



The mechanistic basis linking cytokine storm to thrombosis in COVID-19

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ABSTRACT

It is now well established that infection with SARS-CoV-2 resulting in COVID-19 disease includes a severely symptomatic subset of patients in whom an aggressive and/or dysregulated host immune response leads to cytokine storm syndrome (CSS) that may be further complicated by thrombotic events, contributing to the severe morbidity and mortality observed in COVID-19. This review provides a brief overview of cytokine storm in COVID-19, and then presents a mechanistic discussion of how cytokine storm affects integrated pathways in thrombosis involving the endothelium, platelets, the coagulation cascade, eicosanoids, auto-antibody mediated thrombosis, and the fibrinolytic system.

1. Cytokine storm syndrome in COVID-19

Cytokine storm syndrome (CSS) is a triggered acute hyper-inflammatory response of the immune system with continuous activation of lymphocytes, macrophages and other immune effector cells resulting in hypersecretion of inflammatory cytokines. This response has been observed most notably in association with acute viral infections, including SARS-CoV-2, and can lead to systemic inflammation, hemodynamic instability and multiorgan failure.

SARS-CoV-2 binds angiotensin converting enzyme 2 (ACE-2) to enter host cells. Most patients with SARS-CoV-2 infection and resulting COVID-19 disease present with respiratory symptoms as ACE-2 receptors are highly expressed in vascular endothelial cells of the lower respiratory tract. Infection of pulmonary epithelial cells leads to the release of interferon-gamma (IFN- γ), which acts as a potent and direct anti-viral molecule. However, IFN- γ also activates alveolar macrophages, which produce massive levels of interleukin-6 (IL-6), tumor necrosis factor- α (TNF α), and IL-10, leading to the initiation of a “local”

cytokine storm. IL-6 binds to the soluble form of the IL-6 receptor (IL-6R) on alveolar macrophages that has been cleaved from the cell surface by metalloproteinases, and the IL-6/IL-6R complex binds to gp130 and activates cell types that normally would not express IL-6R, leading to further amplification of the signal. A dramatic elevation of IL-6 is a supportive finding for the diagnosis of CSS. IL-6 contributes to vascular leakage, activation of complement and activation of the coagulation cascade leading to disseminated intravascular coagulation (DIC). In fact, this understanding led to early and subsequent immunotherapy studies targeting IL-6 for inhibition in severely symptomatic COVID-19 patients [1]. Monocytes and macrophages amplify the cytokine and inflammatory response while endothelial cells contribute to capillary damage, hypertension, and coagulopathy, which contribute to increased mortality.

1.1. Alveolar and pre-alveolar involvement in SARS-CoV-2

Pulmonary pathological findings of SARS-CoV-2 to SARS-CoV share

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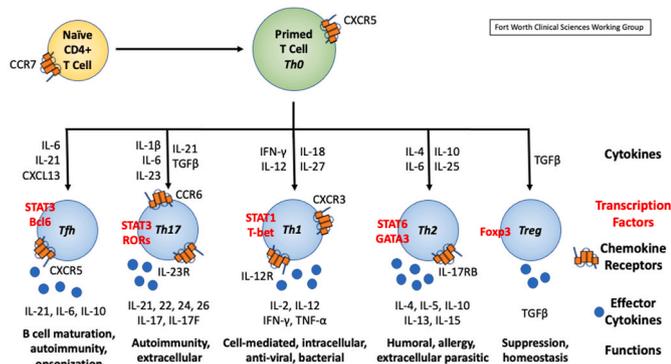


Fig. 1. Overview of T-Cell Differentiation and Cytokine Profiles. Primed Th0 cells differentiate into specific T-cell subsets including Th1 cells that secrete IL-2, IL-12, IFN- γ , and TNF α and Th2 cells that secrete Th2 inflammatory cytokines, including, IL-6, IL-10, IL-13, MIP-1 all of which play crucial roles in the development of COVID-19 cytokine storm. T-Reg cells may also play a role through TGF- β -mediated downstream effects.

many similarities. Both diseases are characterized by heavy damage to type 1 epithelial cells and atypical hyperplasia of type II alveolar cells, with additional focal hemorrhage, exudation, edema, and consolidation of the pulmonary tissue [2]. However, SARS-CoV-2 is unique in that mucous plugs are present in all respiratory tracts, terminal bronchioles and pulmonary alveoli, a finding absent in SARS-CoV [2,3]. Whereas SARS-CoV was only occasionally identified in alveolar macrophages, SARS-CoV-2 was found in the macrophage at all phases of the infection, suggesting that macrophages are a critical component of disease manifestation [4]. These macrophages, termed alternatively activated phenotype (M2) are a shift from the classically activated phenotype (M1), leading to more robust fibrosis of the respiratory tract [5]. Consistent with prior findings, an elevation in IL-6 levels confirms the activation of the cytokine storm [6].

