETP of IgM depleted MV-free plasma triggered by addition of platelet-derived MVs. **(D)** Thrombin generation curves of IgM depleted plasma triggered with THP-1-cell-derived MVs **(E)** Activated partial thromboplastin time of plasma after IgM depletion. **(A-E)** Plots depict representative experiments of at least 4. **(F)** Flow cytometry MV gating strategy for size, Annexin V positivity and MV-IgM positivity. **(G)** Total Annexin V^{+}-MV count of the MV IgM^{low} (n=9) and MV IgM^{high} (n=13) groups, measured by flow cytometry. **(H)** Lag time, peak time, and ETP of pooled MV-depleted plasma reconstituted with isolated IgM^{low} (n=9) and IgM^{high} (n=13) MVs. **(I)** Peak height of pooled MV-depleted plasma reconstituted with isolated IgM^{low} (n=13) and IgM^{high} (n=9) MVs, using CD41a as an MV identifier. **(J)** Total circulating AnnexinV^{+}MV count of WT and Siglec-$G^{-/-}$ mice. **(K)** Lag time, peak time, and ETP of spontaneous thrombin generation of platelet poor plasma from WT and Siglec-$G^{-/-}$ mice. Columns and error bars represent the mean+/-SEM of each group. *P<0.05, **P<0.01, ***P<0.005, Mann-Whitney test.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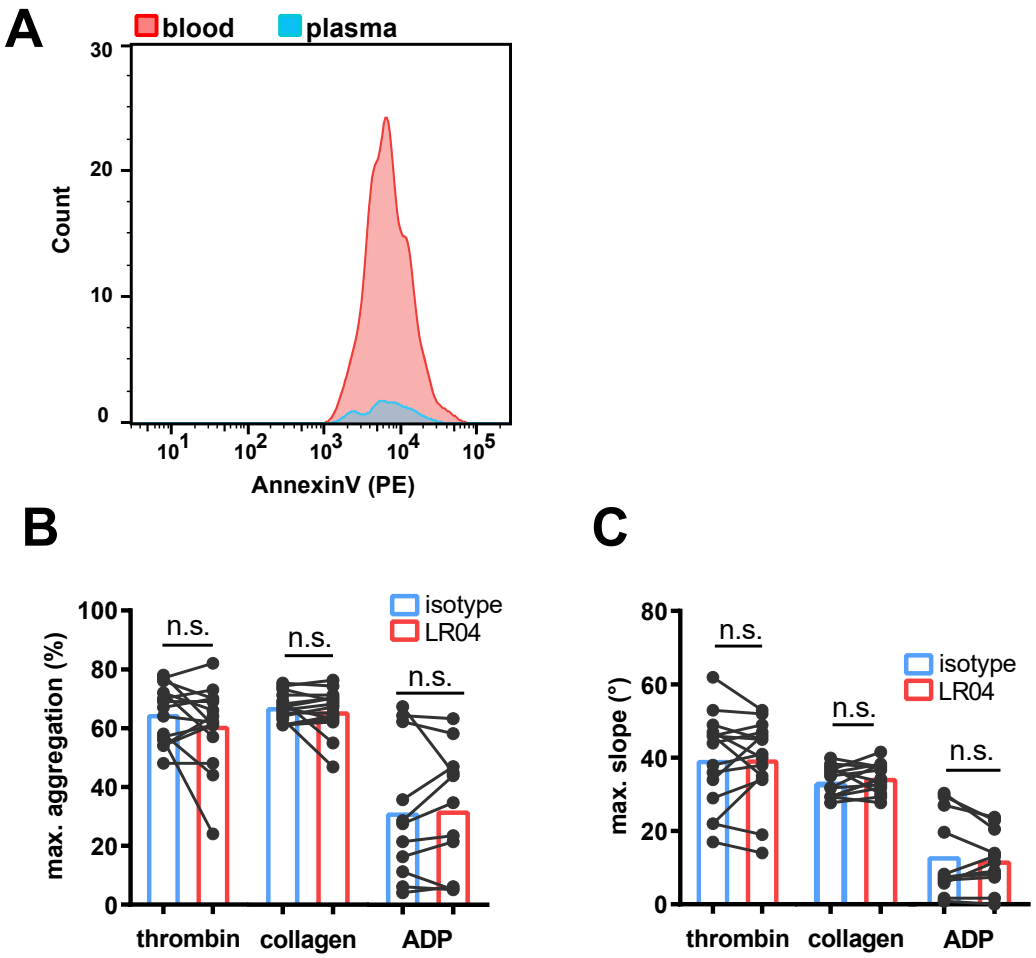
IgM binding (RLU / 100ms)
µg/ml
J
LR04 (RLU / 100ms)
LR04
LR04+P2
K
5. secondary antibody
4. antiFXa antibody
3. plasma (1:1000)
2. IgM preincubation
(5mM CaCl2 buffer)
1. MV coating
= washing step

