Thrombalexin: use of a cytotopic anticoagulant to reduce thrombotic microangiopathy in a highly sensitized model of kidney transplantation.

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Abbreviations:

ABMR Acute Antibody Mediated rejection
EC Endothelial Cell
HLL Hirudin-like peptide

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Abstract

Early activation of coagulation is an important factor in the initiation of innate immunity, as characterized by thrombotic microangiopathy (TMA). In transplantation, systemic anti-coagulation is difficult due to bleeding. A novel ‘cytotopic’ agent, ‘Thrombalexin’, (TLN) combines a cell-membrane bound (mirystoyl tail) anti-thrombin (HLL peptide) which can be perfused directly to the donor organ or cells.

Thromboelastography (TEG) was used to measure time to clot formation (r-time) in both rhesus and human blood, comparing TLN vs. HLL (without cytotopic tail) vs. negative-control. Both TLN and HLL treated rhesus or human whole blood result in significantly prolonged r-time compared to kaolin controls. Only TLN-treated human endothelial cells (EC) and neonatal porcine islets (NPI) prolonged time to clot formation. Detection of membrane-bound TLN was confirmed by immunohistochemistry and FACS. In vivo, perfusion of a NHP kidney TLN-supplemented preservation solution in a sensitized model of transplantation demonstrated no evidence of TLN systemically. Histologically, TLN was shown to be present up to four days after transplantation. There was no platelet deposition and TMA severity, as well as microvascular injury scores (glomerulitis + peritubular capillaritis) were less in the TLN treated animals. Despite promising evidence of localized efficacy, no survival benefit was demonstrated.

Introduction

Thrombotic microangiopathy (TMA) is a systemic pathological phenomenon of systemic thrombocytopaenia, microangiopathic haemolytic anaemia (MAHA) and microvascular thrombosis, with local consequences of potential end-organ damage resulting from deposition of microthrombi. Although primarily a thrombotic disorder, TMA is representative of the crosstalk

IBMIR  Instant Blood Mediated Inflammatory Reaction
MAHA  Microangiopathic Haemolytic Anaemia
NPI  Neonatal Porcine Islets
TEG  Thromboelastography
TLN  Thrombalexin
TMA  Thrombotic Microangiopathy
between coagulation and complement activation, as part of the innate immune system (1). Primary TMA has a wide differential diagnosis (2). Following renal transplantation, TMA is a phenomenon observed in differing clinical circumstances either arising as a result of disease recurrence, such as in haemolytic uraemic syndrome (HUS, and atypical HUS), or in antibody mediated rejection(3). Histological features of TMA in the transplanted organ range from acute fibrin thrombi and endothelial swelling to membranoproliferative lesions, segmental glomerular sclerosis, arteriolar hyalanosis, intimal fibrosis and laminated hypertrophy of the arteriolar walls (4, 5). In the revised Banff classification from 2013, TMA is one of the histological markers of acute tissue injury required for a diagnosis of acute/active antibody mediated rejection (ABMR)(6, 7). Since TMA is one of a number of features of ABMR, and not seen in all cases, it may be difficult to identify recipients at risk of developing TMA, although it is more frequent in highly sensitized models of transplantation (8). Prophylactic treatment at the time of transplantation to prevent TMA is difficult with conventional anti-coagulation therapy (heparin, coumarins, warfarin) due to the risk of bleeding in the post-operative setting and the requirement for systemic treatment and repeated dosing. A localized anticoagulant therapy is therefore highly desirable.

Thrombalexin (TLN) is a novel cytotopic anticoagulant suitable for localized treatment of donor organ, tissues or cells prior to transplantation. It combines a hirudin-like peptide (HLL, based on the thrombin inhibitor, hirudin), with a mirystoyl tail which inserts directly into the cell membrane. TLN inhibits thrombin activation only in the location where it is applied. Previously, variants of the compound have been used in a hepatocyte model, with the goal of overcoming instant blood mediated inflammatory reaction (IBMIR)(9). Thrombalexin has also been tested to treat donor organs in a highly sensitized rodent transplant model wherein it was shown to increase survival when compared to no treatment(10). The mechanism of plasma membrane localization makes Thrombalexin ideally suited for treatment of both solid organ and cell-based transplantation. Here we report our findings of both in vitro and in vivo efficacy of Thrombalexin, in a highly sensitized rhesus model of organ transplantation.