1.2. Lymphocytic activation and Th1 vs Th2 signaling

T-cell “co-activation” is an essential component of CSS and leads to the T-cell mediated release of T-helper cell type (Th)-1 cytokines: IL-1, IL-2, IL-12, TNF α , Type 1 (α and β), 2 (γ), and 3 (λ) interferons [7]. Th1 cytokines, in addition to their anti-viral effects and induction of cell-mediated cytotoxic pathways, also have a role in promoting differentiation of T-cells into subsets that secrete pro-inflammatory cytokines such as IL-6, IL-13, IL-21, and macrophage inflammatory protein

Table 1

Summary of cytokine expression patterns in COVID 19 in early and late stages assessed by serum multiplex particle or bead-based immunoassay expression or peripheral blood mononuclear cell gene expression.

| Cytokine Type | Early Expression | Autoantibodies or Genes Downregulating Host Response | Late Expression |
|--------------------------------|---|--|-----------------|
| Interferons | Type 1 IFNs, Type 2 IFN γ | 30% prevalence of IFN ω and α autoantibodies | Unknown |
| Th 1 Interleukins | IL-2, IL2-R, IL-1 β | No | Unknown |
| Th 2 Interleukins | IL-6, IL-10 | No | IL-6, IL-8 |
| TGF- β and TNF- α | TGF- β , TNF- α | TRAIL | Yes |
| Chemokines | MIP-1 CCL3 CCL4 CXCL2 CXCR5 CXCR9 CXCR9L CXCR2 IP-10 (CXCL10) | No | Unknown |

Numerous studies have reported a consistently elevated expression of Type 1 interferons, Th2 and Th2 interleukins and chemokines early in SARS-CoV2 expression. Recently published studies have reported autoantibodies to interferons present in individuals with severe disease and absent in those with less severe or asymptomatic SARS-CoV2 infection that may underscore development of a strategy to risk stratify individual patients the presences of the autoantibodies [15]. Also of note is a downregulation of TRAIL (TNF related apoptosis inducing ligand), which appears to enable persistently elevated TNF expression, perpetuating the cytokine storm. There appears to be a persistently elevated Th2 interleukin response and ongoing expression of TGF- β , TNF- α late in the disease state following SARS-CoV2 infection.

(MIP)-1 (Fig. 1). In SARS-CoV-2-infected individuals, both Th1 and Th2 cytokines are elevated [8]. Published reports have consistently demonstrated elevations in both pro- and anti-inflammatory cytokines in association with severity of the infection (Table 1). When comparing the cytokine profile of asymptomatic infected patients with symptomatic patients, Long et al. found an elevation of 18 pro- and anti-inflammatory cytokines in symptomatic individuals, with IL-2, IL-2R, TNF α and IFN expression at significantly higher levels in symptomatic patients versus asymptomatic patients [9]. Another study found that patients in the intensive care unit (ICU) setting have significantly higher levels of IL-6, IL-10 and TNF α [10]. When taken together, these studies indicate a more robust immunological response in symptomatic patients compared with asymptomatic patients, and that this response is correlated to the severity of the infection. Although both Th1 and Th2 cytokines have been found to be elevated in severely infected individuals, Th1 cytokines appear to be driving tissue injury in symptomatic patients. Lee et al., studied mRNA expression from peripheral mononuclear cells from healthy donors versus patients with mild and severe infection [11]. Their study demonstrated that in severely infected patients, there is upregulation of the both the type I IFN and the TNF α /IL-1 β inflammatory responses, suggesting that the Th1 pathway might play a more significant role in driving the inflammatory response and severity seen in these patients.

Studies have reported that patients with a more severe presentation of SARS-CoV-2 infection exhibit significantly lower levels of HLA-DR as well as lower CD4 $^{+}$ and CD8 $^{+}$ T-cell counts despite an elevated white blood cell count [12]. Conversely, HLA-DR expression is partially restored in the setting of IL-6 receptor inhibition with tocilizumab, suggesting that the immune dysfunction present in severely infected patients may be attributed to these elevations in cytokines. Furthermore, levels of IL-6, IL-2R, IL-10 and TNF α were mildly elevated or within the normal range in cases of moderate severity, yet markedly elevated in most severe cases, while CD4 $^{+}$ and CD8 $^{+}$ T-cell counts were slightly lower in moderate cases and significantly decreased in severe cases. These findings suggest that elevated cytokine levels and decreased lymphocyte counts are associated with increased disease severity and that CD4 $^{+}$ and CD8 $^{+}$ T-cells may play a protective role in this condition. To summarize, pro-inflammatory cytokines secreted by T-cells recruit defensive “cellular first responders” such as NK cells and macrophages, leading to inflammatory cell infiltration and extensive tissue injury. As IL-2, IL-6, IL-10, TNF α , and neutrophil counts increase with disease severity, CD4 $^{+}$ and CD8 $^{+}$ T-cells decrease, impairing the body’s adaptive immune system and driving CSS [8,13,14].