Supplemental Figure 2

Endogenous IgM antibodies decrease plasma coagulation and procoagulatory properties of circulating MVs.

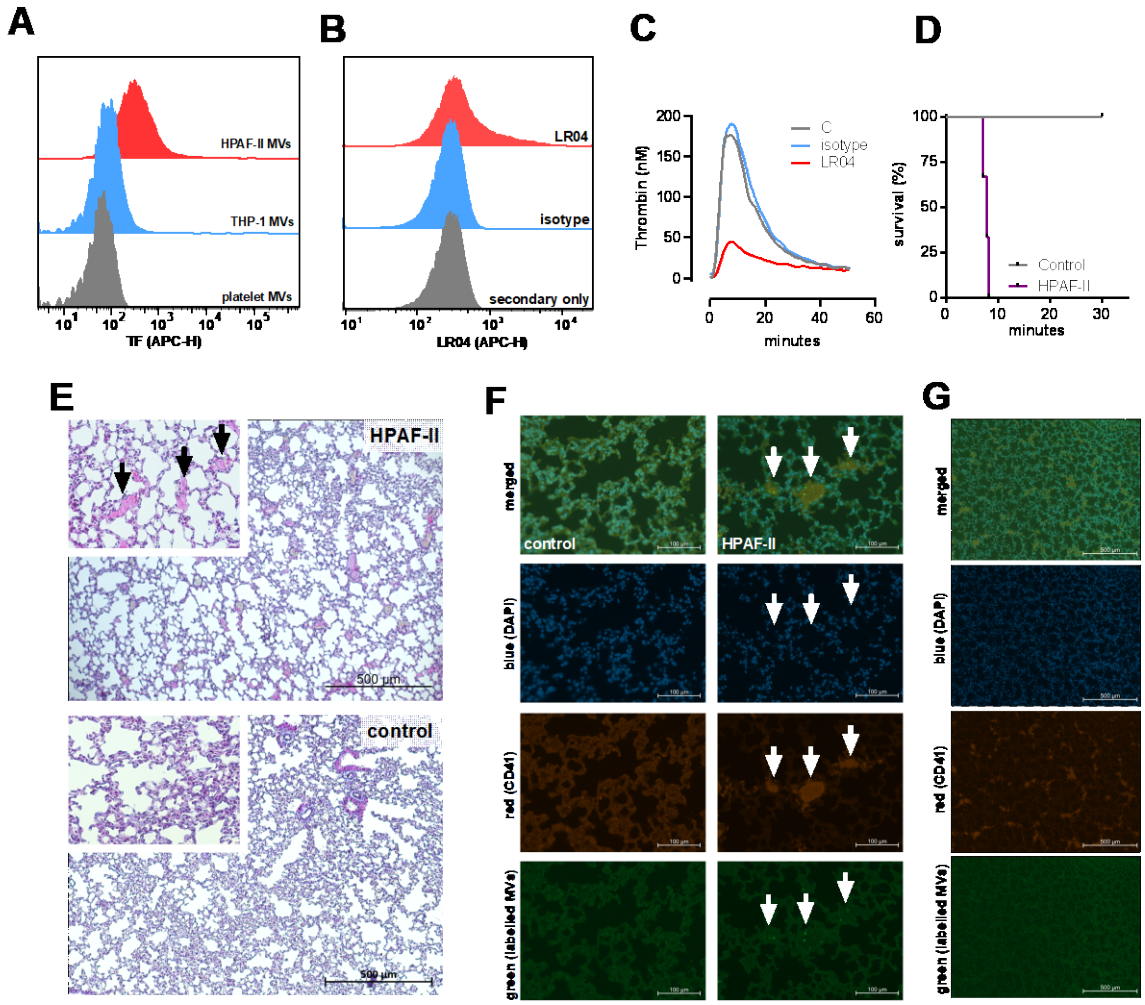
(A) Positivity of platelet derived MVs for LR04, shown by flow cytometry histogram. **(B)** Lag time and peak height quantifications of thrombin generation triggered by PMVs which were preincubated with LR04 or an isotype control antibody (25,50 µg/ml). **(C)** Lag time and peak height quantifications of thrombin generation triggered by platelet derived MVs (2 µg/ml) which were incubated with LR04 (25 µg/ml). LR04 was preincubated with increasing amounts (0,12,25,50,100 µg/ml) of P2 before incubation with MVs. **(D)** Quantifications of lag time and peak height of thrombin generation curves of MV-depleted plasma triggered by platelet derived MVs (2 µg/ml), which were preincubated with 25 µg/ml of either an isotype control antibody or OSE-specific natural IgM antibodies (LR04, NA17: specific for MDA-type epitopes; or E06: specific for the phosphocholine headgroup of oxidized phospholipids). **(E)** Flow cytometry histograms of binding of THP-1 cell derived MVs with TF or LR04 **(F)** Lag time and peak height quantifications of thrombin generation curves of MV-depleted plasma triggered by THP-1 cell derived MVs (40 µg/ml). MVs were preincubated with the OSE-specific murine IgM LR04 or an isotype control antibody (25,50 µg/ml), or a TF blocking antibody (HTF-1, 10 µg/ml). **(G)** Flow cytometry histogram of platelet-derived MVs incubated with LR04 (25 µg/ml), an isotype control antibody (25 µg/ml), or Concanavalin A (ConA) (5 µg/ml). **(H)** Chemiluminescent ELISA showing binding of purified coagulation factor Xa to immobilized MVs and MDA-modified BSA.. **(I)** Chemiluminescent ELISA showing binding LR04 to immobilized MVs. **(J)** Chemiluminescent ELISA showing inhibition of binding LR04 (50 µg/ml) to immobilized MVs by preincubation of LR04 with the peptide mimotope P2 (100 µg/ml). **(K)** Graphical summary of the ELISA method used in Figure 2I. Plots depict representative experiments of at least 4 independent experiments, bars represent mean ± SEM.