Materials and Methods

Cells, plasma, and blood preparation

Citrated blood samples from healthy human volunteers and non-human primates were approved by Duke University institutional review board (IRB Pro00062495) and Duke Institutional Animal Care and Use Committees (IACUC A209-15-07). Platelet poor plasma was obtained from...
citrated whole blood samples, which were spun for 15m at 2700g. Following this, the supernatant was further spun for 15 min at 2700g before being frozen (-20) prior to use (TLN detection & ELISA). Wild-type neonatal porcine islet (NPI) and primary human aortic endothelial cells (EC) were cultured as per supplemental methods.

**Animal selection and care**

Specific pathogen-free, 3 to 6 year old male rhesus macaques (Macaca mulatta) from Alpha Genesis Inc. (Yemassee, SC) were MHC genotyped by deep sequencing at the Genetics Services Unit, Wisconsin National Primate Research Center, Madison, WI. Donor-recipient pairs were selected by choosing maximal disparity for MHC class I & II. As described previously, donor-recipient pairs underwent two serial skin transplants prior to kidney transplantation. All animals received depletional induction with anti-CD4 & anti-CD8 mAbs (CD4R1 & M-T807R1 NHP Reagent Resource, Mass Biologics, MA) at the time of transplant followed by maintenance therapy with tacrolimus, mycophenolate mofetil and prednisolone (8). Rhesus monkey kidneys not required for implantation were retrieved as for a donor nephrectomy, flushed slowly with 5ml of UW (University of Wisconsin) perfusion solution, before being flushed with Thrombalexin (TLN) for ex-vivo detection by IHC, following the slow TLN flush, a further 5ml flush of UW was given to remove unbound compound. All medication and procedures were conducted in accordance with Duke Division of Lab Animal Resources (DLAR), Yerkes National Primate Center, (Atlanta, GA) and the National Institutes of Health (Bethesda, MD) guidelines after approval by both Duke University (Durham, NC) and Emory University (Atlanta, GA) Institutional Animal Care and Use Committees.

**Thrombalexin (TLN; PTL060) infusion**

Thrombalexin (TLN or PTL060, TLN-3) compound consists of membrane-localizing agent (mirystoyl tail) attached to hirudin-derived sequence, ‘HLL’ (a hirudin-like molecule which binds both to the active site of thrombin, and has an exosite which interacts with fibrinogen, see Figure 1A). A stock solution was prepared, 1mg/ml to give a concentration of 2 μM. A compound, (HLL or PTL011) with the hirudin-like molecule but no cytopic tail was used as a control, which was also prepared to a 2μM stock solution. Both agents were generously supplied by Dr R. Smith, MRC Centre for Transplantation, King’s College, London, and were synthesized by Almac Sciences, Craigavon, UK. Following donor nephrectomy, all kidneys were perfused via the renal artery with 5-8mls of University of Wisconsin (UW) perfusion solution until the effluent from the renal vein was clear. Thrombalexin treated animals received
TLN 4uM (500 ul of stock solution (1mg/ml) of PTL060, diluted in 5mls of UW, followed by an additional flush of UW solution (5ml) to remove any unbound TLN.

**Flow cytometric detection of Thrombalexin (PTL060)**

FACS detection of TLN in whole blood or plasma (human or rhesus), EC and PBMC was performed using TLN-specific primary antibody (RICS2, 1:500, gift from R. Smith, King's College) and secondary antibodies (goat anti-mouse FITC, AbD Serotec). For full details see Supplemental Methods. Flow cytometry was performed on BD LSRFortessa x20 and analyzed using FlowJo (Tree Star, San Carlos, CA) software.

**Thromboelastography (TEG)**

Thromboelastography (TEG, Haemonetics, IL) uses mechanical motion to provide a quantification of whole blood coagulation, where r-time represents the time to clot initiation and is a reflection of coagulation factor activity(11). TEG was used to assess the direct effect of TLN (or controls) on whole blood (human or rhesus), TLN (or control) treated cells, as well as post-transplant evidence of changes in time to clot formation. For full details see Supplemental Methods.