1.3. Hemophagocytic lymphohistiocytosis (HLH) as model for cytokine storm in COVID 19

Cytokine storm syndrome is not limited to viral infections [16]. Autoimmune disorders, primary or familial hemophagocytic lymphohistiocytosis (fHLH), and secondary or reactive HLH (rHLH) due to systemic juvenile idiopathic arthritis (JIA) or systemic lupus erythematosus (SLE) have all been linked to CSS [17,18]. Whether familial or reactive, HLH is a hyper-inflammatory syndrome characterized by high levels of cytokines resulting in multiorgan failure. Accumulating evidence suggests that patients with severe SARS-CoV-2 infection mimic rHLH with overlapping clinical and laboratory features [19]. The pathogenesis of fHLH is characterized by aberrant cytokine signaling due to defective cytotoxic functioning of natural killer cells, most often due to a deficiency of perforin resulting from *PRF1* mutations [20]. In contrast with fHLH, the underlying cause of macrophage activation syndrome (MAS) in systemic JIA is less understood, though recent developments in the field link mutations in genes that are also implicated in fHLH, specifically *PRF1* and *MUNC13-4*, that may contribute to MAS development in these patients [21,22]. In a cohort study of fatal Influenza A infection, a high proportion of patients met the modified HLH-2004 criteria (44%) as well as MAS criteria (81%), while whole-exome sequencing revealed 36% of the cohort carried fHLH-associated gene mutations, asserting these mutations could be risk factors for increased mortality among individuals with influenza A infection [21]. Though a link between COVID-19 and longitudinal immunopathies such as rHLH will take time to determine, SARS-CoV-2 infected patients demonstrate a similar cytokine profile to that of rHLH. Some research suggests use of the H-score, often used in assessing individuals with HLH syndrome, may allow the rapid risk stratification necessary to use corticosteroids or other forms of immunotherapy in patients with COVID-19 and CSS [19,23]. Such patients may also be screened for this hyper-inflammatory syndrome using laboratory values such as elevated ferritin, lactate dehydrogenase (LDH), IL-6 and other cytokines, as well as an elevated D-dimer to guide use of therapeutic agents. This initial clue of an elevated D-dimer identified in cases of COVID-19 CSS suggested a linkage between cytokine storm and thrombosis.

2. Linking cytokine storm to thrombosis in COVID-19

Increasing evidence reveals that COVID-19 patients are more likely to develop thrombotic complications that could have a major impact on their clinical course and outcomes, including life-threatening events such as pulmonary emboli [24]. An early COVID-19 autopsy series observed 8 of 10 cases with small fibrinous thrombi in small pulmonary arterioles in both damaged and preserved parenchyma [25]. Furthermore, the study found endothelial tumefaction and an increased number of pulmonary megakaryocytes in pulmonary capillaries, further confirming the presence of the coagulation cascade [25]. Likewise, coagulation disorders are reported in more than 50% of HLH patients, and this is frequently attributed to hypofibrinogenemia. Although the mechanism of hypofibrinogenemia is currently not fully understood, we suspect that plasminogen activator inhibitor type 1 (PAI-1) may have a role, or that this may be linked to fibrinogen consumption in DIC, which can be observed in severe cases of HLH [26].

Numerous studies have reported manifestations of ischemic stroke, pulmonary embolism, and venous thromboembolism in SARS-CoV-2-infected patients, suggesting etiologies including hemostasis, endothelial injury, or hypercoagulability [27]. One study in Strasbourg, France reported 32 of 106 patients with dyspnea in COVID-19 had an acute pulmonary embolus on computerized tomography (CT) angiography completed for either a suspected embolus or for other CT indications [28]. Another retrospective study in New York, USA reported an incidence of stroke in 0.9% based on imaging findings in 3556 patients possibly due to acquired hypercoagulability [29]. An additional autopsy

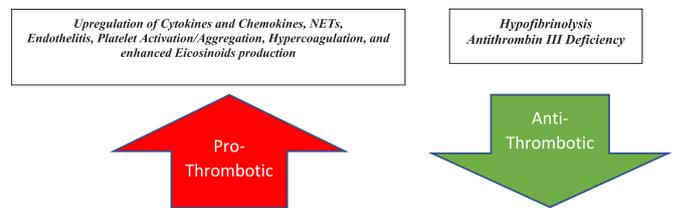


Fig. 2. Relationship Between Procoagulant vs Anticoagulant Factors in COVID 19. Thrombosis in COVID-19 appears to be a complex interplay developing from functional imbalance between pro-thrombotic and anti-thrombotic factors driving significant clinical thrombotic risk. Elevated cytokines and chemokines promote endothelitis, formation of NETs, enhance eicosanoid expression, enhance platelet interactions, and upregulate the coagulation proteins thereby promoting thrombosis. Potentiating this thrombosis is downregulation or “escape” from anti-thrombotic mechanisms through alterations in fibrinolytic pathways and through reduction in anti-thrombin III, the key endothelial derived anticoagulant.

series from New York, USA found all cases to demonstrate evidence of multi-organ thrombosis, in which platelet-rich thrombi were present within the microvasculature of the lungs, kidneys, heart, and liver regardless of anticoagulation received [30]. Likewise, an autopsy series from China describing kidney injury associated with SARS-CoV-2 infection observed severe endothelial injury associated with segmental microthrombi within glomerular capillary loops in a subset of patients presenting with multiorgan failure [31]. SARS-CoV-2 may promote thrombosis through various discrete and integrated effects on endothelial dysfunction, platelet activation and aggregation, induction of coagulation cascade proteins, stimulation of the eicosanoid pathway, and impairment of fibrinolysis. Ultimately, SARS-CoV-2 infection may manifest as a simultaneous “pro-coagulant” and impaired “anti-coagulant” disease state that drives the unique thrombotic risks observed in COVID-19 (Fig. 2).