Supplemental Figure 3

LR04 delays whole blood clotting without affecting platelet aggregation.

(A) MV-numbers in supernatant after ex vivo clotting of recalcified whole blood, compared to supernatant of recalcified plasma, measured by flow cytometry. **(B)** Maximum aggregation rate (%) and **(C)** maximum aggregation slope (°) of platelets derived from healthy volunteers (n=11-15), preincubated with 25 µg/ml of either LR04 or isotype control antibody, then stimulated with either thrombin, collagen or ADP, Wilcoxon matched-pairs signed rank test.

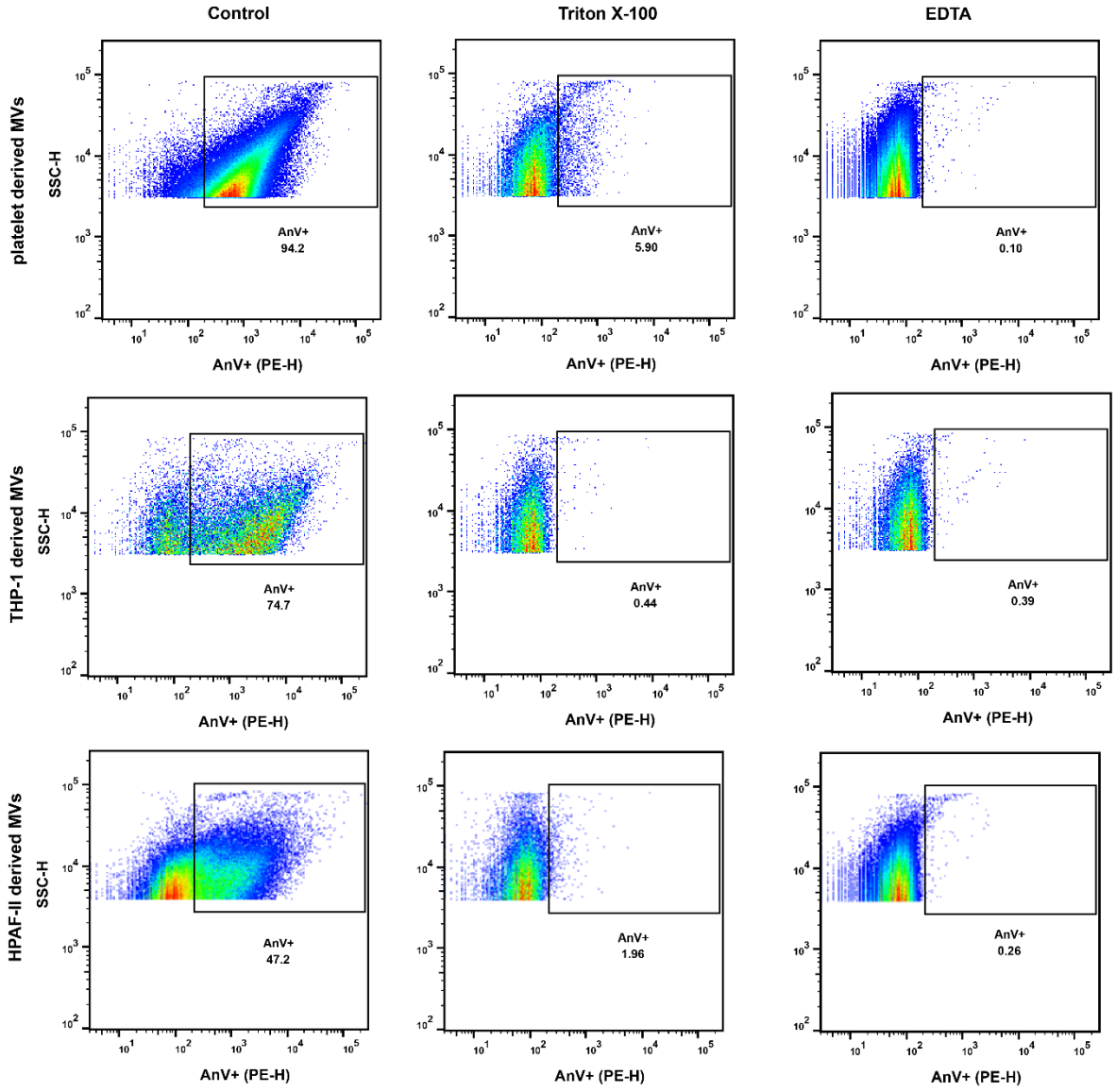


Supplemental Figure 4

LR04 protects mice in an MV-induced pulmonary thrombosis model

(A) Flow cytometric characterization of TF-expression on MVs. Shown are histograms of, HPAF-II derived, THP-1 cell derived and platelet derived MVs. **(B)** Flow cytometric characterization of LR04 and control IgM antibody binding to HPAF-II derived MVs. **(C)** Effect of LR04 on thrombin generation triggered by MVs derived from the pancreatic cancer cell line HPAF-II (0.1 µg/ml). MVs were preincubated with LR04 or an isotype control antibody (50 µg/ml), before adding them to MV depleted plasma. **(D)** Kaplan Meier survival curves of mice intravenously injected with a mixture containing epinephrine (60 ng/g of body weight) and HPAF-II derived MVs (0.1 µg/g bw) or epinephrine alone (control). **(E)** Representative microscopic images of Picro-Mallory stained lung sections of mice, which were injected with a mixture of epinephrine (60ng/g bw) and HPAF-II MVs (0.1 µg/g bw).The

