


# Platelets, Macrophages, and Thromboinflammation in Chagas Disease

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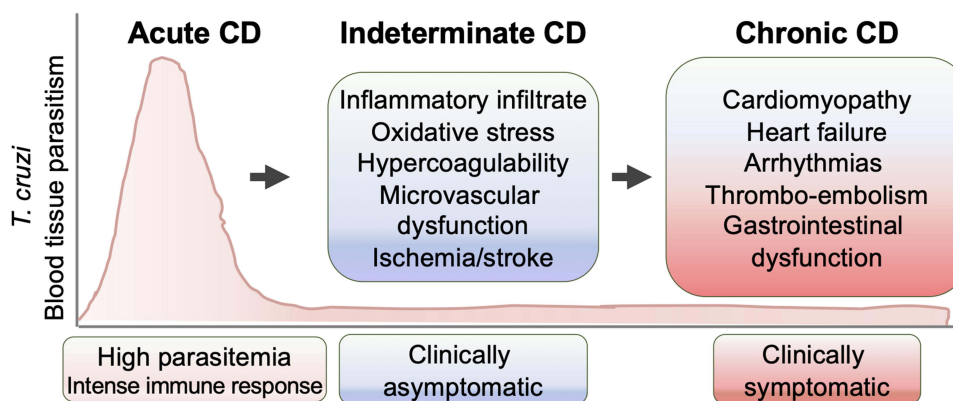
**Abstract:** Chagas disease (CD) is a major health problem in the Americas and an emerging health problem in Europe and other nonendemic countries. Several studies have documented persistence of the protozoan parasite *Trypanosoma cruzi*, and oxidative and inflammatory stress are major pathogenic factor. Mural and cardiac thrombi, cardiac arrhythmias, and cardiomyopathy are major clinical features of CD. During *T. cruzi* infection, parasite-released factors induce endothelial dysfunction along with platelet (PLT) and immune-cell activation. PLTs have a fundamental role in maintaining hemostasis and preventing bleeding after vascular injury. Excessive activation of PLTs and coagulation cascade can result in thrombosis and thromboembolic events, which are recognized to occur in seropositive individuals in early stages of CD when clinically symptomatic heart disease is not apparent. Several host and parasite factors have been identified to signal hypercoagulability and increase the risk of ischemic stroke in early phases of CD. Further, PLT interaction with immune cells and their role in host defense against pathogens and inflammatory processes have only recently been recognized and evolving. In the context of parasitic diseases, PLTs function in directly responding to *T. cruzi* infection, and PLT interactions with immune cells in shaping the proinflammatory or immunoregulatory function of monocytes, macrophages, and neutrophils remains elusive. How *T. cruzi* infection alters systemic microenvironment conditions to influence PLT and immune-cell interactions is not understood. In this review, we discuss the current literature, and extrapolate the mechanistic situations to explain how PLT and innate immune cell (especially monocytes and macrophages) interactions might be sustaining hypercoagulability and thromboinflammation in chronic CD.

**Keywords:** Chagas disease, coagulopathy, inflammation, macrophage activation, platelets, thrombosis

## Introduction

Chagas disease (CD) constitutes a leading health problem in the Americas. Though public health records are not up to date, it is estimated that ~8–10 million individuals are infected with *Trypanosoma cruzi* (*Tc*) and >110 million people are exposed to a risk of infection in endemic countries in the Americas.<sup>1</sup> While direct transmission of the parasite by triatomine vector remains the primary route of infection, consumption of food contaminated with feces of infected triatomines, transfusion of blood from infected donors, and vertical transmission from infected mothers to their newborns are also prominent.<sup>2,3</sup> The oral route of transmission is noted to result in a much higher mortality rate of 8%–35% than is generally observed by vectorial transmission, which is <5%–10%.<sup>4</sup> Due to the high rate of migration from endemic countries, it is estimated that 68,000–120,000 people with CD are currently living in Europe.<sup>5</sup> The Centers for Disease Control and Prevention (CDC) estimates >300,000 immigrants with *T. cruzi* infection are living in the US, with up to 45,000 cardiomyopathy cases occurring yearly.<sup>6</sup> CD is reported to cause >14,000 deaths every year.<sup>1</sup> The global economic burden of CD that can be averted by treatment with antiparasite drugs in the early phase of infection and thereby preventing heart failure and death in young adults is estimated to be US\$8 billion for 2021–2030.<sup>7</sup>

The natural course of CD progression is illustrated in Figure 1. CD is initiated with acute parasitemic phase in which an abundance of *T. cruzi* in circulation, dissemination to tissue, and intracellular replication is noted. With activation of adaptive immunity, infected individuals control the parasitemia and develop to an indeterminate phase in which very low levels of parasites are intermittently detected in circulation and no to mild clinical features are noted.<sup>8</sup> Approximately a



**Figure 1** Clinical phases of Chagas disease (CD). Upon exposure to *Trypanosoma cruzi*, the acute phase of CD presents with high blood parasitemia, strong activation of the immune system, and intense inflammatory infiltrates and tissue parasitism. In indeterminate phases of CD, patients exhibit immune control of parasites and are mostly clinically asymptomatic, though pathological processes, including well-recognized inflammatory and oxidative stress, may continue. With progression to chronic-phase CD, tissue parasitism remains low, yet pathological factors lead to evolution of symptomatic heart disease and systemic, cardiac, pulmonary, or cerebral embolism. Some patients may also develop gastrointestinal and neurological disorders.

third of infected individuals progress to a clinically symptomatic chronic phase and are burdened with major complications of the heart. Cardiomyopathy with progressive heart failure, arrhythmias, and cardiac, pulmonary, and cerebral embolism are believed to be the major cause of death in CD patients.<sup>8</sup> Up to 20% of infected individuals may also develop gastrointestinal disorders that can result in chronic dysphagia, constipation, and malnutrition.

Benznidazole and nifurtimox are the currently available antiparasitic drugs that are used as first-line therapy against *T. cruzi* infection.<sup>9</sup> These drugs are recommended for the treatment of young children and acutely infected adults. Infected children (<14 years old) exhibit a cure rate of >85% upon treatment with nifurtimox.<sup>10,11</sup> Benznidazole is effective in curing up to 90% of acute or recent cases of infection.<sup>12–14</sup> These drugs are contraindicated during pregnancy,<sup>9</sup> and their efficacy in treating infected individuals in indeterminate–chronic stages of CD remains controversial.<sup>15,16</sup> Several efforts have been employed to identify new antiparasitic drugs, yet no new drug has succeeded in clinical trials so far, likely due to gaps in our knowledge of disease pathogenesis and parasite biology.<sup>17</sup>

The complexities of CD and disease-management strategies have been discussed in recent reviews.<sup>18,19</sup> It is generally accepted that parasite persistence at low levels provides consistent antigens that continue to trigger immunoresponses, cause pathological tissue injury, and subsequently lead to cardiac insufficiency.<sup>20</sup> Our work suggests that the myocardium in CD sustains chronic oxidative stress. An increase in macrophage production of superoxide by NADPH oxidase<sup>21</sup> and the mitochondrial generation of reactive oxygen species (ROS) from electron-chain complexes have been noted in the myocardium during CD development.<sup>22–26</sup> Proteins, lipids, and DNA oxidative adducts have been detected in the peripheral blood and/or heart tissue of chronically infected rodents and human CD subjects<sup>27–34</sup> and exacerbated by inefficient antioxidant capacity.<sup>28,31,35–39</sup> Studies demonstrating ROS signaling of cytokine and chemokine production in infected cardiomyocytes and murine hearts<sup>40–43</sup> have provided a mechanistic link between ROS and chronic inflammation in CD. Further details regarding the pathogenesis of chronic inflammation in CD can be found in excellent recent reviews.<sup>8,44</sup>