2.1. Neutrophil-induced extracellular traps and thrombosis

Neutrophils, also known as polymorphonuclear (PMN) cells are the first line of the innate immunity in the defense against invading pathogens. PMNs are the most abundant leukocyte in the human body, and display a wide array of protective functions including pathogen phagocytosis, degranulation, and secretion of cytokines [32]. More recently, PMNs have been shown to perform a novel function amongst immune cells via the formation of neutrophil extracellular traps (NET). The mechanism of NETs is such that PMNs, in response to cytokines such as IL-8, IL-1 β , and TNF α , generally in the setting of inflammation or infection, release an extracellular network of fibers that are composed of degranulation products such as elastase and myeloperoxidase as well as histone-containing DNA complexes which have been shown to be bactericidal [33–35]. Conversely, NET formation in bronchial epithelium has been shown to induce the IL-8 and IL-1 which may contribute to a positive feedback loop especially unchecked in the face of viral infection and cytokine storm [36]. This immunomodulatory mechanism has been indicated in response to multiple inflammatory pathologies, including pulmonary, autoimmune, and cardiovascular diseases, and in general increasing alveolar and plasma NET levels correlate with severity of disease [37–40]. This has been shown to be the case in COVID-19 as well, as reported by Middleton et al., who showed that plasma NET levels significantly increased with increasing COVID-19 severity and decreased to control levels after COVID-19 resolution [41]. Platelets have been shown to be colocalized with neutrophil H3+ citrullinated histones, likely in the form of NETs, in post-mortem microthrombi of COVID-19 patients [41]. This microthrombi infiltration suggests a link between NETs and COVID-19 coagulopathy. In addition to platelet interaction, it has been shown in vitro that NETs induce the apoptosis of lung epithelial cells [42]. NET formation by

PMNs is a potential inducer of coagulopathy in COVID-19 by multiple mechanisms and further elucidation of their role is warranted.

2.2. Systemic endothelial damage and dysfunction

Hypoxia in moderate-to-severe COVID-19 leads to endothelial dysfunction and coagulability. Systemically, hypoxia upregulates P-selectin and adhesion molecules such as intracellular adhesion molecule-1 to induce platelet and leukocyte recruitment [43]. Monocytes may bind to activated endothelial cells through P-selectin glycoprotein ligand-1 and express tissue factor [44]. Furthermore, transcription factors such as hypoxia-induced factors (HIFs) are expressed as a response to hypoxemia and promote thrombosis by increasing endothelial release of PAI-1, inflammatory cytokines, while downregulating thrombomodulin [45]. HIF- α , in particular, downregulates expression of complement regulator CD55, enhancing complement-mediated endothelial damage in COVID-19 [46]. SARS-CoV-2 infects the host through spike protein-mediated binding to the ACE-2 receptor, which is highly expressed in the lungs, heart, gastrointestinal tract, kidney, and the endothelium. SARS-CoV-2 is confirmed to have a direct effect on the both alveolar and system endothelial cells, leading to endothelial dysfunction that shifts the microvascular equilibrium to favor vasoconstriction that subsequently leads to organ ischemia, a procoagulant state, tissue inflammation, and edema [47]. An autopsy series of SARS-CoV-2-infected patients with multiorgan failure revealed the presence of widespread endothelial inflammation as a result of either direct viral-mediated injury to the endothelium alone or in combination with host immune-mediated mechanisms [47]. Of note, this cohort was characterized by high-risk comorbidities for SARS-CoV-2-associated mortality such as hypertension, obesity, diabetes, and coronary artery disease, which also predispose to baseline endothelial dysfunction. Histopathological examination of endothelial cells revealed viral elements, inflammatory cells, and apoptotic bodies in relation to SARS-CoV-2 infection [47].

Another manifestation of endothelial injury reported in COVID-19 is an endothelitis, similar to the pathophysiology of decompression illness (DCI) [48]. A 2011 study reported that angiotensin II (Ang II) receptors are found on the red blood cell membrane [49]. ACE-2, a metalloenzyme responsible for the conversion of Ang II to angiotensin-(1-7), is bound by SARS-CoV and SARS-CoV-2, which downregulates ACE-2 expression and therefore interrupts Ang II clearance. This interference leads to an increase in Ang II levels in the lungs, leading to increases in fibrosis, inflammation, and oxidative stress [50,51]. It is hypothesized that high levels of Ang II cause a rightward shift in the oxygen-hemoglobin curve as red blood cells travel through the lungs [52]. As the blood becomes supersaturated with oxygen, vascular bubbles develop leading to “foam” accumulation in tissues with ACE-2 receptors. Decompression bubbles in the vasculature or tissues may occur in COVID-19, contributing to hemostasis and downstream clotting, as seen in DCI. The inflammatory cascade can be re-activated as a result, leading to a cyclical pattern of pulmonary edema and infarction [52]. A recent case study reported that hyperbaric oxygen, the primary form of treatment in DCI, has been successfully applied in the treatment of COVID-19 patients [53].

SARS-CoV-2 enhanced endothelial production of Ang II could induce PAI-1 through enhanced endothelial secretion and platelet activation [54,55]. Moreover, a SARS-CoV-2-mediated decrease in ACE-2 could lead to decreased activation of tissue plasminogen activator (tPA), further placing the body in a prothrombotic state. Together, high levels of PAI-1 and Ang II can initiate thrombosis leading to detrimental coagulative effects often seen in SARS-CoV-2 infected patients.

