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AÇÃO DA LDL OXIDADA NA ATIVAÇÃO PLAQUETÁRIA DURANTE A DENGUE

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Dissertação de Mestrado submetida à banca examinadora do Curso de Pós-Graduação em Ciências Biológicas – Imunologia e Doenças Infecto-Parasitárias da Universidade Federal de Juiz de Fora, como parte dos requisitos necessários para obtenção do Grau de Mestre em Ciências Biológicas.

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RESUMO

A dengue é causada pelo vírus da dengue (DENV) e transmitida principalmente pela espécie *A. aegypti.* Pode se apresentar desde um quadro assintomático até quadros de aumento do extravasamento vascular, hemorragia grave e comprometimento de órgãos, caracterizando a dengue grave. Concentrações elevadas de citocinas inflamatórias contribuem para o aumento da permeabilidade vascular e gravidade da dengue. Nos pacientes com dengue os níveis de citocinais inflamatórias são correlacionados significativamente com a trombocitopenia, que, por sua vez, está associada positivamente com a fase crítica da doença. Além das funções hemostáticas plaquetárias, as plaquetas também participam das respostas inflamatórias, ao secretar citocinas e quimiocinas, além de interagir com outras células imunológicas. A ativação plaquetária é descrita na dengue, podendo contribuir para a trombocitopenia e também para a inflamação exacerbada e gravidade da doença. Os estímulos de ativação plaquetária ainda não são completamente elucidados. Alterações lipídicas séricas, como redução da lipoproteína de baixa densidade transportadora de colesterol - LDL (do inglês *low-density lipoprotein*) são observadas no curso da infecção e associadas positivamente à gravidade. Esses dados corroboram com o fato do colesterol ser fundamental na replicação dos *Flavivirus*. Além disso, em um ambiente de resposta imunológica e inflamação exacerbada, podemos considerar um alto risco de peroxidação lipídica devido ao aumento do estresse oxidativo. Nesse sentido, é bem esclarecida a formação de compostos bioativos nas LDL oxidadas (oxLDL) que amplificam a resposta inflamatória ao ativar células imunológicas. Portanto, este trabalho teve como objetivo investigar a participação da oxLDL na patogênese da dengue, identificando mecanismos de ativação e apoptose plaquetária e secreção de mediadores inflamatórios pelas plaquetas. As LDL dos pacientes com dengue foram isoladas por gradiente de densidade e ultracentrifugação e avaliadas por teste T-BARS e Western Blot quanto à presença de aldeídos reativos (malondialdeído e 4-HNE, respectivamente) provenientes da peroxidação lipídica. Observou-se um aumento significativo da oxidação nas LDL dos pacientes com dengue. As plaquetas de voluntários saudáveis foram incubadas com LDL de pacientes com dengue em diferentes concentrações (0, 5, 10, 100 e 1000ng/mL) com ou sem trombina (0,02U/mL) e avaliadas por citometria de fluxo quanto à expressão de P-selectina, ativação da GPIIb/IIIa, expressão do receptor CD36 e apoptose (Anexina-V+/TMRE-). Os sobrenadantes após os estímulos plaquetários com LDL de pacientes com dengue ou de indivíduos saudáveis foram recolhidos e analisados por ELISA quanto à secreção de mediadores inflamatórios (IL-1α, IL-1β, PF4/CXCL4, RANTES/CCL5 e MIF). Apesar do maior nível de oxidação das LDLs de pacientes com dengue, estas não foram capazes de induzir ativação, apoptose e secreção de mediadores inflamatórios plaquetários quando comparadas às LDL de voluntários saudáveis. Sendo assim, as oxLDL de pacientes com dengue branda com ou sem sinais de alarme não podem ser apontadas como contribuintes para a ativação plaquetária na dengue. Outros estímulos envolvidos na ativação plaquetária durante a dengue devem ser elucidados.

Palavras-chave: Dengue. Ativação plaquetária. Lipoproteínas. oxLDL. Peroxidaçãolipídica.

ABSTRACT

Dengue is caused by the dengue virus (DENV) and transmitted mainly by the species A. aegypti. The disease can range from asymptomatic to increased vascular leakage, severe hemorrhage and organ damage, characterizing severe dengue.High concentrations of inflammatory cytokines contribute to increased vascular permeability and severity of dengue. In patients with dengue, the levels of inflammatory cytokines are significantly correlated with thrombocytopenia, which, in turn, is positively associated with the critical phase of the disease. In addition to platelet hemostatic functions, platelets also participate in inflammatory responses, by secreting cytokines and chemokines, in addition to interacting with other immune cells. Platelet activation is described in dengue, which may contribute to thrombocytopenia and also to exacerbated inflammation and severity of the disease.Platelet activation stimuli are not yet fully elucidated. Serum lipid changes, such as a reduction in low density lipoprotein - LDL, a cholesterol transporter, are observed in the course of infection and are positively associated with severity.These data corroborate the fact that cholesterol is fundamental in the replication of Flaviviruses. In addition, in an environment of immune response and exacerbated inflammation, we can consider a high risk of lipid peroxidation due to increased oxidative stress.In this sense, the formation of bioactive compounds in oxidized LDL (oxLDL) that amplify the inflammatory response when activating immune cells is well understood. Therefore, this work aimed to investigate the participation of oxLDL in the pathogenesis of dengue, identifying mechanisms of platelet activation and apoptosis and secretion of inflammatory mediators by platelets. LDL from patients with dengue were isolated by density gradient and ultracentrifugation and evaluated by T-BARS and Western Blot tests for the presence of reactive aldehydes (malondialdehyde and 4-HNE, respectively) from lipid peroxidation. There was a significant increase in oxidation in LDL in patients with dengue. Platelets from healthy volunteers were incubated with LDL from dengue patients at different concentrations (0, 5, 10, 100 and 1000ng/mL) with or without thrombin (0.02U/mL) and evaluated by flow cytometry for the expression of P-selectin, GPIIb/IIIa activation, CD36 receptor expression and apoptosis (Annexin-V+/TMRE-). Supernatants after platelet LDL stimulation from dengue patients or healthy individuals were collected and analyzed by ELISA for secretion of inflammatory mediators (IL-1α, IL-1β, PF4/CXCL4, RANTES/CCL5 and MIF). Despite the higher level of oxidation of LDLs in patients with dengue, they were not able to induce activation, apoptosis and secretion of inflammatory platelet mediators compared to LDL from healthy volunteers. Therefore, oxLDL from mild dengue patients with or without warning signs cannot be identified as contributors to platelet activation and apoptosis in dengue. Other stimuli involved in platelet activation during dengue should be elucidated.

Keywords: Dengue. Platelet activation. Lipoproteins. oxLDL. Lipid peroxidation.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

ABC – Transportador de cassete de ligação de ATP;

ACD – Anticoagulante contendo ácido cítrico, citrato de sódio e dextrose;

ADE – Amplificação dependente de anticorpo;

AKT – Proteína quinase B;

APAF-1 - Fator de ativação 1 da protease apoptótica;

APC – Aloficocianina;

APO – Apolipoproteína;

BHT – Hidroxitolueno butilado;

C – Proteína do capsídeo dos Flavivirus;

Caspase – Protease de cisteína-aspartato;

CCL – Quimiocina ligante do motivo CC;

CCR – Receptor de quimiocina do motivo CC;

CD – Cluster de diferenciação;

CETP – Proteína de transferência de colesterol esterificado;

CLR – Receptor de lectina tipo C;

COVID 19 – Doença por coronavírus;

CXCL – Quimiocina ligante do motivo CXC;

CXCR – Receptor de quimiocina do motivo CXC;

DAMP – Padrão molecular associado a dano;

DC-SIGN – Não-integrina acoplada a ICAM-3 específica de célula dendrítica

DENV – Vírus dengue;

DF – Febre do dengue;

DHF – Febre hemorrágica do dengue;

DSS – Síndrome do choque do dengue;

E – Proteína do envelope viral;

EDTA – Ácido etilenodiamino tetra-acético;

ELISA – Ensaio imunoenzimático de captura;

FIT-C – Fluoresceína isotiocinato;

FT – Fator tecidual;

GPCR – Receptor acoplado a proteína G;

GP – Glicoproteína;

HDL – Lipoproteína de alta densidade;

HIV – Vírus da imunodeficiência humana;

HMVEC – Células endoteliais microvasculares humanas;

HPA – Heparanase;

HUVEC – Células endoteliais de veia umbilical humana;

H_2O_2– Hidroperóxido de hidrogênio;

ICAM – Molécula de adesão intercelular;

IDL – Lipoproteína de densidade intermediária;

IFN – Interferon;

IG – Imunoglobulina;

IL – Interleucina;

INOS - Óxido nítrico-sintase induzida;

KBR – Brometo de potássio;

LCAT –Lecitina-colesterol aciltransferase;

LDH –Lactato desidrogenase;

LDL – Lipoproteína de baixa densidade;

