Published in final edited form as:

Br J Haematol. 2020 March ; 188(5): 641–651. doi:10.1111/bjh.16315.

Platelet Biology of the Rapidly Failing Lung

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Abstract

Acute respiratory distress syndrome (ARDS) is characterized by a rapid onset respiratory failure with a mortality rate of approximately 40%. This physiologic inflammatory process is mediated by disruption of the alveolar-vascular interface leading to pulmonary edema and impaired oxygen exchange, which often warrants mechanical ventilation to increase survival in the acute setting. One of the least understood aspects of ARDS is the role of the platelets in this process. Platelets, which protect vascular integrity, play a pivotal role in the progression and resolution of ARDS. The recent substantiation of the age-old theory that megakaryocytes are found in the lungs has rejuvenated interest in and raised new questions about the importance of platelets to pulmonary function. In addition to primary hemostasis, platelets provide a myriad of inflammatory functions that are poised to aid the innate immune system. This review focuses on the evidence for regulatory roles of platelets in pulmonary inflammation, with an emphasis on two receptors, CLEC-2 and TLT-1. Studies of these receptors identify novel pathways through which platelets may regulate vascular integrity and inflammation in the lungs, thereby influencing the development of ARDS.

Introduction:

ARDS presents as a clinical entity in the form of a rapid onset respiratory failure with a mortality rate of 40%. ARDS was first defined in 1967 by Ashbaugh (Ashbaugh, *et al* 1967) and later standardized in 1994 leading to the ARDS Berlin Definition, which described criteria for diagnosing and staging the severity of ARDS. (Bernard, *et al* 1994, Force, *et al* 2012). The actual ARDS conceptual model describes this clinical entity as an acute diffuse inflammatory lung injury that leads to an increased pulmonary vascular permeability, increased lung weight and loss of aerated lung tissue.

Patients at risk of developing ARDS are usually monitored by chest x-ray imaging and arterial blood gas parameters. ARDS is distinguished as a clinical entity by specific criteria: rapid onset (within seven days of initial insult); diffuse bilateral lung infiltrates consistent

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The Authors declare that they have no conflicting interests.

with pulmonary edema and not fully explained by other pulmonary pathologies such as effusions, lobar/lung collapse or consolidation; respiratory failure not explained by heart failure or volume overload; and a decreased ratio of arterial pressure to inspired oxygen ($\text{PaO}_2/\text{FiO}_2$) with a positive end-expiratory pressure (PEEP) or a continuous positive airway pressure (CPAP) ≥ 5 cm H_2O while receiving supplemental oxygen (Force, *et al* 2012). For a comprehensive review on ARDS, we recommend any of several very good reviews on ARDS in the literature (Middleton, *et al* 2018, Ware and Matthay 2000, Yadav, *et al* 2017),

ARDS is not a singular disease, but rather, a complex respiratory sequela arising from an inappropriate inflammatory response to direct or indirect respiratory tissue damage. ARDS develops secondary to preexisting conditions such as chest trauma, near drowning, aspirations of gastric fluid, pneumonia, or sepsis (Rubenfeld *et al* 2005) or as a complication of blood transfusions ((Looney, *et al* 2006) (Looney, *et al* 2009)) or ventilator-induced volutrauma (Carrasco Loza, *et al* 2015). Sepsis, for example, can induce either a direct or an indirect insult. As many as 75% of ARDS cases are derived from sepsis (Bellani, *et al* 2016, Rubenfeld, *et al* 2005). When the bacterial infection originates outside of the lungs, it is considered an indirect insult. However, septic conditions of pulmonary origin are considered a direct insult. Pneumonia is a prime example. In the Lung Safe trial, 59% of the patients with ARDS had cases originating from pneumonia. (Bellani et al. 2016).

The heterogeneity of ARDS and its etiologies has obscured elucidation of its mechanisms. Consequently, after more than 50 years of research and hundreds of clinical trials, improvements have been made in differential diagnosis and clinical staging of ARDS, but no pharmacological agents have demonstrated convincing clinical benefit for the prevention or management of ARDS. The standard of care for ARDS is mechanical ventilation and support for complications and comorbidities and definitive biomarkers are elusive. To identify effective therapeutic targets and biomarkers, we must close the gap of knowledge between the diagnosis and the mechanism. The diagnosis is based on a critical 48-hour period, during which there is rapid fluid infiltration in the lungs and falling $\text{PaO}_2/\text{FiO}_2$. The question becomes, what causes fluid to enter the lungs? In this review we will focus on the overlooked role of platelets in developing ARDS and the evidence for dysregulated platelet activity in the development of ARDS, highlighting two recent studies with clinical implications.

Neutrophils

A prevailing paradigm associates endothelial and alveolar damage to the series of events that end with excessive neutrophil infiltration into the alveolar space. For example, alveolar insult secondary to volutrauma activates nuclear factor-kappa B (NF- κ B) signaling, which consequently leads to the production of interleukin (IL)-6, IL-8, IL-1 β and tumor necrosis factor TNF- α . (Lionetti, *et al* 2005) These contribute to the inflammatory response that attracts neutrophils into the alveoli, where they release antimicrobial factors and proteases such as elastase, myeloperoxidase, cathepsins, and metal metalloproteases (MMPS), which digest the extracellular matrix to aid in neutrophil extravasation from the vasculature into the lung interstitium and alveolar space. (Palmgren, *et al* 1992)

Because neutrophils have a perceived central role in ARDS pathogenesis, inhibiting neutrophil function should offset ARDS progression. Neutrophil elastase is considered a major contributor to the vessel damage that leads to fluid leakage (Carden, *et al* 1998), and the specific inhibitor of elastase, sivelestat has shown promise in reducing lung hemorrhage in animal models of acute lung injury and distress (Guo, *et al* 1995, Iba, *et al* 2006, Kawabata, *et al* 1991). In Japan sivelestat has been approved for treatment of patients with ARDS. Patients treated with sivelestat have reduced time on mechanical ventilation and spend less time in the ICU, however, there is no significant difference in survival between patients who receive sivelestat and those who do not (Aikawa, *et al* 2011, Tamakuma, *et al* 2004). Neutrophils contain high numbers of beta-2-adrenergic receptor receptors, which have the potential to inhibit neutrophil function (de Coupade, *et al* 2004). The β_2 -agonist, salbutamol, delivered intravenously was shown to significantly reduce extravascular fluid during lung injury in the BALTI-1 trial (Perkins, *et al* 2007). In vitro studies suggested that salbutamol would inhibit neutrophil accumulation, however in the small randomized trial, salbutamol demonstrated no beneficial effects in relation to neutrophil function in ARDS (Perkins, *et al* 2007). The BALTI-2 trial was stopped, when interim analysis showed increased mortality among salbutamol-treated patients compared to placebo (Gao Smith, *et al* 2012). The limited clinical efficacy of therapies that target neutrophil accumulation suggests that other mechanisms - secondary to, or even, independent from neutrophils - may be essential in the pathogenesis of ARDS.

At the sites of inflammation, neutrophils can use elastase and myeloperoxidase to break up and release their chromatin extracellularly in a form of cell suicide called netosis, leading to formation of neutrophil extracellular traps (NETS), which can ensnare bacteria (Gan, *et al* 2018, Mikacenic, *et al* 2018, Narasaraju, *et al* 2011, Tadie, *et al* 2013). Neutrophils can undergo two types of netosis: suicidal and vital. When neutrophils undergo suicidal netosis they lose membrane integrity (Fuchs, *et al* 2007). However, activation of platelet toll like receptors (TLR) 2 or 4 induces formation of platelet-neutrophil conjugates and initiates a vital netosis, whereby neutrophils exocytose their DNA but maintain plasma membrane integrity and can still phagocytize bacteria and viruses (Clark, *et al* 2007, Yipp, *et al* 2012). Although NETS are believed to be released to control bacterial infections, there is mounting evidence that NETS exacerbate tissue damage (Lefrançois, *et al* 2018, McDonald, *et al* 2017). Chromatin is highly thrombotic, and release of the chromatin can cause collateral damage (Fuchs, *et al* 2010, Xu, *et al* 2009). In fact, histones were targeted in a recent animal study with C1 esterase inhibitor (C1NIH). It was demonstrated that the anti-histone properties of C1NIH was protective of cellular damage and NET formation (Wygrecka, *et al* 2017). NETS cause vascular occlusion and tissue damage, offering a secondary mechanism by which neutrophils may contribute to vascular damage in ARDS.

Recent work evaluating the effect of thrombocytopenia on the progression of respiratory damage in animal models provides additional evidence for neutrophil-independent mechanisms in the disruption of the capillary - alveolar barrier. Using a mouse model of tracheal infections by *Pseudomonas aeruginosa*, Bain et al found that, while depletion of circulating neutrophils by >60% caused an increase of bacterial load in the lungs, there were no differences in the BALF IgM levels (Bain *et al*, 2019). Moreover, several studies of

respiratory damage in animals, which model the pathology of ARDS, document neutrophil-independent tissue damage and fluid infiltration (Williams and Chambers 2014).