Parasympathetic and sympathetic dysfunction and microvascular impairments have been described to contribute to chronic CD pathology.<sup>8</sup> High incidence of ischemic stroke is frequently noted in CD patients, and prevalence of stroke was higher in CD patients than in the general population of Brazil.<sup>45</sup> Cross-sectional studies in the Americas noted higher frequency of stroke in patients with *T. cruzi* infection and indeterminate or chronic CD than in non-CD individuals (14%–15% vs 2%–6%,  $p < 0.01$  in Brazil; 25% vs 2%,  $p < 0.05$  in Colombia).<sup>46–48</sup> These and other studies infer that ischemic stroke is likely the first clinically relevant sign in indeterminate CD before the clinical symptoms of heart disease become apparent. Cerebral and embolic stroke may occur with the development of chronic CD, involving the left ventricle or left atrium, several types of arrhythmias (right bundle branch block, advanced atrioventricular block, atrial fibrillation, tachycardia), and mural thrombi.<sup>8</sup> Beyond the role of structural alterations of the myocardium and conduction disorders, recent research suggests that *T. cruzi*

presence and inflammation conducive to hypercoagulability also contribute to higher risk of clot formation and thromboembolic events in CD. Accordingly, patients with chronic CD are often prescribed oral anticoagulants; however, long-term efficacy of anticoagulants in CD has not been studied.<sup>49</sup>

In this review, we summarize the current literature on hypercoagulability factors in CD and focus on the potential pathophysiological mechanisms involving platelets (PLTs) and monocytes/macrophages (Mo/M $\phi$ ) in sustaining thrombosis and inflammation in CD.

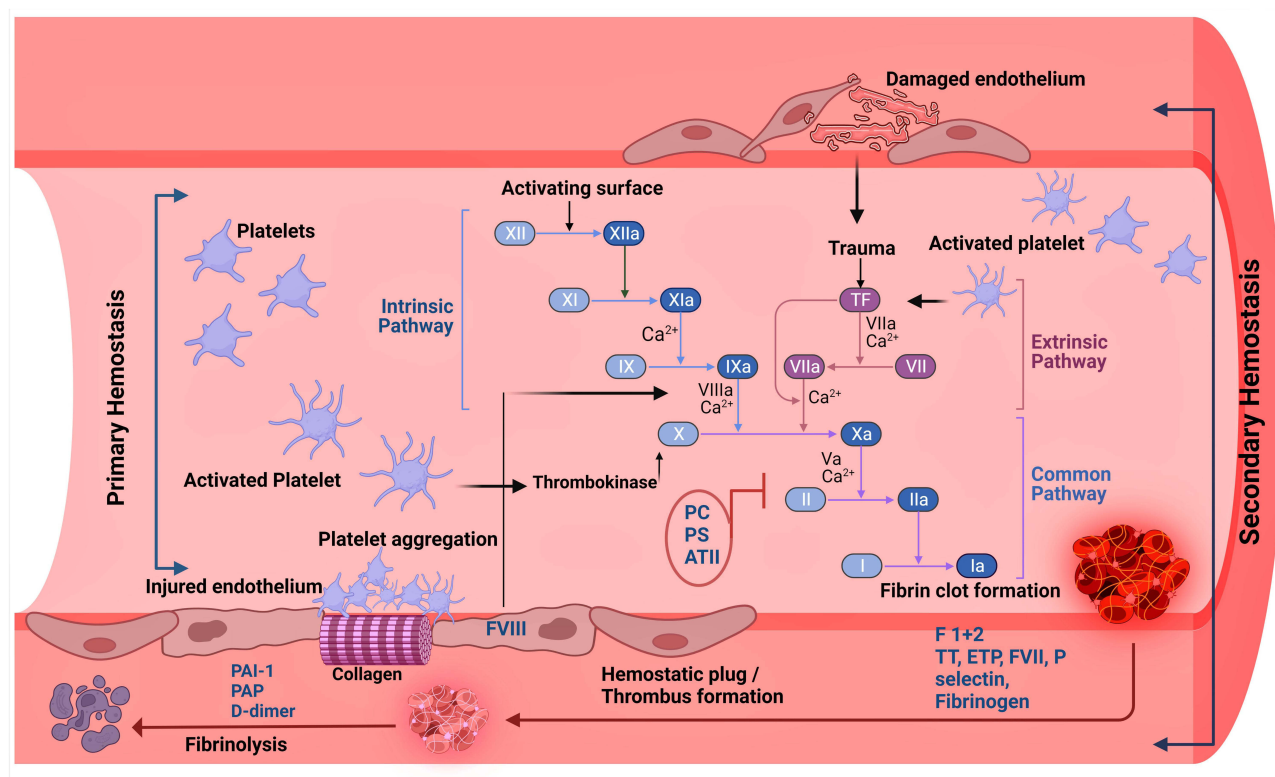
## Integration of Platelet Activation and Coagulation Pathway in Hemostasis

Hemostasis reflects a delicate balance between procoagulant and anticoagulant factors for maintaining the blood flow within the vascular compartment and forming blood clots after vascular injury. There are two main components of hemostasis: primary hemostasis refers to PLT aggregation and plug formation, and secondary hemostasis refers to the deposition of insoluble fibrin to further strengthen the PLT plug.<sup>50,51</sup> These two processes are mechanically and functionally intertwined. Calcium ( $\text{Ca}^{2+}$ ) ions play a major role in tight regulation of the coagulation cascade that is paramount in the maintenance of hemostasis while preventing pathological thrombosis or bleeding.<sup>52</sup>

PLTs play a fundamental role in primary hemostasis via adhesion, activation, and aggregation, which are triggered upon vascular tissue injury. First identified by Schultze in 1865, PLTs' function in hemostasis was elucidated by Bizzazero in 1881,<sup>53</sup> and in 1906, Wright showed that PLTs are generated from megakaryocytes. Lacking a nucleus, PLTs do not need a transcriptional machinery, yet they package carefully selected RNA and translational machinery and are equipped with an abundance of genetic information and processing machinery required for a highly efficient rapid response to vascular injury. Briefly, in the event of vascular injury, endothelial cells (ECs) in the blood vessels and circulating PLTs near the injury site produce von Willebrand factor (VWF). ECs also release the contents of their Weibel–Palade bodies, which enhance local concentrations of VWF. PLTs utilize adhesion molecules/ligands/receptors, including GPIa/GPIIa (collagen), GPIb/GPIX/GPV (VWF), and GPIIb/IIIa (ICAM1/fibrinogen/VWF) to adhere to the endothelium.<sup>54</sup> Adherence and activation of PLTs may also be achieved through agonists (eg, serotonin, thromboxane  $\text{A}_2$  [ $\text{TXA}_2$ ], adenosine diphosphate [ADP], adrenaline, thrombin) that bind to protease-activated receptor 1, GPVI, purinergic receptors (P2Y $1/2$ ), and other G-protein-coupled receptors.<sup>50</sup> Altogether, these signals lead to PLT aggregation and PLT plug formation at the site of vascular injury (Figure 2).

Recruitment of PLTs at the vascular injury site stimulates secondary hemostasis, in which several coagulation mediators undergo cleavage to be activated. Stepwise activation of inactive precursors, or zymogens, is necessary for the formation of insoluble fibrin for vascular healing (Figure 2). The intrinsic pathway is dependent on contact with a negatively charged surface (eg, collagen), and involves cleavage-mediated activation of blood coagulation factors XII, XI, and IX. The extrinsic pathway is dependent on tissue factor (TF) being exposed to the circulation. TF is synthesized and localized in the cell membrane of ECs, fibroblasts, and Mo/M $\phi$ , among others. The TF-bearing extravascular cells scanning the vascular injury encounter plasma factor VII, which is then rapidly activated and brings VIIa/TF in proximity to PLT surfaces. TF inhibitors control factor VIIa–TF complex activity.<sup>54</sup>

Both intrinsic and extrinsic pathways converge to a common pathway to produce cross-linked fibrinogen (fibrin) and stabilization of the PLT plug. Herein, activated PLTs release  $\alpha$ -granules containing factor V and VWF, and promote the assembly of procoagulant complexes, including tenase (factors VIIIa and IXa) and prothrombinase (factors Xa/Va) to propagate large-scale conversion of prothrombin (factor II) to thrombin (factor IIa) and culminating fibrinogen conversion to insoluble fibrin for the clot formation.<sup>54</sup> Naturally occurring anticoagulants, such as protein C (PC), protein S (PS), and antithrombin (AT) keep a check on this process.<sup>52</sup> Anticoagulant drugs including oral inhibitors of factor Xa (eg, rivaroxaban, apixaban, edoxaban) and factor IIa (eg, dabigatran) also check excessive activation of coagulation pathway. Finally, fibrinolysis represents the process in which clots are broken down to permit tissue repair.<sup>55</sup> Disturbances in procoagulant or anticoagulant factors can delay clot formation or dissolution, cause bleeding disorder, or increase blood clotting and thrombosis.<sup>52</sup>



