Interactions of *Treponema pallidum* with human platelets

by

Brigette Monica Church

BSc (Honours), University of Victoria, 2013

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We acknowledge with respect the Lekwungen peoples on whose traditional territory the university stands and the Songhees, Esquimalt and W̱SÁNEĆ peoples whose historical relationships with the land continue to this day.

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Abstract

Treponema pallidum ssp. *pallidum* is the causative agent of syphilis, a multi-stage bacterial infection, transmitted sexually or from mother-to-child, with an unparalleled range of symptoms arising from the ability of treponemes to penetrate any tissue and cross immune privileged endothelial barriers to access the brain, the eye, and the fetus. Further, without treatment *T. pallidum* evades immune clearance and persists within the host to establish a chronic infection. These characteristics suggest that *T. pallidum* may have evolved unique mechanisms for immune escape and to mediate host-cell interactions.

The findings presented in this dissertation contribute to our knowledge of *T. pallidum* pathogenesis by investigating a previously unexplored host-cell interaction, between *T. pallidum* and human platelets. These results validate the hypothesis that, as a pathogen which successfully utilizes vascular dissemination, *T. pallidum* would not only encounter, but interact with human platelets, complex cells now viewed as vascular sentinels that participate in many host-pathogen interactions.

This is the first study to demonstrate that *T. pallidum* interacts with human platelets and to characterize and quantify these interactions using high resolution microscope imaging techniques (video and frame analysis). These interactions were shown to be complex, reversible and mediated by motile treponemes localizing to stationary, (slide-adhered) activated platelets, versus to free-floating, inactive platelets. In addition, it was found that *T. pallidum* discriminates between the *level* of platelet activation and preferentially localized to the most activated platelet. *Treponema pallidum* was also able to induce platelet activation following an extended lag period.

Modified chemotaxis assays quantified by flow cytometry, were used to investigate the migration of *T. pallidum* in response to the plasma of platelets differentially activated with infection-relevant host components (thrombin, collagen). The results herein reveal that *T. pallidum* discriminates between *different mechanisms* of platelet activation, with a significant preference towards the secretions of collagen-activated platelets (under these experimental conditions), compared with that of inactive or thrombin-activated platelets.

Previously, *T. pallidum* chemotaxis had been investigated through genomic characterization and molecular interaction studies with recombinant proteins. This investigation is the first time live *T. pallidum* was utilized for *in vitro* chemotaxis assays and is also the first study of pathogen chemotaxis in response to the secretions of differentially activated platelets.

The body of work in this dissertation provides a foundation to further investigate the role of *T. pallidum*-platelet interactions during infection, adding a new host-cell interaction to our understanding of *T. pallidum* pathogenesis. The evidence that the molecular gradients of host components can affect *T. pallidum* migration suggests an important role for chemotaxis during *T. pallidum* infection. Together, the characterization of platelet-interactions and treponeme chemotaxis in response to host components, adds to our knowledge of *T. pallidum*-host interactions, and eludes to additional pathogenic strategies that may facilitate *T. pallidum* dissemination and immune evasion.

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List of Abbreviations

Abbreviation	Meaning
A2A	Adenosine A2A receptor
ABOs	Adverse birth outcomes
ACD-A	Acid citrate dextrose
ADP	Adenosine disphosphate
ADIPOQ	Adiponectin
AMP	Anti-microbial peptide
ANC	Antenatal care
ANG	Angiogenin
ANGPT	Angiopoietin
ANOVA	Analysis of variance
Apo-A1	Apolipoprotein A1
Arg-1	Arginase-1
ATP	Adenosine triphosphate
Avi	Audio Video Interleave
β_2-AR	β2 adrenergic receptor
BAFF	B-cell-activating factor
BBB	Blood-brain barrier
B. burgdoferi	*Borrelia burgdorferi*
BCCDC	British Columbia Centre for Disease Control
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
B. henselae	*Bartonella henselae*
B. hermsii	*Borrelia hermisii*
Bpa	Binding protein isoform a
BPG	Benzathine penicillin G
BRAK	Breast and kidney-expressed chemokine
BSA	Bovine serum albumin
BSG	Basingen
C	Collagen
C5a	Complement factor 5a
cAMP	Cyclic adenosine monophosphate
CCL	Chemokine C-C motif ligand
CD	Cluster of differentiation
CFD	Complement factor D
CFH	Complement factor H
CFSE	Carboxyfluorescein succinimidyl ester
Che	Chemotaxis proteins
Clf	Clumping factor
CLEC	C-type lectin receptor
CM	Cytoplasmic membrane
CMRL	Connaught Medical Research Laboratories (media)
CNS	Central nervous system
compl	Complement
cP	Centipoise
C. pneumonia	*Chlamydia pneumonia*
CRP	C-reactive protein
cryo-EM	Cryo-electron tomography
CS	Congenital syphilis
CSF	Cerebrospinal fluid
CV	Cardiovascular
CVS	Cardiovascular syphilis
CXCL	Chemokine C-X-C motif ligand

DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DFM	Dark field microscopy
DIC	Disseminated intravascular coagulation
Dkk-1	Dickkopf-1
DPPIV	Dipeptidyl-peptidase IV
DTH	Delayed type hypersensitivity
DTS	Dense tubular system
Eap	Extracellular adherence protein
EB-7	Erythrocyte band-7
EC	Endothelial cell
ECGF	Endothelial cell growth factor
ECM	Extracellular matrix
E. coli	*Escherichia coli*
Efb	Extracellular fibrinogen-binding protein
EGF	Epidermal growth factor
ENA-78	Epithelial neutrophil-activating protein-78
ENG	Endoglin
EP3	PGE2 receptor
ER	Endoplasmic reticulum
ES	Effect size
FasL	Fas ligand
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FLA	Flagellar
Flt	Fms-related tyrosine kinase
FOV	Field of view
F.R.S.	Fellow of the Royal Society
FSC	Forward scatter
GABA	Gamma-aminobutyric acid
G-CSF	Granulocyte colony-stimulating factor
GDF-15	Growth/differentiation factor 15
GH	Growth hormone
GIs	Genomic islands
GLUT	Glucose transport
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP	Granule membrane protein
GO	Gene ontology
GP	Glycoprotein
GPCR	G-protein-coupled receptor
GROα	Growth related oncogene-alpha
GTP	Guanosine-5'-triphosphate
5-HT	Serotonin
HDVSMC	Human dermal vascular smooth muscle cells
HGF	Hepatocyte growth factor
HIV	Human immunodeficiency virus
H. pylori	*Helicobacter pylori*
HRGP	Histidine rich glycoprotein
HSS	Human syphilitic serum
HVS	Human visual system
ICAM	Intercellular adhesion molecule
ID	Infectious dose

IFN-γ	Interferon-γ
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor-binding protein
IL	Interleukin
IM	Inner membrane
IM	Intramuscular
iNOS	Inducible nitric oxide synthase
IP-10	Interferon γ-induced protein 10 kDa
IRS	Immune rabbit serum
ITAC	Interferon-inducible T-cell alpha chemoattractant
IV	Intravenous
JAM	Junctional adhesion molecule
JND	Just noticeable difference
L	Length
-L	Ligand
LAMP	Lysosomal-associated membrane protein
LamR	Laminin receptor
LCs	Langerhans cells
LIF	Leukemia inhibitory factor
LPS	Lipopolysaccharide
LTBP	Latent-transforming growth factor beta-binding protein
Mϕ	Macrophage
M1	Classically-activated macrophage
M2	Alternatively-activated macrophage
Mac-1	Macrophage-1 antigen
Mb	Megabase
MB	Motility buffer
MCP	Monocyte chemoattractant protein
MCPs	Methyl-accepting chemotaxis transmembrane proteins
MDC	Macrophage-derived chemokine
MFI	Medium fluorescence intensity
MHC	Major histocompatibility complex
MIF	Macrophage migration inhibitory factor
MIG	Monokine induced by interferon-gamma
MIP	Macrophage inflammatory protein
MK	Megakaryocyte
MMP	Matrix metalloproteinase
MO_2	Microaerophilic
MPO	Myeloperoxidase
ms	Millisecond
MS	Mass spectroscopy
MSM	Men-who-have-sex-with-men
M. tuberculosis	*Mycobacterium tuberculosis*
NAP-2	Neutrophil-activating peptide-2
NET	Neutrophil extracellular trap
NK	Natural killer cell
NOD	Nucleotide-binding oligomerization domain
NRS	Normal rabbit serum
NS	Normal saline
OCS	Open canaliculi system
OM	Outer membrane

OMP	**Outer membrane protein**
OPN	**Osteopontin**
OSM	**Oncostatin M**
P2Y	**Purinergic (nucleotide) G-protein coupled receptor**
PADGEM	**Plt activation dependent granule-external membrane protein**
PAF	**Platelet-activating factor**
PAFR	**Platelet-activating factor receptor**
P. aeruginosa	***Pseudomonas aeruginosa***
PAI	**Plasminogen activator inhibitor**
PAR	**Proteinase activated receptor**
PAMP	**Pathogen-associated molecular pattern**
PBS	**Phosphate buffered saline**
PDCI	**Platelet derived collegenase inhibitor**
PDGF	**Platelet-derived growth factor**
PDI	**Protein disulfide isomerase**
PE	**Phycoerythrin**
PECAM	**Platelet endothelial cell adhesion molecule**
PE/Cy5	**Phycoerythrin and cyanine 5**
PF4	**Platelet factor4**
PFA	**Paraformaldehyde**
PFP	**Platelet free plasma**
PG	**Prostaglandin**
PG	**Peptidoglycan**
P. gingivalis	***Porphyromonas gingivalis***
Plt	**Platelet**
PMP	**Platelet microparticle**
PN-II	**Protease nexin-II**
polyP	**Polyphosphate**
PP_i	**Pyrophosphate**
PPI	**Protein-protein interactions**
PPP	**Platelet poor plasma**
PRP	**Platelet rich plasma**
PRR	**Pattern recognition receptor**
P- (selectin)	**PADGEM**
PSGL	**Platelet selectin glycoprotein ligand**
PTFE	**Polytetrafluoroethylene**
PVDF	**Polyvinylidene difluoride**
-R	**Receptor**
Rab	**Ras-related protein**
RAGE	**Receptor for advanced glycation endproducts**
RANTES	**Regulated on activation, normal T cell expression & secreted**
Rap1	**Ras-proximate 1**
RBCs	**Red blood cells**
RBP4	**Retinol binding protein 4**
ROS	**Reactive oxygen species**
RT	**Room temperature**
s	**Seconds**
s-	**Soluble-**
S1P	**Sphingosine-1-phosphate**
S. aureus	***Staphylococcus aureus***
SarP	**Staphylococcal accessory regulator protein**
SHBG	**Sex hormone-binding globulin**

Siglec	**Sialic acid recognizing Ig-like lectin**
SLAM	**Signaling lymphocytic activation molecule**
S. pneumoniae	***Streptococcus pneumoniae***
S. pyogenes	***Streptococcus pyogenes***
SRB1	**Scavenger receptor class B type 1**
SRGN	**Serglycan**
SHBG	**Sex hormone-binding globulin**
SD	**Standard deviation**
SDF-1α	**Stemcell derived factor-1 α**
sec	**Second**
SEM	**Standard error of the mean**
Serpin	**Serine protease inhibitor**
S. gordonii	***Streptococcus gordonii***
SD	**Standard deviation**
SDF-1α	**Stem cell derived factor-1 α**
sec	**Second**
SEM	**Standard error of the mean**
S. sanguinis	***Streptococcus sanguinis***
SSC	**Side scatter**
ST2	**Serum stimulation-2**
subsp.	**Subspecies**
T	**Thrombin**
TARC	**Thymus and activation regulated chemokine**
T. carateum	***Treponema carateum***
T. denticola	***Treponema denticola***
TF	**Tissue factor**
TFF3	**Trefoil factor 3**
TFPI	**Tissue factor plasminogen activator inhibitor**
TfR	**Transferrin receptor protein**
TGF	**Transforming growth factor**
Th	**T helper**
Tie	**Tyrosine kinase with Ig-like and EGF-like domains 164**
TIM-3	**T-cell immunoglobulin and mucin domain-3**
TIMP	**Tissue inhibitor of metalloproteinase**
TJ	**Tight junctions**
TLR	**Toll-like receptor**
TLT-1	**Trem-like transcript-1**
TNF	**Tumor necrosis factor**
Tp or *T. pallidum*	***Treponema pallidum* subspecies *pallidum***
tPA	**Thromboxane receptor**
T. paraluiscuniculi	***Treponema paraluiscuniculi***
TPeC	***Treponema paraluiscuniculi* ecovar Cuniculus**
T. p. endemicum	***Treponema palladum* subspecies *endemicum***
TPO	**Thrombopoietin**
***T. p. pertentue or* TPE**	***Treponema palladum* subspecies *pertenue***
Tpr	***Treponema pallidum* repeat proteins**
Treg	**Regulatory T cell**
TREM	**Triggering receptor expressed on myeloid cells**
TSP	**Thrombospondin**
TXA2	**Thromboxane A2**
uPAR	**Urokinase plasminogen activator receptor**
UTP	**Uridine-5'-triphosphate**

UVDFM	**Ultra-low volume dark field micrscopy**
V1a	**Vasopressin receptor**
VAMP	**Vesicle-associated membrane protein**
VCAM-1	**Vascular cell adhesion molecule-1**
VEGF	**Vascular endothelial growth factor**
Vitamin D-Bp	**Vitamin D-binding protein**
VWF	**von Willebrand Factor**
WBCs	**White blood cells**
WHO	**World Health Organization**
ZO	**Zonula occludens**

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Finally, the last acknowledgment I would like to make is to the scientists, doctors and pursuers of knowledge in the past whose shoulders we now all stand upon. While looking for the original source of a famous Sir William Osler quote, I came upon a collection of his original papers where he described so many diseases and conditions with exquisite detail. He investigated a wide range of infectious and genetic conditions as the first “Physician in Chief” of the newly founded Johns Hopkins University and is truly a Great Canadian. Osler published early accounts of platelets, although Prof.

Bizzozero of Turin is credited with their description. The papers show Osler's original painstaking drawings and descriptions, and what started as fact-checking a quote, became a source of inspiration to me. I was inspired reading his detailed descriptions from so long ago that are still so relevant today. Sir William Osler also had a passion for the study of syphilis.

In his honor I have included exerts from his original papers and orations that allow us to see the world through his eyes:

"Let nothing slip by you; the ordinary humdrum case of the morning routine may have been accurately described and pictured, but study each one separately as though it were new-so it is so far as your special experience goes; and if the spirit of the student is in you the lesson will be there....

The observations are made with accuracy and care, no pains are spared, nothing is thought a trouble in the investigation of a problem. The facts are looked at in connection with similar ones, their relationship to others is studied, and the experience of the recorder is compared with that of others who have worked upon the question."

- *The Army Surgeon* by William Osler, M.D., Professor of Medicine, Johns Hopkins University, Baltimore in an address delivered at the closing exercises of the Army Medical School, Washington, D.C., February 28, 1894. (W. S. Osler, 1881)

I have included insights by Sir William Osler into syphilis that continue to be relevant over 100 years later. His original papers have several versions of this quote over the years; however, this is one of the earliest:

> "So, too, with syphilis, which after the first few weeks I claim as a medical affection. I often tell my students that it is the only disease which they require to study thoroughly. Know syphilis in all its manifestations and relations, and what remains to be learned will not stretch the pia mater *of a megalocephalic senior student.*"
>
> - *Internal Medicine as a Vocation* by William Osler, M.D., Professor of Medicine in the Johns Hopkins University, an address before the section on General Medicine at the New York Academy of Medicine, October 19, 1897 (Osler, 1881)

Chapter 1: Introduction

1.1 Syphilis is a world health concern

Syphilis is a curable, bacterial infection caused by *Treponema pallidum* subspecies *pallidum* (*T. pallidu*m*)*, that is transmitted sexually or from mother to fetus, and is estimated by the World Health Organization (WHO) to affect 18 million men and women aged 15-49 worldwide, with 5.6 million new cases annually (Newman et al., 2015). Each year an estimated 1 million pregnant women are affected (Newman et al., 2015; Wijesooriya et al., 2016), yet untreated maternal infection carries a 52% risk of adverse birth outcomes (ABOs) including stillbirth and clinical disease in surviving infants (congenital syphilis) (Korenromp et al., 2019; Patel et al., 2017). Middle- and low-income countries continue to have endemic syphilis within the general population (Kojima & Klausner, 2018), with rates in men and women increasing as income decreases (Newman et al., 2015). In high-income countries syphilis has increased rapidly in specific populations, the greatest increases in men who have sex with men (MSM) and groups with multiple sex partners (Kojima & Klausner, 2018). In Canada the rate of infections increased from 5.0 cases per 100,000 in 2010 to 9.3 in 2015, an increase of 85.6%, and by 2015 the rate was 17.5 per 100,000 males compared with 1.2 per 100,000 females (Choudhri et al., 2018). Infection with syphilis also increases the likelihood of acquiring and transmitting HIV, HIV co-infections in MSM may occur in 50% of cases (Kojima & Klausner, 2018; Marra, 2015) and co-infection with HIV alters the symptoms and may rapidly increase the progression of syphilis (Ahbeddou et al., 2018; Carlson et al., 2011).

Syphilis is a chronic, multi-stage disease with symptomatic phases that resolve and variable asymptomatic periods (latency) (LaFond & Lukehart, 2006). Symptoms may present in any tissue and mimic other infections, autoimmune diseases and malignancies, delaying treatment which may lead to cardiovascular (CV) or central nervous system (CNS) complications and death (LaFond & Lukehart, 2006; O'Byrne & MacPherson, 2019).

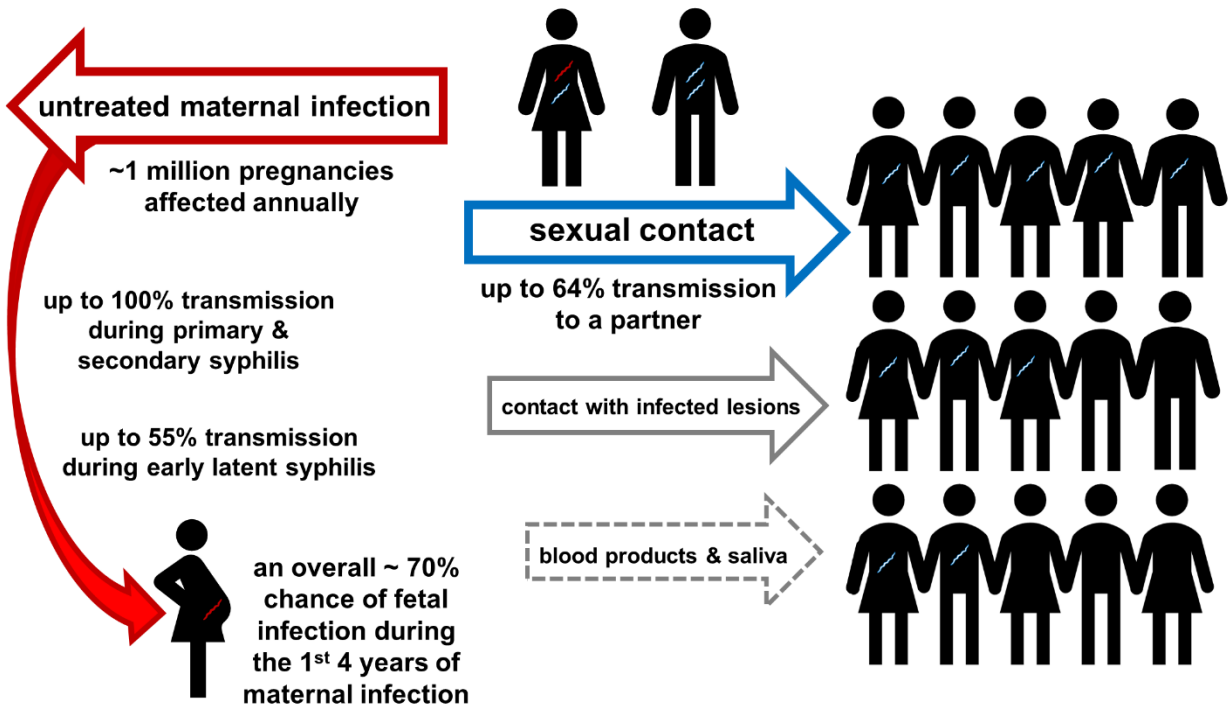

Figure 1: The routes of syphilis transmission. Sexual contact is the primary route of transmission for infectious syphilis, although transmission may also occur through no-sexual contact with infectious lesions or bodily fluids/tissues (LaFond & Lukehart, 2006; Stoltey & Cohen, 2015). Transmission from mother-to-fetus, resulting in congenital syphilis, may occur during any stage of pregnancy (Patel et al., 2017; Stoltey & Cohen, 2015).

Serological diagnosis of syphilis is often made using an algorithm that combines non-(specific) treponemal tests that measure antibodies produced against host cellular damage during infection (rapid plasma regain test, venereal disease research laboratory assay) with treponemal tests which measure antibodies against *T. pallidum* (*T. pallidum* particle agglutination assays, syphilis IgG, enzyme-linked immunosorbent assays) (Dunseth et al., 2017; Naidu et al., 2012). Syphilis can also be diagnosed from lesions by detecting whole organisms (dark-field microscopy, fluorescent antibody *T. pallidum* test) or *T. pallidum* DNA (polymerase chain reaction) (BCCDC, 2016; Dunseth et al., 2017). Algorithms that combine methods help to overcome false-positives, false-negatives, and lack of sensitivity that varies per assay depending on the stage of infection (Dunseth et al., 2017; Naidu et al., 2012). During the early stages of infection (primary, secondary, and early latent) syphilis is treated with 2.4 million units benzathine penicillin G (BPG) by intramuscular (IM) injection, with two additional doses required during late latency (O'Byrne & MacPherson, 2019). Once neurological involvement has occurred curative treatment requires aqueous penicillin G to penetrate the blood-brain barrier (BBB), administered intravenously (IV) over a course of 10 – 14 days (O'Byrne & MacPherson, 2019). When congenital syphilis is suspected IV penicillin is often administered prior to laboratory confirmation due to the increased risk of adverse outcomes with ongoing infection (Lee et al., 2017).

Eliminating syphilis requires access to testing and treatment, a challenge in low income and geographically isolated regions, while high income countries are confronted with social and economic barriers to provide healthcare to key populations where infections

continue to rise (Cameron & Lukehart, 2014; Kojima & Klausner, 2018; LaFond & Lukehart, 2006).

At this time there is no vaccine against *T. pallidum* and the spread of syphilis is only limited through education and treatment (Cameron & Lukehart, 2014; Kojima & Klausner, 2018). In the global effort to eradicate syphilis the most promising strategy remains with prevention versus treatment and underscores the urgency in developing an effective vaccine (Cameron, 2018; Cameron & Lukehart, 2014; Kojima & Klausner, 2018).

1.2 Syphilis: the stages and symptoms

> "...study syphilis thoroughly and you take a knowledge of all others on the way – general medicine, nearly all surgery, and certainly all of the specialties...The syphilis we see but do not recognise everywhere awaits diagnosis, so protean are its manifestations."
>
> - *Annual Oration on The Campaign Against Syphilis* by Sir William Osler, Bart., M.D., F.R.S., delivered before the Medical Society of London, May 14, 1917 (Osler, 1907)

Syphilis is characterized by periods of clinical symptoms (primary, secondary, tertiary) and varying asymptomatic periods (latency) (LaFond & Lukehart, 2006; O'Byrne & MacPherson, 2019). Primary and secondary syphilis are generally marked by highly infectious lesions that potentiate transmission to new individuals, while periods of latency and tertiary syphilis present a reduced risk of transmission (LaFond & Lukehart, 2006).

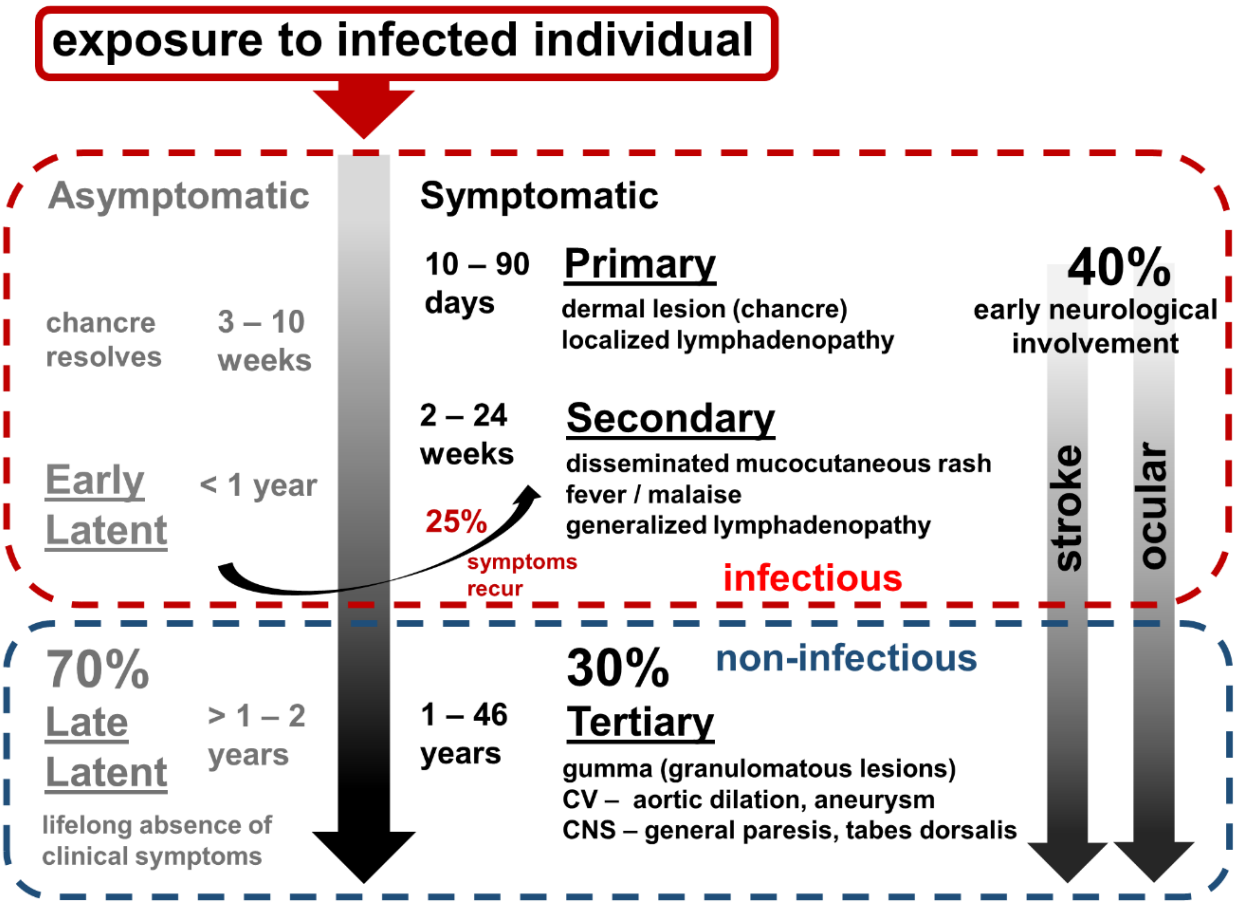

Figure 2: The symptomatic and latent stages of syphilis. Syphilis is a chronic, multistage disease that has symptomatic (primary, secondary, tertiary) and asymptomatic (latent) stages. Neurological involvement occurs in up to 40% of early infections, and during any stage of infection can lead to stroke (LaFond & Lukehart, 2006; O'Byrne & MacPherson, 2019).

1.2.1 Primary syphilis

Infection requires few treponemes for inoculation, the 50% infectious dose is 57 organisms (LaFond & Lukehart, 2006; Magnuson, Harold et al., 1956), which enter micro-abraded keratinized epithelium or intact mucosa (LaFond & Lukehart, 2006) to form a single dermal lesion (chancre) that appears on average 21 days following

exposure (O'Byrne & MacPherson, 2019). The classic symptom of primary syphilis, the chancre, is typically painless, unremarkable, may occur internally and goes unnoticed in 60% of syphilis patients (O'Byrne & MacPherson, 2019). Localized lymphadenopathy may be present (Gaddey & Riegel, 2016; LaFond & Lukehart, 2006) and within 3 -10 weeks the chancre spontaneously heals without scarring (LaFond & Lukehart, 2006; O'Byrne & MacPherson, 2019). During primary syphilis *T. pallidum* rapidly disseminates via blood and lymph vessels and may cross immunologically privileged endothelial barriers that restrict access to the placental, retina, and brain (LaFond & Lukehart, 2006).

1.2.2 Secondary syphilis

The systemic dissemination of *T. pallidum* leads to the symptoms of secondary syphilis 2 – 24 weeks following inoculation, in 15% of cases concurrently with the chancre (LaFond & Lukehart, 2006), or may occur weeks later following an asymptomatic period (O'Byrne & MacPherson, 2019). Systemic infection causes generalized lymphadenopathy in 85% of cases and may include flu-like symptoms and malaise (LaFond & Lukehart, 2006). Migration of treponemes to the epithelium results in the most common symptom, a mucocutaneous rash on the trunk, extremities, and classic of secondary syphilis, frequently also on the palms and soles of the feet (LaFond & Lukehart, 2006). The rash spontaneously resolves and the patient enters a clinically asymptomatic phase (LaFond & Lukehart, 2006; O'Byrne & MacPherson, 2019).

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1.2.3 Latent syphilis and persistence

> "The second point in the biology of the spirochæte is a peculiarity it shares with many other parasites of resting dormant in the body for years. As a rule, such germs, even while retaining their virulence, do little or no damage. Not so the spirochæte, whose capacity to work evil is not to be measured by years. One plain outcome of all recent work is that the untreated or the half-treated syphilitic is a bad life…"
>
> - *Annual Oration on The Campaign Against Syphilis* by Sir William Osler, Bart., M.D., F.R.S., delivered before the Medical Society of London, May 14, 1917 (Osler, 1907)

Asymptomatic periods of variable length occur following the resolution of the chancre in primary syphilis and the rash of secondary syphilis (LaFond & Lukehart, 2006). Early latent syphilis occurs within the first year of infection and remains asymptomatic or reverts to a recurrence of secondary syphilis symptoms in 25% of cases (LaFond & Lukehart, 2006). Late latent syphilis occurs in approximately 70% of cases and the individual remains clinically asymptomatic for the remainder of their lifetime (LaFond & Lukehart, 2006).

1.2.4 Early neurological involvement

Treponemes cross the BBB to invade the central nervous system (CNS) in up to 40% of early infections (LaFond & Lukehart, 2006; Marra, 2015) and while neurologically asymptomatic, 25% to 40% of patients with primary, secondary or early latent syphilis have cerebrospinal fluid (CSF) abnormalities such as pleocytosis and elevated protein

levels (Krishnan et al., 2020; Lukehart et al., 1988; Marra, 2015). Asymptomatic neuroinvasion resolves in 70% of cases, while the remaining 30% continue with chronic infection that may later evolve into symptomatic neurosyphilis (LaFond & Lukehart, 2006; Marra, 2015). During the first few weeks to years of infection, host inflammatory responses can cause syphilitic meningitis or arteritis within cerebral blood vessels (Marra, 2015). This chronic inflammation can cause blood vessel lumens to narrow and platelets to become activated, secrete additional pro-inflammatory factors, and form aggregates that may occlude vessels, and result in ischemic stroke (Fig 3) (Ahbeddou et al., 2018; Golebiewska & Poole, 2015; Marra, 2015; Pagliano et al., 2020; Rayes et al., 2020). In younger patients ischemic stroke is unexpected and may lead to the diagnosis of syphilis (Krishnan et al., 2020), however ocular syphilis, once a major cause of blindness, is frequently the primary neurosyphilis symptom in younger patients during early infection (Landry et al., 2019). Like stroke, ocular syphilis can occur during any stage of infection, leading to inflammatory damage within any ocular structure (Ghanimi Zamli et al., 2019). Although early neurosyphilis is resolved with IV penicillin G for 10 to 14 days (Landry et al., 2019; Marra, 2015), once inflammatory changes have led to stroke, patients may require ongoing anti-platelet therapy and be left with permanent impairment (Ahbeddou et al., 2018; Munshi et al., 2018; Pagliano et al., 2020).

1.2.5 Tertiary syphilis

> "Modern research everywhere leads us to three conclusions:
>
> 1) that there is an immense body of latent syphilis in the community ;
>
> 2) that a very large number of persons have not been thoroughly treated ; and
>
> 3) that to the enormous groups of cerebrospinal and cardiovascular deaths syphilis is an all-important contributor."
>
> - *Annual Oration on The Campaign Against Syphilis* by Sir William Osler, Bart., M.D., F.R.S., delivered before the Medical Society of London, May 14, 1917 (Osler, 1907)

For approximately 30% of untreated syphilis cases serious symptoms appear decades later, taking the form of gummatmous lesions, cardiovascular syphilis (CVS), or late neurosyphilis (LaFond & Lukehart, 2006; Marra, 2015).

Gumma

Chronic infection with *T. pallidum* can lead to gummas, granulomatous inflammatory lesions in skin, bone or internal organs composed of a necrotic central area surrounded by infiltrating macrophages, plasma cells, and fibroblasts (Carlson et al., 2011; LaFond & Lukehart, 2006). Cutaneous lesions represent 70% of gummas and are a classic symptom of tertiary syphilis that responds well to penicillin treatment (Carlson et al., 2011). Gummas occurring within the myocardium, lymph nodes (Carlson et al., 2011), respiratory tract, liver, (LaFond & Lukehart, 2006) and brain, as single or multiple

lesions, can mimic metastasis, delaying diagnosis and treatment (S. Shen et al., 2019). Inflammation of blood vessels within cerebral gummas has resulted in vascular rupture producing massive haemorrhagic stroke (C. Li et al., 2019), unlike ischemic stroke seen in all stages of syphilis (Ahbeddou et al., 2018).

Cardiovascular syphilis

> "For our purpose here, there is but one aortitis - the syphilitic... Syphilitic aortitis is a most distinctive lesion."
>
> - *The Lumleian Lectures on Angina Pectoris*, *Lecture II*, by Sir William Osler M.D., delivered before the Royal College of Physicians of London, on March 15, 1910. (Osler, 1907)

Persistent infection with *T. pallidum* can damage the cardiovascular system when inflamed vessel walls are infiltrated by plasma cells and macrophages, causing the tissue to weaken, become thick, fibrous, less elastic, and prone to aneurysm (Roberts et al., 2009). As the aorta enlarges outwardly, the lumen itself narrows with atherosclerotic plaques (Roberts et al., 2009), fibrous lesions of deposited lipids, leukocytes (Roberts et al., 2009), and platelets (Lebas et al., 2019; Osler, 1881). Syphilitic aneurysm, occurring in up to 50% of cases at the ascending aorta (Paulo et al., 2012), may rupture and lead to sudden death (Fig 3) (Carlson et al., 2011; LaFond & Lukehart, 2006; Roberts et al., 2009).

Platelets play a critical role in the initiation and progression of atherosclerosis by adhering to endothelial cells, leading to platelet activation and secretion of pro-inflammatory cytokines that recruit leukocytes (Nording et al., 2015). Platelets facilitate monocyte extravasation and promote their differentiation to foam cells (Kral et al., 2016;

Nording et al., 2015; Yung-Chih et al., 2016), phagocytic macrophages that enlarge by ingesting lipids and make up a significant portion of an atherosclerotic plaque (Maguire et al., 2019; Mehrpouri et al., 2019). Platelets drive atherogenesis through interactions with endothelial cells that facilitate ongoing leukocyte extravasation and platelet accumulation, leading to plaque growth and later destabilization (Nording et al., 2015; Yung-Chih et al., 2016). Ruptured atherosclerotic plaques can release emboli that may circulate to the brain, lodge within cerebral vessels and cause stroke (Diener et al., 2017; Kral et al., 2016; Rayes et al., 2020; Yung-Chih et al., 2016). Atherosclerosis, driven by inflammatory processes and platelet interactions, plays an important role in the progression of cardiovascular syphilis (Fig 3) (Carlson et al., 2011; Roberts et al., 2009).

Prior to penicillin 5-10% of cardiovascular deaths were attributed to aortic rupture (Paulo et al., 2012) with morphological and histological changes, considered diagnostic, recognized upon autopsy (Paulo et al., 2012; Roberts et al., 2009). In the absence of neurosyphilitic vasculitis, aortic aneurysm has also led to ischemic stroke (Landry et al., 2019). Life-threatening complications of cardiovascular syphilis also include heart failure due to severe aortic regurgitation and myocardial infarction (Roberts et al., 2009). While tertiary syphilis is treated with penicillin, cardiovascular syphilis also requires surgical intervention to repair damaged aortic valves and enlarged aortic aneurysms, which have up to an 80% mortality rate without repair (Paulo et al., 2012).

Late neurosyphilis

After years to decades of asymptomatic syphilis 6.5% of patients will develop late neurosyphilis (LaFond & Lukehart, 2006) with damage to the brain and/or spinal cord

rather than to the meninges and cerebral blood vessels seen during early neurosyphilis (LaFond & Lukehart, 2006; Marra, 2015). General paresis, also known as syphilitic dementia, begins with cognitive changes that progress to psychiatric symptoms, neurological abnormalities (tremor, hypotonia, seizure), and eventually frank dementia (Marra, 2015). Tabes dorsalis follows neural injury and demyelination in the posterior spinal cord and the dorsal root ganglia (Carlson et al., 2011), affecting balance and sensation to cause ataxia, paresthesia, "lightning-like" pain in the legs or abdomen, and dysfunction of the bladder, bowel, and pupils (Argyll- Robertson pupils) (Byard, 2018; Elmouden et al., 2019). The optic nerve is affected in 20% of cases (LaFond & Lukehart, 2006). The degree of nerve and cognitive damage prior to intravenous penicillin treatments limits symptom resolution and regain of function (Elmouden et al., 2019; Marra, 2015).

1.2.6 Congenital syphilis

> "Congenital tuberculosis - how rare! Congenital pneumonia -unknown! - in fact, a killing transmission in the great infections is very rare. In syphilis it stands out less as a biological peculiarity than as a fact of supreme importance in the national health. The spirochete may kill the child in utero, a few days after birth, or within the first two years of life, or the blighted survivor may be subject to innumerable maladies."
>
> - *Annual Oration on The Campaign Against* Syphilis by Sir William Osler, Bart., M.D., F.R.S., delivered before the Medical Society of London, May 14, 1917 (Osler, 1907)

Maternal syphilis, estimated by the WHO to affect 1 million pregnancies worldwide each year (Newman et al., 2015), is the second highest cause of infection-related

stillbirth (Korenromp et al., 2019). During the first four years of infection pregnant mothers have a 70% chance of passing *T. pallidum* to the fetus (Patel et al., 2017) during any stage of pregnancy, making screening and treatment a priority during antenatal care (ANC) visits (Korenromp et al., 2019; LaFond & Lukehart, 2006). Mothers with untreated syphilis are at a 52% risk for ABOs: 21% stillbirth; 6% preterm or low birthweight infants; 9% neonatal death; and 16% of surviving infants will show clinical signs of syphilis (Korenromp et al., 2019). The WHO records the highest number of congenital syphilis (CS) cases, 61% in 2016, in the Africa Region and the lowest numbers in the European Region (Korenromp et al., 2019), demonstrating the importance of access to health care. Adequate treatment of maternal syphilis with benzathine penicillin has been estimated by the WHO to reduce the incidences of stillbirth by 82%, prematurity or low birthweight in infants by 65%, neonatal deaths by 80% and led to a 97% reduction in the incidence of clinical symptoms in infants (Korenromp et al., 2019).

Canada has seen a steady increase in infectious syphilis since the early 2000's, and although prenatal screening and treatment had significantly reduced cases of congenital syphilis, by 2002 Alberta saw its first case in 10 years and was the site of half the cases in Canada from 2002 - 2011 (Verghese et al., 2018). In 2018 British Columbia reached a 30 year high with 919 cases of infectious syphilis, followed by 2 cases of congenital syphilis in early 2019, prompting a BCCDC recommendation and Provincial Health Interim Guideline to increase prenatal testing from a single 1st trimester screen to also include a second test administered any time after 35 weeks during the 3rd trimester or upon admission for delivery (Grennan, Geisbrecht, et al., 2019; Grennan, van

Schalkwyk, et al., 2019). In some cases mothers have tested negatively during the 1st trimester and then test positively in the weeks or months following delivery, delaying treatment, particularly when infants are born asymptomatic (Verghese et al., 2018). Congenital syphilis is divided into early congenital syphilis, within two years of birth and late congenital syphilis, after age two (Choudhri et al., 2018; LaFond & Lukehart, 2006).

Early congenital syphilis

Half of infants born to infected mothers may be asymptomatic at birth, while others demonstrate the classic signs of early congenital syphilis that include snuffles (nasal congestion with highly infectious discharge), lymphadenopathy and mucocutaneous rash (similar to secondary syphilis) (LaFond & Lukehart, 2006; Patel et al., 2017). At birth one-third of infants with congenital syphilis have hepatosplenomegaly, and 40% - 60% have abnormal CSF with elevated protein and/or white blood cells (WBCs) (Arnold & Ford-Jones, 2000; Patel et al., 2017; Verghese et al., 2018). Blood disorders commonly present with abnormal clotting (coagulopathy) (Lee et al., 2017), decreased red blood cells (RBCs) and platelets (thrombocytopenia) (Fig 3) (Patel et al., 2017), while white blood cell (WBC) levels may increase (leukocytosis) or decrease (leukopenia) (Arnold & Ford-Jones, 2000). Abnormal blood cell counts and hepatosplenomegaly mimic congenital leukemia which delays diagnosis and treatment (Lee et al., 2017).

Infants may be born with neurosyphilis symptoms including acute meningitis or chronic meningovascular syphilis that may produce hydrocephalus, cranial nerve palsies or stroke (Arnold & Ford-Jones, 2000). As in adults, congenital syphilis can affect any organ including the kidneys (nephrotic syndrome) and lungs (obliterative fibrosis), or

cause myocarditis, pancreatitis, uveitis or gastritis (Arnold & Ford-Jones, 2000). Diagnosis and treatment is critical to arrest inflammation and limit permanent damage (Arnold & Ford-Jones, 2000; LaFond & Lukehart, 2006).

Late congenital syphilis

Late congenital syphilis, which is defined as disease onset after age two, leads to abnormal growth and development that causes permanent and progressive damage to the eyes, joints, face and skeleton (LaFond & Lukehart, 2006). Neurological involvement can result in developmental delays, seizures, paresis (Arnold & Ford-Jones, 2000), and hearing loss (Marra, 2015), while ocular inflammation can lead to blindness (LaFond & Lukehart, 2006). Facial disfigurement can be caused by abnormal bone development (saddle nose), gummatous erosion of the palate and nasal septum (LaFond & Lukehart, 2006; Pereira et al., 2017), and abnormal tooth development (Hutchinson's incisors, Moon's or Fournier's molars, syphilitic canine) (LaFond & Lukehart, 2006; Nissanka-Jayasuriya et al., 2016). Congenital syphilis can lead to disability due to abnormal development of the long bones (saber shins, Parrot's pseudoparalysis) (LaFond & Lukehart, 2006; Pereira et al., 2017). Although IV BPG clears the infection during late congenital syphilis, the inflammatory damage may be irreversible and continue to progress (LaFond & Lukehart, 2006).

Platelets contribute to syphilis pathophysiology

During different stages of infection, platelets play a role in many of the important pathological outcomes of syphilis, yet aside from thrombocytopenia, which is frequently recognized in congenital syphilis (Fig 3), how infection effects the numerous platelet-

mediated sequelae in adults remains largely uninvestigated (Carlson et al., 2011; LaFond & Lukehart, 2006).

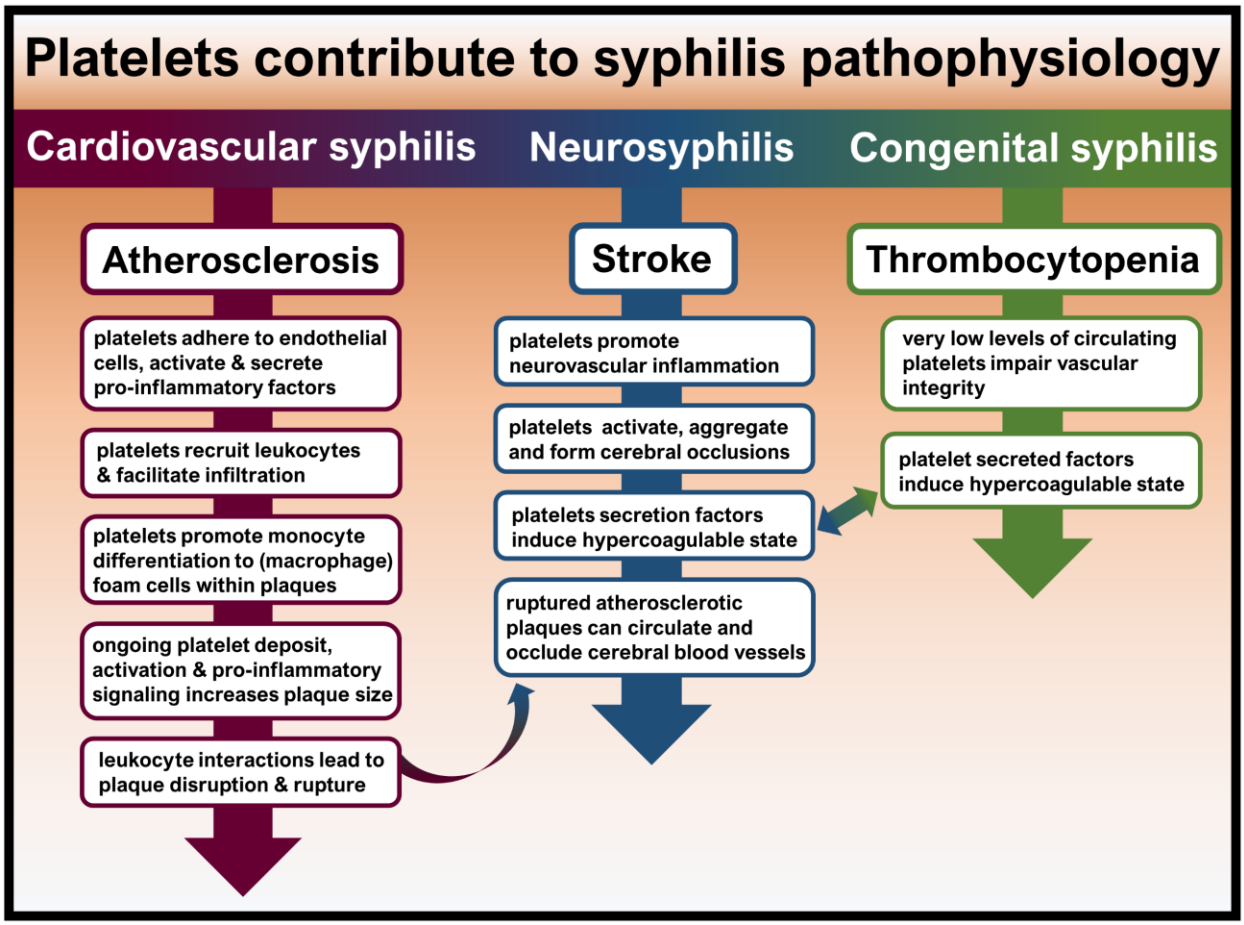

Figure 3: Pathological platelet activation during syphilis. Syphilis demonstrates several examples of infection-induced platelet dysregulation, and while often activated by vascular inflammation, platelet pro-inflammatory responses can lead to chronic inflammation and damage (LaFond & Lukehart, 2006; Marra, 2015; Nicolai & Massberg, 2020). Ischemic stroke can occur following the rupture of atherosclerotic plaques (Yung-Chih et al., 2016) that develop in cardiovascular syphilis, or result from inflammation within cerebral blood vessels during neurosyphilis (Lebas et al., 2019; Marra, 2015;

Nicolai & Massberg, 2020). Infants with congenital syphilis can have many blood abnormalities that often include low platelet count (thrombocytopenia) (Lee et al., 2017).