Further support for neutrophil-independent mechanisms of ARDS pathogenesis comes from a retrospective study of neutropenic ARDS patients (Ognibene, *et al* 1986). Over a thirty-month period, 11 distinct patients were identified that met the criteria for ARDS yet were severely neutropenic at the time of diagnosis. Most of these patients tested positive for either *P. aeruginosa* (45%) or *C. albicans* (27%). The type II secretion system of *P. aeruginosa* directly damages the lung endothelium, and the cell wall β -glycan of *C. albicans* induces apoptosis in lung parenchyma (Inoue, *et al* 2009, Jyot, *et al* 2011). These observations highlight the potential mechanistic differences between direct and indirect insults to the lung.

Neutrophils secrete platelet agonists including cathepsin G (Goel and Diamond 2003) and platelet activating factor (Tence, *et al* 1980). It has been demonstrated that neutrophils polarize during transmigration in a manner that places P-selectin glycosylated ligand-1 (PSGL-1) in uropod protrusions to facilitate platelet capture before transmigration through interaction with P-selectin (Sreeramkumar (Sreeramkumar, *et al* 2014), *et al* 2014). Infiltrations of neutrophils and fluid in lungs are hallmark pathologies of ARDS but activated platelets may actually be pivotal in the control of this process.

Platelets

Platelets are anucleate cells that are most well characterized for their role in the maintenance of vascular integrity. Platelets are released from the polyploid megakaryocytes and are prepared to identify sites of vascular damage and initiate the healing process. Nascent platelets harbor a full complement of receptors to mediate clotting and adherence to the vessel wall (see table 1). Their activation by collagen, or through the tissue factor pathway, following vascular injury results in platelet adherence to the vessel wall and in organization of the platelet-fibrin clot, which protects vascular integrity (Bouchard and Tracy 2001, Butenas and Mann 2002). The details of this process are beyond the scope of this review and can be found in one of several reviews (Estevez and Du 2017, Stefanini and Bergmeier 2018, Tomaiuolo, *et al* 2017)

The traditional view that platelets are produced from megakaryocytes exclusively in the bone marrow was recently challenged. Megakaryocytes are large multinucleate cells that release dumbbell-shaped doublets into the marrow sinusoids, which become platelets in circulation (Italiano, *et al* 1999). In a seminal study published in 2017, LeFrançois *et al* used 2-photon intravital microscopy to film platelet biogenesis from megakaryocytes in murine lungs (LeFrançois, *et al* 2017). The film confirmed mounting evidence that platelet-producing megakaryocytes exist within the pulmonary vasculature. The earliest reports were attributed to Aschoff, who described megakaryocytes in the lungs in 1893 (Aschoff 1893). Subsequent reports noted that more platelets could be observed leaving the lungs than entering the lungs (Howell and Donahue 1937) and that if the blood flow was shunted from one lung in a canine model, there was decreased accumulation of megakaryocytes in that lung (Kaufman, *et al* 1965). Indeed, LeFrançois *et al* demonstrate that megakaryocytes

leaving the bone marrow are caught in the pulmonary circulation, where they mature and form platelets. It was further demonstrated that there are some phenotypic differences, including higher levels of toll like receptor (TLR) transcripts between the megakaryocytes found in the lungs and those in marrow, suggesting that there could be different subpopulations of platelets in circulation (Lefrançois, *et al* 2017). The identification of megakaryocytes in the lungs offers new promise of understanding the role of platelets in lung physiology.

Over the past couple decades, as the immune functions of platelets have been elucidated, we have come to understand that platelets are not simply the cellular arbiters of hemostasis (Ho-Tin-Noe, *et al* 2018). Platelets are disc-shaped cells that contain stored membranes as well as dense, lysosomal, and alpha granules (George 2000). Upon activation platelets undergo a shape change, bringing stored membrane to the surface, thereby expanding their surface area and releasing the contents of the dense and α -granules to the extracellular milieu (Davies, *et al* 1993). Within the α -granules are a wealth of proteins with specific inflammatory functions. These include the receptors, P-selectin (Furie and Furie 1995) and TREM-like transcript-1 (TLT-1) (Washington, *et al* 2004). The cytokines TGF- β , CCL5, CXCL4 are also found in abundance in platelet granules (Heijnen and van der Sluijs 2015, Rowley, *et al* 2011, Simon, *et al* 2014). Identification of CD40 ligand on activated platelets (Henn, *et al* 1998) and the observation that activated platelets translate latent RNAs to make IL-1 β (Lindemann, *et al* 2001) were early indications that platelets were immune regulators. In addition to these inflammatory proteins, the dense granules secrete ADP and serotonin; in fact, platelets are the predominant source of peripheral serotonin (Cloutier, *et al* 2012). ADP activates platelets in an autocrine fashion, while serotonin causes vascular dilation. Serotonin also mediates neutrophil recruitment and extravasation into the lung (Duerschmied, *et al* 2013). It was recently demonstrated that serotonin release from platelets mediates shock after immune complex formation (Cloutier, *et al* 2018).

A novel facet to our understanding of platelet function is that platelets use alternative mechanisms to mediate hemostasis after inflammatory challenge. Goerge *et al* compared vessel integrity after inflammatory challenges in thrombocytopenic mice and mice with normal platelet counts (Goerge, *et al* 2008). Thrombocytopenic mice bleed, when challenged with inflammatory insults such as immune complexes (reverse arthus) or lipopolysaccharide (lung inhalation), which in and of itself is not surprising. What is surprising is the fact that mice that had normal platelet counts but lacked the major components of the hemostatic mechanism (i.e. CalDAG-GEFI, GPVI, or β 3 integrin) were able to maintain hemostasis, demonstrating that there is another pathway - or other pathways - that lead to bleeding, which platelets are mechanistically equipped to manage. It was later shown that one of these mechanisms involved ITAM (immunoreceptor tyrosine-based activation motif) signaling through GPVI or CLEC-2 (Boulaftali, *et al* 2013).

Platelet-depletion studies in animal models have revealed specific roles for platelets in the development of respiratory distress following lung injury. One key observation is that depletion of platelets leads to increased alveolar bleeding, dissemination of bacteria, plasma cytokine counts and disease severity, indicating a primarily protective role for platelets. In separate studies in which investigators used platelet-specific antibodies to decrease platelet

counts before inducing tracheal bacterial infections of *Streptococcus pneumoniae* (van den Boogaard, et al 2015) or *Klebsiella pneumoniae* (de Stoppelaar, et al 2014), higher counts of bacteria were found in distant organs of the platelet-depleted mice, and significantly more thrombocytopenic mice succumbed to the infection. In a separate study using *mpl^{-/-}* mice, which are thrombocytopenic due to a mutation in the thrombopoietin receptor, the authors used *P.aeruginosa* and *K. pneumoniae* to induce respiratory distress. (Bain, et al 2019) In contrast to (de Stoppelaar, et al 2014), they did not see drastic differences using the *K. pneumoniae*, however they did demonstrate that platelets specifically inhibit the lethality of *P. aeruginosa* infection by influencing the bacterial type II secretion system (Bain, et al 2019). Clinical data substantiate the protective role for platelets. Thrombocytopenia is a risk factor for ARDS and prognostic of poor outcomes among patients with ARDS ((Morales-Ortiz, et al 2018, Vanderschueren, et al 2000, Wang, et al 2014).

Platelets are immunomodulatory cells that can be generated in lungs, they carry a vast repertoire of immunomodulatory proteins, and those generated in the lung may be more sensitive to bacterial and viral exposure due to increased presence of TLRs (Lefrançois, et al 2017). Therefore, neutrophils that bind activated platelets have a potent and diversified immune arsenal at their disposal.

Platelets and Neutrophils

Platelet-neutrophil conjugates occur during many inflammatory responses and are well documented, but studies in different systems have generated apparently contradictory models regarding the timing and nature their effects. Some models suggest that platelet activation is neutrophil dependent and that neutrophils guide platelets. A recent publication from Pircher *et al.* demonstrates in a murine ALI model that the neutrophil-derived cathelicidin, CRAMP, activates platelets and subsequently promotes neutrophil extravasation into the tissues. In CRAMP-null mice, both the platelet activation and neutrophil extravasation were reduced (Pircher, et al 2018). Activated platelets display P-selectin, which initiates binding to PSGL-1 on neutrophils. Gros et al used an immune complex model and demonstrated that single platelets seal endothelial breaches caused by leukocyte transmigration (Gros, et al 2015). Extrapolating these findings to neutrophil transmigration, a simple model emerges; in response to chemotactic and activation signals, platelets become activated and bind neutrophils, neutrophils carry the platelets to the sites of inflammation, and the platelets through interaction with collagen and ITAM signaling seal the vessel breach (Boulaftali, et al 2013, Gros, et al 2015).