**Figure 2** Platelets: coagulation cascade cross talk in hemostasis. Primary hemostasis refers to platelets' activation, adhesion, and aggregation at the site of endothelium injury. Adherence of platelets onto the exposed collagen takes place in a multifaceted process, and as a result platelets form a plug at the vascular injury site. Secondary hemostasis refers to the activation of the coagulation cascade. Both the intrinsic and extrinsic pathways converge into a common pathway and involve a series of sequential cleavage and activation of coagulation factors (XII, XI, IX, X, VII, III), ending with zymogen prothrombin (factor II) activation to thrombin (factor IIa). Activated thrombin catalyzes fibrinogen (factor I) cleavage to fibrin (Ia), and polymerization of fibrin monomers into an insoluble fibrin matrix strengthens the clot formation around the platelet plug. Fibrinolysis, through the action of plasmin, prevents unnecessary intravascular fibrin accumulation and enables the removal of thrombi. Protein C (PC), protein S (PS), and antithrombin II (ATII) inhibit the factors Xa/IIa and fibrin (Ia) formation. Tests and biomarkers used in clinical and research settings for examining the activation of platelets and coagulation cascade include, prothrombin fragment F1+2, thrombin time (TT); endogenous thrombin potential (ETP), factor VII, P-selectin, fibrinogen, and tissue factor (TF). Fibrinolysis is measured by plasminogen activator inhibitor I (PAI), plasmin-antiplasmin complex (PAP), and D-dimer tests. Figure created using BioRender.

## Thromboembolism and Stroke in CD

Thromboembolic events have been reported to occur at a high frequency in chronic Chagas heart disease. Samuel et al<sup>56</sup> examined 1345 CD patients, of which 44% exhibited cardiac thrombosis and/or thromboembolic events affecting arteries in lungs, kidneys, and brain. Arteaga-Fernandez et al<sup>57</sup> recorded 73% of CD patients (81 of 111) had cardiac thrombosis and many of them also showed systemic thrombosis. Further, euglobulin lysis time (measures fibrinolysis) in patients with chronic CD differed significantly from the values obtained in the control group, suggesting an occurrence of active thrombosis with decreased fibrinolysis.<sup>56,58</sup> Subsequently, strokes were found to occur at a higher rate in chronic CD patients when compared to patients with heart disease of other etiologies.<sup>46</sup> A history of stroke/transient ischemic attack, atrial fibrillation, and CD-positive serology were strongly associated with the occurrence of ischemic stroke in CD.<sup>59</sup> Multivariate analysis showed cardioembolic CD patients with apical aneurysm and left ventricular (LV) intracavitary thrombi are highly susceptible to develop into ischemic cerebrovascular events.<sup>60</sup> Cardiomyopathy was noted as an independent risk factor for the occurrence of cerebrovascular events in CD patients.<sup>46,59</sup> At a population level, up to 18%–20% of CD patients with chronic heart failure exhibit cerebral infarctions, and nearly half of them have an association between cerebral infarction and death.<sup>49</sup> It is proposed that progressive cardiac segmental contractile changes and aneurysm induce the formation of paradoxical emboli, ie, thrombi from dyskinetic areas of the cardiac cavities detach and are released in the cardiac vena cava and subsequently into the circulation, from where thrombi can reach the brain or other extremities. Therefore, the American Heart Association scientific statement on current knowledge and management of CD concludes that class III–IV LV dysfunction, cardiac dilation, significant electrocardiogram

abnormalities, low ejection fraction, and chronic visceral venous congestion are all risk factors in higher propensity to thromboembolism and ischemic and cerebral stroke in CD patients.<sup>19</sup>

## Role of Hypercoagulation in Induction of Thrombosis in Early Stages of CD

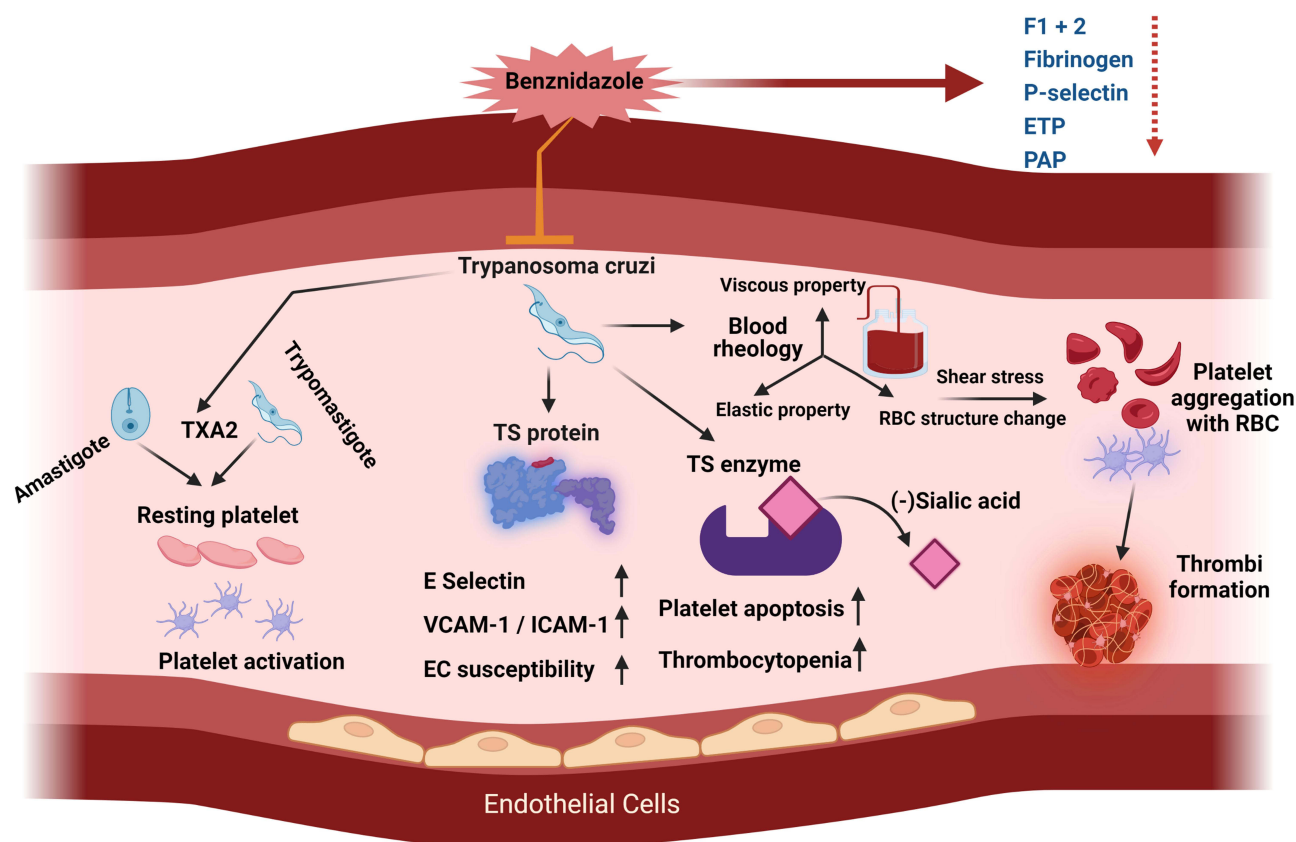
While cardiomyopathy in chronic CD is a known pathological process contributing to thrombosis, several studies have recognized that hypercoagulability, a cause of thrombus formation, occurs in acute-to-indeterminate phases of CD when symptomatic cardiomyopathy is not noted. The presence of procoagulation/prothrombotic phase in early stages of CD has been evidenced by elevated plasma levels of prothrombin fragment F1+2, D-dimer (fibrin degradation product), and the antithrombin enzyme complex in seropositive patients.<sup>58,61</sup> However, Melo et al did not find higher prothrombotic states among heart-failure patients with CD compared to that noted in non-CD patients.<sup>62</sup> Echeverria et al<sup>63</sup> performed a meta-analysis of studies evaluating the pathophysiological aspects of hypercoagulation in CD patients in Argentina, Brazil, and Spain during 1996–2016. Cumulatively, the seven studies included in the meta-analysis enrolled 311 (57%) seropositive CD patients (mostly in the indeterminate phase) and 234 (43%) seronegative, healthy controls. These studies investigated biomarkers of EC activation, ie, sVCAM1, PLT activation (CD41, CD62P, P-selectin after ADP stimulation), thrombosis (F1+2, D-dimer, fibrinogen, FVII), and fibrinolysis (PAI1, PAP). The authors found a significant increase in F1+2 (SMD 5.15, 95% CI 1.92–8.38) and PAI1 (SMD 0.46, 95% CI 0.07–0.89) in CD vs healthy individuals.<sup>63</sup> High levels of F1+2, an important marker of thrombin formation in vivo, may reflect endothelium injury by invading parasites or inflammation. PAI1 is a member of the superfamily of serpin-protease inhibitors and the primary inhibitor of tissue and urokinase-type plasminogen activators (tPA and uPA) that promote plasminogen cleavage to plasmin for its essential role in fibrinolysis. Increased PAI1 expression thus indicates excessive fibrin deposition in indeterminate CD.<sup>64</sup> Four of the seven studies included in the meta-analysis examined PLT function. One study noted decreased frequency of activated PLTs based on the expression levels of CD41a and CD62P and of P-selectin after ADP stimulation in CD patients.<sup>65</sup> Two studies noted significant increases in soluble P-selectin in adults and children with indeterminate CD compared to matched healthy controls.<sup>66,67</sup> In a meta-analysis with a random-effect model, P-selectin was significantly increased in the indeterminate phase of CD (SMD 1.8, 95% CI 0.13–3.47).<sup>63</sup> P-selectin is the largest member of the selectin family of cell-adhesion receptors, and it provides stability to the PLT aggregates, favoring thicker and more compact thrombi.<sup>68</sup> These studies indicate that the high frequency of PLT activation, coagulation disorder, and thrombosis appear in indeterminate CD before the cardiac abnormality of clinical importance is detected in chronic CD patients. Further longitudinal studies will be needed to establish the role of hypercoagulability and prothrombotic status in the development of clinical features of heart failure and cerebral stroke in CD.