1.3 *Treponema pallidum*

> "The spirochete of syphilis is easily the most notable among germs. A protozoon-it is the only protozoon; indeed, it is the only germ of world-wide dominion, irrespective of race or clime."
>
> - *Annual Oration on The Campaign Against Syphilis* by Sir William Osler, Bart., M.D., F.R.S., delivered before the Medical Society of London, May 14, 1917 (Osler, 1907)

1.3.1 *Treponema pallidum* subspecies (subsp.) *pallidum*

Syphilis is caused by *Treponema pallidum* subsp. *pallidum (T. pallidum)*, and like *Borrelia burgdorferi* (*B. burgdorferi*) and *Borrelia hermsii* (*B. hermsii*), the causative agents of Lyme disease and relapsing fever, respectively, belongs to the Spirochaetaceae (spirochete) family (LaFond & Lukehart, 2006). While *B. burgdorferi* and *B. hermsii* can be cultured *in vitro* and genetically modified, *T. pallidum* is fragile, an obligate human pathogen so dependent on its host it not been continuously cultured *in vitro* until very recently (Edmondson et al., 2018; LaFond & Lukehart, 2006). The genus *Treponema* (treponemes) contains other human (e.g. *Treponema denticola*) and animal (e.g. *Treponema paraluiscuniculi*) pathogens as well as symbiotic (e.g. *Treponema primitia*) and environmental species (*Treponema caldaria*) (Abt et al., 2013; Buyuktimkin et al., 2019). Closely related human pathogens include *Treponema carateum* (*Treponema carateum*) the causative agent of pinta, *Treponema pallidum* subsp.

pertenue (*T. p. pertenue*) which causes yaws, and *Treponema pallidum* subsp. *endemicum* (*T. p. endemicum*) which causes bejel (Mitjà et al., 2013). Yaws, pinta and bejel are treponemal diseases endemic to hot tropical or desert climates that are transmitted by skin-to-skin or mouth-to-mouth contact (versus sexual and congenital), that produce destructive lesions in skin and bones, but lack the colonization of immunologically privileged tissues seen in syphilis (LaFond & Lukehart, 2006; Mitjà et al., 2013).

1.3.2 Structure and motility

Spirochetes are named for the characteristic helical shape caused by the wrapping of periplasmic flagella around the cytoplasmic cylinder, between the inner and outer membranes (OM) (LaFond & Lukehart, 2006; J. Liu et al., 2010). *Treponema pallidum* is 6 µm to 15 µm in length and 2 µm wide, so narrow that they must be viewed by dark field microscopy (LaFond & Lukehart, 2006; J. Liu et al., 2010). Recent advances in *T. pallidum* ultrastructure from high-resolution cryo-electron tomography (cryo-EM) have shown the cytoplasmic membrane (CM) to be studded with lipoproteins and overlaid with a layer of peptidoglycan (PG) within the periplasm (Izard et al., 2009; J. Liu et al., 2010). The flagellar ribbon is usually composed of three flagella per treponeme, each flagella attached to a motor near the cell tip (Fig 4), and wraps around the PG enclosed cytoplasmic cylinder (J. Liu et al., 2010). Near the flagellar motors chemoreceptors form arrays (J. Liu et al., 2010; Hongbin Xu et al., 2011) to facilitate rapid responses to chemoeffector gradients (Bi & Sourjik, 2018). Lipoproteins are also located directly below the inner leaflet of the OM, which is sparsely populated with host-exposed proteins (LaFond & Lukehart, 2006; J. Liu et al., 2010). The outer membrane of *T.*

pallidum is fragile and lacks lipopolysaccharide (LPS), used by other bacteria to stabilize the OM, but which also elicits a significant host immune response (Buyuktimkin et al., 2019; LaFond & Lukehart, 2006).

Relative to other pathogens that enter through the skin and disseminate systemically, such as *Pseudomonas aeruginosa* (*P. aeruginosa)* and *Staphylococcus aureus* (*S. aureus*), which can induce massive inflammatory responses, septic shock and death (Nguyen & Soulika, 2019), *T. pallidum* migrates inconspicuously through the host (LaFond & Lukehart, 2006). A mechanism to reduce host immune activation through pathogen-associated molecular patterns (PAMPs) is suggested by the OM structure which lacks LPS (LaFond & Lukehart, 2006), detected by host Toll-like receptor (TLR) 4 (Cox et al., 2011; Kawasaki & Kawai, 2014), and exposes few OM proteins on its outer leaflet (LaFond & Lukehart, 2006) to detection by TLR2 (Anwar et al., 2019; Page & Pretorius, 2020). Further, by locating the flagella and PG, important sources of bacterial PAMPs (Page & Pretorius, 2020), below the OM (LaFond & Lukehart, 2006), the intact outer membrane reduces the exposure of key bacterial components recognized by the host, and is likely critical for *T. pallidum* immune evasion.

Another mechanism that may facilitate immune evasion may arise from the flexible cell body and corkscrew-like motility that allows *T. pallidum* to rapidly penetrate tissue and navigate into immunoprotective niches prior to leukocyte arrival (LaFond & Lukehart, 2006; Liu et al., 2010).

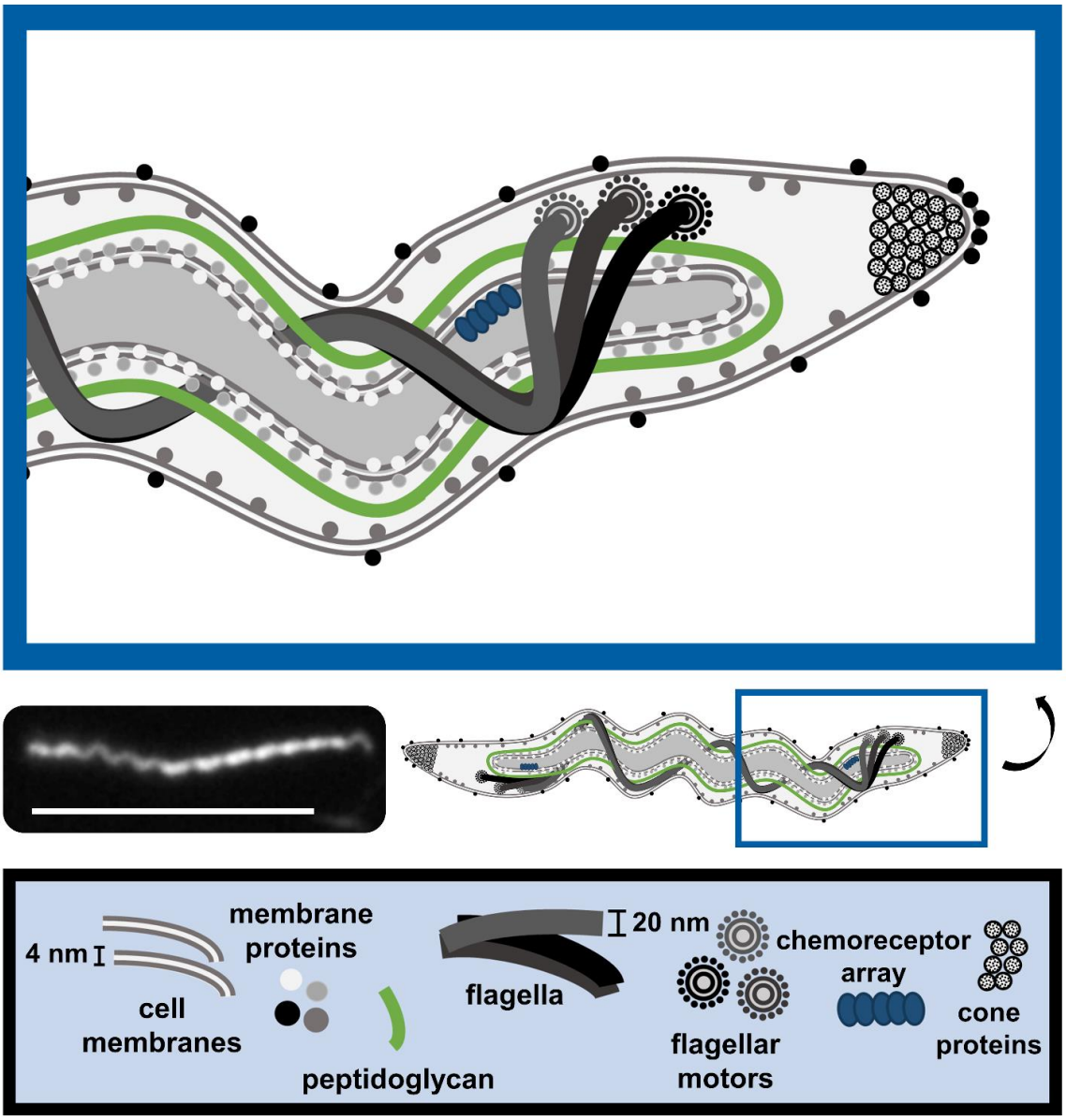

Figure 4: The ultrastructure of *Treponema pallidum*. *Treponema pallidum* has an inner membrane studded with lipoproteins and putative chemoreceptor arrays near the flagellar motors that reside within the periplasm, between a thin layer of peptidoglycan and the outer membrane. Cryo-electron microscopy studies show the presence of a protein cone within the tips of *T pallidum* (J. Liu et al., 2010; Hongbin Xu et al., 2011)**.** Treponeme image scale bar = 10 µm.

1.3.3 Genome

The genome of *T. pallidum* is 1.14 Mb in size, encodes an estimated 1041 open reading frames, and is ~ one-fourth the size of *Escherichia coli* (*E. coli*) K-12's 4.6 Mb genome (LaFond & Lukehart, 2006). Extensive gene loss, particularly of biosynthetic pathways, is common among obligate pathogens which inhabit biomolecule-rich host environments (Kelkar & Ochman, 2013), and *T. pallidum* lacks genes related to the synthesis of nucleotides, amino and fatty acids synthesis and for key enzymes in the tricarboxylic acid cycle (LaFond & Lukehart, 2006). Genes that have been retained by *T. pallidum* encode proteins that facilitate pathogenicity, including multiple *Treponema pallidum* repeat (Tpr) proteins (Addetia et al., 2020; LaFond & Lukehart, 2006). Shown to exhibit intra-patient genetic variations, TprK contributes to evasion of adaptive immune responses and persistence (Addetia et al., 2020; Jaiswal et al., 2020).

Chemotaxis and motility genes make up about 6% of the genome (Charon & Goldstein, 2002; LaFond & Lukehart, 2006), including four sensor genes, methyl-accepting chemotaxis transmembrane proteins (Mcps) and two operons for the downstream regulators, cytoplasmic chemotaxis proteins (Che) (LaFond & Lukehart, 2006).

Genetic diversity within chemotaxis genes occurs at both the intra-patient and intra-strain levels in methyl-accepting chemotaxis protein (*mcp*) 1 (Addetia et al., 2020; Everall & Sánchez-Busó, 2017; Pinto et al., 2016) and chemotaxis protein (*che*) C (Čejková et al., 2015), and may promote survival by chemoreception of host gradients that cue treponeme migration and facilitate immune evasion (Everall & Sánchez-Busó, 2017).

Genomic islands (GIs) within pathogens promote genetic plasticity through the horizontal transfer of genes critical to virulence and chemotaxis genes within *T. pallidum* are located on two GIs (Jaiswal et al., 2020). When compared to the closely related, but not sexually transmitted species, *T. pallidum* subsp. *endemicum* and *T. pallidum* subsp. *pertenue*, the chemotaxis gene *cheA* was detected within only within the GIs of different *T. pallidum* subsp. *pallidum* strains (Jaiswal et al., 2020). Chemotaxis facilitates pathogen motility in response to biomolecular gradients to acquire nutrients and to locate immunoprotective niches to colonize within the host (Charon et al., 2012; Hanyu et al., 2019; Matilla & Krell, 2017).

Given the total dependence on the acquisition of host biomolecules (Brautigam et al., 2016; LaFond & Lukehart, 2006) and localization to an immunoprotective niche (Carlson et al., 2011), and the genetic diversity seen within the *cheA*, *cheC* and *mcp1* genes (Addetia et al., 2020; Čejková et al., 2015; Everall & Sánchez-Busó, 2017; Jaiswal et al., 2020; Pinto et al., 2016), chemotaxis likely plays a crucial role in *T. pallidum* pathogenesis.

1.3.4 The challenges of *T. pallidum* biology in the course of research.

The biology of *T. pallidum* has presented distinctive challenges that have limited both the rate at which research questions have been answered and the techniques available with which to answer them. The most significant challenges are: 1) access to viable organisms, 2) maintaining viable organisms *in vitro* during the experiment/s 3) organism intractability to genetic manipulation, 4) purification/concentration of treponemes without damage to the fragile outer membrane, and 5) difficulties encountered with obtaining a scheduled supply of viable *T. pallidum*.

Most of these challenges have rested upon a single limiting factor - the inability to maintain long-term *in vitro* culture of *T. pallidum*, which restricted viable organisms for research to those propagated *in vivo* within rabbits (Lukehart & Marra, 2007). The challenges of this are ethical, economic, and very practical as the timing of *T. pallidum* extraction is dependent upon the rate of treponeme replication in each outbred rabbit (Lukehart & Marra, 2007). In addition, the extracted treponemes are surrounded by rabbit-derived host components (Cox et al., 1992) and have a limited lifespan *in vitro* (Fitzgerald et al., 1975a; Fitzgerald et al., 1977). Decades of persistent optimization of mammalian cell co-culture systems (Fitzgerald et al., 1977; Norris, 1982; Norris & Edmondson, 1988; Norris & Edmondson, 1986) has led to the recent demonstration of long-term culturing of *T. pallidum in vitro* (Edmondson et al., 2018). This significant development, using rabbit epithelial cells in co-culture, holds the promise of viable treponemes available on-demand for experimental use with far fewer rabbit contaminants. Additionally, the pursuit of *T. pallidum* culture advanced our knowledge of optimal *in vitro* conditions to extend viability during experiments: microaerophilic conditions (~ 1.5% O_2) and a temperature of 34°C (Edmondson et al., 2018).

Ongoing challenges remain the inability to genetically manipulate *T. pallidum*, a basic tool in microbiology to understand the function of particular genes and their role in pathogenesis (LaFond & Lukehart, 2006). To directly bypass this potential virulence factors of *T. pallidum* have been heterologously expressed in related spirochetes, such as *B. burgdorferi* (Kao et al., 2017). Biomolecular techniques, using recombinantly expressed proteins, have facilitated solving the structures of proteins unique to *T. pallidum* (Brautigam et al., 2018; Parker et al., 2016), *T. pallidum* protein-host

component interactions (Cameron, 2003; Cameron et al., 2004, 2005, 2008), and how these may modulate the host response to facilitate pathogenesis (Lithgow et al., 2020).

Although many advances and the use of alternative approaches are leading to a greater knowledge of pathogenesis, researchers will continue to be challenged by aspects of *T. pallidum* biology, particularly the inherently fragile outer membrane, which constrains experimental techniques involving live organisms (Izard et al., 2009; LaFond & Lukehart, 2006).

1.3.5 *Treponema pallidum* interactions with host cells during infection.

While the biology of *T. pallidum* has presented many challenges to researchers, it is exquisitely adapted to survival within the host. *Treponema pallidum* is remarkably invasive, crossing immunologically privileged barriers, resistant to most pathogens, to colonize niches that may facilitate immune evasion and contribute to lifelong persistence within the host (LaFond & Lukehart, 2006). During infection *T. pallidum* interacts with numerous types of tissue and immune cells by adhesion, activation or through interactions that lead to the destruction of the host cell or treponeme (Carlson et al., 2011; LaFond & Lukehart, 2006).

1.3.6 Treponemes within the chancre

Treponema pallidum localizes to the lower layer of the epidermis (Carlson et al., 2011) where it replicates every 30-33 hours (Ho & Lukehart, 2011) until the localized inflammation leads to the development of a 0.5-2 cm chancre (Byard, 2018; Carlson et al., 2011) an average of 21 days after infection (Li et al., 2019). Within the skin treponemes encounter keratinocytes (epithelial), resident myeloid immune cells such as

Langerhans cells (LCs), dendritic cells (DCs) and macrophages (Mϕ), and the endothelial cells (ECs) of capillaries that extend up into the epidermis (Nguyen & Soulika, 2019). Keratinocytes and DCs express pattern recognition receptors (PRRs) that are activated by PAMPS to secrete cytokines (Muraille et al., 2014; Nguyen & Soulika, 2019). Keratinocyte pro-inflammatory cytokines, such as IL-6, recruit and activate T cells, and during syphilis infections keratinocytes proliferate within dermal lesions (Carlson et al., 2011).

Chancres are highly vascularized and show a “pathogenic angiogenesis” characteristic of syphilis (Carlson et al., 2011; Gao et al., 2019), where most treponemes reside in or around the new blood vessels (Juanpere-Rodero et al., 2013). Bacterial-induced angiogenesis facilitates access to host biomolecules, particularly important for pathogens with reduced genomes like *T. pallidum*, and access to the circulatory system for widespread dissemination (Osherov & Ben-Ami, 2016; Pozzobon et al., 2016). *Treponema pallidum* induces a diverse network of blood vessels (Pozzobon et al., 2016), forming a complex vascular web within the chancre, that may provide a nutrient-rich niche that also slows the ability of immune effectors to locate and clear treponemes (Carlson et al., 2011).

As treponemes multiply, however, a delayed type hypersensitivity (DTH) response occurs within the chancre, mediated by a cluster of differentiation (CD) 4+ T helper (Th) 1 and classically-activated (M1) macrophage response (Carlson et al., 2011). Macrophages accumulate and phagocytose treponemes to effectively clear infection within the chancre, allowing the lesion to heal, without scarring, 3 to 8 weeks after it appeared (Carlson et al., 2011; Ho & Lukehart, 2011).

1.3.7 Disseminating treponemes

> "Germs show singular preference for different parts of the body…*Spirochæta pallida* for the nervous system and the blood vessels…The spirochæte attacks the vascular system…"
>
> - *Annual Oration on The Campaign Against Syphilis* by Sir William Osler, Bart., M.D., F.R.S. delivered before the Medical Society of London, May 14, 1917 (Osler, 1907)

While a subpopulation of *T. pallidum* remains at the initial site of infection and replicates, other treponemes rapidly disseminate via lymph and blood vessels, penetrating distant tissues and organs within hours (LaFond & Lukehart, 2006; Salazar, et al., 2007) and crossing the BBB in 40% of early syphilis cases (LaFond & Lukehart, 2006). Dissemination leads to systemic infection and the symptoms of secondary syphilis that include malaise, lymphadenopathy and rash (LaFond & Lukehart, 2006).

Vascular dissemination

Vascular dissemination is critical to the pathogenesis of *T. pallidum* and the mechanisms by which treponemes interact with the endothelium facilitates adhesion to vessel walls, extravasation, and tissue colonization (LaFond & Lukehart, 2006; Lithgow et al., 2020). Treponemes readily interact with endothelial cells *in vitro* (Thomas et al., 1988, 1989; Wu et al., 2017), and have been shown to use outer membrane proteins (OMPs) to mediate interactions with several extracellular matrix (ECM) components (Cameron, 2003; Cameron et al., 2004, 2008; Fitzgerald et al., 1984). *Treponema pallidum* also interacts directly with endothelial cell receptors, recently demonstrated by

OMP Tp0751 interactions with the endothelial laminin receptor (LamR) (Lithgow et al., 2020), a recognized target of neuroinvasive bacterial pathogens (Al-Obaidi & Desa, 2018). In bacteria with reduced genomes proteins frequently demonstrate multiple functions/binding partners (Kelkar & Ochman, 2013), and Tp0751 has also been shown to interact with laminin (Cameron, 2003; Cameron et al., 2005), fibrinogen (Houston et al., 2011), fibronectin, collagen I and collagen IV (Parker et al., 2016). Additionally, the *T. pallidum* adhesins Tp0155 and Tp0483 have been shown to bind to fibronectin (Cameron et al., 2004), and Tp0136 has been shown to bind both fibronectin and laminin (Brinkman et al., 2008).

Within the vasculature *T. pallidum* is exposed to RBCs, which travel centrally within larger vessels, and to platelets, which circulate as immune sentinels close to vessel walls where they interact with endothelial cells and thus would naturally encounter any treponemes adhered during extravasation (Gaertner & Massberg, 2019; Guo & Rondina, 2019; McDonald & Dunbar, 2019). Platelets play important roles not only in haemostasis, but in pathogen sensing, modulating endothelial permeability, immune responses and effector recruitment through rapid secretion of proteins, lipids, cytokines and chemokines (Gaertner & Massberg, 2019; Guo & Rondina, 2019; Manne et al., 2017). During syphilis specific sequelae indicate platelet involvement, including thrombocytopenia in congenital syphilis (Patel et al., 2017), stroke in neurosyphilis, and the atherosclerosis that occurs during cardiovascular syphilis (LaFond & Lukehart, 2006).

Chemotaxis

For motile pathogens chemotaxis is critical during dissemination to locate nutrients and specific host tissues to establish niches that facilitate persistence (Matilla & Krell, 2017; Motaleb et al., 2015). The related pathogenic spirochete *B. burgdorferi* relies on chemotaxis for transmission between the *Ixodes* tick and mammalian hosts, dissemination from the tick bite into deeper mammalian tissues and organs, and to establish a persistent infection (Xu et al., 2017). Chemotaxis likely plays as key a role in *T. pallidum* dissemination and persistence as seen with other pathogenic spirochetes (LaFond & Lukehart, 2006). While disseminating *T. pallidum* must acquire host biomolecules and localize to niches that provide protection from immune clearance and promote persistence (LaFond & Lukehart, 2006). Chemoreceptor arrays sense host biomolecule attractant and repellent gradients and relay signals to flagellar motors that direct treponeme motility (Liu et al., 2010). One example is the *T. pallidum* chemoreceptor Tp0684 that promotes D-glucose acquisition (Brautigam et al., 2016), and other biomolecule gradients likely aide treponemes in locating and crossing immunologically privileged barriers (Jaiswal et al., 2020; LaFond & Lukehart, 2006).

Treponema pallidum migrates rapidly from the initial site of infection using lymph and blood vessels to disseminate, while failing to induce high cytokine levels within the bloodstream (Cruz et al., 2012) that characterize systemic infections by other pathogens (Assinger et al., 2019; Cagliero et al., 2018). The ability of *T. pallidum* to evade immune clearance and establish persistent infection is likely aided by several factors that include: fewer organisms within the blood due to a slow rate of replication and few OMPs, relative to other pathogens (Cruz et al., 2012; LaFond & Lukehart, 2006); and antigenic variation within OMPs which produces treponeme subpopulations (Cruz et al.,

2012; Giacani, Brandt, et al., 2012; LaFond & Lukehart, 2006). In addition, the interaction of *T. pallidum* with host cells through adhesion and activation may not only facilitate access to immune privileged tissues but may also modulate recruitment, polarization, and signaling by immune responsive cells, altering the microenvironment to favour treponeme persistence (Atri et al., 2018; Babolin et al., 2011; Carlson et al., 2011; LaFond & Lukehart, 2006; Osherov & Ben-Ami, 2016). Platelets are now increasingly recognized to play conflicting roles during infection where pathogen interactions may benefit the host through immune activation (Hamzeh-Cognasse et al., 2015; Koupenova et al., 2018; Li et al., 2017; Rayes et al., 2020; Ribeiro et al., 2019), or benefit the pathogen by facilitating dissemination and immune evasion (Hally et al., 2018; Hamzeh-Cognasse et al., 2015; Rayes et al., 2019).

1.3.8 The potential importance of platelets to *T. pallidum* pathogenesis.

Long established as critical players in vascular haemostasis, platelets were disregarded for decades as having any other function (Coller, 2011). More recently, platelets have come to be recognized as important modulators of the host immune response and play significant roles in many disease processes (Gianazza et al., 2020; Hally et al., 2019; Kral et al., 2016; Tamagawa-Mineoka, 2015). Platelets react rapidly to changes in their microenvironment and secrete effector molecules that alter vascular permeability, recruit leukocytes and shift leukocyte polarization (Koupenova et al., 2018; Kral et al., 2016).

Platelets recognize invading pathogens, arrest their dissemination and recruit leukocytes to potentiate immune clearance (Koupenova et al., 2018; Page & Pretorius, 2020; Rosowski & Huttenlocher, 2018), yet when targeted by pathogens they act as

powerful agents that help traffic bacteria, promote dissemination into organs and tissues, and skew the immune response to facilitate persistence (Cox et al., 2011; Petersson et al., 2018). Platelet secretions are also a rich source of rapidly available biomolecules which can be metabolized by bacteria (P. Ketter et al., 2017).

Treponema pallidum is an incredibly invasive pathogen with ample opportunity to interact with platelets during cutaneous inflammation (LaFond & Lukehart, 2006; Tamagawa-Mineoka, 2015) and vascular dissemination (Gaertner & Massberg, 2019; LaFond & Lukehart, 2006). The unique biology of platelets positions them as powerful modulators of their microenvironment (Rayes et al., 2020), which may contribute to the ability of treponemes to enter immune privileged tissue and establish chronic infection (Binsker et al., 2017; LaFond & Lukehart, 2006).

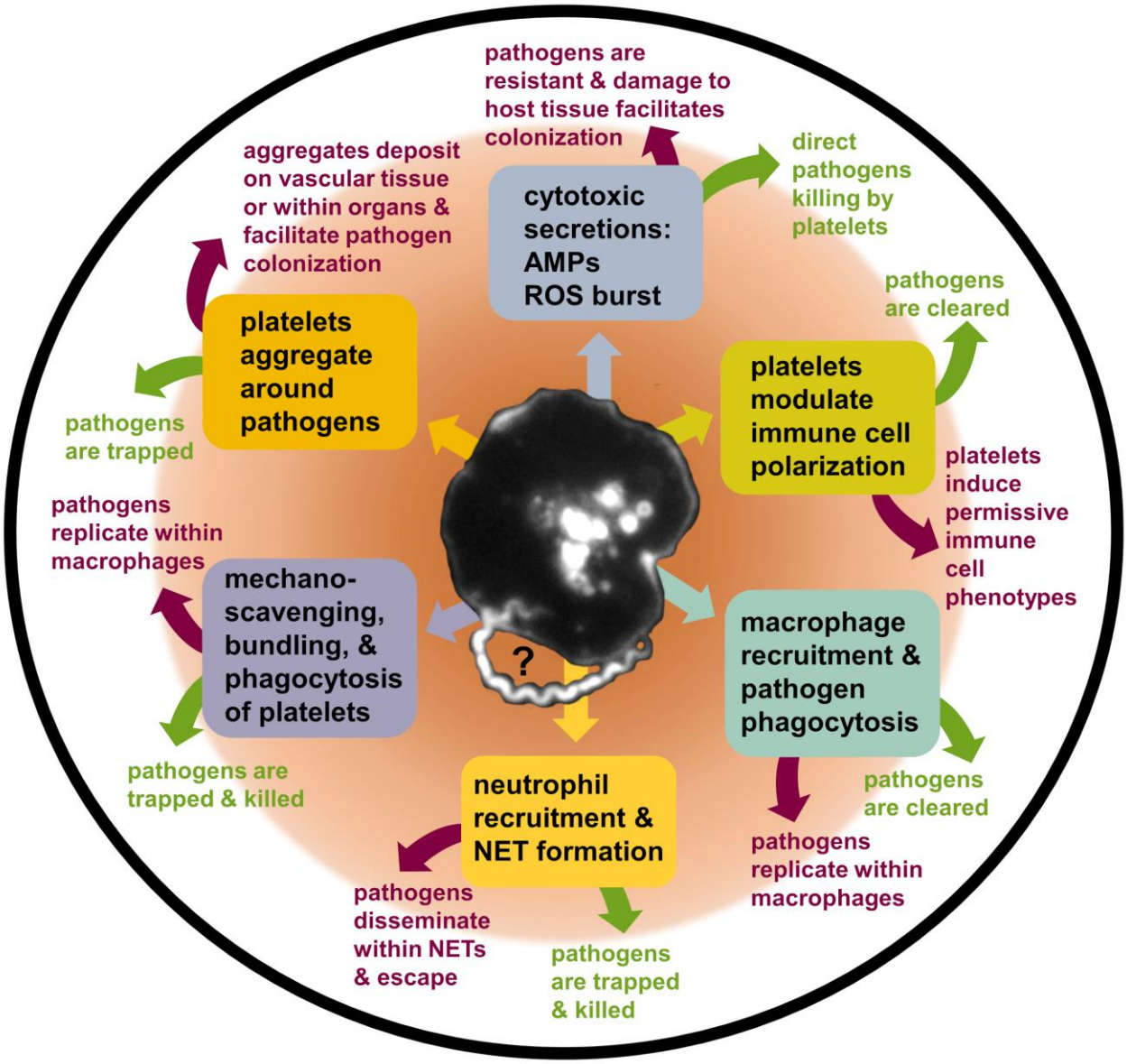

Figure 5: Platelets mediate host immune responses that can clear or promote infections. Activated platelets are inherently able to trap and kill pathogens while mobilizing the host immune response, however co-evolution has allowed pathogens to avoid or subvert many platelet response to facilitate pathogenesis (Liesenborghs et al., 2018; McDonald & Dunbar, 2019). Whether human platelets play a role in containing or aiding *T. pallidum* remains to be elucidated.

1.4 Platelets – "the Third Corpuscle"

To appreciate the potential advantages for pathogens that localize towards platelets activated under certain conditions, or interact directly with platelets themselves, the structure, secretory capacity and vast array of platelet receptors will be introduced. The broad range of platelet functions have made them attractive pathogen targets, and through specific interactions platelet responses can be shaped to suit the requirements of a specific pathogen (Hamzeh-Cognasse et al., 2015; Manne et al., 2017; Osherov & Ben-Ami, 2016; Petersson et al., 2018).

Platelets undergo remarkable cytoskeletal changes and the following is one of the earliest descriptions of platelets as a unique blood cell with an excellent description of the transition from the resting to the early activated platelet:

> "In a small artery or vein, there will be seen with the red and white cells small, pale corpuscles about one-fourth the size of the red ones, often in extraordinary numbers…
>
> The corpuscles are discoid, pale, structureless and often undergo peculiar alterations in shape, elongating or presenting two or three fine hair-like extensions.
>
> To conclude: 1st. There is in mammalian blood a third corpuscular element, one-eight to one-half the size of the red corpuscle… It may be called appropriately the third corpuscle, or 'blood-plate', though the latter expression is not a very satisfactory one."
>
> - *The Third Corpuscle of the Blood* by William Osler M.D., The Medical News, December 29, 1883 (Osler, 1881)

1.4.1 Human platelets

Platelets, also known as thrombocytes (Gaertner & Massberg, 2019), are the second most prevalent blood cell (Ponomarev, 2018) at 1.5 - 4.5 x 10^5 per µL of blood (Gianazza et al., 2020; Melchinger et al., 2019) and circulate 7 – 10 days before being cleared by the spleen and liver (Gianazza et al., 2020; Melchinger et al., 2019). Human (mammalian) platelets, unlike avian thrombocytes, have dispensed with a nucleus (Melchinger et al., 2019) which results in a thin (~ 0.5 µm thick) (Gremmel et al., 2016), small cell (1 - 3 µm), smaller than the average human nucleus (6 µm), with the flexibility to traverse the smallest blood capillaries and lymph vessels (Melchinger et al., 2019).

Platelets arise from megakaryocytes (MKs), large progenitor cells (50 – 100 µm) (Li et al., 2017) within the lungs and bone marrow, that produce ~ 1000 platelets each from cytoplasm-filled membrane extensions that bud off and are released into circulation (Melchinger et al., 2019). The cytoplasm within the budding platelet contains mitochondria, ribosomes, and specialized secretory granules, while the surrounding membrane displays a vast array of receptors (Gianazza et al., 2020; Li et al., 2017; Rayes et al., 2020; Ribeiro et al., 2019). These receptors allow platelets to be activated by numerous mechanisms of receptor-ligand or receptor-protease interactions that signal a change in the status of their microenvironment (Burzynski et al., 2019; Li et al., 2017; Page & Pretorius, 2020; Pokrovskaya et al., 2020).

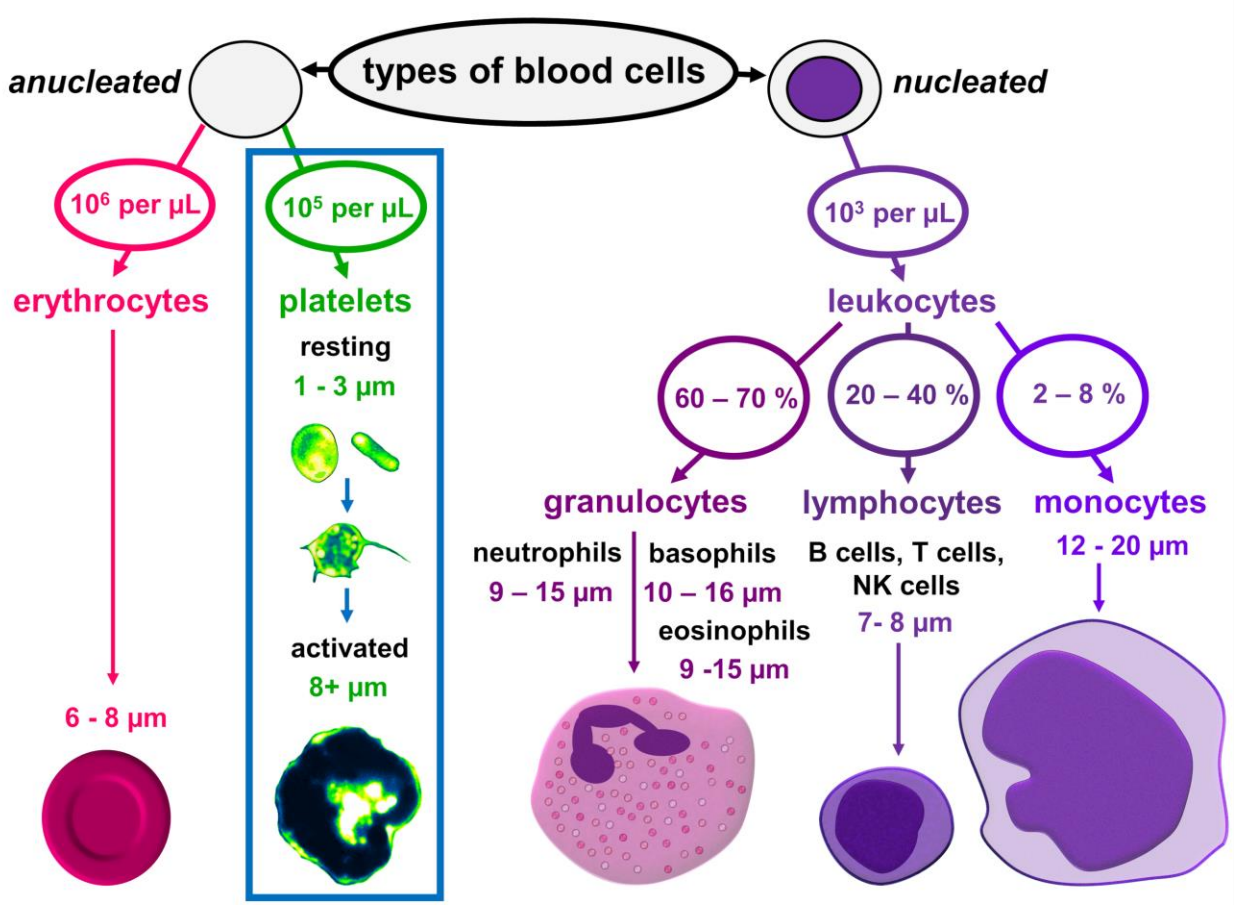

Figure 6: Circulating blood cells. Blood cells are both nucleated (leukocytes) and anucleated (platelets and RBCs). The majority of blood cells are anucleated red blood cells, also known as erythrocytes, and platelets (boxed) that number from 3.5 – 5.9 x 10^6 and 1.5 - 4.5 x 10^5 per µL of blood, respectively (Chang et al., 2018; Dean, 2005; Gianazza et al., 2020). In the absence of infection circulating leukocytes range from 4.0 to 11.0 x 10^3 per µL of blood, the majority of which are neutrophils, a type of granulocyte (Chmielewski & Strzelec, 2018; Prinyakupt & Pluempitiwiriyawej, 2015; Teng et al., 2017).

1.4.2 Platelet structure

Platelets circulate in their resting (inactive) state as flattened discs, with a complex membrane that contains the open canaliculi system (OCS), an invaginated section of membrane that provides an extensive surface area for receptors to interact with and monitor the surrounding milieu (Li et al., 2017; Melchinger et al., 2019; Pokrovskaya et al., 2020). In addition to the OCS, platelets have a specialized, endoplasmic reticulum (ER)-like structure referred to as the dense tubular system (DTS), which may produce a non-canonical platelet “T” granule characterized by the expression of TLR9 and protein disulfide isomerase (PDI) (Gianazza et al., 2020; Sharda & Flaumenhaft, 2018; Yadav & Storrie, 2017).

The most recognized platelet organelles are mitochondria (~ 3 – 11 / platelet) (Melchinger et al., 2019; Pokrovskaya et al., 2020) and the three types of membrane-enclosed storage granules (lysosomal, dense and α-granules) that secrete biomolecules following platelet activation (Gremmel et al., 2016; Li et al., 2017; Manne et al., 2017; Tamagawa-Mineoka, 2015). Platelet granule secretion is regulated through agonist-specific receptor signaling that leads dense granules, which localize close to the plasma membrane (Pokrovskaya et al., 2020), to be secreted rapidly, followed by α-granules, and then lysosomal granules (Sharda & Flaumenhaft, 2018).

Platelets also contain functional ribosomes capable of translating MK-derived or endocytosed mRNA (Li et al., 2017), and upon platelet activation rapidly synthesize and secrete important mediators such as prostaglandin E2 (PGE2) (Linke et al., 2017), Tissue Factor (TF) (Guo & Rondina, 2019), and the cytokine IL-1β (Koupenova et al., 2018).

The key to platelets' unique ability to activate and modulate nearly every other cell type (Clemetson, 2010; Langan et al., 2018; Ponomarev, 2018; Ribeiro et al., 2019; Tamagawa-Mineoka, 2015) rests within two fundamental properties 1) the multitude of receptors platelets express that regulate the mechanism of platelet activation, leading to specific signaling and secretion (Clemetson & Clemetson, 2013; Li et al., 2017; Rayes et al., 2020), and 2) the expansive list of effector molecules platelets secrete that in turn regulate other cells (Koupenova et al., 2018; Pagel et al., 2016; Tamagawa-Mineoka, 2015).

Pathogens that mediate interactions with platelets have the potential to direct how platelets regulate the actions of other cell types, from endothelial to immune cells, during different stages of infection to promote immune evasion and survival (Fox et al., 2018; Haworth et al., 2017).

Platelet granules

In addition to modulation of host cellular responses (Gremmel et al., 2016; Koupenova et al., 2018; Li et al., 2017), platelet granules are sources of readily accessible biomolecules (Ketter et al., 2017)**.**

α-granules

Platelet α-granules play significant roles in pathogenesis as the largest (200 – 400 nm), most abundant (40 – 80 / platelet) granule that contains the vast majority of cytokines, chemokines, plasma proteins, growth factors and receptor ligands secreted by platelets (Li et al., 2017; Pokrovskaya et al., 2020; Tamagawa-Mineoka, 2015; Yadav & Storrie, 2017). Platelet α-granules are an important source of receptors including Glycoproteins

(GP) -VI and GPIb-IX, Platelet and endothelial cell adhesion molecule-1 (PECAM-1), Major histocompatibility complex (MHC) class I, and the integrin heterodimer αIIbβ3, which can be incorporated into the platelet membrane during activation (Yadav & Storrie, 2017). Proteins stored within α-granules are formed through DTS synthesis pathways and by MK/platelet endocytosis of plasma proteins via both receptor-mediated endocytosis and pinocytosis (Sharda & Flaumenhaft, 2018). Endocytosis allows platelets to acquire and secrete the products of other cells, including immunoglobulins from their surrounding milieu, vascular endothelial growth factor (VEGF), endostatin (Sharda & Flaumenhaft, 2018), and fibrinogen (Manne et al., 2017). Platelet α-granules are a rich source of cytokines that recruit leukocytes, induce specific T cell (Margraf & Zarbock, 2019) and monocyte phenotypes and regulate macrophage differentiation (Kral et al., 2016). Proteomic studies have quantified proteins secreted from activated platelets, the majority from α-granules, with mass spectroscopy (MS) results ranging from 300 to 4500 unique proteins, depending on the mechanism of activation and detection (Gianazza et al., 2020). Platelet α-granules release many of the key components (Table 1) for haemostasis, pathogen responses, and immune modulation (Gaertner & Massberg, 2019), yet many of the components they store have conflicting functions in angiogenesis (Yadav & Storrie, 2017), inflammation (Kral et al., 2016; Li et al., 2017), and immune cell polarization (Carestia et al., 2019; Chatterjee et al., 2015; Mehrpouri et al., 2019). Platelet α-granule components that may play a role in *T. pallidum* pathogenesis include receptors that mediate spirochete-platelet interactions (i.e. αIIbβ3 for *Borrelia* species) (Alugupalli et al., 2003; Coburn et al., 1993), and adhesive glycoproteins (i.e. fibrinogen), which are key to many host-pathogen

interactions, not only with platelets (Hamzeh-Cognasse et al., 2015; Koupenova et al., 2018), but also during neuroinvasion (Table 1, Fig 7) (Al-Obaidi & Desa, 2018).

α-granules							
Receptors	Haemostasis	Chemokines	Growth Factors	Proteases	Immunity	Antimicrobial Factors	Other Proteins
α2β1	Factor II	CCL1	VEGF	MMP1	IL-1α	C3 precursor	GMP33
α5β1	Factor V	CCL2	bFGF	MMP2	IL-1β	C4 precursor	Rab-27B
α6β1	Factor VII	CCL3	PDGF	MMP9	IL-4	CFD	
αLβ2	Factor XI	CCL5	FGF	TIMP1	IL-8	IgA	Rab-4
αvβ3	Factor XIII	CCL7	EGF	TIMP4	IL-10	IgG	Rap1
P-selectin	Kininogens	CCL8	HGF	PDCI	IL-13	IgM	
GPIb-IX	Protein S	CCL15	ECGF	PAI-1	IL-17		VAMP-2
GPV	Plasminogen	CCL17	IGF	TFPI	C1 inhibitor	Thymosin-β4	VAMP-3
αIIbβ3			TPO	PN-II	CFH		VAMP-7
GPIV	Fibrinogen	CXCL1	ANG		OSM	Thrombocidin-1	VAMP-8
PECAM-1	Fibronectin	CXCL4	ANGPT-1		PGE2		
CD9/p24	Vitronectin	CXCL5	PAF		TNFα	Thrombocidin-2	HRPG
GLUT-3	VWF	CXCL7	S1P		TNFβ		SRGN
Osteonectin	TSP-1	CXCL8					Albumin
Stomatin		CXCL12	Endostatin				
LTBP-1	α2-antiplasmin	CXCL14	ANGPT-2				
Clusterin							
CD40L							
MHC class I							
TLT-1							

Table 1: Important α-granule components that modulate vascular permeability, haemostasis, and immune responses. Activated platelets upregulate receptors during α-granule secretion (Clemetson & Clemetson, 2013; Gaertner & Massberg, 2019; Smith

et al., 2018), and release a multitude of cytokines and proteins (Amable et al., 2013; Hotta et al., 2019; Koupenova et al., 2018; Mussano et al., 2016).

Dense granules

Dense granules are smaller (<150 nm) and much fewer, usually 3 – 8 per platelet (Gremmel et al., 2016; Pokrovskaya et al., 2020; Tamagawa-Mineoka, 2015), with many located near the cell membrane to facilitate the rapid secretion of small molecules (Pokrovskaya et al., 2020). Many of these molecules act on nearby cells and quickly amplify platelet activation (Cox et al., 2011; Tamagawa-Mineoka, 2015), however, released nucleotides may signal cell damage/stress and induce or inhibit additional platelet activation (Tamagawa-Mineoka, 2015). Platelet dense granules store and secrete Ca^{2+} and Mg^{2+} which stabilize extracellular integrins (Saboor et al., 2013), and release neurotransmitters with pleiotropic effects on immune modulation and vascular integrity (Cloutier et al., 2018; Ponomarev, 2018). Platelet serotonin, for example, increases vascular permeability (Cloutier et al., 2018), alters T cell proliferation (Ponomarev, 2018), and increases *P. aeruginosa* virulence by acting as a quorum sensing molecule (Knecht et al., 2016). *Streptococcus sanguinis* (*S. sanguinis*) platelet activation releases dense granule components, including ATP, which is hydrolyzed to ADP by an ectoATPase on the bacterial membrane, leading to the formation of stable platelet aggregates (Cox et al., 2011) around the bacteria which shields it from the immune system (Haworth et al., 2017). While dense granule secretions may increase vascular permeability and aid in *T. pallidum* dissemination, much remains to be elucidated on host-molecule utilization, and it is possible that *T. pallidum*, like some

bacterial pathogens, is able to take up and utilize host ATP (Fig 7) (Driscoll et al., 2017; Fuchs et al., 2012; Spari & Beldi, 2020).

