Mechanistic Investigation of Coagulation Activation in Childhood Acute Lymphoblastic Leukemia

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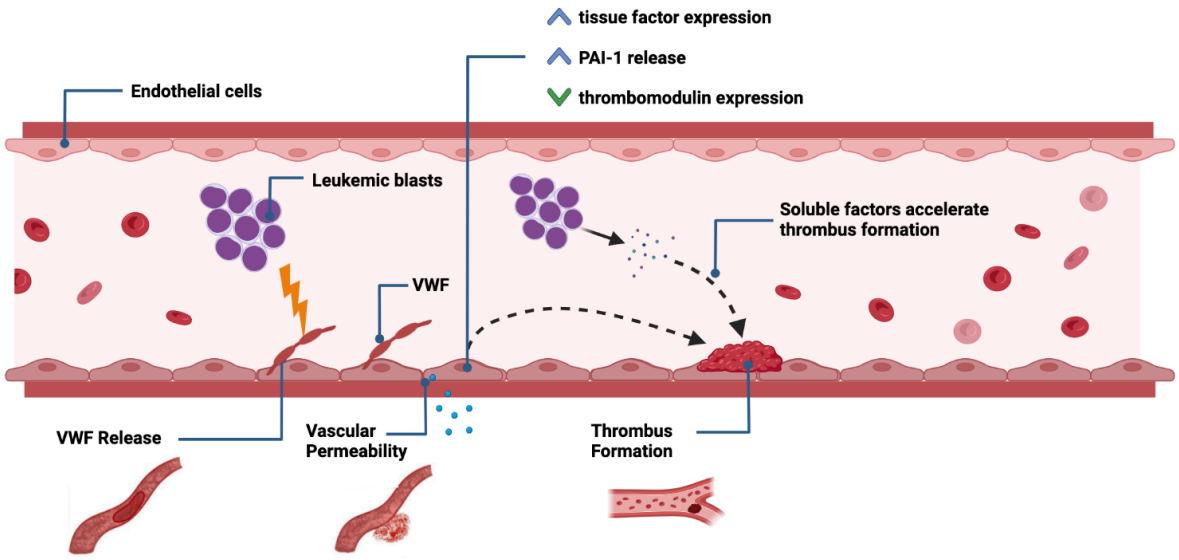
Abstract

Thrombosis is a well-known complication in children with acute lymphoblastic leukemia (ALL) with a reported frequency of up to 36.7%, resulting in significant morbidity and mortality. Children with ALL were found to have persistent increased thrombin generation following diagnosis, yet, the pathogenesis and impact of therapy on thrombin activation are still unknown. Athale et al. previously demonstrated an association between the presence of peripheral lymphoblasts with increased levels of Von Willebrand Factor and parameters of thrombin generation. The objective of this study is to confirm the clinical observation in an *in vitro* co-culture model and to explore the effect of lymphoblasts on hemostasis. Human umbilical vein endothelial cells were co-cultured with peripheral-blood derived lymphoblasts in reduced serum media. Biomarkers of endothelial activation and the expression of endothelial products involved in hemostasis were measured by enzyme-linked immunosorbent assays. Endothelial health was evaluated by endothelial permeability assay and LDH cytotoxicity assay. Thrombin generation on the endothelial surface was monitored with a thrombin substrate. The clotting time of plasma mixed with supernatants derived from lymphoblasts was measured by a plasma recalcification assay. Compared to unstimulated endothelium, the presence of leukemic lymphoblasts significantly increased biomarkers of endothelial activation including VWF and adhesion molecules. In addition, the expression of endothelial products involved in hemostasis was altered towards a prothrombotic phenotype. On endothelium cocultured with leukemic blasts, plasma clotting time was faster, and increased thrombin generation was recorded. When mixed with plasma, secretants from leukemic lymphoblasts increased parameters of thrombin generation. In conclusion, we confirmed the clinical observation that peripheral blasts are capable of activating

endothelium, leading to the elevation of VWF; and may cause the prothrombotic state seen in children with acute lymphoblastic leukemia.

Visual Abstract



Visual Abstract: When cocultured with endothelium, leukemic lymphoblasts induce endothelial activation, resulting in a prothrombotic endothelial phenotype. In addition, soluble factors from lymphoblasts may accelerate thrombus formation. Figure was created in *Biorender.com*.

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List of Abbreviations

ALL: acute lymphoblastic leukemia

APC: activated protein C

aPTT: activated partial thromboplastin time

CNS: central nervous system

ELISA: enzyme-linked immunosorbent assay

EPCR: endothelial protein C receptor

HUVEC: human umbilical vein endothelial cell

MIP-1α: macrophage inflammatory protein-1 alpha

PAI-1: plasminogen activator inhibitor-1

sDPPIV: soluble dipeptidyl peptidase 4

t-PA: tissue plasminogen activator

TAT: thrombin–antithrombin complex

TF: tissue factor

TIMP-1: tissue inhibitor of metalloproteinases-1

TM: thrombomodulin

VCAM-1: vascular cell adhesion molecule-1

VWF: Von Willebrand Factor

WBC: white blood cell count

1. Introduction

1.1 Acute Lymphoblastic Leukemia

Acute lymphoblastic leukemia (ALL) is an aggressive type of leukemia which is characterized by the accumulation of immature lymphoblasts in the bone marrow and blood. The cancer is caused by alterations of the DNA sequence in proto-oncogenes, tumor-suppressor genes, and microRNA genes of hematopoietic stem cells or their committed progenitors (macFröhling & Döhner, 2008; Pui et al., 2004). These mutations influence key regulator processes in a way that gives cells unlimited capacity for self-renewal, or impairs the cellular control of normal proliferation and programmed apoptosis (Fröhling & Döhner, 2008; Pui et al., 2004). A better understanding of how such molecular lesions produce ALL will continue to optimize current treatment. Thanks to advances in risk-directed therapy, survival rate among patients with ALL has improved significantly from less than 50% in the 1970s to more than 80% in the modern day (Howlader et al., 2017). However, undue side effects still result in significant morbidity and mortality (Marina, 1997). Ultimately as we continue to learn more about the molecular mechanisms of leukemic cell transformation and patient response to therapy, personalized ALL therapy with minimal side effects will replace common protocols for large groups of patients (Pui et al., 2004).

1.1.1 Epidemiology

The overall incidence of childhood cancer, including ALL has been increasing since 1975. More than a quarter of all pediatric cancer is ALL, which makes it the most common diagnosed cancer in the pediatric population. The reported incidence of ALL in the United States is 41 cases per 1

million people aged 0 to 14 years and it decreases to 17 cases per 1 million people aged 15 to 19 years (Howlader et al., 2017). There is a sharp peak in ALL incidence among children aged 2 to 3 years (>90 cases per 1 million per year) which is approximately four to five-fold greater than that for infants and for children aged 10 years or older (Howlader et al., 2017). In addition, Hispanic children appear to be most commonly affected (43 cases per 1 million), while the incidence in white children is nearly threefold higher than in black children (Ries, 1999).

1.1.2 Diagnosis and Classification

The French-American-British (FAB) classification of ALL introduced in 1976 was based on the morphology (size, shape and structure) of the leukemic cell and describes three types of blasts in ALL. L1 blasts are small and homogeneous with round nuclei and scanty cytoplasm. L2 type are larger with irregular nuclei, prominent nucleoli and more abundant cytoplasm. L3 blasts are basophilic cells with prominent cytoplasmic vacuoles (Bennett et al., 1976).

On the other hand, the World Health Organization classification for ALL is based on the immunophenotype of the leukemia cells, as determined by flow cytometry and/or cytogenetic tests. By immunophenotyping, 75% of ALL cases arise from B-cell progenitors and 25% from T-cells (Terwilliger & Abdul-Hay, 2017).

In addition, other classification factors include chromosome abnormalities, mixed phenotype acute leukemia and central nervous system involvement. While the Philadelphia (Ph) chromosome translocation is common in 25% of adults with ALL, the frequency in pediatric population is less than 5% (Terwilliger & Abdul-Hay, 2017). Other chromosome abnormalities also exist such as t(4;11), t(9;22), t(12;21), etc. In addition, sometimes the leukemic cells are classified as mixed phenotypes when they have both myeloid and lymphoid characteristics.

Finally, leukemic cells spread to the central nervous system at the time of diagnosis in about 5-10% of patients (Kuo et al., 2018).

1.1.3 Risk Factors

Factors that increase the risk of getting childhood ALL can be categorized into genetic risk factors and environmental risk factors (Schmidt et al., 2021). Having certain genetic conditions may increase a child's risk of developing leukemia. These include trisomy 21, neurofibromatosis type 1, Li-Fraumeni syndrome and Fanconi anemia (Stieglitz & Loh, 2013). There is no known genetic cause linked to ALL. Genomic study reveals that ALL most frequently harbor alterations in WT1, NOTCH1, EZH2, BCORL1, and USP7 genes (Consortium et al., 2017). Siblings of children with ALL have a weakly increased incidence of developing leukemia but the overall risk is still low (Schmiegelow & Hjalgrim, 2006).

On the other hand, environmental risk factors such as being exposed to X-rays before birth, or being exposed to radiation or past treatment with chemotherapy increase the child's risk of getting ALL (Belson et al., 2007). However, most studies have not found strong links between environmental factors and childhood ALL.

1.1.4 Treatment

In childhood ALL, children are classified into risk groups to plan treatment. Study groups worldwide use varying criteria to stratify patients into risk groups (Bhojwani et al., 2009). The Children’s Oncology Group established 4 risk groups: low-risk, standard-risk, high-risk and very-high risk based on clinical and cytogenetic data from more than 6000 patients enrolled in previous trials. The estimated 4-year event-free survival for these 4 groups was 91%, 86%, 76%

and 46%, respectively (Carroll et al., 2008). The standard, or low risk group generally includes children aged 1 to 10 who have a white blood cell count (WBC) less than 50,000/ul at diagnosis. In the high-risk group, children older than 10 years of age or who have WBC over 50,000/ul are included. The very high-risk group includes children less than age 1, or who have with certain genetic changes, or who have a slow response to initial treatment (Schultz et al., 2007). Children in the higher risk group usually receive more or higher doses of anticancer drugs than children in the standard group.

Since ALL is a cancer of the blood, the treatment is systemic through a central line. The treatment of childhood ALL is done in phases, including remission induction, consolidation and maintenance (Pui & Evans, 2006). Induction is the first phase of treatment which usually lasts four weeks. The goal of this phase is to kill the leukemic cells and allow normal blood cells to return. Nearly 98% of children with ALL enter remission at the end of the first month of treatment (Board, 2022). However, this does not mean that the child is cured, because without further treatment, the disease will return. The second phase of treatment is consolidation, which lasts from 12-16 weeks, to kill leukemic cells that may remain after the induction phase. Moreover, in a subgroup of children with central nervous system (CNS) involvement, radiation therapy and intrathecal chemotherapy are delivered to the brain to prevent the growth of leukemic cells in the CNS (Pui & Evans, 2006). The final phase of treatment is maintenance, in which the goal is to prevent a relapse. Although this phase usually lasts two or three years, it is less intensive than the previous phases. In addtion to chemotherapy and radiation, targeted therapy has been developed for some types of childhood ALL, such as the rare "Philadelphia chromosome positive ALL". This treatment targets specific cancer cells, therefore it usually causes less harm to normal cells than chemotherapy or radiation (Portell & Advani, 2014). One

example is tyrosine kinase inhibitor therapy which prevents the enzyme tyrosine kinase from causing stem cells to develop into more white cells. Another example is monoclonal antibodies which directly attach to a specific target on cancer cells to induce apoptosis, block their growth or prevent them from spreading (Cabanillas et al., 2010).

1.2 Coagulation System

1.2.1 Overview

The concept of blood coagulation was developed in the 1960's when Davie, Ratnoff and Macfarlane came up with the "Waterfall" and "Cascade" theories which describe the fundamental principle of cascade of proenzymes leading to activation of downstream enzymes (Macfarlane, 1969). The process is a protective mechanism and meant to control hemorrhage. The cascade culminates in the formation of fibrin clots that close up the damaged site on the blood vessel, arresting the bleeding. The coagulation system under normal function is a delicate balance between the procoagulant pathway which is responsible for clot formation and the anticoagulant mechanisms that inhibit the process beyond the injury site. The system involves numerous enzymatic reactions between clotting factors, cell associated cofactors and receptors, and plasma coagulation protease inhibitors (Dahlbäck, 2000). In addition, the coagulation pathway acts in harmony with the endothelium and platelets in order to form a fibrin clot. A dynamic equilibrium between clot formation and clot lysis maintains the blood fluidity. The classical theory of blood coagulation classifies the coagulation cascade into the intrinsic and extrinsic pathways, both of which converge to form the common pathway. The common pathway ultimately activates fibrinogen into fibrin (Roderique & Wynands, 1967). The resulting fibrin mesh stabilizes the platelet plug.

1.2.2 Extrinsic Pathway

The extrinsic pathway is considered as the first step in plasma-mediated hemostasis. As a result of vascular insult, tissue factor (TF), which is constitutively expressed in the sub-endothelium under normal physiologic conditions, is exposed to blood (Mandal et al., 2006). Exposed TF then binds with circulating activated factor VII (FVIIa) and forms the TF-FVIIa complex. In the presence of a phospholipid surface and calcium ions, the newly formed TF-FVIIa complex promotes the conversion of factor X to factor Xa (Rao & Rapaport, 1987). Factor Xa and its cofactor Va then convert prothrombin into thrombin. In addition, the TF-FVIIa complex can also activate factor IX into factor IXa. Via its cofactor FVIIIa, the newly activated factor IXa can also drive factor Xa generation (Rao & Rapaport, 1987). The resulting thrombin converts fibrinogen into fibrin to form a cross-linked fibrin clot. The extrinsic pathway is clinically evaluated by the prothrombin time (Poller, 1999).

1.2.3 Intrinsic Pathway

Coagulation can also be initiated through the intrinsic or "contact" pathway without the presence of the TF-FVIIa complex. It begins with the activation of circulating factor XII through contact with activated endothelium, the platelet surface, high molecular weight kininogen, kallikrein, polyphosphates or subendothelial collagen (Chaudhry et al., 2018). Activated factor XII then acts as a catalyst to activate factor XI to activated factor XI, which then goes on to activate FIX. Activated FIX then acts with its cofactor FVIII to form the intrinsic tenase complex on a phospholipid surface to activate factor X (Sheehan et al., 2003). As the cascade moves further downstream, the plasma concentration of that factor increases. When prothrombin is converted to

thrombin, the resulting thrombin can reinforce the intrinsic pathway by inducing positive feedback to factors V, VII, VIII and XI (Grover & Mackman, 2019). The intrinsic pathway is clinically evaluated by the activated partial thromboplastin time (aPTT) (Lippi & Favaloro, 2008).

1.2.4 Common Pathway

The common pathway begins at the cleavage of factor X into activated factor X by the tenase complex (Riddel Jr et al., 2007). The extrinsic form of the tenase complex is made up of tissue factor, factor VIIa, and calcium ion. The intrinsic tenase complex consists of factor IXa, its cofactor VIIIa, the substrate factor X, and they are assembled on a negatively charged surface such as phospholipids. The vitamin K-dependant factors dock to the phospholipid surface through their Gla domains involving calcium ion bridges (Chaudhry et al., 2018). The resulting factor Xa then acts with its cofactor Va to cleave prothrombin into thrombin. Thrombin goes on to activate fibrinogen into fibrin, as well as other factor V, VIII, XI and factor XIII. Factor XIIIa crosslinks fibrin strands to form a fibrin mesh to stabilize the platelet plug (Janus et al., 1983).

1.2.5 Negative feedback to hemostasis

Under normal physiological conditions, there is a balance between the pro-coagulant pathway and the mechanisms that inhibit thrombus formation beyond the injury site. For example, thrombin can induce an anticoagulant effect by stimulating secretion of t-PA from endothelial storage, which then converts plasminogen to plasmin. Plasmin is a proteolytic enzyme that degrades fibrin mesh (Gelehrter & Sznycer-Laszuk, 1986). In addition, thrombin can stimulate the production of antithrombin. Antithrombin binds and inhibits the procoagulant functions of

thrombin, FIXa and FXa (Bae et al., 1994; Olson & Björk, 1994) . Furthermore, protein C and protein S elicit their anticoagulant activity through inactivation of FVa and FVIIIa (Gelehrter & Sznycer-Laszuk, 1986).

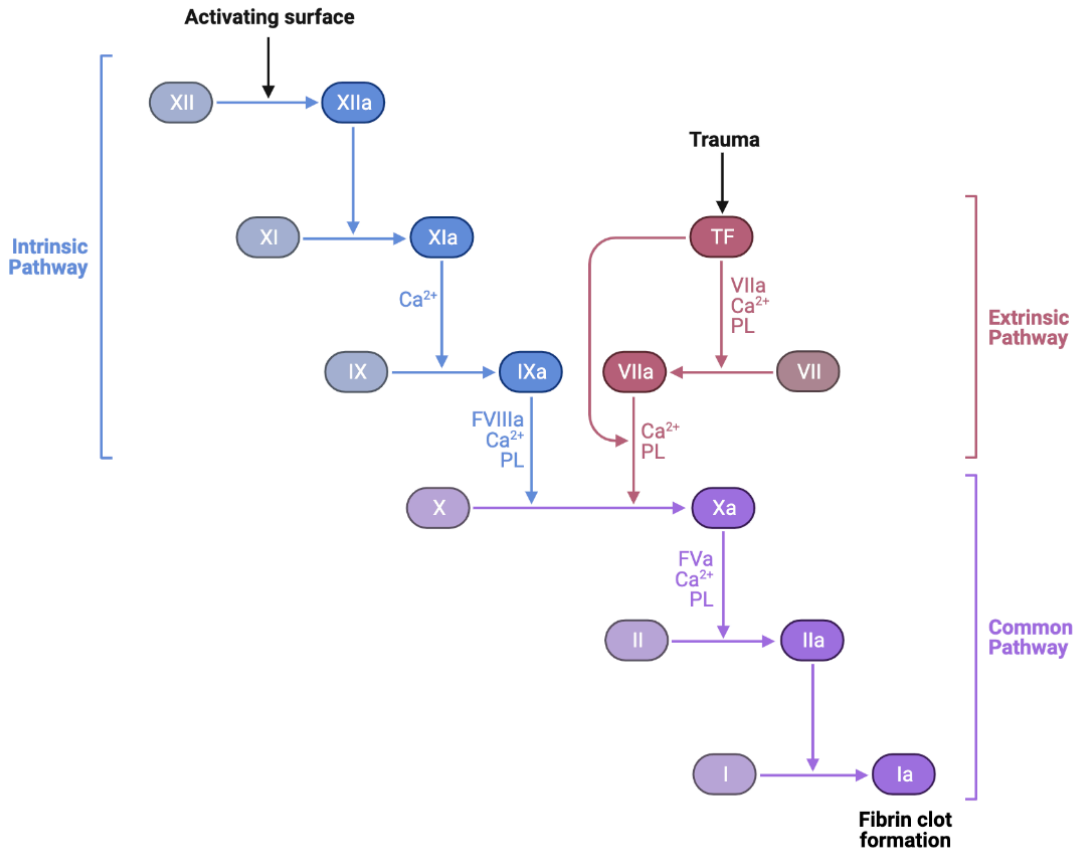

Figure 1. The coagulation cascade. The coagulation cascade refers to the series of steps that leads to hemostasis. There are three pathways: intrinsic, extrinsic, and common. *Reprinted from "Coagulation Cascade", by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates.*

1.3 Endothelial Regulation of Hemostasis

The endothelium is a layer of cells that forms the inner lining of the circulatory system. At the interface between blood and tissues, endothelial cells control blood fluidity and continued tissue

perfusion. The endothelium fine tunes the diameter of blood vessels and regulates vascular tone via its vasodilators including nitric oxide and prostaglandin I_2 as well as vasoconstrictors including endothelin and platelet-activating factor (Katusić & Shepherd, 1991; Shepherd & Katusić, 1991; Tolins et al., 1991).