## Parasite and Host Factors Contributing to Procoagulation State in CD

### Parasite Factors

The literature discussed provides some evidence that an active thrombogenesis process takes place during indeterminate CD when cardiomyopathy and heart failure are not apparent. Some studies have indicated the role of *T. cruzi* factors in hypercoagulation syndrome in early-to-indeterminate CD, and these are discussed next (Figure 3). In 1990s, Tanowitz showed that ECs infected with *T. cruzi* exhibit increased adherence to PLTs in vitro, and PLTs of *Tc*-infected (vs control) mice were two–six-fold more sensitive to aggregation induced by ADP and arachidonic acid.<sup>69</sup> Tanowitz et al also reported that TXA<sub>2</sub> (stimulates PLT activation) is expressed in all developmental forms of *T. cruzi*, maximal TXA<sub>2</sub> is expressed in replicative amastigote form, and *Tc* TXA<sub>2</sub> accounts for up to 90% of the circulating levels of TXA<sub>2</sub> in infected mice.<sup>70</sup> They concluded that increased production of TXA<sub>2</sub><sup>70</sup> and endothelin 1 (EC-activation marker) in CD<sup>71</sup> likely mediate PLT adhesion, activation, and aggregate formation, at least in the acute parasitemic phase of CD. PLT aggregates contributing to transient thrombi have been detected in small epicardial and intramyocardial vessels of *T. cruzi*-infected animals.<sup>72</sup>

*T. cruzi* expresses a large family of *trans*-sialidase (TS) surface glycoproteins, a majority of which are enzymatically inactive.<sup>73</sup> The enzymatically inactive members of the TS family have been shown to signal the expression of E selectin, VCAM1, and ICAM1, and increase EC susceptibility.<sup>74</sup> Others suggested that *T. cruzi* sheds enzymatically active TS that removes sialic acid from PLT surfaces<sup>75</sup> and may contribute to thrombocytopenia in CD.



**Figure 3** *Trypanosoma cruzi* factors contributing to hypercoagulability and thrombosis. *T. cruzi* (all life-cycle stages) releases thromboxane 2 (TXA2), a known activator of platelets, and trans-sialidase (TS) proteins during differentiation from trypomastigote to amastigote form. Endothelial cells (ECs) were susceptible to TS-induced increases in E selectin, VCAM1, and ICAM1 release. The enzymatically active isoform of TS (transfers sialic acid) induced platelet apoptosis and thrombocytopenia. *T. cruzi* infection-induced changes in blood rheological properties causing abnormal red blood cell (RBC) morphology can contribute to increased RBC-platelet aggregates and thrombi formation and affect smooth blood flow. Treatment with the antiparasite benznidazole was shown to reduce the biomarkers of platelet activation (P-selectin) and hypercoagulation (fibrinogen, prothrombin fragment F1+2, endogenous thrombin potential (ETP), plasmin antiplasmin complex (PAP)) in individuals exposed to acute *T. cruzi* infection. Figure created using BioRender.

Blood vessel–wall injury due to circulating parasites and changes in blood viscosity due to parasite-mediated host immune response may also augment hypercoagulability and facilitate the development of thromboembolic events in early-to-indeterminate CD. Berra et al<sup>76</sup> showed increases in hematocrit and mean corpuscular volume, abnormal morphological changes in red blood cells, and increases in plasma viscosity in acutely infected (vs control) rats. The authors concluded that *T. cruzi* infection induced changes in host blood properties and defensive responses determine the blood hyperviscosity that is an important ischemic risk factor capable of altering microvascular blood flow. The role of abnormally shaped red blood cells in promoting thrombus formation and high viscosity of blood in enhancing the stability of thrombi has also been documented in other diseases.<sup>77</sup> Finally, it has been shown that treatment with the antiparasitic benznidazole for 60 days resulted in a significant reduction in plasma levels of P-selectin, F1+2, fibrinogen, PAP, and ETP in acutely infected patients.<sup>66,67</sup>

Together, these findings imply that parasites and parasite-derived factors can contribute to endothelial dysfunction, PLT activation, and hypercoagulability in CD, and highlight the relevance of treatment with benznidazole in early stages of CD. Whether the benefits of antiparasitic treatment in reducing coagulation factors would translate into protection from stroke and chronic severity of CD remains to be investigated in future studies.

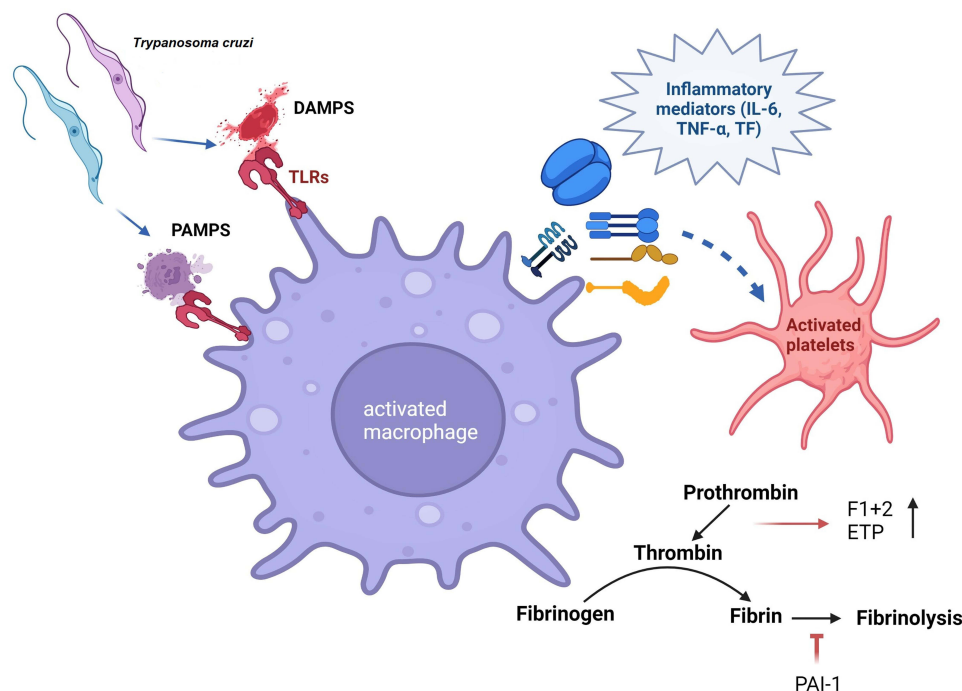
## Host Factors

Circulating and tissue parasite burden is very low in the indeterminate-to-chronic phase of CD. Therefore, we postulate that parasite factors may not solely be responsible for hypercoagulability in indeterminate CD. TF stimulation of