Dense granules			
Granule content			Membrane content
cAMP	PPi	Dopamine	αIIbβ3
ADP	Ca2+	Epinephrine	GPIb
ATP	K+	Glutamine	LAMP-2
GTP	Mg+	Histamine	CD63
UTP	GABA	Serotonin	
polyP	Phosphatases		

Table 2: Dense granules. Release of dense granule contents can alter vascular permeability and cause additional platelets to become activated (Cloutier et al., 2018; Koupenova et al., 2018; Tamagawa-Mineoka, 2015)

Lysosomal granules

Lysosomal granules are ~ 200 nm in diameter, number 1 - 3 per platelet (Gremmel et al., 2016; Sharda & Flaumenhaft, 2018), and contain protein degrading cathepsin D & E, elastase and collagenase (Gremmel et al., 2016). Granules are characterized by expression of lysosomal-associated membrane protein (LAMP)-2 and CD63 (Table 3) (Gremmel et al., 2016). Lysosomal secretion contributes to antimicrobial responses (Yun et al., 2016), cell signaling and inflammation (Tamagawa-Mineoka, 2015), however

much remains to be elucidated about their role/s in haemostasis and platelet function (Gremmel et al., 2016). *Treponema pallidum* dissemination may be enhanced when platelet secreted proteases degrade host structures.

Lysosomal granules		
Granule content		**Membrane content**
Acid phosphatase	Collagenase	LAMP-1
α-arabinosidase	Elastase	LAMP-2
Carboxypeptidase	β-galactosidase	CD63
Cathepsin D	β-glucuronidase	
Cathepsin E	β-hexosaminidase	
n-acetylglucosaminidase		

Table 3: Lysosomal granules. Lysosomal granules contain many enzymes that cleave host proteins and carbohydrates (Gremmel et al., 2016)

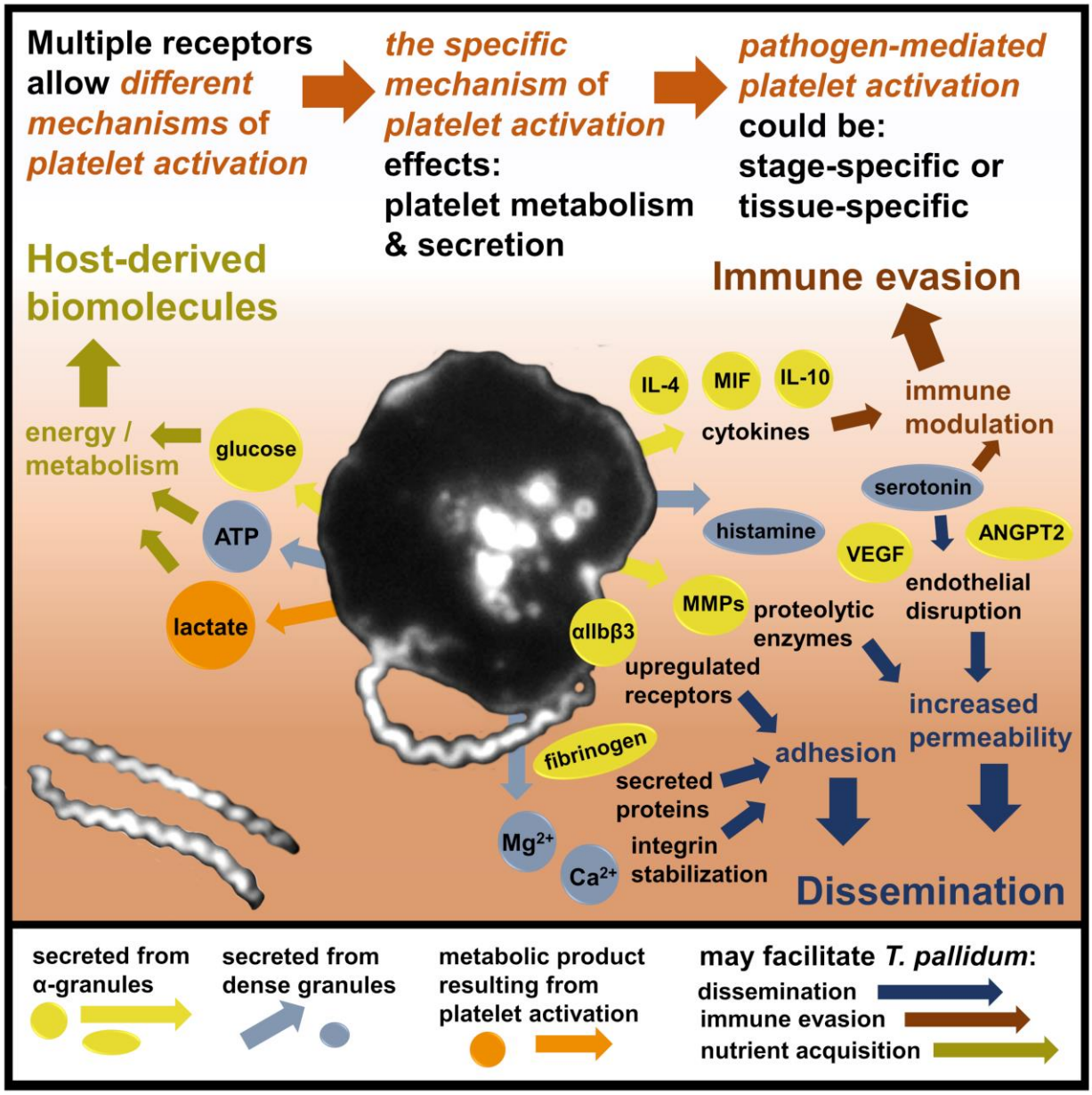

Figure 7: Platelets release effector molecules that may promote *T. pallidum* pathogenesis. Platelet release of lactate may be one source of energy for *T. pallidum* (Deka et al., 2020; Ketter et al., 2017), while α-granule and dense granules may promote adhesion, increased vascular permeability and immune evasion.

.1.4.3 Platelet activation

Platelet activation leads to two fundamental consequences – significant cytoskeletal rearrangement and granule secretion (Gremmel et al., 2016; Li et al., 2017). Platelet secretion is modulated by the species and concentration of agonist and by the receptor/s involved (Gremmel et al., 2016; Li et al., 2017). The platelet “releasate” (secretions) shows agonist- and environmentally-specific biomolecular patterns which alter the surrounding milieu, while the act of secretion itself expands the platelet membrane by two- to three-fold as granule membranes (and receptors) become incorporated (Sharda & Flaumenhaft, 2018).

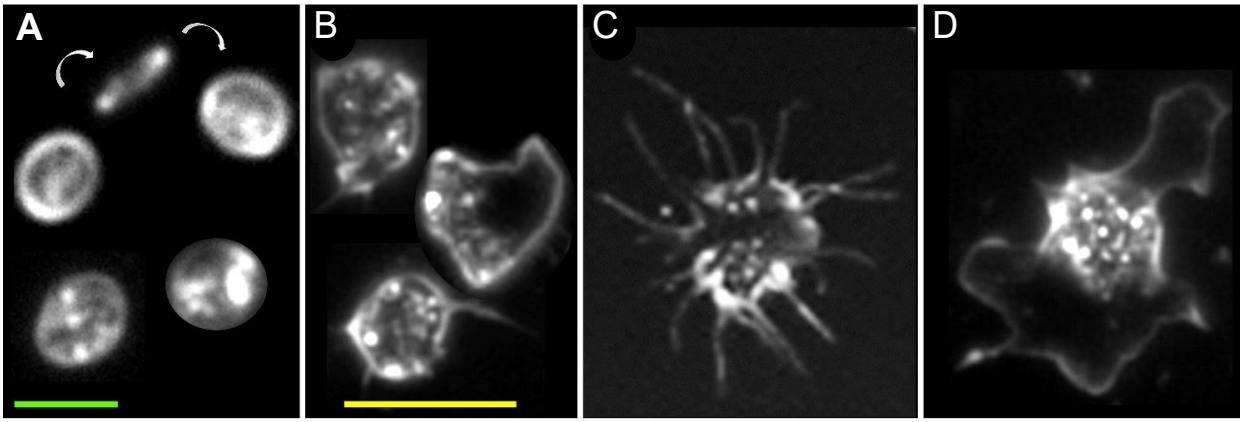

Figure 8: Cytoskeletal changes during platelet activation. Platelets circulate as a flattened disk (A) and undergo cytoskeletal changes as they begin to activate (B) and may become rounded and “star”-like with extended filipodia (C) or spread thinly across a surface (D). Scale bars 3 µm (green), 5 µm (yellow).

Platelets can rapidly transform from a flattened disk to a “star” shape with filopodia, thin tentacle-like extensions containing bundles of actin filament, to adhere to other cells and

structures (Margraf & Zarbock, 2019; Sandmann & Köster, 2016), or spread across a surface via lamellipodia filled with a network of cross-linked actin filaments, to resemble a “fried-egg” (Figs 7 & 8) (Margraf & Zarbock, 2019; Rosowski & Huttenlocher, 2018; Sandmann & Köster, 2016). As vascular sentinels, activated platelets use cytoskeletal rearrangements for chemotaxis towards pathogens, inflammation, injury and leukocytes (Ribeiro et al., 2019; Rosowski & Huttenlocher, 2018).

Spread platelets migrate towards collagen (Sandmann & Köster, 2016), sites of vascular inflammation where they interact with immune cells, facilitate leukocyte extravasation, and can use their OCS to bundle bacteria (Gaertner et al., 2017; Li et al., 2017; Nicolai & Massberg, 2020). Activated platelets can extravasate into inflamed tissue (Gremmel et al., 2016; Kraemer et al., 2010; Ribeiro et al., 2019), and secrete ADP and thromboxane A2 (TX2A) to recruit additional platelets (Rosowski & Huttenlocher, 2018).

Platelet activation can also induce the release of platelet microparticles (pmps), vesicles from 0.1 to ~ 1.0 µm, that can transfer biochemical effector molecules, coding and non-coding RNA, and even mitochondria to ECs, leukocytes, and tumor cells (Li et al., 2017; Melchinger et al., 2019; Milioli et al., 2015).

Platelet activation and aggregation at the site of an injury is critical to haemostasis (Koupenova et al., 2018), however, inflammation can dysregulate platelet activation and promote atherogenesis or lead to the development of emboli that can travel to the brain and cause stroke (Cevik et al., 2016). During sepsis, systemic dysregulation of platelet activation – disseminated intravascular coagulation (DIC) – leads to widespread blockages, organ damage, and platelet depletion (Assinger et al., 2019; Li et al., 2017).

While platelet aggregation can be beneficial or harmful, so too can be the platelet response to pathogens (Page & Pretorius, 2020).

Platelet interactions with pathogens can promote immune clearance by trapping bacteria within aggregates, secreting antimicrobial peptides (Gaertner & Massberg, 2019; Petersson et al., 2018) or reactive oxygen species (ROS) that damage pathogens, and by recruiting leukocytes (Li et al., 2017). Pathogens, however, have developed strategies to escape platelet aggregates (Palace et al., 2020), or to use platelet interactions to facilitate dissemination (within aggregates that circulate to host niches) (Kahn et al., 2013; Petersson et al., 2018), or turn recruited leukocytes into immunoprotective niches for replication (Trotta et al., 2018).

Activated platelets regulate other cell types through the secretion of hundreds to thousands of effectors molecules (Koupenova et al., 2018; Pagel et al., 2016; Tamagawa-Mineoka, 2015), all regulated through the specific mechanism/s of “outside-in” and “inside-out” platelet activation (Gianazza et al., 2020; Gremmel et al., 2016; Rayes et al., 2019).

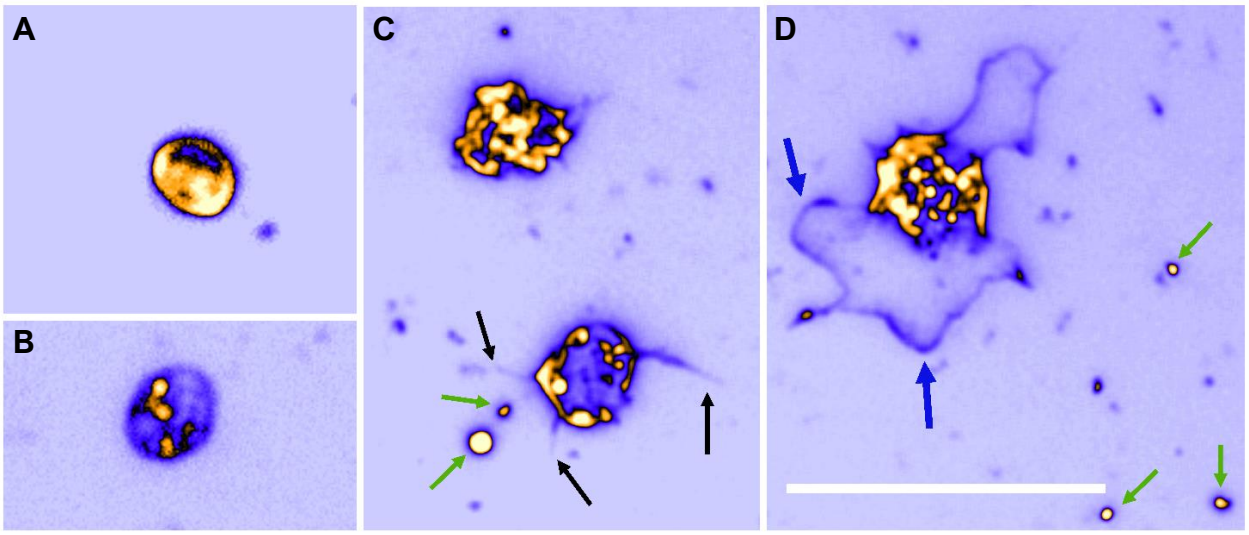

Figure 9: Activated platelets extend filopodia or lamellipodia and secrete microparticles. Pseudo-color enhancement shows disk-shaped resting platelets (A & B), centralized internal granules and few particles in the surrounding milieu. During early activation (C) filopodia extend (black arrows) and particles are released (presumptive pmps green arrows). Activated platelets may extend become fully spread (D) by extending lamellipodia (blue arrows) and PMPs diffuse away (green arrows). Scale bar = 5 µm (white).

1.4.4 Platelet receptors and agonist-specific functions

Platelets accomplish their varied functions as vascular sentinels and are rapidly mobilized through their vast repertoire of receptors, shown in Table 4, that recognize small signaling molecules, chemokines, pathogens, thrombosis, and any perturbance to their local cellular and biochemical milieu (Gremmel et al., 2016; Guo & Rondina, 2019; Li et al., 2017; Mezger et al., 2019). Platelet receptors act by adhesion to ligands bound to surfaces, soluble ligands, and by proteolytic cleavage that initiates activation, migration, and secretion of vascular and immune modulating effector molecules

(Gremmel et al., 2016; Li et al., 2017). Platelets have been shown to induce different patterns of secretion and immune modulation when activated through collagen adhesion receptors (Gianazza et al., 2020; Linke et al., 2017; Vélez et al., 2015) or by thrombin cleavage that exposes the endogenous ligands of protease-activated receptors (PARs) (Eisinger et al., 2018; Gianazza et al., 2020; Vélez et al., 2015).

Platelet receptors						
Activation	Adhesion	Environmental sensing				
	ECs, ECM & Immune Cells	Small Molecules, Hormones & Growth Factors	Pathogens & Antigens	Chemokines	Pathogen (PRRs)	"Self" Recognition & Thrombosis
αIIbβ3	α2β1	P2X1	MHC class I	CCR2	NOD2	Siglec-9
GPVI	α5β1	P2Y1	C1qRp	CCR3	TLR1	Siglec-11
PAR-1	α6β1	P2Y12	C3aR	CCR4	TLR2	SLAM
PAR-4	αLβ2	A2a	C5aR	CCR5	TLR3	SLAM5
GPIb-IX-V	αvβ3	EP_3	FcαRI	CCR6	TLR4	TLT-1
CLEC-2	GPIbα	5-HT2A	FcεRI	CCR7	TLR6	TF
	ICAM-2	Dopamine-R	FcγRIIa	CCR8	TLR7	Siglec-7
	JAM-C	Insulin-R		CCR9	TLR9	Siglec-9
	P-selectin	Leptin-R		CCR10	SRB1	Siglec-11
	PECAM-1	PDGF-Rα		CXCR1		
		PAF-R		CXCR2		
		Tie-1		CXCR3		
		V1a		CXCR4		
		β_2-AR		CXCR5		
		NMDA				

Table 4: Platelets express a vast repertoire of membrane receptors.

1.4.4.1 Platelet activation by adhesion receptors

Platelet receptors promote adhesion to bound plasma proteins or exposed structural proteins at sites of vascular damage or inflammation, with specialized responses to

different levels of “shear” within blood vessels (Koupenova et al., 2018). Platelets are also activated by ligands expressed on cell membranes, such as the glycoprotein podoplanin which interacts with C-type-lectin-like-2 (CLEC-2), and appears to modulate immune activation in opposition to GPVI activation (Rayes et al., 2017, 2019).

	Activating Receptors	mechanism of activation		
		ligand adhesion	proteolytic cleavage	estimate per platelet
	GPVI	collagen		3000 - 4000
	PAR-1		thrombin (low levels)	~ 2,500
	PAR-4		thrombin (high levels)	unknown
	CLEC-2	podoplanin		2000 - 4000
	GPIb-IX-V	VWF		~ 50,000
Activation Markers	αIIbβ3	fibrinogen		80,000 - 100,000
	P-selectin	PSGL-1		8900 – 13,000
	TLT-1	fibrinogen		~ 14,200

Table 5: Key receptors that initiate and mark platelet activation. Platelet activation can be quantified by the level of upregulation of key receptors in comparison to resting platelets (Baaten et al., 2017; Gremmel et al., 2016; Rayes et al., 2019; Smith et al., 2018; Södergren & Ramström, 2018).

Collagen

Collagen is one of the most potent platelet activators that facilitates haemostasis and vascular homeostasis during the host immune response (Boulaftali et al., 2018).

At high shear stress plasma von Willebrand factor (VWF) binds to exposed collagen at sites of endothelial damage (Margraf & Zarbock, 2019). This binding unfolds globular VWF, allowing platelet GPIb to bind and induce platelet activation, which in turn upregulates and activates platelet αIIbβ3, the most highly expressed platelet receptor (Gianazza et al., 2020). Activated αIIbβ3 then binds fibrinogen which promotes additional platelet activation (Rayes et al., 2019), and can now bind VWF, fibrin, fibronectin, vitronectin and thrombospondin to induce platelet aggregation (Gianazza et al., 2020; Saboor et al., 2013). Platelet GPIb and αIIbβ3 are commonly involved in platelet-pathogen interactions, which may involve an intermediary protein such as fibrin, fibrinogen or fibronectin (Hamzeh-Cognasse et al., 2015), and may play a role in *T. pallidum*-platelet interactions.

At low shear stress α2β1 binds to exposed endothelial collagen, which allows platelet GPVI to then bind collagen and induce platelet activation (Table 5,) (Koupenova et al., 2018; Margraf & Zarbock, 2019). Collagen activation may involve a single platelet spreading across an endothelial gap, without inducing the activation of additional platelets (Gros et al., 2015), or can lead to significant activation and aggregation (Rayes et al., 2019). Collagen-activated platelets develop lamellipodia, that unfolds additional membrane, to transform the platelet from a flattened discoid into a significantly larger, flatter form that efficiently seals endothelial gaps (Boulaftali et al., 2018; Sandmann & Köster, 2016). During vascular dissemination a treponeme that interacts with a single,

activated platelet, spread across an endothelial gap, may benefit from platelet-facilitated extravasation similar to the effect seen during leukocyte extravasation (Kral et al., 2016).

Podoplanin

The platelet receptor CLEC-2 activates aggregation and secretion (Rayes et al., 2019) after binding podoplanin, a small, transmembrane glycoprotein expressed on endothelial cells within lymph vessels (Rayes et al., 2019; Welsh et al., 2016), the lungs, the kidneys (Sung & Hsieh, 2019), and the CNS (Rayes et al., 2019; Shibahara et al., 2006). Podoplanin-platelet CLEC-2 interactions induce platelet activation during thromboinflammation (Sung & Hsieh, 2019), yet limit inflammation during bacterial infection (Rayes et al., 2019; Sung & Hsieh, 2019), which may be a factor during *T. pallidum* infection.

1.4.4.2 Platelet activation by proteolytic cleavage.

Platelets express several types of seven transmembrane domain G protein-coupled receptors (GPCRs) that are activated by the binding of ligands such as ADP to the P2Y receptor, or by enzymatic cleavage in the case of protease-activated receptors (PARs) (Heuberger & Schuepbach, 2019). Platelets express two PARs (PAR-1, PAR-4) (Gremmel et al., 2016) which are irreversibly activated by thrombin cleavage of an N-terminal peptide (Heuberger & Schuepbach, 2019). Platelet activation by thrombin occurs during haemostasis and immune responses (Petzold & Massberg, 2019).

Platelet PARs contain multiple cleavage sites that induce specific cellular responses when cleaved by matrix metalloproteases (MMPs), activated protein C, kallikreins, and

by bacterial proteases such as *Porphyromonas gingivalis (P. gingivalis*) gingipain R (Heuberger & Schuepbach, 2019).

Interestingly, proteases such as trypsin, kallikrein-14, and Group A *Streptococcus* exotoxin B have a regulatory role and cleave the N-terminal at sites which remove the ligand domain and therefore, prevent receptor activation (Heuberger & Schuepbach, 2019).

Thrombin

Thrombin is stored as the zymogen prothrombin until vascular wounding or cutaneous inflammation activates TF (Tamagawa-Mineoka, 2015), which in turn cleaves and activates thrombin, resulting in rapid activation of coagulation factors and/or platelets (Koupenova et al., 2018). Thrombin activates platelets through PAR-1 and PAR-4 cleavage, and to some extent by binding GPIb, though this mechanism remains to be fully elucidated (Margraf & Zarbock, 2019; Popović et al., 2012). Thrombin induces significant platelet activation, thrombin-specific secretions (Milioli et al., 2015; Vélez et al., 2015), and modifies the functions of secreted proteins through cleavage (Gianazza et al., 2020). Thrombin-activated platelets release pmps (Milioli et al., 2015), rapidly upregulate TLR9 (Nording et al., 2015), and activation markers TLT-1 and P-selectin (Smith et al., 2018) (Table 5).

Enzymatic cleavage by thrombin produces ongoing platelet activation and therefore, lacks the potential for limited, single platelet activation seen with collagen adhesion (Gros et al., 2015). Thrombin-activated platelets develop long filopodia that engage surrounding cells and form dense aggregates (Sandmann & Köster, 2016).

As thrombin activation of platelets can occur during thromboinflammation to trap pathogens in dense platelet aggregates, this mechanism of platelet activation may inhibit *T. pallidum* dissemination.

PAR-1

Thrombin-cleaved PAR-1 induces rapid filipodia development (Sandmann & Köster, 2016) while thrombin concurrently cleaves soluble fibrinogen to produce fibrin strands, leading to dense, platelet-fibrin aggregates (Koupenova et al., 2018). PAR-1 is responsive to nanomolar concentrations of thrombin (Koupenova et al., 2018) and induces platelets secretion that differs from that induced by PAR-4 (K. A. Nguyen et al., 2015) or by collagen activation (Ollivier et al., 2014; Vélez et al., 2015).

PAR-4

PAR-4 is activated only by higher concentrations of thrombin and reacts up to 70-fold more slowly than PAR-1 (Gremmel et al., 2016), however there is extended Ca^{2+} release and signaling (Sang et al., 2020). PAR-4-mediated activation increases expression of CD154/ CD40L (K. A. Nguyen et al., 2015) which can lead to EC activation (Koupenova et al., 2018), and accelerates platelet activation by thrombin when dimerized with PAR-1 (Heuberger & Schuepbach, 2019).

Given the different concentrations of thrombin required to activate PAR-4 versus PAR-1, and the differences in the resulting platelet secretions, additional physiological functions may be associated with these two mechanisms (Gianazza et al., 2020).

1.4.5 Platelet immunomodulation

Platelets are frontline sentinels with differential responses during haemostasis, inflammation, and when encountering pathogens (Koupenova et al., 2018), that affect secretion and host immune modulation (Gremmel et al., 2016; Koupenova et al., 2018; Li et al., 2017). Platelets are almost 100-fold more prevalent than mononuclear cells within the blood (Ponomarev, 2018), and within post-capillary venules platelets are the first cells to adhere to inflamed tissue, release chemokines to recruit leukocytes, and then facilitate leukocyte arrest and extravasation (Rayes et al., 2020).

Platelets have been shown to interact with and modulate the responses of neutrophils, DCs, NK cells, eosinophils, B cells, T cells, monocytes, macrophages (Koupenova et al., 2018), and keratinocytes (Tamagawa-Mineoka, 2015). The interactions of platelets with immune cells relies on specific receptor interactions that 1) mediate platelet activation and signaling that induces pro- versus anti-inflammatory leukocyte responses (Rayes et al., 2020), and 2) mediates platelet-leukocyte complexes (Koupenova et al., 2018). Platelets form complexes with monocytes that induce monocyte ROS secretion (Gianazza et al., 2020) and promote monocyte recruitment (Kral et al., 2016). Within lymph nodes platelets activate DCs (Li et al., 2017) and participate in lymphocyte trafficking (Rayes et al., 2019). Platelets secrete neurotransmitters that modulate CD4 T cells by enhancing (serotonin) or inhibiting (GABA) proliferation (Ponomarev, 2018).

During bacterial infections platelet-neutrophil interactions can induce neutrophil extracellular trap (NET) formation, which capture pathogens (Koupenova et al., 2018) within a sticky mass of secreted histones, DNA, enzymes and peptides (Nguyen & Soulika, 2019) Platelet-mediated NETosis plays an important role in immunothrombosis to limit the dissemination of pathogens (Nicolai & Massberg, 2020).

Collagen-activated platelets have been shown to promote anti-inflammatory responses (Rayes et al., 2020), converting circulating monocytes to regulatory cells and in co-cultures released significant levels of PGE2, inducing monocyte secretion of IL-10 to increase by almost 3-fold, while also reducing levels of TNFα (Linke et al., 2017).

Thrombin-activated-platelets complex with circulating monocytes via platelet P-selectin and monocyte PSGL-1, leading to a pro-inflammatory phenotype (Linke et al., 2017) and secretion of TNFα, IL-8, and IL-1b (Stephen et al., 2013). *In vitro*, thrombin-activated platelets induced the activation of co-cultured monocytes and neutrophils, leading to a burst of ROS, while upregulation of platelet P-selectin promoted monocyte secretion of IL-8 and monocyte chemoattractant protein (MCP) -1 (Gros et al., 2015).

1.4.6 Platelet interactions with pathogens

Platelets interact with viral and bacterial pathogens and eukaryotic parasites via several mechanisms that can lead to pathogen clearance, or promote immune dysregulation, host damage, and pathogen dissemination (Kahn et al., 2013; Page & Pretorius, 2020)**.** Platelets express numerous receptors to sense PAMPs and danger-associated molecular patterns (DAMPs) that generate host immune responses, yet many pathogens mediate interactions with platelets and platelet activation (Kerrigan et al., 2019; Page & Pretorius, 2020; Ribeiro et al., 2019).

Platelet receptors such as αIIbβ3, GPIb, and FcγRIIa, with important roles in adhesion, activation, and immune cell interactions are commonly targeted by bacterial pathogens (Hamzeh-Cognasse et al., 2015; Page & Pretorius, 2020). The most prevalent platelet receptor, platelet-specific integrin αIIbβ3, interacts with fibrinogen and other plasma

proteins (Gremmel et al., 2016; Södergren & Ramström, 2018) but is also thought to mediate platelet-CD4+ T cell interactions (Ponomarev, 2018). The αIIbβ3 receptor is targeted by many pathogens including *B. burgdorferi* (Coburn et al., 1993), *B. hermsii* (Alugupalli et al., 2001), *Chlamydia pneumonia*, *Streptococcus gordonii (S. gordonii*), *S. pyogenes*, and *Streptococcus pneumoniae* (*S. pneumoniae*) (Page & Pretorius, 2020).

Another platelet-specific receptor, GPIb, is activated by VWF binding, but facilitates immune modulation via Macrophage-1 antigen (Mac-1) on DCs (Nording et al., 2015), and promotes pro-inflammatory monocytes via CD11b (Rayes et al., 2020). This receptor is targeted by many species including *Helicobacter pylori* (*H. pylori), P. gingivalis*, *S. gordonii,* (Haworth et al., 2017; Page & Pretorius, 2020)*, S. sanguinis* (Haworth et al., 2017), and *S. aureus* (Hamzeh-Cognasse et al., 2015; Page & Pretorius, 2020)*.* Platelet interactions may play important roles in bacterial pathogenesis given that several pathogens, including *S. aureus* and *S. gordonii*, have multiple ligands for platelet receptors (Hamzeh-Cognasse et al., 2015; Page & Pretorius, 2020)*.*

Platelet interactions mediated by *S. aureus,* among the most well studied, can occur by numerous membrane and secreted effectors that include direct adhesion by clumping factor (ClfA) or CflB to platelet αIIbβ3, staphylococcal accessory regulator protein (SarP) to GPIb, Protein A to platelet FcγRIIa, or by Protein A binding VWF to facilitate GPIb interactions (Hamzeh-Cognasse et al., 2015). Some secreted mediators of *S. aureus* have opposing functions, whereas extracellular adherence protein (Eap) binds platelet αIIbβ3 to induce activation; granule secretion, and aggregation, extracellular fibrinogen-binding protein (Efb) inhibits platelet activation and aggregation (Page & Pretorius, 2020). This potential to mediate opposing platelet responses is seen again

with *S. aureus* staphylothrombin which induces fibrin polymerization and platelet aggregation, while release of staphylokinase inhibits aggregation and degrades fibrinogen (Page & Pretorius, 2020). Interacting with platelet receptors can induce platelet dysregulation, limiting their functioning as vascular sentinels (Hamzeh-Cognasse et al., 2015), and importantly, inhibiting platelets from interacting with host immune effectors via these receptors, which further hinders their functioning (Hamzeh-Cognasse et al., 2015; Page & Pretorius, 2020).

1.4.7 The challenges of platelet biology in the course of research.

Platelet biology plays a significant role in the approach to experiments and the interpretation of results as some techniques used to isolate platelets alters their sensitivity to biochemical and physical stimulation (Gros et al., 2015). Platelet sensitivity to mechanical stressors and any number of stimulants during purification or handling can produce unintended activation (Gianazza et al., 2020; Pokrovskaya et al., 2020).

Frequently, the approach to purifying platelets for study uses powerful agents that prevent platelet activation and allow significant forces to be applied during centrifugation and isolation, before the platelets are resuspended in media or buffer (Gianazza et al., 2020; Milioli et al., 2015). In contrast, minimally anti-coagulating agents, such as acid citrate dextrose (ACD) solution A, allow platelets to remain in a more natural, physiologically active state but require low centrifugation speeds, limiting shear during transfers, and awareness of temperature changes, etc. (Bausset et al., 2012; Qureshi et al., 2009). The choice of anti-coagulant and handling techniques substantially affect platelet activation and secretion *in vitro*, as does the experimental set-up and agonist concentration/s, and importantly, consistent application of techniques between

experiments must be applied. While the use of mild anti-coagulants in purifying platelets is a more labour intensive approach, requiring careful handling and selection of downstream methodology, limiting biochemical interventions ultimately benefits the researcher by facilitating a more physiologically relevant platelet response.

The degree of reactivity exhibited by platelets, relative to other cell types, makes them challenging to work with and long impeded investigation of platelets' non-haemostatic functions (Coller, 2011). Now, there is exponential growth advancing the understanding of platelet modulation of immune responses, host-pathogen interactions (Gaertner et al., 2017; Guo & Rondina, 2019; Koupenova et al., 2018; Page & Pretorius, 2020; Ribeiro et al., 2019; Rosowski & Huttenlocher, 2018), inflammatory diseases (Gianazza et al., 2020; Ponomarev, 2018; Rayes et al., 2020) and cancer (Koupenova et al., 2018; Pokrovskaya et al., 2020; Schlesinger, 2018).

1.5 Research objectives:

Treponema pallidum has evolved to rapidly disseminate, colonize any host tissue, and evade immune clearance, yet there remains much to be understood regarding the role of specific host-cell interactions in facilitating this. As a pathogen that exploits vascular dissemination, a key interaction is likely to occur between *T. pallidum* and platelets, now regarded as innate immune cells that are highly involved in host-pathogen interactions.

Prior to this dissertation, the field of research surrounding *T. pallidum* interactions with host cells has lacked any investigation into the existence or characteristics of interactions with human platelets. However, investigation into the nature of *T. pallidum*-platelet interactions may provide insight into host-cell interactions that promote pathogenesis.

In this work, I began with the hypothesis that, as a vascular pathogen *T. pallidum* would demonstrate an interaction with platelets, and a characterization of these interactions would lead to a greater understanding of *T. pallidum* dissemination and immune evasion strategies. As the foundation for a new line of inquiry into *T. pallidum* pathogenesis, this body of work aims to be the first to establish that *T. pallidum* interacts with platelets with the main objectives as follows:

(1) Provide the first documentation and characterization of interactions between *T. pallidum* and human platelets.
(2) Establish whether *T. pallidum* demonstrates the ability to activate human platelets.

In Chapter 2 of this dissertation, these objectives were investigated using high resolution microscopy to characterize, analyze and quantify interactions. This technique allowed detailed characterization of *T. pallidum*-platelet interactions which demonstrated that the *level of platelet activation* was important in *T. pallidum* interactions and provided the basis for additional investigation into the treponeme response to platelet activation. The analysis of *T. pallidum*-mediated platelet activation was approached using flow cytometry to quantify the response in large populations of platelets. The results in this chapter prompted an additional area of focus with the following objectives:

(3) Develop a methodology to characterize whether *T. pallidum* demonstrates a to *different mechanisms* of platelet activation.
(4) Identify potential components secreted by platelets activated by different mechanisms that may have a chemoattractive or chemorepellent effect on *T. pallidum* migration.

In Chapter 3 of this dissertation, these objectives were approached by the modification of a capillary tube chemotaxis setup to quantify the response of *T. pallidum* to different platelet secretions, induced through two physiologically distinct, infection-relevant mechanisms of platelet activation (by collagen or by thrombin).

This approach was novel for the following two reasons:

(1) platelets were isolated in a minimally invasive manner that allowed the resulting PRP to be used as an alternative model, intermediate between *in vivo* experiments *and in vitro* assays with cell cultures in media; and

(2) the response of bacteria to different platelet secretomes had not yet been investigated (within an extensive literature search)

Differences in the resulting platelet secretions were quantified using a 105 component human cytokine assay.

Chapter 2: Interaction of *Treponema pallidum*, the syphilis spirochete, with human platelets

Contributions: BC performed all experiments and analysis.

Adapted from:

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2.1 Abstract

Extracellular bacteria that spread via the vasculature employ invasive mechanisms that mirror those of metastatic tumor cells, including intravasation into the bloodstream and survival during hematogenous dissemination, arrestation despite blood flow, and extravasation into distant tissue sites. Several invasive bacteria have been shown to exploit normal platelet function during infection. Due to their inherent ability to interact with and influence other cell types, platelets play a critical role in alteration of endothelial barrier permeability, and their role in cancer metastasis has been well established. The highly invasive bacterium and causative agent of syphilis, *Treponema pallidum* subspecies *pallidum*, readily crosses the endothelial, blood-brain and placental barriers. However, the mechanisms underlying this unusual and important aspect of *T. pallidum* pathogenesis are incompletely understood. In this study we use darkfield microscopy in combination with flow cytometry to establish that *T. pallidum* interacts with platelets. We also investigate the dynamics of this interaction and show *T. pallidum* is able to activate platelets and preferentially interacts with activated platelets. Platelet-interacting treponemes consistently exhibit altered kinematic (movement) parameters compared to

free treponemes, and *T. pallidum* -platelet interactions are reversible. This study provides insight into host cell interactions at play during *T. pallidum* infection and suggests that *T. pallidum* may exploit platelet function to aid in establishment of disseminated infection.

2.2 Introduction

Syphilis, caused by the spirochete *Treponema pallidum* subsp. *pallidum* (hereafter *T. pallidum*), is a chronic, sexually transmitted infection affecting an estimated 36 million people worldwide, with 11 million new cases occurring annually (LaFond & Lukehart, 2006; Newman et al., 2015). In recent years rates of primary and secondary syphilis have risen sharply in particular populations, most prominently amongst men who have sex with men, while a general increase in infectious syphilis cases in both heterosexual men and women has been observed in cities across North America, Europe, and Asia (Centers for Disease Control and Prevention, 2017; de Voux et al., 2017). Paralleling the rise in syphilis cases amongst women, congenital syphilis cases have increased in middle and high income countries and continue to be a leading cause of stillbirth in low income nations, affecting an estimated 1.36 million pregnant women each year and resulting in over 500,000 adverse outcomes from maternal syphilis (Newman et al., 2013).

Syphilis is a multistage disease punctuated by asymptomatic periods of latency. The primary and secondary stages of syphilis present with a painless chancre at the initial site of infection followed by a non-pruritic rash, respectively, both of which spontaneously resolve (LaFond & Lukehart, 2006). Treponemes rapidly disseminate from the initial site of infection via the circulatory system, with invasion of the central

nervous system occurring in up to 40% of early infections (Lukehart et al., 1988). Indeed, neurological symptoms such as stroke may occur at any stage of infection, as can ocular involvement which is often accompanied by abnormal cerebrospinal fluid (CSF) (Ahamed et al., 2009; Dai et al., 2016; Liu et al., 2017; Shen et al., 2015). In the absence of antibiotic treatment, the majority of infected individuals exhibit lifelong latency while approximately 30% of patients exhibit symptoms of tertiary syphilis, which can include gummatous lesions, neurosyphilis and/or cardiovascular syphilis (LaFond & Lukehart, 2006; Paulo et al., 2012; Radolf et al., 2016).

Circulating near the blood vessel walls as flattened disks, inactive platelets are optimally situated to rapidly respond to deviations in the vascular environment and perform central roles in hemostasis and in the modulation of inflammation, immune responses and endothelial permeability (Menter et al., 2017). Ligand binding to platelet receptors induces specific signaling and secretion of granule components (Berthet et al., 2012; Yun et al., 2016). Cytoskeletal restructuring occurs and the platelet transforms from the inactive state to the active spheroid state, which becomes spherical and characterized by pseudopod extensions and up-regulation of key receptors on the platelet surface including P-Selectin, αIIbβ3 integrin and LAMP-3 (lysosomal-associated membrane protein 3) (Menter et al., 2017). Platelets tether to the endothelium via the pseudopods and may further activate to a spread fully activated form (Menter et al., 2017). Platelet aggregates may be composed of both spheroid and spread platelets (Shin et al., 2017). Activated platelets have been shown to loosen endothelial cell junctions (Petri et al., 2010) and promote leukocyte recruitment via secreted platelet chemokines (Ho-Tin-Noé et al., 2011). Responding leukocytes localize to these foci of increased endothelial

permeability and extravasate. This strategy has been shown to be exploited by tumor cells to promote perivascular infiltration (Leblanc & Peyruchaud, 2016; Tesfamariam, 2016; Zuchtriegel et al., 2016).

Platelets have also been shown to be a target of several invasive pathogens including *Staphylococcus aureus, Streptococcus pyogenes* and *Borrelia* species (Binsker et al., 2018; Coburn et al., 1993, 1994; J. R. Fitzgerald et al., 2006; Kerrigan, 2015; McNicol, 2015). Upon pathogen interaction, platelets can become activated by direct or indirect receptor contact with bacterial virulence factors, with the latter occurring via a bridging plasma protein (Cognasse, 2015). Further, platelets have been shown to promote *S. pyogenes* dissemination in a murine sepsis model, where significantly fewer bacteria disseminated to the blood, lungs and spleen in platelet-depleted animals (Kahn et al., 2013). Surprisingly, despite the potential importance of platelet interactions in bacterial pathogenesis, pathogen-platelet investigations remain a relatively unexplored field of study.

Treponema pallidum is an obligate human pathogen that can rapidly invade the circulatory system and traverse the blood-placenta, blood-retina and blood-brain barriers (LaFond & Lukehart, 2006). This invasive capacity, together with the known ability of several other invasive pathogenic bacteria to target platelets, prompted us to investigate whether platelet exploitation may also play a role during *T. pallidum* infection. Previous studies have determined that *T. pallidum* binds a variety of nucleated mammalian cells in culture (Fieldsteel et al., 1981; Fitzgerald et al., 1975b; Fitzgerald et al., 1977; LaFond & Lukehart, 2006), but to date no studies have been undertaken to investigate the capacity of *T. pallidum* to interact with platelets. Disease symptoms

associated with syphilis that are consistent with potential *T. pallidum*-platelet interactions include occlusive stroke, congenital thrombocytopenia, and aortic aneurism (Ahamed et al., 2009; De Santis et al., 2012; Roberts et al., 2015; Szanto, 1971).

In this study we investigate the potential interaction of *T. pallidum* with human platelets using a modified method of darkfield video microscopy to compile high resolution datasets of live treponemes with fresh human platelets and flow cytometry to quantitate treponeme-platelet interactions and determine the dependence of this interaction upon treponeme viability. Herein we demonstrate that *T. pallidum* interacts with platelets in a manner that correlates with the degree of platelet activation. We investigate treponeme-platelet interactions during stationary adhesion and show that *T. pallidum* displays phenotypic changes during platelet interactions by forming a more compact shape and by increasing its axial rotation and the velocity of its translational motility. We also demonstrate that platelet-tethered treponemes exhibit reduced displacement under the force of moving plasma and that treponemes are able to induce platelet activation. This study may reveal a role for treponeme-platelet interactions in *T. pallidum* pathogenesis.

2.3 Materials and methods

Ethics statement

All human blood studies were approved by the local Institutional Review Board at the University of Victoria and samples were obtained by informed consent from volunteer donors. All animal studies were approved by the local Institutional Review Board at the University of Victoria and were conducted in strict accordance with standard accepted principles as set forth by the Canadian Council on Animal Care, National Institutes of

Health and the United States Department of Agriculture in a facility accredited by the Canadian Council on Animal Care and the American Association for the Accreditation of Laboratory Animal Care.

T. pallidum **propagation and extraction**

Propagation and harvesting of *T. pallidum* was performed as per Lukehart and Marra (Lukehart & Marra, 2007), with the exception that testicular extractions were performed under microaerophilic conditions of ~5% oxygen in a Coy Laboratory Products anaerobic chamber (Mandel Scientific Company Inc., Guelph, ON, Canada) to enhance *T. pallidum* viability (Cox et al., 1990; Cox, 1994; Radolf et al., 2016).

T. pallidum **heat treatment**

A subset of viable treponemes were incubated at 56˚C for 45 minutes to abrogate motility (Fitzgerald et al., 1984; Thomas et al., 1988). Heat-treated treponemes were then assessed by darkfield microscopy to ensure they were non-motile yet remained morphologically consistent with viable treponemes.

Platelet purification

Donor blood was extracted from healthy volunteers into BD Vacutainers ACD-A tubes (BD Canada, Mississauga, ON), transferred into sterile 15 mL conical tubes (Sarstedt Inc., Montreal, QC) and centrifuged at 180 x *g* for 15 minutes at 22˚C (with the centrifuge rapid acceleration and rapid brake functions disabled to reduce platelet activation through shear force, and contamination of the platelet rich plasma layer by the buffy coat, respectively). The platelet rich plasma (PRP) portion was transferred, with care not to disturb the buffy coat, into fresh conical tubes, and allowed to slowly run

down the inside of the tube. This process yielded an average of 5.0 x 10^5 platelets/µL. Platelet poor plasma (PPP) was obtained from the supernatant of PRP centrifuged at 15,000 x *g* for 15 minutes. All PRP and PPP samples were stored at room temperature. Platelet activation stages were assigned as described in Zhao et al. (Zhao et al., 2015).

***T. pallidum*-platelet coincubation**

All coincubations of treponemes and platelets were initiated in a microaerophilic chamber (5% oxygen). Samples were then either maintained at 34˚C and 5% oxygen or tightly sealed and transferred to coincubate at 37˚C in atmospheric oxygen. All live treponeme treatments, such as staining or fixation, occurred only under microaerophilic conditions.

Darkfield Microscopy

To achieve optimal magnification and high resolution, the *T. pallidum*-platelet sample volume was limited to 4 µL per 1.0-1.2 mm thick glass slide with a 22 x 36 mm cover glass (Fisher Scientific Company, Ottawa, ON) gently pressed into place. Slides were viewed at 1000x total magnification, using a 10x ocular lens with a 100x oil pan-fluor objective lens set to 0.7 on a Nikon Eclipse 80i darkfield microscope with a Nikon DS-Qi1Mc digital camera with NIS-Elements imaging software (Nikon Canada Inc., Mississauga, ON). For video microscopy (high resolution real-time imaging), exposure times were set at 3 - 40 ms. For additional brightness, gain was increased to 9 - 16. Image clarity was directly related to the level of platelet activation and platelet microparticle (pmp) secretion. As activation and pmp secretion increased, additional light was scattered, resulting in a brighter field, and exposure times were further

reduced to compensate. The quality of imaging samples with reduced light scattering was improved by increasing the exposure time to ≥ 60 ms and opting for still imaging rather than videos.