LOX-1 – Receptor 1 de LDL oxidado do tipo lectina;

LPA – Ácido lisofosfatídico;

LPC – Lisofosfatidilcolina;

LPL – Lipoproteína lipase;

LXR – Receptores hepático X;

M – Proteína da membrana viral;

MCP – Proteína quimioatraente de macrófagos;

MDA – Malondialdeído;

MHC – Complexo principal de histocompatibilidade;

MIF – Fator inibidor da migração de macrófagos;

MMP– Metaloproteinase de matriz;

MPTP - Poro de transição de permeabilidade mitocondrial;

MV – Microvesícula;

NACL – Cloreto de sódio;

NETs– Armadilhas extracelulares dos neutrófilos;

NLR – Receptor do tipo NOD;

NLRP3 – NLR contendo domínio pirina 3;

NOX - Nicotinamida adenina dinucleótido fosfato oxidase;

NS – Proteína não estrutural de Flavivírus;

OMS – Organização Mundial da Saúde;

OPAS – Organização Panamericana de Saúde;

OSBP – Proteína de ligação ao oxysterol;

oxLDL – LDL oxidada;

oxPL – Fosfolipídio oxidado;

PAF – Fator de ativação plaquetário;

PAF-AH – PAF acetil hidrolase;

PAMP – Padrão molecular associado a patógeno;

PAR – Receptor ativado por proteases;

PBMC – Células mononucleares do sangue periférico;

PBS – Tampão fosfato e salina;

PGE – Prostaglandina E;

PMA – Agregados plaquetas-monócitos;

PON – Paraoxonase;

PPP – Plasma pobre em plaquetas;

PrM – Proteína pré-membrana viral

PRP – Plasma rico em plaquetas;

PRR – Receptor de reconhecimento de padrão;

PS – Fosfatidilserina;

PSG – Tampão PIPES, salina e glicose;

PSGL-1 – Ligante 1 da glicoproteína p-selectina;

PUFAs – Ácidos graxos poliinsaturados;

PVDF – Fluoreto de Polivinilideno;

RNA – Ácido ribonucléico;

RNS – Espécies reativas de nitrogênio;

ROS – Espécies reativas de oxigênio;

RXRA – Receptor retinóide X alfa;

SCD – Síndrome do choque por dengue;

SDS-PAGE – Eletroforese gel de poliacrilamida com dodecil- sulfato de sódio;

SREBP – Proteínas de Ligação a Elemento Regulador de Esterol;

TAG – Triglicerídeos;

TBA – Ácido tiobarbitúrico;

TBS – Tampão tris e salina;

TLR –Receptores do tipo Toll;

TMRE – Tetrametilrodamina;

TNF – Fator de necrose tumoral;

TP – Receptor para tromboxano-prostanóide;

T-BARS – Substâncias reativas ao ácido tiobarbitúrico;

VCAM – Molécula de adesão de células vascular;

VEGF – Fator de crescimento do endotélio vascular;

VLDL – Lipoproteína de muito baixa densidade;

VWF - Fator de von Willebrand;

4-HNE – 4-Hydroxynonenal.

SUMÁRIO

1 INTRODUÇÃO

A dengue consiste em uma arbovirose causada pelo vírus da dengue (DENV) e transmitida pela picada de fêmeas de mosquitos do gênero *Aedes,* principalmente pela espécie *A. aegypti* (CHEN; VASILAKIS, 2011). Estima-se que ocorram entre 50 e 100 milhões de infecções sintomáticas anualmente com 500.000 casos de dengue grave e 10.000 casos fatais, com um custo global anual total de R$ 8,9 bilhões (BHATT *et al*., 2013; SHEPARD *et al.*, 2016; STANAWAY *et al.*, 2016). Um amplo espectro de manifestações clínicas é observado na dengue, a qual pode se apresentar desde um quadro assintomático até quadros de aumento do extravasamento vascular, hemorragia grave e comprometimento de órgãos, caracterizando a dengue grave (WILDER-SMITH *et al.*, 2019). Os mecanismos que desencadeiam a dengue grave não são completamente elucidados. Contudo, as evidências apontam para o grande potencial da tempestade de citocinas inflamatórias, as quais podem contribuir para ativação endotelial vascular e consequentemente para o aumento da permeabilidade vascular crítica na dengue grave (CIPITELLI *et al.*, 2019; MALAVIGE; OGG, 2017).

A trombocitopenia é uma importante alteração hematológica no curso da infecção e um importante parâmetro associado à evolução para a fase crítica da doença (HUY *et al.,* 2013; MOURÃO *et al.*, 2007). Nos pacientes com dengue os níveis de citocinais inflamatórias são correlacionados significativamente com a trombocitopenia, que, por sua vez, possui uma correlação positiva significativa com o extravasamento vascular (BOZZA *et al., 2008;* HUY *et al., 2013;* KRISHNAMURTI *et al*., 2001; MOURÃO *et al*., 2007; WILLS *et al*., 2009). Alguns mecanismos são sugeridos como contribuintes para a trombocitopenia na dengue e envolvem a redução na trombopoiese com supressão da medula óssea e também o aumento da depuração de plaquetas do sangue periférico (HOTTZ *et al*., 2011; SRICHAIKUL; NIMMANNITYA, 2000). Nesse contexto, a ativação e a apoptose plaquetária são sugeridos como mecanismos associados à depuração periférica plaquetária durante a infecção pelo DENV. Marcadores de ativação e apoptose, como translocação da P-selectina, ativação da αIIbβ3, translocação do CD63, exposição da fosfatidilserina e disfunção mitocondrial estão associados à trombocitopenia e gravidade da

infecção (HOTTZ et al., 2013b; MICHELS et al., 2014; OJHA et al., 2017; TRUGILHO et al., 2017). A ativação e a apoptose plaquetária descritas *ex vivo* e em plaquetas infectadas com o DENV *in vitro* contribuem para a interação de plaquetas e monócitos e para a fagocitose plaquetária (ALONZO *et al.,* 2012; HOTTZ *et al.*, 2014; OJHA *et al.,* 2017). Agregados plaquetas-monócitos são descritos em pacientes com dengue e são associados positivamente com a trombocitopenia (HOTTZ *et al.*, 2014). Além disso, a interação de plaquetas com outros leucócitos como, linfócitos e neutrófilos, assim como com células endoteliais, também é descrita no sangue periférico de pacientes infectados com DENV contribuindo para a trombocitopenia observada na dengue (HOTTZ *et al*., 2014; KRISHNAMURTI *et al.*, 2002; PÓVOA *et al.*, 2014; TSAI *et al.*, 2011).

Nesse cenário, no qual a ativação e apoptose plaquetárias são apontadas como potentes mecanismos associados à trombocitopenia descrita na dengue, as plaquetas poderiam contribuir para a amplificação da inflamação, aumento da permeabilidade endotelial e extravasamento plasmático (HOTTZ *et al.*, 2014; KOUPENOVA *et al*., 2018; VAN DER MEIJDEN; HEEMSKERK, 2019). As plaquetas possuem funções hemostáticas bem estabelecidas, envolvendo a interação com células endoteliais, agregação plaquetária e formação de trombos. No entanto, perspectivas emergentes apontam para a participação das plaquetas na resposta imune e em processos inflamatórios ao serem capazes de secretar citocinas e quimiocinas inflamatórias, além de amplificar a reposta inflamatória ao interagir com outras células imunológicas (CLARK *et al.,* 2007; DIB *et al.*, 2020; HOTTZ *et al.*, 2014; KOUPENOVA *et al*., 2018; VAN DER MEIJDEN; HEEMSKERK, 2019). É descrito que a infecção plaquetária pelo DENV induz ativação plaquetária com secreção de citocinas e quimiocinas como RANTES/CCL5, PF4/CXCL4 e MIF(BARBOSA-LIMA et al., 2020; HOTTZ et al., 2013b; TRUGILHO et al., 2017). Nesse sentido, a proteína viral não-estrutural NS1 demonstrou induzir a secreção de RANTES/CCL5 e MIF pelas plaquetas (QUIRINO-TEIXEIRA et al., 2020). De maneira interessante, o DENV induz a expressão plaquetária de moléculas do complexo principal de histocompatibilidade (MHC) classe I de forma dependente da atividade do proteassoma (TRUGILHO et al., 2017). É descrito também a liberação de microvesículas (MVs) contendo IL-1β por plaquetas ativadas pelo DENV, as quais demonstraram induzir hiperpermeabilidade endotelial de forma dependente de IL-1R

(HOTTZ et al., 2013a). O aumento da permeabilidade endotelial decorrente de microvesículas secretadas por plaquetas estimuladas com o DENV também pode envolver a interação observada dessas MVs com neutrófilos, induzindo a formação de armadilhas extracelularesde DNA denominadas NETs (do inglês, *neutrophil extracellular traps*)(SUNG; HUANG; HSIEH, 2019). Além da interação de MVs plaquetárias com neutrófilos, as próprias plaquetas ativadas com DENV interagem com monócitos, induzindo a secreção de mediadores inflamatórios pelos mesmos, biogênese de corpúsculos lipídicos e secreção de PGE_2 de forma parcialmente dependente de MIF plaquetário (BARBOSA-LIMA et al., 2020). Outros estímulos para ativação plaquetária na dengue, além da ativação direta pelo DENV, ainda não são completamente elucidados. A queda da viremia na fase crítica, em que há o pico de ativação plaquetária, nos sugere a importância de investigar outros estímulos responsáveis por essa ativação.