The endothelial cell layer acts as a physical barrier between circulating blood and the subendothelial layers that constitutively express tissue factor (Mandal et al., 2006). Upon damage to the endothelium, platelets from the flowing blood rapidly adhere to the exposed subendothelial surface through an interaction between the glycoprotein (GP) Ib-IX-V complex on the platelets and VWF immobilized on the subendothelial surface. Platelets then firmly bind to collagen and get activated through the GP VI, $\alpha_2\beta_1$ and GP Ib-IX-V complexes (Chen & López, 2005). During inflammation, intact endothelial cells also release VWF and P-selectin from their Weibel-Palade bodies, which are ligands for GP Ib-IX-V and take part in platelet recruitment. On the other hand, exposed tissue factor from the subendothelium initiates secondary hemostasis, resulting in a fibrin mesh which stabilizes the newly formed platelet plug at the site of injury.

Upon adequate formation of fibrin clots, endothelial cells promote anticoagulant properties and counteract platelet activation and aggregation, in order to prevent thrombus formation on adjacent, uninjured endothelium. For instance, intact endothelial cells express thrombomodulin which binds to thrombin generated at the site of injury. This complex facilitates the activation of protein C bound to the endothelial protein C receptor (EPCR). Activated protein C (APC) downregulates blood coagulation by inactivating the proteolytic cleavage of factor Va and factor VIIIa via proteolytic cleavage (Walker, 1980). This results in a reduction in newly generated thrombin. The physiological importance of the anticoagulant protein C is demonstrated by the

severe thrombotic disease, purpura fulminans, which affects neonates with protein C deficiency (Price et al., 2011). Endothelial cells play an important role in protein C regulation and activation through the expression of thrombomodulin and EPCR.

While antithrombin is mainly synthesized in the liver, endothelial cells are also capable of synthesizing antithrombin (Chan & Chan, 1981). Circulating antithrombin binds to the endothelial surface through various receptors, including heparan sulphate glycosaminoglycan (Catieau et al., 2018; Chan & Chan, 1981). Endothelial cells also express syndecan-4 on their cell surface, which interacts with the heparin-binding site of antithrombin and thus increases the affinity of antithrombin for thrombin as well as its enzymatic inhibition (Iba & Saitoh, 2014). Furthermore, endothelial cells provide a surface for tissue factor pathway inhibitor (TFPI) to anchor via the glycosylphosphatidylinositol group (Ameri et al., 1992; Maroney & Mast, 2008). TFPI is a potent anticoagulant protein that inhibits the activity of the TF-FVIIa complex and factor Xa (Girard et al., 1989). Endothelial cells can synthesize and release TFPI upon treatment with heparin (Hansen et al., 2000; Sandset et al., 1988). Patients with low TFPI levels of less than the 10^{th} percentile of the normal reference range are at increased risk for venous thrombosis (Dahm et al., 2003). This highlights the importance of TFPI in hemostasis.

Endothelial cells participate in fibrinolysis by secreting tissue plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1). tPA is involved in the breakdown of blood clots by converting plasminogen to the active enzyme plasmin, which then cleaves fibrin clots (Suzuki et al., 2009; Van den Eijnden-Schrauwen et al., 1995). PAI-1 binds to tPA and inhibits its fibrinolytic function. Endothelial PAI-1 has been shown to increase the fibrinolytic resistance of an *in vitro* fibrin clot (Handt et al., 1996). In patients with hypertension, endothelial dysfunction is found to be associated with abnormalities in fibrinolysis (Tomiyama et al., 1998).

Endothelial cells control platelet adhesion and activation by regulating the release and cleavage of VWF (Ruggeri, 2003). The endothelium also secretes ectonucleotidase CD39/NTPDase1, which metabolizes the platelet agonist ADP, and platelet inhibitors, such as nitric oxide and prostacyclin (Marcus et al., 1997; Mitchell et al., 2008). Overall, endothelial cells are essential in maintaining hemostasis and preventing thrombosis. The endothelium can provide new avenues for exploration and novel therapies for the prevention and management of thromboembolisms (Yau et al., 2015).

1.3.1 Endothelial products involved in hemostasis

1.3.1.1 Endothelial tissue factor

Tissue factor is a transmembrane glycoprotein that binds to coagulation factor VII/VIIa and initiates the extrinsic coagulation pathway, leading to thrombus formation (Martin et al., 1995). It consists of a single polypeptide chain of 263 amino acids with a molecular mass of approximately 46 kDa (Morrissey, 2001; Morrissey et al., 1987; Paborsky et al., 1989). Tissue factor is normally not expressed by endothelial cells to prevent improper coagulation cascade activation (Cimmino et al., 2015). In endothelial cells, tissue factor expression is regulated through a promoter which is a binding site for activator protein-1, nuclear factor-κB and SP-1 transcription factors (Moll et al., 1995). Endothelial cells only express tissue factor when they are stimulated. Tissue factor expression can be induced by bacterial lipolysaccharide, proinflammatory cytokines or membrane microparticles released from activated host cells (Ameri et al., 1992; Bevilacqua et al., 1984; Chan et al., 2001; Conway et al., 1989). When tissue factor is exposed to the bloodstream, it binds to native factor VIIa and forms the TF/FVIIa

complex (Rao & Rapaport, 1988). This newly formed complex activates both factor IX and factor X, which initiates thrombus formation (Rao & Rapaport, 1988).

1.3.1.2 Endothelial thrombomodulin

Thrombomodulin is a multidomain integral membrane protein consisting of 557 amino acids (Esmon, 1982). The protein has five domains: D1, an N-terminal C-type lectin domain; D2, a chain of six extracellular EGF-like repeats; D3, an extracellular serine/threonine-rich region; D4, a transmembrane spanning region; and D5, a short cytoplasmic tail. Studies have revealed the functions of these domains, which range from mediating the hemostatic properties of thrombomodulin (D2/D3) to regulation of inflammatory responses (D1) (Conway et al., 1994; Ito & Maruyama, 2011; Kokame et al., 1998). Thrombomodulin is a high affinity thrombin receptor and a cofactor of thrombin-catalyzed activation of protein C. The binding of thrombin to thrombomodulin blocks the interaction of thrombin with circulating procoagulant substrates (Light et al., 1999). In addition, the thrombin-thrombomodulin complex substantially elevates the rate of protein C activation (Adams & Huntington, 2006). Activated protein C possesses potent anticoagulant effects through the proteolytic inactivation of factor Va and factor VIIIa (Dahlbäck & Villoutreix, 2005). Furthermore, thrombin binding to thrombomodulin also enhances the activation of thrombin-activatable fibrinolysis inhibitor (TAFI) (Adams & Huntington, 2006). Activated TAFI is a suppressor of fibrinolysis by removing the C-terminal lysines from the fibrin clot, which is the binding site for plasminogen, plasmin and t-PA (Leung et al., 2008). This stabilizes the fibrin clot and ensures greater thrombus localization during an injury. Overall, thrombomodulin is a key player in maintaining a healthy vascular endothelium by preventing unwanted blood clotting and potentially lethal thrombus formation (Cines et al.,

1998; Pearson, 1999). Its anti-inflammatory role beyond hemostasis is discussed in this review by Conway (Conway, 2012). Endothelial injury and/or activation of the endothelium results in reduced thrombomodulin expression (Boehme et al., 1996; Grey et al., 1998). Elevated levels of soluble thrombomodulin has also been reported as a feature of vascular diseases (Kikuchi et al., 2021; Matsuyama et al., 2008).

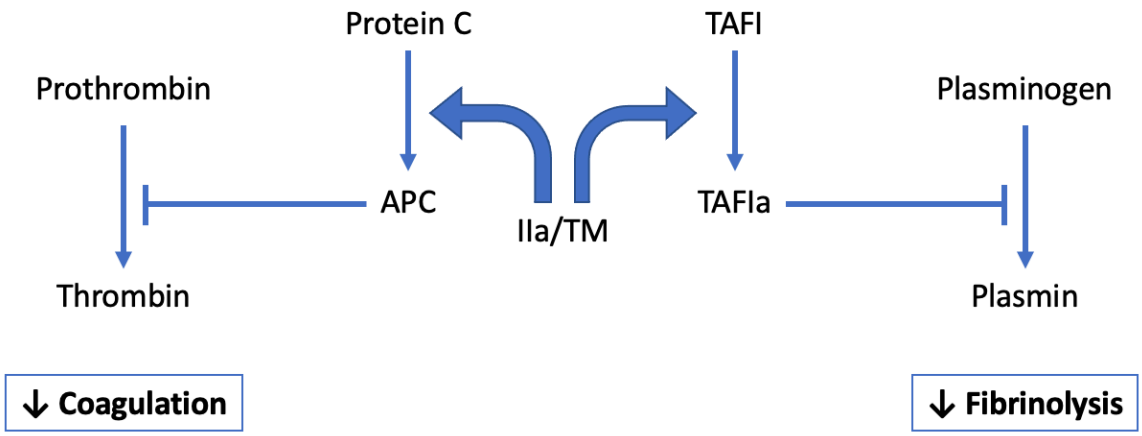

Figure 2. The role of thrombin-thrombomodulin complex role in hemostasis. The thrombin-thrombomodulin complex substantially elevates the activation rate of protein C, a natural anticoagulant. In addition, the complex is the physiologic activator of TAFI, which inhibits plasmin-mediated fibrinolysis.

1.3.1.3 Endothelial Plasminogen Activator Inhibitor-1

Plasminogen activator inhibitor 1 (PAI-1) is a serine protease expressed by endothelial cells, hepatocytes, adipocytes, megakaryocytes and neuronal cells (Dellas & Loskutoff, 2005). It is considered a critical regulator of the fibrinolytic system due to its role as the inhibitor of tissue- and urokinase-type plasminogen activators (Fay et al., 1997). PAI-1 has been identified as the

primary inhibitor of t-PA in plasma (Damare et al., 2014). By inhibiting t-PA, PAI-1 prevents the formation of plasmin from plasminogen and therefore inhibits the degradation of fibrin. Elevated PAI-1 levels are associated with thrombotic vascular diseases, such as myocardial infraction and deep venous thrombosis (Hamsten et al., 1987; Hamsten et al., 1985; Prisco et al., 1993).

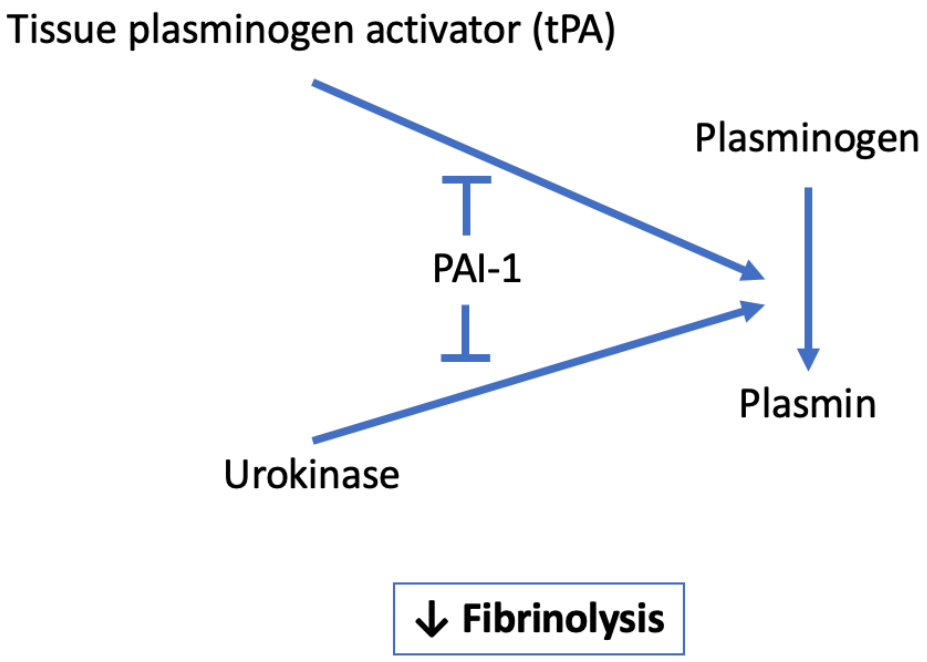

Figure 3. Plasminogen activator inhibitor-1 (PAI-1) role in hemostasis. PAI-1 functions as the primary blocker of plasminogen activators, such as tPA and urokinase. As a result, plasmin-mediated fibrinolysis is effectively inhibited.

1.4 Markers of endothelial activation

1.4.1 Von Willebrand factor

The plasma multimeric glycoprotein Von Willebrand factor plays a central role in primary hemostasis. It mediates platelet adhesion to damaged vascular subendothelium, platelet aggregation and thrombus growth at the site of injury. Additionally, it is the carrier of pro-coagulant FVIII, which is activated by thrombin and takes part in the tenase (FVIIIa/FIXa) complex (Eikenboom et al., 2002; Lip & Blann, 1997; Ruggeri, 2007; Sousa et al., 2007). The protein is synthesized by vascular endothelial cells and megakaryocytes. VWF is either constitutively secreted or stored in Weibel-Palade bodies in endothelial cells or in alpha granules in megakaryocytes and platelets. Under normal physiological conditions, megakaryocytes and platelets do not contribute an important amount of plasma VWF. This conclusion was made based on an observation that transplantation of normal bone marrow into a pig with severe VWF deficiency produced platelets containing normal amounts of the glycoprotein but did not normalize its VWF plasma level (Bowie et al., 1986). Thus, most of the plasma VWF is thought to be of endothelial origin. Elevated VWF is considered to be an indicator of endothelial activation since damage to endothelial cells leads to the release of VWF (Lip & Blann, 1997; Ruggeri, 2007).

1.4.2 E-selectin

E-selectin is a cell surface adhesion molecule that is expressed in endothelial cells. The protein has a cassette structure: an N-terminal, C-type lectin domain, an EGF-like domain, six Sushi domain units, a transmembrane domain and an intracellular cytoplasmic tail (Graves et al., 1994). Endothelial cells only express E-selectin in response to induction by cytokines including

interleukin-1 beta, TNF-alpha and lipopolysaccharides (Bevilacqua et al., 1987; Bevilacqua et al., 1989). Unlike P-selectin, which is stored in the Weibel-Palade bodies, E-selectin has to be transcribed, translated, and transported to the cell surface in response to inflammation. Therefore, it takes about two hours after cytokine recognition for E-selectin to be expressed on the endothelial surface (Leeuwenberg et al., 1992). In addition, the expression is only observed at sites of acute and chronic inflammation (Lasky, 1992). During inflammation, E-selectin plays an important part in the recruitment and extravasation of circulating leukocytes into sites of inflammation (Butcher, 1991). Local macrophages release cytokines IL-1β and TNF-alpha to induce the over-expression of E-selectin on endothelial cells (Ley et al., 1998). E-selectin then recognizes and binds to sialylated carbohydrate ligands on the surface of certain leukocytes. Under shear stress of blood flow, the leukocytes "roll" along the endothelium until they are activated by chemokines released by injured tissue, which enhances their firm adhesion to the endothelial surface (Ley et al., 1998). In summary, E-selectin is expressed by activated endothelial cells and mediates leukocyte adhesion to the site of inflammation.

1.4.3 Vascular cell adhesion molecule-1

Vascular cell adhesion molecule 1 (VCAM-1) is a 90-kDA glycoprotein that is predominantly expressed in endothelial cells (Osborn et al., 1989; Rice & Bevilacqua, 1989). The structure of human VCAM-1 contains an extracellular domain with six or seven immunoglobulin-like domains, a transmembrane domain and a cytoplasmic domain (Cook-Mills et al., 2011). VCAM-1 is expressed in endothelial cells in response to induction by pro-inflammatory cytokines, reactive oxygen species, oxidized low density lipoprotein, high glucose concentration, toll-like receptor agonists and shear stress (Cook-Mills et al., 2011). The VCAM-1 protein plays an

important role in leukocyte recruitment and facilitates the adhesion of lymphocytes, monocytes, eosinophils and basophils to vascular endothelium (Deem & Cook-Mills, 2004). In asthma, VCAM-1 signalling is required for eosinophil infiltration into the lung (Chin et al., 1997). In allergic encephalomyelitis, T-cell infiltration across the blood-brain barrier is dependent on VCAM-1 cell surface expression (Baron et al., 1993). Furthermore, cancer cells can utilize VCAM-1 to adhere to the endothelium (Schlesinger & Bendas, 2015). In summary, activated endothelial cells express VCAM-1 which facilitates leukocyte adhesion and infiltration into tissues.

1.5 Thrombin Generation Assay

Evaluation of a patient's hemostatic profile reflecting the risk of hemorrhage or thromboembolism is critical in many clinical situations. Thrombin is the pivotal enzyme in the coagulation cascade. Generation of thrombin can be measured with the use of a thrombin generation assay (TGA) (Lancé, 2015). TGA is a dynamic assay which is capable of continuously measuring both thrombin generation and inhibition. The principle of the assay involves the cleavage of a thrombin-specific substrate over time. In the presence of either relipidated tissue factor or a negatively charged surface such as kaolin, the TGA is initiated with calcium ions. Upon initiation, lag phase occurs when small amounts of thrombin are generated but are not detectable by the assay. A rapid increase in the thrombin generation curve indicates formation of measurable amounts of thrombin. A peak in thrombin generation is reached when there is an equilibrium between thrombin generation and thrombin inhibition. The generation of thrombin can be quantified on thrombograms (Tripodi, 2016). There are four main parameters that are quantified: lag time, time to peak thrombin, peak thrombin generated and the area under

the curve (Figure 4). Area under the curve, also known as endogenous thrombin potential, indicates the total amount of thrombin generated throughout the course of the assay.