Recent work has further enhanced our understanding of how the endothelium contributes to advanced disease in SARS-CoV-2 cases [56]. IL-1 downregulates VE-cadherin allowing for vascular leakage and microvascular dysfunction, thereby linking the cytokine storm to endothelial damage [57]. Endothelial damage, furthermore, plays a role in protein accumulation in the alveolar space leading to fluid

accumulation and pneumonitis experienced in COVID-19 infection [57], and dysfunction in permeability may contribute to peripheral thrombosis and microvascular complications [58]. As such, Libby and Lüscher described COVID-19 as a disease of the endothelium [57].

2.3. Interactions between damaged endothelium and platelets

Animal models studying viral infections, such as influenza A virus (IAV)-mediated acute respiratory distress syndrome (ARDS) have shown increased platelet aggregation and endothelial damage mediated through hyperinflammatory cytokine responses [59]. The cytokine storm in IAV stimulates lung damage leading to the release of a variety of inflammatory cytokines as a result of both epithelial and endothelial dysfunction within the lungs. While elevated TNF α levels have shown to stimulate vascular endothelial cell (EC) apoptosis, TNF α , IL-1 β and IL-6 can upregulate trypsin in endothelial cells, resulting in the loss of zonula occludens-1 (ZO-1), a tight junction protein, and vascular hyperpermeability via protease-activated receptor-2 (PAR-2) [60]. Furthermore, hypoxia due to IAV stimulates endothelial cell activation, which releases pro-inflammatory IL-1, IL-6, platelet-activating factor (PAF), intercellular adhesion molecule1 (ICAM-1), P-selectin and von Willebrand Factor (vWF) [60]. All of these are powerful activators of platelets, are critical to adhesion, or play crucial roles in aggregation. As a result, SARS-CoV-2 infection can lead to pulmonary hemorrhage and pulmonary edema due to the direct damage of endothelial cells, loss of tight junctions, and hyperpermeability of the cell due to inflammatory factors eventually leading to apoptosis of endothelial cells.

Using mice as an animal model, one study found that IAV activates coagulation by increasing thrombin generation, fibrin deposition, and fibrinolysis while D-dimer, which is a marker of coagulation and fibrinolysis, and vWF activity were also elevated [61]. It has been shown that COVID-19 patients with severe disease often have abnormally increased vWF, as well [62]. The most potent pathway of platelet activation is through protease activated receptor (PAR-1 and PAR-4) mediated thrombin induction, and there is evidence that viral infection can induce this exceptionally important pro-coagulant and platelet activator [61, 63]. When the endothelial barrier is disrupted, this leads to the expression of collagen, tissue factor (TF), and vWF [64]. These in turn enhance platelet binding to endothelial cells, which further induces platelet activation and aggregation in a crescendo-like effect. This “white clot” associated with the endothelium activates the extrinsic coagulation cascade, leading to fibrin interlacing and maturation. This process left unchecked, especially with further disruptions in fibrinolytic mechanisms, leads to DIC and triggers decompensated thrombocytopenia, which is induced by hyper-activation, excessive aggregation, and passive exhaustion of platelets [60]. In severe COVID-19, Hottz et al. demonstrated not only that activated platelets aggregate with monocytes, but that these aggregations increase monocyte TF expression, which positively correlated with elevating D-dimer levels [65]. Interestingly, activated platelets have been shown previously to induce NET formation in alveolar endothelial cells in transfusion-related acute lung injury, which potentially may exacerbate the pathologic effects of platelet dysfunction seen in COVID-19 [66]. Highlighting the importance of platelet function in COVID-19, a retrospective study from Wuhan showed that thrombocytopenia on admission to the hospital for COVID-19 was associated with a 4.24-fold increased risk of mortality [67].

2.4. Effects on the coagulation cascade

The observed increase in IL-6 associated with CSS in the setting of SARS-CoV-2 infection may contribute to vascular leakage, activation of complement and coagulation cascade activation leading to DIC. DIC is an advanced complication of a severe infection that arises from the disturbances of hemostatic balance and is characterized by activation of the coagulation cascade with simultaneous inhibition of fibrinolysis. In

the extrinsic pathway of the coagulation cascade, TF has been implicated in DIC, with increased levels of TF expressed in states of upregulated pro-inflammatory cytokines such as CSS. TF is a glycoprotein that is expressed in vascular endothelial cells, that binds and activates factor VII, subsequently activating clotting factor X and IX. Cytokines and chemokines that increase TF expression are TNF α , IL-1, IL-6, IL-8, IFN- γ , and monocyte chemoattractant protein (MCP)-1, whereas cytokines like transforming growth factor (TGF)- β , IL-4, IL-10, and IL-13 have been shown to decrease expression of TF [68]. In addition, Th1 T-cells produce IFN- γ which induces monocytic TF factor production while Th2 cytokines like IL-4 and IL-10 inhibit production [69]. IL-6 has been implicated in the severity of CSS, which suggests that it could play a role in coagulation pathway activation by increasing the expression of TF. The role of IL-6 in TF expression is further supported by studies that show that in chimpanzees exposed to low dose lipopolysaccharide (LPS), treatment with an *anti*-IL-6 antibody prevented activation of the coagulation cascade [68]. Interestingly, IL-10, a cytokine that has been shown to have inhibitory effects on TF expression and anti-coagulant properties in the setting of endotoxemia in humans infected with LPS, is one of the cytokines that is consistently elevated in patients with CSS [70]. This might suggest a compensatory mechanism to regulate the expression of TF through induction of cytokines like IL-6.