Alterações lipídicas séricas, como redução de lipoproteínas de baixa densidade (LDL, do inglês *low-density lipoprotein*), são descritas na dengue, havendo uma ligação direta com a progressão para casos graves da infecção. As LDL consistem em agregados macromoleculares responsáveis pelo transporte de colesterol do fígado para os tecidos extra-hepáticos (CUI *et al.*, 2013; DURÁN *et al.*, 2015; ENGELKING, 2015; VOGE *et al.*, 2016). Essa redução das LDL corrobora com o fato do colesterol ser fundamental na replicação dos *Flavivirus*, estando envolvido em etapas desde a endocitose do vírus até a formação da partícula viral no retículo endoplasmático das células permissivas (OSUNA-RAMOS; REYES-RUIZ; DEL ÁNGEL, 2018). Além do consumo lipídico aumentado, a participação de diversas células da imunidade inata e adquirida, como macrófagos, neutrófilos, células NK e linfócitos e a síntese de diversas citocinas e quimiocinas pró inflamatórias já é bem descrita em infecções virais e na patogênese da dengue (BRACIALE; HAHN, 2013). Nesse sentido, sugere-se que uma resposta imunológica exacerbada frente ao agente viral da dengue, ao desencadear altos níveis de mediadores inflamatórios contribua para disfunção endotelial e condições associadas aos sinais de alarme e aos sintomas característicos da dengue grave (CIPITELLI et al., 2019). De fato, a presença de antígeno viral, RNA viral e citocinas em tecidos periféricos de pacientes com casos fatais de dengue indicam a dispersão viral com migração de leucócitos, contribuindo para inflamação, aumento de

permeabilidade endotelial e dano tecidual (PÓVOA et al., 2014, 2016). Nesse contexto, a associação de atividades inflamatórias, estresse oxidativo e consumo lipídico na dengue nos leva a considerar um cenário propício à peroxidação lipídica e circulação de LDL oxidadas (oxLDL). De fato, é descrito um aumento significativo de malondialdeído (MDA) sérico, um aldeído reativo produzido na peroxidação lipídica, e de proteínas carboniladas séricas (estas são formadas por aldeídos provenientes da peroxidação lipídica que formam adutos com as proteínas) em pacientes com dengue em relação ao controle (SOUNDRAVALLY *et al.*, 2008), sugerindo que os lipídeos oxidados estão aumentados na dengue e, portanto, poderiam contribuir para a gravidade da doença.

É bem esclarecida a formação de compostos bioativos nas oxLDL que funcionam como padrões moleculares associados a danos (DAMPs), ativando receptores de reconhecimento de padrões (PRRs) da imunidade inata e induzindo respostas imune inflamatória (MILLER; SHYY, 2017). Os mecanismos inflamatórios desencadeados pela oxLDL em doenças cardiovasculares estéreis são amplamente estudados, envolvendo, por exemplo, a formação de macrófagos espumosos e a ativação e agregação plaquetária (CHISTIAKOV *et al*., 2017; H.-C. *et al*., 2013). Nesse sentido, em um artigo de revisão que publicamos recentemente, a oxidação da LDL é apontada como um fator possivelmente envolvido no aumento do risco cardiovascular e pior prognóstico de obesos com influenza ou doença por corona vírus 2019 (ANEXO A). No entanto, os mecanismos inflamatórios desencadeados pela oxLDL ainda não foram elucidados em doenças infecciosas como a dengue. Nesse sentido, além da peroxidação lipídica se correlacionar negativamente com a contagem de plaquetas na dengue grave (SOUNDRAVALLY *et al.*, 2008), a trombocitopenia possui uma correlação positiva com a diminuição de LDL sérico na dengue (DURÁN *et al*., 2015). Sendo assim, este trabalho tem como objetivo investigar a participação da oxLDL na patogênese da dengue, a partir da identificação de mecanismos de ativação plaquetária associados à resposta inflamatória na infecção.

2 REVISÃO DA LITERATURA

2.1 Dengue

2.1.1 Epidemiologia

A dengue é uma doença viral transmitida por mosquito com grande impacto para a saúde pública. Consiste em uma doença negligenciada com disseminação geográfica significativa do vírus e de seu vetor, com inúmeros casos graves da infecção e carga onerosa subseqüente a doença (OMS, 2012). A transmissão da dengue é altamente presente em países localizados nos trópicos, sendo América do Sul, Sudeste Asiático e África central zonas de alto risco (BHATT *et al.*, 2013; MESSINA *et al.,* 2019) (Figura 1A). Associações positivas foram demonstradas em escala global entre o elevado risco de transmissão da doença e a proximidade a centros urbanos e peri-urbanos de baixa renda, assim como características locais de chuvas e temperatura também demonstraram influenciar o risco de transmissão (BHATT *et al.,* 2013). Calcula-se que quase metade da população do mundo viva em países onde a dengue é endêmica (MESSINA *et al.*, 2019; OMS, 2012) e que ocorram de 50 milhões a 100 milhões de infecções sintomáticas a cada ano (BHATT *et al.,* 2013; SHEPARD *et al.,* 2016; STANAWAY *et al.,* 2016) (Figura 1B). Além disso, estimam-se cerca de 500.000 casos anuais de dengue grave (OMS, 2017), 10.000 mortes por ano (STANAWAY *et al.,* 2016) e um custo global anual total de R$8,9 bilhões (SHEPARD *et al.*, 2016). O número de infecções sintomáticas pela dengue mais do que duplicou a cada 10 anos entre 1990 e 2013 (STANAWAY *et al.,* 2016), assim como os números de casos de dengue reportados para a OMS aumentaram 15 vezes nas últimas duas décadas (OMS, 2020).

Durante os séculos XVIII, XIX e início do XX, epidemias de doenças semelhantes à dengue foram relatadas e registradas globalmente, tanto em regiões tropicais quanto em algumas temperadas (OMS, 2011). A origem do primeiro mosquito vetor da dengue é sugerida como sendo Ásia (*Ae. Albopictus*) e África (*Ae. Aegypti*) (GUBLER, 1998; MAYER; TESH; VASILAKIS, 2017). O mosquito e o vírus

foram difundidos devido ao uso de embarcações na expansão comercial para todas as cidades costeiras tropicais do mundo. Sugere-se posteriormente que a Segunda Guerra Mundial contribuiu para a expansão global da doença, devido à dispersão de tropas para os interiores e à urbanização precária no período pós-guerra (GUBLER, 1998; MAYER; TESH; VASILAKIS, 2017; OMS, 2011). Durante as décadas de 1950 e 1960 houve o controle da expansão da dengue na América, devido à campanha de controle da febre amarela em 1947, proposta pela Organização Pan-Americana da Saúde (OPAS). O projeto teve como objetivo a eliminaçãodo *Ae. aegypti* da América Central e do Sul. No entanto, com o foco no controle vetorial por aspersão de inseticidas, a interrupção da campanha levou ao reinfestamento do continente em 1970 com posterior reintrodução do vírus da dengue, um prelúdio para as epidemias subseqüentes (GUBLER, 1998; MAYER; TESH; VASILAKIS, 2017; OMS, 2011).