thrombin is quantified by endogenous thrombin potential that is increased in indeterminate CD.<sup>66,67</sup> TF is expressed by several cells, including ECs, neutrophils, and Mo/Mφ, after exposure to inflammatory cytokines, eg, TNFα and IL6, and TF is the primary mediator of interactions between inflammatory and coagulation pathways.<sup>78</sup> Patients with indeterminate CD exhibit higher plasma levels of TNFα<sup>79</sup> and IL6<sup>80</sup> than uninfected healthy controls. We have documented a significant increase in peripheral and myocardial levels of CD11b<sup>+</sup>CD68<sup>+</sup>TNFα<sup>+</sup> Mo/Mφ in CD.<sup>81,82</sup> In vitro studies have demonstrated significantly elevated levels of proinflammatory cytokines (IL6, IL1β) in the supernatants of *T. cruzi*-infected ECs.<sup>83</sup> Parasite-infected ECs exhibit NFκB transcriptional activation and increased expression of E-selectin, ICAM1, and VCAM1.<sup>83,84</sup> It is thus conceivable that ECs or circulating Mo/Mφ, after encountering *T. cruzi*, *Tc* pathogen-associated molecular patterns (PAMPs), or *Tc*-induced damage associated molecular patterns (DAMPs) and proinflammatory cytokines, begin to express TF that then promotes coagulation cascade and thrombin production in CD (Figure 4). This hypothesis, however, remains to be tested in experimental and clinical studies.

P-selectin mediates adhesion of PLTs with endothelium and leukocytes, and soluble P-selectin signals recruitment of cells and cell particles containing TF to the injury site.<sup>85</sup> Likewise, PAI1 has been identified as a key factor linking fibrinolysis with subclinical (eg, inflammation, atherosclerosis) and clinical (eg, metabolic syndrome) conditions.<sup>64</sup> Circulating proinflammatory cytokines can directly activate PLTs and release of P-selectin and PAI1, among others.<sup>86,87</sup> An increase in soluble P-selectin and PAI1 associated with an increase in PLT death has been noted in patients with indeterminate CD.<sup>63</sup> It is possible that inflammatory responses that continue to persist in all stages of *T. cruzi* infection and disease progression signal activation and exhaustion of PLTs in CD. If such is the case, these events will contribute to PAI1-mediated increases in fibrin deposition and soluble P-selectin-mediated enhanced stability of PLT-leukocyte aggregates and formation of thicker thrombi. Subsequently, P-selectin may also contribute to apoptosis of activated PLT.

Summarizing, proinflammatory mediators and *Tc*-activated EC and Mo/Mφ potentially contribute to TF-induced PLT activation and hypercoagulability in acute-to-indeterminate CD. However, mechanistic studies documenting PLT-



**Figure 4** *Trypanosoma cruzi*-macrophage interactions and induction of thrombosis. *T. cruzi* releases pathogen-associated molecular patterns (PAMPs), and *T. cruzi*-mediated cellular injury generates damage-associated molecular patterns (DAMPs). Macrophage uptake of PAMPs and DAMPs through Toll-like receptors (TLRs) may signal the release of inflammatory mediators, eg, IL6, TNFα, and tissue factor (TF) to induce the activation of platelets and coagulation cascade. Increase in prothrombin fragment F1+2, endogenous thrombin potential (ETP), and PAI1 is noted in Chagas disease. Figure created using BioRender.

immune cell interactions and the signals that contribute to disturbances of hemostasis in early-to-indeterminate CD have not yet been conducted.

## Platelet Role in Inflammation

A connection between PLTs and inflammation is very well recognized in invertebrate arthropods, in which a single cell type (hemocytes) carries out the function of innate immune cells (eg, phagocytosis, antimicrobial release), as well as wound plugging and repair.<sup>88</sup> Considering that PLTs constantly scan the endothelium and vascular injury, it is not surprising that they are well positioned to act as first responders to detect and respond to invading pathogens. We briefly discuss PLT involvement in shaping host immunodefense and inflammatory responses and extrapolate the literature findings in the context of CD here.

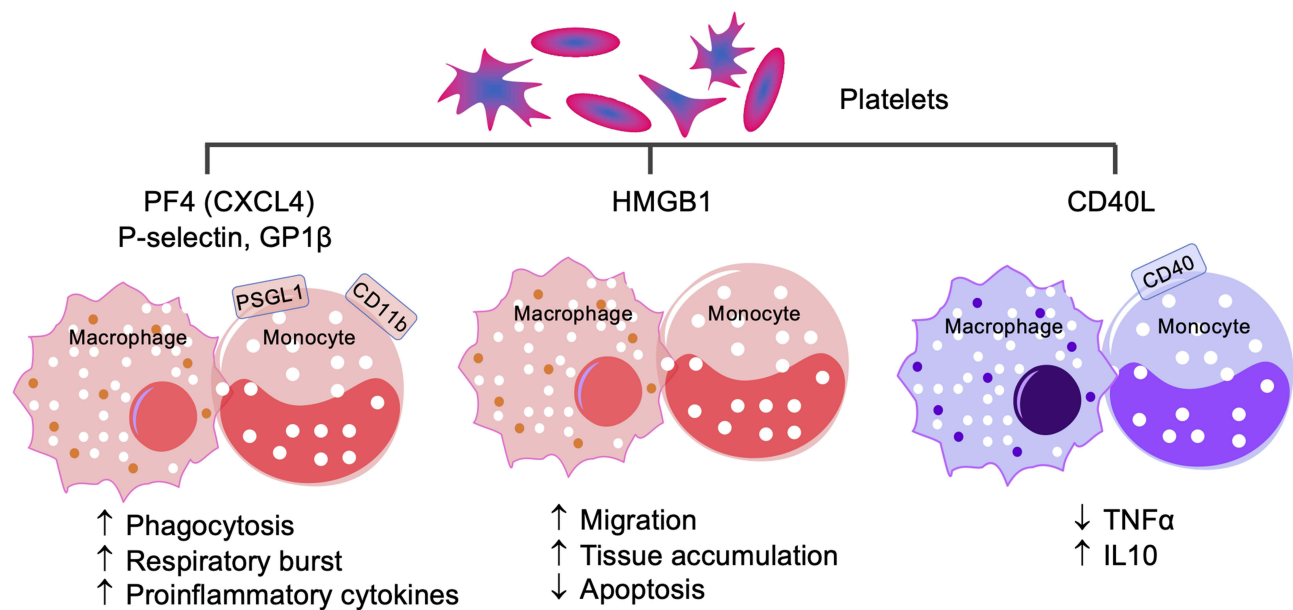
PLTs synthesize and package molecules in dense  $\alpha$ -granules and selectively release prothrombotic, proinflammatory, or immunoregulatory molecules depending on the microenvironment. Activated PLTs have an arsenal of inflammation inducers, including P-selectin, sCD40L, IL1 $\beta$ , HMGB1, C–C motif chemokine ligand (CCL) family members, such as CCL3 (MIP1 $\alpha$ ), CCL5 (RANTES), and CCL7 (MIP3), and C–X–C chemokine ligand (CXCL) family members, eg, CXCL1, CXCL4 (PF4), CXCL5, and CXCL7.<sup>89</sup> PLTs also release the mediators of tissue repair, such as TGF $\beta$ , depending on the microenvironment.<sup>90,91</sup> Activated PLTs can rapidly bind to and engulf microbes, and their direct role in the control of pathogens is discussed elsewhere.<sup>92</sup>