Other models suggest that platelets are activated independent of neutrophils and guide neutrophils to trans migratory sites in inflamed vessels (Zuchtriegel, et al 2016). Monocytes and alveolar macrophages secrete CCL2 which leads to increased levels of ICAM, VCAM, and von Willebrand's factor (vWF) at the endothelial junctions. Platelet Gp1b binds vWF, mediating platelet adherence at the junctions of inflamed vessels (Andrews, et al 2003), thus providing a molecular framework on which platelet adherence would guide leukocytes to trans migrate at the endothelial junctions (Zuchtriegel, et al 2016). While CCL2 has a demonstrated role in lung inflammation (Maus, et al 2003), a caveat to the work by Zuchtriegel et al is this study was completed in the cremaster muscle of mice, What happens

in one vascular bed may not translate to another. Similar caution should be taken when considering work of Gos et al 2015 which uses immune complexes in the skin. Taken together, platelets complex with neutrophils upon their activation, guide the neutrophils to the sites of transmigration, or seal the breaches caused by neutrophil extravasation.

Simple models, however, rarely describe the complexities found in any particular vascular bed. Early studies in animal models delivered conflicting accounts on whether neutrophils brought platelets to sites of inflammation or, if in fact, platelets recruited neutrophils. Early studies on acid-induced injury suggested that if platelets were depleted, there was a significant reduction in neutrophil recruitment to the lungs (Zarbock, *et al* 2006). In contrast, Looney et al using a two hit model of transfusion related acute lung injury (TRALI), reported that platelet recruitment to the lungs was neutrophil dependent (Looney, *et al* 2009). These seemingly controversial findings may highlight the difference between direct (acid injury) and indirect (TRALI) insults to the lung as well as demonstrate the interdependent nature of these two end-stage cells.

Recent studies have added to the complexity of understanding of platelets and neutrophils in lung injury. It was demonstrated that high ventilator tidal volume increases chemokine CXCL2 levels and neutrophil sequestration in the lungs (Belperio, *et al* 2002). Blocking of the CXCL2 receptor or using CXCL2^{-/-} mice reversed the neutrophil accumulation in the lung. Hwaiz *et al* demonstrated that platelet secretion of CXCL4 induces alveolar macrophages to secrete the neutrophil chemoattractant CXCL2, suggesting that platelets may be indirectly responsible for a secondary wave of neutrophil recruitment (Hwaiz, *et al* 2015).

Platelets play important roles in protection of lung, but dysregulated activity may contribute to inflammation in lung. Clinical studies with generalized platelet inhibitors reflect this dual nature of activity. Aspirin, which prevents release of the platelet agonist thromboxane A₂ through inhibition of cyclooxygenase 1, reduced neutrophil infiltration and lung permeability following LPS challenge in animal models (Tilgner, *et al* 2016). However, in a multicenter, double-blind, placebo-controlled, randomized clinical trial (LIPS-A), aspirin demonstrated limited benefits as a preventative measure for ARDS (Kor, *et al* 2012). Meta-analysis of 8 trials involving aspirin suggests an overall beneficial effect of aspirin on the risk of developing ARDS, but the authors note great heterogeneity between studies and suggest that further clinical analysis is warranted (Panka, *et al* 2017). In a second meta-analysis, similar results supporting aspirin therapy in ARDS were also reported (Yu, *et al* 2018). Because platelets are essential for protection in early phases of inflammation, a more rational strategy for platelet-directed intervention should consider both mechanism and timing of disease. Rather than generalized prophylactic antiplatelet therapy, which may inhibit essential protective activity of platelets, specific biomarkers may indicate dysregulated platelet function and provide rational therapeutic targets for dysregulated mechanisms.

Delineating the mechanisms of platelet involvement in ARDS may lead to novel biomarkers of ARDS progression as well as new modalities of treatments. Recent studies from animal models suggest that the interaction between platelet CLEC-2 and podoplanin on the surface

of alveolar macrophages can limit neutrophil infiltration and prevent hypoxemia following lung injury, while studies with the platelet molecule TLT-1 suggest that TLT-1 may use fibrinogen to guide neutrophil transmigration. We will expound on these two platelet receptors below.

CLEC -2

CLEC -2 is the primary target for the toxin, rhodocytin, isolated from venom of the Malayan pit viper (*Calloselasma rhodostoma*). CLEC-2 has a hemi-immunoreceptor tyrosine-based activation motif (hemITAM)(YXXL) in its cytoplasmic domain that binds Syk and mediates signaling through LAT, SLP76 and PLC γ (Suzuki-Inoue, *et al* 2006). The CLEC -2 endogenous ligand, podoplanin (PDPN: also known as T1-alpha), was identified on podocytes in the kidney (Christou, *et al* 2008) and is expressed on fibroblasts, alveolar epithelial type I cells, renal tubular epithelial cells, keratinocytes, alveolar macrophages, and osteoblasts (Breiteneder-Geleff, *et al* 1997, Dobbs, *et al* 1988, Gandarillas, *et al* 1997, Wetterwald, *et al* 1996).

CLEC-2 and ARDS

Recently, the role of CLEC-2 has been evaluated in animal models of lung injury. Using null and conditional null mice, Lax *et al* demonstrated a clear role for CLEC-2 and PDPN in the prevention of inflammation and respiratory damage after intratracheal LPS instillation. Mice lacking platelet CLEC-2 had reduced arterial oxygenation, increased alveolar neutrophil infiltration, and increased protein and cytokine concentrations in the bronchial alveolar lavage (Lax, *et al* 2017b). PDPN is found on the type1 alveolar epithelial cells, which are not normally exposed to platelets. Accordingly, removal of PDPN from the alveolar cells did not affect the progression of respiratory damage. However, specific removal of PDPN from alveolar macrophages phenocopied the platelet-specific CLEC-2 deficient mice, showing reduced lung function and increases in proinflammatory cytokines in response to LPS (Lax, *et al* 2017b). Likewise, treatment with a crosslinking/activating anti-podoplanin antibody (α -PDPN) also led to reduced lung inflammation in this mouse intratracheal LPS model (Lax, *et al* 2017a).

While the mechanism behind the reduced inflammation is not yet known, the CLEC-2/PDPN interaction represents a putative therapeutic target for ARDS. Enhancement of the hemostatic properties by the antibody or activation of CLEC-2 by PDPN could lead to reduced vascular damage by neutrophil diapedesis. This would fit the model in which neutrophils pass through the vessel wall and the platelets are able to block the passage of fluids and prevent further damage by adhering to the vessel wall. A caveat that must be considered for therapeutic intervention is that CLEC-2 and PDPN are found on several cell types and targeting these proteins may have adverse effects.

TLT-1.

Another particularly interesting receptor found on platelets is TLT-1. Recent studies of TLT-1 have demonstrated its potential, both as a biomarker for ARDS and potential target

for ARDS intervention. Interestingly, TLT-1 also seems to play a role in immune-derived bleeding, however, it is not believed to work through the same signaling pathways as CLEC-2 and GPVI, since it does not have an ITAM signaling motif.

TLT-1 is a type 1 receptor found mainly on platelets and encoded by the *trem1* gene on chromosome 6 in humans and 17 in mouse (Allcock, *et al* 2003, Barrow, *et al* 2004, Washington, *et al* 2002). There is a splice variant that lacks the extracellular domain found on pre-osteoclasts (Yoon, *et al* 2012), but the IgG domain-containing variants have only been identified on platelets to date. TLT-1 is stored in the α -granules and is abundant on the platelet surface after activation. Attempts to quantify the number of TLT-1 on the platelet surface estimate an average of 50,000 copies (Morales-Ortiz, *et al* 2018). In pull-down studies using a recombinant human sTLT-1, fibrinogen was identified as its ligand (Washington 2009), which is interesting because the major platelet receptor α IIB β 3 is estimated to have 80,000 copies (Wagner, *et al* 1996) and also binds fibrinogen. The presence of these two fibrinogen receptors on the activated platelets suggests that they may functionalize fibrinogen differently for platelets. Where α IIB β 3 mediates platelet-platelet aggregation, TLT-1 seems to be important for fibrin deposition (Morales, *et al* 2010).

A soluble fragment of TLT-1 (sTLT-1) is released from activated platelets. While there is a soluble splice variant, the majority of sTLT-1 is believed to be cleaved from the platelet surface; there is drastic reduction in the amount of TLT-1 on the surface of the platelet over time after platelet activation (Morales-Ortiz, *et al* 2018). Increased levels of sTLT-1 have been associated with sepsis severity (Washington 2009) and with ARDS in two separate cohorts (Morales-Ortiz, *et al* 2018, Morales-Ortiz, *et al* 2018b). Studies using the soluble fragment show that sTLT-1 enhances platelet aggregation and increases platelet adhesion to fibrinogen-coated coverslips in a concentration-dependent manner (Morales, *et al* 2010).