**IHC and Immunofluorescence staining for Thrombalexin**

Tissue sections were submitted for formalin fixed paraffin embedding (FFPE). And stained for Haematoxylin & Eosin, C4d (American Research Products, Inc, Whaltham, MA), CD61 (human platelet glycoprotein IIIa, Sigma-Aldrich, USA) by the core histopathology research lab (Duke University, Durham, NC). Interpretation of immunohistochemistry specimens was performed by Dr David Howell (Duke University, Durham) this included TMA & CD61 scoring (1 = mild; 2 = moderate; 3 = severe, 0 = none). Dr Brad Farris (Emory University, Atlanta) undertook blinded review of Banff scoring at a secondary institution. For a TLN detection, tissue sections were embedded in OCT, rapidly frozen and maintained at -80 degrees. Frozen section slides were fixed in cold acetone for 20 minutes and air dried. After autoquench and blocking steps, primary staining was performed with RICS2 (1:100, primary antibody raised in mouse against PTL060, gift from R Smith) and a secondary FITC ab (goat-anti-mouse FITC, AD Serotec). For full details, see Supplemental methods. For detection of PTL060 on NPI, treated NPI were resuspended in FACS buffer (2% FBS in PBS), and treated with primary (RICS, 1: 500) and secondary antibody (goat anti-mouse FITC, AbD Serotec, 1: 200) before being added transferred to a slide, using a Cytospin machine. For full details, see Supplemental methods.
Microscopy was performed on a Leica DMRA2 Compound Microscope with OpenLab Software. 10x and 20x objectives were used in conjunction with the following fluorescent cubes: DAPI: UV excitation (Ex. DAPI/Hoechst), HQ:R/Dil: Green excitation (Ex. Rhodamine RITC, Dil and HQ:F: Blue excitation (Ex. FITC). Digital images were captured using Volocity software. Confocal imaging was performed on a Leica SP5 confocal microscope.

**ELISA analysis of C3a, C4d and TCC (C5b-9)**

For quantitative analysis, Enzyme linked immunoassays (ELISAs) were performed on commercially available kits, according to manufacturers instructions: serum samples were tested for C3a (MicroVue, Quidel, San Diego); C4d (MicroVue, Quidel, San Diego); SC5b-9 (MicroVue, Quidel, San Diego). Plasma samples were tested for TAT (Thrombin-anti-Thrombin, Enzygnost, TAT, Siemens); Asserachrom vWF:Ag (Diagnostica Stago, NJ ,USA). Limited control plasma samples (n = 1) were available for analysis.

**Statistical analysis**

All data are presented as mean ± standard deviation (error bars in graphs) or as otherwise indicated. Sample comparisons of different animals and/or time points were achieved by two-tailed (unpaired) t-test in normally distributed data. In case of unequal variances we used Welch’s t-test (15). For survival analysis we used the Kaplan-Meier method and log-rank test. Values of p<0.05 were considered to be statistically significant. We used Prism 6.0 (GraphPad Software, San Diego, CA)

**Results (1123)**

**Detection of cell membrane-bound Thrombalexin (PTL060) in whole blood**

To confirm the binding of TLN to cell membranes, whole blood (NHP and human) was spiked with TLN (2µM, 500µM, 125µM, 31.25µM, 7.8µM,1.94µM, 0.4µM, 0.1µM). As shown in Figure 1B and C, TLN was detected by RICS2 mAb in both NHP (n=6), and human blood (n=6). Membrane bound TLN is detectable at very low levels (~0.1µM, p<0.05), which allows for systemic detection of the compound. Time to clot formation, was assessed by Thromboelastography (TEG). Citrated blood was treated with TLN (PTL060), HLL (PTL011) or control and time to clot initiation (r-time) measured. Interestingly, rhesus blood showed a reduced time to clot initiation, compared to human blood, indicating greater thrombogenic
potential of NHP blood. When added directly to whole blood, both PTL060 and PTL011 prolonged the r-value in both NHP (Figure 2A) and human blood (Figure 2B), indicating anticoagulant effect compared to control. To demonstrate membrane binding of Thrombalexin, RICS2 mAb was used to detect PTL060 after endothelial cell (EC) treatment with PTL060, PTL011 or PBS alone, (Figure S1). PTL060 alone is detectable on EC after treatment and washing. NPIs are not amenable to flow detection without first being lysed, hence the immunofluorescent method of detection. Similarly, evidence of PTL060 binding to NPI is demonstrated in compared to control or PTL011 treatment (Figure S1).