Live treponeme and platelet analysis

Treponeme motility and platelet interactions were monitored by darkfield microscopy. For field of view (FOV) counts, 20 random fields per slide were counted. Treponeme viability was associated with vigorous to moderate activity consisting of axial rotation, flexing, bending and translational motility (forward and backward motion). Platelet sub-populations spontaneously adhere to glass slides during microscopic observation; the number of treponemes bound to slide-adhered platelets were enumerated as above. Treponeme speed and relative displacement in plasma currents were calculated from microscopy video segments obtained prior to the plasma reaching steady state.

Image and video acquisition and analysis

Micrographs and videos were captured with a Nikon DS-Qi1Mc digital camera (Nikon Camera Inc.). Images were saved in uncompressed JPEG format or as AVI movies. ImageJ (ImageJ v1.6.0_24/1.51h, embedded in Fiji) (Schindelin et al., 2012; Schneider et al., 2012) and GIMP software (GIMP 2.8.18, http://www.gimp.org/) were used to adjust brightness and contrast, scale, dpi and sharpness. Contrast in multi-panel images was harmonized during figure construction in Excel 2016 (version 1806, Microsoft, Redmond, WA). For optimum definition of the treponeme to measure wavelength, amplitude and bacterial cell length, images were imported into Excel, enlarged to 400% and contrast-enhanced. Images were selected with all or the majority

of the treponeme flat-wave in the imaging plane (Harman et al., 2013). Wavelengths were measured from peak apex to peak apex and averaged. Amplitude was measured from peak apex to trough and divided by two at five separate points along the cell body and averaged per treponeme. Cell length was measured from pole to pole. Time-stamped image capture for relative displacement and speed calculations was performed with Frameshots software (version 3.1.3, EOF Productions, http://www.frame-shots.com/) or ImageJ then imported into Excel and measured with an imported ruler calibrated to the scale bar on each image. Speed was calculated by dividing the displacement by the elapsed time (Bhat et al., 2014). This measurement technique enabled greater image magnification and was validated by comparison to displacement measurements in both ImageJ and Tracker (version 4.11.0, http://www.cabrillo.edu/~dbrown/tracker/), an Open Source Physics tool (https://physlets.org/tracker/). To compare the velocity, rotation, and displacement of platelet-interacting versus non-interacting treponemes, AVIs were imported into Tracker with frame by frame manual image position correction. Velocity and displacement were calculated as a function in Tracker, and full peak rotations were manually counted. Treponeme rotation was calculated only when an individual peak remained in focus and was averaged per treponeme (mean = 160.90 ± 14.59 frames/ video). Video contrast, brightness, and/or sharpness was enhanced, and video format was change from AVIs to MP4s with PowerPoint 2016 (version 1806, Microsoft, Redmond, WA).

Flow cytometry

All treatments occurred in microaerophilic conditions with treponemes in tightly closed sterile conical (Sarstedt) or BD Falcon 5 mL polystyrene tubes (BD Canada) when

removed from the microaerophilic chamber. For platelet binding assays, viable treponemes were stained with 10 µM carboxyfluorescein succinimidyl ester (CFSE, AAT Bioquest, Sunnyvale, CA) for 30 minutes at room temperature in the dark with gentle rocking. Treponemes were then quenched with one to two volumes of PPP, divided in two, and half the volume was then heat-treated (Jun et al., 2008; Tuominen-Gustafsson et al., 2006) (heat-treated treponemes retain CFSE staining). Treponemes and platelets were combined at a ratio of 3:5 treponemes per platelet in PPP at room temperature in the microaerophilic chamber in 5 mL polystyrene tubes (BD Canada), tightly sealed and incubated in the dark at 37˚C. Platelets were pre-activated with 0.1 U/mL bovine thrombin (Sigma-Aldrich, Oakville, ON) for two minutes prior to use. Following 19 – 24 hours of co- incubation, sample tubes were returned to 5% oxygen and the mixture was incubated with a 1/20 dilution of platelet-specific mouse anti-human CD41a (αIIb integrin) monoclonal antibodies labeled with either PE or PECy5 (clone HIP8, BioLegend, San Diego, CA). After 45 minutes at room temperature, samples were fixed with one volume of ice-cold 2% paraformaldehyde (PFA) in PBS, stored at 4˚C, then diluted with sterile PBS just prior to flow cytometry. Samples were acquired on a BD Fascalibur (BD Canada) with platelet and treponeme populations co-localizing and identified with BD CellQuest acquisition software (BD Canada). Software was set to log scale for both forward scatter (FSC E-1) and side scatter (SSC), and gating was aided by using the SPHERO Flow Cytometry Size Standard Kit (containing polystyrene beads between 2.0 and 9.96 µm from Spherotech, Inc., Lake Forest, IL). Forward and side scatter gating was used to acquire at least 5000 events, and up to four biological replicates were analyzed per sample type. After initial gating by forward and side

scatter, FlowJo V10 (FlowJo, LLC, Ashland, OR) analysis further separated subpopulations by the mean fluorescence intensity (MFI) of both platelet (PE or PECy5) and treponeme (CFSE) markers. The shift of platelet and treponeme events positive for both markers to quadrant 2 was counted as a treponeme-platelet binding event. Heat-treated treponemes with or without platelet coincubation exhibited an auto fluorescence signal that was gated and removed from all samples. For platelet activation assays, coincubations were conducted in PPP under microaerophilic conditions at either 34°C or 37°C for 2, 4, 8, 20 or 24 hours at a ratio of 5 treponemes to 1 platelet and samples were stained, fixed and prepared for flow cytometry as above. Unlabeled treponemes and platelets were coincubated, followed by platelet staining with a 1/20 dilution of mouse anti-human monoclonal anti-CD62P (clone AK4, BioLegend, San Diego, CA) labeled with FITC. At least three biological and technical replicates of each sample type were used per experiment. Forward and side scatter gating was conducted as above, followed by quantification of the relative median fluorescence intensity (MFI) of either the activation marker CD41a (PE) or CD62P (FITC). MFI comparisons included platelets that were resting, pre-activated with either 160 µM SFLLRN peptide (Biomatik Corporation, Cambridge, ON) or 5 µg/mL rat collagen I (R & D Systems, Inc., Minneapolis, MN) or coincubated with *T. pallidum.*

Plate-based fibrin clot production assay

Fibrin clot production by activated platelets was measured in 96 well tissue culture plates (Thermo Scientific Nunc, Waltham, MA) using a method modified from Vinholt *et al* [48]. Control wells included 150 µL of resting PRP + 50 µL of NS/10% NRS. To prepare control activated platelets, either 0.5 U/mL bovine thrombin or 5 µg/mL rat

collagen I prepared in NS/10%NRS was added to PRP. Test wells included 50 µL of *T. pallidum* (~4.0 x 10^7 treponemes/mL) in NS/10% NRS added to 150 µL of resting PRP. Plates were incubated at 34°C for 18 hours under microaerophilic conditions, after which the absorbance at 600 nm was read with a BioTek Synergy HT plate reader (Fisher Scientific, Waltham, MA), Results were analyzed using Excel.

Statistics

A two-way ANOVA analysis evaluated the overall statistical significance of three independent binding experiments assessed by flow cytometry with results shown as means ± the standard deviation (SD). The statistical significance of three independent binding experiments assessed by darkfield microscopy was calculated using an unpaired, two-tailed Student's t-test and shown as mean ± SD. A Student's two-tailed t-test was used to assess statistical significance of differences in *T. pallidum* morphology, kinematics and levels of platelet activation in individual experiments and is shown as mean ± standard error of the mean (SEM). Statistics were performed, and graphs were constructed, using GraphPad Prism (version 7.04) (GraphPad Software, La Jolla California, USA).

2.4 Results

***Treponema pallidum* interacts with human platelets**

Numerous bacterial species are recognized to interact with human platelets during infection, including the spirochetes *Borrelia burgdorferi* and *Borrelia hermsii* (Alugupalli et al., 2001; Coburn et al., 1993; Malawista & de Boisfleury Chevance, 2008), but to date this potential host cell interaction has remained unexplored for *T. pallidum*. To

identify potential interactions, human platelets and viable treponemes were coincubated and observed by darkfield microscopy. High resolution video imaging demonstrated that treponemes interacted with platelets primarily with tip-mediated contact (Fig 10 green arrows). This analysis also revealed interactions to be dynamic with frequent coiling and rotation against (Fig 10 cyan curved arrow) or gliding across the platelet.

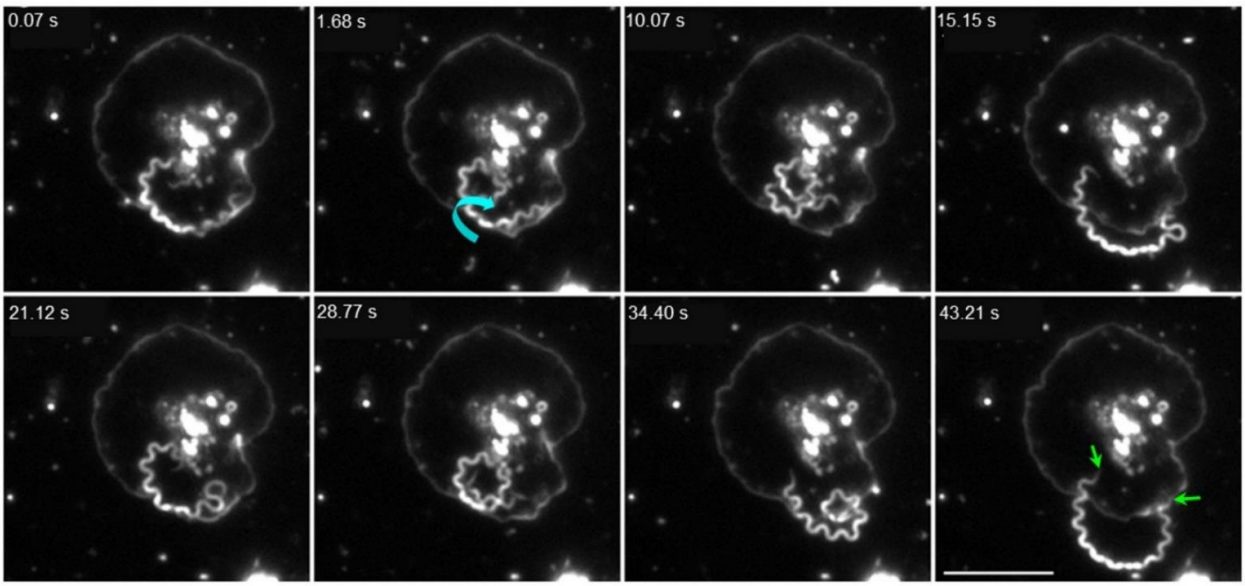

Figure 10: *Treponema pallidum* exhibits a dynamic interaction with human platelets. Darkfield video microscopy panels (1000x total magnification) show a treponeme attached to a fully activated platelet by both tips (green arrows 43.21 s). This treponeme alternated between coiling with vigorous axial rotation (2.68 Hz) against the platelet membrane (cyan curved arrow indicates the direction of rotation) (1.68 - 10.07 s, 28.77 s) and extending above the platelet (15.15 s, 21.12 s, 34.40 s), then remained attached by both tips (43.21 s) for a further 18 min 23 s of observation. Scale bar = 10 µm.

***Treponema pallidum*-platelet interactions correlate with *T. pallidum* viability**

To determine if *T. pallidum*-platelet interactions were dependent upon viable treponemes, we compared the binding of heat-treated (nonviable) treponemes and viable treponemes to human platelets by flow cytometry (Fig 11A-D). Platelets and treponemes co-localize and this population formed the first gate on the FSC by SSC plot (blue broken line Fig 11A). The binding events (lime gates) of platelets with either heat-treated (Fig 11B) or viable (Fig 11C) treponemes were compared. Viable treponeme-platelet binding events (mean = 55.13% ± 2.91 [SD] $P<0.0001$, Figs 10C and D) were significantly higher than events associated with heat-treated treponemes (mean = 19.05% ± 2.29 [SD], Fig 11 B and D). Microscopic analysis consisted of determining the percent of platelet-interacting treponemes out of the total treponemes observed in 20 random locations per slide and confirmed viable treponemes coincubated with human platelets bound significantly more platelets (mean = 31.73% ± 0.96 [SD] $P<0.0001$, Fig 11E) compared with heat-treated treponemes (mean = 6.47% ± 0.71 [SD], Fig 11E). Viable treponemes were also observed to disengage from interactions with platelets (Fig 11G panels 0.01, 8.10 & 9.05 s, Figs 16 and 17. Heat treatment resulted in non-motile treponemes (Fig 11F top) that remained morphologically consistent with viable treponemes (Fig 11F bottom).

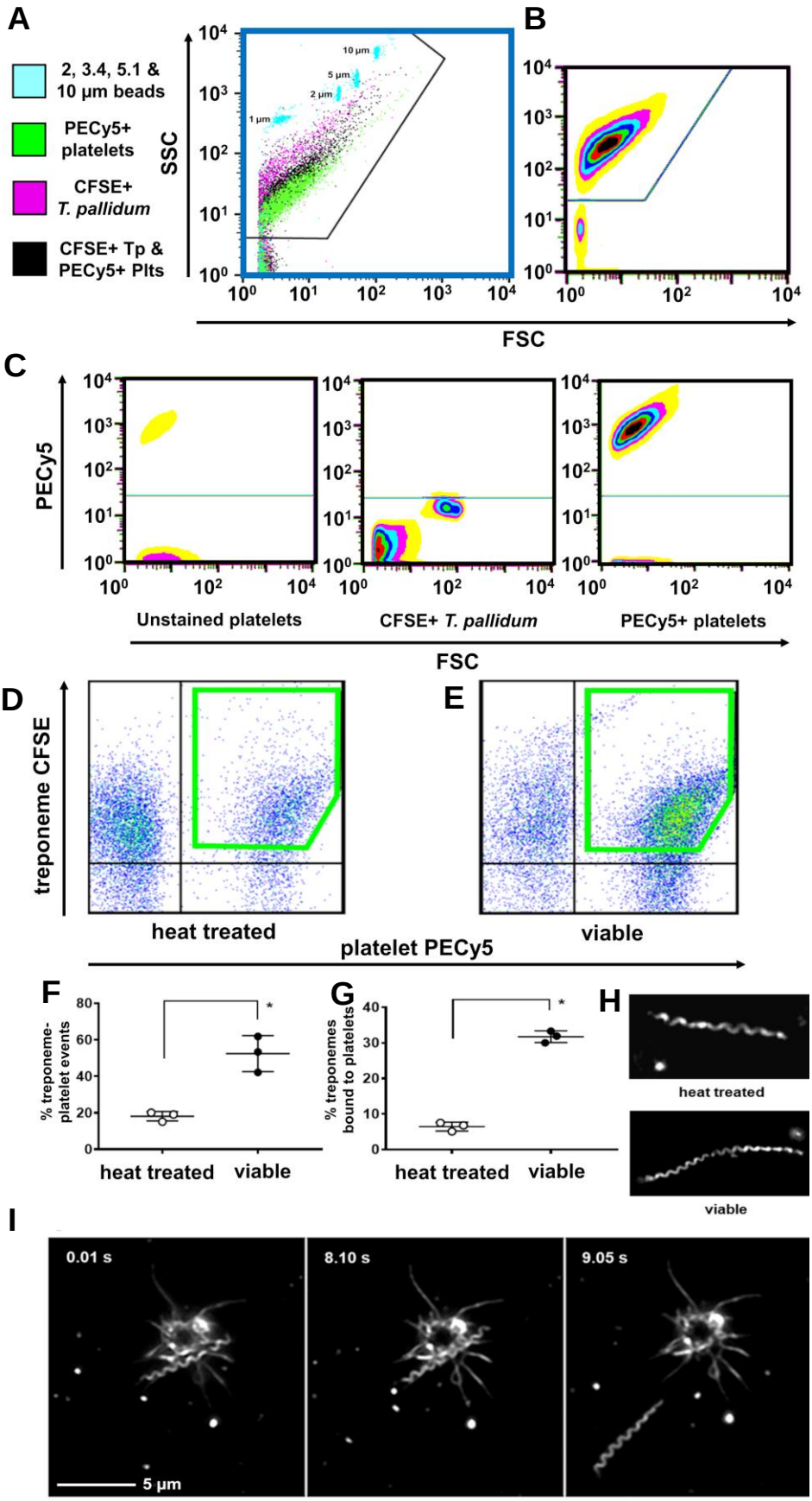
A
2, 3.4, 5.1 & 10 µm beads
PECy5+ platelets
CFSE+ T. pallidum
CFSE+ Tp & PECy5+ Plts
SSC
FSC
10 µm
5 µm
2 µm
1 µm
B
C
PECy5
Unstained platelets
CFSE+ T. pallidum
PECy5+ platelets
FSC
D
treponeme CFSE
heat treated
E
viable
platelet PECy5
F
% treponeme-platelet events
heat treated
viable
G
% treponemes bound to platelets
heat treated
viable
H
heat treated
viable
I
0.01 s
8.10 s
9.05 s
5 µm

Figure 11: *Treponema pallidum*-platelet interactions. (A) A flow cytometry overlay of SSC by FSC dot plots of individual samples of PECy5+ platelets (lime), CFSE+ *T. pallidum* (rose), PECy5+ platelets and CSFE+ *T. pallidum* (black), together with 2 to 10 μm sized beads (cyan) shows population colocalization by size facilitates initial gating. (B) Flow cytometry gating of PECy5+ platelets on a PECy5+ (FL3) by FSC plot. (C) Flow cytometry negative control plots show unlabeled platelets (left) and CFSE+ *T. pallidum* (middle) do not demonstrate PECy5 fluorescence (FL3) compared with the positive control PECy5 labeled platelets (right). Dot plots of (D) CFSE-labeled heat-treated treponemes demonstrate reduced binding to human platelets labeled with PECy5 anti-CD41a compared with (E) CSFE-labeled viable treponemes. (F) CFSE-labeled viable treponemes bound significantly more human platelets (mean = 55.13% ± 2.91 [SD] *P<0.0001) compared with heat-treated treponemes (mean = 19.05% ± 2.29 [SD]) following coincubation for 16 hours at 37˚C and ~ 5% oxygen. Results represent the mean of three independent experiments with statistical significance computed by two-way ANOVA, with a minimum of 3 replicates per sample type per experiment. (G) Darkfield microscopy FOV counts (20 random locations/slide) demonstrate viable treponemes bind significantly more human platelets (mean = 31.73% ± 1.19 [SD] *P<0.0001) than heat-treated treponemes (mean = 6.47% ± 1.19 [SD]) following coincubation at 5% oxygen at 34˚C for 48 hours. Results represent the mean of three independent experiments with statistical significance computed by unpaired two-tailed Student's t test, n = 3. (H) Heat-treated treponemes (top) are non-motile yet morphologically similar to viable treponemes (bottom). (I) Darkfield microscopy at 1000x magnification demonstrates platelet interactions are reversible. Image capture from

video micrographs show edgewise attachment of a treponeme to an activated platelet (0.01 s to 8.10 s) which then detaches and moves away (9.05 s).

Treponema pallidum–platelet binding positively correlates with increasing platelet activation

We hypothesized that fully activated, spread platelets would be preferentially targeted by treponemes given the direct adhesion of spread platelets *in vivo* to the endothelium during hemostasis and modulation of vascular permeability (Gay & Felding-Habermann, 2011). Indeed, when the collection of darkfield images was analyzed there were 125 treponeme-platelet interactions (75 individual images sampled from coincubations of platelets with 11 independent treponeme extractions), and the majority of treponeme-platelet interactions (57.8%, n = 72) occurred between fully activated, spread platelets (Fig 12A and B, right panel) followed by 37.6% to the next most activated state, the spheroid form with fully extended pseudopods (n = 47) (Fig 12A and 9B [middle panel]. Platelets in the earliest visible stage of activation, with at least one pseudopod bud (Fig 11A and 9B [left panel], yellow), were observed to interact with treponemes to a much lesser degree at 4.8% (n = 6), whilst at no time were fully inactive, discoid platelets (Fig 12B left panel, grey) observed to interact with *T. pallidum*. The preferential interaction between treponemes and spread, activated platelets regularly occurred adjacent to platelets in lesser states of activation in the same FOV, confirming specificity (Figs 14 and 15). The relative binding of treponemes to pre-activated platelets was close to double that observed for resting platelets (mean = 1.98 ± 0.08 [SEM] P = 0.0003; Fig 21).

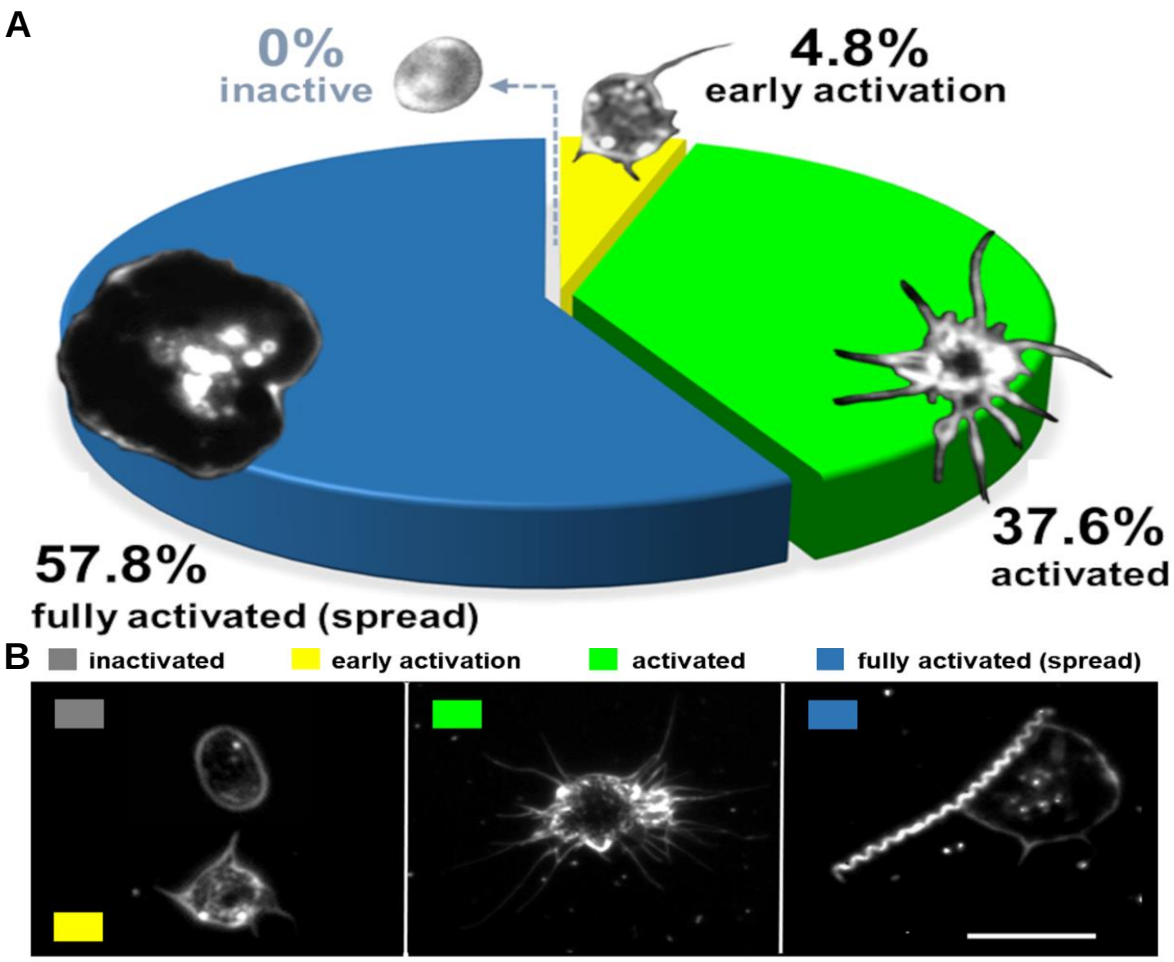

Figure 12: Binding of *T. pallidum* to platelets increases with platelet activation. (A) Treponemes displayed preferential binding to fully activated spread platelets (blue), with 57.8% of viewed images showing an interaction between fully activated platelets and *T. pallidum.* Treponemes bound to activated spheroid platelets (lime) in 37.6% of images, while platelets in early activation (yellow) constituted only 4.8% of the observed interactions. *Treponema pallidum* was never observed to bind to inactive platelets in 125 interactions analyzed from 75 images containing a total of 422 platelets (Table 6). Interactions and activation states were observed at 1000x magnification using darkfield microscopy. (B) Images of platelets at different stages of activation at 1000x

magnification using darkfield microscopy. Inactive platelets circulate as disks (grey) and bud pseudopods during early activation (yellow). Activated platelets may be spheroid with extended pseudopods (lime) or fully activated with an enlarged surface area and a very thin spread form (blue). Scale bar = 5.0 microns.

The interaction of *T. pallidum* with platelets is dynamic

Darkfield and electron microscopy studies have previously demonstrated that 90% of *T. pallidum* cells adhere to cultured rabbit epithelial by one or both tips, with over a third of treponemes using both tips (Izard et al., 2009). Initial darkfield observations revealed a complexity to treponeme-platelet interactions beyond simple adhesion. Over the course of platelet coincubations with 11 independent treponeme extractions we assembled a collection of live, high resolution images. We categorized and quantified four major types of interactions (227 interactions in 158 micrographs); (1) adhesion by one or both tips (Fig 13A and 10B [left panel]) occurring at a rate of 69% (n = 227); (2) “edgewise” binding along the axial plane of the cell body (Fig 13A and 10B [middle panel]) occurring at a rate of 14%; (3) “indirect” binding by adhesion to a platelet-interacting treponeme or extended platelet pseudopod (Fig 13A and 10B [right panel]) occurring at a rate of 14% and; (4) “dynamic” binding, characterized by coiling and uncoiling against the platelet membrane and/or rapid back and forth translations, (Fig 13A and C) comprised approximately 3% of interactions. Binding by both tips was observed in less than 1% of *T. pallidum*-platelet interactions. “Dynamic” binding involved continuous contact with the platelet but rather than “tip” or “edgewise“ adhesion, the treponeme remained in constant motion (Fig 13C).

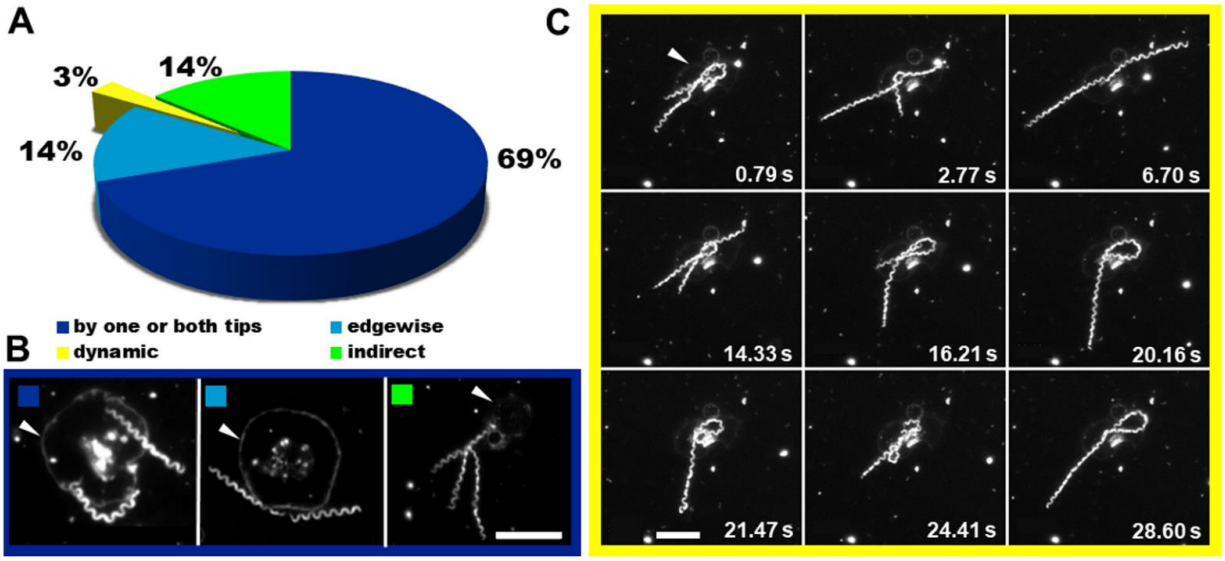

Figure 13: Treponemes bind in multiple ways to activated platelets. (A) Image analysis of 227 interactions in 158 micrographs demonstrated that 69% of treponemes bind platelets using one or both tips (dark blue). Both edgewise binding along the axial plane of the cell body (medium blue) and indirect binding via a platelet-bound treponeme or platelet pseudopod (lime) occurred in 14% of interactions, followed by "dynamic" binding (yellow), which occurred in 3% of interactions. (B) Treponemes interact with one or both tips (left), edgewise (middle) or indirectly via another treponeme (right). (C) Image capture of two treponemes with an activated platelet. Coiling of the treponeme on the platelet (0.79, 14.33, 16.21, 20.16, 21.47 and 24.41 s) with intervening periods of translation and extension away from the platelet by one (2.77, 20.16, 21.47 and 28.60 s) or both (6.70 s) treponemes was observed. White arrowheads indicate fully activated platelets. Scale bars = 5 µm.

Treponema pallidum **increases rotation and translational velocity upon interacting with platelets**

The ability to observe the interaction of treponemes and platelets at high resolution prompted us to compare the motility characteristics of platelet-interacting and non-interacting treponemes in the same FOV. By performing frame-by-frame kinematic comparisons, we detected a significant increase in both the translational velocity (forward and backward motility) and axial rotation of treponemes when engaging with platelets (Fig 14A-D shows axial rotation of both a platelet-interacting and a non-interacting treponeme). The translational velocity of platelet-interacting treponemes (mean = 1.68 ± 0.10 µm/s [SEM] $P < 0.0001$) was observed to be over two-fold that of non-interacting treponemes (mean = 0.65 ± 0.01 µm/s [SEM]), with a maximum observed velocity of 2.58 µm/s (Fig 13C). Accompanying this, the rotation rate increased (mean = 2.47 ± 0.86 hertz [SEM] **$P = 0.0060$) in contrast with non-interacting treponemes (mean = 1.58 ± 0.77 hertz [SEM]) (Fig 14D). Subtle phenotypic changes occurred as the cell body became more compact with a significantly shorter wavelength (ʎ) (mean = 0.79 ± 0.02 µm [SEM] $P = 0.0237$) compared to that of non-interacting treponemes (mean = 0.86 ± 0.02 µm [SEM]) (Fig 14A green bracket and Fig 14E top). Platelet-interacting treponemes also showed a trend towards a correspondingly higher amplitude (A) (mean = 0.30 ± 0.02 µm [SEM], Fig 14A yellow bracket and Fig 14E middle) and shorter cell body (L) (mean = 9.56 ± 0.65 µm [SEM], Fig 14B magenta bracket and 6E bottom) than non-platelet interacting treponemes (A mean = 0.26 ± 0.01 µm [SEM], Fig 7E middle, L mean = 10.46 ± 0.66 µm [SEM], Fig 14E bottom).

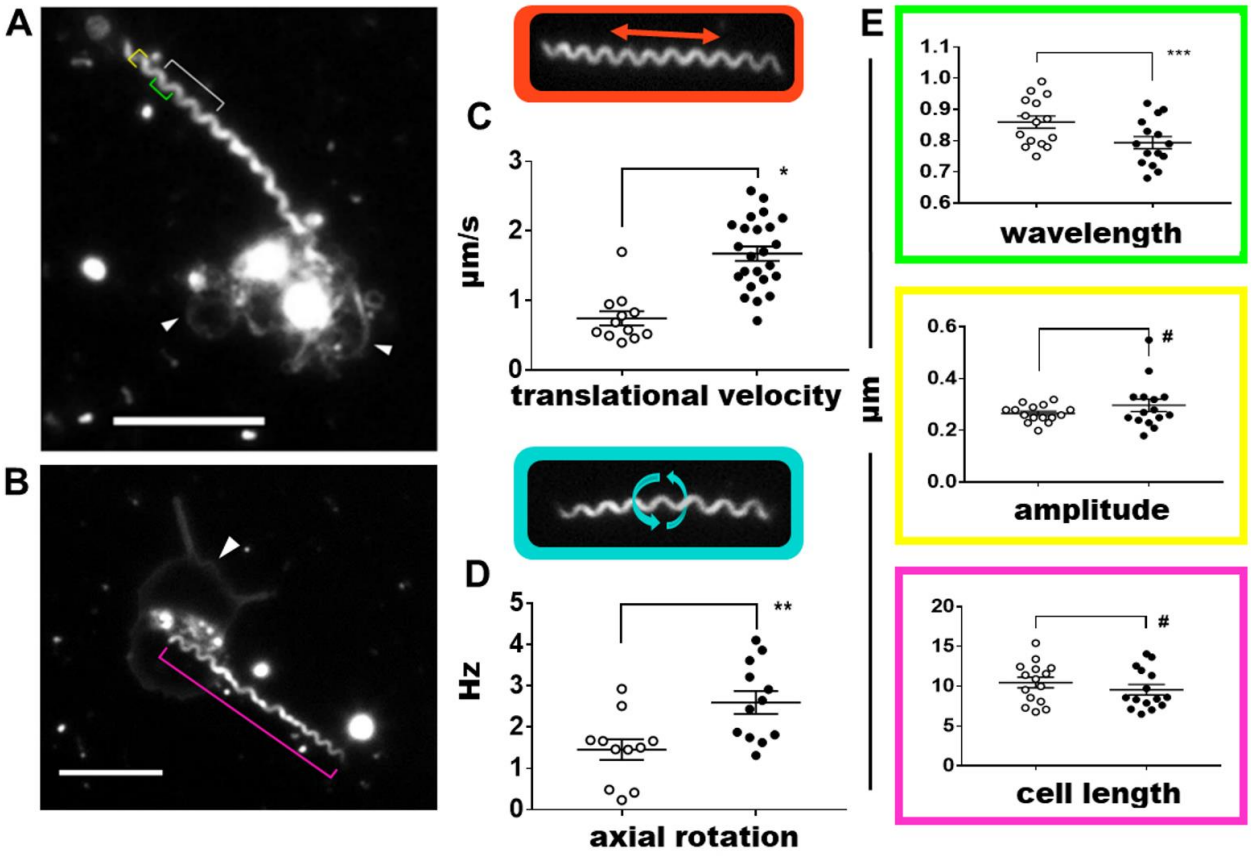

Figure 14: Motility parameters associated with *Treponema pallidum*-platelet interactions. The movement and cell characteristics were contrasted between platelet-interacting (shaded circles) and non-interacting (open circles) treponemes utilizing high-resolution darkfield videos at 1000x magnification. (A) Treponeme wavelength (green bracket) and amplitude (yellow bracket) were measured only when the cell body waveform was in the plane of focus (grey bracket). Two activated platelets (white arrowheads) are bound by one treponeme. The grey bracket shows a segment of the treponeme with cell peak and troughs in the same focal plane. (B) Treponeme length was measured when the full treponeme was in the same focal plane (magenta bracket). (C) Platelet-interacting treponemes demonstrated a higher translational velocity (mean = 1.68 ± 0.10 µm/sec [SEM] *P < 0.0001, n = 24) compared with non-interacting

treponemes (mean = 0.65 ± 0.10 μm/sec [SEM], n = 12). (D) Frame by frame analysis determined platelet-interacting treponemes rotate at a higher frequency (mean = 2.60 ± 0.27 Hz [SEM] **P = 0.0060, n = 12) versus non-interacting treponemes (mean = 1.46 ± 0.25 Hz [SEM], n = 11). (E) Top panel: Platelet-interacting treponemes maintained a more tightly coiled helix resulting in a shorter wavelength (mean = 0.79 ± 0.02 μm [SEM] ***P = 0.0237) versus non-interacting treponemes (mean = 0.86 ± 0.02 μm [SEM]), n = 15. Middle panel: Platelet-interacting treponemes demonstrated a trend towards a higher amplitude (mean = 0.30 ± 0.02 μm [SEM]) compared with non-interacting treponemes (mean = 0.27 ± 0.01 μm [SEM]), n = 15. Bottom panel: Platelet-interacting treponemes also displayed a trend towards a shorter, more compact coiled shape (mean cell length = 9.56 ± 0.65 μm [SEM]) compared with non-interacting treponemes (mean cell length = 10.46 ± 0.66 μm [SEM]), n = 15. Student's unpaired two-tailed t tests were used to calculate statistical significance. Scale bars = 5 μm.

***T. pallidum*-platelet interactions reduce treponemal speed and displacement upon plasma movement**

To measure the ability of *T. pallidum*-platelet interactions to reduce treponeme displacement, we used high resolution videos to record *T. pallidum* movements across the FOV on slides. Prior to reaching equilibrium, plasma currents propelled non-adherent treponemes and platelets across the FOV. Three video image sets that contained both short-term platelet-interacting and non-interacting treponemes were used to compare the effect of platelet adhesion on the ability of treponemes to resist fluid motion until plasma reached a steady state. Treponemes bound to platelets prior to plasma movement maintained attachment, and treponemes that established an

interaction with a platelet reduced their speed and overall displacement across the FOV (Figs 14, 18 and Table 9). For these measurements the ratio of values for non-interacting to interacting treponemes was compared to determine the differences in relative speed and displacement between different FOV (Tables 7 and 8). Non-interacting treponemes experienced a higher speed (mean = 2.40 ± 0.16-fold [SEM]) and larger displacement (mean = 2.10 ± 0.30-fold [SEM]) across the FOV compared to platelet-interacting treponemes (Fig 18 and Tables 7 and 8). The interactions observed in one representative video are shown in Fig 15. By establishing an interaction with a platelet, treponeme 1 (Fig 15 green) reduced its overall speed to 3.64 μm/sec and displacement to 48.64 μm during 13.35 s of viewing. In comparison, treponeme 2 (Fig 15 cyan), which did not interact with platelets as it passed across the FOV, had a speed of 8.73 μm/sec and displacement of 82.98 μm during the 9.51 s it was visible in the FOV (Table 8).

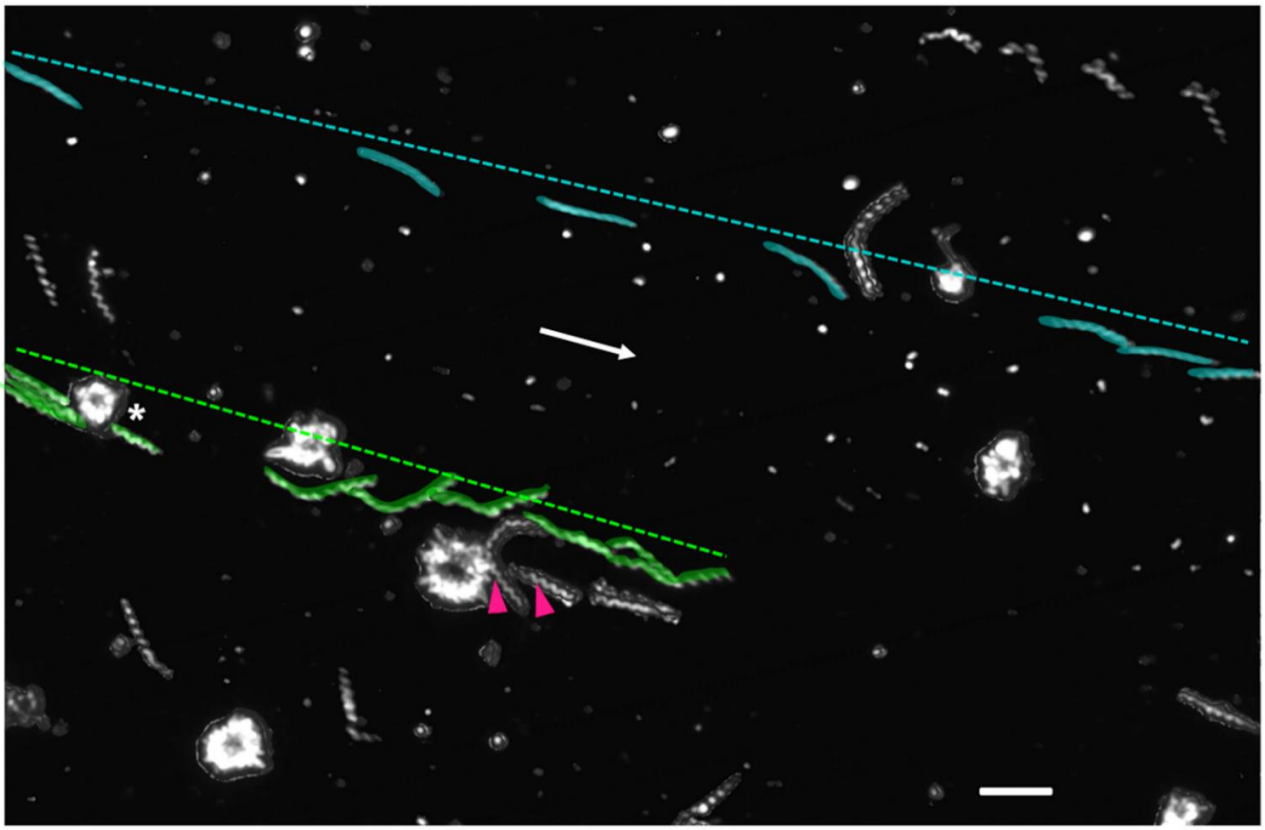

Figure 15: Platelet-tethered treponemes experience reduced displacement and a lower speed than non-interacting treponemes. An overlay of several video microscopy frames demonstrates the reduced speed and displacement experienced by a treponeme (green) that interacted with a slide-adhered platelet (asterisk). This interaction reduced its overall speed to 3.64 µm/sec and displacement to 48.64 µm (green dotted line) compared with a treponeme that did not interact (cyan) which has a speed of 8.73 µm/sec and displacement of 82.98 µm (cyan dotted line). Treponemes engaged in stationary adhesion (magenta arrowheads) maintained attachment when slide movement induced plasma movement. White arrow indicates the direction of flow. Scale bar = 5 µm.

***Treponema pallidum* activates human platelets**

The potential for *T. pallidum* to activate platelets was investigated by quantifying platelet receptor CD41a expression by flow cytometry. Coincubation of *T. pallidum* with resting platelets elicited a significant increase in CD41a expression (mean = 806.0 ± 37.76 MFI [SEM], P = 0.0118) compared to resting platelets (mean = 545.5 ± 104.4 MFI [SEM]) (Fig 16A). The level of platelet activation by treponemes was comparable to that seen with agonist-activated platelets (mean = 746.5 ± 124.3 MFI [SEM], Fig 16A).

Platelet activation by *T. pallidum* was also assessed by probing for the production of fibrin clots using a plate-based assay to measure absorbance at 600 nm. In this assay, an increase in absorbance is indicative of fibrin clot formation, which occurs as a result of platelet activation. As shown in Fig 16B, incubation of resting platelets with *T. pallidum* resulted in an absorbance increase (mean = 0.4137 ± 0.007 [SEM], P = 0.0221) compared to that of resting platelets alone (mean = 0.3730 ± 0.018 [SEM]). As with the flow cytometry experiments, the level of platelet activation by treponemes was comparable to that seen with agonist-activated platelets (mean = 0.3791 ± 0.027 [SEM], Fig 16B).

Upon initial exposure to *T. pallidum*, platelets displayed no significant increase in the CD62P MFI (Fig 23, blue box, mean = 59.69 ± 1.11 [SEM]) compared to inactive platelets (mean = 56.18 ± 0.37 [SEM]). After this 4 hour lag period, treponeme coincubated platelets expressed significantly more CD62P (mean = 64.77 ± 1.57 [SEM], P = 0.0002) compared with inactive platelets (mean = 53.62 ± 0.72 [SEM]|) and this trend continued with significant results at 8 hours (P < 0.0001), 20 hours (P < 0.001) and 24 hours (P = 0.0013).

A similar lag period was observed in a separate experiment which tested the platelet binding potential of heat-treated versus viable treponemes, with no significant difference in platelet binding after 1 hour coincubation (Fig 23, blue box) with viable (mean = 23.39 ± 3.92 [SEM]) or heat-treated treponemes (mean = 17.36 ± 0.73 [SEM]). Significantly increased platelet binding was seen after 3 hours (mean = 31.65 ± 4.75 [SEM], P = 0.0342) and 15 hours (mean = 31.12 ± 1.67 [SEM], P = 0.0016) of coincubation with viable treponemes, compared to the level of binding observed with heat-treated treponemes at 3 hours (mean = 16.44 ± 0.83) and 15 hours (mean = 17.17 ± 0.77 [SEM]).

While exposure to *T. pallidum* induced platelet activation, shown by the 1.48-fold increase in CD41a (Fig. 15A) and the 1.21-fold increase in CD62P expression (Fig 23), the lag period of 4 hours is notably longer than the 5 to 30-minute lag period typical of bacterial pathogens (Matus et al., 2017; Shannon, 2017). In comparison, enterohemorrhagic *E. coli* O111, a highly virulent and prevalent serotype that induces DIC, has been shown to increase platelet CD62P expression by 2.84-fold after 30 minutes of coincubation (Matus et al., 2017). Together, the long lag period and comparatively lower levels of platelet activation demonstrated in this study *in vitro*, reflect the pathogenesis seen during the rapid vascular dissemination of *T. pallidum in vivo,* which fails to induce systemic, pathological platelet activation (LaFond & Lukehart, 2006).