TLT-1 and ARDS

Our studies suggest that TLT-1 mediates a non-classical hemostasis. While manipulation of TLT-1 induces elements of hemostasis (Gattis, *et al* 2006, Giomarelli, *et al* 2007, Washington 2009, Washington, *et al* 2004), and we describe our results in terms of hemostatic parameters, the effect of TLT-1 on classic hemostasis is mild. However, the effect seen after LPS challenges in *trem1*^{-/-} mice is dramatic (Washington 2009) and lends credence to the concept that platelets use alternative mechanisms to mediate hemostasis after inflammatory challenge.

While establishing a role for TLT-1 in the progression of sepsis and disseminated intravascular coagulation (DIC) we discovered that TLT-1 plays a role in inflammation-derived bleeding. Using the Shwartzman reaction, which uses sequential subcutaneous injections of LPS and tumor necrosis factor alpha (TNF- α), we demonstrated a significant and visible difference in hemorrhage between *wild type* and *trem1*^{-/-} mice (Washington 2009). Similarly, using the reverse arthus model, we demonstrated significant differences in bleeding within the lesion between *wild type* and *trem1*^{-/-} mice (Morales-Ortiz, *et al* 2018a).

We have further evaluated the effect of TLT-1 on inflammatory bleeding using the inhaled LPS model of acute lung injury (Goerge, *et al* 2008). In this model LPS is introduced intranasally and the bronchial alveolar lavage (BAL) fluid is evaluated for bleeding at 24 hours. Compared to *wild-type* mice, there is markedly more blood in the *trem1*^{-/-} mouse demonstrated visibly, by flow cytometry, and histologically (Morales-Ortiz, *et al* 2018). Additionally, null mice had reduced fibrin deposition in the damaged tissue compared to *wild-type* mice. Curiously, there were also differences in neutrophil transmigration between null and *wild-type* mice. At 12 hours the *wild-type* mice had higher numbers of neutrophils in the BAL than the *trem1*^{-/-} mice. At 24 hours the *wild-type* mice had rectified the neutrophil influx and demonstrated only baseline levels of neutrophils. The null mouse was overwhelmed with neutrophils, at higher levels than the *wild type* mice had at 12 hours (Morales-Ortiz, *et al* 2018). Our interpretation of these results is that in the *trem1*^{-/-} mouse fibrin deposition is impaired and neutrophil transmigration is delayed.

These animal studies have been corroborated by studies demonstrating that the soluble fragment of TLT-1 is indeed a prognostic factor for ARDS in the clinical setting (Morales-Ortiz, *et al* 2018). TLT-1 was retrospectively measured in serum samples from 799 patients who had been enrolled in the ARDS Network clinical trials comparing ventilation volumes in management of ARDS (Acute Respiratory Distress Syndrome, *et al* 2000). There was a significant correlation between higher serum concentrations of sTLT-1 and mortality. In a distribution analysis for all the patient samples, the peak serum concentration of sTLT-1 was 1200 pg/ml; using that value as a single cutoff, we found that patients with serum sTLT-1 1200 pg/mL were twice as likely to succumb to ARDS as those whose serum concentrations fell below the cutoff. Patients whose sTLT-1 concentrations exceeded the cutoff had significantly shorter survival time and spent more time on the ventilator compared to those with lower sTLT-1 concentrations. These data demonstrated that sTLT-1 was a prognostic factor and also suggested that TLT-1 played a role in the progression of ARDS (Morales-Ortiz, *et al* 2018).

Using the null mouse model, the addition of the soluble fragment reduced inflammation-associated bleeding into the lungs and restored the fibrinogen accretion to levels seen in wild type mouse (Morales-Ortiz, *et al* 2018). Based on the delay that is seen in both the cremaster muscle and in the LPS inhalation model, fibrinogen may be important for neutrophil transmigration. Recent findings with the $\gamma 390-396A$ (fib γ^{390-6A}) mouse demonstrate that fibrinogen deposition is important for leukocyte transmigration (Flick, *et al* 2004, Kopec, *et al* 2017). Mutation of the MAC-1 binding site on fibrinogen ($\gamma 390-396A$) reduces leukocyte transmigration demonstrating an important role for fibrinogen in neutrophil extravasation into the tissue.

Thus, fibrinogen deposition on inflamed tissue by TLT-1 may facilitate neutrophil transmigration and quench the inflammatory response in the early stages of ARDS progression. In the later stages of ARDS, high levels of sTLT-1 may promote aberrant fibrinogen deposition leading to DIC, which could explain the poorer prognosis for ARDS patients with elevated TLT-1. This concept is consistent with the seemingly paradoxical point that addition of sTLT-1 is beneficial. The addition of sTLT-1 rescued the null phenotype conceivably by focusing fibrinogen deposition. In our early sepsis studies, we

demonstrated that elevated TLT-1 levels are associated with DIC. Peak levels of 1200 pg/mL are consistent with the presence of DIC and may be an indication to block TLT-1/sTLT-1 function to control DIC and aberrant fibrinogen deposition in ARDS patients.

Unanswered Questions

The role of platelet-neutrophil conjugates remains a perplexity. There is a wealth of data implicating this tandem pair in many aspects of inflammation, including ARDS. However, there is not a singular role or order of interaction for these two end stage cells. There remain many questions: how much do platelets influence neutrophil transmigration? How differently do neutrophils function in the presence or attached to platelets than alone? Do the conjugates play different roles in direct versus indirect lung injury? The order of events, and outcomes may be dependent on the model being used and on the nature of the damage (whether direct or indirect?). Nonetheless, collectively, it appears that platelets have a protective role essential for orderly transmigration of neutrophils at sites of damage. Unlocking the secrets of platelet- neutrophil conjugates may give us key insights to understanding the progression of ARDS and many other aspects of inflammation.

A second set of questions arises from our understanding, or lack thereof, of NETs. While reducing NETs in vivo has been associated with decreased thrombosis, which in part leads to tissue damage (McDonald, *et al* 2017), it is also associated with increased bacterial load (Lefrançois, *et al* 2018, McDonald, *et al* 2012). Are NETs inherently beneficial, but the associated histones detrimental? How do platelets affect netosis? Histones cause thrombosis and tissue damage. (Xu, *et al* 2009). The C1INH studies demonstrate inhibition of the histone interactions lowers the thrombosis and tissue damage, thus targeting histones may provide viable treatment for ARDS (Wygrecka, *et al* 2017). Platelets have been reported to alter vital vs suicidal netosis (Pieterse, *et al* 2016). The question becomes is vital more beneficial than suicidal NETS?

Finally, the finding that megakaryocytes make platelets in the lungs (Lefrançois, *et al* 2017) brings great excitement as well as many questions. For example, in humans, are there megakaryocytes in the lungs and if so, what portion of platelets are produced in the lungs? Can we exploit this finding for treatment? While megakaryocytes in the human lung remain underdefined, the possibility to develop these platelets into a means to target the innate immune system or regulate cytokine production have become a prospect. If the platelets from megakaryocytes in human lungs are different from those in the bone marrow, they may offer new biomarkers specific to the development of ARDS.

Conclusions

ARDS continues to plague the medical community with high mortality, no effective pharmacological therapies, and an absence of commonly accepted biomarkers to give indication of the onset of ARDS or its prognosis. Even though the recent Berlin definition of ARDS standardizes diagnosis allowing clinicians to gauge the severity of cases and investigators to deliver clearer insights into progression and resolution of ARDS, it remains an enigma. While it is clear that neutrophils are one of the primary causes of fluid

infiltration of the lungs, there are multiple pathways leading to ARDS and platelets are also key players in the pathophysiology of ARDS. The animal models presented here suggest that platelets have immunological activity that is independent of neutrophil control. These findings are consistent with the fact that thrombocytopenia is associated with poor prognosis in ARDS (Morales-Ortiz, *et al* 2018, Vanderschueren, *et al* 2000, Wang, *et al* 2014). Individuals that are thrombocytopenic would lose the ability to deposit fibrinogen, to guide neutrophil transmigration, and to seal the damaged vasculature after diapedesis. Platelets may provide therapeutic targets of treatment and novel biomarkers to help predict the onset of ARDS.