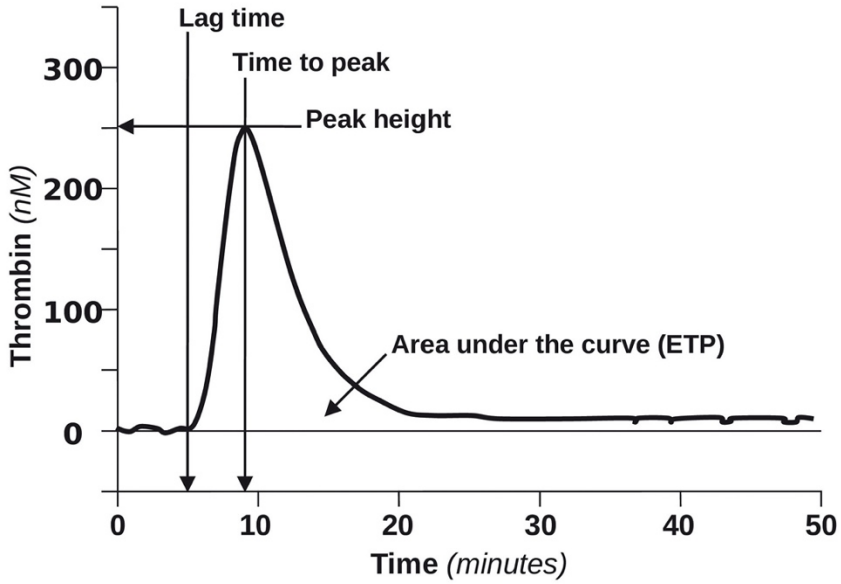

Figure 4. Thrombin generation parameters. Typical thrombin generation curve with related parameters obtained after *in vitro* activation of coagulation in plasma. Figure from Tripodi 2020, *https://doi.org/10.3324/haematol.2020.253047*.

1.6 Hemostasis and Cancer

Cancer affects the hemostatic system in multiple ways. Cancer cells have been shown to release procoagulant factors and microparticles that can directly activate the coagulation cascade (Falanga et al., 2009). Inflammatory cytokines released from cancer cells or direct adhesive contact may also activate the endothelium and platelets, which further enhances clotting activation. Tissue factor is a well-characterized procoagulant expressed on the malignant cell surface which can directly initiate the extrinsic blood coagulation cascade (Kasthuri et al., 2009). Cancer cells can also shed membrane microvesicles which provides an anionic phospholipid

surface for the assembly of tenase and prothrombinase complexes. Elevated levels of blast-derived microvesicles have been reported in patients with leukemia (van Aalderen et al., 2011). Cancer procoagulant is another protein produced by cancer cells that directly activates factor X independently of factor VII (Auwerda et al., 2011; Molnar et al., 2007). In patients with leukemia, the presence of cancer procoagulants is associated with the number of blast cells (Falanga et al., 2009). Furthermore, cancer cells had been shown to interact with the host fibrinolytic system through their expression of plasminogen activators (uPA and t-PA) as well as their inhibitors (PAI-1 and PAI-2) (Danø et al., 2005; Falanga et al., 2009). As a result, a subclinical activation of blood coagulation is typically present in patients with cancer, as demonstrated by abnormalities of circulating thrombotic biomarkers (Falanga et al., 2015).

1.6.1 Cancer procoagulants

Tumor cells can produce a variety of procoagulant substances, including cancer procoagulant that is capable of directly activating the clotting mechanism (Connolly & Khorana, 2019). Cancer procoagulant is a cysteine proteinase with a molecular weight of 68 kDa and initiates coagulation by directly activating factor X in the coagulation cascade (Falanga & Gordon, 1985; Gordon & Cross, 1981; Gordon et al., 1985). Its enzymatic activity is calcium-dependent and factor VII-independent. The protein has been extracted in adenocarcinomas (Kazmierczak et al., 2005) and carcinoma tissue (Gordon et al., 1975), but not normally differentiated tissues (Gordon et al., 1979; Gordon & Lewis, 1978). Gordon et al. extensively characterized the protein and demonstrated that it is distinct from other coagulation enzymes such as tissue factor (Falanga & Gordon, 1985; Gordon & Cross, 1981). Besides coagulation, this protein is postulated to have a role in tumor growth and metastasis (Gordon & Mielicki, 1997). For example, cancer

procoagulant-mediated deposition of fibrin around the malignant colony protects it from intrinsic immune system response. Due to its proteolytic activity, cancer procoagulant can facilitate cell matrix degradation to assist cancer metastasis (Tafazoli, 2022). With regards to hematological malignancy, blasts from acute nonlymphoid leukemia and acute lymphoblastic leukemia has been shown to express cancer procoagulants (Alessio et al., 1990; Donati et al., 1990; Falanga et al., 1988). While tissue factor is a well-recognized procoagulant associated with leukemic cells (Hair et al., 1996; Kubota et al., 1991; Tanaka et al., 1989), the contribution of cancer procoagulant to hemostasis in leukemia still remains to be investigated.

1.7 Thrombosis in Acute Lymphoblastic Leukemia

Survival rate among patients with ALL has improved significantly from less than 50% in the 1970s to more than 80% in the modern day (Howlader et al., 2017). However, toxicities from therapy for ALL results in significant morbidity and mortality (Marina, 1997). Thrombosis is a well-recognized serious complication in patients with ALL. More than half of thromboses are anatomically reported in potentially life-threatening sites such as the central nervous system, the right atrium and the lungs. Couturier et al. reported worse survival in patients who developed CNS thrombosis compared to those without (Couturier et al., 2015). The incidence of thrombosis in childhood ALL varied from 1.1% to 36.7% depending on treatment protocols and study design (Athale & Chan, 2003). The presence of inherited thrombophilia, the use of central venous catheters and the intensity of treatment are risk factors for thrombosis. In addition, children classified as high risk on the Dana-Farber Cancer Institute (DFCI) protocol had at least four times higher risk of developing thrombosis compared to those with standard risk ALL (Athale et

al., 2004). Overall, the occurrence of thrombosis in childhood ALL emerges from both the treatment-related and disease-related factors.

1.7.1 Pathogenesis of Thrombosis in Acute Lymphoblastic Leukemia

The endothelium is a thin layer of cells that forms the inner lining of the circulatory system (van Hinsbergh, 2012). At the interface between blood and tissues, endothelial cells control blood fluidity and continued tissue perfusion. Endothelial cells maintain hemostatic balance by promoting anti-coagulant properties and counteracting platelet activation and aggregation. For instance, endothelial cells express endothelial protein C receptor and thrombomodulin which plays an important role as a natural anticoagulant. The direct injury of endothelial cells by tumor-derived products or by chemotherapeutic agents leads to the loss of endothelial antithrombotic properties, contributing to increased thrombosis risk. In addition, cancer cells release pro-inflammatory (i.e., TNF-α and IL-1β) and pro-angiogenic (i.e., VEGF and bFGF) factors which can stimulate the prothrombotic features of the endothelium (Falanga et al., 2009). Furthermore, the adhesion of cancer cells to the vasculature promotes localized blood clotting activation and thrombus formation.

1.8 Clinical Observation of Impact of Peripheral Blasts on VWF levels and Thrombin Activation in Children with ALL

A cohort study by Athale et al. in children with ALL observed a significant association between VWF levels and the presence of peripheral lymphoblasts (Athale et al., 2010). Specifically, children with peripheral blasts had significantly higher levels of plasma VWF compared to those without peripheral blasts. This could be explained by increased inflammatory response in

patients with peripheral blasts. However, the study observed comparable levels of factor VIII in both groups, and factor VIII is also an inflammatory marker and a protein partner of VWF. From the disproportional level of VWF to factor VIII in patients with peripheral blasts, the authors of the study speculated that circulating blasts may stimulate VWF release through endothelial activation. The observation that VWF levels were significantly decreased following steroid therapy which reduces blast count, further supported the correlation between peripheral blasts and elevated levels of VWF.

In addition, the study demonstrated evidence of thrombin activation at diagnosis in children with ALL, which was in accordance with previous studies (Giordano et al., 2000; Semeraro et al., 1990; Uszyński et al., 2000). The study correlated increased thrombin activation with the presence of peripheral blasts. In other words, children with peripheral blasts had significantly higher levels of TAT and D-Dimer compared to those without peripheral blasts. Following steroid therapy that reduced peripheral blast count, parameters of endogenous thrombin generation also decreased; suggesting the procoagulant effect of leukemic lymphoblasts on hemostasis. In summary, Athale et al. observed an association of peripheral blasts with increased VWF level and parameters of thrombin generation in children with ALL (Athale et al., 2010). The hypothesis that endothelial cell activation by peripheral blasts represents one of the mechanisms of coagulation activation in acute lymphoblastic leukemia requires additional *in vitro* study to confirm the phenomenon.

1.9 Endothelial dysfunction in children with ALL

The endothelium plays a pivotal role in the regulation of hemostatic balance. Activation of endothelial cells is a common feature of early complications in hemato-oncologic therapy

(Soultati et al., 2012). Treatment-related causes of endothelial injury include chemotherapeutics, steroids and certain aspects of the bone marrow transplant procedure (Andrulis et al., 2012; Szczepaniak et al., 2022; Vion et al., 2015). ALL is an aggressive disease characterized by the accumulation of immature malignant cells. Higher levels of adhesion molecules, soluble thrombomodulin and VWF has been reported in children with ALL at the time of diagnosis, which suggests endothelial damage before the treatment begins (Doroszko et al., 2018; Hatzipantelis et al., 2011; Hatzistilianou et al., 1997; Tacyildiz et al., 1999). By monitoring biomarkers of endothelial health such as VWF, thrombomodulin and adhesion molecules, Doroszko et al. and Hatzipantelis et al. demonstrated severe endothelial dysfunction throughout the treatment course of ALL (Doroszko et al., 2018; Hatzipantelis et al., 2011). In addition, the authors suggest the prognostic ability of these endothelial markers. For example, high baseline levels of thrombomodulin, soluble E-selectin and PAI-1 may indicate poorer prognosis in children with ALL. In addition, endothelial dysfunction in childhood ALL has been linked to the risk of thrombosis in a retrospective study where increased levels of thrombomodulin and VEGFR-1 were independently linked to the odds of developing thromboembolism (Andrés-Jensen et al., 2022).

1.10 Hypothesis

The first hypothesis is that leukemic lymphoblasts can induce endothelial activation, which then alters the endothelium to have a prothrombotic phenotype. The second hypothesis is that soluble factors secreted from leukemic lymphoblasts can directly activate the coagulation system.

1.11 Rationale

1.11.1 Aim 1: Explore the effects of leukemic lymphoblasts on endothelial cells

The endothelium plays a pivotal role in the regulation of hemostatic balance. Activation of endothelial cells is a common feature of early complications in hemato-oncologic therapy (Soultati et al., 2012). The accumulation of immature malignant cells in ALL may have an impact on the endothelium. Higher levels of adhesion molecules, soluble thrombomodulin and VWF have been reported in children with ALL at the time of diagnosis, which suggests endothelial damage before the treatment begins (Doroszko et al., 2018; Hatzipantelis et al., 2011; Hatzistilianou et al., 1997; Tacyildiz et al., 1999). Furthermore, a cohort study by Athale et al. demonstrated significantly higher levels of VWF in a subgroup of ALL children with peripheral blasts comparing to those without peripheral blasts (Athale et al., 2010). This observation inspires us to test the hypothesis that circulating blasts may stimulate VWF release through endothelial activation.

1.11.2 Aim 2: Evaluate the *in vitro* prothrombotic effect of leukemic lymphoblasts

Increased thrombin generation potential and evidence of thrombin activation were demonstrated in children with ALL at diagnosis (Athale et al., 2010; Yau et al., 2015). Athale et al. correlated increased parameters of thrombin generation with the presence of peripheral blasts. Children with peripheral blasts had significantly higher levels of TAT and D-Dimer compared to those without peripheral blasts. Therefore, this clinical observation prompts us to investigate the prothrombotic effect of leukemic blasts *in vitro*.

2. Experimental Procedure

2.1 Materials

2.1.1 Cell lines and media

Human umbilical vein endothelial cells (HUVECs) were obtained from Lonza (C2519A, pooled donor) and maintained in EBM-2 Basal Medium (CC-3156), supplemented with EGM-2 SingleQuots (CC-4147), which contains 2% fetal bovine serum (FBS), hydrocortisone, hFGF-B, VEGF, R3-IGF-1, Ascrobic Acid, hEGF and GA-1000. Cells were used from passage 2 to 6. The growth conditions were 37 degrees Celsius and 5% CO_2 in a humidified incubator. Cells were frozen in complete media containing 10% DMSO (ATCC). Cell viability was assessed by the Trypan Blue stain (0.4%) method (Gibco, 15250-061).

Peripheral-blood derived B lymphoblasts were obtained from ATCC (CCL-120), and were isolated from a buffy coat preparation from a 11.5-year-old male patient with acute lymphoblastic leukemia. Cells were maintained in Iscove's modified Dulbecco's medium supplemented with 10% FBS. The growth conditions were 37 degrees Celsius, 5% CO_2 in a humidified incubator. Cells were used from passage 2 to 8. Cells were frozen in complete media containing 10% DMSO. Viability was assessed by the Trypan Blue method.

Lyophilized platelets were obtained from BioData and resuspended in the provided TBS buffer.

2.1.2 Tissue culture hardware

The following tissue-culture treated hardware was used: 10cm dish (Fisherbrand, FB012924), 24-well plate (Fisherbrand, FB012929), 96-well plate (Thermo, 167008), 0.4 micron cell culture insert (VWR, 734-2742), and T75 flasks (Sarstedt, 83.3911.502).

2.1.3 ELISA reagents

Biomarkers of endothelial activation were measured by an enzyme-linked immunosorbent assay (ELISA) method. The following paired antibody sets were used: 1. Von Willebrand Factor (VWF) Paired Antibody Set (Affinity Biologicals), 2. Human Tissue Factor Pair Kit (Abcam), 3. PAI-1 matched antibody pair set (Sino Biological) and 4. VCAM-1 matched antibody pair set (Sino Biological). Visualize Buffer Pak (Affinity Biologicals) was used for the preparation and performance of ELISA assays. The components of the kit were: 1. Coating buffer (50mM Carbonate, pH 9.6, plus preservative); 2. Blocking buffer (1% BSA in PBS, pH 7.4, plus preservative); 3. PBS-Tween (2% (v/v) Tween-20 in PBS, pH 7.4, plus preservative); 4. HBS-BSA-T20 (0.5% (v/v Tween-20, 5% BSA in HBS, pH 7.2, plus preservative); 5. Substrate Buffer (Citrate-phosphate buffer, pH 5.0 plus preservative); and 6. OPD tablets (o-Pheylenediamine.2HCl). Thrombomodulin and E-selectin antibodies were obtained from R&D System to develop an in-house cell-based ELISA.

2.1.4 Thrombin generation assay reagents and thrombin substrate

Commercial platelet-poor plasma and factor-deficient plasma were obtained from Affinity Biologicals and Precision Biologic. Pacific Hemostasis™ Activated Partial Thromboplastin Time (APTT) Reagents and calcium chloride were obtained from ThermoFisher (100402TS and 100314TS). The thrombin substrate Z-Gly-Gly-Arg-AMC · HCl was obtained from Bachem (4002155) as substrate. Human alpha thrombin was obtained from Enzyme Research Laboratories (HT 1002a) to construct the thrombin generation calibration curve. Thrombin generation assays were conducted in Corning™ 96-Well Clear Bottom Black Microplates.

2.2 Methods

2.2.1 Culturing HUVECs

HUVECs were cultured on gelatin-coated surfaces. 2% gelatin solution was prepared from bovine gelatin powder (Sigma, G6650) and autoclaved. The 2% gelatin was added to the tissue culture hardware and incubated at 37 degrees Celsius in a humidified incubator for 15 minutes. Then, the gelatin solution was aspirated off the culture surface. The plates were then ready for culturing HUVECs. Vials of cells (approximately 1 million cells in 1ml) were thawed from frozen and grown in a 10cm dish, supplemented with media for a total volume of 10ml. At confluency, cells were trypsinized with 0.05% Trypsin (Gibco, 15400-054), incubated in the incubator for 5 minutes before the reaction was inactivated with 10 times the volume of media. Trypan blue dye was used to check cell viability and to ensure over 90% cell viability for experiments (see section 2.2.4). Cell count was determined with a hematocytometer and seeded at 10,000 cells/ cm^2 to continue to culture.

2.2.2 Culturing peripheral-blood derived lymphoblasts

Peripheral-blood derived B lymphoblasts were thawed from frozen (1 million cells per vial) and grown in a T75 flask, supplemented with media for a total volume of 10ml. Trypan blue dye was used to ensure over 90% cell viability. Cell count was determined with a hematocytometer; and cells were maintained at 500,000 viable cells per ml.

2.2.3 Isolating and culturing human healthy lymphocytes

Blood from healthy donors was collected into Vacutainer® Plus Citrate Tubes (BD Biosciences). Informed consent was obtained from donors. Approval for blood collection from healthy donors was obtained from the Hamilton Integrated Research Ethics Board (HiREB). Lymphoprep density gradient medium was used for the isolation of mononuclear cells. Detailed protocol can be found in document #10000000358 from STEMCELL Technologies. Briefly, blood was mixed with 2% FBS in PBS (Gibco, 10010023) and Lymphoprep. Then the mixture was centrifuged at 800xg for 20 minutes. The mononuclear cell layer at the plasma:Lymphoprep interface was collected without disturbing the erythrocyte/granulocyte pellet. The mononuclear cells were resuspended with Iscove's modified Dulbecco's medium supplemented with 10% FBS once and collected by centrifuging at 500xg for 5 minutes. The cells were resuspended in Iscove's modified Dulbecco's medium supplemented with 10% FBS and cultured in a 10cm tissue-culture treated dish. After an overnight incubation in a humidified incubator, the cell suspension, containing lymphocytes, was collected and washed once with Iscove's modified Dulbecco's medium supplemented with 10% FBS as described above. Cell count was determined with a hematocytometer. Cell viability was assessed by Trypan Blue stain to ensure that cells were over 90% viable for experiment.

2.2.4 Cell viability assay (Trypan blue and LDH cytotoxicity assay)

Cell viability was assessed by the Trypan blue stain method and LDH cytotoxicity assay. In the Trypan blue stain method, 10ul of cell suspension was mixed with 10ul of Trypan blue stain, 0.4%, for one minute. Then, 10ul from the mixture was pipetted onto the hematocytometer and cell count was conducted. Cells were considered not viable if they were stained blue. The total

number of stained cells were subtracted from the total number of stained and unstained cells; then divided by the total number of stained and unstained cells to determine the cell viability. In addition, the CyQUANT LDH Cytotoxicity Assay Kit (ThermoFisher, C20300) was utilized to determine cell viability in the coculture experiment. Reaction mixture was prepared according to the manufacturer's instructions. Then 50ul of conditioned medium from HUVECs grown with or without leukemic lymphoblasts was mixed with 50ul of the reaction mixture in a 96 well plate and incubated for 30 minutes at room temperature. After the stop solution was added, absorbance was measured at 490nm and 680nm. To determine the LDH activity, the 680nm absorbance was subtracted from the 490nm absorbance value.