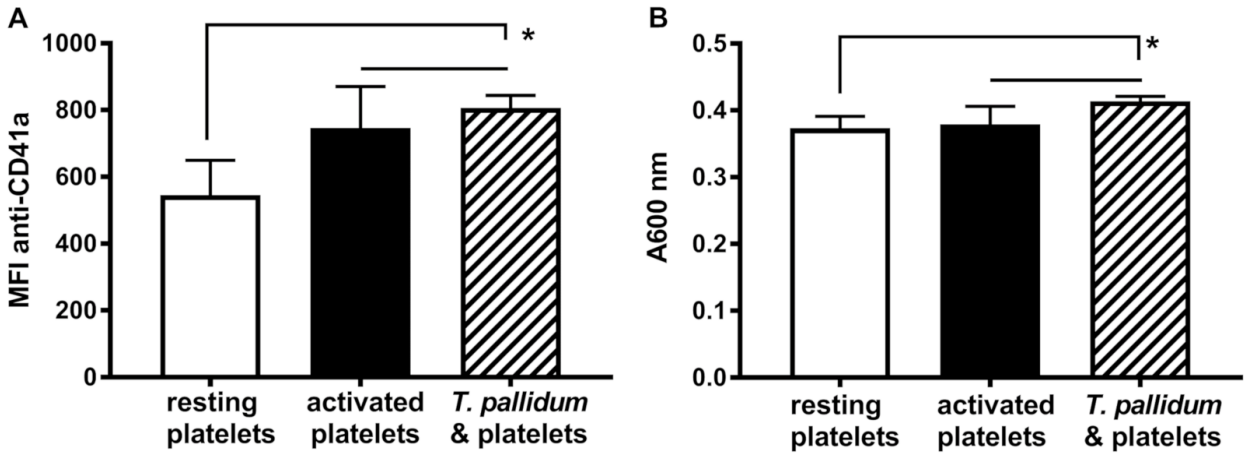

Figure 16: *Treponema pallidum* activates human platelets. The potential for treponemes to activate platelets was assessed by flow cytometry (A) and using a plate-based assay (B). (A) Following 18 hours coincubation at 37°C the results of two independent flow cytometry assays quantified platelet activation by the median fluorescence intensity (MFI) associated with CD41a receptor up-regulation. Platelets coincubated with treponemes (hatched bar) demonstrated a significantly higher MFI (mean = 806.0 ± 37.76 [SEM], *P = 0.0118, n = 25) compared to resting platelets (white bar) (mean = 545.5 ± 104.4 [SEM], n = 18). There was no significant difference between treponeme coincubated platelets and agonist pre-activated platelets (black bar) (mean = 746.5 ± 124.3 [SEM], n = 18). (B) Following coincubation for 18 hours at 34°C the results of two independent platelet activation experiments were assessed by measuring the absorbance at 600 nm as a surrogate measure of fibrin clot formation. Platelets coincubated with *T. pallidum* (hatched bar) had a higher absorbance (mean = 0.4137 ± 0.007 [SEM], *P = 0.0221, n = 20) compared to resting platelets (white bar) (mean = 0.3730 ± 0.018 [SEM], n = 12). There was no significant difference between treponeme-

coincubated platelets and activated platelets (black bar) (mean = 0.3791 ± 0.027 [SEM], n= 7).

2.5 Discussion

In this study we have established, for the first time, that live *T. pallidum* interacts with human platelets. By using a modified darkfield microscopy method, wherein the slide volume is reduced to enhance resolution, we were able to compare physical and kinematic parameters between platelet-interacting and non-interacting treponemes. Platelet-interacting treponemes increased their axial rotation and translational velocity and decreased their wavelength compared with non-interacting treponemes, to an extent that achieved statistical significance. A trend towards increased amplitude and decreased length was also observed with platelet-interacting treponemes compared to non-interacting treponemes, although these parameters did not achieve statistical significance. Interestingly, *B. burgdorferi* has demonstrated decreased cell length following coincubation with soluble fibronectin, present in plasma at 300-400 µg/mL (Mosesson, 2005; Niddam et al., 2017).

By comparison with a previous report that investigated the translational velocity, wavelength and amplitude of free *T. pallidum* at a 400x magnification, our measured amplitudes were comparable (0.30 ± 0.02 µm and 0.26 ± 0.01 µm compared to 0.28 ± 0.01 µm reported by Harman et al (Harman et al., 2013)). However, notable differences in translational velocity and wavelength were observed. In our study, the translational velocity of free treponemes in human plasma was slower (mean = 0.65 ± 0.01 µm/sec) compared to the translational velocity previously reported for *T. pallidum* in CMRL medium (mean = 1.9 ± 0.2 µm/s) (Harman et al., 2013). Similarly, we observed a

wavelength of 0.86 ± 0.02 μm for free treponemes while Harman et al reported a wavelength of 1.56 ± 0.04 μm (Harman et al., 2013). Possible reasons for these differences include the fact that our study was conducted at a higher magnification, potentially allowing for increased accuracy of measurements, as well as the possibility of altered environmental conditions increasing treponemal health, with our use of a microaerophilic chamber for *T. pallidum* extraction and nutrient-rich platelet suspensions. Further, Harman et al reported a reduction of *T. pallidum* translational velocity upon increasing viscosity of the treponemal suspension buffer (Harman et al., 2013). Since human plasma has a higher viscosity than CMRL medium (1.10-1.60 cP (Harman et al., 2013; Schindelin et al., 2012) compared to 0.89 cP (Bhat et al., 2014), respectively), this may explain the observed difference in translational velocity between the two studies.

The current study shows that *T. pallidum* exhibits an altered phenotype upon interacting with human platelets. Overall, we observed that *T. pallidum* achieves a more compressed spiral shape and increased activity upon interaction with human platelets, and that *T. pallidum* preferentially interacts with spread, activated platelets. Further, we observed that *T. pallidum*-platelet interactions occurred only with viable (and not heat-killed) treponemes, were reversible, and increased the translational velocity of *T. pallidum*. Collectively these findings suggest the interaction of *T. pallidum* with platelets is an active process that could contribute to treponemal pathogenesis, rather than a platelet-initiated process to aid in treponemal elimination during infection.

Relevantly, the current study suggests *T. pallidum* both interacts with activated platelets and activates platelets from an unactivated state over a time period of approximately 4

hours after the time of first interaction. Regardless of the route of activation, the interaction of *T. pallidum* with spread, activated platelets could contribute to treponeme persistence and extravasation. Activated platelets adhere to vascular breaches in a manner dependent upon the interaction of the platelet receptor Glycoprotein VI with collagen, facilitating platelet retainment *in situ* for several hours (Boulaftali et al., 2018; Gros, Syvannarath, et al., 2015); *in vivo* this would avoid the clearance observed with circulating, activated platelets (Handtke et al., 2018; Manfredi et al., 2010; Maugeri et al., 2009). Further, during inflammation endothelial cell engulfment of adherent platelets occurs (Ma et al., 2017), which could aid in the extravasation of co-adherent *T. pallidum*.

The significance of the demonstrated association of *T. pallidum* with platelets, and in particular activated platelets, is expected to be two-fold. First, the association may provide *T. pallidum* with nutrients which enable enhanced fitness, since activated platelets secrete a vast array of small molecules, ions and enzymes into the surrounding cellular milieu [46,48–50]. Second, the activated platelet secretome has also been demonstrated to facilitate platelet-endothelial cell adhesion and alter vascular permeability (Alugupalli et al., 2001; Ed Rainger et al., 2015; Gay & Felding-Habermann, 2011; Izard et al., 2009; Jun et al., 2008; Zuchtriegel et al., 2016). Endothelial-bound activated platelets play a key role in mediating *Streptooccus gordonii* and *Streptococcus oralis* adhesion during infective endocartitis (Haworth et al., 2017; A. K. Singh et al., 2017; Yakovenko et al., 2018). Metastatic tumor cells have been shown to directly interact with, and rely on, platelets to faciltiate extravasation (Leblanc & Peyruchaud, 2016; Schlesinger, 2018; Zuchtriegel et al., 2016), and extravasation and

dissemination of *T. pallidum* may be similarly aided by *T. pallidum*-platelet interactions. In this scenario, the interaction of *T. pallidum* with platelets would reduce treponemal speed and limit treponemal displacement within the bloodstream, as suggested by the observations reported in this study, allowing *T. pallidum* to be poised for extravasation. The *T. pallidum*-platelet interactions would further assist with extravasation via the natural association of activated platelets with endothelial cells and the capacity of the secretome from activated platelets to increase vascular permeability (Rainger et al., 2015; Golebiewska & Poole, 2015). In the current study nearly one third of treponemes bound to platelets in a heterogeneous manner, which differs from the preferential tip binding noted to dominate direct binding of *T. pallidum* to endothelial cells from prior studies (Izard et al., 2009). When considered in the context of the increased translational velocity observed for platelet-interacting treponemes, this may provide an opportunity for *T. pallidum* to navigate platelet-endothelial cell associations and access areas of increased vascular permeability.

In addition to increasing vascular permeability, platelets also increase blood-brain barrier permeability through secretion of sCD40L, VEGF, IL-1, CXCL4/PF4 and serotonin (Cloutier et al., 2012; Jones et al., 2016; Nair et al., 2015; Srivastava et al., 2008). Further, maternal platelets are incorporated into the lumen of blood vessels during placental formation and are found in the placental villi and fetal endothelium during the first trimester of pregnancy (Blaschitz et al., 2015; Bódis et al., 2014). The ability of *T. pallidum* to interact with platelets may therefore also play a role in the capability of *T. pallidum* to cross the blood-brain and placental barriers.

While the molecular details of the observed *T. pallidum*-platelet interactions have yet to be elucidated, prior investigations focused on *T. pallidum* and other pathogens suggest multiple mechanisms may contribute to this interaction (Hamzeh-Cognasse et al., 2015; McNicol, 2015; Rondina et al., 2017). The related spirochetes *B. burgdorferi* and *B. hermsii* bind directly to the platelet receptor β3 integrin via an outer membrane protein (Alugupalli et al., 2003; Coburn et al., 1993), and *T. pallidum* proteins bind the plasma proteins fibrinogen and (Parker et al., 2016) fibronectin (Dickerson et al., 2012), which in turn bind to the αIIbβ3, αvβ3 and α5β1 integrins found on platelets (Hamzeh-Cognasse et al., 2015; Sánchez-Cortés & Mrksich, 2009). Platelet activation is both paracrine and autocrine, mediated by granule secretion and synergy between signaling pathways (Estevez & Du, 2017). Activation of platelets via collagen engagement of the Glycoprotein VI platelet receptor induces specific signaling, hemostasis-independent single platelet adhesion to inflamed endothelium (Boulaftali et al., 2018; Gros, Syvannarath, et al., 2015) and ADP secretion, which in turn promotes autocrine activation, high affinity integrin RGD-ligand interactions and platelet spreading (Estevez & Du, 2017; Joo, 2012; O'Brien et al., 2012). The significance of platelet-mediated extravasation to metastasis also suggests an important role for increased platelet sensitivity to ADP during late metastasis (Haemmerle et al., 2018; Li, 2016), a feature also reported during syphilis infections (Szanto, 1971). Thus, *T. pallidum* may interact with platelets via plasma proteins and platelet receptors using a mechanism common to many bacterial pathogens, and at the same time exploit mechanisms used by metastatic cells to facilitate hematogenous dissemination.

The current study reports the novel finding that *T. pallidum* attaches to platelets, with a preference for activated platelets. Determination of the relevance of this association to the process of *T. pallidum* pathogenesis will require further investigations that extend beyond observational findings to probe the molecular details, and *in vivo* relevance, of the *T. pallidum*-platelet interaction.

Acknowledgments

We would like to thank Charmaine Wetherell for technical support, Julie Schroeder and Bradley Bachmeier for providing phlebotomy services, Simon Houston for critical manuscript review, and Rebecca Hof and Alloysius Thomas for *T. pallidum* propagation.

2.6 Supporting Information

Additional Figures

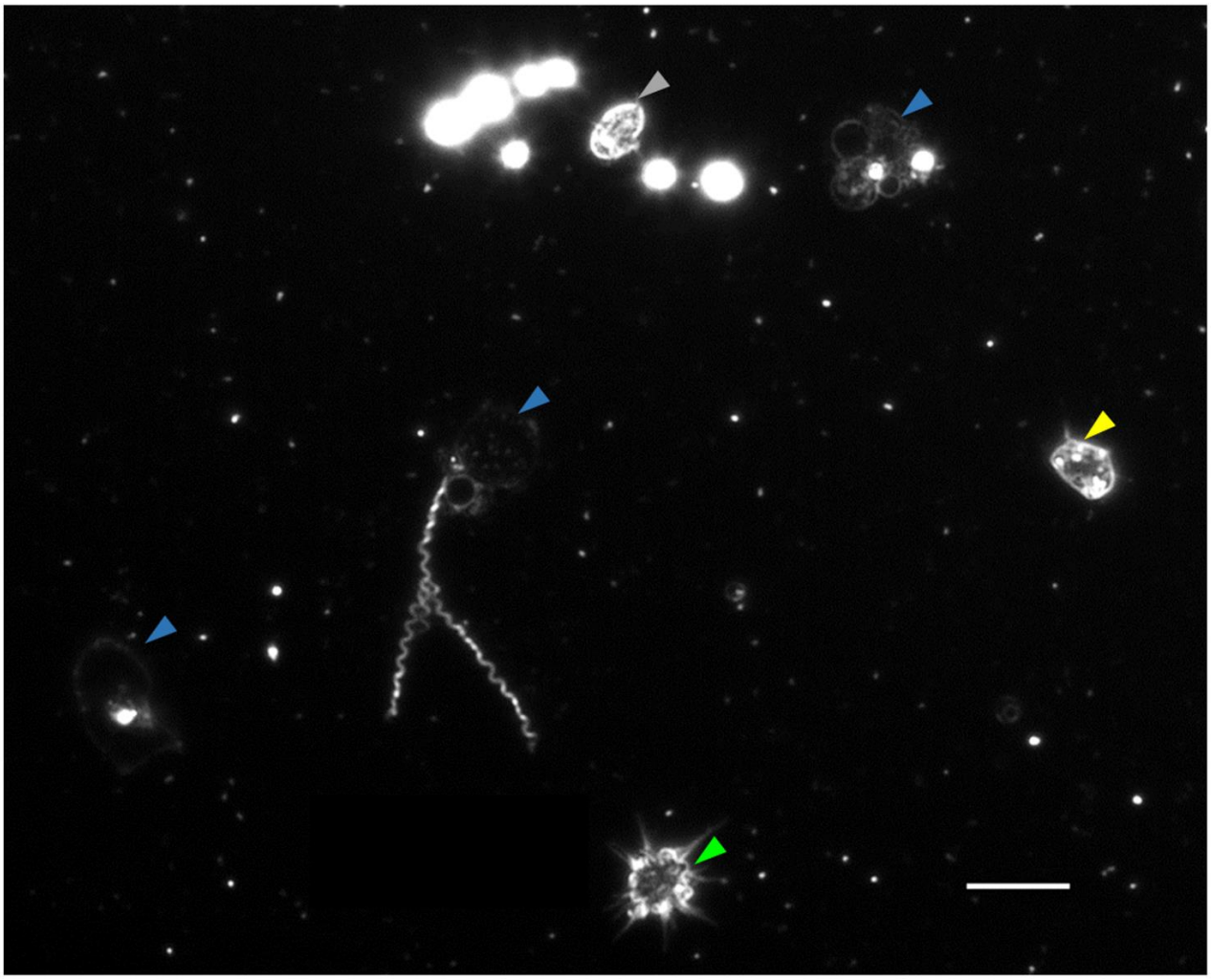

Figure 17: *Treponema pallidum* preferentially binds activated platelets. UVDFM image of platelets in the same FOV in both the inactive state and all stages of activation: inactive (grey arrowhead), early activation (yellow arrowhead), activated spheroid (lime arrowhead) and fully activated spread (blue arrowheads) with both direct and indirectly bound treponemes. Scale bar = 5 µm.

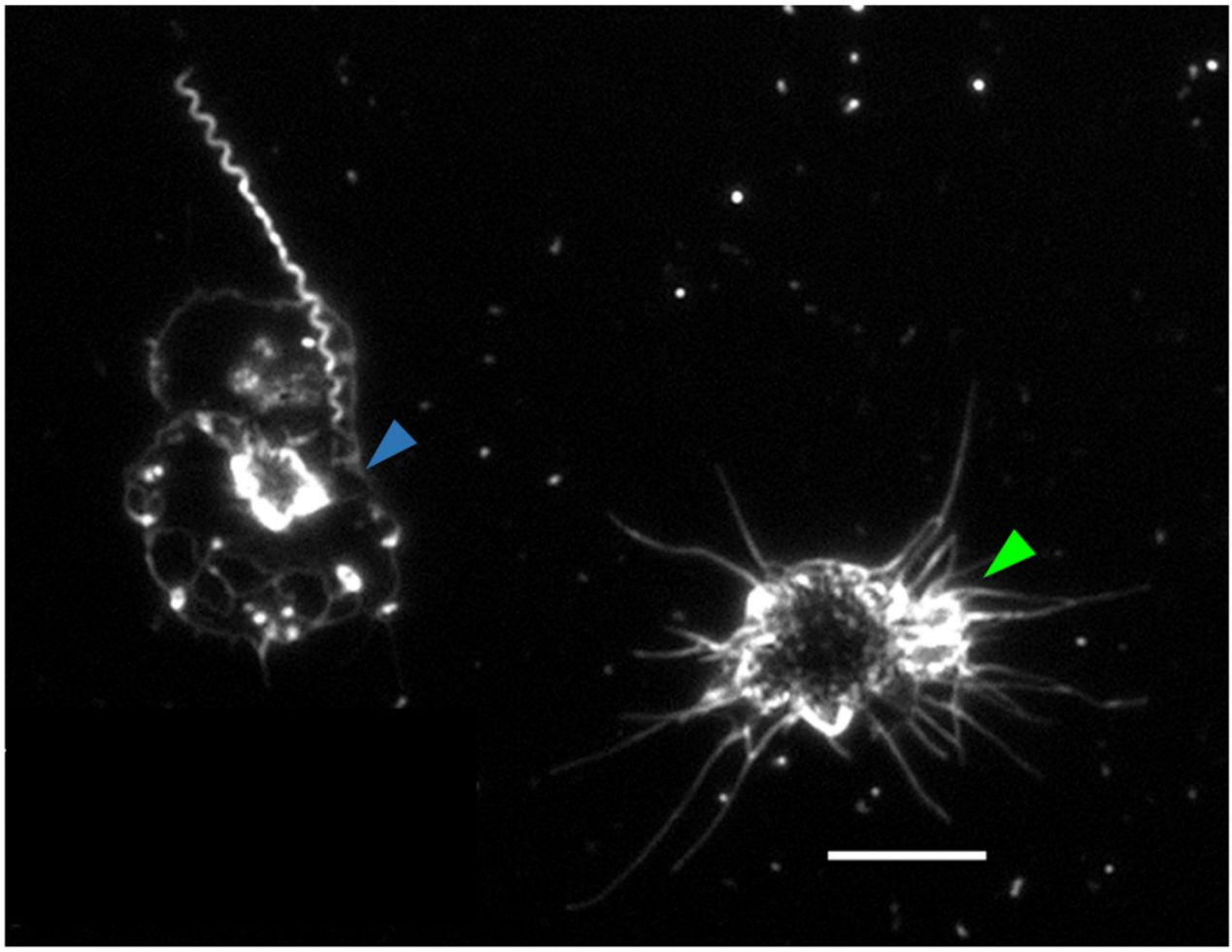

Figure 18: *Treponema pallidum* attached to a fully activated spread platelet. A treponeme is attached by one tip to a fully activated, spread platelet (blue arrowhead) with an activated spheroid platelet adjacent (lime arrowhead). Scale bar = 5 µm.

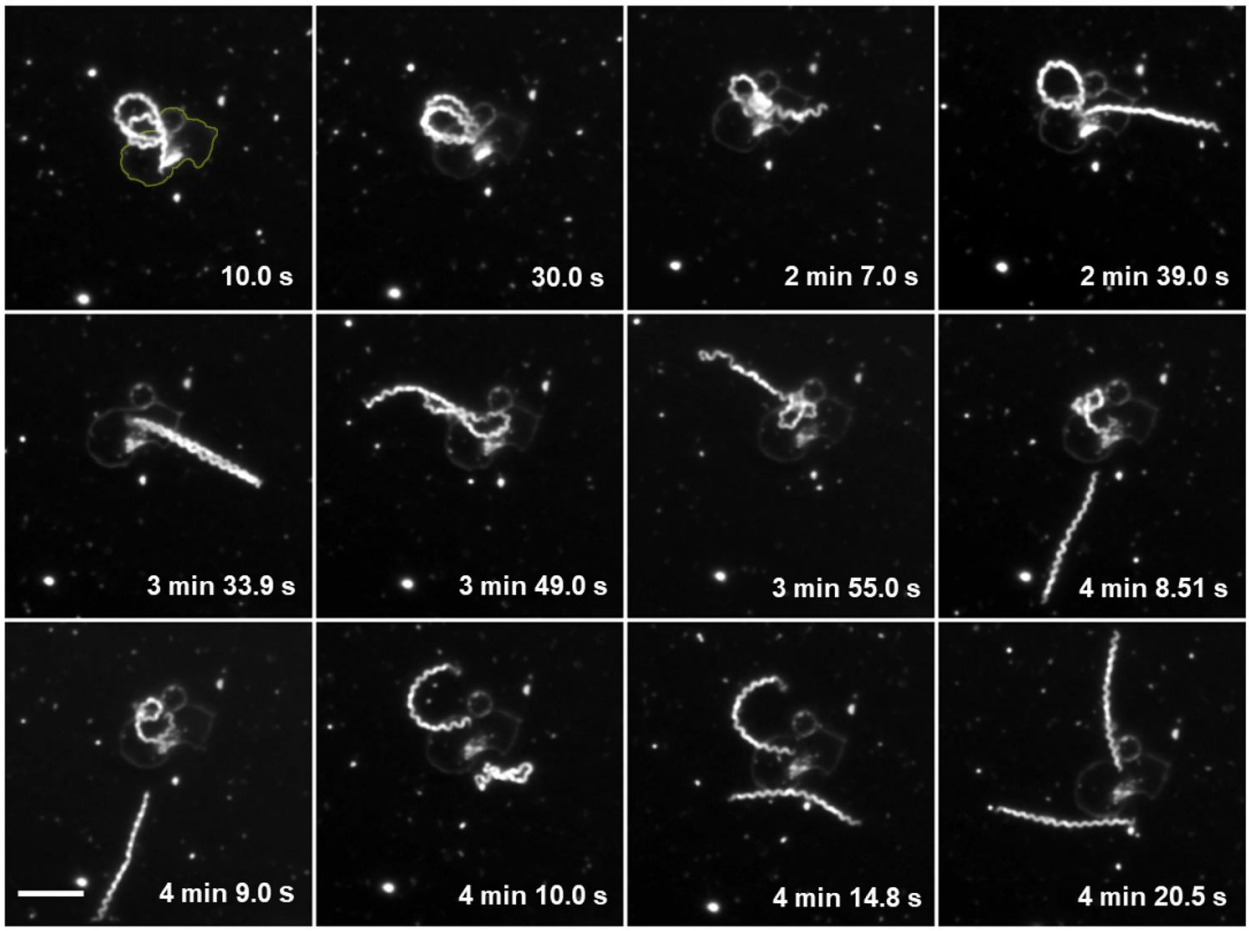

Figure 19: *Treponema pallidum* adhesion to platelets is a reversible interaction. Frame capture from UVDFM videos demonstrate interactions between two treponemes and a slide-anchored activated platelet (yellow outline). The treponemes engage in dynamic binding with one treponeme leaving the platelet at 4 min 6.5 s and returning to re-engage the platelet at 4 min 10.1 s. Scale bar = 5 µm.

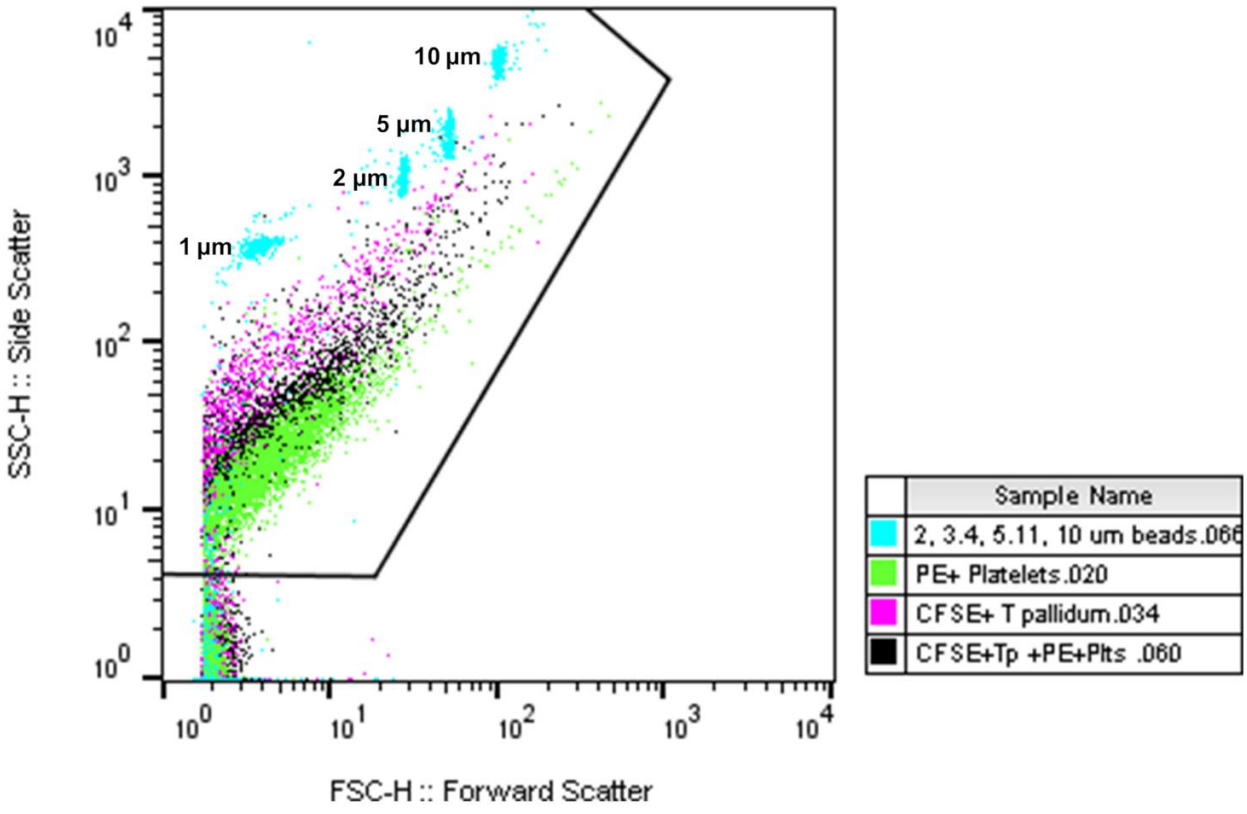

Figure 20: Treponemes and platelets co-localize by size. Treponeme-platelet co-localization is demonstrated in flow cytometry FSC x SSC dot plots showing the position of 1, 2, 5, and 10 micron sized beads (cyan) overlaid with representative dot plots of *T. pallidum* only (magenta), platelet only (lime) and *T. pallidum*-platelet coincubation (black) samples.

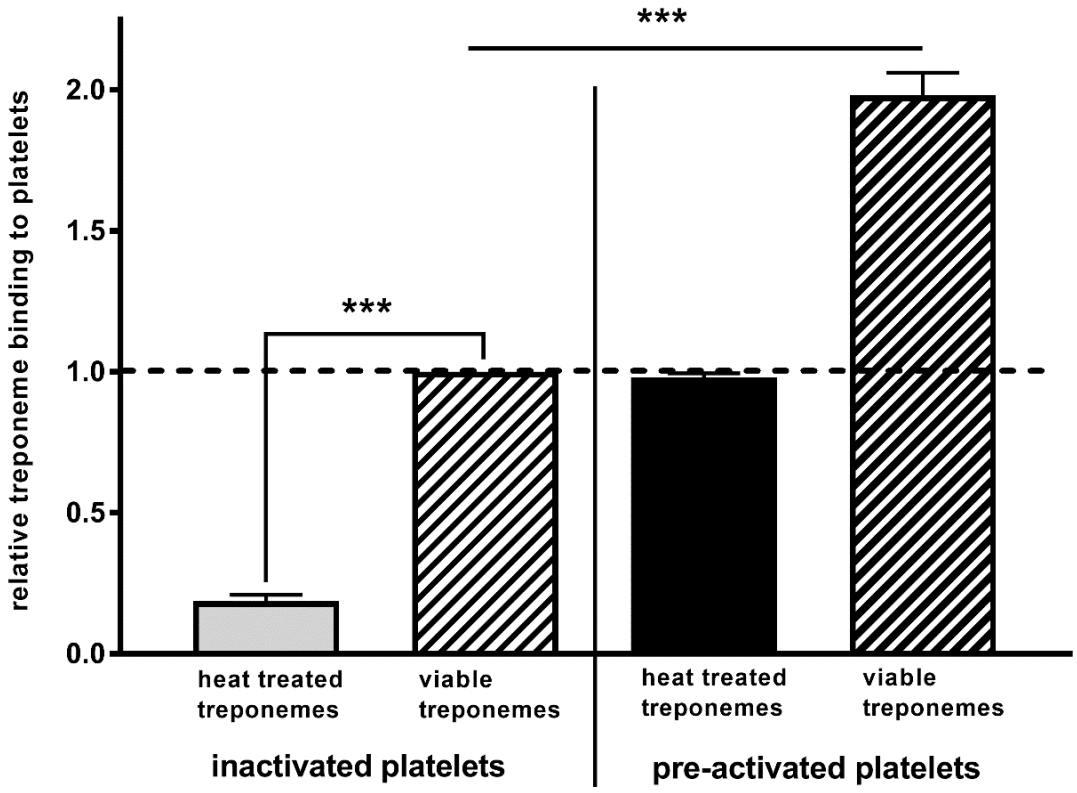

Figure 21: Pre-activating platelets results in higher treponeme binding. Flow cytometry quantified the binding of CFSE-labeled viable or heat treated treponemes to either resting (defined as resting prior to coincubation) or pre-activated platelets stained with PE-labeled anti-CD41a (three biological replicates per sample type). The number of viable treponeme (hatched bars)-resting platelet interactions was designated as the baseline (and set at 1.0) and used to compare viable treponeme binding to pre-activated platelets. Platelet pre-activation nearly doubled the binding events (mean = 1.98 ± 0.08 [SEM] ***P = 0.0003). Compared to viable treponemes, heat treated treponemes bound significantly fewer resting platelets (grey bar) (mean = 0.187 ± 0.02 [SEM] ***P = 0.0003) and pre-activated platelets (black bar) (mean = 0.98 ± 0.02 [SEM] P = 0.0003).

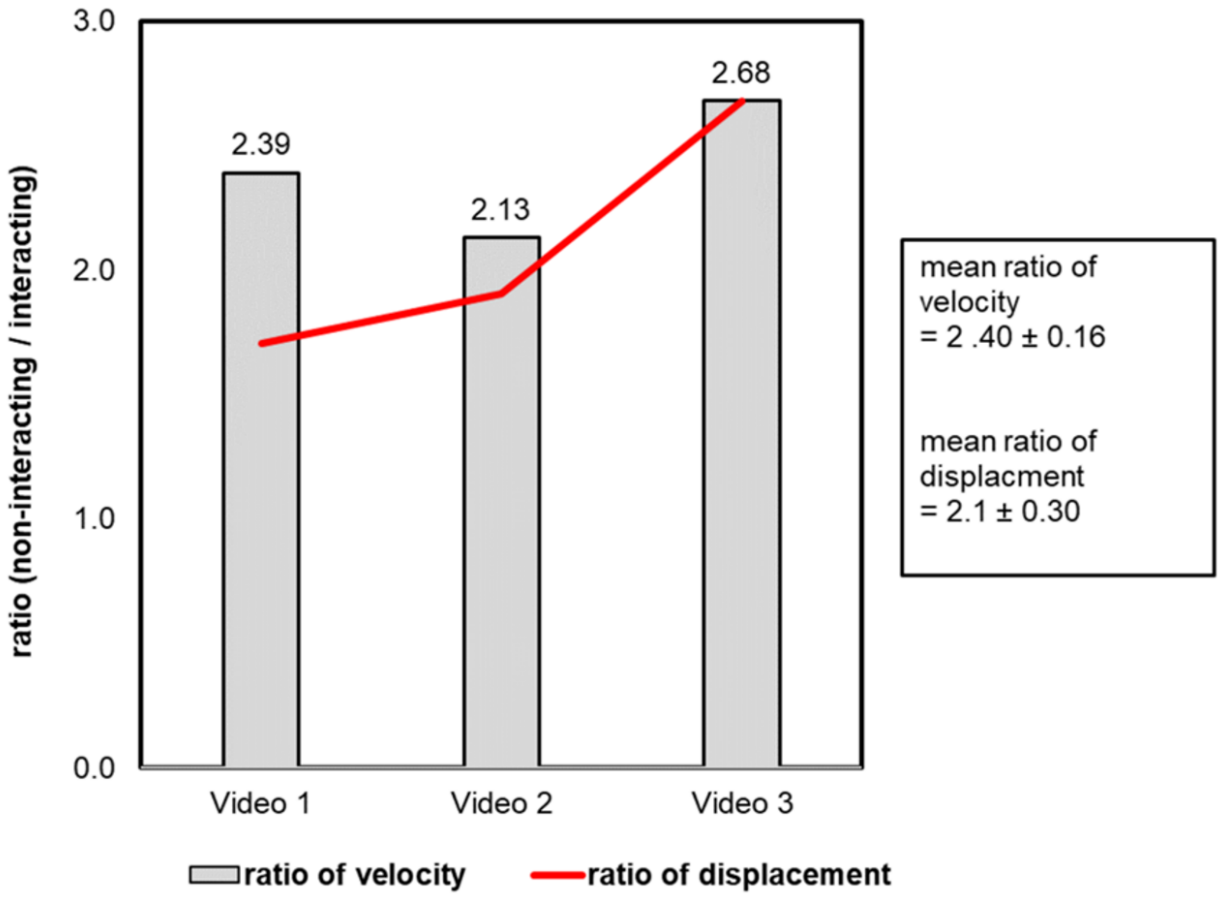

Figure 22: Platelet interactions reduce the relative velocity and relative displacement of platelet-interacting treponemes. The ratio of non-interacting treponemes to platelet-interacting treponemes was utilized to normalize the values for relative velocity and displacement of treponemes between three different videos. Treponemes that did not engage platelets experienced a greater than two-fold relative velocity (grey bars) and displacement (red line) increase compared to treponemes engaged in platelet tethering.

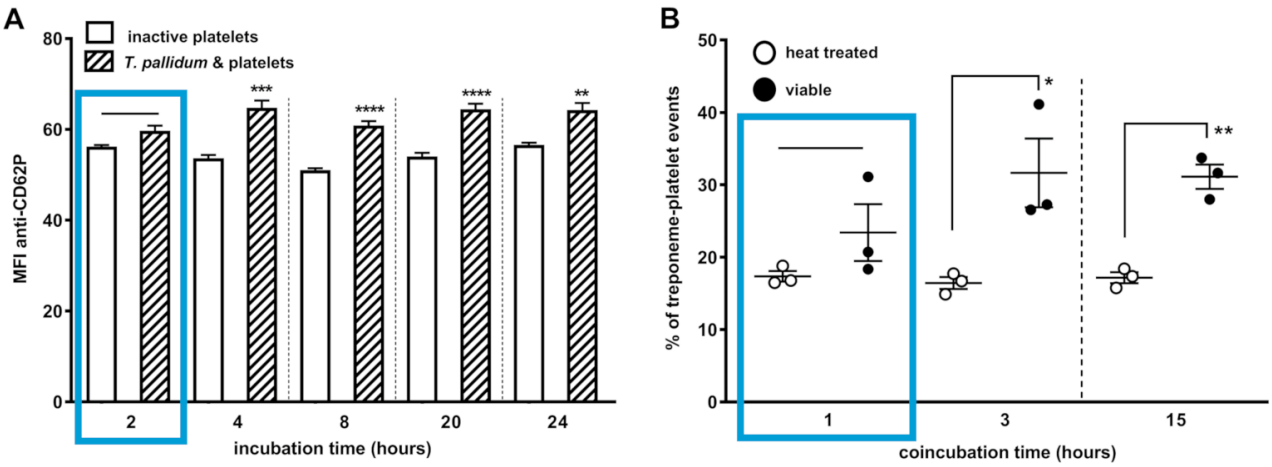

Figure 23: Treponemes bind to and activate platelets after a lag period. Two time course experiments demonstrate a lag period (blue boxes) for platelet activation (A) and maximal binding (B). (A) The MFI associated with the expression level of platelet activation marker CD62P was compared between initially inactive platelets (white bars) and those coincubated with viable *T. pallidum* (hatched bars) after 2, 4, 8, 20 and 24 hours at 37°C. After 2 hours (blue box) there was no significant increase in CD62P up-regulation between inactive platelets (mean = 56.18 ± 0.37 [SEM], n = 4) and treponeme coincubated platelets (mean = 59.69 ± 1.11 [SEM], n = 14). Significant CD62P expression was seen in platelets coincubated with treponemes after 4 hours (mean = 64.77 ± 1.57 [SEM], ***P = 0.0002, n = 7), 8 hours (mean = 60.84 ± 0.95 [SEM], ****P < 0.0001, n = 5), 20 hours (mean = 64.45 ± 1.20 [SEM], ****P < 0.0001, n = 5) and 24 hours (mean = 64.23 ± 1.56 [SEM], **P = 0.0013, n = 4) compared to inactive platelets at 4 hours (mean = 53.62 ± 0.72 [SEM], n = 5), 8 hours (mean = 51.0 ± 0.42 [SEM], n = 5), 20 hours (mean = 53.98 ± 0.83 [SEM], n = 5) and 24 hours (mean = 56.56 ± 0.48 [SEM], n = 5). (B) Platelet binding was compared by flow cytometry for

CFSE-labeled viable (black circles) or CSFE-labeled heat-treated treponemes (open circles) to platelets stained with PE-labeled antiCD41a after 1, 3, or 15 hours coincubation at 37 °C. After 1 hour coincubation (blue box) there was no significant difference in platelet binding between viable (mean = 23.39 ± 3.92 [SEM], n= 3) and heat-treated (mean = 17.36 ± 0.73 [SEM], n = 3) treponemes. Viable treponemes bound significantly more platelets after 3 hours (mean = 31.65 ± 4.75 [SEM], *P = 0.0342, n = 3) and 15 hours (mean = 31.12 ± 1.67 [SEM], **P = 0.0016, n = 3) compared to heat-treated treponemes after 3 hours (mean = 16.44 ± 0.83 [SEM], n = 3) or 15 hours (mean = 17.17 ± 0.76 [SEM], n = 3).

Tables

Table 6: Treponeme interactions with platelets increases with platelet activation state.

platelet activation state	inactivated	early activation	activated	fully activated (spread)	overall total	avg/FOV
# platelets observed	26	90	135	171	422	5.6
% of total	6.2	21.3	32.0	40.5		
# interactions with treponemes	0	6	47	72	125	1.7
% of total	0	4.8	37.6	57.8		

Table 7: A comparison of the physical and kinematic parameters of platelet-interacting and non-interacting treponemes.

	mean +/- SEM platelet-interacting	mean +/- SEM non-interacting
mean ʎ wavelength (μm)	0.79 ± 0.02	0.86 ± 0.02
mean A amplitude (μm)	0.30 ± 0.02	0.26 ± 0.01
mean ʎ/A	2.86 ± 0.18	3.24 ± 0.09
mean length (μm)	9.56 ± 0.65	10.46 ± 0.66
mean axial rotation (Hz)	2.47 ± 0.86	1.58 ± 0.77
mean velocity (μm/sec) (μm/min) N	1.68 ± 0.10 100.52 ± 6.18 24	0.65 ± 0.01 39.00 ± 5.81 15
max velocity (μm/sec) (μm/min)	2.58 154.67	1.7 102.08
mean acceleration ($\mu m/s^2$)	27.4	19.01
phenotype	compact helix rapid axial rotation higher velocity	elongated helix slower axial rotation slower velocity

Table 8: Relative velocity and displacement of platelet-interacting and non-interacting treponemes in three representative videos.

Video		Microns moved across FOV	sec	microns/ sec	ratio speed	ratio displacement
1	Treponeme 1 Treponeme 2	48.64 82.98	13.35 9.51	3.64 8.73	2.39	1.71
2	Treponeme 1 Treponeme 2	41.92 79.80	28.74 25.71	1.46 3.10	2.13	1.90
3	Treponeme 1 Treponeme 2	18.19 48.70	31.42 31.42	0.58 1.55	2.67	2.68
	Treponeme 1 = platelet-interacting treponeme			Treponeme 2 = non-interacting treponeme		

Table 9: Ratios of non-interacting to platelet-interacting treponemes compares relative velocity and relative displacement.

	ratio of velocity	ratio of displacement
Video 1	2.39	1.71
Video 2	2.13	1.90
Video 3	2.68	2.68
mean	2.40	2.10
SEM	0.16	0.30
N	3	3

Chapter 3:*Treponema pallidum,* the syphilis spirochete, demonstrates positive chemotaxis to the secretome of collagen-activated platelets

3.1 Abstract

Introduction. *Treponema pallidum* ssp. *pallidum*, the causative agent of syphilis, has previously been shown to interact with platelets, host cells that serve an important role as cutaneous and vascular immune sentinels. Platelets modulate local host microenvironments by releasing agonist-specific, opposing secretion gradients that are recognized by host cells and induce a differential immune response, with thrombin and collagen serving as the primary platelet-specific agonists. Thrombin-activated platelets primarily release pro-inflammatory cytokines, while collagen-activated platelets predominantly secrete anti-inflammatory modulators.

Aims. To determine if *T. pallidum* exhibits preferential migration towards the secretomes of collagen- and/or thrombin-activated platelets and to identify candidate secretome components that may mediate treponemal chemotaxis.

Methodology. Treponeme chemoattraction was assessed using capillary tube chemotaxis assays and virulent *T. pallidum* exposed to platelet rich plasma (PRP) filtrates prepared from either resting, collagen-activated or thrombin-activated human platelets, followed by treponemal quantitation via flow cytometry. Cytokine, chemokine, and acute phase protein profiles specific to collagen- versus thrombin-activated PRP filtrates were determined using a human cytokine array, and gene ontology analysis was used to identify candidate *T. pallidum* chemoattractants.

Results. Filtrates from collagen-activated platelets induced higher levels of *T. pallidum* chemotaxis compared to the level observed with filtrates from resting or thrombin-activated filtrates, and the extent of chemotaxis increased over an 8 hour incubation period. Differential cytokine array analyses identified a predominance of cytokines/factors associated with anti-inflammatory, growth factor-inducing, and M2 macrophage-activating responses contained in the collagen-activated PRP filtrate. Conversely, the thrombin-activated PRP filtrate contained higher levels of the acute phase protein myeloperoxidase.

Conclusions. The observed preferential chemoattraction of *T. pallidum* to the secretions of collagen-activated platelets, which displayed a higher proportion of host anti-inflammatory, growth and M2 macrophage polarizing molecules, suggest these host factors may contribute to treponemal pathogenesis during infection. *Treponema pallidum* chemoreception of collagen-activated platelet secretions may enhance treponemal dissemination and contribute to niche localization and/or immune evasion.

3.2 Introduction

Syphilis, caused by the spirochete *Treponema pallidum* ssp. *pallidum* (*T. pallidum*), is a multistage infection spread via sexual contact or vertically from mother to fetus (LaFond & Lukehart, 2006). Infectious syphilis is a sexually transmitted infection estimated by the WHO to have a global prevalence of 18 million cases in men and women aged 15-49, with 5.6 million new infections annually (Newman et al., 2015). Congenital syphilis affects an estimated 1 million pregnancies per year (Wijesooriya et al., 2016), resulting in fetal death or significant fetal damage if left untreated (Korenromp et al., 2019; LaFond & Lukehart, 2006; Wijesooriya et al., 2016). Although untreated infectious

syphilis can result in tertiary syphilis symptoms that can lead to irreversible tissue damage and in some cases death (Korenromp et al., 2019; LaFond & Lukehart, 2006; O'Byrne & MacPherson, 2019), approximately 70% of infections will enter latency and remain asymptomatic for the lifetime of the individual (LaFond & Lukehart, 2006; Sheila A. Lukehart et al., 1992; Peeling et al., 2017). *Treponema pallidum* is highly efficient and invasive, with a 50% infectious dose (ID_{50}) in humans of 57 organisms (Magnuson, Harold et al., 1956), an observed traversal of the blood-brain barrier in up to 40% of primary stage infections (LaFond & Lukehart, 2006; S. Lukehart et al., 1988), and colonization in every tissue or organ (LaFond & Lukehart, 2006; Peeling et al., 2017). Infection with *T. pallidum* can lead to diverse symptoms commonly misdiagnosed as herpes, psoriasis, viral retinitis, dementia (Watts et al., 2016), cancer (Darwish et al., 2008; Marra et al., 1991), and stroke (Ahbeddou et al., 2018; Liu et al., 2012).

Treponema pallidum enters the body through epithelial microabrasions or mucosal barriers (LaFond & Lukehart, 2006; Yi et al., 2013). (LaFond & Lukehart, 2006; Yi et al., 2013). Within the skin, *T. pallidum* encounters immune surveillance cells including keratinocytes and dendritic cells (DCs) which identify pathogens via TLRs (Carlson et al., 2011; Groeger & Meyle, 2019; Quaresma, 2019). Keratinocyte/DC TLR5 recognizes bacterial flagellin, and TLR1, 2, and 6 recognize bacterial PG and lipoproteins (Eyerich et al., 2018; Groeger & Meyle, 2019; Grover & Mackman, 2018). One response of pathogen-activated keratinocytes/DCs is decryption (activation) of cutaneous tissue factor (TF), initiating a rapid thrombin-mediated host response of pro-inflammatory signaling, and pathogen clearance (Burzynski et al., 2019; Delabranche et al., 2017; Grover & Mackman, 2018; Margraf & Zarbock, 2019; Petzold & Massberg, 2019). In a

departure from conventional Gram-negative pathogens, intact *T. pallidum* has few surface-exposed proteins and masks both peptidoglycan (PG) and flagella with its outer membrane (OM) (Izard et al., 2009; LaFond & Lukehart, 2006), imparting low surface immunogenicity and limiting immune activation as treponemes enter the dermis (Edmondson et al., 2018; Izard et al., 2009; LaFond & Lukehart, 2006).

However, despite this “stealthy” pathogenic strategy an immune response against *T. pallidum* clearly occurs, as demonstrated by the dermal lesion (chancre) that forms at the site of initial infection. Chancres are located in close proximity to proliferating blood vessels that result from the angiogenesis stimulation observed with syphilis (Carlson et al., 2011; Gao et al., 2019; Juanpere-Rodero et al., 2013; LaFond & Lukehart, 2006). While a portion of treponemes remain within the chancre and are later cleared by M1 polarized macrophages (Hawley et al., 2017; Lin et al., 2018; Liu et al., 2018), others rapidly disseminate via blood vessels and the lymphatic system to colonize distant sites and cross the blood-placenta, blood-retina and blood-brain barriers (Everall & Sánchez-Busó, 2017; LaFond & Lukehart, 2006; Lukehart et al., 1988; Lukehart et al., 1992).