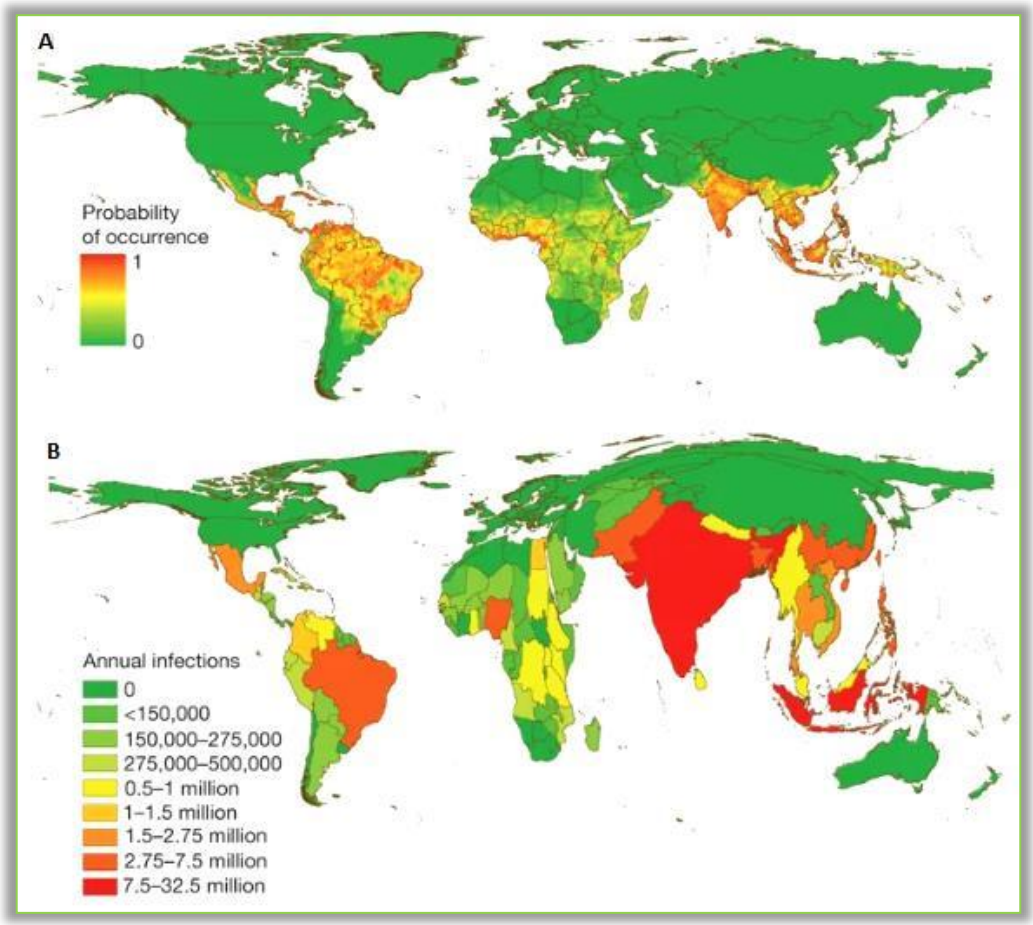

Figura 1 – **Representação global das áreas endêmicas.**
Figura **1A:** Demonstração das regiões com maior probabilidade de ocorrência da dengue, sendo as áreas de maior probabilidade na cor vermelha e as de menor probabilidade na cor verde. Figura **1B:** Demonstração quantitativa dasinfecções anuais por área.Fonte: BHATT *et al.*, 2013.

Na década de 1980 ocorreu a primeira epidemia no Brasil, em Boa Vista/RR, causada pelos sorotipos DENV-1 e DENV-4. Após 6 anos, em 1986, o DENV-1 foi reintroduzido no Rio de Janeiro, assim como o DENV-2 em 1990, os quais foram dispersados para outros estados (MS/SVS, 2019; NUNES *et al.,* 2019). Após esse período, os anos de 2002, 2008, 2010, 2013 e 2015 tiveram números consideráveis de casos, os quais foram compilados em uma revisão por Nunes e colaboradores (2019) (Figura 2). Em 2002, a epidemia causada principalmente pelo DENV-3 detectado em 2000 no Rio de Janeiro, levou em média a 700.000 casos, com 2700 casos de dengue grave e 150 mortes. Em 2008, devido à reemergência do DENV-2 em 2007, foram reportados em torno de 700.000 casos e 561 mortes. Já a epidemia de 2010, após a reermengência do DENV-1 em 2009, 1.000.000 de casos foram reportados com 650 óbitos. Em 2010, o DENV-4 foi reintroduzido no país e em 2013 e 2015 ocorreu uma explosão de casos, com em média 1.500.000 casos e 674 mortes em 2013 e com 1.649.008 casos reportados e 986 mortes confirmadas em 2015. Em 30 anos (1985-2015) somam-se 11.084.755 de casos no Brasil e em torno de 6.000 mortes confirmadas (NUNES *et al.*, 2019). Em 2019, os dados voltam a ficar preocupantes com 1.544.987 casos prováveis de dengue notificados e 1.419 casos de dengue grave, 18.740 casos de dengue com sinais de alarme e 782 óbitos. Destacam-se os estados de Minas Gerais, São Paulo e Goiás que concentraram 67,9% dos casos prováveis do país(MS/SVS, 2020).

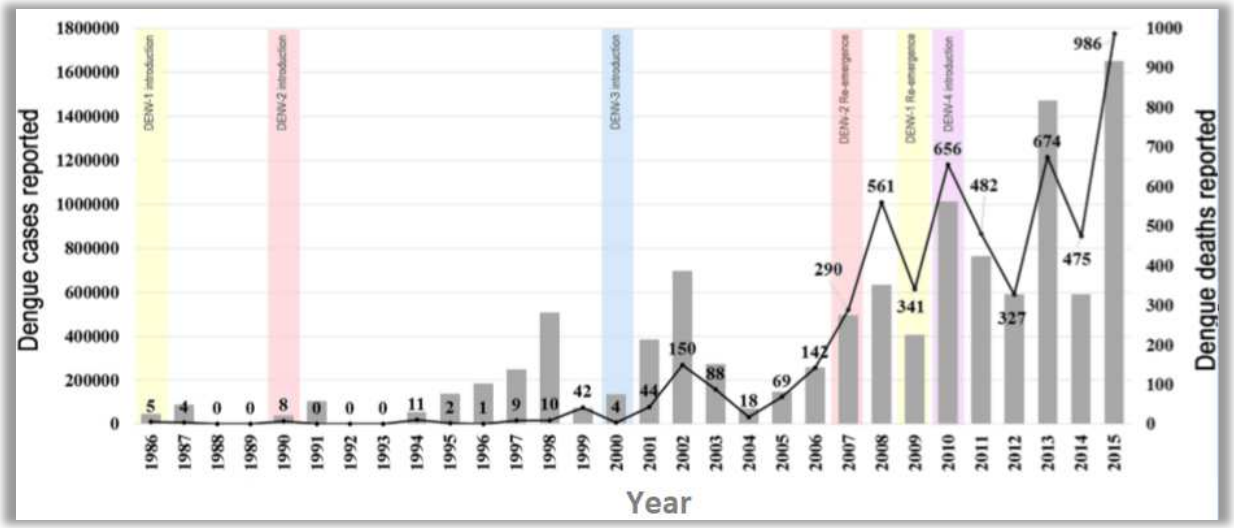

Figura 2 –Casos de dengue reportados e óbitos por dengue no Brasil (1986-2015). As barras em cinza mostram os números de casos de dengue (no eixo y à esquerda) por ano. As barras coloridas mostram o ano da introdução ou reemergência dos vírus no Brasil (Amarelo DENV1/ Rosa DENV2/ Azul DENV3/ Roxo DENV4).A linha mostra os casos de óbitos por dengue (no eixo y à direita) por ano. Fonte:NUNES *et al.,* 2019.

Em um estudo recente foram aplicadas técnicas de mapeamento estatístico para prever a adequação ambiental global para o vírus em 2020, 2050 e 2080 a partir de 2015, com uso de projeções climáticas, populacionais e socioeconômicas, considerando temperatura, umidade, precipitação, urbanização, PIB e distribuição dos mosquitos vetores da dengue (MESSINA *et al.,* 2019). Foi previsto uma elevação no total da população em risco para mais de 6,1 bilhões, ou seja, 60% da população do mundo. No geral as áreas já consideradas endêmicas permanecem em alto risco de dengue, com o risco se estendendo para a região do Sahel na África em 2020 e para o sul da África, sudeste dos EUA, norte da Argentina, interior da Austrália e leste da China em 2050 e 2080 (MESSINA *et al.*, 2019).

2.1.2 Vetor, agente etiológico e ciclo de replicação viral

O DENV é transmitido aos seres humanos através das picadas de mosquitos fêmeas *Aedes* infectados, as quais ingeriram sangue humano infectado anteriormente (CHEN; VASILAKIS, 2011; OMS, 2009). Os vírus são mantidos em dois ciclos de transmissão: o ciclo silvestre no oeste da África e Sudeste da Ásia, que envolve primatas não humanos e mosquitos *Aedes* arbóreos; e o ciclo urbano em ambientes tropicais e subtropicais, que envolve o homem e os mosquitos domésticos *Aedes aegypti* e peridomésticos *Aedes albopictus* e *Ae. polynesiensis*(CHEN; VASILAKIS, 2011). Com a proximidade ecológica e da relação filogenética entre as espécies doadora (primatas não humanos) e receptora (humanos) houve a mudança de vetor (mosquitos arbóreos para mosquitos domésticos e peridomésticos) e a mudança da espécie hospedeira (*cross-species*) resultando em quatro sorotipos do vírus da dengue (DENV-1 a DENV-4) que circulam hoje (CHEN; VASILAKIS, 2011).