PLT binding shapes the proinflammatory or immunoregulatory activation of immune cells. In general, PLTs utilize the classical PSGL1 axis to adhere to all types of immune cells and employ selective receptors to adhere to specific population of immune cells. Nonclassical pathways also activate PLT–immune cell interactions via engagement of a vast repertoire of pattern-recognition receptors including Toll-like receptors (TLR2, TLR4, TLR9), C-type lectin-like receptors, and TNF $\alpha$  and IL1 $\beta$  receptors.<sup>93,94</sup>

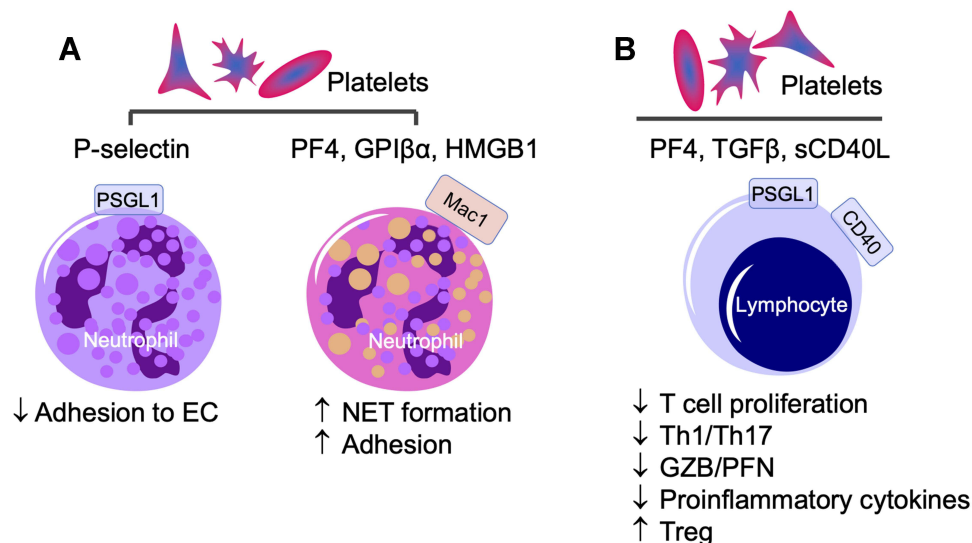
With reference to influence on Mo/M $\phi$ , PLT interactions with Mo/M $\phi$  through P-selectin–PSGL1 induce the expression of MCP1 and IL8 in Mo/M $\phi$ , while GPIIb–CD11b induces proinflammatory response with increased TNF $\alpha$  production<sup>95</sup> (Figure 5). Circulatory PF4/CXCL4 is increased in chronic inflammation of diverse etiologies.<sup>96,97</sup> In experimental models, PF4/CXCL4 and P-selectin have been shown to potentiate phagocytosis, respiratory burst, and a three–ten-fold increase in proinflammatory cytokines expression in human monocytes.<sup>98,99</sup> PLT release of HMGB1 induced monocyte migration and accumulation in tissue by inhibiting apoptosis in a TLR-dependent manner.<sup>100</sup> CD40L is primarily secreted by activated PLTs, which signals a CD40-mediated immunoregulatory phenotype in human monocytes by enhancing IL10 and suppressing TNF $\alpha$  production (Figure 5).<sup>101</sup>

Regarding PLTs' role in shaping the neutrophil response, PLT and neutrophil interactions through P-selectin–PSGL1 or GPIIb–integrin  $\alpha$ M $\beta$ 2 (Mac1) axes have been shown to modulate neutrophil adhesion to ECs and neutrophil extracellular trap (NET) release (Figure 6A).<sup>102</sup> Likewise, PLT release of HMGB1 enhances neutrophil activation and NET formation (Figure 6A).<sup>103,104</sup> PLT–neutrophil interactions facilitate NET release, providing protection from poxvirus infection.<sup>105</sup> Human PLT release of  $\beta$ -defensin–induced NET formation impairs the growth of clinical strains of *Salmonella aureus*.<sup>106</sup> PLT–neutrophil interactions may also inform the pathogenesis of invasive *S. pyogenes* infection.<sup>107</sup> Conversely, PF4/CXCL4 inhibition decreases vasculature inflammation and preserves heart function by reducing leukocyte recruitment, adhesion, and extracellular nuclear DNA trap release by activated neutrophils.<sup>108</sup>

The specific role of PLT-adhesion molecules is, however, context-dependent. For example, PF4 has been shown to inhibit the proinflammatory, cytotoxic activation of CD8<sup>+</sup> T cells<sup>109</sup> and limit the T<sub>h</sub>1 and T<sub>h</sub>17 cell differentiation and cardiac allograft rejection by suppressing ROR $\gamma$  expression (Figure 6B).<sup>110</sup> Likewise, elevated CD40L levels induce immunosuppression by mediating the regulatory T cell (T<sub>reg</sub>) expansion in HIV-infected patients.<sup>111</sup> Some studies have shown that PLT expression of TGF $\beta$  suppresses lymphocyte function, while IL1 $\alpha$ /IL1 $\beta$  plays a crucial role in systemic and cerebrovascular inflammation.<sup>112,113</sup> Specifically, PLT TGF $\beta$  inhibited the expression of proinflammatory cytokines (IFN $\gamma$ ) and cytotoxic molecules (granzyme B and perforin) in CD8<sup>+</sup> T cells, and TGF $\beta$  inhibitors improved adoptive T-cell therapy in multiple cancers in mice.<sup>114</sup> PLT release of TGF $\beta$  also activates the T<sub>reg</sub> phenotype and suppresses the T<sub>h</sub>1/T<sub>h</sub>17 response in human CD4<sup>+</sup> T cells<sup>115</sup> (Figure 6B). Likewise, PLT–CD4<sup>+</sup> T cell aggregates exhibit reduced



**Figure 5** Platelet–monocyte/macrophage interactions. PF4, also known as CXCL3, P-selectin, and GPI $\beta$  interact with PSGL1 and CD11b receptor–positive monocytes/macrophages to signal proinflammatory activation, while platelet expression and release of HMGB1 signals monocyte/macrophage migration to tissue and cell apoptosis. Conversely, CD40L interacts with CD40 to drive the anti-inflammatory, immunomodulatory monocyte/macrophage response by suppressing proinflammatory cytokine TNF $\alpha$  and increasing anti-inflammatory IL10.



**Figure 6** Platelet activation of neutrophils and lymphocytes. (A) Platelets signal neutrophils via platelet factor 4 (PF4), GPI $\beta$ , or HMGB1 interactions with the macrophage 1 (Mac1) antigen, which enhances neutrophil extracellular trap (NET) release. P-selectin–PSGL1-mediated platelet–neutrophil interaction suppresses neutrophil adhesion to endothelial cell (ECs) and subsequent activation. (B) Platelet–lymphocyte interactions via PF4–PSGL1, CD40L–CD40, or platelet release of TGF $\beta$  result in a decline in T-cell proliferation, decreased expression of T helper 1/T helper 17 (T<sub>h</sub>1/T<sub>h</sub>17) proinflammatory cytokines and granzyme (GZB) and perforin (PFN) cytotoxic molecules, and upregulation of immunomodulatory T<sub>h</sub>2 regulatory T-cell response.

proliferation and proinflammatory cytokine production in rheumatoid arthritis patients, and higher levels of PLT–CD4<sup>+</sup> T-cell aggregates correlate with reduced risk of cardiovascular disease.<sup>116</sup>

In summary, PLTs can exert dual function, ie, proinflammatory and immunoregulatory on Mo/M $\phi$ , neutrophils, and distinct subsets of T lymphocytes. How the expression of different receptors and ligands on PLTs and immune cells is determined in different conditions, how microenvironmental conditions influence the PLT and immune cells interactions