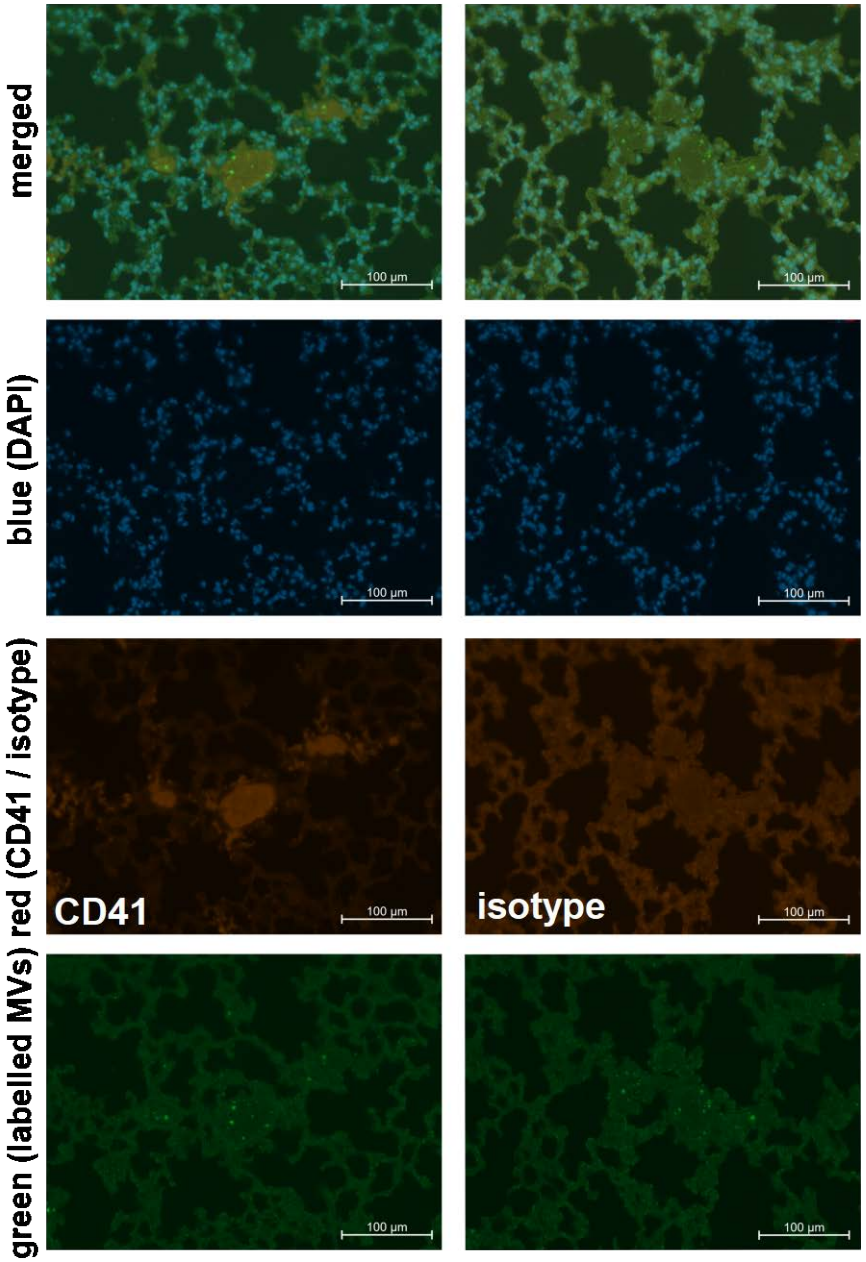
mixture was preincubated either with 25 µg/ml of estimated blood volume (2.25 µg/g bw) of LR04 or isotype control antibody. 5X and 20X magnification (top left of each panel), arrows indicate thrombi (fibrin = bright pink). **(F)** Representative microscopic fluorescent image of lung sections of mice injected with a mixture containing epinephrine (60 ng/g bw) and fluorescently labelled HPAF II MVs (0.1 µg/g bw; right panels) or PBS (control; left panels). Nuclei were stained with DAPI (blue) and platelets with anti-CD41 antibody (red), injected labelled MVs (green) (20X magnification). Arrows indicate thrombi containing fluorescent MVs. (**G)** Representative microscopic fluorescent image of lung sections of mice injected with a mixture containing epinephrine (60 ng/g bw) and fluorescently labelled HPAF II MVs (0.1 µg/g bw) at 5x magnification (platelets and nuclei visualized as in **F)**.



Supplemental Figure 5

Annexin V staining specificity

Representative dot plots of platelet-derived MVs (upper panels), THP-1 MVs (middle panels) and HPAF-II MVs (lower panels) stained with Annexin V either in the absence or presence of 0.1% Triton X-100 or EDTA (100 µM). MVs were preincubated with EDTA or Triton X-100 for 30 min at 37°C before addition of Annexin V. SSC-H = side scatter, PE = Annexin V.