Despite the small size of the *T. pallidum* genome, about 6% of the encoded proteins are dedicated to chemotaxis (Charon & Goldstein, 2002; Lux et al., 2000), highlighting the importance of these proteins to treponeme survival and/or pathogenesis. This set of proteins include multiple sensory methyl-accepting chemotaxis proteins (Mcp1-4) (Fraser, 1998; Greene & Stamm, 1998; LaFond & Lukehart, 2006; Matilla & Krell, 2017), chemotaxis regulatory proteins (CheA, CheB, CheR, CheW1, CheW2, CheY) (Benson et al., 2015; Szklarczyk et al., 2019), and motility proteins (FliD, FliY) (Szklarczyk et al., 2019). Chemotaxis requires a substantial energy output, yet in other pathogens has

been shown to be a critical virulence strategy that aids in establishment of infection (Matilla & Krell, 2017), including guiding *Borrelia burgdorferi* (*B. burgdorferi*) dissemination (Sze et al., 2012) and facilitating tissue penetration and niche colonization by *Treponema denticola* (Lux et al., 2001), *Helicobacter pylori* (*H. pylori*) (Johnson & Ottemann, 2018; Perkins et al., 2019), and *Campylobacter jejuni* (Korolik, 2019). Motile bacteria quickly respond to attractant gradients to acquire host-derived nutrients (Frank et al., 2016) and localize to favourable microenvironments (Bi & Sourjik, 2018; Matilla & Krell, 2017; Waite et al., 2018), while avoiding toxins, the defenses of commensal microbes and host immune effectors (Eyerich et al., 2018) to establish chronic infections (Johnson & Ottemann, 2018).

Treponema pallidum has been shown to interact with many different types of human cells. The majority of *T. pallidum*-host cell interaction studies have focused on macrophage opsonophagocytosis and monocyte migration assays (Cruz et al., 2012; De Melo et al., 2010; Gao et al., 2019; Lin et al., 2019; Lukehart et al., 1992), permeability assays with endothelial monolayers in transwell plates (Gao et al., 2019) or adhesion/activation to cultured epithelial (Edmondson et al., 2018; Izard et al., 2009; Norris & Edmondson, 1988) or endothelial cells (Lithgow et al., 2020; Parker et al., 2016; Riley et al., 1992; Thomas et al., 1988) . Key advances have been made in understanding cutaneous macrophage-mediated clearance (De Melo et al., 2010), and *T. pallidum*-endothelial interactions that facilitate adhesion and extravasation (Lithgow et al., 2020; Parker et al., 2016), yet much remains to be elucidated on the interaction of treponemes with blood cells and treponeme chemotaxis within the vasculature (Church

et al., 2019; Cruz et al., 2012; Podwinska et al., 2000; Salazar et al., 2007; Xu et al., 2017).

The cellular component of blood, a “specialized” connective tissue (Ogawa et al., 2010), consists of red blood cells (RBCs) and resting platelets. Red blood cells are larger (6-8 µm) (Kim et al., 2015), prevalent ($3.5 – 5.9 \times 10^6$ per µL) (Dean, 2005), and travel centrally within blood vessels (Gaertner & Massberg, 2019), while resting platelets are smaller (1-2 µm), fewer ($1.5 – 4.0 \times 10^5$ per µL) (Margraf & Zarbock, 2019; Montague et al., 2020), and circulate along the endothelial wall where they respond to disturbances in the glycocalyx, the presence of microbes (Gaertner & Massberg, 2019), and are able to extravasate into inflamed tissue (Gaertner et al., 2017; Li et al., 2017; Rosowski & Huttenlocher, 2018). Platelets serve as sentinel cells that mediate haemostasis, host/pathogen interactions and immunomodulation through a vast receptor array that allows platelets to rapidly respond to stimuli and alter their microenvironment (Gaertner & Massberg, 2019; Rosowski & Huttenlocher, 2018). Platelets express major histocompatibility complex (MHC) class I, TLR1- 10, immunoglobulin receptors, integrins, glycoproteins (GPs), and GPCRs for C-C and C-X-C chemokine ligands in addition to important GPCRs that are protease-activated receptors (PARs) (Claushuis et al., 2019; Hughes & Nibbs, 2018; Manne et al., 2017; Tamagawa-Mineoka, 2015). Engagement of platelet receptors induces activation where platelets rapidly (1) change shape to become “star-like” with protruding filopodia that adhere to neighboring cells or “fried egg-like” via lamellipodia to spread across endothelial breaches (Margraf & Zarbock, 2019; Rosowski & Huttenlocher, 2018; Sandmann & Köster, 2016), and (2)

release “secretomes” of proteins, cytokines, lipids and small molecules specific to the activation pathway (Li et al., 2017; Manne et al., 2017).

Of the numerous platelet agonists two of the most important are collagen type I (collagen) and thrombin, which modulate how platelets influence vascular permeability/angiogenesis (Eisinger et al., 2018) and the host immune response (Linke et al., 2017; Stephen et al., 2013). Collagen is a key platelet agonist and major structural protein within the extracellular matrix (ECM) (Arseni et al., 2018; Sayers et al., 2019), but is also highly expressed within the dermis, cornea, bones, and cartilage (Sayers et al., 2019), tissues where *T. pallidum* is known to localize (Carlson et al., 2011; LaFond & Lukehart, 2006). Platelet receptors α2β1 and GPVI bind collagen which induces platelet chemotaxis (Rosowski & Huttenlocher, 2018), activation, secretion (Koupenova et al., 2018), and cytoskeletal changes with or without aggregation (Boulaftali et al., 2018). Collagen-activated platelets regulate innate (Ribeiro et al., 2019) and adaptive immune responses (Boulaftali et al., 2018; Rossaint et al., 2018), inflammation, macrophage polarization (Linke et al., 2017; Rayes et al., 2020), and secrete prostaglandin (PG) E_2 which promotes anti-inflammatory monocytes, interleukin (IL) -10 secretion and down-regulates tumor necrosis factor alpha (TNFα) (Linke et al., 2017; Rayes et al., 2020).

Thrombin, the most potent platelet activator, acts by cleaving PAR1 (at low concentrations) and PAR4 (high concentrations) (Gremmel et al., 2016) to induce swift pro-inflammatory (Popović et al., 2012) and procoagulant secretions (Gremmel et al., 2016) and the development of filopodia (Sandmann & Köster, 2016). Intravascular thrombin mediates haemostasis by rapidly activating the coagulation cascade and

platelets to form dense, fibrin-rich aggregates (Induruwa et al., 2018). Thrombin-activated platelets bind monocytes and induce monocyte pro-inflammatory secretion of TNF-α, IL-8 and IL-1β (Linke et al., 2017; Stephen et al., 2013).

Platelets are also activated through direct interactions with pathogens (Gaertner & Massberg, 2019; Guo & Rondina, 2019; Li et al., 2017) and by secreted pathogen virulence factors (Li et al., 2017). Pathogen-mediated platelet responses may be pro- or anti-inflammatory and modulate leukocyte recruitment and differentiation, angiogenesis, and endothelial permeability (Kral et al., 2016; Li et al., 2017; Manne et al., 2017; Rayes et al., 2020; Tamagawa-Mineoka, 2015).Bacterial pathogens, including multiple *Streptococcal* species (Kahn et al., 2013; Yakovenko et al., 2018),target particular receptors to regulate the mechanism of platelet activation to promote immune evasion, dissemination, and localization to specific host niches (Hamzeh-Cognasse et al., 2015; Kerrigan, 2015; Rossaint et al., 2018). For pathogens, such as the spirochetes *B. burgdorferi* (Coburn et al., 1993) and *Borrelia hermsii* (Alugupalli et al., 2001) the pathogenic advantage of platelet activation is less understood.

We have recently demonstrated that *T. pallidum* actively engages human platelets, with contact significantly increasing the rotational frequency and translational speed of treponemes (Church et al., 2019). *Treponema pallidum/* platelet interactions are reversible and can occur via any part of the treponeme in addition to the tip, indicating that platelet-interacting components can remain distributed along the cell body (Church et al., 2019) in contrast to migrating to the tips and “capping” (LaFond & Lukehart, 2006) seen in endothelial (Quist et al., 1983) and epithelial cell adhesion (Izard et al., 2009). Pathogen binding is a fundamental step towards platelet activation (Singh et al., 2017;

Yakovenko et al., 2018), and we observed that *T. pallidum* induced platelet activation following a 4-hour lag period (Church et al., 2019). In addition, we identified that *T. pallidum* discriminates between resting platelets and varying degrees of activation to selectively bind the most activated platelets available (Church et al., 2019).

The discrimination between platelets by their *level of activation* prompted us to explore whether *T. pallidum* would also distinguish between the *mechanisms of activation*. We investigated this using a modified capillary tube chemotaxis assay (Bakker et al., 2007) that exposed treponemes to the cell free secretions (filtrate) of platelets that were either resting or activated by collagen or thrombin, which *in vivo* induce divergent host microenvironments (Boulaftali et al., 2018; Ollivier et al., 2014; Rossaint et al., 2018). Plasma is a nutrient rich bacterial chemoattractant (Brautigam et al., 2016; Ketter et al., 2019; Lux et al., 2000; Mussano et al., 2016), thus migration into the resting platelet filtrate was used as a baseline to normalize multiple experiments (Flamm et al., 2012; Garner et al., 2017; Rossaint et al., 2018). We utilized a cytokine array to quantify differences in specific factors when we activated platelets with collagen or thrombin (Rossaint et al., 2018), followed by Gene Ontology (GO) analysis to provide context for the physiological effect of these secretions on host cells and treponeme chemotaxis (Szklarczyk et al., 2019). *Ex vivo*, platelet rich plasma (PRP) can retain many of its physiological characteristics as connective tissue (Ogawa et al., 2010) to provide a biologically relevant *in vitro* model to explore host-pathogen interactions (Barrila et al., 2018; Ketter et al., 2019). This represents a novel approach to investigating pathogenic strategies in *T. pallidum* and to our knowledge, the first study of pathogen chemotaxis to

host molecules secreted by platelets activated by contrasting mechanisms, representing distinctive host microenvironments.

3.3 Methods

T. pallidum **propagation and extraction**

Propagation and harvesting of *T. pallidum* was performed as per Lukehart and Marra (Lukehart & Marra, 2007), with the exception that testicular extractions were performed under microaerophilic (MO_2) conditions of 1.5-5% oxygen in a Coy Laboratory Products anaerobic chamber (Mandel Scientific Company Inc.) to enhance *T. pallidum* viability (Cox, 1994; Edmondson et al., 2018).

Ethical approval

All platelets in this study were obtained from human blood as approved by the local Institutional Review Board at the University of Victoria and obtained by informed consent from volunteer donors. To obtain viable *T. pallidum* animal studies were approved by the local Institutional Review Board at the University of Victoria and were conducted in strict accordance with standard accepted principles as set forth by the Canadian Council on Animal Care, National Institutes of Health and the United States Department of Agriculture in a facility accredited by the Canadian Council on Animal Care and the American Association for the Accreditation of Laboratory Animal Care.

Minimally invasive platelet purification

Donor blood, from healthy volunteers, was extracted with a 21-gauge butterfly needle into BD Vacutainers ACD-A tubes, then to avoid platelet activation, was transferred by

pouring slowly down the inside of sterile 15 mL conical tubes (Sarstedt Inc.) and centrifuged at 180 x *g* for 15 minutes at 22˚C (with the centrifuge rapid acceleration and rapid brake functions disabled to reduce platelet activation through shear force, and contamination of the platelet rich plasma layer by the buffy coat, respectively). To avoid leukocyte contamination the pipette tips were held at least 2 mm above the buffy coat to withdraw the platelet rich plasma (PRP) layer, which was then slowly pipetted down the inside of new, sterile tubes. Samples with disrupted buffy coat or erythrocyte layers were discarded. Platelets were enumerated by hemocytometer and averaged 5.0×10^5 platelets per µL of PRP. All PRP was stored at room temperature (RT), allowed to rest at least 30 minutes prior to stimulation, and was inspected extensively by microscopy and found to be free from erythrocyte and leukocyte contamination. No additional reagents were added to inhibit platelet activation. Platelet free plasma (PFP) was obtained from the supernatant of PRP centrifuged at 17,000 x *g* for 15 minutes then gently filtered using a 0.2 µm Polytetrafluoroethylene (PTFE) membrane filter (Millex-LG, Millipore).

Platelet activation and filtrate preparation for chemotaxis assays

Agonist concentration was previously optimized under our experimental conditions, to induce a comparable level of platelet activation (measured by flow cytometry) and macroscopic aggregation (shown Fig 23) using thrombin or collagen. Plasma and buffer filtrates were prepared in sterile tubes using 0.5 U mL^{-1} bovine thrombin (Sigma-Aldrich) to activate the PRP or added to Motility Buffer (MB) [136.9 mM NaCl, 8.10 mM Na_2HPO_4, 2.7 mM KCl,1.47 mM KH_2PO_4, 2% BSA w/v, pH 7.4] as a control, then vortexed, incubated for 15 min at 37°C, and very gently filtered using a 0.2 µm PTFE

filter. Cell free plasma was verified by microscopy. Collagen filtrates were prepared by adding 100 µg mL^{-1} collagen I, rat tail (3 mg mL^{-1} Gibco, Thermo Fisher Scientific) to activate the PRP or as a control to MB, vortexed, incubated for 3 hours at 37°C and filtered as above. Resting plasma, defined as untreated PRP stored at RT, or MB was filtered, as above. Once activated the plasma was filtered immediately to eliminate ongoing platelet secretion and prevent treponeme-platelet binding interactions (Church et al., 2019). Plasma and buffer filtrates were then transferred to the MO_2 chamber for use in the chemotaxis assay.

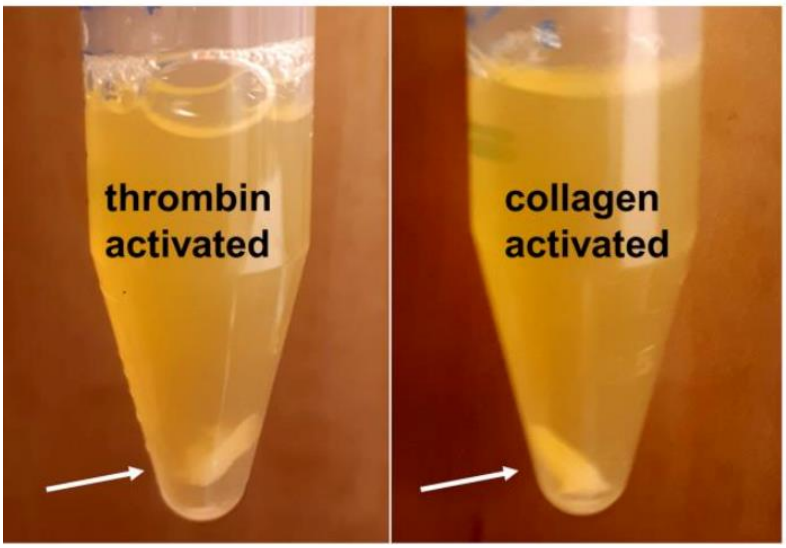

Figure 24: Platelet activation by collagen and thrombin optimized to produce similar levels of platelet aggregation / activation.

Adaptation of the modified capillary tube chemotaxis assay

Materials and methods were adapted from the modified capillary tube chemotaxis assay per Bakker, *et al*. (Bakker et al., 2007) to accommodate *T. pallidum* and plasma filtrates. Prior to experiment day capillary tubes were marked 1 cm from one end, carefully

scored and sterilized by autoclave or 70% ethanol. Two 96-well polystyrene tissue culture plates (Thermo Scientific Nunc) were selected, aligned face-to face and the corresponding well bottoms were marked (Fig 25A). The “top” plate had holes punched through the well bottoms, with a flamed dissecting needle, large enough to accommodate the diameter of the capillary tubes. The 96-well plates were sterilized with 70% ethanol and stored.

One day prior to the chemotaxis assay methylcellulose (400 cP Alfa Aesar) was dissolved in MB to 5% w/v and 3% w/v concentrations and stored in the MO_2 chamber at 34°C. Under aseptic conditions, the “bottom” plate was prepared by adding 200 µL of the 5% w/v methylcellulose solution to each marked well using 1 mL syringes due to viscosity (Fig 25B). The plate was covered to prevent contamination and evaporation and stored under experimental conditions (microaerophilic conditions, 34°C). All subsequent procedures were conducted under microaerophilic conditions, prior to flow cytometry.

On experiment day 100 µL of 2×10^7 mL^{-1} well-suspended and viable *T. pallidum* was added to the methylcellulose solution in each sample well (bottom plate) (Fig 25C) and gently mixed with a pipette tip. The “top” plate was aligned, face-to-face and the plates were taped together and incubated for 1-2 hours at 34°C to allow the treponemes to acclimatize. The filtered plasma and buffer were mixed 1:1 with 3% w/v methylcellulose in buffer, wicked into sterilized glass capillary tubes (1.5 x 100 mm borosilicate glass melting point tubes, DWK Life Sciences) and the top ends were sealed with plasticine (Fig 25C). The 96-well plates were secured on an incubator rack at a 50-60° angle such that the capillary tubes could be inserted through the “top” plate holes to enter the

"bottom" plate" sample wells and were then coincubated for 4 or 8 hours at 34°C. Following coincubation, the contents of each capillary tube was added to 200 µL cold 2% w/v paraformaldehyde in PBS (pH 7.4) in a BD Falcon 5 mL polystyrene tube (BD Canada) (Fig 25C) and stored at 4°C until flow cytometry.

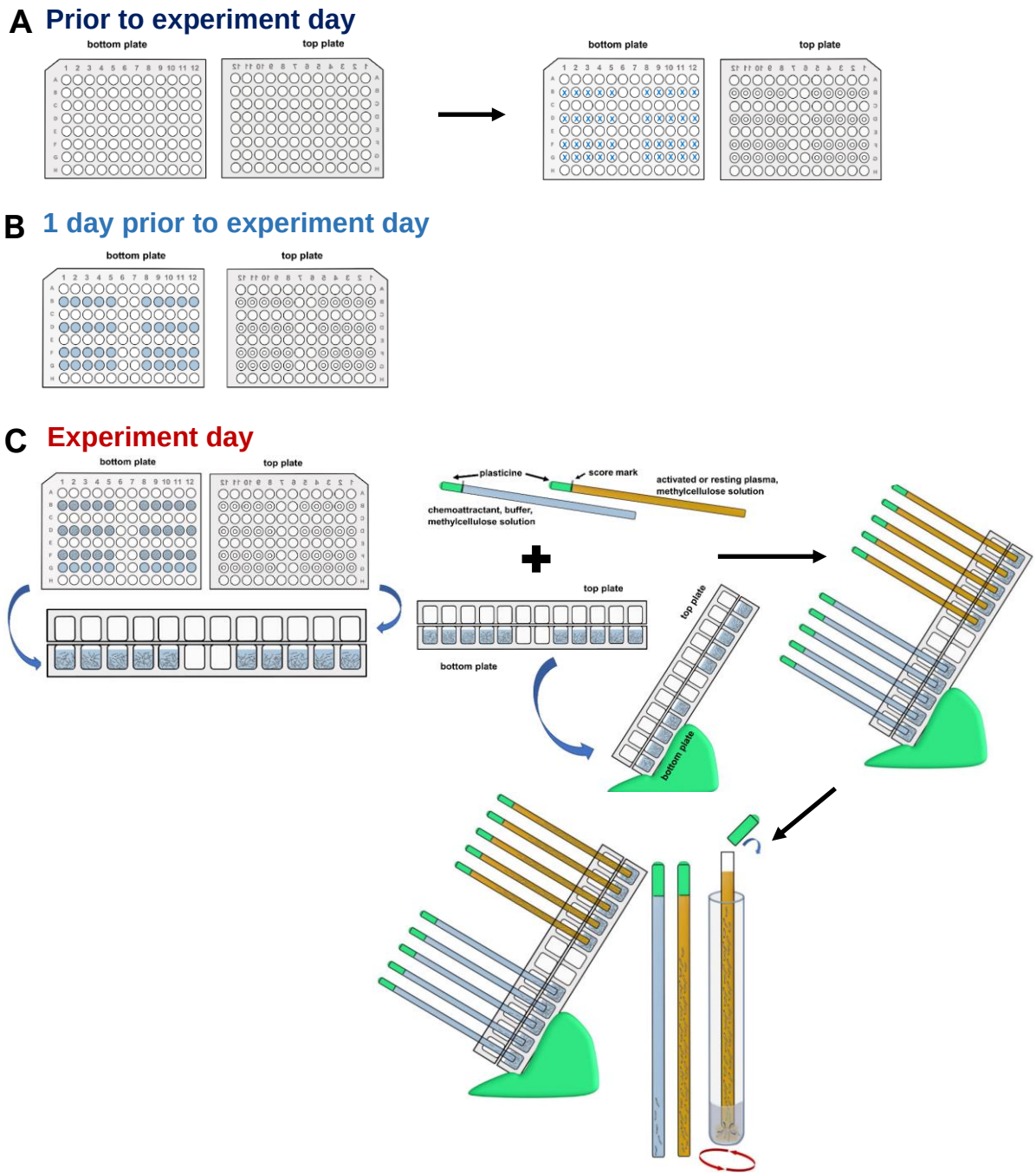

Figure 25: Adaptation of the modified capillary tube chemotaxis assay. (A) Corresponding wells on two 96-well plates are marked and holes are punched in the "top" plate well bottoms. (B) The methylcellulose solution is added to the marked wells of the "bottom" plate, ensuring an accurate volume to each well. (C) To the methylcellulose wells of the "bottom" plate viable *T. pallidum is* added. Once the "top" and "bottom" plates are taped together and the set-up is positioned appropriately, the capillary tubes loaded with sample plasma or control solutions are inserted into the plates. Following coincubation, the capillary tubes are removed, wiped clean, inserted into 2% PFA containing flow cytometry tubes, the tops snapped off, swirling the contents as it drains.

Flow cytometry

Samples were analyzed on a BD FACScalibur (BD Canada) with BD CellQuest acquisition software (BD Canada). Software was set to log scale for both forward scatter (FSC E-1) and side scatter (SSC), the treponeme gate was set using SPHERO Flow Cytometry Size Standard Kit (Spherotech, Inc.) 2.0, 3.4, 5.1 and 10.0 µm beads and a sample of fresh *T. pallidum*. Events were acquired for 30 s on low speed with three technical replicates averaged for each biological replicate. FlowJo V10 (FlowJo, LLC) analysis was used to refine gating and quantify migrated treponemes. The "fold increase" was used to compare independent results by setting the averaged number of treponemes migrating into the resting filtrate as the baseline (set to 1.0) for each assay, then dividing the averaged migration for each sample type by this for a ratio of the relative migration.

Human XL Cytokine Screen

Aliquots of activated plasma filtrate, or plasma filtrate from platelets coincubated with NS/ NRS as a negative control for activation, were prepared as above for the chemotaxis assays, were immediately flash frozen in 500 µL aliquots and stored at -80°C pending analysis. Thawed samples were centrifuged at 9 000 x *g* for 10 min at 4°C and the top 200 µL of each sample was removed for the Proteome Profiler Human XL Cytokine Array Kit (cat no ARY022B, R & D Systems) and assayed as per instructions. Chemiluminescent signals were detected with a LI-COR Odyssey CLx Imager with C-DiGit blot scanner (Fig 26), quantified with Image Studio Lite Software (LI-COR INC.) and exported into Excel 2016 (version 1806, Microsoft) for final analysis.

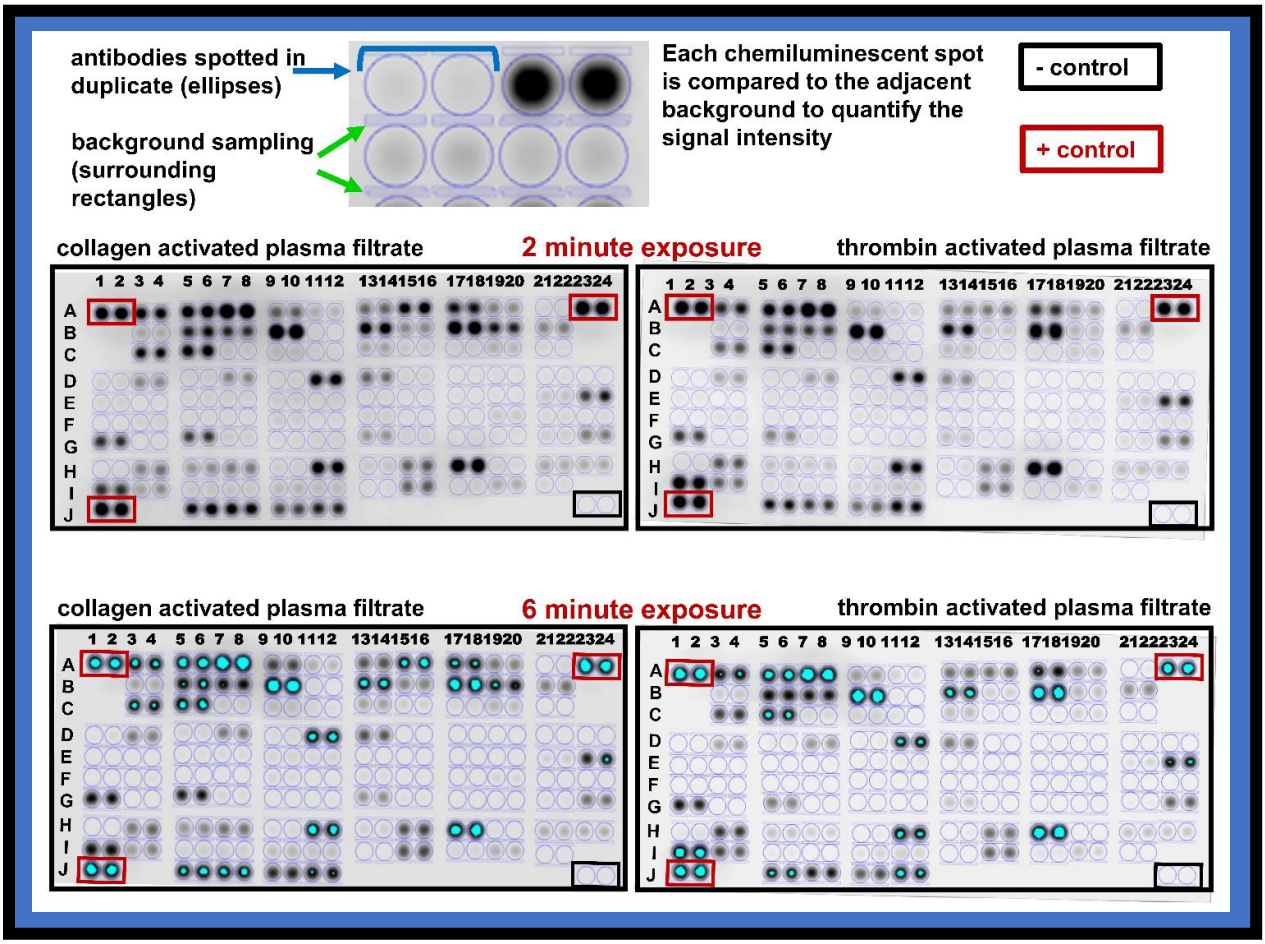

Figure 26: Proteome Profiler Human XL Cytokine Array. Exposures at both 2 and 6 minutes were analyzed to optimize the analysis of both stronger and weaker chemiluminescent signals.

Statistics

A two-way ANOVA with Dunnett's multiple comparisons test was used to determine the statistical significance when comparing treponeme migration into the plasma or buffer filtrates. A one-way ANOVA with Tukey's multiple comparisons test was used to evaluate the statistical significance of independent experiments, compared using the fold increase relative to resting plasma, following 4- or 8-hour chemotaxis coincubations. The chemiluminescent signal intensities from the Human XL Cytokine Array were assessed for statistical significance pairwise for corresponding thrombin-activated or collagen-activated filtrate analytes at both 2 min and 6 min exposures by grouped analysis multiple unpaired t-tests, and row means with SD and SEM. Statistical analysis was performed and graphs were generated with GraphPad Prism (version 8.1.2) (GraphPad Software).

Human XL Cytokine Array Analysis

The duplicate signal intensity values exported into Excel were analyzed with an algorithm (Fig 27), evaluating four aspects before assigning "upregulation" (Claridge-Chang & Assam, 2016; Coe, 2002; Maher et al., 2013).

(1) the duplicate values were imported to generate the mean, SD, SEM and P value for each pair at both 2 min and 6 min exposures. The maximum signal intensity assigned by Image Studio Lite was 26 031 (thrombin-activated filtrate, 6 min exposure, spot 2, IL-

18Bpa) and spots exceeding this were designated “infinite” (inf) by the software. To include all values “inf” spots were assigned the value of 26 000. The use of 2 min exposure pairs versus 6 min exposure pairs for further comparison was made by excluding over- and under-exposed pairs, and then by determining which exposure demonstrated the best “signal to back to background”, and “signal separation” values.

(2) The means, SD and sample size (n = 2) were entered into an on-line Effect Size (ES) Calculator (Ellis, 2009) which provided the ES, r and r^2 values used to determine the magnitude of the difference in platelet secretion when activated by thrombin or collagen (Claridge-Chang & Assam, 2016; Ialongo, 2016; Maher et al., 2013; Sawilowsky, 2009). The ES with Glass’s Δ was used based on substantially different SDs between sample pairs (Lakens, 2013; Maher et al., 2013). As the results comprised both P values < 0.05 (which gives very high ES results) and P values ≥ 0.05, the ES scale was modified to use results with a difference in magnitude ≥ 1.90(Maher et al., 2013; Sawilowsky, 2009). In the case of both a P value < 0.05 and an ES ≥ 1.90, the final check was to ensure positive contrast discrimination (Step 4).

(3) The fold increase was calculated using the mean signal intensity for each specific pair of duplicate spots on one membrane (i.e. “C” collagen-activated filtrate) divided by the mean for the corresponding duplicate spots on the other membrane (i.e. “T” thrombin-activated filtrate) at the same exposure time. Given the additive/synergistic effect of many cytokines (Thaiss et al., 2016), and to further define secretion trends, upregulation was designated by an increase of at least 20%, a ratio ≥ 1.20. The calculation was performed in both directions for a ratio, C/T ≥ 1.20 = upregulation by

collagen-activated platelets and T/C ≥ 1.20 = upregulation for thrombin-activated platelets, shown as the “fold increase”.

(4) Finally, pairs were required to be sufficiently different for human visual system (HVS) contrast discrimination, with a minimal standard of a Just Noticeable Difference (JND) (Burton, 1981), i.e. sufficient contrast was present to visually recognize differences.

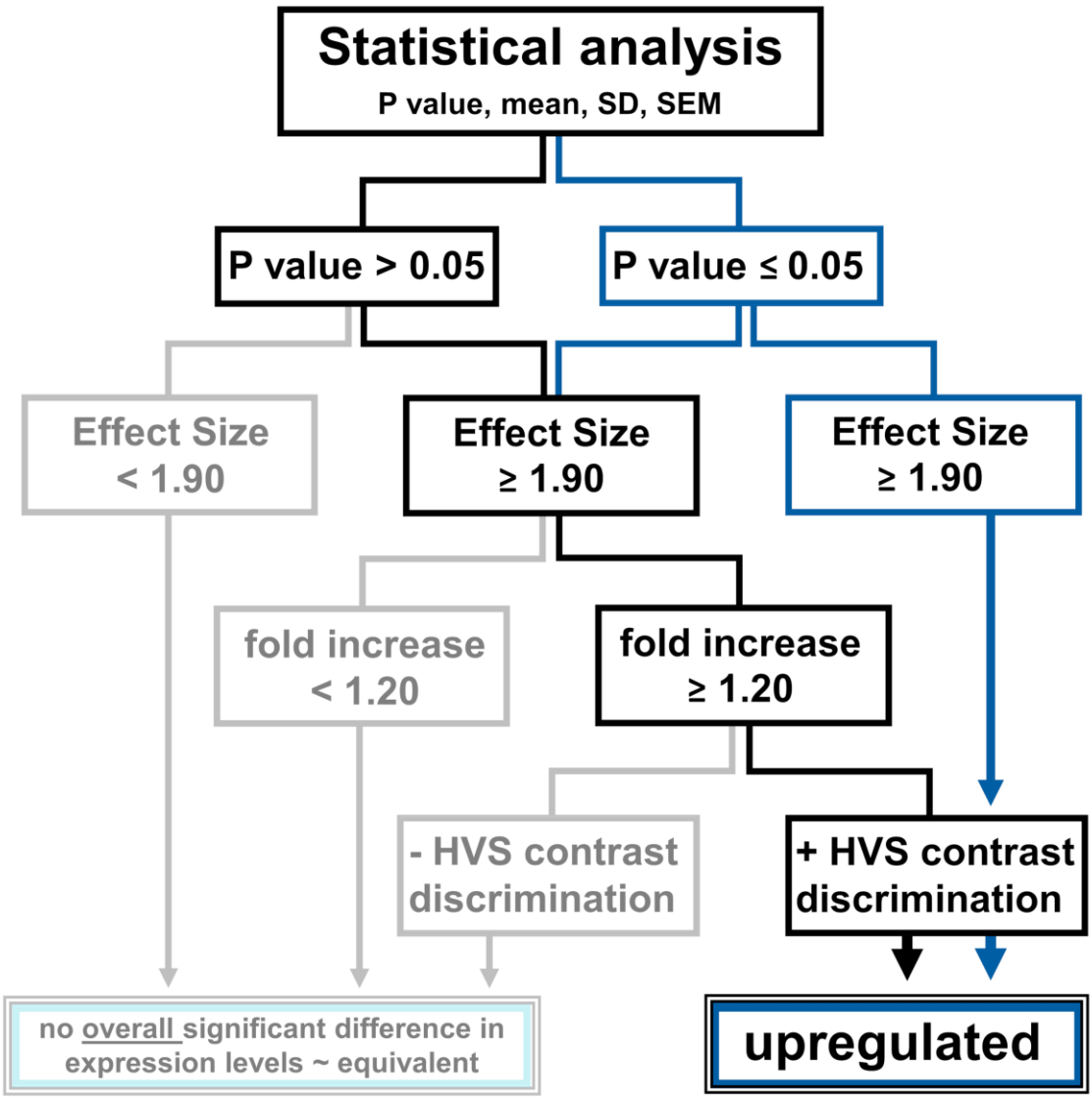

Figure 27: Algorithm determination of Human XL Cytokine Array factor upregulation. The mean signal intensity of duplicate chemiluminescent spots to

compare the results of collagen-activated platelet filtrate to thrombin-activated platelet filtrate first with statistical analysis to determine the P value. Effect Size was then calculated, and the mean signal intensity was used to determine the fold increase of a factor in one filtrate versus the other. Finally, a visual inspection was used for the lower intensity signals and results without a visually apparent difference were not designated as “upregulated”. When pairs failed to meet the algorithm baseline to be designated as “upregulated”, the overall difference was considered insignificant and those components were considered to be expressed at a relatively equivalent level.

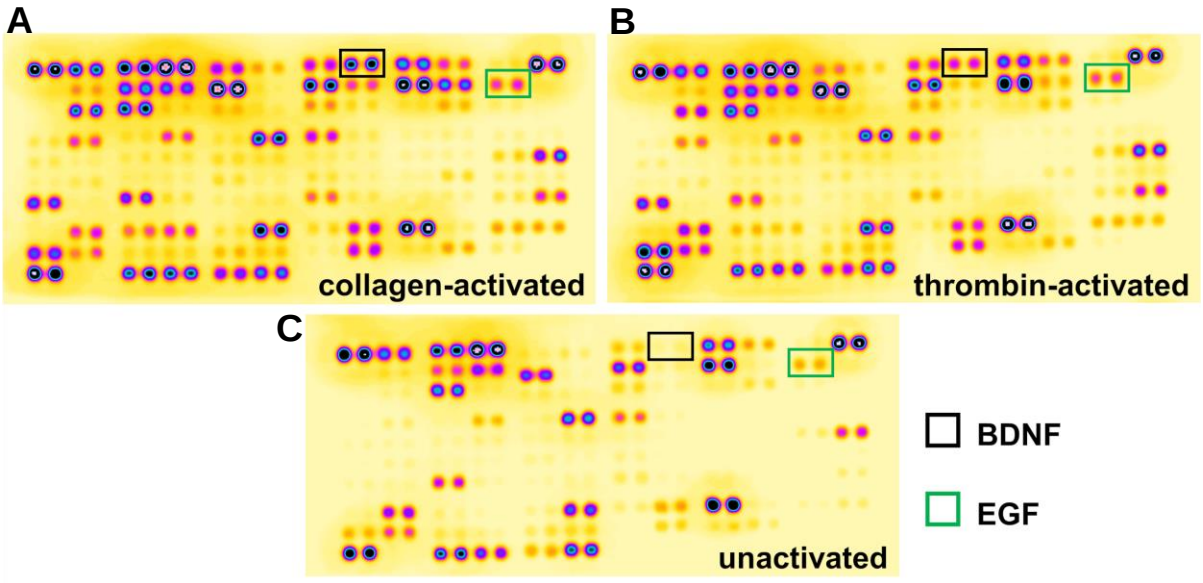

Figure 28: Comparison of collagen-activated and thrombin-activated platelet filtrates to the filtrate of unactivated platelets. False colour imaging, though not used during analysis, allows the comparison of the (A) collagen-activate filtrate, (B) thrombin-activated filtrate, and (C) unactivated filtrate duplicate pairs. The collagen-activated filtrate demonstrated 2.47-fold higher level of BDNF (black boxes) than the thrombin-activated filtrate, and 39.01-fold higher level in comparison to the filtrate of unactivated platelets. Comparison of EGF (green boxes) expression showed the collagen-activated

filtrate was 4.23-fold higher than the thrombin-activated filtrate, and 50.95-fold higher than in the unactivated filtrate. When low intensity results were evaluated, included, and marked (*****) when designated upregulated, however, when the comparison spots on one membrane were absent or negligible, the result was a large difference in the fold increase, and a limitation of this method.

3.4 Results

Secretions from collagen-activated platelets induced the highest levels of positive chemotaxis by *T. pallidum*

Collagen activation produced the highest response after 4-hours coincubation (mean = 6124 ± 710.3 [SEM] n=12) compared with the resting filtrate (P = 0.0227, mean 4148 ± 625.7 [SEM] n=11) and the thrombin-activated filtrate (P = 0.0006, mean = 3393 ± 449.2 [SEM] n=12) (Fig 29B). Significantly fewer treponemes migrated into the buffer control filtrates compared with the resting filtrate ($P < 0.05$), confirming chemoattraction to plasma but not to the agonists (collagen and thrombin) Fig 29B-D). Subsequent assays showed an overall 2-fold increase in treponemes migrating into the collagen-activated filtrates at 4-hours (P = 0.0166, mean = 2.013 ± 0.2990 [SEM]) (Fig 29D), and a 4-fold increase at 8-hours (P = 0.0043, mean = 4.063 ± 0.3436 [SEM]) (Fig 29D) relative to the resting filtrates (baseline). At 4-hours the thrombin-activated filtrates attracted fewer treponemes than the baseline (mean = 0.6400 ± 0.1069 [SEM]), which was significantly fewer than the collagen-activated filtrates (P = 0.0018) (Fig 29C). Interestingly, after 8-hours the thrombin-activated filtrates attracted 2-fold more treponemes than the resting filtrates (P = 0.0307, mean = 2.027 ± 0.4836 [SEM]), suggesting chemorepellent factors in the thrombin-activated filtrate may have degraded after the 4-hour mark.

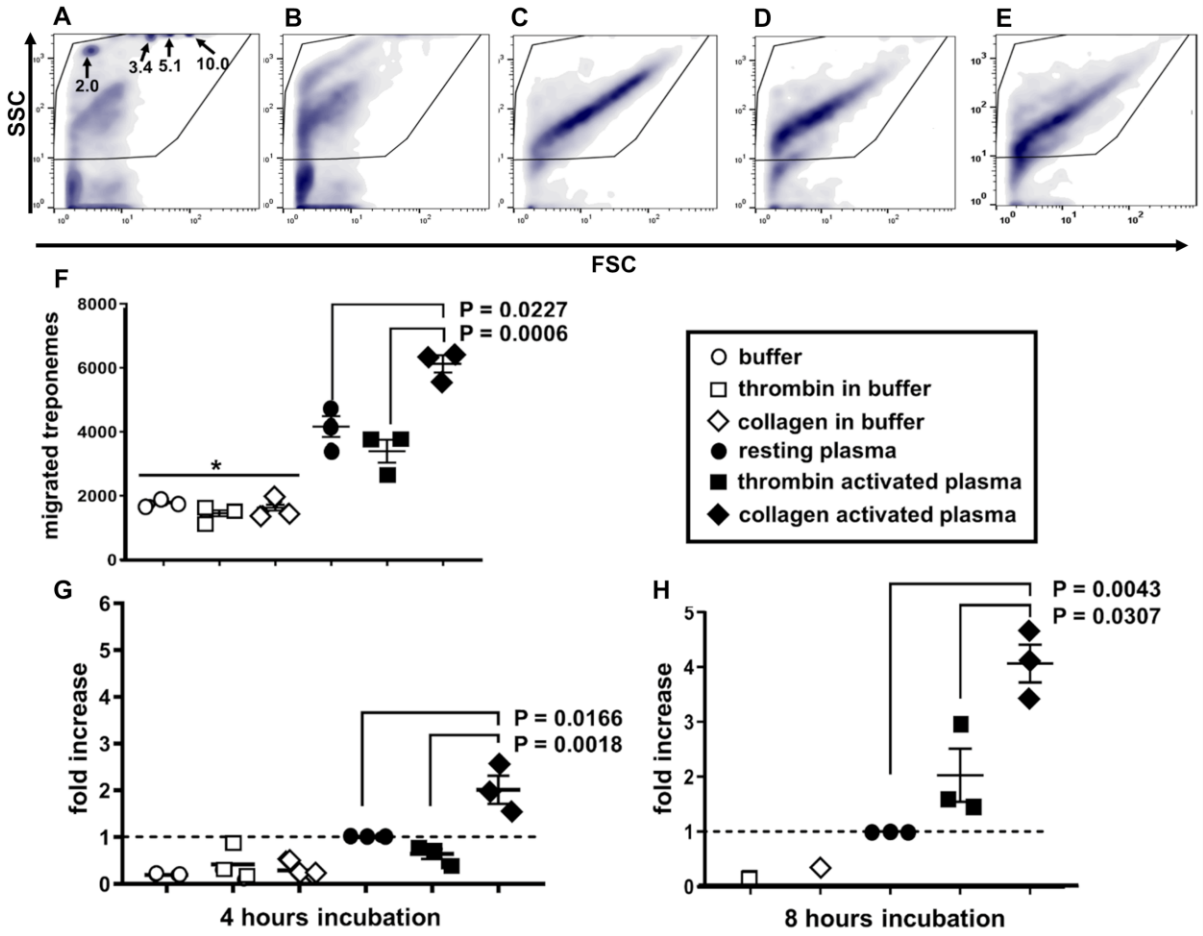

Figure 29: Flow cytometry analysis of treponeme migration into platelet-free human plasma. (A) A capillary tube chemotaxis assay quantified by flow cytometry was gated with 2.0 to 10.0 µm beads and viable treponemes. (B) *T. pallidum* in NS/NRS to confirm gating. (C) The migration of *T. pallidum* into 0.2 µm filtered plasma (filtrate) of resting (unactivated) platelets, (D) thrombin-activated, or (E) collagen-activated platelets. (F) Treponeme migration was then quantified into resting (●), thrombin-activated (■), or collagen-activated (♦) plasma filtrate or buffer (○), buffer + thrombin (□), or buffer + collagen (◇) after 4-hour (shown) and 8-hour coincubations at 34°C MO_2. Each marker is a biological replicate averaged from 3 technical replicates ± SEM (F-H). The results of three independent experiments after either 4-hours (G) or 8-hours (H)

coincubation were compared by using migration to the resting filtrate in each experiment as the baseline (set to 1.0-fold) and plotting the averaged relative response as the fold increase. Each marker represents the mean of technical and biological replicates ± SEM. * $P < 0.05$.

The collagen-activated filtrate contained higher levels of anti-inflammatory mediators when quantified by human cytokine array

We investigated what factors differed between the collagen-activated and thrombin-activated filtrates using a 105-component human cytokine array (Fig 30A) (*Proteome Profiler Array Human XL Cytokine Array Kit*, 2017), two chemiluminescent exposures and an algorithm to assign upregulation (Fig 27) or equivalent expression.

The thrombin-activated filtrate demonstrated 110.74-fold higher levels of myeloperoxidase (MPO), pro-inflammatory/antimicrobial cytokine IL-23* increased 1.47-fold; osteopontin (OPN) increased by 1.49-fold, and matrix metalloprotease (MMP) 9 was 1.21-fold higher (Fig 30C, Table 10). The serine protease inhibitor (serpin) E1 was 1.64-fold higher and the plasma protein sex hormone-binding globulin (SHBG) increased by 1.99-fold (Fig 30C, Table 10).

Table 10: Platelet components up-regulated by thrombin activation.