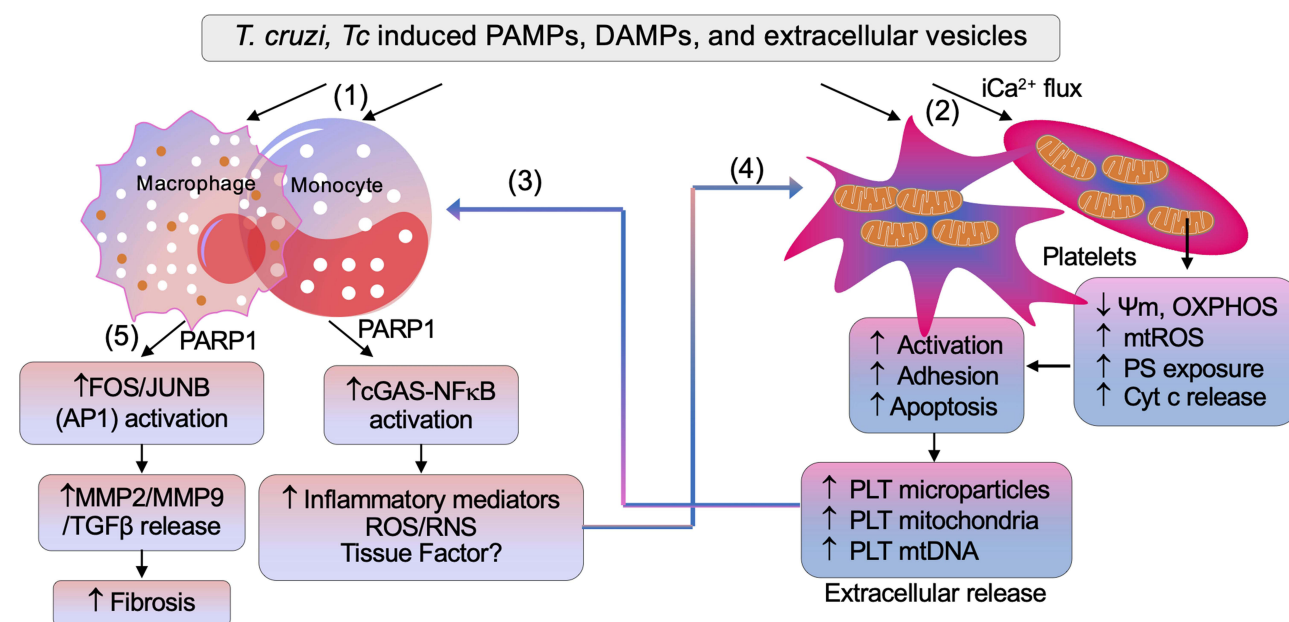
and whether PLT stimulated immune cells migrate to tissue to exert their effects is not completely understood and requires further investigation.

## A Perspective on the Role of Platelets' Partnership with Macrophages in Chronic Inflammation in CD

There is practically no information in literature on PLT-neutrophils partnership in CD and the limited literature on the potential role of neutrophils in CD is summarized recently.<sup>117</sup> Herein, we will discuss our perspectives on potential role of PLT – M $\phi$  interactions in chronic inflammation in CD (Figure 7).

### Proinflammatory Macrophage Response in CD

Studies in human and mouse macrophages show that *T. cruzi* elicits  $\text{Ca}^{2+}$  flux for invasion of immune and non-immune cells, and signals low levels of NADPH oxidase mediated oxidative burst, inducible nitric oxide synthase mediated nitric oxide production and delayed inflammatory cytokines/chemokines response in innate immune cells.<sup>118,119</sup> Because of this, M $\phi$  are not able to clear the parasites, and instead may support parasite dissemination to tissue.<sup>120,121</sup> With the activation of adaptive B and T cell immunity, parasitemia is controlled, and very low levels of circulating parasites are infrequently detected in the patients progressing from indeterminate to chronic CD. Yet, CD patients present systemic increase in inflammatory markers (N-terminal pro-brain natriuretic peptide, C-reactive protein), inflammatory cytokines (eg, IL1 $\beta$ , IL6, IFN $\gamma$ , TNF $\alpha$ ) and chemokines (eg, CXCL9, CXCL10), and free radical induced oxidative lesions of lipids (lipid hydroperoxides, 4-hydroxynonenal), DNA (8-oxo-7,8-dihydro-2'-deoxyguanosine) and proteins (protein carbonyls, cysteine nitrosylation) during indeterminate-to-chronic CD (reviewed in)<sup>122–124</sup>. CD patients also display a proinflammatory transcriptomic and proteomic profile in peripheral blood mononuclear cells,<sup>125–128</sup> and increased levels of TNF $\alpha$ <sup>+</sup>



**Figure 7** Potential cross talk between platelets and macrophages in Chagas disease. (1) *Trypanosoma cruzi* (Tc), and Tc-induced pathogen associated molecular patterns (PAMPs), damage associated molecular patterns (DAMPs), and extracellular vesicles signal initial proinflammatory activation of macrophages. (2) Platelets encountering *T. cruzi*-induced molecules undergo intracellular  $\text{Ca}^{2+}$  flux-mediated mitochondrial stress presented by a decline in membrane potential ( $\Psi_m$ ) and oxidative phosphorylation (OXPHOS) capacity, and an increase in mitochondrial reactive oxygen species (mtROS), Cyt c release, and phosphatidylserine (PS) exposure. These events signal platelet activation, aggregation, and subsequent apoptosis. (3) Extracellular vesicles, microparticles, mitochondria, and mtDNA released by Tc, Tc-infected cells, and platelets are phagocytized by macrophages, and elicit transcriptional activation of mediators of inflammation and oxidative stress (ie, ROS and reactive nitrogen species [RNS]). (4) Macrophage inflammatory mediators, along with tissue factors, can sustain platelet activation and apoptosis. (5) PARP1-API synergy also signals release of profibrotic molecules, ie, matrix metalloproteinase 2 (MMP2), MMP9, and TGF $\beta$ , leading to tissue fibrosis in Chagas disease.

**Abbreviation:** cGAS, cyclic GMP-AMP synthase.

monocytes<sup>129–131</sup> in circulation. Tissue distribution of proinflammatory Mo/M $\phi$  is also increased in chronic CD.<sup>132</sup> Thus, Mo/M $\phi$  are recognized as major drivers of chronic inflammation in CD.

## Extracellular Vesicles Signal Proinflammatory Activation of Macrophages in CD

Recent literature documents that *T. cruzi* sheds compositionally different membrane vesicles, also referred as extracellular vesicles (EV), depending on their developmental stage.<sup>133,134</sup> EV of infective trypomastigote form of *T. cruzi* were fused to host cell membranes and promoted EV release from THP1 M $\phi$ .<sup>135–138</sup> We showed that EV are released in media of cells, eg, human peripheral blood mononuclear cells (PBMC), mouse and human M $\phi$ , and human cardiac myocytes in vitro incubated with *T. cruzi* and in peripheral blood of chronically infected mice and humans, and these *Tc*-induced EV signaled the proinflammatory gene expression in primary and cultured M $\phi$ .<sup>81,139</sup> Compositional and molecular analysis showed that EV released in plasma of CD patients were of leukocytes and endothelial origin and packaged oxidized DNA (oxDNA) of host and parasite.<sup>81,139</sup> Treatment of these EV with DNase abrogated the inflammatory signature, thus indicating that EV<sub>oxDNA</sub> is necessary for proinflammatory activation of M $\phi$  in CD.<sup>81</sup>