Supplemental Figure 6

Specificity of Anti-CD41 antibody staining

Representative microscopic fluorescent images of lung sections of mice injected with a mixture containing epinephrine (60 ng/g bw) and fluorescently labelled HPAF II MVs (0.1 µg/g bw). Nuclei were stained with DAPI (blue), injected labelled MVs (green) (5x magnification). Anti-CD41 antibody (red) was used to stain platelets (left panel) and an isotype antibody was used to assess staining specificity (right panel); 20x magnification.

3. Discussion

The immune system is nowadays mostly considered a villain in the context of coagulation and thrombosis. For example, recent efforts have focussed on how to inhibit the prothrombotic role of neutrophil extracellular traps (Thålin et al., 2019) in both arterial and venous thrombosis. Antibodies in particular have a bad reputation among researchers and clinicians when it comes to thrombosis. Indeed, a procoagulant state induced by lupus anticoagulant, anti-apolipoprotein antibodies, or anti-cardiolipin antibodies, causes the antiphospholipid syndrome (Schreiber et al., 2018). Platelet-targeting antibodies are the culprit behind heparin induced thrombocytopenia (and thrombosis) (HIT/HITT) (Arepally, 2017) and have also recently been investigated in the context of both COVID-19 associated thrombosis (Althaus et al., 2021) and in regards to the potential link between rare cases of thrombosis found in people vaccinated with the Oxford–AstraZeneca COVID-19 vaccine (Greinacher et al., 2021). Furthermore, overactivation of the complement system can also lead to pathologies with thrombotic aspects such as the atypical hemolytic uremic syndrome (aHUS) (Noris and Remuzzi, 2014) or paroxysmal nocturnal haemoglobinuria (PNH) (Hill et al., 2017). On the other hand, autoantibodies targeting coagulation factor VIII can cause a bleeding phenotype in acquired hemophilia A (Kruse-Jarres et al., 2017).

Our study provides a counterpoint to these findings, in demonstrating that innate immunity can also be a beneficial inhibitor of thrombosis by limiting the procoagulatory properties of microvesicles. I therefore provide the mechanistic basis behind previous publications that found an inverse association between the level of OSE-binding IgM antibodies and the risk of venous thrombosis. Future studies should try to replicate these findings in bigger cohorts, and more specifically in diseases in which MVs are potential drivers of thrombosis, such as pancreatic cancer (Stark et al., 2018). Ultimately, measurements of OSE-binding IgM could contribute to improve current risk-stratification systems such as the Khorana score that is used in cancer patients (Mulder et al., 2019) to decide which at-risk patients need anticoagulation.

Since MVs are proven mediators of thrombosis, but not the main drivers in hemostasis (Owens and Mackman, 2011), targeting them therapeutically could be the “holy grail” of anticoagulation, by inhibiting thrombosis without risking bleeding. Modifying MVs therapeutically presents an additional interesting avenue by inhibiting both their proinflammatory and procoagulatory role at the same time. However, due to the dynamic balance of constant release and uptake/clearance of MVs, targeting them directly over a longer period of time may not be feasible, as the drug may be cleared just as quickly. In addition, IgM antibodies themselves have a comparably short half-time in vivo compared to IgG antibodies, as they lack the neonatal Fc receptor (FcRn) mediated “escape pathway” from lysosomal degradation. Therefore, lowering

the procoagulatory potential of MVs in acute situations such as myocardial infarction of VTE may be more feasible. Intravenous immunoglobulins (IVIG) enriched in IgM have been used in treatment of severe sepsis (Neilson et al., 2005), a pathology accompanied by a state of coagulopathy (Simmons and Pittet, 2015). Indeed, a recent trial found that infusion of this IgM enriched IVIG positively affected microcirculation in septic patients (Domizi et al., 2019). Considering our findings, one could hypothesize that this beneficial effect is due to the fraction of OSE-binding IgM antibodies that should be present in IgM enriched IVIG.