Os vírus da dengue (DENV) são pequenos vírus esféricos com 50nm de diâmetro, pertencentes ao gênero *Flavivirus* e à família *Flaviviridae* (OMS, 2009). As partículas virais maduras são envelopadas com glicoproteínas em uma bicamada

lipídica envolvendo um capsídeo icosaédrico, o qual envolve o RNA de cadeia linear simples com polaridade positiva (5’ - 3’)(OMS, 2009; PERERA; KUHN, 2008; WILDER-SMITH *et al.,* 2019).

Após transmissão do vírus pelos mosquitos ao hospedeiro vertebrado, os vírus da dengue se ligam nas células hospedeiras e são endocitados(CLYDE; KYLE; HARRIS, 2006). O DENV demonstrou ser capaz de infectar células em diversos órgãos como pele e fígado, replicando-se em hepatócitos, células endoteliais, macrófagos e células dendríticas (BEGUM et al., 2019; DALRYMPLE; MACKOW, 2011; PÓVOA *et al.*, 2014; SCHAEFFER *et al.,* 2015). Após internalização e acidificação do endossomo ocorrem mudanças conformacionais na proteína E viral constituinte do envelope, levando à fusão da membrana viral com a membrana do endossomo. Sendo assim, há a entrada do nucleocapsídeono citoplasma e liberação do genoma (CLYDE; KYLE; HARRIS, 2006). O genoma com polaridade positiva atua como um RNA mensageiro e é traduzido em uma poliproteína no retículo endoplasmático (RE) (Figura 3A). A poliproteína será clivada pelas proteases hospedeiras e virais em três proteínas estruturais: proteína C (capsídeo); proteína prM (precursora da proteína de membrana) e proteína E (envelope); e sete proteínas não-estruturais: NS1; NS2a; NS2b; NS3; NS4a; NS4b e NS5 (CLYDE; KYLE; HARRIS, 2006; OMS, 2009; PERERA; KUHN, 2008; WILDER-SMITH *et al.,* 2019). Após subseqüentes traduções, as proteínas não estruturais como NS5, NS3 e seu cofator NS2b, vão desempenhar papéis importantes na replicação do RNA viral. A partir da fita molde de RNA positivo há a transcrição de fitas de RNA negativas, as quais servem de molde para replicações sucessivas de fitas de RNA positivas (CLYDE; KYLE; HARRIS, 2006; PERERA; KUHN, 2008). Estas novas fitas de polaridade positiva serão encapsidadas pelas proteínas C formando nucleocapsídeos que, por sua vez, serão endocitados por membranas lipídicas do retículo endoplasmático. Com a endocitose do nuclecapsídeo pelo retículo forma-se uma partícula viral imatura, estando as proteínas E e prM ancoradas na bicamada lipídica do retículo endoplasmático formando o envelope viral (CLYDE; KYLE; HARRIS, 2006). O envelope viral é bem definido e consiste em 180 cópias de proteínas E e prM, as quais possuem diferenças conformacionais na partícula viral imatura e madura (PERERA; KUHN, 2008). O peptídio ‘pr’ impede as mudanças conformacionais na proteína E, não permitindo que ocorra a fusão do envelope nas

organelas durante o transporte da partícula imatura para o complexo de Golgi (YU *et al.,* 2008). O peptídeo 'pr' é clivado de prM pela protease furina durante a maturação no complexo de Golgi de forma pH-dependente e M permanece na partícula madura como uma proteína transmembranar sob o invólucro da proteína E (Figura 3B). A partícula madura do vírus é então liberada por exocitose (PERERA; KUHN, 2008; YU et al., 2008).

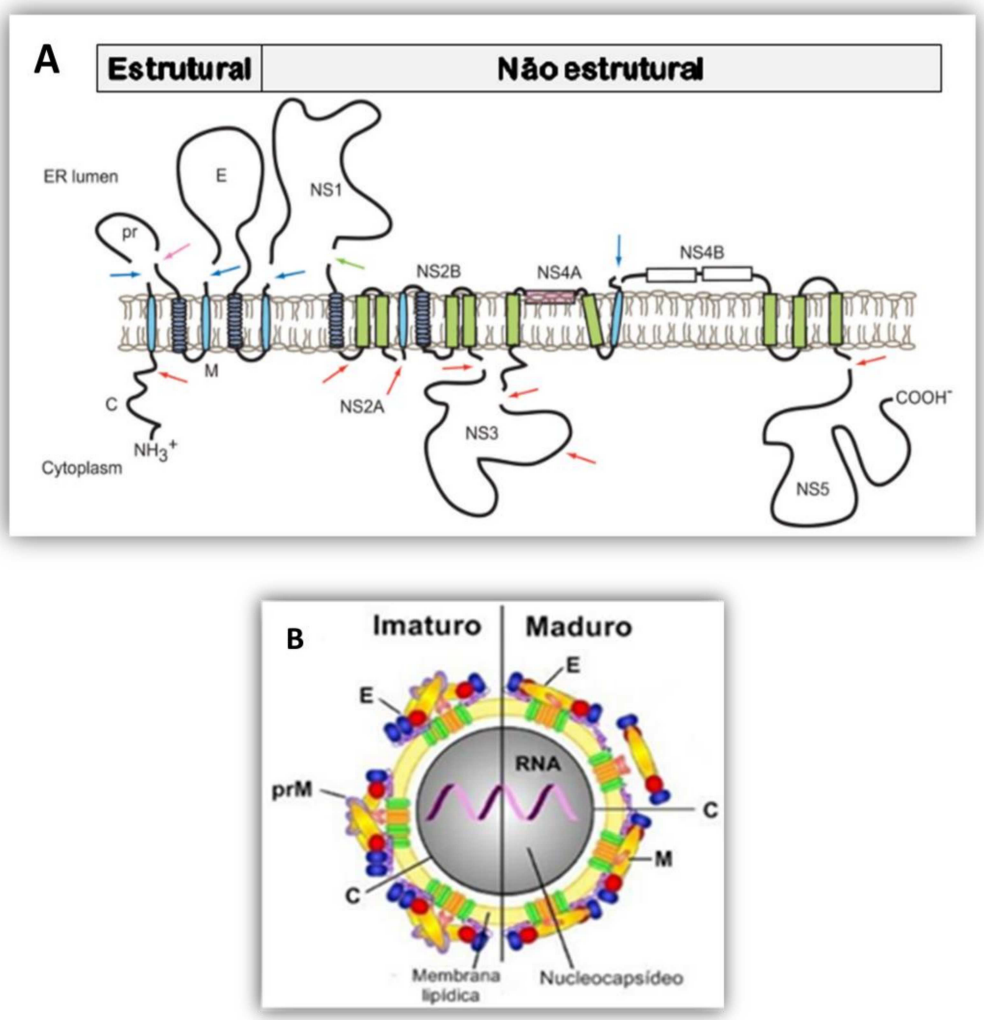

Figura 3 – Representação da estrutura e poliproteína viral. (A) Poliproteína viral no retículo endoplasmático da célula hospedeira. As proteínas estruturais (C, prM e E) e não estruturais (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) codificadas do RNA viral são processadas por proteases celulares e virais (sítios de clivagem indicados por setas). Fonte: Adaptado de PERERA; KUHN, 2008 **(B) Representação da estrutura da partícula viral imatura e madura.** E – proteína do envelope viral; prM – proteína percursora da proteína da membrana (vírus imaturo); M – proteína de membrana; C – proteína do capsídeo. Fonte: Adaptado de LIRA, 2016.

2.1.3 Sinais e sintomas

Um bom esquema de classificação da dengue permite a triagem adequada dos pacientes, facilitando o manejo clínico e a avaliação de possíveis intervenções para melhor prognóstico. É de grande valia uma classificação que permita a identificação de pacientes com probabilidade de evolução para doença grave, assim como facilite as notificações e vigilância epidemiológica (DEEN *et al.*, 2006). Tendo em vista a importância de um bom esquema de definições dos casos de dengue, a classificação apresentada em 1997 pela OMS foi questionada. Tal classificação baseou-se em casos de dengue diagnosticados em crianças tailandesas e consistia em Febre do Dengue (DF) ou Febre Hemorrágica do Dengue (DHF), sendo a DHF caracterizada por hepatomegalia, plaquetopenia, hemoconcentração e hipoproteinemia e classificada em quatro graus de gravidade (FHD I –IV), com os graus III e IV definidos como Síndrome do Choque por Dengue (DSS)(OMS, 1997). Com o aumento do número de casos globalmente e diversas apresentações clínicas da doença, pesquisadores relataram dificuldades em enquadrar os casos diagnosticados nas classificações vigentes. Alegou-se, por exemplo, uma sobreposição de sintomas entre DF e DHF, que casos graves nem sempre preenchiam todos os critérios de DHF e que a hemorragia enfatizada no termo classificatório mascarava a importância de monitorar o extravasamento plasmático e choque (DEEN *et al.*, 2006). Sendo assim, em 2009, a OMS revisou a classificação facilitando o manejo clínico e melhorando a qualidade dos dados epidemiológicos (OMS, 2009). Esta classificação será a abordada por nós no presente estudo, no entanto, as análises realizadas por outros estudos que optaram em utilizar a classificação anterior serão citadas de acordo com suas respectivas referências.