2.2.5 Coculture method and sample collection

A confluent layer of HUVECs was initially established. The endothelial surface was washed one time with PBS. Then, leukemic lymphoblasts were added to the culture at a concentration of 1 million cells per ml in reduced serum medium (OptiMEM, Thermo 31985070). At various timepoints, the media was collected and centrifuged to remove cell debris. The samples were then referred to as the conditioned medium. In addition, cell lysates were harvested for the purpose of analyzing internal cellular content. RIPA lysis buffer with protease inhibitors (Roche, 11836153001) was used to collect cell lysates. The RIPA buffer composition was 150mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate , 0.1% SDS, 50mM Tris and 5mM EDTA). Sonication of cells were conducted to shear the DNA. The amplitude setting was 70%. Samples were sonicated for 2 seconds, rested on ice for 1 minutes, then sonicated again for 2 seconds for a total of 3 rounds. Samples were stored at -80 degrees Celsius until further analysis.

2.2.6 Antigenic ELISA method

Biomarkers of endothelial activation were measured by enzyme-linked immunosorbent assay (ELISA) methods. The following paired antibody sets were used: 1. Von Willebrand Factor (VWF) Paired Antibody Set (Affinity Biologicals), 2. Human Tissue Factor Pair Kit (Abcam), 3. PAI-1 matched antibody pair set (Sino Biological), and 4. VCAM-1 matched antibody pair set (Sino Biological). Reference plasma (Precision Biologics) was used to construct the standard curve for the VWF ELISA. Lyophilized protein with known amounts of antigen were resuspended in sample diluents as per manufacturer's instructions and used to construct the standard curves for the tissue factor, PAI-1 and VCAM-1 ELISA. Visualize Buffer Pak (Affinity Biologicals) was used for the preparation and performance of ELISA assays. Samples were diluted in commercial diluent from the Visualize Buffer Pak, HBS-BSA-T20 (0.5% (v/v Tween-20, 5% BSA in HBS, pH 7.2, plus preservative). Four parameter logistic regression statistical method was employed to determine the protein concentration in the cell supernatants and lysates.

2.2.7 Cell-based ELISA method

Endothelial cells grown in 96 well-plate were washed with PBS and fixed with 50ul of 2% paraformaldehyde in PBS for 15 minutes at room temperature. Then the wells were washed with PBS and blocked overnight with 5% milk in PBS at 4 degrees Celsius. The next day, the wells were washed twice with 1% milk in PBS, and then incubated with primary antibody diluted 1:1000 in 5% milk for 2 hours at room temperature. The primary antibodies were goat anti-human E-selectin (R&D System, BBA18) and sheep anti-human thrombomodulin (R&D System, AF3947). Then each well was washed thrice with 1% milk, followed by incubation in secondary antibody diluted 1:10000 in 5% milk for 1 hour at room temperature. The secondary antibodies

are rabbit anti-goat IgG, peroxidase conjugate (Sigma, A5420) and donkey anti-sheep IgG, peroxidase conjugate (Sigma, A3415). Then each well was washed thrice with 1% milk and 100ul of TMB substrate (Abcam, ab171523) was added into each well. After 15 minutes of colour development, 50ul 2N sulfuric acid was added into each well to stop the reaction. Absorbance at 450nm was measured. Data was expressed as relative differences in absorbance values between experimental groups.

2.2.8 Endothelial permeability assay

The procedure was adapted from a validated protocol (Chen & Yeh, 2017). The assay evaluates the leakage of HRP-conjugated streptavidin through endothelial cell layer. HUVECs were seeded onto a 0.4 micron cell culture insert (VWR, 734-2742) at 20,000 cells/cm^2. The amount of media in the cell culture insert and the bottom chamber was 0.5ml and 0.5ml, respectively. Confluency was checked under the microscope and by evaluating the leakage of media into the bottom chamber. Once confluent, media in the cell insert was removed. Leukemic lymphoblasts were added to the culture insert at the concentration of 1 million cells per ml in reduced serum medium at 0.5ml. After coculturing for 3 hours, the media in the culture insert was replaced by HRP-conjugated streptavidin (ThermoFisher, N100), diluted 1:4,000,000 in OptiMEM. After 5 minutes, the culture insert was removed and 20ul of media in the lower chamber was added to a well of a 96-well with 50ul TMB substrate and allowed to develop for 20 minutes. The reaction was stopped with 50ul 2N sulfuric acid. Absorbance at 450nm was measured. Data was expressed as relative differences in absorbance values between experimental groups.

2.2.9 Proteome profiler array

Proteome Profiler Human Angiogenesis Array Kit was purchased from R&D Systems (ARY007) and the manufacturer's instructions were followed. Leukemic lymphoblasts were grown in T175 flasks for 24 hours in OptiMEM at the seeding density of $1x10^6$ cells/ml. The conditioned medium was collected, centrifuged at 500xg for 5 minutes to remove cellular debris and stored at -80 degrees Celsius until further analysis. The Proteome Profiler Human Angiogenesis Array Kit was performed on this sample. ImageJ was used to analyze the results based on pixel density of the blot.

2.2.10 Generation of leukemic cell conditioned medium

Leukemic lymphoblasts were grown in T175 flasks in OptiMEM at the seeding density of $1x10^6$ cells/ml. After 24 hours in the humidified incubator, the supernatants were collected and centrifuged at 500xg for 5 minutes to remove cellular debris. The conditioned medium from leukemic cell culture was stored at -80 degrees Celsius until further use.

2.2.11 Plasma clotting assay

Normal platelet-poor plasma (Affinity Biologicals), or factor VII, IX and X-deficient plasma (Affinity Biologicals) were thawed from frozen for 5 minutes at 37 degrees Celsius. The plasma were mixed with either OptiMEM or leukemic cell-derived supernatants at a 3:1 ratio (plasma: supernatants) in a 96-well plate for a total volume of 100ul per well. Then 100ul of 0.02M calcium chloride was added to each well. Absorbance was measured at 405nm and time to half max was recorded. In addition, plasma clotting assay was conducted on HUVECs grown in 96-

well plate. Media in each well was replaced by 100ul plasma. Clotting was then induced by calcium chloride and absorbance at 405nm was measured.

2.2.12 Thrombin generation assay

Normal platelet-poor plasma (Affinity Biologicals), or factor VII, IX and X-deficient plasma (Affinity Biologicals) were thawed from frozen for 5 minutes at 37 degrees Celsius. The plasma were mixed with either OptiMEM or leukemic cell-derived supernatants at a 3:1 ratio (plasma: supernatants) in a 96-well clear bottom black microplate for a total volume of 40ul per well. Then 10ul of ellagic acid activator, diluted 1:16 in 0.02M HEPES, pH 7.4, was added into each well and incubated for 15 minutes at 37 degrees Celsius. A reaction mixture was prepared by adding 1 part of the thrombin substrate Z-Gly-Gly-Arg-AMC · HCl to 9 part of 0.02M calcium chloride. After the incubation period, 50ul of the reaction mixture was added into each well and the microplate was read at 1 minute interval for 90 minutes at 360nm excitation and 460nm emission in a SpectraMax M4 Microplate Reader. Standard curves were generated with commercial human alpha thrombin. Various concentration of thrombin diluted in 0.02M HEPES to a total volume of 50ul were mixed with 50ul of the reaction mixture, and absorbance was measured as previously described. Thrombin generation curves were analyzed using the Technothrombin® TGA Software (Technoclone, Vienna, Austria).

To evaluate whether soluble factors released from leukemic lymphoblasts are capable of cleaving the thrombin substrate, 100ul of secretants from leukemic lymphoblasts were mixed with 50ul of the reaction mixture (1 part of the thrombin substrate Z-Gly-Gly-Arg-AMC · HCl and 9 part of 0.02M calcium chloride) in a well of a 96-well plate. Absorbance was then measured as previously described.

2.2.13 Blast activation of platelets

Lyophilized platelets were resuspended in TBS buffer at a concentration of 200,000 platelets per µl. Lymphoblasts were incubated with platelets for 30 minutes at 37 degrees Celsius with agitation at 600 rpm. Phorbol 12-myristate 13-acetate (PMA) was used as a positive control at 0.1 µM. The cell mixture was then centrifuged at 500xg for 5 minutes and the supernatant was collected and stored at -80 degrees Celsius until further use. This sample was then thawed and analyzed for VWF content by ELISA as per section 2.2.6.

2.2.14 Removal of microvesicles from leukemic cell conditioned media

Leukemic cell conditioned media were aliquoted into Eppendorf tubes at a total volume of 750 per tube. The tubes were then centrifuged at 12000xg for 10 minutes at 4 degrees Celsius. The upper two-thirds of the liquid volume (500ul) was carefully removed into another tube. This 500ul amount was then referred to as microvesicles-free secretants. The remaining 250ul amount was vortexed and then referred to as microvesicles. The samples were stored at -80 degrees Celsius until further use.

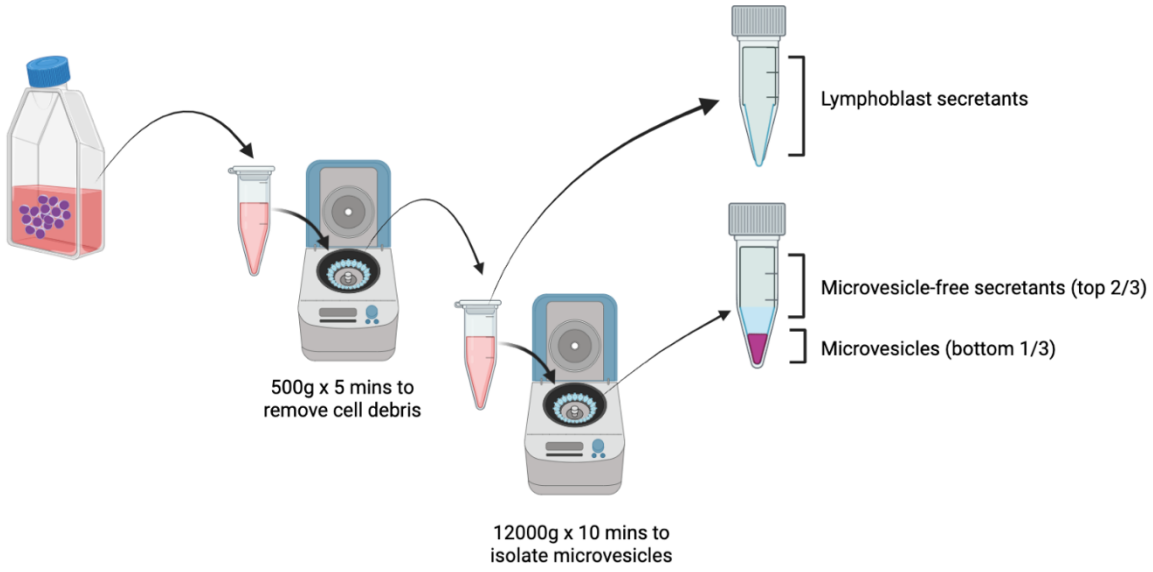

Figure 5. Preparation of microvesicle-free media from leukemic lymphoblast. Conditioned medium were first centrifuged at 500xg for 5 minutes to remove cell debris. The supernatant was then referred to as lymphoblast secretants. Then the lymphoblast secretants were centrifuged at 12000g for 10 minutes. The top two third of the volume was referred to as microvesicle-free secretants and the bottom one third of the volume was referred to as the microvesicles.

2.2.15 Blocking tissue factor from microvesicles by antibody

Leukemic cell conditioned media was incubated with polyclonal anti-tissue factor antibody (100ug/ml, Affinity Biologicals, SATF-IG) for 30 minutes at 37 degrees Celsius. This mixture was then mixed with platelet-poor plasma at a 3:1 ratio (plasma: supernatants) and subjected to thrombin generation assay as previously described in section 2.2.12.

2.2.16 Statistics

All graphs were produced in GraphPad Prism 4 and Microsoft Excel 2019. Comparisons of the data were done using GraphPad Prism 4 in which One Way ANOVA and t-tests were conducted. Tukey's HSD was used as the post hoc test. Values of $p < 0.05$ were deemed significant.

3. Result

3.1 Aim 1: Explore the effects of leukemic lymphoblasts on endothelial cells

The first hypothesis is that leukemic lymphoblasts can induce endothelial activation, which then alters the endothelium to have a prothrombotic phenotype.

3.1.1 Lymphoblasts are capable of activating resting endothelial cells

To evaluate the effect of lymphoblasts on the endothelium, lymphoblasts were cocultured with HUVECs for 3 hours in reduced serum media. Evidence of endothelial activation was analyzed by measuring the levels of the activation biomarkers including VWF, E-selectin and soluble VCAM-1. Compared to the endothelium cultured alone, coculture of lymphoblasts significantly elevated biomarkers of endothelial activation.

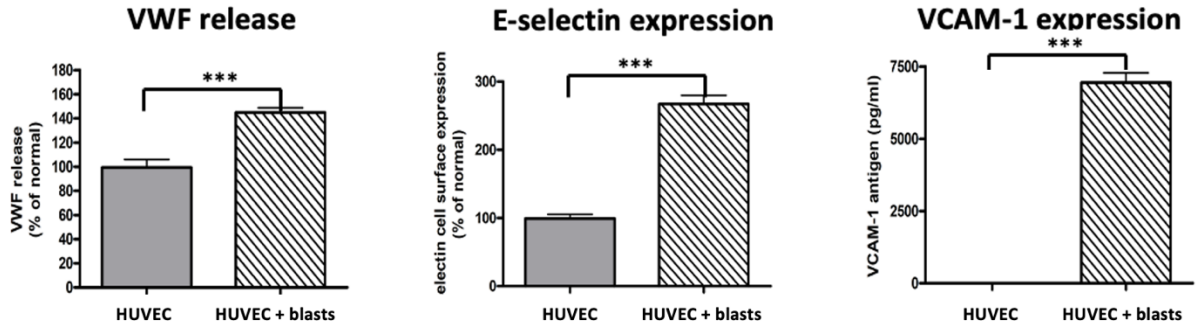

Figure 6. Expression levels of biomarkers of endothelial activation. Endothelial VWF released into the medium, E-selectin cell surface expression and soluble VCAM-1 level in cell lysates were measured by ELISAs. Elevated levels of biomarkers of endothelial activation were observed in the coculture group. Results are shown relative to levels in HUVEC cultured alone. Data was expressed as mean and error bar represented standard deviation. VWF: $p<0.001$; Mean

difference (95% CIs): 45.5% (28.29 to 62.70); n=6; E-selectin: $p<0.001$; Mean difference (95% CIs): 167% (136 to 199); n=6. ***: $p<0.001$; **: $p<0.01$; *: $p<0.05$.

3.1.2 Lymphoblasts induce endothelial cytotoxicity and increase endothelial permeability

To evaluate the effect of coculturing HUVECs with lymphoblasts on endothelial health, cytotoxicity and endothelial permeability assays were conducted. A higher signal for LDH, a cytosolic enzyme, was recorded in the coculture group compared to the endothelium cultured alone. In addition, coculture with lymphoblasts caused the endothelial cell to become more permeable to HRP-conjugated streptavidin. HRP has a molecular mass of ~44 kDa and steptavidin has a molecular mass of ~60 kDa (Haeuptle et al., 1983; Welinder, 1979).

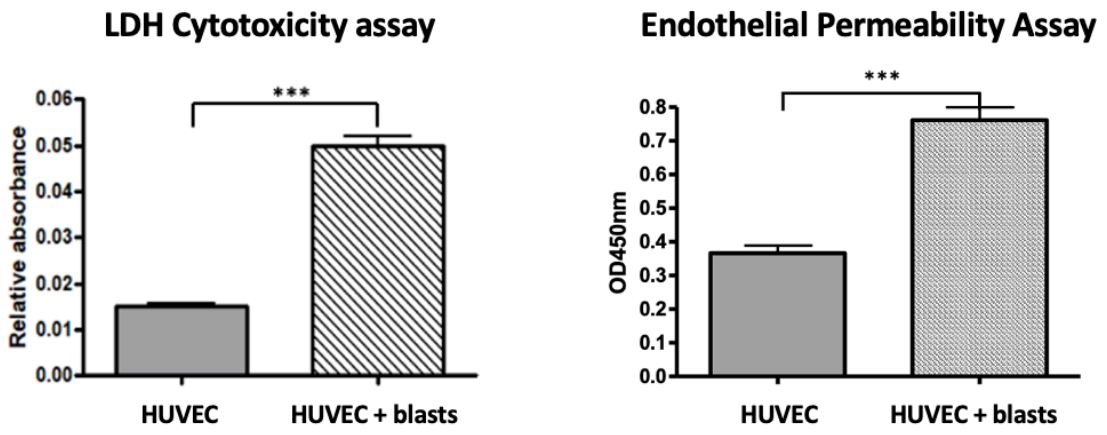

Figure 7. Endothelial health and permeability. HUVECs were cocultured in the presence or absence of leukemic lymphoblasts for 3 hours in reduced serum media. In the cytotoxicity assay, increased levels of LDH, a marker of cellular damage, were observed in HUVECs cultured with leukemic lymphoblasts. In the permeability assay, HUVECs cultured in the presence of leukemic lymphoblasts allows more HRP-conjugated streptavidin through their membranes, which

indicates increased permeability. Results are shown relative to levels in HUVEC cultured alone. Data was expressed as mean and error bar represented standard deviation. LDH assay: $p<0.001$; Mean difference (95% CIs): 0.03 (0.029 to 0.041); n=3. Endothelial permeability assay: $p<0.001$; Mean difference (95% CIs): 0.39 (0.28 to 0.5); n=4. ***: $p<0.001$; **: $p<0.01$; *: $p<0.05$.

3.1.3 Expression of endothelial products involved in hemostasis is altered by lymphoblasts

To evaluate the possible impacts on hemostasis when the endothelium is activated by lymphoblasts, the expression level of endothelial products involved in hemostasis was investigated. Tissue factors levels in endothelial cell lysate, PAI-1 levels in the conditioned medium and thrombomodulin expression on cell surface were measured by ELISAs. While the level of tissue factor and PAI-1 were elevated in the coculture group, cell surface expression of thrombomodulin was reduced.