Another mechanism that may contribute to DIC in COVID-19 patients is disruption on cell membrane surface proteins. Hemostasis requires local regulation at the site of injury, a process which is impaired in DIC. Localization is achieved through tight regulation of cell membrane proteins within cellular lipid membrane bilayers. Among these membrane surface proteins, phosphatidylserine (PS) and phosphatidylethanolamine (PE) have been implicated in the coagulation cascade. In the quiescent state, PS and PE reside in the inner surface of the membrane; however, with injury, scramblase shuffles phospholipids to the extracellular surface through intracellular cytosol calcium dependent signaling. PS and PE have been shown to accelerate coagulation by increasing the binding affinity of factor VIIa for TF increasing in a concentration dependent manner [71]. SARS-CoV-2 infection contributes to this pathway through the upregulation of phospholipid scramblase-1 (PLSCR1). In a study by Tang et al., in 2005, SARS-CoV infection resulting in SARS was shown to upregulate PLSCR1, PAI-1 and other elements of the pro-coagulation pathway [72]. Upregulation of scramblase leading to an increase in PS and PE on the outer membrane, may play an important role in COVID-19 thrombosis.

D-dimer elevation is frequently observed in patients infected with SARS-CoV-2 and can be associated with increased mortality [73]. In their study, Yao et al., measured D-dimer levels at initial presentation and found that median D-dimer levels were significantly higher in the non-survivor group compared to the survivor group, suggesting an association between elevated levels and increased mortality in these patients. Furthermore, D-dimer levels of >2.14 mg/L predicted inpatient mortality with a sensitivity of 88.2% and a specificity of 71.3%. D-dimers are a product of cross-linked fibrin degradation by plasmin cleavage. Plasminogen is converted into plasmin by tPA, which is released from endothelial cells via activation of its G-protein coupled receptor (GPCR). This GPCR is activated through thrombin and factor Xa, along with many others [74]. SARS-CoV-2 virulence and infectivity may be influenced by cleavage of its spike proteins by plasmin among other endothelial proteases [75]. Finally, elevated levels of cytokines including IL-1, IL-6, IFN- γ , and TNF α . seen in COVID-19 patients activate the coagulation cascade through increased expression of TF, leading to microangiopathic thrombosis.

2.5. Effects on eicosanoids

Eicosanoids (prostaglandins, leukotrienes, and lipoxins) are bioactive lipid signaling molecules derived from 20-carbon unsaturated fatty acids that play a large role in pathological and physiological responses, including tissue homeostasis, host defense, inflammation, and cancer

[76,77]. De novo synthesis of eicosanoids involves metabolism of polyunsaturated fatty acids, most prominently arachidonic acid (AA), by cyclooxygenases, lipoxygenase, cytochrome p450, or nonenzymatic isoprostane pathways [49,50]. Prostaglandins (PGE₂, PGI₂, PGD₂, PGF₂) and TXA₂ are pro-inflammatory factors while leukotrienes and lipoxins contribute to both pro- and anti-inflammatory pathways [78]. Pro-inflammatory factors TXA₂ and PGE₂ are potent activators of platelets and are implicated in the initiation of thrombosis [77,79]. COX-1 and COX-2 enzymes are of interest due to their regulation by pro-inflammatory nuclear factor kappa-light-chain-enhancer (NF-kappaB). COX-1 and COX-2 are upregulated by NF-kappaB in the presence of infection, atherosclerosis, and varying cancers [77]. Furthermore, upregulation of COX-2 results in the increased production of prostaglandins, which have been discovered in bronchodilation, pain signaling, atherosclerosis, and viral infection [77,79]. Among those prostaglandins is PGE₂, which induces inflammation, while alternative non-cyclooxygenase derived isoprostanes are used as indication of significant oxidative stress [80].

SARS-CoV-2 infection leads to significant release of pro-inflammatory cytokines (IL-6, IL-1 β , TNF α), but also results in production of eicosanoids, which have been identified as significantly elevated in bronchioalveolar lavage samples of intubated COVID-19 patients [81, 82]. It has been demonstrated by Gross et al. that PGE₂ upregulation during inflammatory stress helps facilitate the initiation of arterial thrombosis and atherothrombosis [79]. Among all prostaglandins, PGE₂ is significantly upregulated in response to viral infection, including SARS-CoV-2, making this specific eicosanoid a therapeutic target [83]. It is clear that CSS is a significant factor in the pathophysiology of severe COVID-19 disease, leading to a hypercoagulable state, thrombotic disease, and DIC supported by autopsy findings [81]. Significant upregulation of eicosanoids in COVID-19, perhaps creating an "eicosanoid storm", may create an amplification effect that potentiates CSS [83]. Interestingly, Pace et al. found that elevated PGE₂ and COX-2 levels were seen in male mice, older mice, and obese mice, consistent with the evidence that SARS-CoV-2 infections are more severe in males, older populations, and patients with comorbidities, such as obesity [84,85].