Um amplo espectro de manifestações clínicas é observado na infecção pelo DENV, a qual pode ser classificada como dengue branda, dengue branda com sinais de alarme ou dengue grave. A dengue branda pode se apresentar com curso clínico autolimitado ou com progressão para dengue grave (OMS, 2009). Após um período de incubação de 2 a 7 dias, inicia-se uma fase febril com alta viremia. Esta fase é caracterizada por um início repentino de febre alta acompanhada de rubor

facial, eritema cutâneo, dor generalizada no corpo, mialgia, artralgia, dor de cabeça, anorexia e vômitos. Em torno de 3 dias observa-se a defervescência com queda da viremia, marcando o período de melhora ou evolução para fase crítica. Este período deve ser monitorado atentando-se para os sinais de alarme como: letargia e/ou inquietação, dor abdominal intensa e contínua, hepatomegalia > 2cm, vômitos persistentes, acúmulo clínico de líquidos, sangramento de mucosa e aumento do hematócrito com queda abrupta na contagem de plaquetas (OMS, 2009; WILDER-SMITH et al., 2019). Alterações severas na permeabilidade vascular levarão à importante extravasamento plasmático na dengue grave com hipoperfusão dos órgãos, resultando no comprometimento progressivo dos mesmos, graves hemorragias e coagulação intravascular disseminada. Quando o paciente sobrevive à fase crítica, entra-se na fase de recuperação onde ocorre uma reabsorção gradual do líquido do compartimento extravascular e estabilização do estado hemodinâmico, com progressiva melhora do quadro clínico(OMS, 2009; WILDER-SMITH et al., 2019) (Figura 4).

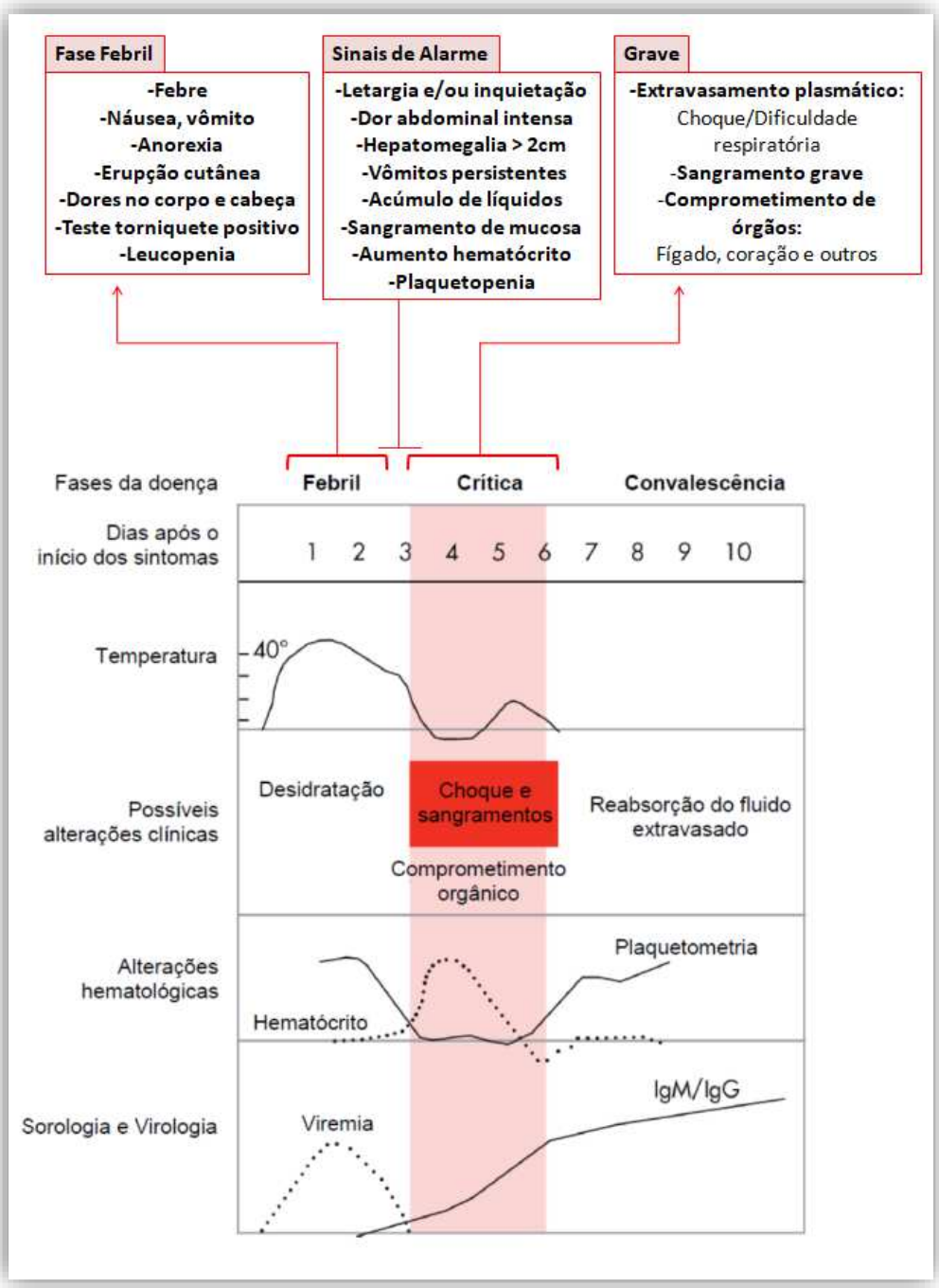

Figura 4 – Sinais e Sintomas da Dengue – Curso das alterações clínicas e laboratoriais. Fonte: Adaptado de OMS, 2009.

2.1.4 Patogênese

Os mecanismos descritos associados à patogênese da dengue corroboram com os mecanismos imunológicos descritos em infecções virais, onde as células da imunidade inata, como macrófagos, células dendríticas e células NK, reconhecem os padrões moleculares associados aos vírus e desencadeiam cascatas inflamatórias e respostas da imunidade adquirida (BRACIALE; HAHN, 2013). Os receptores citoplasmáticos tipo RIG, como RIG-I e MDA5, e os receptores endossomais tipo Toll, como TLR3, foram descritos como receptores de reconhecimento de padrões moleculares associados ao DENV, desencadeando respostas de interferon tipo I (IFN α e β)em células infectadas (NASIRUDEEN *et al.,* 2011; SPROKHOLT *et al.,* 2017). Os interferons α e β levam ao estabelecimento de um estado antiviral que restringe a replicação do DENV em células não infectadas (DIAMOND *et al.,* 2000). A ativação dos receptores RIG-I e MDA5 frente à replicação do DENV além de desencadear a transcrição de IFN α e β, também induziu a expressão de citocinas pelas células dendríticas como IL-6, IL-1β e TNF, de quimiocinas como CCL2/MCP-1, CCL3/MIP-1α e CCL4/MIP-1β e na indução da resposta imune adaptativa com diferenciação para células Th1, as quais possuem alta produção de IFN-γ (SPROKHOLT *et al.,* 2017). Foi descrito um aumento da citocina IL-15 no início da infecção pelo DENV, o qual foi associado com os níveis aumentados das células NK ativadas nos estágios iniciais de casos de DF(AZEREDO *et al.,* 2006). As células NK da imunidade inata com atividade citotóxica também são importantes secretoras de IFN-γ (BOEHM *et al.,* 1997). Nesse cenário, sabe-se que os macrófagos são ativados pelo IFN-γ, levando à síntese de citocinas como IL-12 e síntese de óxido nítrico pela enzima iNOS (BOEHM *et al.,* 1997). O óxido nítrico, por sua vez, já demonstrou inibir a replicação do DENV *in vitro* (CHARNSILPA *et al.,* 2005). Os macrófagos estimulados por IFN-γ também sintetizam diversas quimiocinas quimioatraentes para linfócitos e monócitos, como CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP1-β eCCL5/RANTES, além do aumento de ligantes de integrinas,como ICAM-1 e VCAM-1 nas células endoteliais (BOEHM *et al.,* 1997). Os linfócitos T na infecção pelo DENV contribuem para o controle da replicação viral, com células T CD8+ DENV-específicas produzindo IFN-γ, TNFα e exibindo atividade citotóxica *in vivo,* e com células T CD4+ DENV-específicas

produzindo IFN-γ, TNFα e IL-2 (LINDOW *et al.*, 2012; YAUCH *et al.,* 2009). A resposta humoral também é descrita na infecção pelo DENV, com anticorpos para proteínas estruturais e não estruturais, como, por exemplo a NS1, fornecendo proteção *in vivo* e prevenção da infecção sintomática por cepas homólogas do DENV (HENCHAL; HENCHAL; SCHLESINGER, 1988; JOHNSON; ROEHRIG, 1999; VALDES *et al.,* 2000).