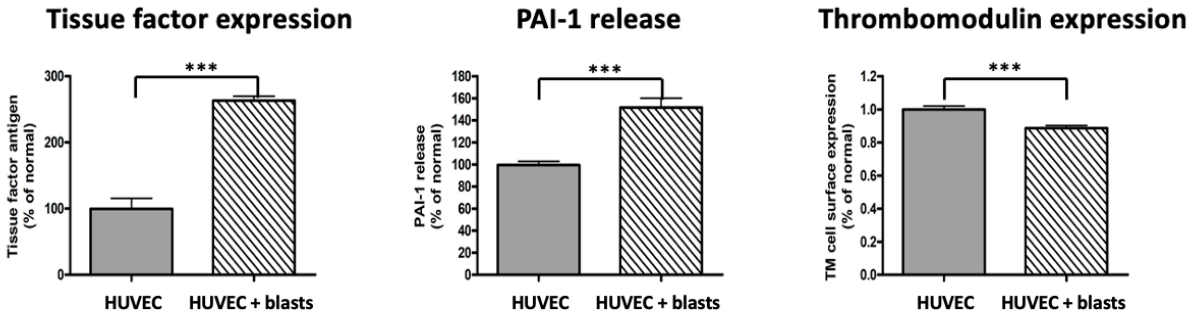

Figure 8. Altered expression of endothelial products involved in hemostasis. HUVECs were cocultured with/ without leukemic lymphoblasts for 3 hours in reduced serum media. The expression of endothelial products which are involved in hemostasis were altered so as to impart a prothrombotic phenotype to the endothelium in the coculture group. Results are shown relative

to levels in HUVEC cultured alone. Data was expressed as mean and error bar represented standard deviation Tissue factor: : $p<0.001$; Mean difference (95% Cis): 163.5% (124.5 to 202.5); n=6; PAI-1: $p<0.001$; Mean difference (95% Cis): 52% (31.39 to 72.6); n=6; Thrombomodulin: $p<0.001$; Mean difference (95% Cis): 11.4% (6.4 to 16.5); n=12. ***: $p<0.001$; **: $p<0.01$; *: $p<0.05$.

3.1.4 Healthy lymphocytes do not activate endothelium

To evaluate the effect of healthy lymphocytes on endothelial cells, as a comparison to leukemic lymphoblasts, HUVECs were cocultured with healthy lymphocytes. The expression of some of the same biomarkers for endothelial activation was measured. While the level of PAI-1 was slightly elevated in the coculture group compared to endothelium cultured alone, there were no significant differences between the VWF and tissue factor levels measured under the two culture conditions.

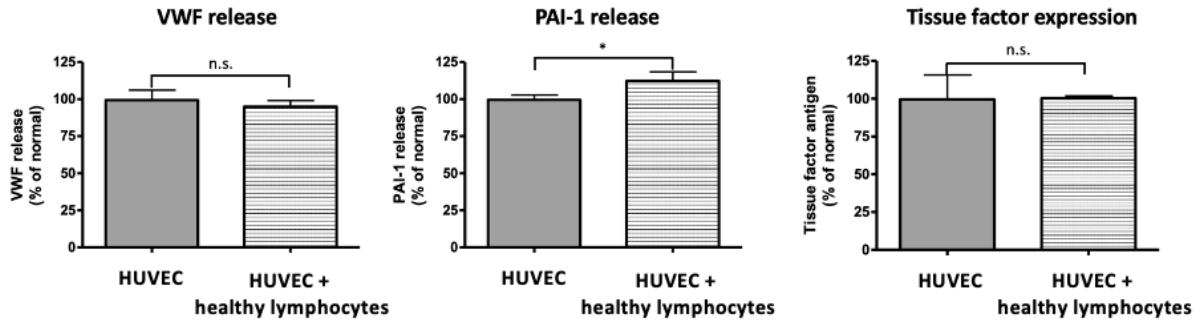

Figure 9. Endothelial cells cocultured with healthy lymphocytes. HUVECS were cocultured with healthy lymphocytes. Supernates were collected and markers of endothelial activation were measured by ELISAs. Results are shown relative to levels in HUVEC cultured alone. Data was expressed as mean and error bar represented standard deviation. VWF: $p=0.43$; Mean difference (95% CIs): -4.37% (-16.3 to 7.6); n=6; PAI-1: $p=0.02$; Mean difference (95% CIs): 12.79% (2.38

to 23.2); n=6; Tissue factor: p=0.97; Mean difference (95% CIs): -0.5% (-33.5 to 32.4); n=6. ***: $p<0.001$; **: $p<0.01$; *: $p<0.05$, n.s.: not significant.

3.1.5 VWF was not detected from activated platelets

To evaluate the effect of leukemic lymphoblasts on platelet activation, commercial platelets were incubated with lymphoblasts or PMA, and conditioned media was collected and analyzed for VWF levels. All signals fell below the standard curve; therefore, no VWF was detected in either experimental group with lymphoblasts or the positive control group with PMA. The VWF ELISA detection limit was 0.01728 U/ml according to the manufacturer of the matched antibody pair.

3.1.6 Lymphoblasts secrete factors that are known to affect endothelial cell health

A global proteomic analysis on endothelial-cell related proteins was conducted on leukemic lymphoblast conditioned media to identify secreted factors from leukemic lymphoblasts that may affect endothelial health. Several factors known to affect endothelial health, including soluble dipeptidyl peptidase 4 (sDPPIV), macrophage inflammatory protein-1 alpha (MIP-1α/CCL3), tissue inhibitor of metalloproteinases-1 (TIMP-1) and angiopoietin-2, were detected in secretants from leukemic lymphoblasts.

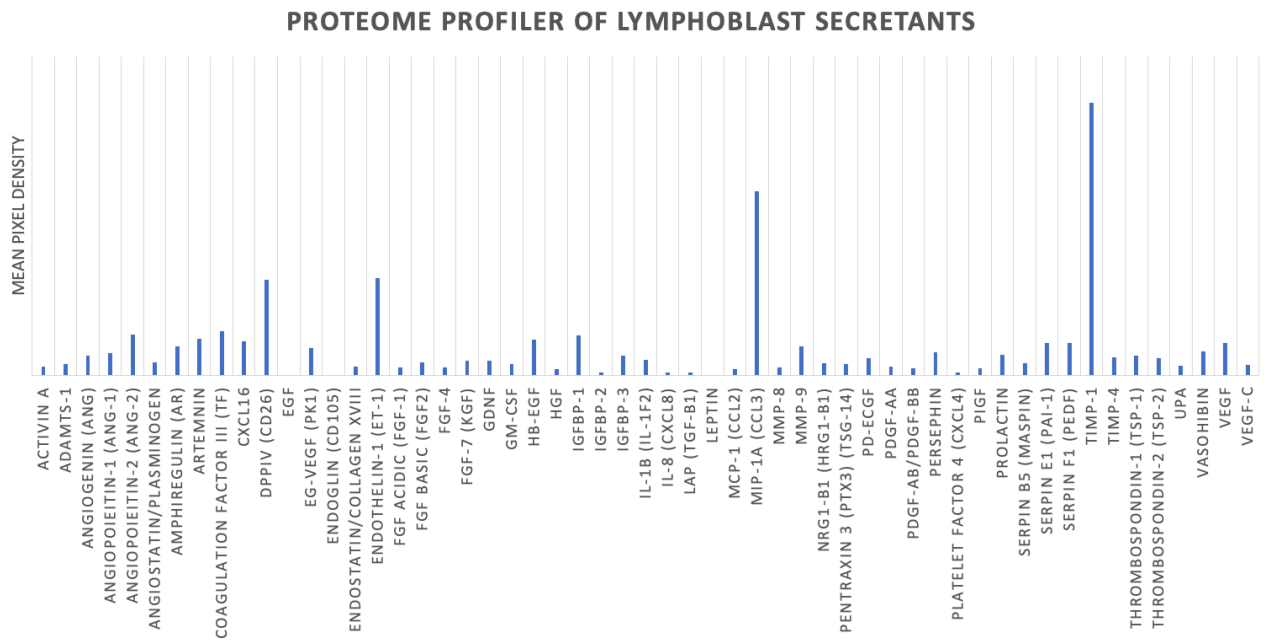

Figure 10. Proteome profiler of lymphoblast secretants. The R&D Human Angiogenesis Proteome Profiler Array Kit was used on the lymphoblast conditioned media. Mean pixel density on the blot was calculated by ImageJ.

3.1.7 Leukemic lymphoblast-induced endothelial activation promoted faster clotting time and increased thrombin generation

This experiment was designed to evaluate the prothrombotic phenotype of the endothelium when endothelial cells are activated with either leukemic lymphoblasts or 5μM histamine. Plasma was added to wells containing an endothelial surface and clotting was induced with calcium chloride. Clot formation was monitored with a spectrophotometer. Plasma took significantly less time to clot on endothelial cells which had been previously cocultured with leukemic lymphoblasts compared to endothelium cultured in the absence of leukemic lymphoblasts. In another set of experiments, thrombin generation was measured in recalcified plasma exposed to endothelium. A

statistically significant increase in thrombin peak height and a non-statistically significant increase in velocity index were measured on endothelial cells which had been previously cocultured with leukemic lymphoblasts compared to endothelium cultured in the absence of leukemic lymphoblasts. There were no significant differences between groups with regards to velocity index, time to peak, lag time and area under the curve.

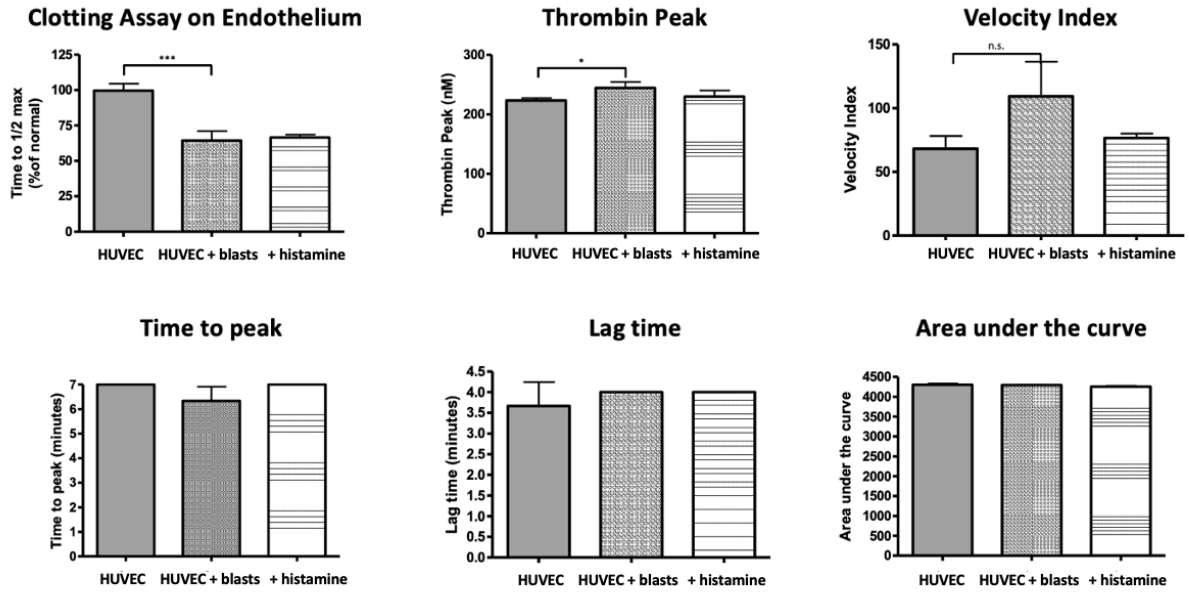

Figure 11. Clotting assay and thrombin generation on endothelium. Plasma took less time to clot on endothelium which was cultured with leukemic lymphoblasts or exposed to 5µM histamine, compared to untreated endothelium. A statistically significant increase in thrombin peak height and a non-statistically significant increase in velocity index were recorded on endothelial cells which had been previously cocultured with leukemic lymphoblasts compared to endothelium cultured in the absence of leukemic lymphoblasts. Data was expressed as mean and error bar represented standard deviation. Clotting assay: $p<0.001$; Mean difference (95% CIs): 35.34% (18.87 to 51.8); n=12. Thrombin peak height: $p=0.026$; Mean difference (95% CIs): -

21.06% (-38.06 to -4.07); n=3. Velocity index: p=0.07; Mean difference (95% CIs): -41.08% (-87.55 to 5.38); n=3. ***: p<0.001; **: p<0.01; *: p<0.05.

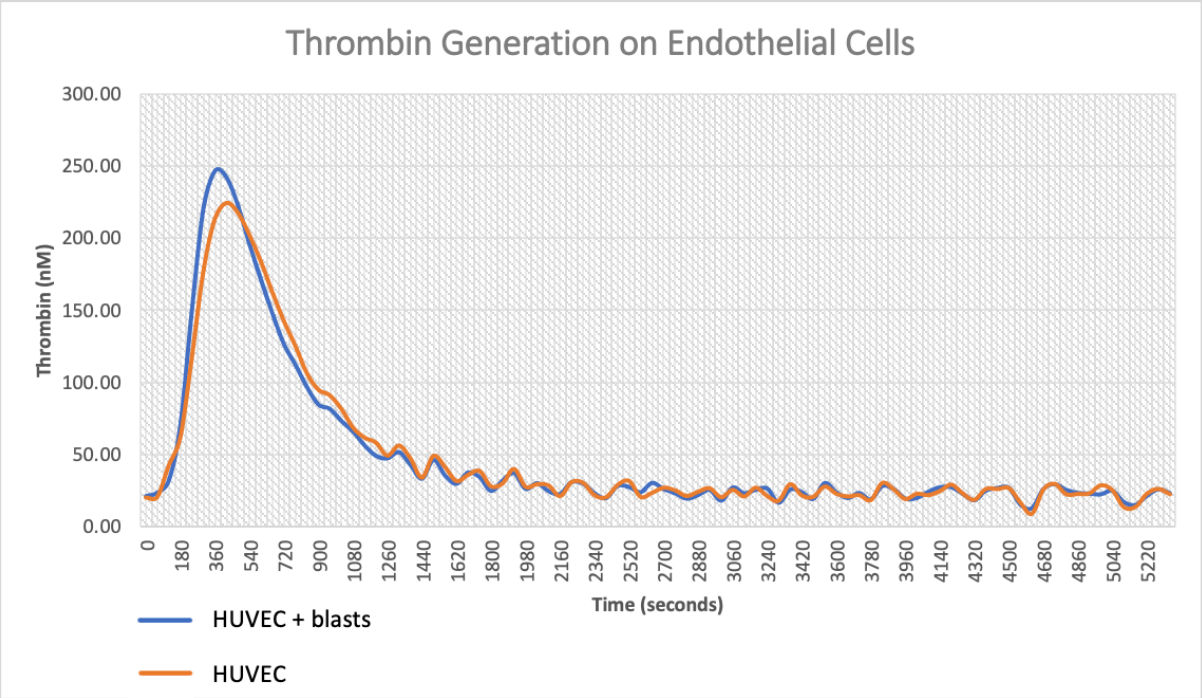

Figure 12. Thrombogram of plasma clotting on endothelial cells. Thrombin generation was measured in recalcified plasma exposed to endothelium. Evidence of increased thrombin generation was observed on endothelial cells which had been previously cocultured with leukemic lymphoblasts compared to endothelium cultured in the absence of leukemic lymphoblasts. Representative thrombograms of one experiment from each experimental group were chosen for display in the figure.

3.2 Aim 2: Evaluate the *in vitro* prothrombotic effect of leukemic lymphoblasts

The second hypothesis is that soluble factors secreted from leukemic lymphoblasts can directly activate the coagulation system.

3.2.1 Secretants from leukemic lymphoblasts induce faster clotting time and increased thrombin generation in plasma

To evaluate the effect of secreted factors from leukemic lymphoblasts on coagulation activation, secretants from leukemic lymphoblasts were mixed with plasma and clotting was induced with calcium chloride. Clotting time was decreased when plasma was mixed with conditioned media from leukemic lymphoblast culture, compared to plasma mixed with medium alone. In addition, elevated thrombin peak height and higher velocity index were recorded when plasma was mixed with secretants from leukemic lymphoblasts, compared to plasma mixed with medium alone. There were no significant differences between groups with regards to time to peak, lag time and area under the curve.

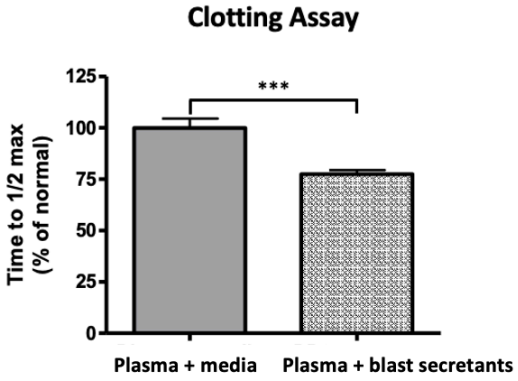

Figure 13. Clotting assay on plasma mixed with secretants from leukemic blasts. Shorter time to clot was observed when plasma was mixed with secretants from leukemic lymphoblasts, compared to plasma mixed with medium alone. Data was expressed as mean and error bar represented standard deviation. $p<0.001$; Mean difference (95% CIs): 22.56% (12.12 to 33.02); n=9. ***: $p<0.001$; **: $p<0.01$; *: $p<0.05$.

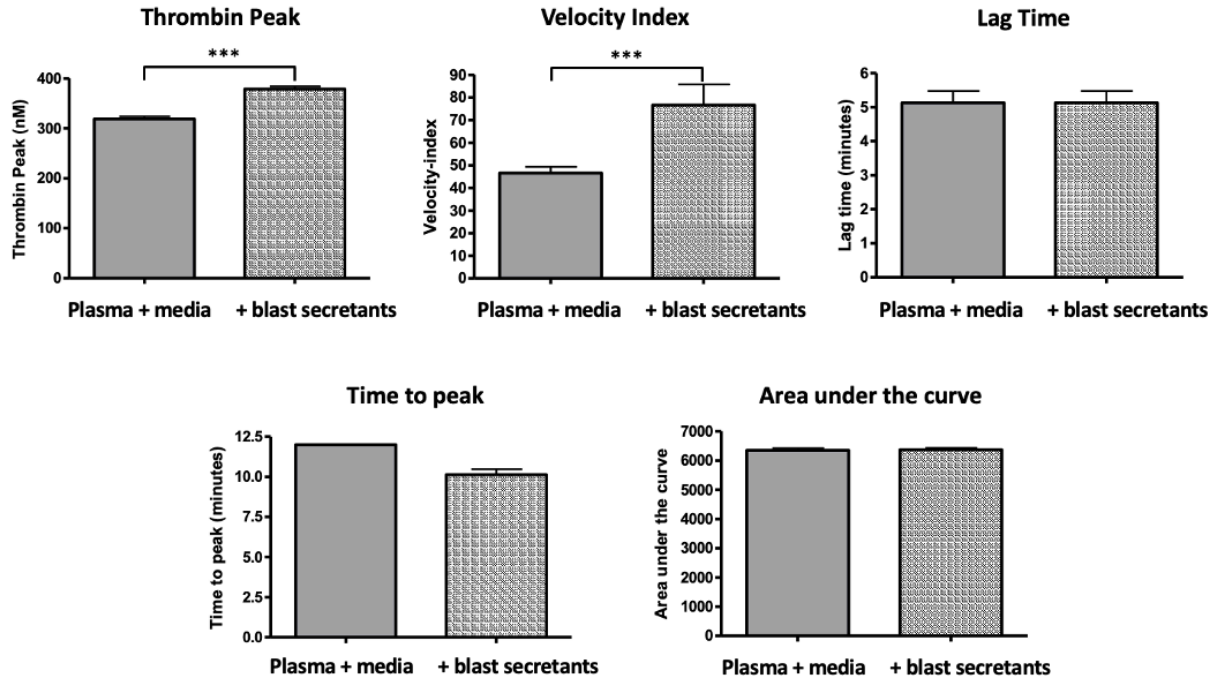

Figure 14. Thrombin generation assay on plasma mixed with secretants from leukemic lymphoblasts. Increased thrombin peak height and velocity index were recorded when plasma was mixed with secretants from leukemic lymphoblasts, compared to plasma mixed with medium alone. Data was expressed as mean and error bar represented standard deviation. Thrombin peak height: $p<0.001$; Mean difference (95% CIs): -59.8 nM (-65.07 to -54.52); n=12. Velocity index: $p<0.001$; Mean difference (95% CIs): -30.1 (-36.91 to -23.29); n=12. ***: $p<0.001$; **: $p<0.01$; *: $p<0.05$.