**Prolonged coagulation time by cell-bound Thrombalexin (PTL060) on Endothelial Cells and Neonatal Porcine Islets**

To confirm the effect of cells incubated with Thrombalexin, r-time of human blood was measured with human endothelial cell (EC) alone, PTL011 incubated EC, and PTL060 incubated EC, as well as NPI. PTL060 incubated EC significantly increased r-time while PTL011 incubated EC did not prolong the coagulation time compared to EC control (Figure 2C). Compared to PBS controls, ECs themselves shorten r-time, indicating a pro-thrombotic effect of EC controls (data not shown), this effect was reversed by PTL060 treatment. Similar findings were replicated using wild type porcine NPI alone, compared to PTL060 or PTL011 (Figure 2D). It should be noted that, compared to human EC in human blood, porcine NPI alone induce a shorter time to clot initiation in human blood.

**Detection of Thrombalexin (TLN) after kidney transplantation and reperfusion**

Thrombalexin is able to bind to kidney endothelium following ex-vivo perfusion within preservation solution. We perfused NHP kidneys with UW solution alone, UW solution supplemented with PTL060 (2μM, 4μM and 8μM) and UW solution supplemented with PTL011 (4μM) and performed immunohistochemistry. After 1 hour ex-vivo, we detected RICS2 staining only from the PTL060 infused kidneys. The RICS2 staining patterns changed overtime: deposition was predominantly within the glomeruli at 1hr post infusion but detected in tubular area at 72 hr post-infusion (Figure S2).
As a membrane-localising anticoagulant, one of the primary safety concerns in using TLN was the possibility of systemic elution, resulting in an increased bleeding risk. We confirmed that there is no detectable TLN in circulation by flow cytometry using RICS2 antibody (Figure 3A), either in cell bound (whole blood), or in plasma (Figure 3B, detected by secondary PBMC capture). To evaluate its systemic effect, we performed TEG with whole blood and plasma samples from untreated and TLN-treated animals. As shown in Figure 3C, at 1-hour post-transplant samples from all animals show a variable prolonged coagulation time, although it should be noted that per our operative protocol, all animals receive IV heparin sulfate (100-200 units/kg) shortly before explantation of the donor kidney. Surgically, no difference in anastamotic bleeding was observed between TLN treated and untreated animals.

All TLN treated donor kidneys were perfused with TLN (4uM) in UW-solution, and received a secondary UW flush to eliminate any unbound TLN prior to implantation. Kidney biopsies from TLN treated animals, taken post reperfusion, were compared to those of untreated animals. TLN treated animals showed consistently positive TLN staining in the graft (Figure 3D), which was visible on post-transplant day 4 and still positive by POD9 (Data not shown). These data suggest that the tested concentration of TLN (4uM) with UW-solution localizes to the graft but does not induce systemic anti-coagulation.

**The impact of localised anti-coagulation on histological evidence of AMR**

Previously, we reported that early antibody-mediated rejection (AMR) in the sensitized rhesus after kidney transplantation was present, with half of the animals demonstrating histopathological evidence of TMA within the transplanted kidney within the first week post-transplant(12). We hypothesized that coagulation induced by donor-specific antibody (DSA) initiates TMA and thereby induces AMR. Interestingly, TLN treatment (n=3) did not prolong graft survival compared to all untreated controls (controls n = 7, Figure 4A). In order to investigate the effect of TLN on histological evidence of TMA, time matched controls (early rejectors, n = 4, graft survival: 1, 7 and 8 days) were compared to the TLN treated monkeys (n=3, graft survival: 4, 7 and 9 days). TLN treated animals showed significantly reduced (p=0.03) microvascular inflammation scores (MVI = glomerulitis, g score + peritubular capillaritis, ptc score; Figure 4B) and a trend of reduced C4d deposition in the graft (Figure 4C). Thus total Banff 2013 AMR scores were reduced in TLN-treated animals (Table 1), although this did not reach significance. TMA was also not entirely eliminated in the TLN treated group but it showed strong trend of reduction compared to the early rejector controls (p = 0.07; Figure 4D), platelet deposition –
measured by CD61 staining – was also reduced in the treated group, compared to controls, as was fibrinogen staining by immunofluorescence (Figure 4E).