Sendo assim, a participação de diversas células da imunidade inata e adquirida com a síntese de diversas citocinas e quimiocinas pró inflamatórias já é bem descrita em infecções virais e na patogênese da dengue. No entanto, os mecanismos fisiopatológicos que levam à dengue grave ainda não são totalmente estabelecidos. Postula-se que estejam envolvidos com altos níveis de citocinas e quimiocinas inflamatórias, as quais atuam na ativação endotelial vascular e na migração celular (CIPITELLI *et al.,* 2019; MALAVIGE; OGG, 2017). Algumas hipóteses foram propostas para explicar o desenvolvimento da dengue grave, todas convergindo para o mesmo fator: elevados níveis de mediadores inflamatórios.

Duas hipóteses, não mutuamente exclusivas, explicam as maiores chances observadas de infecções secundárias culminarem na dengue grave (ALVAREZ *et al.,* 2006; GUZMÁN *et al.,* 2000). Na hipótese da amplificação dependente de anticorpo (ADE, do inglês *antibody-dependent enhancement*), os anticorpos reativos cruzados de uma infecção anterior são incapazes de neutralizar completamente o novo sorotipo DENV infectante. Além disso, aumentam a captação do DENV pelas células portadoras de receptores Fc, ampliando as oportunidades de entrada dos vírus nas células para replicação, aumentando, portanto, as cargas virais e as cascatas inflamatórias (HALSTEAD; NIMMANNITYA; COHEN, 1970). Na segunda hipótese, referida como 'pecado antigênico original', postula-se que as células T de memória são ativadas preferencialmente sobre células T *naive*, produzindo um aumento das citocinas mais rapidamente. No entanto, as células reativadas durante a infecção secundária podem não ter avidez ideal para os epítopos do novo sorotipo do vírus infectante, não sendo eficazes na redução da carga viral, apesar de contribuir para o aumento de mediadores inflamatórios(MONGKOLSAPAYA *et al.,* 2003).

Há também a hipótese que considera os fatores virais, elucidando possíveis causas da ocorrência da dengue grave também em infecções primárias. Sugere-se que diferentes sorotipos e genótipos variam em virulência e imunopatogenia, desenvolvendo, por exemplo, mecanismos diversos e algumas vezes mais eficientes para evitar respostas antivirais do hospedeiro(COLOGNA; ARMSTRONG; RICO-HESSE, 2005; FRIED *et al.,* 2010; MANOKARAN *et al.*, 2015). Fatores genéticos dos hospedeiros também foram associados à forma grave da dengue, como, por exemplo, polimorfismos nos genes codificadores das proteínas OSBPL10 (proteína de ligação ao oxysterol) e RXRA (receptor retinóide X alfa), entre outros, que foram associados com a menor suscetibilidade de pessoas de ascendência africana para a dengue grave (SIERRA *et al.*, 2017). Essas proteínas consistem em receptores plasmáticos e nucleares envolvidos na sinalização e metabolismo lipídico, além do RXRA estar envolvido também na regulação da expressão de mediadores inflamatórios (MA *et al.,* 2015; PERTTILÄ *et al.,* 2009). No entanto, as vias que ligam o metabolismo lipídico à resposta imune na dengue ainda não foram bem estabelecidas.

Nesse contexto, sugere-se que altos níveis de mediadores inflamatórios contribuam para condições associadas aos sinais de alarme e aos sintomas característicos da dengue grave, como vigoroso aumento da permeabilidade endotelial, intenso extravasamento vascular e choque (CIPITELLI *et al.*, 2019). De fato, é descrito um aumento significativo de citocinas inflamatórias em pacientes com dengue grave em relação à dengue branda, como IL-4, IL-6, IL-8, IL-1β, INF-γ, MCP-1, TNF-α e MIF (BOZZA *et al.,* 2008; BUTTHEP *et al.,* 2012; CHEN *et al.,* 2018, 2006; LEE *et al.,* 2006; PRIYADARSHINI *etal.*, 2010). Além disso, análises histopatológicas e imunohistoquímicas de tecidos periféricos de pacientes com casos fatais de dengue demonstraram infecção e replicação viral em hepatócitos, pneumócitos, fibras cardíacas, monócitos e em células endoteliais circulantes e residentes, além de uma frequência significativamente aumentada de células que expressam IFN-ɣ, TNF-α e CCL5/RANTES nos mesmos tecidos, validando o aumento da permeabilidade vascular na dengue grave com conseqüente dispersão viral, migração de leucócitos, inflamação e dano tecidual (PÓVOA *et al., 20*14, 2016).

Diversos estudos já demonstraram a associação das citocinas com alteração da permeabilidade endotelial na dengue, como, por exemplo, as citocinas TNF-α e IL-8, as quais foram secretadas no sobrenadante de linhagens monocíticas infectadas por DENV. A exposição das células endoteliais microvasculares humanas (HMVEC) ao sobrenadante neutralizado para essas citocinas reduziu significativamente a hiperpermeabilidade das células em comparação à exposição ao sobrenadante sem neutralização (KELLEY; KAUFUSI; NERURKAR, 2012). A redução da permeabilidade de células endoteliais da veia umbilical humana (HUVEC) incubadas com sobrenadante de monócitos infectados com DENV-2também foi significativa quando neutralizada a quimiocina MCP-1 (LEE *et al.,* 2006). A citocina MIF também demonstrou ser crítica no processo de hiperpermeabilidade em HUVECs. A NS1 viral induziu a secreção da citocina MIF por leucócitos e células endoteliais, contribuindo para o aumento da permeabilidade endotelial. O mecanismo envolveu a degradação do glicocálice celular devido secreção de HPA-1 (heparanase) pelas células endoteliais e de MMP-9 (metaloproteinase) pelos leucócitos estimulados por MIF(CHEN *et al.,* 2018). Além da TNF-α, IL-8, MCP-1 e MIF, também é descrito que a infecção pelo DENV em células mononucleares do sangue periférico (PBMCs) e em macrófagos induz a secreção de IL-1β a partir da interação da proteína M do DENV com o NLRP3 nessas células. A infiltração de células inflamatórias e as lesões teciduais foram induzidas por M nos tecidos de camundongos selvagens, mas não ocorreram nos tecidos de camundongos deficientes para NLRP3, sugerindo que a ativação de NLRP3 por M e consequente secreção de IL-1β contribuem para o aumento da permeabilidade vascular em camundongos (PAN et al., 2019a). De fato, um estudo do mesmo grupo demonstrou que a IL-1β induz hiperpermeabilidade endotelial em HUVECs *in vitro* e também *in vivo*, contribuindo para a infiltração por células inflamatórias e lesão tecidual (PAN *et al.*, 2019b).

No estudo realizado por Bozza *et al.* 2008 em que avaliou-se as relações entre os níveis de citocinas e várias manifestações clínicas identificou-se que a IL-1β foi associada significativamente à trombocitopenia acentuada (≤50.000 plaquetas/mm^3), assim como as citocinas TNF-α, IL-8 e MCP-1 foram associadas inversamente à trombocitopenia. A trombocitopenia é uma importante alteração hematológica no curso da infecção pelo DENV, com contagens mais baixas de

plaquetas descritas em casos mais graves da dengue, havendo uma correlação positiva da trombocitopenia com o aumento do extravasamento vascular (BOZZA *et al.*, 2008; HUY *et al.,* 2013; KRISHNAMURTI *et al.*, 2001; MOURÃO *et al.*, 2007; WILLS *et al.,* 2009). Sugere-se que a diminuição na contagem de plaquetas envolva mecanismos de destruição periférica plaquetária devido à ativação das mesmas, contribuindo não apenas para a trombocitopenia, mas também para a amplificação da inflamação e extravasamento plasmático(HOTTZ *et al.,* 2011; OJHA *et al.,* 2017). No entanto, apesar de a plaquetopenia ser descrita no curso da infecção pelo DENV, os mecanismos associados a essa alteração hematológica na dengue e à participação das plaquetas na patogênese ainda não são completamente elucidados.

2.2 Plaquetas e suas funções imunoreguladoras

As plaquetas são pequenas células em tamanho (~4μm), mas estão altamente presentes em número (em média 200.000 plaquetas/μL, totalizando em média 1 trilhão de plaquetas no sangue humano), ocorrendo uma produção diária de 100 bilhões de novas plaquetas a partir de megacariócitos da medula óssea, as quais possuem uma meia vida de 8-10 dias(SEMPLE; ITALIANO; FREEDMAN, 2011). A proposta de plaquetas atuantes como células do sistema imune na dengue faz parte de perspectivas emergentes da atividade plaquetária, tendo em vista que as atividades descritas para essas pequenas células anucleadas se restringiram durante muito tempo ao envolvimento das mesmas na hemostasia e trombose patológica (VAN DER MEIJDEN; HEEMSKERK, 2019).