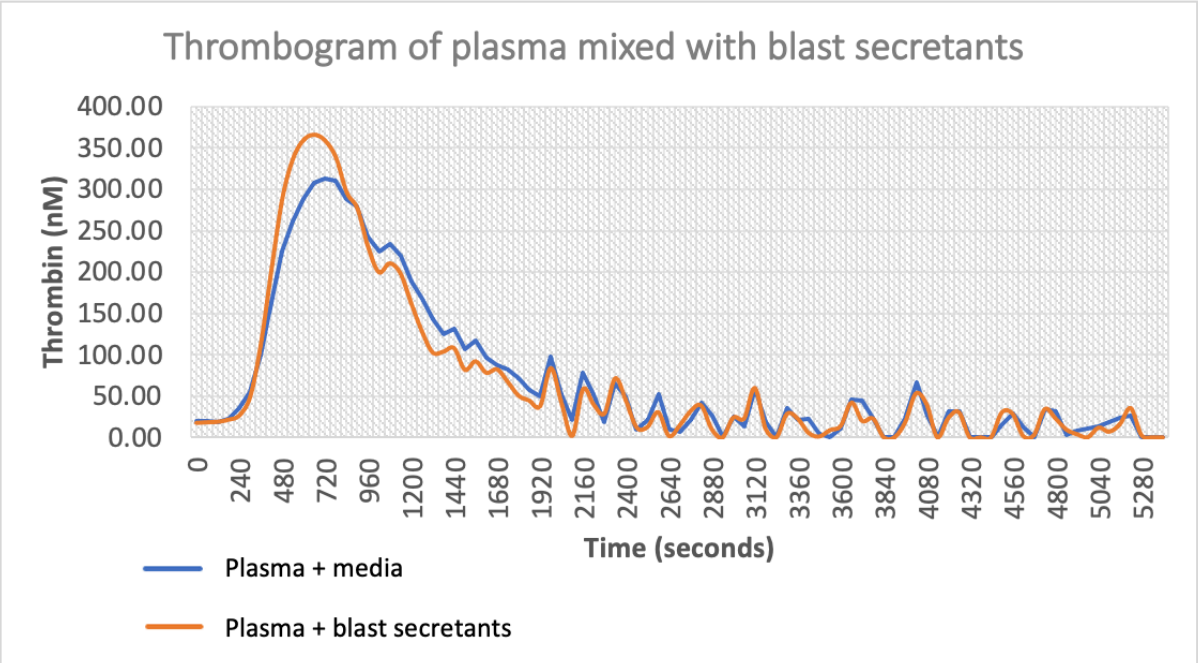

Figure 15. Thrombogram of plasma mixed with secretants from leukemic lymphoblasts. Evidence of increased thrombin generation was observed in recalcified plasma mixed with secretants from leukemic lymphoblasts, compared to plasma mixed with medium alone. Representative thrombograms of one experiment from each experimental group were chosen for display in the figure.

3.2.2 Secretants from leukemic lymphoblasts may induce increased thrombin generation in plasma in a FVII-independent manner

To investigate the involvement of the extrinsic pathway in leukemic lymphoblast-mediated coagulation activation, secretants from leukemic lymphoblasts or media were mixed with FVII-deficient plasma and thrombin generation assays were conducted. Elevated thrombin peak height and higher velocity index were recorded when FVII-deficient plasma was mixed with secretants from leukemic lymphoblasts, compared to plasma mixed with medium alone. There were no

significant differences between groups with regards to time to peak, lag time and area under the curve.

With reference to the results obtained on normal plasma mixed with secretants from leukemic lymphoblasts (Figure 14), the degree of elevation in thrombin peak height was similar between normal plasma (-59.8 nM, $p<0.001$) and FVII-deficient plasma (-67.7 nM, $p<0.001$). In addition, the difference in velocity index was similar between normal plasma (-30.1, $p<0.001$) compared to FX-deficient plasma (-27.3, $p<0.001$). Therefore, lymphoblast secretants may increase thrombin generation in plasma in a FVII-independent manner.

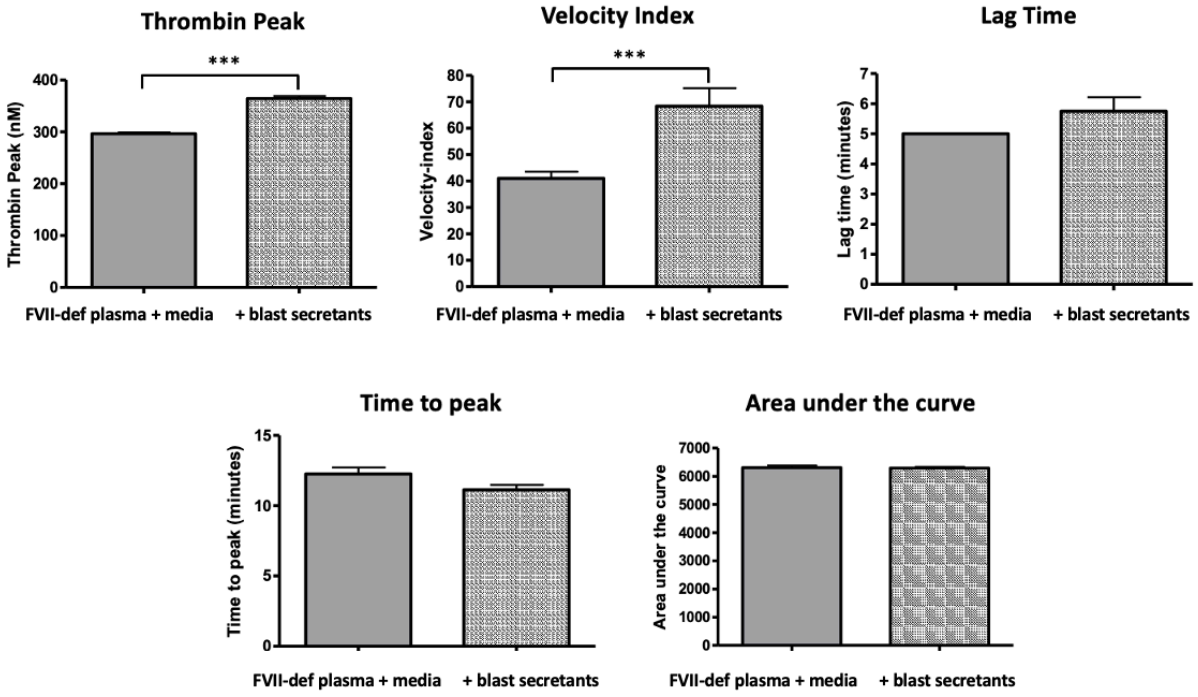

Figure 16. Thrombin generation assay on FVII-deficient plasma mixed with secretants from leukemic lymphoblasts. Increased thrombin peak height and velocity index were recorded when FVII-deficient plasma was mixed with secretants from leukemic lymphoblasts, compared to plasma mixed with medium alone. Data was expressed as mean and error bar represented

standard deviation. Thrombin peak height: p<0.001; Mean difference (95% CIs): -67.7 nM (-71.59 to -63.8); n=12. Velocity index: p<0.001; Mean difference (95% CIs): -27.3 (-32.46 to -22.13); n=12. ***: p<0.001; **: p<0.01; *: p<0.05.

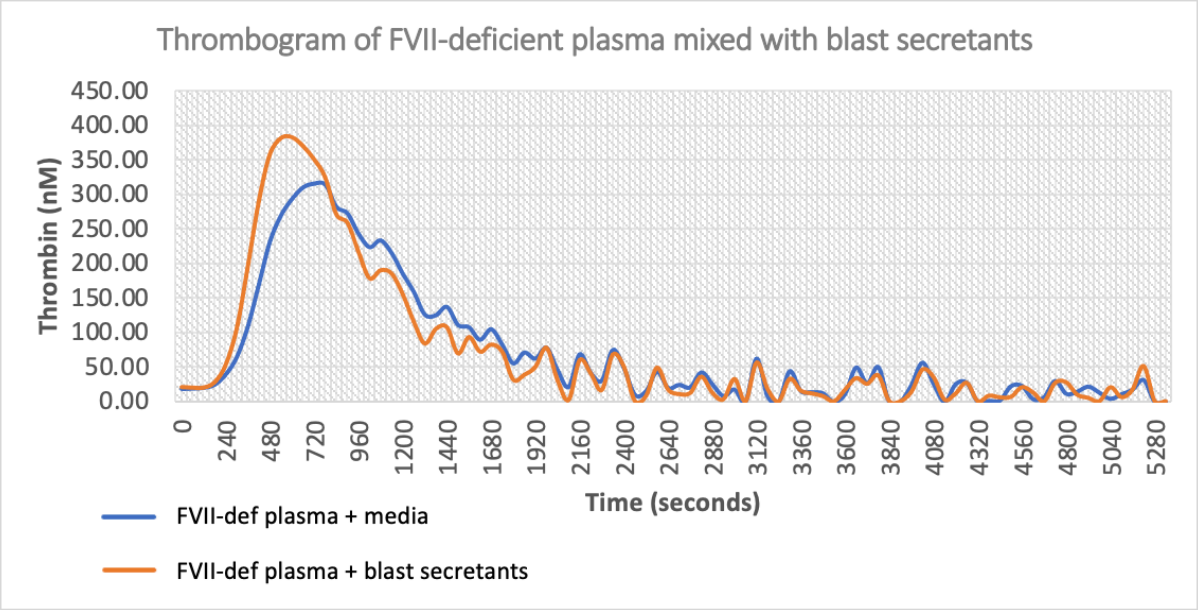

Figure 17. Thrombogram of FVII-deficient plasma mixed with secretants from leukemic lymphoblasts. Evidence of increased thrombin generation was observed in recalcified FVII-deficient plasma mixed with secretants from leukemic lymphoblasts, compared to plasma mixed with medium alone. Representative thrombograms of one experiment from each experimental group were chosen for display in the figure.

3.2.3 Increased thrombin generation due to lymphoblast secretants may be a FIX-dependent process

To investigate the involvement of the intrinsic pathway in leukemic lymphoblast-mediated coagulation activation and the possible presence of cancer procoagulants from leukemic cells, FIX-deficient plasma was mixed with secretants from leukemic lymphoblasts or media, and

thrombin generation assays were conducted. Elevated thrombin peak height, but no statisitcally significant difference in velocity index were recorded when FIX-deficient plasma was mixed with secretants from leukemic lymphoblasts, compared to plasma mixed with medium alone. There were no significant differences between groups with regards to velocity index, time to peak, lag time and area under the curve.

With reference to the results obtained on normal plasma mixed with secretants from leukemic lymphoblasts (Figure 14), the degree of elevation in thrombin peak height was higher in normal plasma (-59.8 nM, $p<0.001$) compared to FIX-deficient plasma (-6.9 nM, $p=0.002$). In addition, the difference in velocity index was higher in normal plasma (-30.1, $p<0.001$) compared to FIX-deficient plasma (-0.5, $p=0.1$). Therefore, lymphoblast secretants may increase thrombin generation in plasma in a FIX-dependent manner.

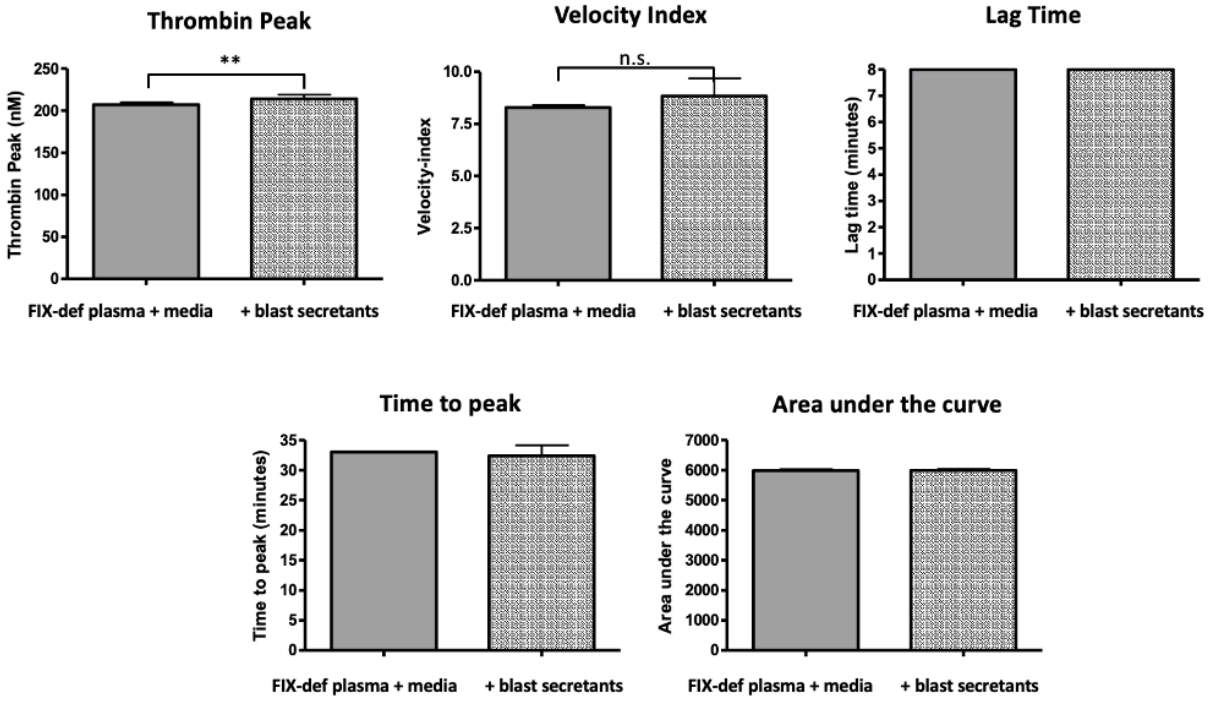

Figure 18. Thrombin generation assay on FIX-deficient plasma mixed with secretants from leukemic lymphoblasts. Increased thrombin peak height was recorded when FIX-deficient plasma was mixed with secretants from leukemic lymphoblasts, compared to plasma mixed with medium alone. Data was expressed as mean and error bar represented standard deviation. Thrombin peak height: p=0.002; Mean difference (95% CIs): -6.9 nM (-10.8 to -2.94); n=12. Velocity index: p=0.1; Mean difference (95% CIs): -0.5 (-1.11 to 0.11); n=12. ***: p<0.001; **: p<0.01; *: p<0.05, n.s.: not significant.

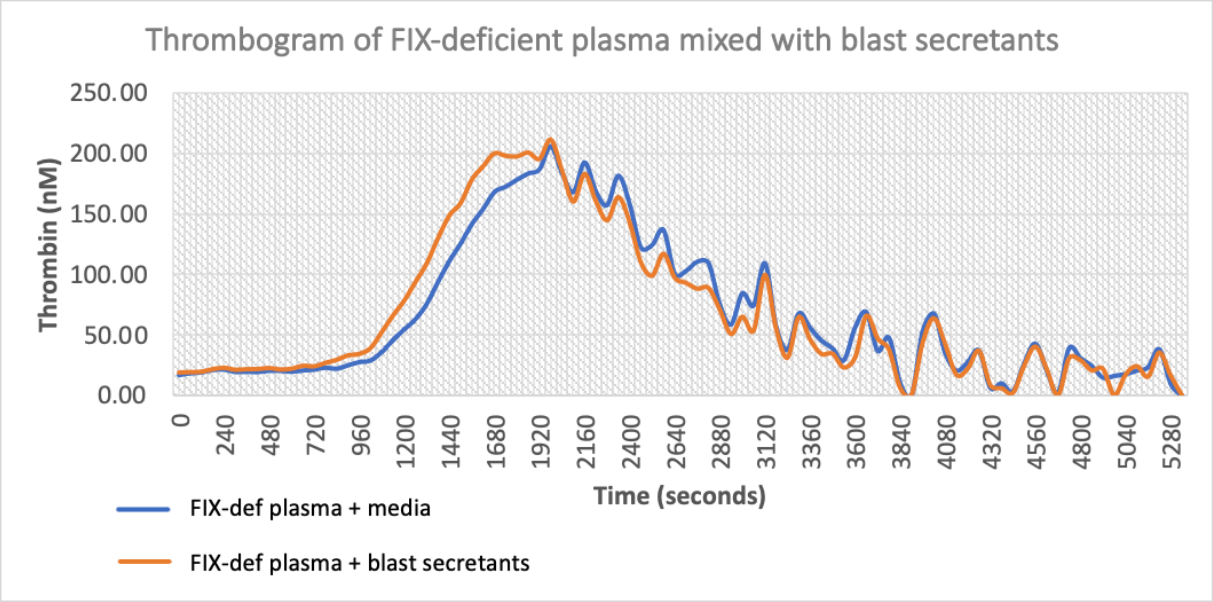

Figure 19. Thrombogram of FIX-deficient plasma mixed with secretants from leukemic lymphoblasts. Little evidence of increased thrombin generation was observed in recalcified FIX-deficient plasma mixed with secretants from leukemic lymphoblasts, compared to plasma mixed with medium alone. Representative thrombograms of one experiment from each experimental group were chosen for display in the figure.

3.2.4 Increased thrombin generation due to lymphoblast secretants may be a FX-dependent process

To investigate the possibility of the presence of a prothrombin protease from leukemic cells, secretants from leukemic lymphoblasts or media were mixed with FX-deficient plasma, and thrombin generation assays were conducted. No statistically significant differences in thrombin generation parameters were recorded when FX-deficient plasma was mixed with secretants from leukemic lymphoblasts, compared to plasma mixed with medium alone.

With reference to the results obtained on normal plasma mixed with secretants from leukemic lymphoblasts (Figure 14), the degree of elevation in thrombin peak height was higher in normal plasma (-59.8 nM, $p<0.001$) compared to FX-deficient plasma (-8.1 nM, $p=0.06$). In addition, the difference in velocity index was higher in normal plasma (-30.1, $p<0.001$) compared to FX-deficient plasma (-0.5, $p=0.1$). Therefore, lymphoblast secretants may increase thrombin generation in plasma in a FX-dependent manner.