Poly(ADP-ribose) polymerase 1 (PARP1) is the enzymatically active member of the PARP family of 18 proteins. PARP1 senses DNA oxidative damage to catalyze NAD<sup>+</sup> dependent poly-ADP-ribosylation of target histone proteins and functions in DNA repair.<sup>140</sup> PARP1 expression and activity were increased in CD;<sup>141</sup> however, PARP1 enhanced the mitochondrial ROS production and nuclear factor kappa B (NF $\kappa$ B) transcriptional activation of inflammatory gene expression in cardiac myocytes and M $\phi$  in response to *T. cruzi* infection.<sup>81</sup> PARP1 synergized with cytosolic DNA response element cyclic GMP-AMP synthase (cGAS) in sensing the EV<sub>oxDNA</sub> shed in plasma of *T. cruzi* infected animals and humans to transmit the signal for NF $\kappa$ B-dependent inflammatory gene expression in human and mouse M $\phi$ .<sup>81</sup> Further, PARP1 signaled the FOS/JUNB (members of the activator protein 1 (AP1) complex) transcriptional activation of matrix metalloproteinases (MMP2, MMP9) and TGF $\beta$  expression in M $\phi$  infected with *T. cruzi*.<sup>82</sup> Consequently, PARP1 deficiency by genetic, chemical, or siRNA approaches was beneficial in inhibiting the inflammatory and fibrotic response in M $\phi$  incubated with *T. cruzi* or *Tc*-induced EV, decreasing the oxidative and inflammatory stress in infected mice, and improving the mitochondrial and cardiac function in chronic CD mice.<sup>81,82,141</sup> These studies conclude that EV<sub>oxDNA</sub>/PARP1 crosstalk with cGAS-NF $\kappa$ B and AP1 signaling pathways in Mo/M $\phi$  to sustain inflammation and fibrosis with progression of chronic CD. The source of oxidized DNA that triggers chronic proinflammatory/profibrotic M $\phi$  response in CD was not defined in these studies.

## Platelet Shedding of Mitochondria and Its Potential Role in Chronic Inflammation in CD

PLTs lack chromosomes but pack 5–8 mitochondria to meet their energy demand. PLT are highly flexible in energy source utilization for their classical function in hemostasis.<sup>142</sup> Double knockout of glucose transporters GLUT1 and GLUT3 led to mitochondria reprogramming, and reduced thrombosis, PLT activation, and thrombocytopenia.<sup>143</sup> Likewise, PLT treated with pharmacological inhibitors of mitochondrial respiratory complexes exhibited reduced thrombosis activity,<sup>142</sup> though mechanisms were not discussed.

Recent studies indicate that mitochondria influence the PLT health and activation via their role in regulation of cell survival and apoptosis. Briefly, disturbances in mitochondrial Ca<sup>2+</sup> hemostasis can signal mitochondrial membrane permeability transition, increased ROS production, cytochrome c release, and involvement of pro- and anti-apoptotic members of B cell lymphoma 2 (Bcl-2) family to guide the activation of caspase cascade and cell death decision.<sup>144,145</sup> B cell lymphoma extra-large (Bcl-xl) protein was shown to govern the lifespan of healthy PLT,<sup>146</sup> while iCa<sup>2+</sup> flux facilitated the collagen, thrombin, and ADP mediated PLT activation.<sup>147</sup> PLT expression of Bcl-2 family proteins and exposure of phosphatidylserine that facilitates recognition and removal of the apoptotic cell by phagocytes were deemed essential for PLT adhesion and apoptosis.<sup>146,148</sup> Apoptotic PLT (and their microparticles) induced 50–100-fold higher procoagulant activity than the quiescent, healthy PLTs.<sup>149</sup> Overall, these studies indicate that mitochondrial signals drive the thrombotic function and subsequent apoptosis of activated PLTs.

*T. cruzi* induction of mitochondrial dysfunction is well documented in cardiac myocytes (reviewed in<sup>8,26</sup> and briefly discussed in Introduction). Considering that PLT encounter circulating parasites, especially in acute phase, *T. cruzi* signals  $\text{Ca}^{2+}$  flux during invasion, and *Tc*-released PAMPs and DAMPs signal macrophage proinflammatory response and possibly PLT aggregation and activation, it is conceivable that *T. cruzi* may also initiate mitochondrial dysfunction in PLTs, and consequently, enhance the PLT apoptosis and PLT mitochondria derived EV release in circulation.

Indeed, cell-derived mitochondrial components including mtDNA can be found free or enclosed by membrane vesicles in the peripheral blood of healthy subjects and patients with various diseases.<sup>150–152</sup> Meddeb et al<sup>153</sup> reported that ~50,000 fold more copies of mitochondrial genome than the nuclear genome is present in circulation of healthy individuals, and these further increase in disease conditions. The cell free mtDNA is less fragmented than the nuclear genome,<sup>154</sup> and, therefore, circulatory mtDNA has emerged as biomarker of multiple diseases including diabetes, myocardial infarction, cancer, and trauma.<sup>150–152</sup> Despite the clinical implications, knowledge regarding the origin and function of cell free mtDNA and how they are protected from degradation in circulation is lacking. Some studies have reported that monocytes and PLT are the primary source of cell free mitochondria and mitochondrial fragments found in circulation, and free and EV-encapsulated mitochondrial fragments and mtDNA released by activated monocytes and PLT serve as inflammatory trigger.<sup>155,156</sup> Indeed, mitochondria isolated from lipopolysaccharide (LPS) activated Mo/M $\phi$ , EV released by LPS-activated Mo/M $\phi$ , as well as EV isolated from plasma of human volunteers receiving low-dose LPS-injections induced type I IFN and TNF $\alpha$  response in ECs.<sup>157</sup> Activated PLTs released respiratory-competent mitochondria, both within EV and as free organelles, and mitochondrial membranes and mtDNA present in these PLT-derived extracellular mitochondria promoted leukocytes activation, neutrophils adhesion and inflammatory responses.<sup>158</sup> The mtDNA encapsulated in circulating EV of chronic CD patients signaled proinflammatory M $\phi$  activation (discussed above), and we postulate that the mtDNA in circulation of CD patients are primarily derived from activated PLT contributing to hypercoagulability. Overall, despite several gaps in our knowledge, we surmise that a crosstalk between PLT and macrophages via circulating mtDNA (oxidized or native) might feed the chronic inflammatory and oxidative stress in progressive CD development.

## Concluding Remarks

In this review, we have summarized the current literature describing the general and CD-specific features of PLTs' role in coagulopathy and inflammation. While inflammation, oxidative stress and hypercoagulability are well-recognized characteristics of CD; the literature discussed here suggests that these pathological events might occur because of the crosstalk between monocytes/macrophages and PLTs. While *T. cruzi* and *Tc*-derived PAMPs may initially elicit Mo/M $\phi$  and PLT activation, we propose that a feedback cycle of inflammation and hypercoagulation persists in CD because PLT activation for signaling the coagulation cascade is followed by PLT apoptosis and release of mitochondria, mitochondrial particles and mtDNA in circulation, that then serve as DAMPs in proinflammatory activation of Mo/M $\phi$  in CD. Thus, we envision that new therapies modulating PLT activation and mitochondria dysfunction or reprogramming the Mo/M $\phi$  towards immunomodulatory phenotype will arrest excessive thrombus formation as well as inflammation in chronic CD.

Although some evidence is available regarding the beneficial effect of antiparasitic benznidazole therapy in correcting the hypercoagulable state in acutely infected children and young adults, these findings need to be repeated and verified in larger cohorts. Further, extensive preclinical and clinical research efforts are needed for evaluating the benefits and risks of anti-parasite, anti-PLT, and anticoagulant therapies in arresting the hypercoagulable state and thromboembolic events in indeterminate stage of CD. Longitudinal studies in which seropositive indeterminate CD patients are offered these therapies and followed for several years to monitor the progression to chronic CD would potentially offer new treatments to prevent the clinical manifestation of CD.

## Abbreviations

AT, antithrombin; CD, Chagas disease; CCL, C–C motif chemokine ligand family; CXCL, C–X–C chemokine ligand family; EC, endothelial cell; ETP, endogenous thrombin potential; F1+2, prothrombin factor 1 and 2; GP, glycoprotein; Mo/M $\phi$ , monocytes/macrophages; MMP, matrix metalloproteinase; NET, neutrophil extracellular traps; PAP, plasmin–

antiplasmin complex; PAMPs, pathogen-associated molecular patterns; ROS, reactive oxygen species; SMD, standard mean difference; TF, tissue factor; TXA2, thromboxane 2; VWF, von Willebrand factor.

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## Disclosure

The authors report no conflicts of interest in this work.

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