Substances that confer the same sterical hindrance effect, may also be used to inhibit the pro-coagulatory effect of MVs. However, substances which do not share this effect, may actually competitively bind to MVs and prevent their anticoagulatory effect. Another aspect that I could not take into consideration in this study is whether the presence of natural IgM on MVs increases their clearance. If that is the case, targeting MVs with other substances may affect the total number in circulation by interfering with IgM mediated clearance, even if they confer a similar sterical hindrance effect. If the clearance of MVs is indeed affected by the presence of IgM antibodies on their surface, increasing physiological IgM production could increase MV clearance in addition to directly decreasing their procoagulatory effects. As previously discussed, molecular mimicry between the phosphocholine present in the pneumococcal capsule and the phosphocholine group of oxidized phospholipids present on oxidized LDL leads to an increase in anti-oxLDL antibodies. Similar molecular mimicries may be exploited in the future to boost natural IgM levels to supraphysiological levels and therefore confer beneficial effects in inflammatory and thrombotic diseases.

Summary and conclusion

Natural IgM antibodies have been shown to possess various beneficial properties, especially in atherosclerotic cardiovascular disease and other inflammatory-driven diseases. In this study, I expand the knowledge on these beneficial effects of natural IgM antibodies by demonstrating their inhibitory role in MV-mediated coagulation and thrombosis. While there are many unanswered questions, I believe that these findings are of great relevance in the field of thrombosis. Future studies building on this knowledge have the potential to impact clinical practice by exploring the therapeutic and diagnostic potential of OSE-binding natural IgM antibodies in coagulation and thrombosis.

4. Bibliography

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5. Curriculum Vitae

Georg Obermayer, MD

EDUCATION

2014 - 2021
MEDICAL UNIVERSITY OF VIENNA / CEMM
PhD-Program

- Translational scientific projects in thrombosis, inflammation and innate immunity
- Scientific writing (grants/publications)
- Presentations at national & international conferences
- Organization of scientific retreats and conferences

2007 - 2013
MEDICAL UNIVERSITY OF GRAZ
Human medicine / MD

EMPLOYMENT

2019 - current
SWEDISH ORPHAN BIOVITRUM
Medical Science Manager

- medical/scientific support of internal departments in hematology and immunology
- support of prelaunch and launch activities of rare disease, hematology and immunology products
- conducting internal and external scientific education
- organization of advisory boards and scientific meetings in rare disease, rheumatology, immunology, hematology, dermatology and the complement system
- allocation and supervision of medical grants
- Informationsbeauftragter gem. § 56 AMG

2014
AUSTRIAN ARMED FORCES
Medical Doctor in military service

- Admission and treatment of recruits and military staff
- Anatomy, physiology and first aid course teaching

2012 - 2013
GREEN BEAT (Institute of Nutrient Research)
Scientific assistant

- Clinical study design
- Study subject recruitment
- Support of clinical study subjects
- Data analysis

PUBLICATIONS

Obermayer et al.
Natural IgM antibodies inhibit microvesicle-driven coagulation and thrombosis
Blood, 2020

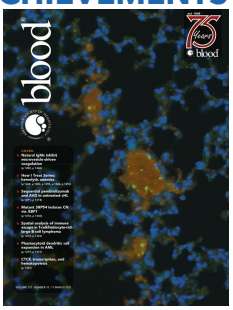
Kovacevic et al.
Von Willebrand factor Antigen levels predict major adverse cardiovascular events in patients with asymptomatic carotic stenosis of the ICARAS study
Atherosclerosis, 2019

Puhm et al.
Mitochondria are a subset of extracellular vesicles released by activated monocytes and induce Type I IFN and TNF responses in endothelial cells
Circulation Research, 2019

Obermayer et al.
Oxidized low-density lipoprotein in inflammation-driven thrombosis
Journal of Thrombosis and Hemostasis, 2017

Lamprecht et al.
Supplementation with a juice powder concentrate and exercise decrease oxidation and inflammation, and improve the microcirculation in obese women: randomised controlled trial data
British Journal of Nutrition, 2013

ACHIEVEMENTS



2021
COVER PAGE FEATURE
Blood, Volume 137 Issue 10, March 11th

2017
ISTH CONGRESS - YOUNG INVESTIGATOR AWARD
Natural IgM Binding Oxidation-specific Epitopes Protect from Microvesicle-driven Thrombosis

2016
ASH ANNUAL MEETING - ABSTRACT ACHIEVEMENT AWARD
Natural IgM Against Oxidation-Specific Epitopes Inhibit Microvesicle-Driven Coagulation

2012
ENA POSTER AWARD
Effects of a juice powder concentrate and walking exercise on systemic markers of oxidation, inflammation and skin microcirculation in obese women