2.6. Development of pro-thrombotic autoantibodies

Recent observations have led researchers to investigate the role of autoantibodies in the hypercoagulable state of patients with COVID-19, with special attention to antiphospholipid antibodies [86]. Antiphospholipid antibody syndrome (APS) is an acquired systemic autoimmune disease that often presents clinically with thrombotic complications; it is diagnosed by a combination of serologic testing for autoantibodies and clinical manifestations [87]. Patients with APS form autoantibodies to phospholipids and phospholipid-binding proteins, binding to cell surfaces and activating neutrophils, endothelial cells, and platelets, which results in a hypercoagulable state; common targets of these antibodies are prothrombin and beta-2-glycoprotein, which are found in plasma [88]. A study of 74 critically ill COVID-19 patients found that 88% demonstrated an elevated level of antiphospholipid antibodies, although this specific study found no significant association with thrombotic complications [89]. Zuo et al. measured the levels of eight types of antiphospholipid antibodies in the serum of 172 patients hospitalized with COVID-19 and found that over half of these patients became at least temporarily positive for antiphospholipid antibodies, which have the potential to cause severe pathogenic effects through neutrophil and platelet overactivation and subsequent thrombotic events [88].

COVID-19 patients have also been observed to have abnormalities in coagulation studies, including a prolonged prothrombin time (PT) and activated partial-thromboplastin time (aPTT) [90]. One type of autoantibody involved in APS, lupus anticoagulant, is associated with both thrombotic risk and effects on in vitro blood coagulation studies [91]. Lupus anticoagulants are heterogenous IgG and IgM autoantibodies that

target phospholipids in the cell membrane, and are also associated with a prolonged clotting process, making them a risk factor for serious thrombotic events like stroke and transient ischemic attacks [92]. To investigate the relationship between lupus anticoagulant and the prolonged coagulation studies, specifically aPTT, Bowles et al. performed lupus anticoagulant assays on 34 COVID-19-positive patients confirmed to have a prolonged aPTT and found that 31 of those 34 patients (91%) were positive for the lupus anticoagulants [91]. Importantly, they also found that the lupus anticoagulants were not in any way associated with a bleeding tendency, but rather with thrombosis via their role in APS. Although the specific role of lupus anticoagulant autoantibodies in thrombosis associated with COVID-19 requires further study, these findings support the concept that anticoagulation therapy should be used as treatment and that a prolonged aPTT should not be a reason to withhold therapy [91].

Anticoagulant treatment with heparin or immunosuppression with corticosteroids are both common therapies used to improve outcomes in patients with APS [93]. While both treatment options have been explored in patients with COVID-19, further investigation into combination therapies and their effects on serum antiphospholipid antibody levels in these infected patients is needed.

2.7. Effects on the fibrinolytic system

Although integrated pathways promoting thrombus formation by cytokine induction in COVID-19 may tip the scales towards “pro-coagulation”, evidence is emerging that impaired, endogenous “anti-coagulation” secondary to hypo-fibrinolysis plays an important role. Plasminogen is an inactive zymogen that is converted to plasmin by tissue plasminogen activator (t-PA) or urokinase plasminogen activator (u-PA). Plasmin is the molecular knife that cleaves fibrinogen into fibrin degradation products (FDP) and fibrin into fibrin split products (FSP), which are then cleared from the circulation through the reticular endothelium system (RES) in the spleen [94]. PAI-1 is a serine protease inhibitor (SERPIN) produced in the endothelium and uroepithelium that is the endogenous inhibitor of the plasminogen activators, tPA and uPA [95]. Given its influential role in human hemostasis as a physiologic anti-fibrinolytic molecule, PAI-1 is a critical determinant of pathophysiology in thrombosis. For instance, it is well established that the 4G/5G polymorphism leading to over-expression of PAI-1 is associated with early stroke and myocardial infarction [94]. Conversely, PAI-1 deficiency is associated with delayed clinical bleeding phenotypes. PAI-1 has also been implicated in cell migration and in particular, mobilization and migration of stem cells in models of vascular injury [95,96]. Given its influential role in human hemostasis as a physiologic anti-fibrinolytic molecule, PAI-1 plays a crucial role in thrombosis.

In systemic infection, PAI-1 is over-expressed resulting in a suppression of t-PA leading to a state of “hypo-fibrinolysis”. Sepsis and DIC are two life-threatening complications that are often concomitantly seen in critically ill, infected patients, and recent studies have shown that the presence of significantly elevated PAI-1 indicates a poor prognosis for such patients [97–99]. Cytokine mediated induction of PAI-1 is an area of great interest to our group, with evidence linking PAI-1 to TNF α , IL-1 β , IL-1 α , IL-6, and TGF- β . TNF α is known to induce PAI-1 [100,101], with one mechanism related to enhancement of NF-kappaB sites of the PAI-1 gene [102]. Another mechanism of induction is through TNF α -induced superoxides that directly activate NF-kappaB which then upregulates the promoter region of PAI-1 gene [103]. A 2019 study demonstrated that inhibition of TNF α with Infliximab, a chimeric monoclonal anti-TNF α antibody medication, significantly reduces levels of PAI-1 in patients with inflammatory bowel diseases [104]. IL-1 β indirectly promotes anti-fibrinolytic activity through Ang II-mediated activation of PAI-1 [105]. IL-1 α and IL-6, have also been implicated in PAI-1 upregulation via gene promoter activation through independent protein kinase signaling cascade pathways [106]. To further support the role of IL-1 β in the induction of PAI-1, a very recent study demonstrated