Cytokine array coordinates	uniprot	Cytokine/Protein	Collagen activated mean	Thrombin activated mean	Fold increase (T/C ratio) ◊◊	P value signal	Effect Size* Glass's Δ	r	r^2
H1/H2	**P05164**	**myeloperoxidase**	**12**	**1274**	**110.74**	**0.0001**	**800.03**	**1.000**	**1.000**
I3/I4	P04278	SHBG	2198	4372	1.99	0.0633	3.49	0.884	0.782
I1/I2	**P05121**	**serpin E1 (PAI-1)**	**7365**	**12070**	**1.64**	**0.0286**	**26.62**	**0.945**	**0.893**
H3/H4	**P10451**	**osteopontin**	**12912**	**19189**	**1.49**	**0.0397**	**3.55**	**0.925**	**0.856**
F5/F6	Q9NPF7	IL-23 *	204	300	1.47	0.1523	2.38	0.749	0.561
G23/G24	**P14780**	**MMP-9**	**10791**	**13020**	**1.21**	**0.0109**	**7.89**	**0.979**	**0.958**

*** effect size thrombin activated plasma = group 1** **◊◊ ratio of thrombin activated signal mean / collagen activated signal mean**

bold = P < 0.05

In contrast to the thrombin-activated, the collagen-activated filtrate (Table 11) had higher levels of pro-angiogenic factors: vascular endothelial growth factor (VEGF) 4.27-fold, platelet-derived growth factor (PDGF) -AA 2.83-fold, PDGF-AB/BB 6.38-fold, epidermal growth factor (EGF) 4.23-fold, angiopoietin (ANGPT) -2 1.85-fold, and hepatocyte growth factor (HGF) 1.97-fold (Fig 30B, Table 11). The collagen-activated platelets secreted several fold higher levels of immunomodulators: IL-10 5.40-fold, IL-8 4.51-fold, interferon (IFN) ϒ 2.42-fold, thymus and activation regulated chemokine (TARC) 95.36-fold, Fms-related tyrosine kinase (Flt) 3 ligand 6.52-fold, transforming growth factor (TGF) -α 18.64-fold*****, granulocyte colony-stimulating factor (G-CSF) 3.89-fold*****, pentraxin-3 5.16-fold and soluble receptor for advanced glycation endproducts (RAGE) 9.19-fold (Fig 30B, Table 11).

Table 11: Platelet components up-regulated by collagen activation

Cytokine array coordinates	uniprot	Cytokine/Protein	Collagen activated mean	SD	Thrombin activated mean	SD	Fold Increase (C/T ratio) ◊	P value signal	Effect Size* Glass's Δ	r	r^2
I7/I8	**Q92583**	**TARC (CCL17)**	**1335**	**278.60**	**14**	**19.09**	**95.36**	**0.0216**	**69.22**	**0.958**	**0.918**
I13/I14	**P01135**	**TGF-alpha ✱**	**261**	**31.11**	**14**	**19.80**	**18.64**	**0.0110**	**12.47**	**0.978**	**0.957**
H13/H14	**Q15109**	**RAGE**	**1254**	**301.23**	**137**	**193.04**	**9.19**	**0.0476**	**5.79**	**0.911**	**0.830**
C15/C16	**P49771**	**Flt-3 ligand (FLT3LG)**	**1463**	**264.46**	**225**	**160.51**	**6.52**	**0.0298**	**7.73**	**0.943**	**0.889**
H7/H8	**P01127**	**PDGF-AB/BB**	**12592**	**139.30**	**1973**	**62.23**	**6.38**	**0.0001**	**170.63**	**1.000**	**1.000**
E9/E10	P22301	IL-10	1324	356.38	245	243.24	5.40	0.0715	4.44	0.870	0.758
H9/H10	P26022	pentraxin-3	863	364.16	167	220.62	5.16	0.1471	3.15	0.756	0.572
E7/E8	**P10145**	**IL-8 (CXCL8)**	**618**	**127.99**	**137**	**67.88**	**4.51**	**0.0426**	**7.08**	**0.920**	**0.846**
I21/I22	P15692	VEGF	478	121.62	112	65.05	4.27	0.0642	5.63	0.882	0.779
B19/B20	**P01133**	**EGF**	**7283**	**463.86**	**1722**	**24.75**	**4.23**	**0.0035**	**224.68**	**0.993**	**0.986**
C17/C18	P09919	G-CSF (CSF3)	1291	318.91	332	77.07	3.89	0.0538	12.44	0.900	0.810
I5/I6	**Q01638**	**ST2 (IL-1 R4)**	**1510**	**31.11**	**415**	**45.96**	**3.64**	**0.0013**	**23.84**	**0.997**	**0.995**
D1/D2	**P09341**	**GRO-α (CXCL1)**	**620**	**41.72**	**197**	**101.12**	**3.16**	**0.0317**	**4.18**	**0.939**	**0.882**
E11/E12	**P20809**	**IL-11**	**1447**	**9.19**	**507**	**52.33**	**2.85**	**0.0016**	**17.95**	**0.997**	**0.994**
H5/H6	**P04085**	**PDGF-AA**	**9404**	**1163.19**	**3323**	**9.19**	**2.83**	**0.0178**	**661.69**	**0.965**	**0.932**
B15/B16	**O94907**	**Dkk-1 (Dickkopf-1)**	**2857**	**205.77**	**1122**	**62.23**	**2.55**	**0.0076**	**27.88**	**0.985**	**0.970**
B3/B4	P29965	CD40 ligand (CD154)	1009	207.89	400	111.72	2.52	0.0676	5.45	0.877	0.769
A15/A16	**P23560**	**BDNF**	**10420**	**651.95**	**4213**	**14.14**	**2.47**	**0.0055**	**438.96**	**0.989**	**0.978**
D9/D10	P01579	IFN-Y	1218	349.31	504	374.06	2.42	0.1870	1.91	0.702	0.493
G13/G14	**P14174**	**MIF**	**7454**	**447.60**	**3141**	**132.94**	**2.37**	**0.0058**	**32.44**	**0.988**	**0.977**
F19/F20	**P02778**	**IP-10 (CXCL10)**	**1029**	**96.17**	**435**	**62.23**	**2.37**	**0.0181**	**9.55**	**0.965**	**0.931**
F21/F22	O14625	I-TAC (CXCL11)	494	92.63	237	85.56	2.09	0.1022	3.00	0.822	0.675
A9/A10	**Q15389**	**angiopoietin-1**	**4868**	**157.68**	**2447**	**26.87**	**1.99**	**0.0022**	**90.10**	**0.996**	**0.991**
D5/D6	**P14210**	**HGF**	**1291**	**200.11**	**654**	**53.03**	**1.97**	**0.0490**	**12.01**	**0.909**	**0.826**
A11/A12	**O15123**	**angiopoietin-2**	**1538**	**142.84**	**832**	**79.90**	**1.85**	**0.0258**	**8.84**	**0.950**	**0.903**
G5/G6	**P80188**	**lipocalin-2**	**20590**	**1180.87**	**11357**	**651.25**	**1.81**	**0.0105**	**14.18**	**0.979**	**0.959**
I11/I12	P02786	TfR (CD71)	2203	352.85	1253	133.64	1.76	0.0706	7.11	0.872	0.760
G15/G16	Q07325	MIG	581	7.07	338	94.05	1.72	0.0675	2.58	0.877	0.768
I9/I10	**Q07654**	**TFF3**	**1198**	**55.15**	**722**	**26.16**	**1.66**	**0.0081**	**18.20**	**0.984**	**0.968**
J7/J8	**P16284**	**PECAM-1 (CD31)**	**9744**	**67.18**	**6338**	**81.32**	**1.54**	**0.0005**	**41.88**	**0.999**	**0.998**
D3/D4	P01241	growth hormone	10324	584.07	7107	1125.01	1.45	0.0696	2.86	0.894	0.799
C3/C4	**P42830**	**ENA-78 (CXCL5)**	**7746**	**444.77**	**5572**	**278.60**	**1.39**	**0.0279**	**7.80**	**0.946**	**0.896**
H23/H24	**P48061**	**SDF-1α (CXCL12)**	**3491**	**28.28**	**2551**	**103.24**	**1.37**	**0.0064**	**9.10**	**0.987**	**0.975**
E1/E2	P05112	IL-4	520	45.96	384	69.30	1.35	0.1477	1.96	0.756	0.572
C13/C14	**O95750**	**FGF-19**	**6073**	**136.47**	**4575**	**219.20**	**1.33**	**0.0145**	**6.83**	**0.972**	**0.944**
I19/I20	Q03405	uPAR	2115	239.00	1599	31.82	1.32	0.0939	16.22	0.834	0.696
C9/C10	**P09038**	**FGF basic (FGF-2)**	**1076**	**65.05**	**820**	**5.66**	**1.31**	**0.0310**	**45.23**	**0.941**	**0.885**
H21/H22	**Q9HD89**	**resistin**	**3698**	**130.11**	**2928**	**144.96**	**1.26**	**0.0305**	**5.32**	**0.942**	**0.887**
J9/J10	**Q8TDQ0**	**TIM-3**	**6082**	**222.03**	**4817**	**328.80**	**1.26**	**0.0458**	**3.85**	**0.914**	**0.836**
H15/H16	P13501	RANTES (CCL5)	16633	1188.65	13243	223.45	1.26	0.0582	14.52	0.893	0.797
C19/C20	Q99988	GDF-15 (MIC-1)	1611	142.84	1298	43.13	1.24	0.0971	7.26	0.829	0.688
J5/J6	P02774	vitamin D BP	9707	1264.31	7868	84.15	1.23	0.1765	21.85	0.716	0.513
H11/H12	P02776	PF4 (CXCL4)	11852	316.08	9726	878.23	1.22	0.0844	2.42	0.850	0.722
C21/C22	P04141	GM-CSF (CSF2) ✱	465	53.74	383	39.60	1.21	0.2245	2.07	0.656	0.430
D7/D8	**P05362**	**ICAM-1 (CD54)**	**10390**	**53.74**	**8822**	**16.26**	**1.18**	**0.0006**	**96.46**	**0.999**	**0.997**
A13/A14	**Q9Y275**	**BAFF**	**4763**	**55.86**	**4092**	**136.47**	**1.16**	**0.0232**	**4.92**	**0.955**	**0.912**
A17/A18	**P01031**	**complement C5/C5a**	**8553**	**70.00**	**7572**	**125.87**	**1.13**	**0.0106**	**7.79**	**0.979**	**0.959**

*** effect size collagen activated plasma = group 1 ◊ ratio of collagen activated signal mean/thrombin activated signal mean bold = P < 0.05**

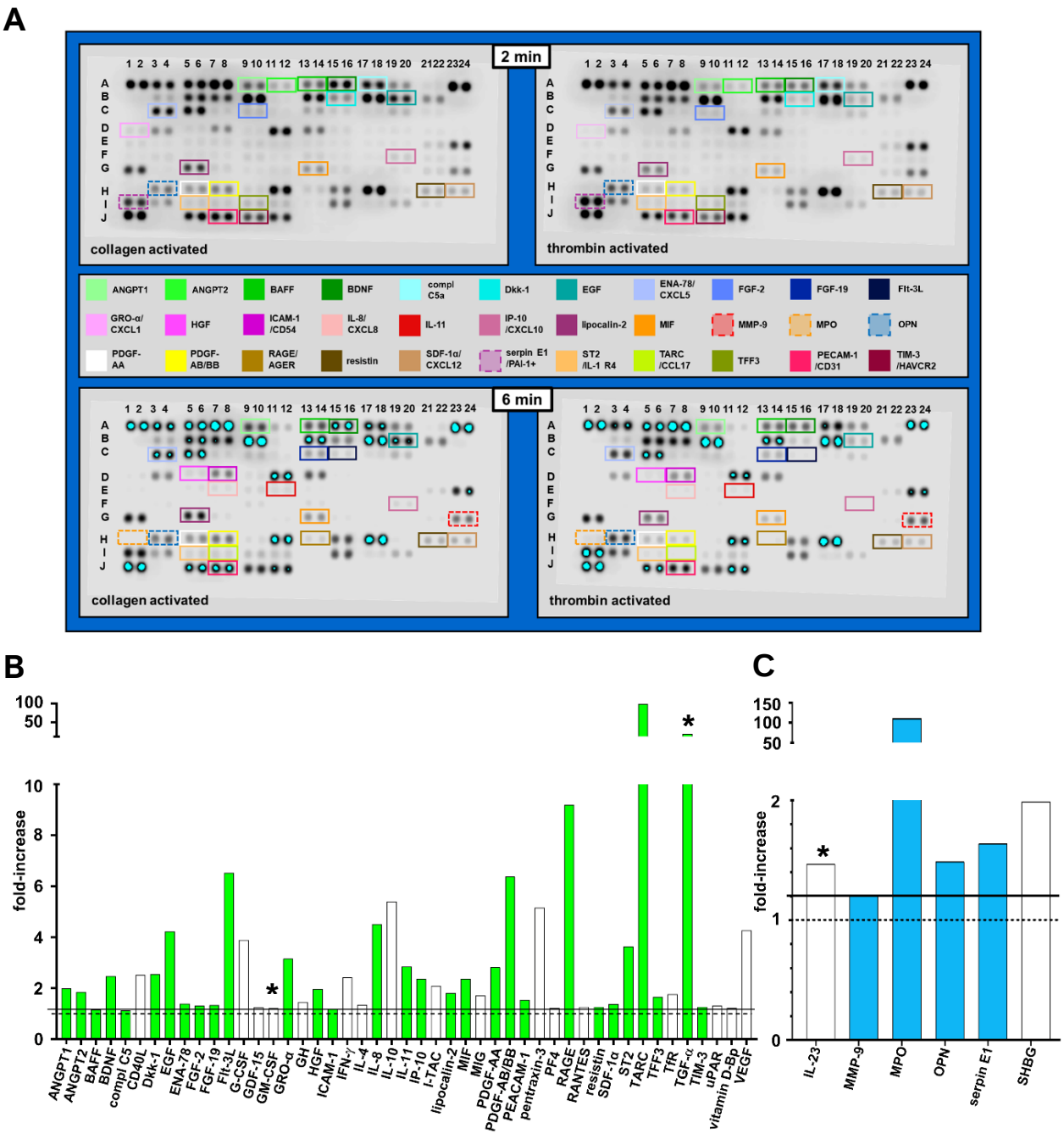

Figure 30: A human XL cytokine array quantified differences between the collagen activated and thrombin activated platelet filtrates used in the chemotaxis assays. (A) Chemiluminescent detection of 105 factors, spotted in duplicate, was

measured following 2- (top) and 6-minute (bottom) exposures after coincubation with the collagen activated (left) or thrombin activated (right) filtrates. For comparison, duplicate spots with a P value < 0.05, one component used to assess overall upregulation, are shown surrounded by colour coded boxes, factors upregulated by collagen (solid lines) or thrombin (dashed lines). (B-C) Signal value means were used to determine the relative expression levels and up-regulation in either the (B) collagen activated filtrate or (C) thrombin activated filtrate using Effect Size (ES), visual contrast discrimination, and the fold increase (shown) with a P value < 0.05 (coloured bars) or ≥ 0.05 (white bars). Dashed horizontal lines indicate 1-fold, solid lines 1.20-fold. P values were calculated using the GraphPad Prism Student's two-tailed t-test

Platelets secreted a pool of cytokines at equivalent levels when activated by collagen or thrombin

While we quantified 47 factors upregulated in the collagen-activated filtrate, and 6 in the thrombin-activated filtrate, platelet activation also resulted in 33 components secreted in both filtrates at a relatively equivalent level of expression (Fig 31A top, Tables 9 - 11). We used GO to investigate protein-protein interactions (PPI) and produced a 28 node PPI network of these factors involved in modulating host inflammation/immune responses, leukocyte chemotaxis and vascular permeability (Fig 31A bottom) (Brown et al., 2013; Szklarczyk et al., 2019). Following 8-hour coincubations there was a distinct preference for treponemes to migrate into *activated* plasma, whether by collagen-activated (4-fold higher, mean = 4.063 ± 0.3436 [SEM], P = 0.0108) or thrombin-activated platelets (2-fold higher, mean = 2.027 ± 0.4836 [SEM], P = 0.0488) relative to the plasma from *resting* platelets (Fig 31B). This suggests that *T. pallidum* may

recognize factors common to both these mechanisms of platelet activation (Fig 31B) and that *T. pallidum* exhibits chemotaxis towards activated platelets. Factors coexpressed in the activated filtrates may be chemoattractants that cue *T. pallidum* towards host microenvironments that may present favourable conditions, such as areas demonstrating immune or vascular dysregulation (Eisinger et al., 2018; Gao et al., 2019; Gros et al., 2015; Rayes et al., 2019; Xie et al., 2017).

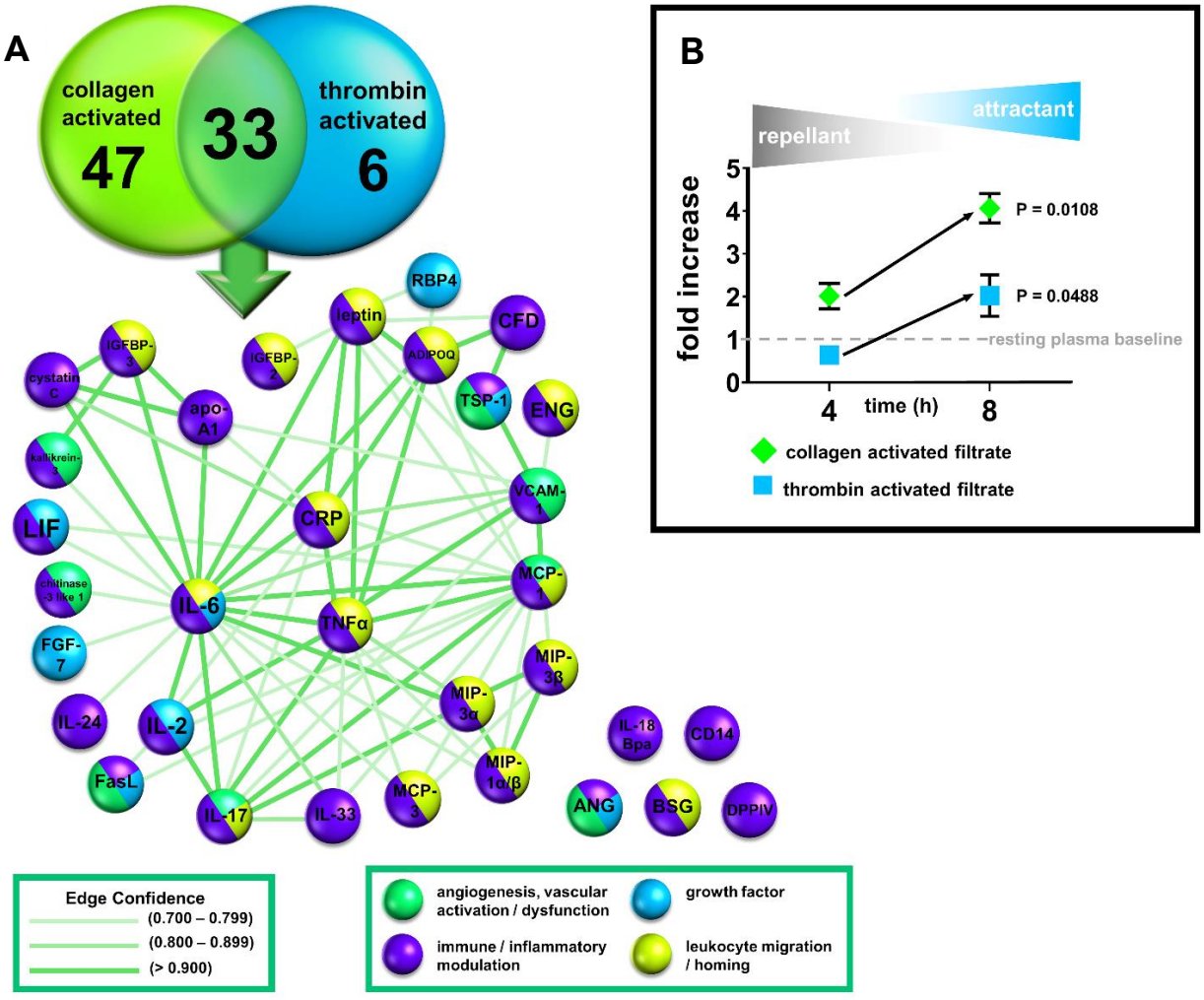

Figure 31: Platelet activation results in a pool of equivalently expressed factors that may act as chemoattractants for *T. pallidum*. (A) Human XL cytokine array

results quantified factors upregulated by collagen-activated or thrombin-activated platelets, and those expressed at similar levels in both activated filtrates. GO analysis of equivalently expressed factors shows immune and growth factor activity in a 28 node protein-protein interaction (PPI) (Szklarczyk et al., 2019). (B) The collagen-activated platelet filtrates (green diamonds) induced 2-fold and 4-fold higher migration than the resting platelet filtrates (baseline - dashed grey line) after 4-hour and 8-hour coincubations, respectively. The thrombin-activated filtrates (blue squares) failed to attract as many treponemes as the baseline after 4-hour coincubations, then surpassed the baseline by 2-fold after 8-hours. The results of 3 independent experiments are shown as the mean ± SEM of biological and technical replicates. P values were calculated using the GraphPad Prism Student's two-tailed t-test.

Table 12: Platelet components expressed at similar levels in both filtrates.

Components that failed to meet the full criteria for upregulation (Fig 27), in some cases had a fold increase > 1.20 must failed to have a minimal difference in visual constrast

cytokine array coordinates	uniprot	cytokine/protein	collagen activated mean	SD	thrombin activated mean	SD	fold increase (C/T ratio) ◊	P value signal	Effect Size Glass's Δ
A3/A4	A8K660	adiponectin (ADIPOQ)	9169	459.62	8242	522.55	1.11	0.2001	1.77
A5/A6	P02647	apolipoprotein A-1 (apo A1)	11840	525.38	10978	142.84	1.08	0.1547	6.05
A7/A8	P03950	angiogenin (ANG)	21072	117.38	20200	411.54	1.04	0.1023	2.12
A19/A20	P08571	CD14	3252	297.69	3223	106.07	1.01	0.9071	0.27
B5/B6	P36222	chitinase 3-like 1	23982	2854.59	21198	1006.21	1.13	0.3230	2.77
B7/B8	P00746	complement factor D (CFD)	5948	198.70	5962	199.40	1.00	0.9521	0.07
B9/B10	P02741	C-reactive protein (CRP)	21265	98.99	19763	9.19	1.08	0.0022	163.43
B13/B14	P01034	cystatin C	10486	40.31	9822	386.79	1.07	0.1371	1.72
B17/B18	P27487	DPPIV (CD26)	15755	153.44	14817	500.63	1.06	0.1269	1.84
B21/B22	P35613	emmprin (basingen, CD147)	11827	1175.92	10178	1455.23	1.16	0.3389	1.13
C5/C6	P17813	endoglin (ENG, CD105)	9531	289.91	8692	403.76	1.10	0.1395	2.08
C7/C8	P48023	Fas ligand (CD178)	411	84.85	322	160.51	1.28	0.5579	0.55
C11/C12	P21781	FGF-7	350	43.84	176	12.02	1.99	0.0323	14.48
D11/D12	P18065	IGFBP-2	10426	379.01	8952	384.67	1.16	0.0610	3.83
D13/D14	P17936	IGFBP-3	4379	208.60	3748	50.91	1.17	0.0532	12.39
D21/D22	P60568	IL-2	211	62.93	203	144.96	1.04	0.9431	0.06
E5/E6	P05231	IL-6	420	5.66	466	0.71	0.90	0.0078	0.98
E21/E22	Q16552	IL-17	1292	171.83	1265	183.14	1.02	0.8931	0.15
E23/E24	O95998	IL-18 Bpa	7542	1188.65	8250	158.39	0.91	0.4913	0.60
F7/F8	Q13007	IL-24	141	17.68	158	10.61	0.89	0.3638	0.96
F15/F16	O95760	IL-33	123	73.54	101	63.64	1.22	0.7794	0.35
F23/F24	P07288	kallikrein 3	99	65.05	78	110.31	1.27	0.8382	0.19
G1/G2	P41159	leptin	7263	354.26	7015	477.30	1.04	0.6149	0.52
G3/G4	P15018	LIF	219	92.63	133	77.07	1.65	0.4191	1.12
G7/G8	P13500	MCP-1 (CCL2)	517	25.46	485	19.09	1.07	0.2855	1.42
G9/G10	P80098	MCP-3 (CCL7)	235	55.15	244	154.86	0.97	0.9484	0.16
G17/G18	Q8NHW4	MIP-1α/MIP-β (CCL3/CCL4)	323	217.08	374	92.63	0.86	0.7888	0.23
G19/G20	P78556	MIP-3α (CCL20)	353	108.19	200	176.07	1.77	0.4050	0.87
G21/G22	Q99731	MIP-3β (CCL19)	840	38.89	888	0.00	0.95	0.2198	1.23
H17/H18	P02753	RBP4	16845	508.41	19210	912.87	1.14	0.0853	4.65
I15/I16	P07996	thrombospondin-1 (TSP-1)	16815	1072.68	14253	73.54	1.18	0.0779	34.84
I17/I18	P01375	TNF-α	1543	98.29	1358	202.23	1.14	0.3657	0.91
J11/12	P19320	VCAM-1 (CD106)	7601	152.74	8915	530.33	1.17	0.0780	8.60

The collagen-activated platelets secreted more M2 than M1 macrophage polarizing factors

We continued our investigation of the collagen-activated filtrate GO results by dividing the factors into two broad immunological categories: pro-inflammatory and/or M1 macrophage polarizing; or anti-inflammatory and/or M2 polarizing (Fig 32). The highly antibacterial M1 macrophage is “classically activated” by interferon (INF) -Ɣ (2.42-fold higher) and granulocyte-macrophage colony stimulating factor (GM-CSF, 1.21-fold***** higher) (Fig 32 top left). M1 macrophages are associated with a Th1 response, high

levels of surface expressed inducible nitric oxide synthase (iNOS), major histocompatibility complex (MHC II), and cluster of differentiation (CD) 64, CD80, and CD86 (Fig 32 lower left) (Atri et al., 2018; Shapouri-Moghaddam et al., 2018; Yao et al., 2019). Fms-like tyrosine kinase (Flt) 3 ligand (6.52-fold higher), recently recognized to induce M1 polarization (Gao et al., 2018), was upregulated together with several pro-inflammatory mediators (Fig 32 left, Table 10).

In contrast, the “alternatively activated” M2 macrophage, associated with a Th2 response (Atri et al., 2018), differentiates into subtypes: M2a (anti-inflammatory), M2b (immunoregulatory and infection promoting), M2c (immunosuppressive) and M2d (angiogenic) (Fig 32 bottom right) (Atri et al., 2018; Shapouri-Moghaddam et al., 2018; Wang et al., 2019; Yao et al., 2019). M2 macrophages express surface arginase (Arg) -1, CD86, CD163, CD206 and CD209 and often play a role in immune tolerance and/or pathogen persistence (Atri et al., 2018; Shapouri-Moghaddam et al., 2018; Wang et al., 2019; Yao et al., 2019), thus we grouped M2 inducers and associated factors together with anti-inflammatory and growth factors.

Factors associated with anti-inflammatory, growth and/or M2 polarizing host modulation were overrepresented relative to pro-inflammatory and/or M1 macrophage inducing factors (Fig 32, Table 11) (Akdis et al., 2016; Atri et al., 2018; Musolino et al., 2017; Szklarczyk et al., 2019). Core M2 macrophage polarizers IL-4 (1.35-fold) and IL-10 (5.40-fold) were increased in addition to recently recognized M2 polarizers: TARC serum stimulation (ST) -2, VEGF, granulocyte colony-stimulating factor***** (G-CSF), macrophage migration inhibitory factor (MIF), and stromal cell-derived factor (SDF) -1α (Fig 32 top right, Table 11).

Angiogenesis plays an important role in altering vascular permeability, and many pathogens induce dysregulated angiogenesis to facilitate nutrient acquisition, dissemination, and immunoprotective niche localization (Osherov & Ben-Ami, 2016). Angiogenic modulators at higher levels included: TGF-α*****, EGF, PDGF-AA, PDGF AB/BB, VEGF, FGF2, and HGF (Fig 32 right middle, Table 11).

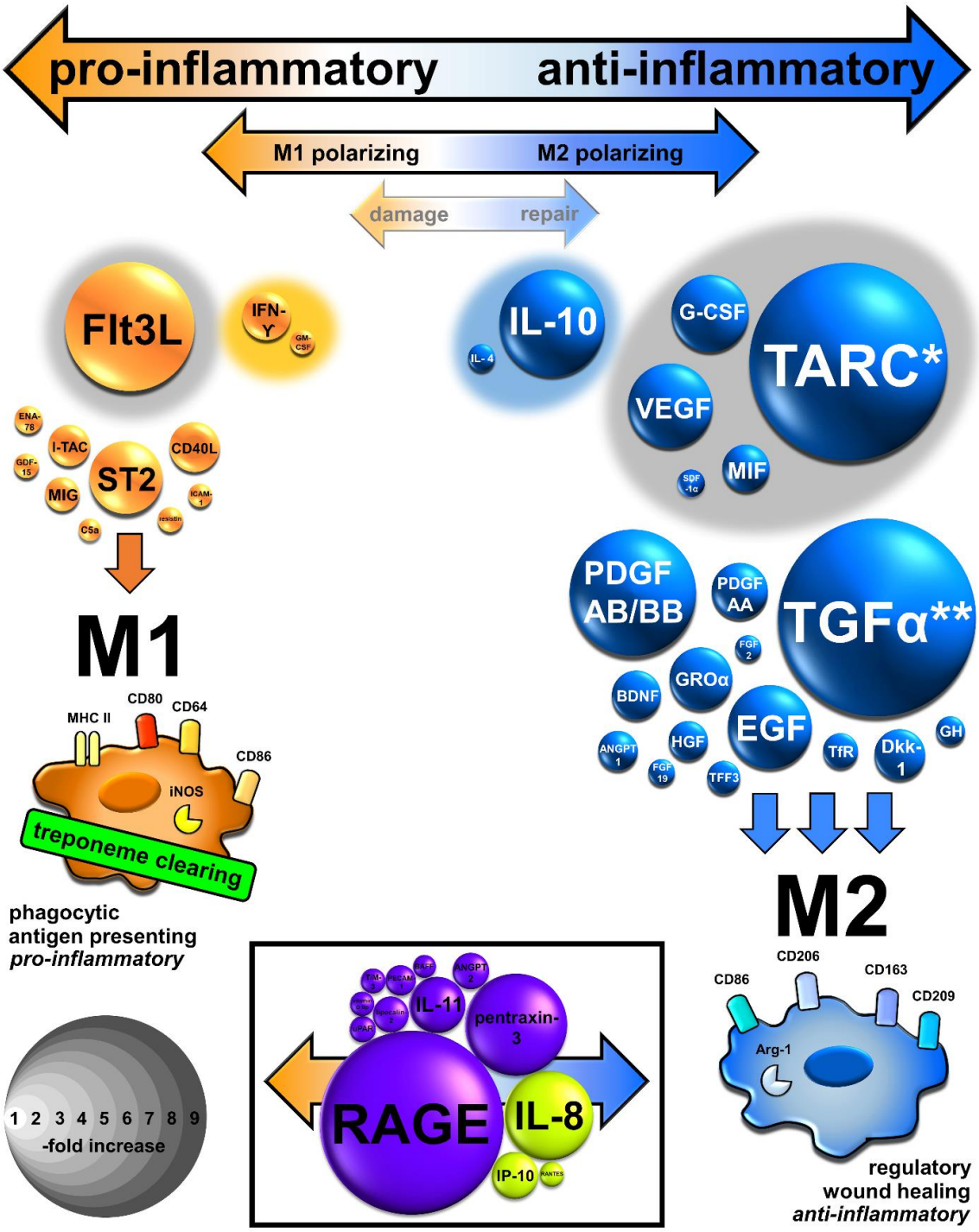

Figure 32: Anti-inflammatory, M2 polarizing and growth factors were more highly represented in the collagen activated platelet filtrate than pro-inflammatory mediators. (left - orange spheres) Core M1 macrophage polarizing factors are IFN-Ɣ

and GM-CSF***** (orange shading), secondary M1 modulator (grey shading), and pro-inflammatory or M1 associated mediators. (bottom left) M1 macrophages up-regulate expression of MHC class II, iNOS, and may include CD64, CD80 and CD86. (right - blue spheres) Core M2 macrophage polarizing factors are IL-10 and IL-4 (blue shading), secondary M2 modulators (grey shading), and anti-inflammatory or M2 associated mediators and growth factors (blue spheres). (bottom right) M2 macrophages upregulate Arg-1, and markers may include CD86, CD163, CD206 and CD209. (inset) Factors with variable, milieu-dependent functions modulate inflammation (purple) and leukocyte recruitment (lime). (bottom left -key) Sphere diameters correspond to the mean fold-increase in the collagen activated filtrate. *TARC 95.36-fold increase, **TGF-α 18.64-fold increase, low signal intensities.

Gene ontology results:

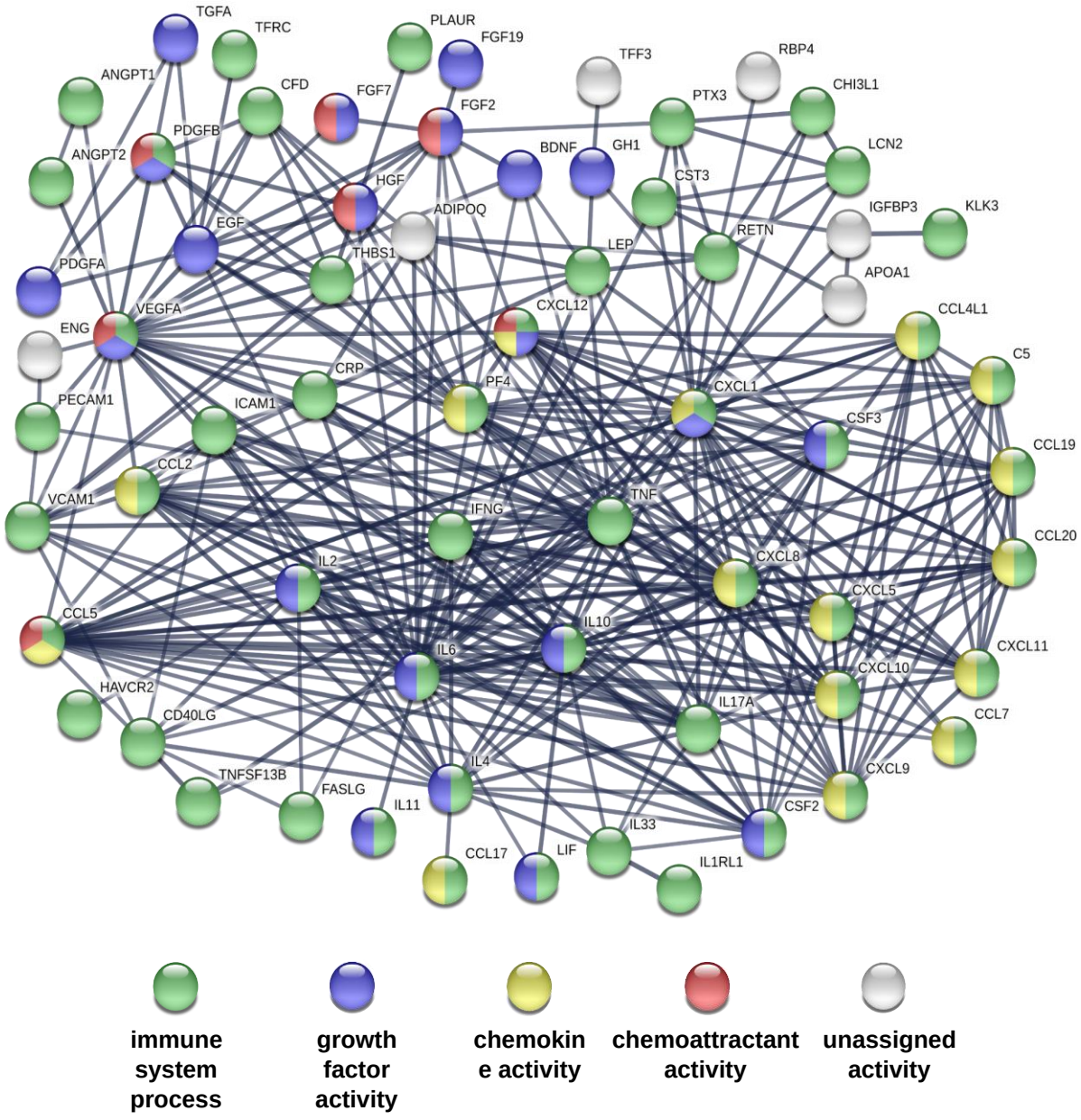

Figure 33: Gene ontology and protein-protein interaction networks of the collagen-activated platelet filtrate. Minimum interactions score = 0.900 (highest confidence). Cytokine assay components not within PPI networks not shown. (Szklarczyk et al., 2019)

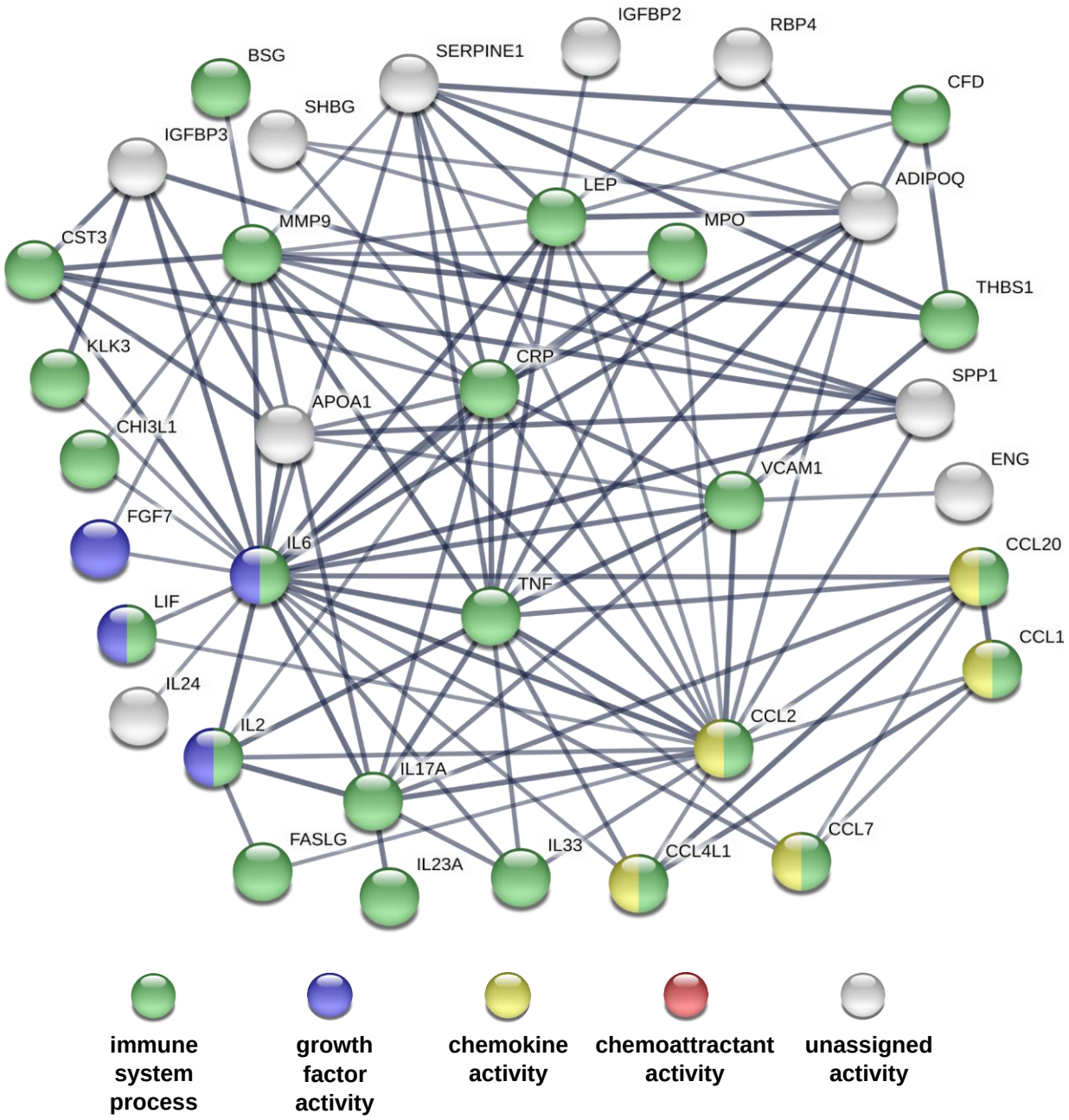

Figure 34: Gene ontology and protein-protein interaction networks of the thrombin-activated platelet filtrate. Minimum interactions score = 0.700 (high confidence). Cytokine assay components not within PPI networks not shown. (Szklarczyk et al., 2019)

3.5 Discussion

Chemotaxis allows motile pathogens to respond to molecular gradients for nutrient acquisition (Lopes & Sourjik, 2018; Matilla & Krell, 2017), recognize cues to their anatomical location within the host (Karavolos et al., 2013; Lopes & Sourjik, 2018; Olive & Sassetti, 2016), and to changes in the host immune response (Olive & Sassetti, 2016). This study demonstrates that *T. pallidum* chemotaxis is altered in response to the different biomolecules present in human plasma obtained from platelets that are resting, activated by collagen or activated by thrombin. In addition, treponemes demonstrated a significant preference for the secretions of collagen-activated platelets compared with that of resting platelets.

These results demonstrate that *T. pallidum* differentiates not only between different *states* of platelet activation (Church et al., 2019) but also between different *mechanisms* of platelet activation. To our knowledge this is the first investigation of bacterial chemotaxis in response to secretions within human plasma that result from platelets activated by different mechanisms.

Thrombin and collagen induce platelet immunomodulation relevant to *T. pallidum*

Thrombin and collagen, two of the most potent platelet agonists that induce platelet immunoregulation (Gros et al., 2015; Koupenova et al., 2018; Linke et al., 2017; Milioli et al., 2015; Rayes et al., 2017, 2020; Ribeiro et al., 2019), were selected for their relevance to the pathogenesis of *T. pallidum* (Carlson et al., 2011; LaFond & Lukehart, 2006; Pozzobon et al., 2016).

Thrombin, stored as its precursor prothrombin, is present within keratinized and mucosal epithelia (Burzynski et al., 2019; Motta et al., 2019), the vasculature (Langerak et al., 2019; Miao et al., 2018), and within platelet α-granules (Golebiewska & Poole, 2015). Host immune responses to treponemes have the potential to induce thrombin activation pathways within the skin (Cugno et al., 2019; Motta et al., 2019) during primary and secondary syphilis, and within the vasculature during dissemination (Carlson et al., 2011; LaFond & Lukehart, 2006).

Collagen is important for the structure of bone, tendon, connective tissues, and the cornea, in addition to its role as a major sub-endothelial component and platelet activator at endothelial disruptions (Arseni et al., 2018; Boulaftali et al., 2018; Linke et al., 2017). *Treponema pallidum* has the potential to disrupt endothelial tissue and promote collagen activation within the dermal capillaries of the highly angiogenic syphilitic skin lesions (Gao et al., 2019; Juanpere-Rodero et al., 2013; Macaron et al., 2003) which would be accompanied by the influx of platelets (Eisinger et al., 2018; Menter et al., 2017).

Platelet rich plasma as a model system

By utilizing cell-free plasma obtained from the PRP of resting, collagen-activated or thrombin-activated human platelets, this work advances previous findings that *T. pallidum* preferentially interacts with activated versus resting platelets. Here, demonstrated through increased chemotaxis, this preference is shown to extend to the plasma obtained from collagen-activated platelets.

In addition, this experimental set-up provides a novel model to study bacterial pathogenesis, as a system partway between *in vivo* animal and *in vitro* cell culture techniques. By focussing strict attention to methodology that reduced the need for powerful anti-coagulants, the platelets remained inactive, and were marginally concentrated, while removing the red and white blood cells. The resulting PRP retained host components and remained similar to its native state as a specialized connective tissue (Montague et al., 2020). This system facilitated our previous treponeme-platelet interactions studies (Church et al., 2019), and here facilitated platelet activation within the complex background of host plasma to mimic host microenvironments (Etulain, 2018; Renn et al., 2015). Key to this model was the optimization of consistent platelet handling and activation under our experimental conditions (Fig 21) which lead to consistent responses by *T. pallidum* (Fig 29).

Thrombin-activated platelet secretions significantly reduced chemotaxis

The first notable demonstration that the mechanism of platelet activation may be of importance to *T. pallidum* chemotaxis came from the significantly reduced migration into the thrombin-activated plasma that was consistently apparent after four hours coincubation (Fig 29B-C). Thrombin-activated platelets produce a unique set of secretions (De Candia, 2012; Gianazza et al., 2020; Vélez et al., 2015) that induce coagulation during haemostasis with additional platelet activation and aggregation, pathogen trapping via immunothrombosis (Burzynski et al., 2019; Koupenova et al., 2018), and antimicrobial ROS generation through MPO secretion which leads to the generation of $HOCl^-$, $HOBr^-$, and $HOSCN^-$ (Aratani, 2018; Koupenova et al., 2018). The enzymatic actions of thrombin (Gianazza et al., 2020; Parsons et al., 2018) and MMP9

(Shao et al., 2017) alter the functions of secreted peptides/proteins, including OPN (Kobori et al., 2018), within the platelet releasate. Thrombin-activated platelets induce leukocyte recruitment via MCP-1 and IL-8 (Koupenova et al., 2018) and mediate neutrophil induced MPO, ROS and NET formation (Aratani, 2018; Gros, et al., 2015; Koupenova et al., 2018). Thrombin is activated during host immune responses within the skin (Cugno et al., 2019; Tamagawa-Mineoka, 2015), intestinal mucosa (Motta et al., 2019), and within the vasculature (Koupenova et al., 2018). Thrombin activation increases platelet responsiveness to pathogens by upregulating TLR4, TLR9, and the chemokine C-X3-C motif receptor 1 (CX3CR1) (Koupenova et al., 2018; Linke et al., 2017). In turn, thrombin-activated platelets may cue pathogens to hostile microenvironments.

Cytokine analysis of the thrombin-activated filtrate identified anti-microbial components

Under these conditions the levels of the powerful antimicrobial enzyme, MPO, were 110.74-fold higher in thrombin-activated filtrate (Fig 30C). Myeloperoxidase may have been an important contributor to the significant chemorepellent effect demonstrated during the first four hours of treponeme coincubation (Fig 29C) given its prominent role in antimicrobial responses (Aratani, 2018; Delabranche et al., 2017; Gros et al., 2015). Myeloperoxidase is a recognized target of the bacterial virulence factors LipL21 and Lip45 in *Leptospira interrogans* (*L. interrogans*) (Vieira et al., 2018) and by *S. aureus* staphylococcal peroxidase inhibitor (SPIN) (de Jong et al., 2017).