Literature Cited

- Acute Respiratory Distress Syndrome, N., Brower RG, Matthay MA, Morris A, Schoenfeld D, Thompson BT & Wheeler A (2000) Ventilation with lower tidal volumes as compared with traditional tidal volumes for acute lung injury and the acute respiratory distress syndrome. *N Engl J Med*, 342, 1301–1308. [PubMed: 10793162]
- Aikawa N, Ishizaka A, Hirasawa H, Shimazaki S, Yamamoto Y, Sugimoto H, Shinozaki M, Taenaka N, Endo S, Ikeda T & Kawasaki Y (2011) Reevaluation of the efficacy and safety of the neutrophil elastase inhibitor, Sivelestat, for the treatment of acute lung injury associated with systemic inflammatory response syndrome; a phase IV study. *Pulm Pharmacol Ther*, 24, 549–554. [PubMed: 21540122]
- Allcock RJ, Barrow AD, Forbes S, Beck S & Trowsdale J (2003) The human TREM gene cluster at 6p21.1 encodes both activating and inhibitory single IgV domain receptors and includes NKp44. *Eur. J. Immunol*, 33, 567–577. [PubMed: 12645956]
- Andrews RK, Gardiner EE, Shen Y, Whisstock JC & Berndt MC (2003) Glycoprotein Ib-IX-V. *Int. J Biochem. Cell Biol*, 35, 1170–1174. [PubMed: 12757754]
- Aschoff L (1893) Über capillare Emboie von riesen kernhaltigen Zellen. *Virchows Arch. Path Anat*, 134.
- Ashbaugh DG, Bigelow DB, Petty TL & Levine BE (1967) Acute respiratory distress in adults. *Lancet*, 2, 319–323. [PubMed: 4143721]
- Bain W, Olonisakin T, Yu M, Qu Y, Hulver M, Xiong Z, Li H, Pilewski J, Mallampalli RK, Nouraie M, Ray A, Ray P, Cheng Z, Shanks RMQ, St Croix C, Silverstein RL & Lee JS (2019) Platelets inhibit apoptotic lung epithelial cell death and protect mice against infection-induced lung injury. *Blood Adv*, 3, 432–445. [PubMed: 30733303]
- Barrow AD, Astoul E, Floto A, Brooke G, Relou IA, Jennings NS, Smith KG, Ouwehand W, Farndale RW, Alexander DR & Trowsdale J (2004) Cutting edge: TREM-like transcript-1, a platelet immunoreceptor tyrosine-based inhibition motif encoding costimulatory immunoreceptor that enhances, rather than inhibits, calcium signaling via SHP-2. *J. Immunol*, 172, 5838–5842. [PubMed: 15128762]
- Bellani G, Laffey JG, Pham T, Fan E, Brochard L, Esteban A, Gattinoni L, van Haren F, Larsson A, McAuley DF, Ranieri M, Rubenfeld G, Thompson BT, Wrigge H, Slutsky AS, Pesenti A, Investigators LS & Group ET (2016) Epidemiology, Patterns of Care, and Mortality for Patients With Acute Respiratory Distress Syndrome in Intensive Care Units in 50 Countries. *JAMA*, 315, 788–800. [PubMed: 26903337]
- Belperio JA, Keane MP, Burdick MD, Londhe V, Xue YY, Li K, Phillips RJ & Strieter RM (2002) Critical role for CXCR2 and CXCR2 ligands during the pathogenesis of ventilator-induced lung injury. *J Clin Invest*, 110, 1703–1716. [PubMed: 12464676]
- Bernard GR, Artigas A, Brigham KL, Carlet J, Falke K, Hudson L, Lamy M, LeGall JR, Morris A & Spragg R (1994) Report of the American-European consensus conference on ARDS: definitions, mechanisms, relevant outcomes and clinical trial coordination. The Consensus Committee. *Intensive Care Med*, 20, 225–232. [PubMed: 8014293]
- Bouchard BA & Tracy PB (2001) Platelets, leukocytes, and coagulation. *Current Opinion in Hematology*, 8, 263–269. [PubMed: 11604560]

- Boulaftali Y, Hess PR, Getz TM, Cholka A, Stolla M, Mackman N, Owens AP III, Ware J, Kahn ML & Bergmeier W (2013) Platelet ITAM signaling is critical for vascular integrity in inflammation. *J Clin. Invest*, 123, 908–916. [PubMed: 23348738]
- Breiteneder-Geleff S, Matsui K, Soleiman A, Meraner P, Poczewski H, Kalt R, Schaffner G & Kerjaschki D (1997) Podoplanin, novel 43-kd membrane protein of glomerular epithelial cells, is down-regulated in puromycin nephrosis. *Am J Pathol*, 151, 1141–1152. [PubMed: 9327748]
- Butenas S & Mann KG (2002) Blood coagulation. *Biochemistry (Mosc.)*, 67, 3–12. [PubMed: 11841335]
- Carden D, Xiao F, Moak C, Willis BH, Robinson-Jackson S & Alexander S (1998) Neutrophil elastase promotes lung microvascular injury and proteolysis of endothelial cadherins. *Am J Physiol*, 275, H385–392. [PubMed: 9683424]
- Carrasco Loza R, Villamizar Rodriguez G & Medel Fernandez N (2015) Ventilator-Induced Lung Injury (VILI) in Acute Respiratory Distress Syndrome (ARDS): Volutrauma and Molecular Effects. *Open Respir Med J*, 9, 112–119. [PubMed: 26312103]
- Christou CM, Pearce AC, Watson AA, Mistry AR, Pollitt AY, Fenton-May AE, Johnson LA, Jackson DG, Watson SP & O'Callaghan CA (2008) Renal cells activate the platelet receptor CLEC-2 through podoplanin. *Biochem. J*, 411, 133–140. [PubMed: 18215137]
- Clark SR, Ma AC, Tavener SA, McDonald B, Goodarzi Z, Kelly MM, Patel KD, Chakrabarti S, McAvoy E, Sinclair GD, Keys EM, Ien-Vercoe E, Deviney R, Doig CJ, Green FH & Kubes P (2007) Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. *Nat Med*, 13, 463–469. [PubMed: 17384648]
- Cloutier N, Allaey I, Marcoux G, Machlus KR, Mailhot B, Zufferey A, Levesque T, Becker Y, Tessandier N, Melki I, Zhi H, Poirier G, Rondina MT, Italiano JE, Flamand L, McKenzie SE, Cote F, Nieswandt B, Khan WI, Flick MJ, Newman PJ, Lacroix S, Fortin PR & Boilard E (2018) Platelets release pathogenic serotonin and return to circulation after immune complex-mediated sequestration. *Proc Natl Acad Sci U S A*, 115, E1550–E1559. [PubMed: 29386381]
- Cloutier N, Pare A, Farndale RW, Schumacher HR, Nigrovic PA, Lacroix S & Boilard E (2012) Platelets can enhance vascular permeability. *Blood*, 120, 1334–1343. [PubMed: 22544703]
- Connolly AJ, Ishihara H, Kahn ML, Farese RV & Coughlin SR (1996) Role of the thrombin receptor in development and evidence for a second receptor. *Nature*, 381, 516–519. [PubMed: 8632823]
- Davies MJ, Richardson PD, Woolf N, Katz DR & Mann J (1993) Risk of thrombosis in human atherosclerotic plaques: role of extracellular lipid, macrophage, and smooth muscle cell content. *Br. Heart J*, 69, 377–381. [PubMed: 8518056]
- de Coupade C, Gear RW, Dazin PF, Sroussi HY, Green PG & Levine JD (2004) Beta 2-adrenergic receptor regulation of human neutrophil function is sexually dimorphic. *Br J Pharmacol*, 143, 1033–1041. [PubMed: 15477226]
- de Stoppelaar SF, van 't Veer C, Claushuis TA, Albersen BJ, Roelofs JJ & van der Poll T (2014) Thrombocytopenia impairs host defense in gram-negative pneumonia-derived sepsis in mice. *Blood*, 124, 3781–3790. [PubMed: 25301709]
- Dobbs LG, Williams MC & Gonzalez R (1988) Monoclonal antibodies specific to apical surfaces of rat alveolar type I cells bind to surfaces of cultured, but not freshly isolated, type II cells. *Biochim Biophys Acta*, 970, 146–156. [PubMed: 3382696]
- Duerschmied D, Suidan GL, Demers M, Herr N, Carbo C, Brill A, Cifuni SM, Mauler M, Cicko S, Bader M, Idzko M, Bode C & Wagner DD (2013) Platelet serotonin promotes the recruitment of neutrophils to sites of acute inflammation in mice. *Blood*, 121, 1008–1015. [PubMed: 23243271]
- Estevez B & Du X (2017) New Concepts and Mechanisms of Platelet Activation Signaling. *Physiology (Bethesda)*, 32, 162–177. [PubMed: 28228483]
- Flick MJ, Du X, Witte DP, Jirouskova M, Soloviev DA, Busuttill SJ, Plow EF & Degen JL (2004) Leukocyte engagement of fibrin(ogen) via the integrin receptor alphaMbeta2/Mac-1 is critical for host inflammatory response in vivo. *J Clin Invest*, 113, 1596–1606. [PubMed: 15173886]
- Force ADT, Ranieri VM, Rubenfeld GD, Thompson BT, Ferguson ND, Caldwell E, Fan E, Camporota L & Slutsky AS (2012) Acute respiratory distress syndrome: the Berlin Definition. *JAMA*, 307, 2526–2533. [PubMed: 22797452]

- Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, Weinrauch Y, Brinkmann V & Zychlinsky A (2007) Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol*, 176, 231–241. [PubMed: 17210947]
- Fuchs TA, Brill A, Duerschmied D, Schatzberg D, Monestier M, Myers DD, Wroblewski SK, Wakefield TW, Hartwig JH & Wagner DD (2010) Extracellular DNA traps promote thrombosis. *Proc Natl Acad Sci U S A*, 107, 15880–15885. [PubMed: 20798043]
- Furie B & Furie BC (1995) The molecular basis of platelet and endothelial cell interaction with neutrophils and monocytes: role of P-selectin and the P-selectin ligand, PSGL-1. *Thromb Haemost*, 74, 224–227. [PubMed: 8578462]
- Gan T, Yang Y, Hu F, Chen X, Zhou J, Li Y, Xu Y, Wang H, Chen Y & Zhang M (2018) TLR3 Regulated Poly I:C-Induced Neutrophil Extracellular Traps and Acute Lung Injury Partly Through p38 MAP Kinase. *Front Microbiol*, 9, 3174. [PubMed: 30622526]
- Gandarillas A, Scholl FG, Benito N, Gamallo C & Quintanilla M (1997) Induction of PA2.26, a cell-surface antigen expressed by active fibroblasts, in mouse epidermal keratinocytes during carcinogenesis. *Mol Carcinog*, 20, 10–18. [PubMed: 9328432]
- Gao Smith F, Perkins GD, Gates S, Young D, McAuley DF, Tunnicliffe W, Khan Z, Lamb SE & investigators B. s. (2012) Effect of intravenous beta-2 agonist treatment on clinical outcomes in acute respiratory distress syndrome (BALTI-2): a multicentre, randomised controlled trial. *Lancet*, 379, 229–235. [PubMed: 22166903]
- Gattis JL, Washington AV, Chisholm MM, Quigley L, Szyk A, McVicar DW & Lubkowski J (2006) The structure of the extracellular domain of triggering receptor expressed on myeloid cells like transcript-1, and evidence for a naturally occurring soluble fragment. *J. Biol. Chem*
- George JN (2000) Platelets. *Lancet*, 355, 1531–1539. [PubMed: 10801186]
- Giomarelli B, Washington VA, Chisholm MM, Quigley L, McMahon JB, Mori T & McVicar DW (2007) Inhibition of thrombin-induced platelet aggregation using human single-chain Fv antibodies specific for TREM-like transcript-1. *Thromb Haemost*, 97, 955–963. [PubMed: 17549298]
- Goel MS & Diamond SL (2003) Neutrophil cathepsin G promotes prothrombinase and fibrin formation under flow conditions by activating fibrinogen-adherent platelets. *J Biol Chem*, 278, 9458–9463. [PubMed: 12524437]
- Goerge T, Ho-Tin-Noe B, Carbo C, Benarafa C, Remold-O'Donnell E, Zhao BQ, Cifuni SM & Wagner DD (2008) Inflammation induces hemorrhage in thrombocytopenia. *Blood*, 111, 4958–4964. [PubMed: 18256319]
- Gros A, Syvannarath V, Lamrani L, Ollivier V, Loyau S, Goerge T, Nieswandt B, Jandrot-Perrus M & Ho-Tin-Noe B (2015) Single platelets seal neutrophil-induced vascular breaches via GPVI during immune-complex-mediated inflammation in mice. *Blood*, 126, 1017–1026. [PubMed: 26036804]
- Guo L, Yamaguchi Y, Ikei S, Sugita H & Ogawa M (1995) Neutrophil elastase inhibitor (ONO-5046) prevents lung hemorrhage induced by lipopolysaccharide in rat model of cerulein pancreatitis. *Dig Dis Sci*, 40, 2177–2183. [PubMed: 7587785]
- Heijnen H & van der Sluijs P (2015) Platelet secretory behaviour: as diverse as the granules ... or not? *J Thromb Haemost*, 13, 2141–2151. [PubMed: 26391322]
- Henn V, Slupsky JR, Grafe M, Anagnostopoulos I, Forster R, Muller-Berghaus G & Kroczeck RA (1998) CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature*, 391, 591–594. [PubMed: 9468137]
- Ho-Tin-Noe B, Boulaftali Y & Camerer E (2018) Platelets and vascular integrity: how platelets prevent bleeding in inflammation. *Blood*, 131, 277–288. [PubMed: 29191915]
- Howell WH & Donahue DD (1937) The Production of Blood Platelets in the Lungs. *J Exp Med*, 65, 177–203. [PubMed: 19870594]
- Hwaiz R, Rahman M, Zhang E & Thorlacius H (2015) Platelet secretion of CXCL4 is Rac1-dependent and regulates neutrophil infiltration and tissue damage in septic lung damage. *Br J Pharmacol*, 172, 5347–5359. [PubMed: 26478565]
- Iba T, Kidokoro A, Fukunaga M, Takuhiro K, Yoshikawa S & Sugimoto K (2006) Pretreatment of sivelestat sodium hydrate improves the lung microcirculation and alveolar damage in

- lipopolysaccharide-induced acute lung inflammation in hamsters. *Shock*, 26, 95–98. [PubMed: 16783204]
- Inoue K, Takano H, Oda T, Yanagisawa R, Tamura H, Adachi Y, Ishibashi K & Ohno N (2009) Soluble cell wall beta-glucan of *Candida* induces/enhances apoptosis and oxidative stress in murine lung. *Immunopharmacol Immunotoxicol*, 31, 140–145. [PubMed: 19951074]
- Italiano JE Jr., Lecine P, Shivdasani RA & Hartwig JH (1999) Blood platelets are assembled principally at the ends of proplatelet processes produced by differentiated megakaryocytes. *J. Cell Biol*, 147, 1299–1312. [PubMed: 10601342]
- Jyot J, Balloy V, Jouvion G, Verma A, Touqui L, Huerre M, Chignard M & Ramphal R (2011) Type II secretion system of *Pseudomonas aeruginosa*: in vivo evidence of a significant role in death due to lung infection. *J Infect Dis*, 203, 1369–1377. [PubMed: 21502078]
- Kaufman RM, Airo R, Pollack S, Crosby WH & Doberneck R (1965) Origin of Pulmonary Megakaryocytes. *Blood*, 25, 767–775. [PubMed: 14282046]
- Kawabata K, Suzuki M, Sugitani M, Imaki K, Toda M & Miyamoto T (1991) ONO-5046, a novel inhibitor of human neutrophil elastase. *Biochem Biophys Res Commun*, 177, 814–820. [PubMed: 2049103]
- Kopec AK, Abrahams SR, Thornton S, Palumbo JS, Mullins ES, Divanovic S, Weiler H, Owens AP 3rd, Mackman N, Goss A, van Ryn J, Luyendyk JP & Flick MJ (2017) Thrombin promotes diet-induced obesity through fibrin-driven inflammation. *J Clin Invest*, 127, 3152–3166. [PubMed: 28737512]
- Kor DJ, Talmor DS, Banner-Goodspeed VM, Carter RE, Hinds R, Park PK, Gajic O, Gong MN, Lips-A USCI & Injury Trials Group: Lung Injury Prevention with Aspirin Study, G. (2012) Lung Injury Prevention with Aspirin (LIPS-A): a protocol for a multicentre randomised clinical trial in medical patients at high risk of acute lung injury. *BMJ Open*, 2.
- Lax S, Rayes J, Thickett DR & Watson SP (2017a) Effect of anti-podoplanin antibody administration during lipopolysaccharide-induced lung injury in mice. *BMJ Open Respir Res*, 4, e000257.
- Lax S, Rayes J, Wichaiyo S, Haining EJ, Lowe K, Grygielska B, Laloo R, Flodby P, Borok Z, Crandall ED, Thickett DR & Watson SP (2017b) Platelet CLEC-2 protects against lung injury via effects of its ligand podoplanin on inflammatory alveolar macrophages in the mouse. *Am J Physiol Lung Cell Mol Physiol*, 313, L1016–L1029. [PubMed: 28839100]
- Lefrançois E, Mallavia B, Zhuo H, Calfee CS & Looney MR (2018) Maladaptive role of neutrophil extracellular traps in pathogen-induced lung injury. *JCI Insight*, 3.
- Lefrançois E, Ortiz-Munoz G, Caudrillier A, Mallavia B, Liu F, Sayah DM, Thornton EE, Headley MB, David T, Coughlin SR, Krummel MF, Leavitt AD, Passegue E & Looney MR (2017) The lung is a site of platelet biogenesis and a reservoir for haematopoietic progenitors. *Nature*, 544, 105–109. [PubMed: 28329764]
- Leon C, Hechler B, Vial C, Leray C, Cazenave JP & Gachet C (1997) The P2Y₁ receptor is an ADP receptor antagonized by ATP and expressed in platelets and megakaryoblastic cells. *FEBS Lett*, 403, 26–30. [PubMed: 9038354]
- Lindemann S, Tolley ND, Dixon DA, McIntyre TM, Prescott SM, Zimmerman GA & Weyrich AS (2001) Activated platelets mediate inflammatory signaling by regulated interleukin 1 β synthesis. *J Cell Biol*, 154, 485–490. [PubMed: 11489912]
- Lionetti V, Recchia FA & Ranieri VM (2005) Overview of ventilator-induced lung injury mechanisms. *Curr Opin Crit Care*, 11, 82–86. [PubMed: 15659950]
- Looney MR, Nguyen JX, Hu Y, Van Ziffle JA, Lowell CA & Matthay MA (2009) Platelet depletion and aspirin treatment protect mice in a two-event model of transfusion-related acute lung injury. *J Clin Invest*, 119, 3450–3461. [PubMed: 19809160]
- Looney MR, Su X, Van Ziffle JA, Lowell CA & Matthay MA (2006) Neutrophils and their Fc gamma receptors are essential in a mouse model of transfusion-related acute lung injury. *J Clin Invest*, 116, 1615–1623. [PubMed: 16710475]
- Maus UA, Waelsch K, Kuziel WA, Delbeck T, Mack M, Blackwell TS, Christman JW, Schlondorff D, Seeger W & Lohmeyer J (2003) Monocytes are potent facilitators of alveolar neutrophil emigration during lung inflammation: role of the CCL2-CCR2 axis. *J Immunol*, 170, 3273–3278. [PubMed: 12626586]