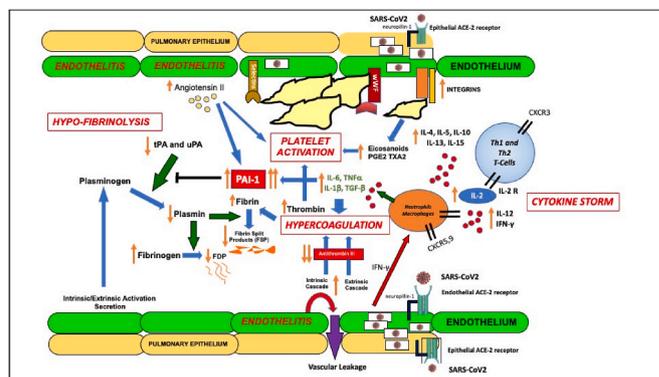


Fig. 3. Pathways Linking Cytokine Storm to Thrombosis in COVID-19. SARS-CoV2 induction of cytokine storm leads to alterations in the endothelium, platelets, coagulation cascade and fibrinolytic system. Infection with SARS-CoV2 results in very early hyper-expression of Type 1 interferons, Th1, and Th2 cytokines leading to activation of neutrophils and macrophages, resulting in secretion of pro-inflammatory cytokines such as TNF α and IL-6. This “cytokine bath” can induce platelet activation, secretion of eicosanoids, result in direct endothelial damage, and induce thrombin generation through reductions in anti-thrombin III activity. Enhanced thrombin promotes thrombosis through potent platelet activation and generation of mature clot through production of fibrin monomers that cross link to become fibrin polymers. Angiotensin II release from SARS-CoV2 activated endothelium leads to additional activation of platelets, direct endothelial damage, and induction of PAI-1. PAI-1 is also significantly enhanced through mechanisms linked to cytokine storm. The resulting over-expression of PAI-1 results in reduced tPA and uPA secretion from the endothelium, leading to a decrease in plasmin generation and resulting potentiation of fibrin polymers.

that attenuation of IL-1 β via administration of omentin-1 (an anti-inflammatory, anti-IL-1 β medication used in the treatment of osteoarthritis) significantly decreases PAI-1 release in chondrocytes [107]. These studies imply a very potent role for the cytokine-induced promotion of PAI-1 protein signaling exacerbated by cytokine storm syndrome or in states of sepsis, correlating with poorer outcomes.

TGF- β is also implicated in the upregulation of PAI-1. TGF- β acts through phosphorylation of Smad2/3, which leads to increased expression of PAI-1 as well as profibrotic genes [108]. A study examined the role of SnoN in the expression of phospholipid scramblase 1 (PLSCR1) [109]. SnoN is a Smad-interacting protein that negatively regulates TGF- β pathways by inhibiting the formation of Smad4/Smad-R complexes. Given the proximity of PLSCR1 to SnoN at the 3q locus, the study found that both a SnoN knockdown and treatment with TGF- β led to a reduction of PLSCR1 mRNA levels. Interestingly, the study also found that in mutants with the knockdown SnoN, as well as in those treated with TGF- β , PAI-1 was upregulated. The results of this study illustrate the importance of TGF- β in PAI-1 expression, as well as a possible role of PLSCR1 in the modulation of TGF- β signaling pathway. These findings highlight the importance of TGF- β in PAI-1 expression, as well as a possible role of PLSCR1 in the modulation of the TGF- β signaling pathway, linking CSS to hypo-fibrinolysis through this intracellular signaling pathway.

TGF- β and PAI-1 can also be further linked by the peroxisome proliferator-activated receptor (PPAR- γ). PPAR- γ agonists may offer a protective role in thrombosis through both downregulation of P-selectin expression and leukocyte-endothelial interactions and inhibition of the TGF- β /Smad signal transduction cascade and may reduce coagulopathy seen in SARS-CoV-2 infected individuals by indirect inhibition of PAI-1 [110]. Taken together, the results of these studies suggest that PAI-1 could be an important mediator to many of the pathologic processes seen in SARS-CoV-2 infection, and therefore disruption of this pathway may be an important mediator of outcomes in COVID-19 thrombosis.

3. Conclusion

In summary, SARS-CoV-2 induces the cytokine storm through direct and indirect effects on a broad array of immune cells. This process then orchestrates injury of the endothelium, activation of protease activated receptors on platelets and leukocytes, and induction of coagulation factors as we have described in this report. This “pro-coagulant” state is simultaneously worsened by hypo-fibrinolysis, i.e. reduced degradation of fibrin polymerized clot through mechanisms including induction of PAI-1. In this report, we propose the first comprehensive and integrated review of mechanisms that drive cytokine induced thrombosis in COVID-19 using a cell-based model of thrombosis (Fig. 3). Further studies are needed to investigate the pathways discussed in this review to advance our understanding of these proposed mechanisms linking the cytokine storm and thrombosis in COVID-19. We believe this review provides a strategic template for investigators to help visualize hypothesis driven investigations in COVID-19 and other similar syndromes that may further our understanding of how cytokine storm may induce clinical thrombosis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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