Treponema pallidum is highly sensitive to oxidative damage (LaFond & Lukehart, 2006) which may be encountered during cutaneous inoculation of a new host, and through

antimicrobial responses of keratinocytes (Aratani, 2018), T cells (Aratani, 2018; Burzynski et al., 2019; Gros et al., 2015), and neutrophils (Aratani, 2018; Rosowski & Huttenlocher, 2018) that lead to MPO and ROS production. To mitigate ROS damage *T. pallidum* contains neelaredoxin (Tp0823) to convert O_2^- to H_2O_2 (Aratani, 2018; Edmondson et al., 2018; LaFond & Lukehart, 2006) and hydrogen reductase C (Tp0509) to reduce H_2O_2 to H_2O (LaFond & Lukehart, 2006), but lacks genes encoding superoxide dismutase (SOD) and catalase detoxifying enzymes (Edmondson et al., 2018; Giacani et al., 2013). Superoxide dismutase expressed by bacteria, such as *B. burgdorferi* SodA, are important virulence factors to manage host immune responses and low pH microenvironments (Broxton & Culotta, 2016). An alternative approach by pathogens is to utilize chemotaxis to sense host generated ROS and avoid those microenvironments (Collins et al., 2018). Given the inherent sensitivity of *T. pallidum* to oxidative stress (Edmondson et al., 2018; Giacani et al., 2013), the detection of MPO suggests *T. pallidum* may utilize chemotaxis to avoid host ROS.

In addition to MPO, the thrombin-activated filtrate contained higher levels of pro-inflammatory MMP9 (Aratani, 2018; Kral et al., 2016), IL-23* (Bunte & Beikler, 2019), and OPN which recruits leukocytes to inflamed tissue (Singh et al., 2017) (Fig 30C, Table 10).

Extended exposure to the thrombin-activated filtrate resulted in positive chemotaxis and supports MPO as a chemorepellent

While exposure to the thrombin-activated filtrate for four hours dependably reduced *T. pallidum* chemotaxis to levels significantly below those seen levels to the resting and collagen-activated plasma filtrates (Fig 29B-C), coincubation for eight hours resulted in

a sharp increase in positive chemotaxis (Fig 29D). A plausible explanation for this is the plasma half-life of MPO which is ~ four hours (Pek et al., 2019), at which point its ability to generate ROS would also be reduced. Chemosensory pathways that facilitate responses to oxidative stress are a recognized strategy for pathogens (García-Fontana et al., 2019; Matilla & Krell, 2017). Taken together, the negative chemotaxis demonstrated at four hours coincubation, the positive chemotaxis then seen at eight hours coincubation, with the four-hour plasma half-life of MPO, these results suggest *T. pallidum* utilizes chemotaxis to avoid MPO and/or ROS during infection.

Collagen-activated platelet secretions induced highly positive chemotaxis

During the first four hours of coincubation, while *T. pallidum* exhibited the lowest plasma-mediated migration into the thrombin-activated filtrate, the collagen-activated platelet filtrate induced significant positive chemotaxis (Fig 29B-D). The response to the collagen-activated filtrate consistently significantly exceeded that to the resting plasma filtrate (Fig 29B-D), and aligns with the previous findings that *T. pallidum* preferentially interacts with activated platelets (Church et al., 2019).

Collagen activation induces different signaling patterns than thrombin activation (Poulter et al., 2017; Vélez et al., 2015), may occur from localized disturbance to the glycocalyx/endothelium (Gaertner & Massberg, 2019; Li et al., 2017), and may involve a single platelet without induction of aggregation or coagulation (Boulaftali et al., 2018). Collagen-activated platelets facilitate leukocyte adhesion to and transmigration through blood vessels walls, induce secretion of platelet ANGPT-1, serotonin and ATP which modulate vascular permeability and endothelial activation (Boulaftali et al., 2018), and

have been shown to mediate anti-inflammatory host immune responses (Rayes et al., 2020).

Angiogenic mediators in the collagen-activated filtrate promote *T. pallidum* chemotaxis

The cytokine array analysis revealed that when activated platelets by collagen, there was a 1.31- to 18.64-fold increase in several pro-angiogenic mediators: EGF (4.23), VEGF (4.27), FGF-2 (1.31), angiopoietin-2 (ANGPT-2,1.85), TGF-α (18.64)*****, PDGF (-AA 2.83, -AB/BB 6.38), SDF-1 (1.37) and chemokine IL-8 (4.51) (Fig 31B, Table 11) (Eisinger et al., 2018; Kumar, 2019; Rosowski & Huttenlocher, 2018).

Angiogenesis is tightly regulated during homeostasis, but pathogens induce dysregulation to increase vascular permeability and access to host metabolites, expedite dissemination, and to facilitate niche localization (Osherov & Ben-Ami, 2016; Pozzobon et al., 2016). Pathological angiogenesis reduces immune clearance and facilitates the persistence of *Mycobacterium tuberculosis* (*M. tuberculosis*) within infected macrophages via VEGF secretion (Osherov & Ben-Ami, 2016) while *Bartonella bacilliformis* (*B. bacilliformis)* upregulates VEGF, ANGPT-2 (Pozzobon et al., 2016), EGF and IL-8 to promote small vessel proliferation and chronic cutaneous infection within lesion endothelial cells (Pons et al., 2017).

Treponema pallidum induction of vascular inflammation and angiogenesis is considered vital to pathogenesis (Gao et al., 2019) and occurs during all stages of syphilis (Xie et al., 2017). During primary syphilis treponemes localize in and around the prolific, nutrient-rich blood vessels that form complex networks within chancres (Carlson et al.,

2011; Macaron et al., 2003; Pozzobon et al., 2016). The mucocutaneous lesions of secondary syphilis are also highly angiogenic (Juanpere-Rodero et al., 2013; Pozzobon et al., 2016) and vascular inflammation plays a significant role in both neuro- and cardiovascular syphilis sequelae (Carlson et al., 2011; LaFond & Lukehart, 2006; Pozzobon et al., 2016; Xie et al., 2017). Patients with active syphilis exhibit higher levels of VEGF (Kojima et al., 2019), another indication of dysregulated angiogenesis.

Taken together, the importance of angiogenesis to the pathogenesis of *T. pallidum* (LaFond & Lukehart, 2006; Pozzobon et al., 2016), the increased levels of pro-angiogenic factors within the collagen-activated filtrate (Fig 30, Table 11), and the observed chemotaxis towards collagen-activated platelets (Fig 29), suggest treponemes may utilize chemotaxis to localize to areas of active angiogenesis.

Factors common to the thrombin-activated and collagen-activated filtrates may signal platelet activation

Cytokine analysis demonstrated that not only were specific factors expressed more highly when platelets were activated by collagen or by thrombin, but a set of factors were also expressed at similar levels (Fig 31, Table11). Factors that were equivalently expressed included interleukins (-2, -6, -17 and -33), vascular cell adhesion protein (VCAM) -1, macrophage migration inhibitory factor (MIP) -1α/β, C-reactive protein (CRP), angiogenin (ANG), and MCP-1 (Fig 31, Table 12).

The specific preference for activated versus resting platelets previously demonstrated (Church et al., 2019), in addition to the results of the eight hour coincubations that show a significant preference to activated versus resting platelet secretions (Fig 29D),

suggests treponemes may utilize chemotaxis to generally localize to activated platelets in the absence of chemorepellent factors.

Syphilis infections elevate cytokines also secreted by activated platelets

Activated platelets can secrete immunomodulators and angiogenic factors that induce dysregulation (Cognasse, 2015; Furukawa et al., 2017; McDonald & Dunbar, 2019), and many of these cytokines have been also been detected during syphilis infections (Cruz et al., 2012; Ho & Lukehart, 2011; Kenyon et al., 2018; LaFond & Lukehart, 2006; Wang et al., 2014).

During cytokine analysis certain factors present in the collagen-activated or thrombin-activated filtrates were noted to also be present at higher levels during syphilis infection or induced *in vitro* using recombinant *T. pallidum* proteins (Fig 35).

Syphilis elevates plasma/serum levels of TNF-α, IFN-Ɣ, IP-10, MIP-3β, (Kenyon et al., 2018), IL-6 (Yan et al., 2017), IL-10 (Kenyon et al., 2018; Yan et al., 2017) and IL-17 (C. Wang et al., 2014). Dermal lesions upregulate IL-2 in primary syphilis (LaFond & Lukehart, 2006) and IL-17, TNF-α, IFN-Ɣ, IP-10 and MCP-1 in secondary syphilis (Cruz et al., 2012).

In vitro viable *T. pallidum* induces DCs to secrete TNF-α, IL-6; liver macrophages to secrete TNF-α (LaFond & Lukehart, 2006); and undifferentiated THP-1 derived Mϕ macrophages to polarize and upregulate M1-assocated TNF-α (Lin et al., 2018). Viable *T. pallidum* also induces human dermal vascular smooth muscle cells (HDVSMCs) to upregulate IL-6, MCP-1, and ICAM-1 (Xie et al., 2017).

These factors can contribute to dysregulation by providing simultaneous anti- and pro-inflammatory and/or anti- and pro-angiogenic signaling and the inability to clear pathogens or limit (Gao et al., 2019; Muraille et al., 2014; Xie et al., 2017).

Whether these factors play a role in *T. pallidum* chemotaxis to cue dissemination to permissive host microenvironments (Muraille et al., 2014; Olive & Sassetti, 2016; Osherov & Ben-Ami, 2016), or if platelets contribute to higher levels of these important mediators during syphilis infection, remains an interesting question for future studies.

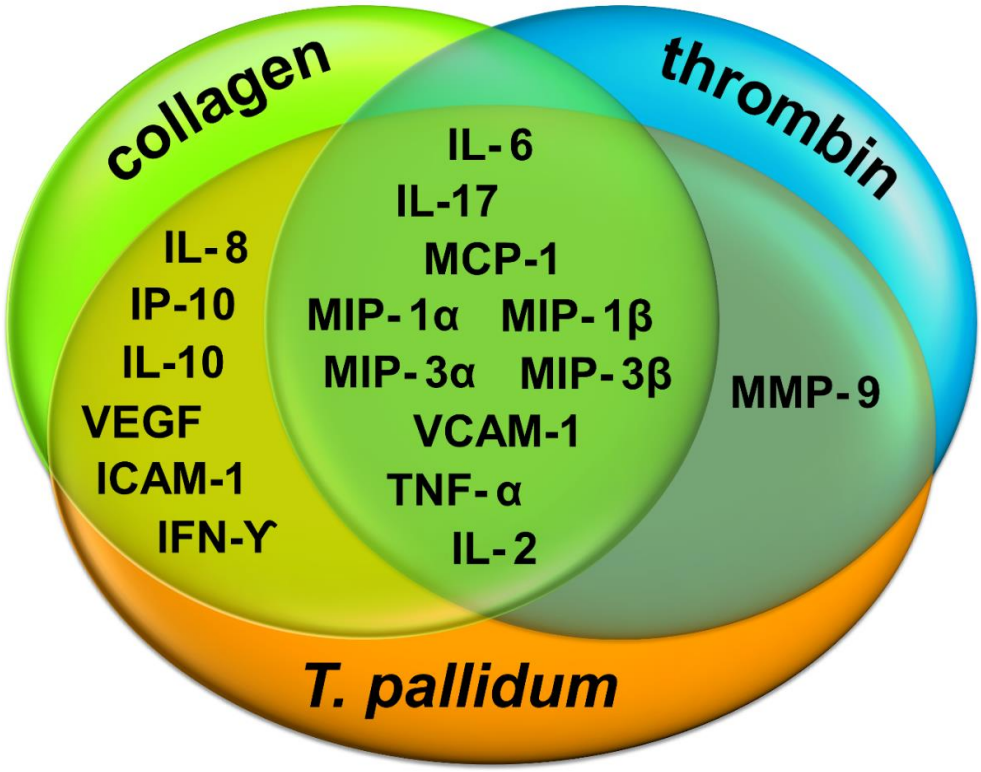

Figure 35: *Treponema pallidum* induces human cells to secrete several factors *in vitro* and during infection that are also secreted by collagen-activated and/or thrombin-activated platelets.

Treponema pallidum **may be attracted to the high proportion of M2 macrophage polarizing factors in the collagen-activated filtrate**

Further GO analysis of the collagen-activated filtrate revealed an overrepresentation of M2 polarizing factors in comparison to M1 polarizing factors (Fig 36). Deployed in early infection, M1 macrophages are highly phagocytic and bactericidal, they promote a pro-inflammatory microenvironment by secreting Th1/CD4+ associated cytokines and chemokines (Muraille et al., 2014), express CD80 (Atri et al., 2018), iNOS (Atri et al., 2018), and upregulate both the FcϒR receptor 1 (CD64) (Akinrinmade et al., 2017; Kumar, 2019) and MHCI (Kumar, 2019). Both M1 macrophages and CD4+ T cells secrete cytokines that maintain pro-inflammatory signaling and differentiation (Atri et al., 2018).

Macrophage polarization is vital to clear *T. pallidum* from within primary syphilis chancres and is mediated through a DTH response characterized by (Th1) CD4+ T cells (Carlson et al., 2011; LaFond & Lukehart, 2006), with the prominent dermal macrophage CD64 receptor (Nguyen & Soulika, 2019) key to the antibody-mediated opsonophagocytic clearance (Akinrinmade et al., 2017; Hawley et al., 2017). Immature macrophages exposed to Th1/CD4+ T cell cytokines polarize to pro-inflammatory M1 macrophages (Atri et al., 2018) which effectively clear treponemes (Carlson et al., 2011). *In vitro* viable *T. pallidum* has been shown to induce M1 polarizing TNF-α expression by human DCs (LaFond & Lukehart, 2006) and by THP-1 derived macrophages which secreted additional TNF- α following M1 polarization (Lin et al., 2018). Cutaneous lesions during secondary syphilis also exhibit CD4+ T cells, but in

these lesions the CD8+ T cell phenotype is more prevalent (Cruz et al., 2012; Stary et al., 2010).

Th2 and CD8+ T cells are common within skin infections (Nguyen & Soulika, 2019), induce a humoral response with non-complement fixing antibodies, and are ineffective at clearing treponemes from primary syphilis lesions (Carlson et al., 2011). Immature macrophages exposed to Th2 / CD8+ cytokines differentiate into M2 macrophages which reduce inflammation, induce growth and wound healing, but can also lead to pathological angiogenesis (Kobori et al., 2018). Collagen-activated platelets have been shown *in vitro* to release significant amounts of PGE_2 (not quantified in this study) that induced macrophages to polarize to the M2 phenotype and secrete 2.8-fold more anti-inflammatory and M2 promoting IL-10 (Linke et al., 2017).

Macrophage M2 differentiation contributes to a permissive niche for infection (Atri et al., 2018) and inducing an M2 host response is an immune escape strategy used by numerous pathogens (Muraille et al., 2014). Oral commensal bacteria also promote M2 polarization to promote a permissive environment by inducing gingival cells to secrete IL-4 and IL-13 (Huang et al., 2016), while intracellular pathogens induce IL-10 expression to promote a favourable M2 polarized host niche and promote immunosuppression (Muraille et al., 2014).

Given the role of a Th1/M1 polarized immune response in clearing treponemes within chancres during early infection, the ability to recognize host components associated with a Th2 or M2 polarized macrophage response may contribute to immune evasion (Fig. 30).

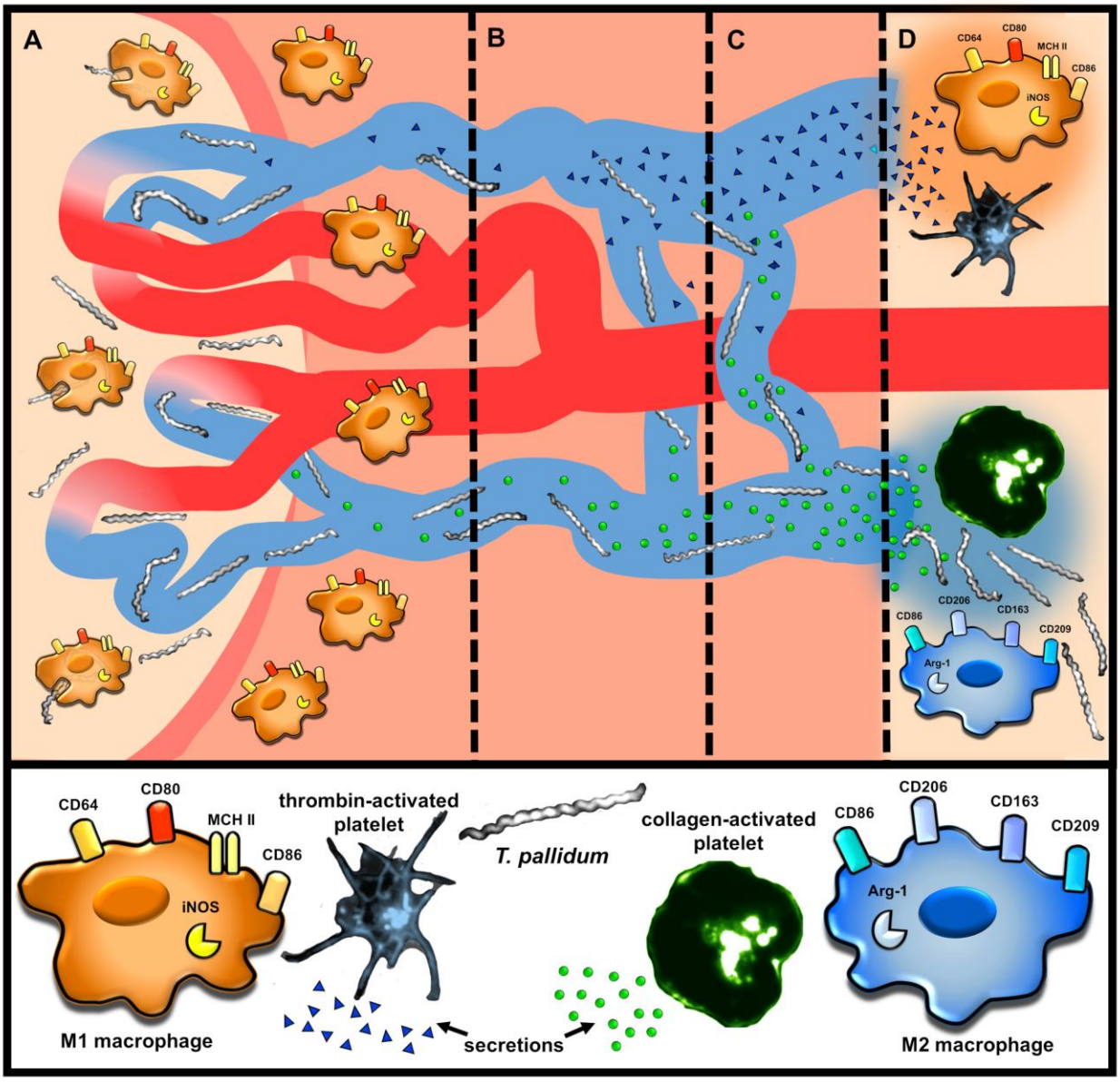

Figure 36: Model of the potential influence of platelet secretion on *T. pallidum* migration from the primary chancre in syphilis. (A) Treponemes localize at dermal blood vessel as M1 polarized macrophages clear treponemes via phagocytosis. (B) Dissemination from the chancre exposes treponemes to gradients of host molecules within blood vessels. (C) Treponemes may use chemotaxis to avoid pro-inflammatory host responses and locate favourable microenvironments. (D) Chemotaxis may promote

treponeme localization to M2 polarized microenvironments which may facilitate evasion of macrophage clearance.

Roles for chemotaxis in *T. pallidum* pathogenesis

The metabolic cost of motility and chemotaxis (Matilla & Krell, 2017) must be offset by its advantages and genome analysis suggests chemotaxis may be crucial to *T. pallidum* pathogenesis (Everall & Sánchez-Busó, 2017; Pětrošová et al., 2012; Pinto et al., 2016). Chemotaxis genes are among the few genomic regions where sequences differ from homologues in closely related but less infective treponemes (p*araluisleporidarum*) (Graves & Downes, 1981; Mikalová et al., 2010; Šmajs et al., 2012) and other *T. pallidum* subspecies (*endemicum, pertenue*) (Maděránková et al., 2019; Mikalová et al., 2010). Infectious *T. pallidum* shows positive selection and strain variability in chemotaxis genes similar to that seen in the TprK virulence factor (Giacani et al., 2012; Giacani et al., 2012; Maděránková et al., 2019). In addition, during infection *T. pallidum* expresses chemotaxis genes at high levels; detected within the transcriptome (Smajs et al., 2005), the proteome (Osbak et al., 2016), and as antigens by both immune rabbit serum (IRS) and the sera of patients with syphilis (McGill et al., 2010).

Treponema pallidum is entirely dependent upon host derived biomolecules (LaFond & Lukehart, 2006) and chemotaxis is likely critical in obtaining glucose (Brautigam et al., 2016), nutrients such as amino acids and nucleotides (Matilla & Krell, 2017), and may fulfil multiple functions during infection (Matilla & Krell, 2017; Olive & Sassetti, 2016).

Chemotaxis plays a critical role in facilitating persistence during *M. tuberculosis* and *Chlamydia trachomatis* infections where chemoreceptor sensing of host immune or

metabolic signals prompts a shift to a quiescent, non-replicative state and chronic infection (Olive & Sassetti, 2016). This may be a potential mechanism that facilitates the persistence characteristic of *T. pallidum* infection (LaFond & Lukehart, 2006).

***Treponema pallidum*-platelet interactions may facilitate dissemination, nutrient acquisition and enhance our understanding of pathogenesis**

This study expands on previous findings that *T. pallidum* preferentially interacts with activated versus resting platelets while significantly increasing their energy output during interactions (Church et al., 2019), and suggests chemoreception of platelet secreted factors may cue treponeme motility (Hamzeh-Cognasse et al., 2015).

These results validate the preference of *T. pallidum* to interact with activated versus resting platelets (Church et al., 2019) using chemotaxis assays to quantify the migration of *T. pallidum* into the cell-free plasma of activated compared with that of resting platelets. Further, this provides strong evidence that *T. pallidum* does in fact differentiate between subsets of platelet secretions, in response to collagen or thrombin activation, demonstrated through positive and negative chemotaxis, respectively.

This study also demonstrates the sensitivity of *T. pallidum* chemoreception to detect platelet activation-specific molecules amid the complex milieu of lipids, proteins, cytokines and small molecules within human plasma (Morrell et al., 2018; Mussano et al., 2016; Nguyen et al., 2015).

Here, *T. pallidum* demonstrated a distinct and consistent response to each of the three plasma filtrates, with the most positive chemotaxis towards the plasma of collagen-activated platelets, which contained higher levels of angiogenic and M2 macrophage

associated factors. Activated platelets generate metabolite and cytokine gradients that modulate the activation, recruitment and maturation of immune cells that may result in a hostile or favourable host microenvironment (David & Kubes, 2019; Galperin, 2018; Giacani et al., 2013).

This study validated the hypothesis that *T. pallidum* preferentially migrates towards specific subsets of platelet secretions and has shown that the secretions of collagen-activated platelets were the most chemoattractive under these experimental conditions. Many factors within the collagen activated filtrate promote angiogenesis and contribute to M2 macrophage polarization which may be recognized by *T. pallidum* as molecular cues during dissemination to evade immune clearance and establish a persistent infection (Fig 36) (Carlson et al., 2011; Rayes et al., 2020).

Limitations

For the cytokine array analysis, the comparison of spots with very high signal intensities may lack the sensitivity to fully quantify the difference in expression levels. The low intensity signal data was analyzed and included to evaluate the secretion patterns and signaling networks in context. These limitations can be improved in future studies by including cytokine-specific quantitation assays.

Chapter 4: Concluding Chapter

4.1 New chapters in the study of syphilis (and platelets).

> "The improved technique by which the spirochæte is demonstrated in the tissues and the serum reactions have opened a new chapter in our knowledge of the prevalence of the disease. The profession has read it with amazement, the sanitary authorities with bewilderment, but best of all the public is actually reading the chapter in the open!
>
> - *Annual Oration on The Campaign Against Syphilis* by Sir William Osler M.D., delivered before the Medical Society of London, on May 14, 1917. (Osler, 1907)

Over 100 years after Sir William Osler gave this address techniques for diagnosing syphilis have leapt forward, yet research is ongoing to develop methodology to *directly* detect *T. pallidum* within bodily fluids (Kaur & Kaur, 2015; Naidu et al., 2012). Technical challenges have significantly complicated the study of *T. pallidum*, particularly the historical inability to maintain long-term *in vitro* culture (Edmondson et al., 2018) and the limitations imposed by the fragile outer membrane (LaFond & Lukehart, 2006). New biochemical methods are providing opportunities to isolate and characterize the mechanisms of dissemination, access to immune privileged niches, and immune evasion. *In vivo* work is piecing together stage-specific cytokines during infection (Cruz et al., 2012; Kenyon et al., 2018), while *in vitro* assays continue to elucidate *T. pallidum* molecular interactions with host cells and modulation of the host response (Carlson et al., 2011; LaFond & Lukehart, 2006). To this point *T. pallidum* has been demonstrated to interact with epithelial (Djokic et al., 2019; Edmondson et al., 2018; Fitzgerald,

Johnson, et al., 1977; Izard et al., 2009), endothelial (Lithgow et al., 2020; Macaron et al., 2003; Thomas et al., 1988, 1989), and immune cells (Hawley et al., 2017; Liu, et al., 2018; Lukehart et al., 1992; Xu et al., 2017), and to adhere and/or activate secretory responses. While pathological evidence of platelet involvement during syphilis is evident during ischemic stroke and by the presence of platelets within artherosclerotic plaques (LaFond & Lukehart, 2006; Landry et al., 2019), these aspects fail to inform us on the interactions between *T. pallidum* and platelets beyond vascular inflammation. The work in this dissertation provides the first investigation and characterization of interactions between *T. pallidum* and human platelets.

In parallel to the historic challenges and recent advances in the study of *T. pallidum,* the study of platelets has overcome research challenges and experienced an exponential growth in understanding platelet biology beyond haemostasis (Gianazza et al., 2020; Gremmel et al., 2016; Li et al., 2017; Ribeiro et al., 2019). Investigations into non-haemostatic platelet functions were limited until biochemical and isolation techniques advanced to study cells that within seconds may change in size, shape, and receptor expression, while rapidly secreting hundreds of biomolecules, and shifting from individual cells to aggregates (Lesyk & Jurasz, 2019). Another key historic barrier to platelet research was the outlook that, as an anucleated cell, platelets' sole function was to participate in haemostasis (Lesyk & Jurasz, 2019).

The work in this dissertation builds on the knowledge and methodologies to investigate *T. pallidum* pathogenesis, in combination with the expanding knowledge of platelet functions, to open an important new chapter in the study of *T. pallidum*-host interactions

and contributes a novel approach to investigating bacterial pathogenesis in response to host signaling.

4.2 Investigating *T. pallidum* interactions with host cells.

Treponema pallidum is an obligate human pathogen, however the susceptibility of rabbits to the closely related pathogen, *T. paraluiscuniculi* (Buyuktimkin et al., 2019; Šmajs et al., 2012), has contributed to the recent breakthrough of long-term *in vitro* culture of *T. pallidum* using rabbit (Sf1Ep) epithelial cells (Edmondson et al., 2018). Early study of *T. pallidum* pathogenesis began with analysis of treponeme *adhesion* to cells (Fitzgerald et al., 1977; Izard et al., 2009; Repesh et al., 1982) and *localization* within tissues (Juanpere-Rodero et al., 2013), which led to investigations of *host cell responses* to intact treponemes (Carlson et al., 2011; LaFond & Lukehart, 2006; Lin et al., 2019; Lukehart & Miller, 1978; Osbak et al., 2016; Shin et al., 2004) and recombinant treponemal proteins (Djokic et al., 2019; Gao et al., 2019; Kao et al., 2017; Lithgow et al., 2020; Zhang et al., 2014).

***Treponema pallidum* and human platelets**

In Chapter 2 of this dissertation, the first documented interactions between *T. pallidum* and human platelets were identified and analyzed, by treponeme *adhesion* and by the selective *localization* of *T. pallidum.* An important distinction lies in the difference between platelet-mediated interactions – suggestive of an immune, host protective response; and bacteria-mediated interactions – suggestive of a pathogenic strategy. Microscopic analysis demonstrated *T. pallidum*-platelet *adhesion* is reversible and that treponeme *localization* was greatest to fully activated platelets, while resting platelets were disregarded.

In addition, the *platelet response* to treponeme interactions was investigated and platelets were shown to activate following a remarkably long lag period, which suggests treponemes may rely more on *locating* activated platelets compared with *inducing* platelet activation. The work in this dissertation also provides important insights into *T. pallidum* pathogenesis by investigating the *response of T. pallidum* to host cells expressing different physical (size, shape, receptor content) and biochemical (secretions) parameters, uniquely facilitated by studies involving platelets.

This alternative approach provides a foundation for the studies performed in Chapter 3, which focus upon whether the selective response of *T. pallidum* to different states of platelet activation also extends to different mechanisms of platelet activation.

In Chapter 3 this approach is expanded to investigate whether the detection and selective response of *T. pallidum* to *different states* of platelet activation would also be demonstrated to *different mechanisms* of platelet activation. Chemotaxis assays, adapted to exploit the distinctive cytokine and protein subsets platelets secrete when exposed to different stimuli, were used to reveal consistent and *specific responses* by *T. pallidum*. By quantifying treponeme migration into the cell-free plasma from resting, collagen-activated or thrombin-activated platelets it became evident that host signaling gradients play an important role in *T. pallidum* localization. While greater analysis of treponeme responses to host signaling molecules is an important next step, the cytokine array analysis in this dissertation revealed interesting differences in secretome content following the different mechanisms of platelet activation. The positive chemotaxis demonstrated by *T. pallidum* to the collagen-activated filtrate, higher in expression of M2 macrophage polarizing factors (under these experimental conditions),

provides a foundation for further research towards understanding the response of *T. pallidum* to cytokines and what role, if any, they play in immune evasion during infection. Of equal significance is the distinct chemorepellent response by *T. pallidum* to the thrombin-activated filtrate during the first four hours of coincubation, and while the analysis of the filtrates was limited to the cytokine array in this work, suggests particular host molecular gradients are recognized by *T. pallidum* and avoided to enhance survival. Greater understanding of what factors *T. pallidum* recognizes and avoids within the host has the potential to aid in the development of new, alternative treatments and augment future vaccines. In addition, the chemoattraction towards the thrombin-activated filtrate after eight-hour coincubations suggests:

(1) sensitive and ongoing chemoreception by treponemes,

(2) some factors present in the filtrate provided “activated-platelet” cues that attracted treponemes and correlates with the preference to interact with platelets already in an activated state, and,

(3) a possible repellent may be MPO, which is known to degrade over time, and is an important factor released during host immune responses with the potential to be particularly damaging to the fragile outer membrane of *T. pallidum.*

An important next step in understanding the responses of *T. pallidum* to host generated repellents will be through greater analysis of, not only the components within the thrombin-activate filtrate (under these experimental conditions), but in comparison of which factors change over time, analysis that was not undertaken in this initial investigation.

Understanding bacterial pathogenic strategies through new approaches

Throughout the work in this dissertation minimally invasive platelet isolation techniques were optimized and used to develop a new model to better reflect *in vivo* conditions (Cox et al., 2011). This methodology provides a foundation for future studies of bacterial pathogenesis, not only with *T. pallidum*, but of other pathogens that interact with platelets or use vascular dissemination. Ongoing research continues to demonstrate a pattern of multiple mechanisms by which bacteria interact with platelets, to induce or inhibit platelet activation, or mediate adhesion (Cox et al., 2011; Gaertner & Massberg, 2019; Page & Pretorius, 2020). Much remains to be elucidated on how multiple mechanisms are used to promote pathogenesis, and whether they are tissue-specific, stage-of-infection-specific, or redundant to ensure bacterial-mediated platelet interactions.

4.3 Platelets – an "asset" for the host or the pathogen?

Platelets have many protective roles within innate and adaptive immunity, from trapping and killing pathogens to recruiting other immune effectors (Li et al., 2017; Menter et al., 2017; Page & Pretorius, 2020; Rossaint et al., 2018) however, opportunistic pathogens have evolved strategies to subvert platelet responses (Kahn et al., 2013; Petersson et al., 2018). Pathogen-mediated platelet activation, aggregation and even phagocytosis of bacteria does not necessary inhibit pathogenesis, but may regulate platelet signaling and secretion outcomes (Cox et al., 2011). For example, during infective endocarditis the non-motile pathogens *S. aureus* and *P. gingivalis* enter the vasculature and induce platelet activation, traveling within a platelet cloak that facilitates survival, immune evasion, and niche localization as platelet aggregates deposit on the endothelium (Cox et al., 2011).

Platelets have a remarkable capacity to influence endothelial and immune cells (Gaertner & Massberg, 2019; Li et al., 2017; Page & Pretorius, 2020), with additional modulation enabled by the ingestion, storage, and secretion of any factor produced by other cells and released into the bloodstream (Li et al., 2017; Manne et al., 2017; Parsons et al., 2018; Pokrovskaya et al., 2020; Sharda & Flaumenhaft, 2018). Further complexity to pathogen interactions is likely as circulating platelets have subpopulations with different biochemical and functional properties (Lesyk & Jurasz, 2019), from maintaining blood/lymph separation and leukocyte recruitment to lymph nodes (Li et al., 2017; Nayar et al., 2014), to populations that develops within the lungs and functions as innate immune cells (Gomez-Casado et al., 2019). In addition to entering lymph nodes and lung tissue, platelets leave blood vessels during inflammation to enter skin (Linke et al., 2017), and tissue (Kraemer et al., 2010), and during neuroinflammation cross the BBB to enter brain parenchyma (Cloutier et al., 2018; Kniewallner et al., 2020; Ponomarev, 2018).

Given that platelets interact and regulate numerous cell types, secrete hundreds to thousands of biomolecules in patterns specific to the mechanism of activation, and are not restricted to the circulatory system, there are countless potential benefits to a pathogen that:

(A) specifically interacts with platelets,

(B) *mediates the mechanism of activation*, and/or

(C) recognizes *specific signaling gradients* from activated platelets.

The work in this dissertation establishes that *T. pallidum* mediates specific and reversible platelet interactions, preferentially interacts with activated platelets, will

induce platelet activation during sustained interactions with resting platelets, and has sensitive chemotactic responses to distinct sets of platelet secretions.

Platelets, dissemination, and immune evasion

In Chapter 2 of this dissertation, a key discovery was the characteristic of *T. pallidum* to localize and interact with the most activated platelet in its vicinity which suggests a powerful mechanism that could have several advantages for an opportunistic pathogen:

(1) A significant amount of time is saved during dissemination and extravasation by interacting with an activated platelet already adhered to the endothelium, compared with step-wise adhesion to endothelial cells (Fig 37D & E).

(2) Activated platelet secretions may cue treponemes to sites of endothelial dysfunction / disrupted endothelial junctions to aide in entering or leaving the vasculature (Fig 37C-E).

(3) Activated platelets are less likely to be secreting cytokines and chemokines during *T. pallidum* interactions compared with resting platelets activated by pathogens interactions.

(4) Activated platelets are often localized to endothelial gaps, which may allow interacting treponemes to rapidly extravasate into host niches and evade immune clearance.

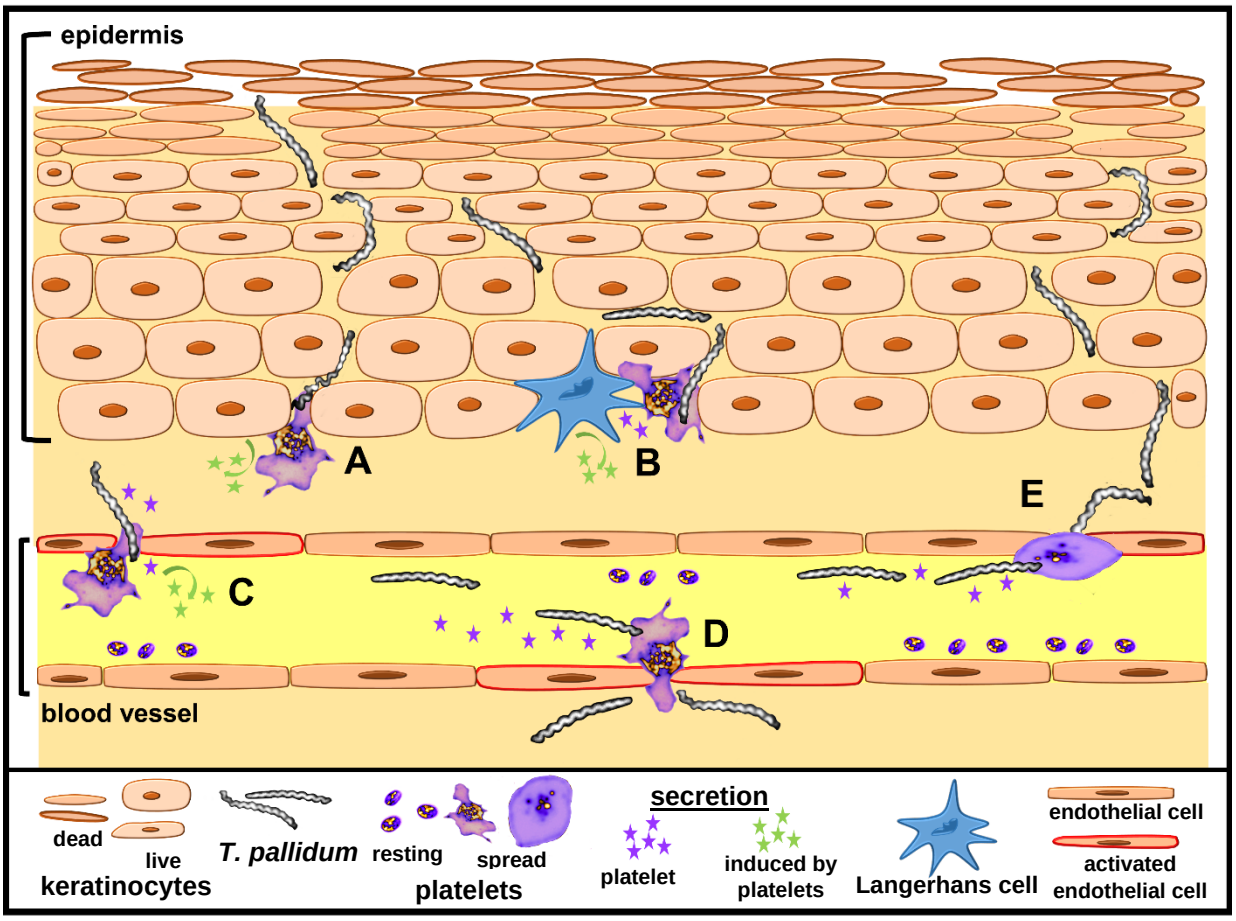

Figure 37: Activated platelets may facilitate *T. pallidum* dissemination and immune evasion. During primary syphilis *T. pallidum* disseminates locally through the skin, then systemically through blood and lymph vessels. During secondary syphilis treponemes migrate to the epidermis where they mainly reside between keratinocytes. Platelets can leave blood vessels and modulate the responses of cells *T. pallidum* encounters: (A) keratinocytes, (B) Langerhans cells in the epithelium or DCs in the dermis, and while extravasating may secrete chemoattractants that draw *T. pallidum* to disrupted endothelial junctions to quickly enter (C) or leave the blood stream (D & E).

Chemoattraction to “pre”-activated platelets, activated through various physiological pathways, may be a mechanism that greatly enhances the speed of *T. pallidum* dissemination in accord with the rapid penetration of the BBB seen during early infection (LaFond & Lukehart, 2006; Marra et al., 1991).

Treponeme motility may potentiate favourable platelet interactions

In Chapter 2 platelet-interacting treponemes were shown to significantly increase their rotational frequency and the speed of their translational movements across the surface of the platelet. This facet of platelet interactions may directly alter platelet signaling or via reorganization of platelet lipid rafts, detergent-resistant domains rich in glycolipids, that contain membrane receptors such as GPVI, which participate in activation and signaling (Gianazza et al., 2020). Platelet lipid-raft receptor and signaling proteins reorganize after activation, incorporating proteins present in α-granule membranes including stomatin (Gianazza et al., 2020), which was recently identified to interact with the *T. pallidum* protein Tp0751 (Lithgow et al., 2020). Platelets also recognize lipid rafts present on other cells such as T cells, neurons, and BBB astroglial cells (Ponomarev, 2018).

Numerous pathogens interact with host lipid rafts to facilitate adhesion and/or cell entry, and many modify lipid rafts to alter signaling that regulates immune responses to create a permissive microenvironment (Bukrinsky et al., 2020). Pathogens utilize host lipid rafts to acquire biomolecules they cannot synthesize: the spirochete *B. burgdorferi* incorporates host-derived cholesterol into its outer membrane, resulting in domains that resemble host lipid rafts (Toledo & Benach, 2016).

While not explored further in this dissertation, the surge in activity, quantified by a significant increase in rotational frequency and translational velocity, holds interesting questions for future investigations, and may:

(1) play a role in rapidly locating and entering an endothelial gap covered by an activated platelet (Fig 37),

(2) facilitate reorganization of platelet receptors within lipid rafts to alter platelet signaling and secretion to regulate the immune response of adjacent cells to aide in immune evasion (Fig 37 A & B), or

(3) promote the release or acquisition of host-derived molecules T. pallidum cannot synthesize.

Taken together, the preference for *T. pallidum* to interact with activated platelets, to migrate towards the secretions of collagen-activated platelets (under these experimental conditions), and to demonstrate a significant increase in activity during platelet interactions, these results suggest that platelets likely play an important role in promoting *T. pallidum* pathogenesis.

Platelets and neuroinvasion

One of the hallmarks of syphilis is the ability of *T. pallidum* to cross the BBB during early infection (LaFond & Lukehart, 2006) and platelets can either reduce or promote cerebral vessel inflammation and vascular permeability (Sotnikov et al., 2013; Wachowicz et al., 2016). In fact, platelets promote cerebral malaria caused by *Plasmodium falciparum* (Gramaglia et al., 2017; Nacer et al., 2012) and many types of bacterial meningitis (Zhao, 2015). Inducing pro-inflammatory cytokine secretion is one mechanism used by neuroinvasive pathogens such as *S. pneumoniae* and *N. meningitidis* to increase

permeability and penetrate the BBB, along with targeting the endothelial laminin receptor (LamR) (Al-Obaidi & Desa, 2018; Doran et al., 2016). When these pathogens invade the brain they produce severe clinical symptoms and may leave lasting neural deficits (Doran et al., 2016), which differ from the stealth penetration of the BBB often seen during syphilis (LaFond & Lukehart, 2006). Neuroinvasion by *T. pallidum* is often asymptomatic and does not always lead to meningitis (LaFond & Lukehart, 2006; Landry et al., 2019). This difference in clinical presentation suggests a pathogenic strategy to enter the CNS that does not induce wide-spread neural inflammation and a potential role for treponeme-platelet interaction in modulating localized BBB permeability.

4.4 Platelet interactions may contribute to the unique pathogenesis of *T. pallidum*

Treponema pallidum is a pathogen with exceptional access to sites within the human body unavailable to colonization by commensal bacteria while circumventing the intense immune activation that occurs when other bacterial pathogens infect these sites. Host responses dictate the difference between immune tolerance to commensal bacteria versus the cytokine storms that characterize sepsis, and therefore, the host response is key to *T. pallidum* pathogenesis. *Treponema pallidum* demonstrates characteristics of both commensal and pathogen:

(1) the lack of immune clearance leads to persistent infections, while regional or systemic lymphadenopathies resolve (Carlson et al., 2011), and

(2) for ~ 70% of patients infected with *T. pallidum,* once secondary syphilis symptoms resolve, no further clinical symptoms will occur, even in the absence of curative treatment (LaFond & Lukehart, 2006).

Within this contradiction are clues to a pathogenic strategy that influences the host response to benefit *T. pallidum* immune evasion and persistence. When symptoms develop, sometimes decades later, it may be the result of specific changes in host tissue or signaling that triggers a pathogenic bacterial response (Petersson et al., 2018). *Treponema pallidum* spreads through the vasculature without inducing severe complications, such as disseminated intravascular coagulopathy (DIC) during sepsis where widespread platelet activation leads to aggregation, organ dysfunction and platelet depletion (Guo & Rondina, 2019; Page & Pretorius, 2020). The platelet response to *T. pallidum* is unlike the responses seen to other bacterial pathogens during vascular dissemination, and platelet interactions may be particularly important given the role of platelets in regulating the activities other cell types (Li et al., 2017; Manne et al., 2017; Nicolai et al., 2019; Stocker et al., 2017). Pathogen-mediated platelet interactions, therefore, have considerable potential for many downstream effects to facilitate *T. pallidum* pathogenesis.

4.5 Future perspectives

> "There is only one syphilis, one and indivisible, with many manifestations."
>
> The Schorstein Lecture *ON SYPHILIS AND ANEURSYM* by Sir William Osler M.D., delivered at the London Hospital, October 1909. (British Medical Journal, 2(2552), 1509-1514)

The work within this dissertation has established a previously unrecognized *T. pallidum*-host interaction and provides the foundation for future investigations. The importance of platelet interactions to bacterial pathogenesis leads to key research questions that should be considered in future work to better understand *T. pallidum* pathogenesis. First, an area of focus for further study should identify how activation by *T. pallidum* modulates platelet secretion to determine if platelet-secreted factors play a role in lesion angiogenesis, endothelial transmigration, and immune modulation. Chemotaxis, as an area of focus for further study, should include the role of chemoattractants in *T. pallidum* niche localization and whether host factors act as chemorepellents that aid in immune evasion. Finally, given the ability of *T. pallidum* to cross privileged endothelial barriers, an area of focus for further study should be to examine a potential role for platelets in trafficking treponemes across the endothelium.

This work lays the foundation for ongoing investigation into how the distinctive biology of *T. pallidum* interacts with the unique biology of platelets, and how this may contribute to pathogenesis.

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Appendix

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(1) Church, B., Wall, E., Webb, J. R., & Cameron, C. E. (2019). Interaction of Treponema pallidum, the syphilis spirochete, with human platelets. PLOS ONE, 14(1), e0210902. https://doi.org/10.1371/journal.pone.0210902