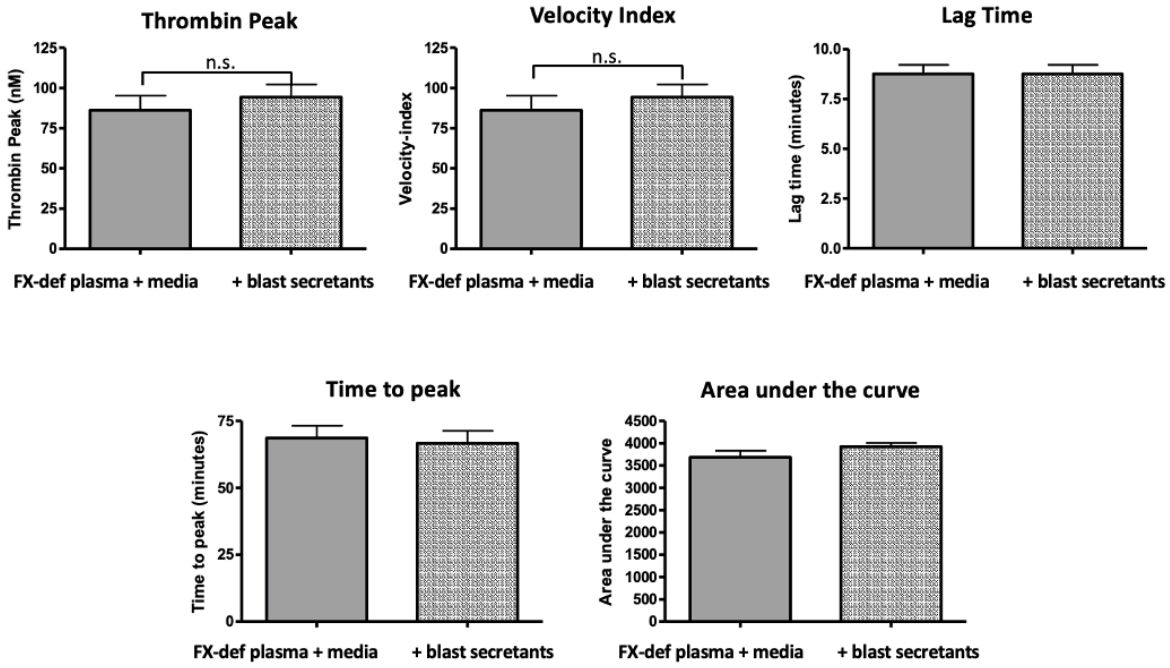

Figure 20. Thrombin generation assay on FX-deficient plasma mixed with secretants from leukemic lymphoblasts. No statistically significant differences in thrombin peak height and velocity index were recorded when FX-deficient plasma was mixed with secretants from leukemic lymphoblasts, compared to plasma mixed with medium alone. Data was expressed as mean and error bar represented standard deviation. Thrombin peak height: p=0.06; Mean difference (95% CIs): -8.1 nM (-16.6 to 0.4); n=12. Velocity index: p=0.37; Mean difference (95% CIs): -0.1 (-0.33 to 0.13); n=12. ***: p<0.001; **: p<0.01; *: p<0.05, n.s.: not significant.

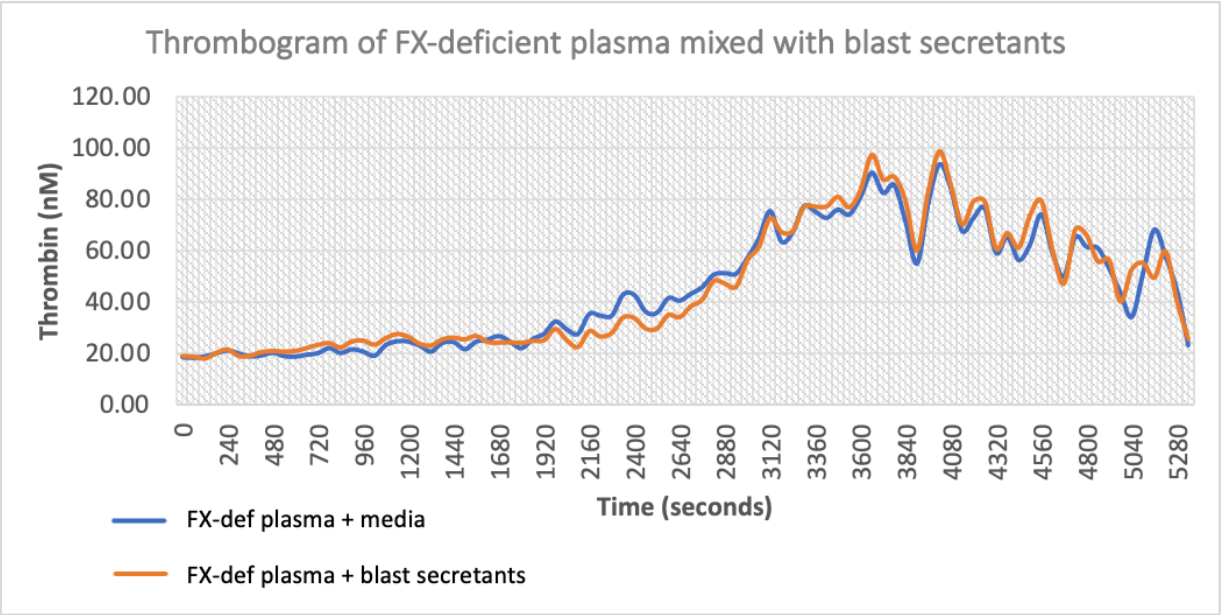

Figure 21. Thrombogram of FX-deficient plasma mixed with secretants from leukemic lymphoblasts. Little evidence of increased thrombin generation was observed in recalcified FX-deficient plasma mixed with secretants from leukemic lymphoblasts, compared to plasma mixed with medium alone. Representative thrombograms of one experiment from each experimental group were chosen for display in the figure.

3.2.5 Microvesicles released from leukemic lymphoblast may cause increased thrombin generation

To investigate the contribution of microvesicles released from leukemic cells towards coagulation activation, microvesicles were removed from lymphoblast secretants by centrifugation. Thrombin generation assays were conducted on plasma mixed with the remaining microvesicle-free secretants. A statistically significant reduction in thrombin peak height and velocity index were recorded when microvesicles were removed from the leukemic cell-derived secretants. There was an increase in thrombin and velocity index when microvesicles previously

separated from leukemic cell-derived secretants were added to the plasma. There were no significant differences between groups with regards to time to peak, lag time and area under the curve.

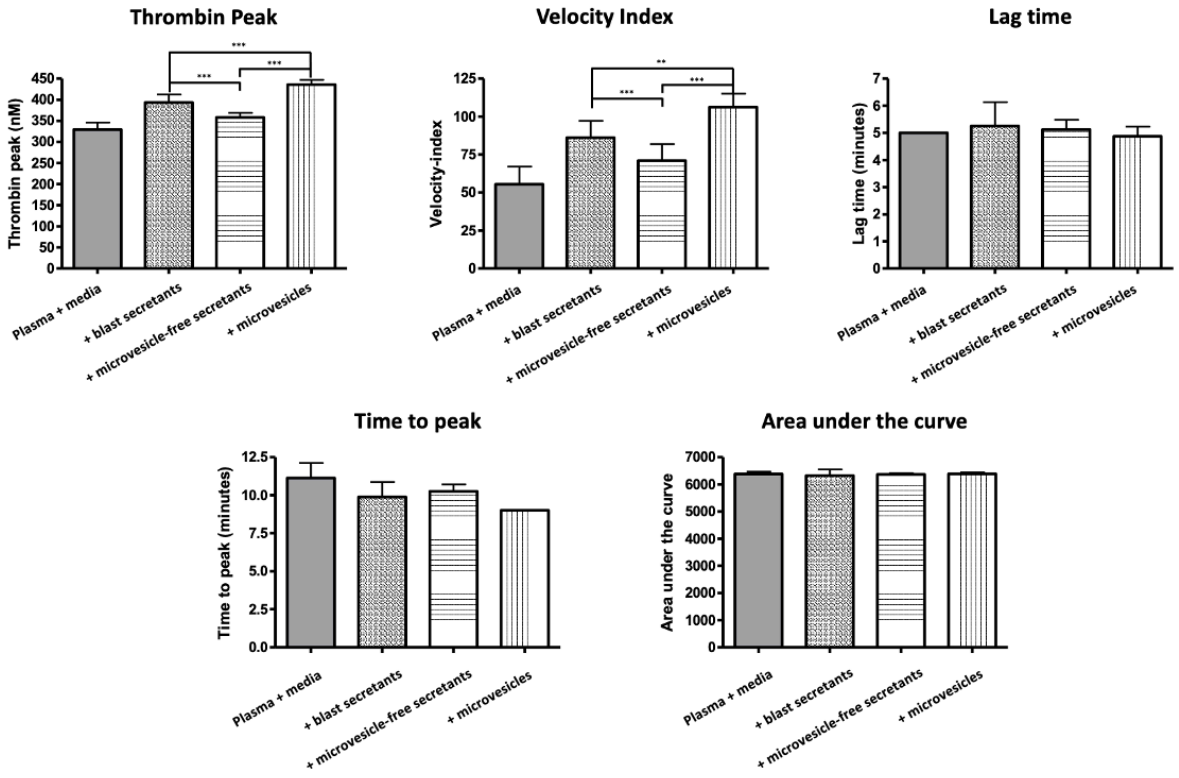

Figure 22. Thrombin generation assay on plasma mixed with secretants from leukemic lymphoblasts with or without microvesicles. A reduction in thrombin peak height and velocity index were recorded when microvesicles were removed from the leukemic cell-derived secretants. There was an increase in thrombin and velocity index when microvesicles previously separated from leukemic cell-derived secretants were added to the plasma. Data was expressed as mean and error bar represented standard deviation. ***: $p<0.001$; **: $p<0.01$; *: $p<0.05$.

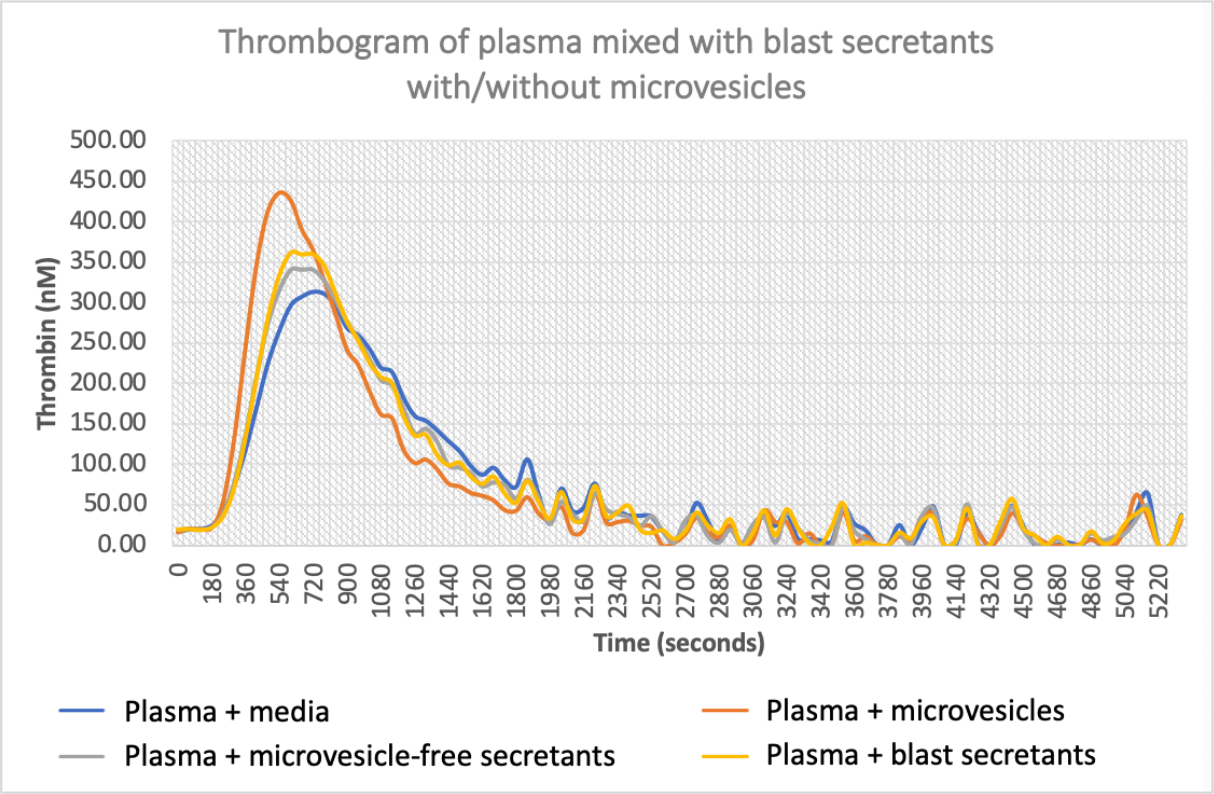

Figure 23. Thrombogram of plasma mixed with secretants from leukemic lymphoblasts with or without microvesicles. Evidence of increased thrombin generation was observed in recalcified plasma mixed with microvesicles released from leukemic lymphoblasts, compared to plasma mixed with medium alone, or plasma mixed with secretants from leukemic lymphoblast. Representative thrombograms of one experiment from each experimental group were chosen for display in the figure.

3.2.6 Secretants from leukemic lymphoblasts may induce increased thrombin generation in plasma in a tissue factor-independent manner

To investigate the contribution of tissue-factor bearing microvesicles towards coagulation activation, lymphoblast secretants were incubated with anti-tissue factor antibody. Thrombin

generation assay was conducted on plasma mixed with the secretants with or without anti-tissue factor antibody pre-treatment. Slightly decreased thrombin peak height and non-significantly different velocity index were recorded when plasma was mixed with secretants from leukemic lymphoblasts pretreated with anti-tissue factor antibody, compared to plasma mixed with lymphoblast secretant. There were no significant differences between groups with regards to velocity index, time to peak, lag time and area under the curve. Therefore, lymphoblast secretants may increase thrombin generation in plasma in a tissue factor-independent manner.

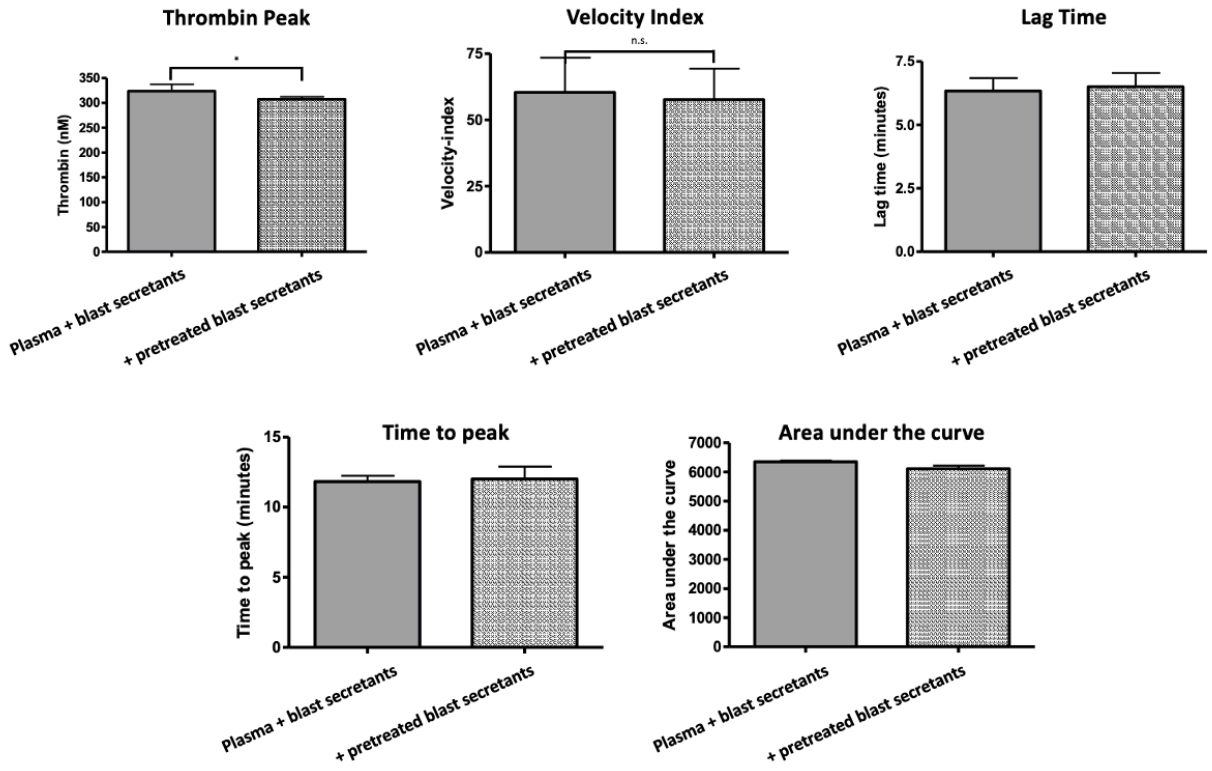

Figure 24. Thrombin generation assay on plasma mixed with secretants from leukemic lymphoblasts pretreated with anti-tissue factor antibody. Slightly decreased thrombin peak and non-significantly different velocity index were recorded when plasma was mixed with secretants from leukemic lymphoblasts pretreated with anti-tissue factor antibody, compared to

plasma mixed with lymphoblast secretant. Data was expressed as mean and error bar represented standard deviation. Thrombin peak height: p=0.014; Mean difference (95% Cis): -16.37 nM (-4.09 to -28.64); n=6. Velocity index: p=0.68; Mean difference (95% Cis): 2.75 (-11.76 to 17.26); n=6. ***: p<0.001; **: p<0.01; *: p<0.05, n.s.: not significant.

3.2.7 Leukemic cell-derived supernatants are capable of cleaving the thrombin substrate

To investigate the non-specific cleavage of the thrombin substrate by secretants from lymphoblasts, the thrombin substrate was mixed with lymphoblast secretants. Compared to the control with sample diluent, secretants from leukemic lymphoblasts showed some cleaving activity to the thrombnin substrate.