**Systemic coagulation factors and complement activation after TLN treatment**

Systemic haemoglobin levels and circulating platelet counts were not different between groups (Figure 5A). These data suggest that TLN reduces intragraft thrombosis but it does not alter any systemic effects - anemia or thrombocytopenia - seen with systemic TMA. In order to demonstrate the efficacy of thrombin blockade by thrombalexin, we measured circulating vWF levels, as well as thrombin-anti-thrombin (TAT) complexes. (Figure 5B), no difference in circulating vWF was seen in treated animals, compared to untreated control, however there was a trend for reduced plasma TAT levels in the early post-operative course of the TLN treated animals, suggesting effective thrombin inhibition, however these data should be interpreted with caution in view of the limited availability of control plasma samples.

The role of thrombin in complement activation has been identified(13). To evaluate whether the complement activation cascade was affected by targeting thrombin via Thrombalexin, we measured serum C3a, C5b-9, C4d, and MBL. In general, there was a trend for reduced evidence of complement activation products in the first 24 hours post-transplant in the TLN treated group, which was most evident, but not significant for serum C3a on day 1 (p=0.07) (Figure 5C).

**Discussion**

The use of an anticoagulant, in the form of anti-thrombin, to prevent TMA is a novel application. TMA is a poorly described phenomenon post kidney transplantation, although clinicians involved with positive crossmatch (HLAi) are familiar with this aggressive phenotype of AMR, which may necessitate dramatic intervention in order to prevent acute graft loss in the early post-operative period(14). TMA is a descriptive term, for the observed clinical presentation of microangiopathic haemolytic anaemia (MAHA), thrombocytopenia and microvascular thrombosis occurring as a result of endothelial cell activation. It has a range of predisposing hereditary and acquired underlying causes, which are important to distinguish with respect to optimal treatment(15, 16). In renal transplantation, de novo histological evidence of TMA was found to be most commonly associated with C4d positive AMR (55%), of whom 84% had a high PRA. TMA is often considered to be a complement mediated disease(17), however it more accurately represents the interface between complement and coagulation(1).
The use of complement inhibition in the setting of highly sensitized kidney transplantation has gained considerable interest with the advent of anti-C5 inhibition with Eculizumab. Initial results in reducing AMR in highly sensitized kidney transplant recipients were very promising(18), and many case reports of the ability of complement inhibition to reverse severe AMR demonstrate great benefit to salvage grafts(19), although this is in the absence of long-term benefit(20), and may therefore represent the ability of complement inhibition to reverse early AMR features, which are associated with TMA.

Systemic anticoagulation has, for the most part, been avoided in the immediate post-operative period in kidney transplantation, as a result of the potential for increased bleeding risk, hence the demonstration of no systemic elution of drug, or change in time to clot formation (Figure 1). Alternative means to control the thrombotic process of TMA through inhibition or down regulation of coagulation are therefore of interest. Recombinant soluble thrombomodulin (TBM) – a co-factor of thrombin important for the down regulation of coagulation and inflammation - has been used to treat TMA in the setting of liver transplantation(21). The manipulation of the coagulation system has also been used in xenotransplantation, as exogenously administered coagulation factors (human prothrombin concentrate complex, or recombinant FVIIa)(22). Interestingly, the genetic manipulation of TBM expression on cells within the transplanted organ has shown promise in both mitigating the systemic thrombocytopaenia associated with TMA in xenotransplantation(23), as well as abrogating the activation of complement (24).

Our hypothesis was that TLN, as a topical anti-thrombin agent, would provide local protection of the kidney, or treated cells from the effects of thrombosis (Figure 2), as well as mitigate complement activation and, potentially down regulate inflammation and graft infiltration with adaptive immune cells. Thrombin itself has a role in both primary haemostasis –with respect to platelet activation, and secondary haemostasis, in which fibrin is crosslinked to form a stable haemostatic clot, which is later degraded by fibrinolysis. Previously, bivalirudin, a synthetic analog of hirudin currently in use in cardiac surgery, which is most analogous to the active element of TLN, has been demonstrated to decrease thrombin-induced platelet activation(25). The importance of platelet deposition in TMA, has previously been demonstrated with CD61 staining of platelets which was positive in TMA induced by CNI toxicity in kidney transplant recipients(26). In our study, the absence of platelet deposition, as demonstrated by the lack of CD61 positivity in the TLN treated kidney grafts supports a biological effect of thrombin inhibition with TLN to prevent platelet activation and deposition. Additionally, histology clearly demonstrated less fibrin deposition and a less histologically severe form of TMA (Figure 4).
The effect of thrombin on complement activation is currently a field of active study. It has been recognized that thrombin is an activator of complement at the level of C3 however, in addition, thrombin has also been shown to be able to cleave C5 directly, independent of C3 (27). In our model, serum measurements of complement activation products, principally C3a showed a trend for lower levels in the immediate post-operative period (Figure 5), however this was a transient observation. In the kidney, evidence of complement activation, in the form of C4d deposition was somewhat lower (p = 0.09) in treated vs untreated animals, but this not significant. In addition, there was no histological difference in graft infiltrating cells, T-cell (CD3, CD4) or B-cell (CD20) (data not shown). Taken together, these data indicate a possible effect of thrombin-inhibition on complement activation, although this is not conclusive.