- McDonald B, Davis RP, Kim SJ, Tse M, Esmon CT, Kolaczowska E & Jenne CN (2017) Platelets and neutrophil extracellular traps collaborate to promote intravascular coagulation during sepsis in mice. *Blood*, 129, 1357–1367. [PubMed: 28073784]
- McDonald B, Urrutia R, Yipp BG, Jenne CN & Kubes P (2012) Intravascular neutrophil extracellular traps capture bacteria from the bloodstream during sepsis. *Cell Host. Microbe*, 12, 324–333. [PubMed: 22980329]
- Middleton EA, Rondina MT, Schwertz H & Zimmerman GA (2018) Amicus or Adversary Revisited: Platelets in Acute Lung Injury and Acute Respiratory Distress Syndrome. *Am J Respir Cell Mol Biol*, 59, 18–35. [PubMed: 29553813]
- Mikacenic C, Moore R, Dmyterko V, West TE, Altemeier WA, Liles WC & Lood C (2018) Neutrophil extracellular traps (NETs) are increased in the alveolar spaces of patients with ventilator-associated pneumonia. *Crit Care*, 22, 358. [PubMed: 30587204]
- Moore KL, Eaton SF, Lyons DE, Lichenstein HS, Cummings RD & McEver RP (1994) The P-selectin glycoprotein ligand from human neutrophils displays sialylated, fucosylated, O-linked poly-N-acetylactosamine. *J Biol Chem*, 269, 23318–23327. [PubMed: 7521878]
- Morales-Ortiz J, Deal V, Reyes F, Maldonado-Martinez G, Ledesma N, Staback F, Croft C, Pacheco A, Ortiz-Zuazaga H, Yost CC, Rowley JW, Madera B, St John A, Chen J, Lopez J, Rondina MT, Hunter R, Gibson A & Washington AV (2018) TLT-1 is a Prognostic Indicator in ALI/ARDS and Prevents Tissue Damage in the Lungs in a Mouse model. *Blood*.
- Morales-Ortiz J, Reyes F, Santiago O, Rivera L, Ledesma N, Manne BK, Madera B, Rondina M & Washington AV (2018a) TREM Like Transcript-1 controls early thrombus formation and stability by facilitating $\alpha\text{IIb}\beta_3$ outside-in signaling. *International Journal of Applied Research*, 6, 1143–1149.
- Morales-Ortiz J, Rondina MT, Brown SM, Grissom C & Washington AV (2018b) High Levels of Soluble TREM like transcript (TLT) – 1 Are Associated with Acute Respiratory Distress Syndrome (ARDS). *Clin Appl Thromb Hemost In Press*.
- Morales J, Villa K, Gattis J, Castro W, Colon K, Lubkowski J, Sanabria P, Hunter R & Washington AV (2010) Soluble TLT-1 modulates platelet-endothelial cell interactions and actin polymerization. *Blood Coagul. Fibrinolysis*, 21, 229–236. [PubMed: 20093931]
- Moroi M, Jung SM, Okuma M & Shinmyozu K (1989) A patient with platelets deficient in glycoprotein VI that lack both collagen-induced aggregation and adhesion. *J Clin Invest*, 84, 1440–1445. [PubMed: 2808700]
- Nachman RL & Leung LL (1982) Complex formation of platelet membrane glycoproteins IIb and IIIa with fibrinogen. *J Clin Invest*, 69, 263–269. [PubMed: 6460044]
- Narasaraju T, Yang E, Samy RP, Ng HH, Poh WP, Liew AA, Phoon MC, van Rooijen N & Chow VT (2011) Excessive neutrophils and neutrophil extracellular traps contribute to acute lung injury of influenza pneumonitis. *Am J Pathol*, 179, 199–210. [PubMed: 21703402]
- Ognibene FP, Martin SE, Parker MM, Schlesinger T, Roach P, Burch C, Shelhamer JH & Parrillo JE (1986) Adult respiratory distress syndrome in patients with severe neutropenia. *N Engl J Med*, 315, 547–551. [PubMed: 3736638]
- Palmgren MS, deShazo RD, Carter RM, Zimny ML & Shah SV (1992) Mechanisms of neutrophil damage to human alveolar extracellular matrix: the role of serine and metalloproteases. *J Allergy Clin Immunol*, 89, 905–915. [PubMed: 1560171]
- Panka BA, de Grooth HJ, Spoelstra-de Man AM, Looney MR & Tuinman PR (2017) Prevention or Treatment of Ards With Aspirin: A Review of Preclinical Models and Meta-Analysis of Clinical Studies. *Shock*, 47, 13–21. [PubMed: 27984533]
- Paul BZ, Jin J & Kunapuli SP (1999) Molecular mechanism of thromboxane A₂-induced platelet aggregation. Essential role for p2t(ac) and alpha(2a) receptors. *J Biol Chem*, 274, 29108–29114. [PubMed: 10506165]
- Perkins GD, Nathani N, McAuley DF, Gao F & Thickett DR (2007) In vitro and in vivo effects of salbutamol on neutrophil function in acute lung injury. *Thorax*, 62, 36–42. [PubMed: 16928710]
- Pieterse E, Rother N, Yanginlar C, Hilbrands LB & van der Vlag J (2016) Neutrophils Discriminate between Lipopolysaccharides of Different Bacterial Sources and Selectively Release Neutrophil Extracellular Traps. *Front Immunol*, 7, 484. [PubMed: 27867387]