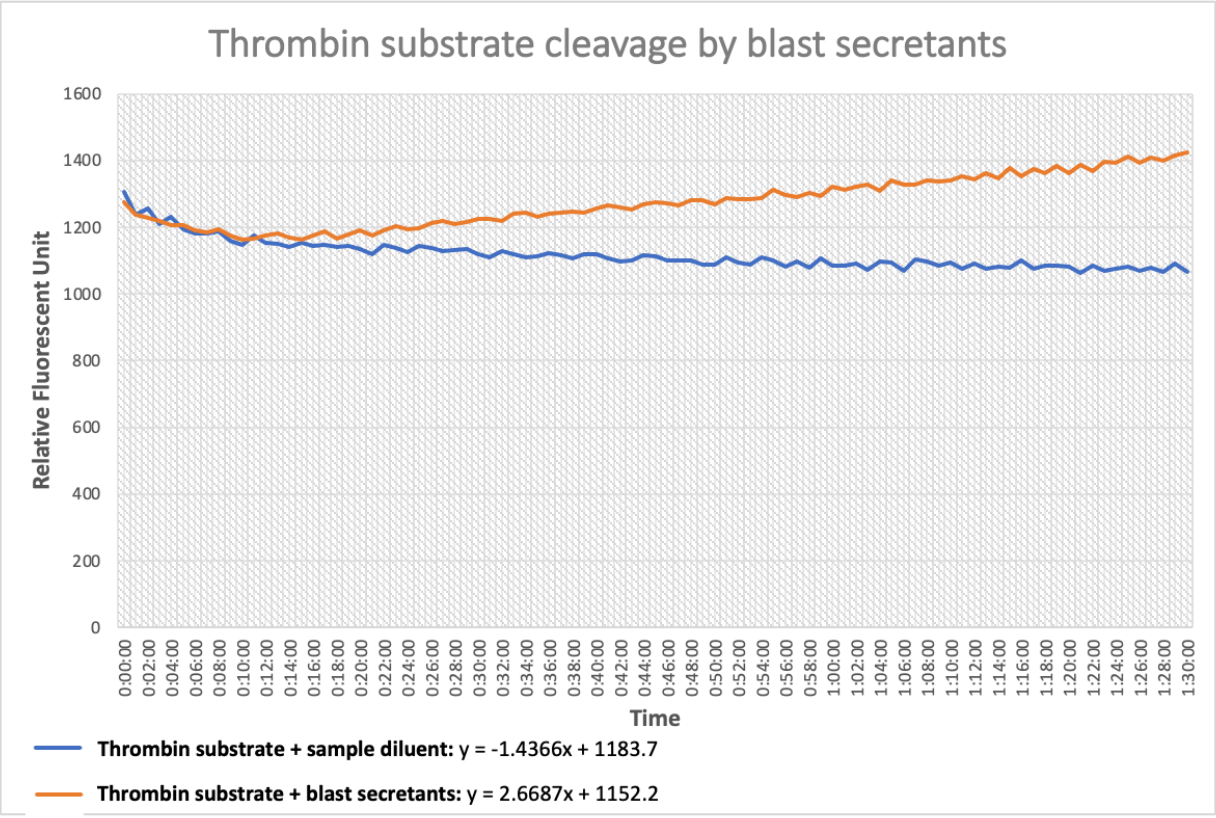

Figure 25. Fluorescent reading of thrombin substrate mixture with secretants from leukemic lymphoblasts or sample diluent . 2mM Z-GGR-AMC thrombin substrate was mixed with 100ul of either lymphoblast secretants or 20mM HEPES buffer. Cleavage activity was monitored in a fluorescent spectrophotometer.

4. Discussion

4.1 Aim 1: Explore the effects of leukemic lymphoblasts on endothelial cells

The first aim of this study was to explore the effect of leukemic lymphoblasts on endothelial cells. It was hypothesized that leukemic lymphoblasts could activate the endothelium, which would then impart a prothrombotic phenotype to the endothelium. The endothelium plays a pivotal role in the regulation of hemostatic balance. Endothelial cells maintain hemostatic balance by promoting anti-coagulant properties and counteracting platelet activation and aggregation. For instance, endothelial cells express endothelial protein C receptor and thrombomodulin which play important roles as natural anticoagulants (Nan et al., 2005). Activation of endothelial cells is a common feature of early complications in hemato-oncologic therapy (Mancuso et al., 2001). In ALL, the accumulation of immature malignant cells may have an impact on the endothelium. Higher levels of adhesion molecules, soluble thrombomodulin and von Willebrand factor has been reported in children with ALL at the time of diagnosis, which suggests endothelial damage before the treatment begins (Hatzipantelis et al., 2011). Our study attempted to investigate the effect of leukemic lymphoblasts on endothelial cells in an *in vitro* coculture model.

4.1.1 Lymphoblasts are capable of activating resting endothelial cells

Endothelial activation is a proinflammatory state of the endothelium characterized by the release of the Weibel-Palade bodies' contents and elevated expression of adhesive molecules for leukocyte recruitment. VWF is stored in the Weibel-Palade bodies and released upon endothelial activation. Elevated VWF is an independent risk factor for VTE (Edvardsen et al., 2021). E-selectin and VCAM-1 are adhesion molecules expressed in endothelial cells in response to inflammation. When leukemic lymphoblasts were cocultured with human endothelial cells, significant increases in the amounts of these endothelial activation biomarkers were measured, which indicates that leukemic lymphoblasts are capable of activating resting endothelial cells (Figure 6). The increase in VWF levels is in accordance with the previous clinical observation that children with circulating blasts had a significantly higher level of plasma VWF (Athale et al., 2010). Higher levels of adhesion molecules, soluble thrombomodulin and von Willebrand factor has been reported in children with ALL at the time of diagnosis, which suggests endothelial damage before the treatment begins (Doroszko et al., 2018; Hatzipantelis et al., 2011; Hatzistilianou et al., 1997; Tacyildiz et al., 1999). These markers of endothelial dysfunction could prove to be prognostic indicators. For example, high baseline levels of thrombomodulin, soluble E-selectin and PAI-1 were shown to indicate poorer prognosis in children with ALL (Doroszko et al., 2018; Hatzipantelis et al., 2011). In addition, endothelial dysfunction in childhood ALL has been linked to the risk of thrombosis in a retrospective study where increased levels of thrombomodulin and VEGFR-1 were independently linked to the odds of developing thromboembolism (Andrés-Jensen et al., 2022).

Vascular leakage is another hallmark of endothelial activation. Under normal physiological conditions, endothelial cells actively regulate the exchange of molecules between blood and

tissue. In severe cases of vascular damage, platelets can adhere to the exposed subendothelium and become activated (van Hinsbergh, 1997). In our study, endothelial cells which were cocultured with leukemic lymphoblasts displayed increased endothelial permeability (Figure 7). Vascular damage was also suggested from the observation that increased LDH, a cytosolic enzyme, was measured at a higher amount in the endothelium cocultured with leukemic lymphoblasts compared to the endothelium cultured alone (Figure 7). Damaged vascular subendothelium could expose binding sites for platelet adhesion, aggregation and thrombus growth. In acute myeloid leukemia, leukemic cells have been shown to take advantage of endothelial permeability for their extravasation into the subendothelium and avoid chemotherapeutic agents (Barbier et al., 2020). Future work could investigate the endothelial transmigration of lymphoblasts and its significance in relapse.

4.1.2 Endothelial expression of products involved in hemostasis is altered by coculturing with lymphoblasts, resulting in a prothrombotic phenotype.

Endothelial cells maintain hemostatic balance by promoting anti-coagulant properties and counteracting platelet activation and aggregation. Tissue factor is a cell surface glycoprotein normally not expressed by the endothelium to prevent improper coagulation cascade activation (Cimmino et al., 2015). Activated endothelial cells express tissue factor on the cell surface. Endothelial cells also affect the fibrinolytic pathway through the release of PAI-1, which increases fibrinolytic resistance of the fibrin clot (Handt et al., 1996). In our *in vitro* experiments, coculturing with lymphoblasts significantly increased endothelial expression of tissue factor and PAI-1 (Figure 8). On the other hand, thrombomodulin is a high affinity thrombin receptor on the endothelial cell surface and helps inhibit the procoagulant functions of thrombin (Boffa &

Karmochkine, 1998). A significant reduction in thrombomodulin cell surface expression was recorded in the endothelium cocultured with leukemic lymphoblasts compared to the endothelium cultured alone (Figure 8).

Overall, the reduction in thrombomodulin cell surface expression and increase in endothelial tissue factor suggest that when endothelial cells were cocultured with leukemic lymphoblasts, they gained a prothrombotic phenotype. In addition, activated endothelium affects the fibrinolytic pathway through the release of PAI-1, which increases fibrinolytic resistance of the fibrin clot. Under normal physiological conditions, the endothelium is considered to possess an anticoagulant property to maintain blood fluidity and prevent thrombosis. This anticoagulant effect has been demonstrated *in vitro* where endothelial cell confluence was negatively correlated with thrombin generation (Billoir et al., 2018). We conducted thrombin generations on endothelium in our study to evaluate the hemostatic property of endothelial cells after coculturing with leukemic lymphoblasts. We observed an increase in thrombin generation parameters and faster time to clot on endothelial cells which were cocultured with leukemic blasts, compared to the endothelium cultured alone (Figures 11, 12). These findings support and provide mechanistic insight into the clinical observation that children with circulating blasts had significantly higher parameters of thrombin generation (Athale et al., 2010).

4.1.3 Healthy lymphocytes isolated from peripheral blood did not activate endothelium

Coculturing lymphocytes from healthy adult donors with endothelial cells did not increase endothelial expression of VWF or tissue factor (Figure 9). However, there was a modest increase

in the level of PAI-1 under these conditions. These results suggest that healthy lymphocytes do not induce endothelial activation to the same degree as leukemic lymphoblasts.

4.1.4 Leukemic lymphoblasts secreted soluble factors that are known to affect endothelial health

The protein content in the secretants from leukemic lymphoblasts were evaluated by the R&D Human Angiogenesis Proteome Profiler Array Kit (Figure 10). Several factors known to affect endothelial health, including soluble dipeptidyl peptidase 4 (sDPPIV), macrophage inflammatory protein-1 alpha (MIP-1α/CCL3), tissue inhibitor of metalloproteinases-1 (TIMP-1) and angiopoietin-2, were detected in secretants from leukemic lymphoblasts. sDPPIV was shown to trigger endothelial cell senescence and promote endothelial dysfunction (Valencia et al., 2022). MIP-1α is involved in the recruitment of leukocytes to the endothelium during inflammation (Lukacs et al., 1994). TIMP-1 plays a role in the regulation of endothelial barrier integrity (Tang et al., 2020). Finally, angiopoietin-2 was demonstrated to activate the β1-integrin pathway, leading to endothelial destabilization (Hakanpaa et al., 2015). The result suggests that lymphoblasts are capable of secreting soluble factors which affect endothelial health.

4.2 Aim 2: Evaluate the *in vitro* prothrombotic effect of leukemic lymphoblasts

The second aim of this study is to evaluate the coagulation activation ability of leukemic lymphoblasts. Cancer cells have been shown to release procoagulant factors and microparticles that can directly activate the coagulation cascade (Gordon et al., 1985). Inflammatory cytokines released from cancer cells or direct adhesive contact may also activate the endothelium and platelets, which further enhances clotting activation (Mancuso et al., 2001). Athale et al.

demonstrated increased thrombin generation potential in children with ALL at diagnosis (Athale et al., 2010). There was a strong correlation of increased parameters of thrombin generation with the presence of peripheral blasts. Therefore, this clinical observation prompted us to investigate the prothrombotic effect of leukemic blasts *in vitro*.

4.2.1 Secreted factors from lymphoblasts are capable of enhancing thrombin generation *in vitro*

Leukemic lymphoblasts were cultured in reduced serum media, and the conditioned media were harvested, centrifuged to remove cellular debris and aliquoted to avoid repeated freeze-thaw cycles. Platelet-poor plasma was aliquoted and stored at minus 80 degrees Celsius to prevent any confounding effect of repeated freezing and thawing on coagulation assay (Philip et al., 2013). We recorded significantly faster clotting times and significantly increased thrombin peak height and velocity index when the lymphoblasts conditioned media were added to the plasma, compared to medium alone (Figures 13-15). This observation is in accordance with the clinical observation that children with circulating blasts had a larger increase in thrombin generation compared to those without (Athale et al., 2010).

4.2.2 Secretants from leukemic lymphoblasts may induce increased thrombin generation in plasma in a FVII-independent manner

We postulated that the phenomenon could be driven by the extrinsic pathway activation of the coagulation system where tissue factor on the surface of blast microvesicles forms TF:FVIIa complexes and activates factor X. To test this hypothesis, factor VII-deficient plasma was mixed with the conditioned media from leukemic lymphoblasts and subjected to a thrombin generation

assay. Interestingly, in the absence of FVII, secretants from leukemic lymphoblasts still increased thrombin peak height and velocity index compared to plasma mixed with medium alone (Figures 16, 17). Similar magnitude of differences in thrombin peak height and velocity index in plasma mixed with conditioned media from leukemic lymphoblasts or medium alone were observed between normal plasma and FVII-deficient plasma. Therefore, we hypothesize that coagulation activation by blast secretants may be a FVII-independent process.

4.2.3 Secretants from leukemic lymphoblasts may induce increased thrombin generation in a FIX and FX-dependent manner

From the previous observation that coagulation activation by blast secretants might be a FVII-independent process, we postulated that soluble factors from blasts could directly activate factor X to Xa. To evaluate this hypothesis, we employed FIX-deficient plasma. When secretants from leukemic lymphoblasts were added to FIX-deficient plasma, a statistically significant increase in thrombin peak height, but not velocity index, was observed compared to FIX-deficient plasma mixed with media in the thrombin generation assay (Figures 18, 19). Although the magnitude of increase in thrombin peak height was small in FIX-deficient plasma, a factor, which can directly activate factor X to Xa, may be present in the lymphoblast secretants. One possibility for the identity of this factor is cancer procoagulant. To further confirm and validate this observation, future work could employ a factor Xa substrate or flow cytometry. In the literature, it has been demonstrated that tumor cells can produce a variety of procoagulant substances, including cancer procoagulant that is capable of directly activating the clotting mechanism (Connolly & Khorana, 2019). Blasts from acute nonlymphoid leukemia and acute lymphoblastic leukemia can express cancer procoagulants (Alessio et al., 1990; Donati et al., 1990; Falanga et al., 1988).

Alternatively, since there is a possibility that a prothrombin activator is present in the lymphoblasts secretants, we employed FX-deficient plasma to test the hypothesis. When secretants from leukemic lymphoblasts were added to factor X-deficient plasma, no difference in thormbin peak height and velocity index were recorded compared to the control mixed with media (Figure 20, 21). As a result, the possible presence of a prothrombin activator in the lymphoblasts secretants can likely be ruled out.

4.2.4 Secretants from leukemic lymphoblasts were capable of cleaving the thrombin substrate

Next we investigated the non-specific cleavage of the thrombin substrate in our experimental model by mixing the thrombin substrate with secretants from leukemic lymphoblasts. Compared to the control with sample diluent which showed no cleaving activity, the secretants from leukemic lymphoblasts were capable of cleaving the thrombin substrate, Z-GGR-AMC (Figure 25). The thrombin substrate can also be cleaved by other proteases including t-PA, trypsin, u-PA and proteasome endopeptidase complex (Bachem, product number: 4002155). Therefore, there is a possibility that these proteins were present in the secretants from leukemic lymphoblasts and hence, cleaved the thrombin substrate in our experimental model.

Furthermore, to evaluate the contribution of this non-specific cleavage to our obtained result, we analyzed the thrombin generation data set on FX-deficient plasma. When secretants from leukemic lymphoblasts were added to FX-deficient plasma, no difference in thormbin peak height and velocity index were observed compared to plasma mixed with media (Figures 20, 21). If the lymphoblast secretants can greatly cleave the thrombin substrate, we would expect to observe a siginificant difference in the thrombin generation parameters. However, the alternate

hypothesis was refuted when no significant difference was detected. Therefore, we propose that the non-specific cleavage of the substrate by the lymphoblast secretants had little impact in our thrombin generation experiments.

4.2.5 Microvesicles in secretants from leukemic lymphoblasts may induce increased thrombin generation in plasma

We postulate that a soluble factor released from leukemic lymphoblasts could involve and drive up the thrombin generation when lymphoblast secretnants were added to the plasma. Cancer cells are capable of releasing microvesicles that can directly activate the coagulation cascade (Falanga et al., 2009). In our study, we employed centrifugation at high speed to separate the lymphoblast secretants into two parts: the bottom one third fraction containing the microvesicles and the top two thirds fraction depleted of microvesicles (Figure 5). Then these two fractions were added to plasma and thrombin generation was evaluated. The removal of microvesicles from the lymphoblast secretants significantly reduced thrombin peak height and velocity index (Figures 22, 23). These results suggested a significant contribution of microvesicles towards coagulation activation by lymphoblast secretants. We postulate that microvesicles from leukemic blasts could either provide an anionic phospholipid surface for the contact factors and/or carry tissue factor on their surface which eventually forms the TF:FVIIa complexes and activatea factor X.

To test the hypothesis that tissue factor on the microvesicle surface is enhancing thrombin generation activity, we employed an anti-human tissue factor antibody to block tissue factor (Affinity Biologicals, CAT#: SATF-IG). Lymphoblast secretants were incubated with the antibody before being added to the plasma. The pre-treatment with tissue factor antibody caused a slight decrease in thrombin peak height and no difference in velocity index compared to the

control group without the tissue factor antibody (Figure 24). These results indicated a minor contribution of tissue factor in the lymphoblast secrentants towards the increased thrombin generation observed when plasma was mixed the the lymphoblast secretants. This supports our observation that the increase in thrombin generation was a FVII-independent process. Furthermore, phosphatidylserine on the microvescle surface may contribute to the increased procoagulant activity in patients with cancer (Abdol Razak et al., 2018; Yu et al., 2020). Future work could investigate the procoagulant characteristic of phosphatidylserine on lymphoblast microvesicles. A better understanding of the pathogenesis of cancer-related thrombosis may improve patient management and outcomes. Treatment of cancer-related thrombosis, especially hematological malignancy is difficult because of an increased risk of both recurrent VTE and anticoagulant or thrombocytopenia-induced bleeding complications.

4.2.6 Limitations

The lack of a relevant healthy control did not allow us to definitively conclude that the observed effects on endothelial activation and hemostasis were solely caused by leukemia. Ideally, we would like to compare between lymphoblasts derived from patients with leukemia and lymphoblasts derived from healthy donors. Due to the technical and ethical challenges in conducting bone marrow aspirates from healthy age-matched donors, lymphocytes isolated from adult peripheral blood were instead used as a comparator. Nevertheless, we showed that leukemic lymphoblasts induced endothelial activation while healthy lymphocytes did not cause any elevation of endothelial dysfunction markers. Furthermore, only one type of endothelial cells was used in this study, which were derived from umbilical veins. The phenotypes of endothelial cells vary between organs in terms of structure and function (Aird, 2012). Therefore, the

observed impact on endothelium by lymphoblasts may be limited to HUVECs. In addition, the proteomic analysis provided an insight on soluble factors secreted by lymphoblasts that are known to affect endothelial health. Future work could compare the expression of these proteins between leukemic and healthy lymphoblasts. Another limitation of this study is the non-specific cleavage of the thrombin substrate, Z-GGR-AMC. Besides thrombin, the substrate can also be cleaved by other proteins including t-PA, trypsin, u-PA and proteasome endopeptidase complex, which may be present in the secretants from leukemic lymphoblasts.

5. Conclusion

In this study, the clinical postulation that circulating lymphoblasts may impact the endothelium has been supported by an *in vitro* coculturing model. Biomarkers for endothelial activation were elevated when leukemic lymphoblasts were cocultured with the endothelium. In addition, we demonstrated the direct coagulation activation by soluble factors from ALL lymphoblasts. This could explain the clinical observation that children with circulating blasts had evidence of increased thrombin generation.

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