The lack of survival benefit in TLN treated animals, compared to controls, contrasts with previous findings in a sensitized rat model(10). This is likely to be attributable to a number of factors. Firstly, although a survival benefit in the rat model was demonstrated, all survival times, in the absence of any immunosuppression, were short: TLN increased the MST in the rat model from 1.5d to 5.1. MST in our TLN treated group was 6.6 days, compared to 4.25d in the early rejector control group, and 18.7d with all controls. This may represent the dwell time of TLN on the cell-membrane target, and therefore period of efficacy.

Secondly, it may be a consequence of underdosing, although drug elution experiments (data not shown) suggest that effluent from the renal vein directly after TLN administration is positive for TLN after 4uM dosing. Alternatively, the expression of the compound on the cell membrane is insufficient to provide activity, either as a consequence of the dwell time on the cell surface, or as a result of cellular uptake. Repeated dosing of TLN is not possible, due to the route of administration arterially to the organ itself.

In present form the role of this compound remains to be established, although isolated treatment of the transplanted organ is a pragmatic option, particularly in light of the new role of ex-vivo normothermic perfusion treatments. A potential application of TLN may be, as Parajuli et al. advocate, as part of preventative strategies for patients at increased thrombotic risk (ie. Anti-phospholipid antibodies protein C & S deficiencies etc), this may be a protective strategy in the first 24 hours post-transplant (28) that avoids the management difficulties of post-operative anticoagulation.
Further study of the interactions between coagulation and complement may yield further evidence which would support combination therapy with Mirococept (cytotoxic C3-convertase inhibitor) in the perioperative setting, however these data are demonstrate promising efficacy, safety and detection of a novel agent within a large animal model.

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Author contributions
M.M. designed experiments, performed ex vivo and in vivo experiments, interpreted data and prepared the manuscript.

J.K. designed experiments, conducted in vitro and in vivo experiments interpreted data and prepared the manuscript.

K.S. prepared porcine islet, conducted in vitro experiments, prepared the manuscript.

C.B. and M.M. participated in in vivo experiments.

J.Y. conducted in vitro experiments.

H.X. cultured human endothelial cells, performed in vitro experiments.

A.L.M. prepared porcine islet, conducted in vitro experiments.

K.F., V.C., and E.B. conducted in vitro experiments.


R.A.S., S.S., A.D. and N.M. designed experiments, interpreted data and prepared the manuscript.

S.K. designed experiments, interpreted data and prepared the manuscript.
Disclosure
The authors of this manuscript have no conflicts of interest to disclose as described by the American Journal of Transplantation.

Figure legend

Figure 1. A. Schematic illustration of the structure and binding site of Thrombalexin (PTL060; TLN). Thrombalexin is anchored to the cell membrane with a misorystoyl tail, and prevents activation of Thrombin (Factor IIa) via inhibition of both the active and exo-sites. Detection of Thrombalexin using RICS2 mAb (mouse anti-Thrombalexin), in both rhesus (B) and human (C) whole blood.

Figure 2. Efficacy of Thombalexin (PTL060; TLN). A. Thromboelastography (TEG) using rhesus whole blood comparing adding TLN or HLL (PTL011, active Hirudin-like molecule) directly to blood, compared to kaolin control. B. TEG using human whole blood comparing kaolin control, Thombalexin (PTL060; TLN) or HLL (PTL011). C. TEG demonstrating efficacy of Thrombalexin treated EC, compared to HLL in prolonging time to clot initiation (r time, mins) compared to EC alone. PBS alone (no cell suspension) controls in black are shown for representative comparison. D. TEG demonstrating efficacy of Thrombalexin treated NPI, compared to HLL in prolonging time to clot initiation (r time, mins) compared to NPI alone. NS>0.05, *p<0.05, **p<0.01, and ***p<0.001. PBS alone (no cell suspension) controls in black are shown for representative comparison.