- Pircher J, Czermak T, Ehrlich A, Eberle C, Gaitzsch E, Margraf A, Grommes J, Saha P, Titova A, Ishikawa-Ankerhold H, Stark K, Petzold T, Stocker T, Weckbach LT, Novotny J, Sperandio M, Nieswandt B, Smith A, Mannell H, Walzog B, Horst D, Soehnlein O, Massberg S & Schulz C (2018) Cathelicidins prime platelets to mediate arterial thrombosis and tissue inflammation. *Nat Commun*, 9, 1523. [PubMed: 29670076]
- Rowley JW, Oler AJ, Tolley ND, Hunter BN, Low EN, Nix DA, Yost CC, Zimmerman GA & Weyrich AS (2011) Genome-wide RNA-seq analysis of human and mouse platelet transcriptomes. *Blood*, 118, e101–111. [PubMed: 21596849]
- Rubenfeld GD, Caldwell E, Peabody E, Weaver J, Martin DP, Neff M, Stern EJ & Hudson LD (2005) Incidence and outcomes of acute lung injury. *N Engl J Med*, 353, 1685–1693. [PubMed: 16236739]
- Simon LM, Edelstein LC, Nagalla S, Woodley AB, Chen ES, Kong X, Ma L, Fortina P, Kunapuli S, Holinstat M, McKenzie SE, Dong JF, Shaw CA & Bray PF (2014) Human platelet microRNA-mRNA networks associated with age and gender revealed by integrated plateletomics. *Blood*, 123, e37–45. [PubMed: 24523238]
- Seeramkumar V, Adrover JM, Ballesteros I, Cuartero MI, Rossaint J, Bilbao I, Nacher M, Pitaval C, Radovanovic I, Fukui Y, McEver RP, Filippi MD, Lizasoain I, Ruiz-Cabello J, Zarbock A, Moro MA & Hidalgo A (2014) Neutrophils scan for activated platelets to initiate inflammation. *Science*, 346, 1234–1238. [PubMed: 25477463]
- Stefanini L & Bergmeier W (2018) Negative regulators of platelet activation and adhesion. *J Thromb Haemost*, 16, 220–230. [PubMed: 29193689]
- Suzuki-Inoue K, Fuller GL, Garcia A, Eble JA, Pohlmann S, Inoue O, Gartner TK, Hughan SC, Pearce AC, Laing GD, Theakston RD, Schweighoffer E, Zitzmann N, Morita T, Tybulewicz VL, Ozaki Y & Watson SP (2006) A novel Syk-dependent mechanism of platelet activation by the C-type lectin receptor CLEC-2. *Blood*, 107, 542–549. [PubMed: 16174766]
- Tadie JM, Bae HB, Jiang S, Park DW, Bell CP, Yang H, Pittet JF, Tracey K, Thannickal VJ, Abraham E & Zmijewski JW (2013) HMGB1 promotes neutrophil extracellular trap formation through interactions with Toll-like receptor 4. *Am J Physiol Lung Cell Mol Physiol*, 304, L342–349. [PubMed: 23316068]
- Tamakuma S, Ogawa M, Aikawa N, Kubota T, Hirasawa H, Ishizaka A, Taenaka N, Hamada C, Matsuoka S & Abiru T (2004) Relationship between neutrophil elastase and acute lung injury in humans. *Pulm Pharmacol Ther*, 17, 271–279. [PubMed: 15477122]
- Tence V, Polonsky J, Le Couedic JP & Benveniste MJ (1980) Release, purification, and characterization of platelet-activating factor (PAF). *Biochimie*, 62, 251–259. [PubMed: 17941181]
- Tilgner J, von Trotha KT, Gombert A, Jacobs MJ, Drechsler M, Doring Y, Soehnlein O & Grommes J (2016) Aspirin, but Not Tirofiban Displays Protective Effects in Endotoxin Induced Lung Injury. *PLoS One*, 11, e0161218. [PubMed: 27583400]
- Tomaiuolo M, Brass LF & Stalker TJ (2017) Regulation of Platelet Activation and Coagulation and Its Role in Vascular Injury and Arterial Thrombosis. *Interv Cardiol Clin*, 6, 1–12. [PubMed: 27886814]
- van den Boogaard FE, Schouten M, de Stoppelaar SF, Roelofs JJ, Brands X, Schultz MJ, van't Veer C & van der Poll T (2015) Thrombocytopenia impairs host defense during murine *Streptococcus pneumoniae* pneumonia. *Crit Care Med*, 43, e75–83. [PubMed: 25627210]
- Vanderschueren S, De Weerd A, Malbrain M, Vankerschaever D, Frans E, Wilmer A & Bobbaers H (2000) Thrombocytopenia and prognosis in intensive care. *Crit Care Med*, 28, 1871–1876. [PubMed: 10890635]
- Vicente V, Kostel PJ & Ruggeri ZM (1988) Isolation and functional characterization of the von Willebrand factor-binding domain located between residues His1-Arg293 of the alpha-chain of glycoprotein Ib. *J Biol Chem*, 263, 18473–18479. [PubMed: 3263968]
- Vu TKH, Hung DT, Wheaton VI & Coughlin SR (1991) Molecular-Cloning of A Functional Thrombin Receptor Reveals A Novel Proteolytic Mechanism of Receptor Activation. *Cell*, 64, 1057–1068. [PubMed: 1672265]

- Wagner CL, Mascelli MA, Neblock DS, Weisman HF, Collier BS & Jordan RE (1996) Analysis of GPIIb/IIIa receptor number by quantification of 7E3 binding to human platelets. *Blood*, 88, 907–914. [PubMed: 8704248]
- Wang T, Liu Z, Wang Z, Duan M, Li G, Wang S, Li W, Zhu Z, Wei Y, Christiani DC, Li A & Zhu X (2014) Thrombocytopenia is associated with acute respiratory distress syndrome mortality: an international study. *PLoS One*, 9, e94124. [PubMed: 24732309]
- Ware LB & Matthay MA (2000) The acute respiratory distress syndrome. *N Engl J Med*, 342, 1334–1349. [PubMed: 10793167]
- Washington AV (2009) TREM-like transcript-1 protects against inflammation-associated hemorrhage by facilitating platelet aggregation in mice and humans. *The Journal of Clinical Investigation*, 119, 1489–1501. [PubMed: 19436112]
- Washington AV, Quigley L & McVicar DW (2002) Initial characterization of TREM-like transcript (TLT)-1: a putative inhibitory receptor within the TREM cluster. *Blood*, 100, 3822–3824. [PubMed: 12393607]
- Washington AV, Schubert RL, Quigley L, Disipio T, Feltz R, Cho EH & McVicar DW (2004) A TREM family member, TLT-1, is found exclusively in the alpha-granules of megakaryocytes and platelets. *Blood*, 104, 1042–1047. [PubMed: 15100151]
- Wetterwald A, Hoffstetter W, Cecchini MG, Lanske B, Wagner C, Fleisch H & Atkinson M (1996) Characterization and cloning of the E11 antigen, a marker expressed by rat osteoblasts and osteocytes. *Bone*, 18, 125–132. [PubMed: 8833206]
- Williams AE & Chambers RC (2014) The mercurial nature of neutrophils: still an enigma in ARDS? *Am J Physiol Lung Cell Mol Physiol*, 306, L217–230. [PubMed: 24318116]
- Wygrecka M, Kosanovic D, Wujak L, Reppe K, Henneke I, Frey H, Didiasova M, Kwapiszewska G, Marsh LM, Baal N, Hackstein H, Zakrzewicz D, Muller-Redetzky HC, de Maat S, Maas C, Nolte MW, Panousis C, Schermuly RT, Seeger W, Witzernath M, Schaefer L & Markart P (2017) Antihistone Properties of C1 Esterase Inhibitor Protect against Lung Injury. *Am J Respir Crit Care Med*, 196, 186–199. [PubMed: 28005404]
- Xu J, Zhang X, Pelayo R, Monestier M, Ammollo CT, Semeraro F, Taylor FB, Esmon NL, Lupu F & Esmon CT (2009) Extracellular histones are major mediators of death in sepsis. *Nat Med*, 15, 1318–1321. [PubMed: 19855397]
- Yadav H, Thompson BT & Gajic O (2017) Fifty Years of Research in ARDS. Is Acute Respiratory Distress Syndrome a Preventable Disease? *Am J Respir Crit Care Med*, 195, 725–736. [PubMed: 28040987]
- Yipp BG, Petri B, Salina D, Jenne CN, Scott BN, Zbytniuk LD, Pittman K, Asaduzzaman M, Wu K, Meijndert HC, Malawista SE, de Boisleury Cheavance A, Zhang K, Conly J & Kubes P (2012) Infection-induced NETosis is a dynamic process involving neutrophil multitasking in vivo. *Nat Med*, 18, 1386–1393. [PubMed: 22922410]
- Yoon SH, Lee YD, Ha J, Lee Y & Kim HH (2012) TLT-1s, alternative transcripts of triggering receptor expressed on myeloid cell-like transcript-1 (TLT-1), Inhibits the triggering receptor expressed on myeloid cell-2 (TREM-2)-mediated signaling pathway during osteoclastogenesis. *J Biol Chem*, 287, 29620–29626. [PubMed: 22761415]
- Yu H, Ni YN, Liang ZA, Liang BM & Wang Y (2018) The effect of aspirin in preventing the acute respiratory distress syndrome/acute lung injury: A meta-analysis. *Am J Emerg Med*, 36, 1486–1491. [PubMed: 29804790]
- Zarbock A, Singbartl K & Ley K (2006) Complete reversal of acid-induced acute lung injury by blocking of platelet-neutrophil aggregation. *J. Clin. Invest*, 116, 3211–3219. [PubMed: 17143330]
- Zuchtriegel G, Uhl B, Pühr-Westerheide D, Pornbacher M, Lauber K, Krombach F & Reichel CA (2016) Platelets Guide Leukocytes to Their Sites of Extravasation. *PLoS Biol*, 14, e1002459. [PubMed: 27152726]

Table 1.

Major Platelet receptors and their ligands.

Major Platelet Receptors	Ligand(s)	References
(Protease Activated Receptor (PAR) 1 and 4	thrombin	(Connolly, <i>et al</i> 1996, Vu, <i>et al</i> 1991)
P2Y ₁₂ -	ADP	(Leon, <i>et al</i> 1997)
TPa and b	Thromboxane A ₂	(Paul, <i>et al</i> 1999)
GPVI/Fcγ	Collagen	(Moroi, <i>et al</i> 1989)
C-type lectin-like 2 (CLEC-2)	Podoplanin	(Christou, <i>et al</i> 2008)
GP1b/IX/V	von Willebrand factor thrombin	(Vicente, <i>et al</i> 1988)
αIIbβ ₃	Fibrinogen	(Nachman and Leung 1982)
TLT-1	Fibrinogen	(Washington 2009)
P-selectin	P-selectin Glycosolated ligand 1	(Moore, <i>et al</i> 1994)