Figure 3. Systemic and In-situ detection of Thrombalexin (TLN) in kidney following transplantation A. Systemic detection of TLN following transplantation of a kidney infused with TLN at the time of retrieval in rhesus whole blood using RICS2 mAb. B. Systemic capture of TLN following transplantation of a kidney infused with TLN at the time of retrieval from rhesus plasma using an alternative source of cells (rhesus PBMC) & RICS2 mAb. C. Post-transplantation TEG of rhesus whole blood. D. Post-transplant Immunofluorescence demonstrating the presence of Thrombalexin (TLN) in 1hr post-reperfusion biopsy, and terminal kidney samples in TLN treated animals, compared to untreated controls.

Figure 4. Post-transplant survival and histology after Thrombalexin (TLN) treatment of the donor kidney. A. Survival of TLN (4uM) treated kidneys, compared to untreated controls B. Banff (2013) Microvascular inflammation scores (glomerulitis + peritubular capillaritis) between time matched controls and TLN treated kidneys. C. Banff (2013) C4d deposition between time matched controls and TLN treated kidneys. D. Histological thrombotic microangiopathy (TMA) scores between time matched controls and TLN treated kidneys. E. Histological evidence of platelet (CD61) deposition between time matched controls and TLN treated kidneys and representative histological fibrinogen deposition between time matched controls and TLN treated kidneys. NS>0.05 and *p<0.05.

Figure 5. Post-transplant systemic evidence of coagulation & complement inhibition. A. Systemic haemoglobin & platelet levels of TLN treated animals, compared to controls. B. Systemic (plasma) detected von Willebrand Factor (vWF) & Thrombin-anti-Thrombin complexes (TAT) of TLN treated animals, compared to controls. C. Systemic detected complement products: C3a, C5b-9, C4d containing activation fragments & Mannose Binding Lectin (MBL) in TLN treated animals, compared to controls. *p<0.05.
Supporting Information

Additional Supporting Information may be found in the online version of this article.

Supplemental Methods

**Figure S1.** A. Detection of membrane bound on EC after incubation with Thrombalexin (PTL060; TLN), HLL (PTL011) or PBS control, using PTL060 detection by RICS2 mAb by flow cytometry. B. Immunofluorescence detection of Thrombalexin binding to neonatal porcine islets (NPI) using RICS2 mAb compared to HLL or control.

**Figure S2.** In-situ detection of Thrombalexin. A. Immunofluorescence demonstrating the presence of Thrombalexin (PTL060; TLN), at various concentrations (2mM, 4mM and 8mM) at 1hr post-perfusion, compared to HLL (PTL011, 4mM). B. Immunofluorescence demonstrating the presence of Thrombalexin (PTL060; TLN) following infusion of 4mM solution after 1hr, 24hrs, 48hrs and 72 hrs.

References:


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Table 1: Pre-transplant T-cell and B-cell FXCM, survival and antibody mediated rejection histology scoring (Banff 2013) for early rejecting control NHP who experienced thrombotic microangiopathy (TMA), compared to Thrombalexin (TLN) treated animals. Following 2 sensitising skin transplants, all animals received induction therapy with CD4+mAb & CD8+mAb depletion at the time of kidney-swopping transplantation, in addition to triple therapy maintenance immunosuppression (tacrolimus, steroid & mycophenolate mofetil) post transplant.

<table>
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<tr>
<th>Treatment</th>
<th>Animal</th>
<th>DSA T Cell XM (MFI ratio)</th>
<th>DSA B Cell XM (MFI ratio)</th>
<th>Survival (days)</th>
<th>AMR Score (Banff)</th>
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<td>Pre-Tx</td>
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Figure 1

A

- Active site inhibitory sequence
- Exosite ligand sequence
- Cytotopic membrane-anchoring elements
- Thrombin
- Exosite
- Active site
- 5 kDa
- Endothelial Cells

B

Rhesus-TLN titration

MFI

C

Human-TLN titration

MFI

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Figure 4

A  Survival

B  Microvascular inflammation

C  C4d deposition

D  Thrombotic Microangiopathy

E  Fibrogen

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Figure 5

A  Haemoglobin (Hb)  Platelets

B  vWF  TAT Complex

C  C3a  C5b-9

C4d-containing activation fragments

MDL

Control  TLN