Diversos são os estímulos pró-coagulantes (colágeno, fibrinogênio, fator de von Willebrand - vWf) e os receptores envolvidos com a atividade plaquetária na hemostasia, como, por exemplo, os GPCRs (do inglês, *G protein-coupled receptors*) para trombina (receptores ativados por proteases do tipo 1 e 4 - PAR1 e PAR4), para ADP (receptores purinérgicos do tipo 2 metabotrópicos- P2Y12 e P2Y1) e para tromboxano A2 (receptor para tromboxano prostanóide - TP). Os estímulos agonistas levam à sinalizações intracelulares com amplificação da resposta

plaquetária e interações plaqueta-plaqueta, plaqueta-endotélio e plaqueta-leucócitos a partir de integrinas e selectinas (VAN DER MEIJDEN; HEEMSKERK, 2019). No entanto, plaquetas também são capazes de reconhecer PAMPs (padrões moleculares associados a patógenos) e DAMPs (padrões moleculares associados a dano) através de receptores de reconhecimento de padrões (PRRs), como, por exemplo: os receptores do tipo toll (TLRs), incluindo TLR1, 2, 4 e 6 na superfície plaquetária, e TLR 3, 7 e 9 nos endossomosos; os receptores do tipo NOD (NLRs), como NLRP3; e os receptores de lectina tipo C (CLRs), como DC-SIGN (ANABEL *et al.,* 2014; COGNASSE *et al.,* 2005; HOTTZ *et al.,* 2013a, 2013b; KOUPENOVA *et al.,* 2014; SHIRAKI *et al.,* 2004). As plaquetas também expressam receptores para citocinas e quimiocinas, como IL-1R, IL-8R, CXCR6 e CXCR7, contribuindo para a amplificação das respostas plaquetárias (BORST *et al.,* 2012; CHATTERJEE *et al.*, 2014; SCHAUFELBERGER *et al.,* 1994). Além dos receptores para agonistas clássicos, dos PRRs e dos receptores de citocinas e quimiocinas, as plaquetas também expressam receptores que reconhecem o fator de ativação de plaquetas (PAF) e certos fosfolipídios oxidativamente modificados, como o PAFR, o receptor de oxLDL do tipo lectina (LOX-1) e o receptor scavenger CD36 (CHEN et al., 2001; KEATING; SCHNEIDER, 2009; SILVERSTEIN, 2009).

A ativação plaquetária pode desencadear a secreção de moléculas estocadas em grânulos citoplasmáticos, como os grânulos densos que estocam ADP, ATP, serotonina e cálcio; e os α-grânulos que são os mais abundantes e armazenam reguladores da coagulação (Fator V, Fator VIII, PAI-I), moléculas de adesão (P-selectina, vWf, fibrinogênio, integrina $\alpha IIb\beta_3$), a molécula co-estimulatória CD40L, peptídios antimicrobianos, fatores de crescimento como o VEGF e citocinas e quimiocinas inflamatórias, como CCL3/MIP-1α, PF4/CXCL4, RANTES/CCL5, MIF, CXCL8/IL-8, CCL7/MCP-3 e TGF-β(FITCH-TEWFIK; FLAUMENHAFT, 2013; SEMPLE; ITALIANO; FREEDMAN, 2011). Além disso, apesar de serem anucleadas, as plaquetas possuem pré moléculas de RNA mensageiro (mRNA) provenientes de megacariócitos e pró-plaquetas na trombopoiese. Os pré-mRNAs são processados por um spliceossoma funcional que produz o mRNA traduzível após estímulo. O mRNA maduro é traduzido em proteínas como IL-1β, fator tecidual (FT) e COX-2(DENIS *et al.,* 2005; SCHWERTZ *et al.,* 2006; SHASHKIN *et al.,* 2008). As plaquetas ativadas também formam agregados com leucócitos a partir da P-

selectina (CD62-P) translocada dos α-grânulos pós estímulo, a qual interage com a glicoproteína ligante 1 de P-selectina (PSGL-1) nos leucócitos (KLING *et al.,* 2013). Além da interação P-selectina e PSGL-1, o fibrinogênio e o CD40L solúvel funcionam como uma ponte para a interação plaquetas-leucócitos através dos receptores αIIbβ3 em plaquetas e Mac-1 em leucócitos(PATKO *et al.,* 2012) (Figura 5).

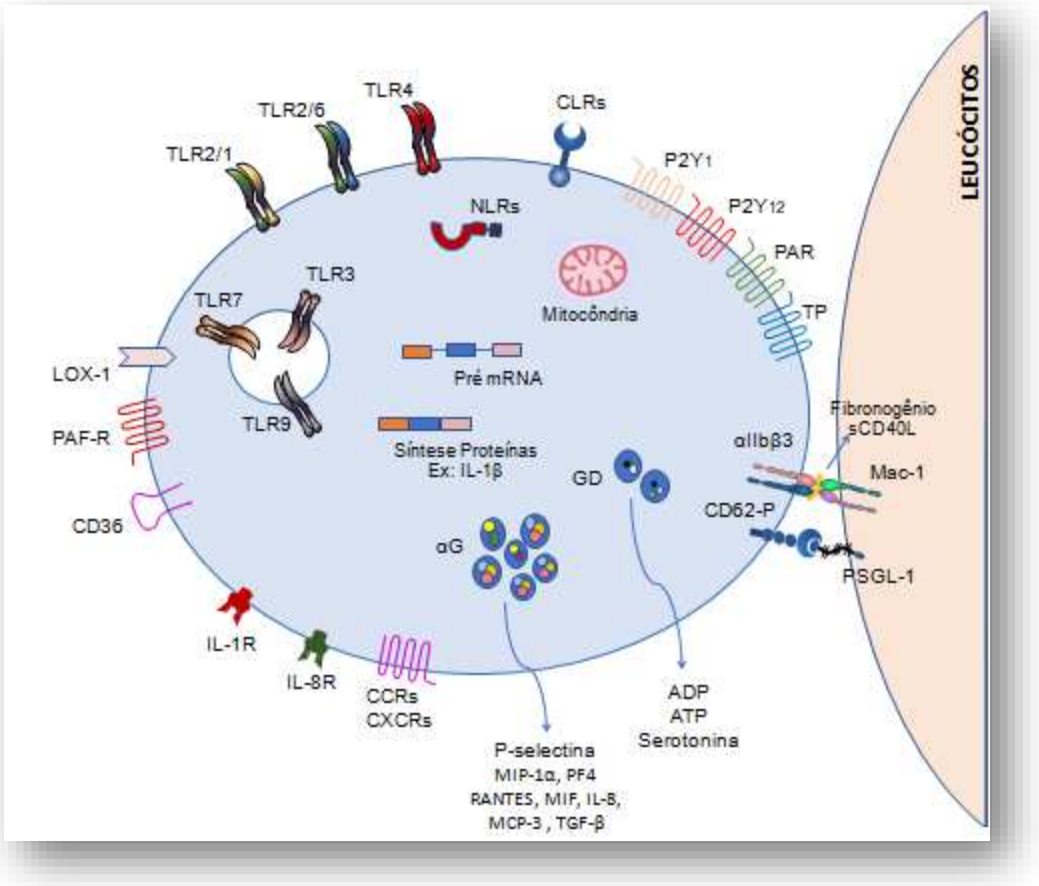

Figura 5 – Representação de elementos celulares plaquetários e interação plaqueta-leucócitos. TLR 1, 2, 4 e 6: Receptores do tipo toll de membrana plasmática. **TLR 3, 7 e 9:** Receptores do tipo toll endossomais. **NLRs:** Receptores citoplasmáticos do tipo NOD, como NLRP3. **CLRs:** Receptores lectina tipo C, como DC-SIGN. **P2Y1/P2Y12:** Receptores de ADP. **PAR:** Receptor de trombina. **TP:** Receptor de TBXA2. **LOX1, PAF-R e CD36**: Receptores de PAF e/ou lipídios oxidados. **IL-1R, IL-8R, CCRs, CXCRs**: Receptores de citocinas e quimiocinas. **CD62-P:** P-selectina. **PSGL-1:** Glicoproteína ligante 1 de P-selectina. **αIIbβ3 e Mac-1:** Ligantes de fibrinogênio. **αG:**α-grânulos. **GD:** Grânulos densos. **Pré mRNA**: RNA mensageiro com íntrons e exóns pré ação de spliceossomas. Fonte: Adaptado de Dib *et al.,* 2020 – ainda não publicado.

Além dos mecanismos apresentados desencadeados pela ativação plaquetária, é descrito que as plaquetas sofrem apoptose, um processo biológico de

morte celular sem perda da integridade da membrana, que limita a vida útil das células (MASON *et al.*, 2007). O balanço antagônico entre as proteínas anti-apoptóticas, como Bcl-2 e Bcl-xL, e pró-apoptóticas, como Bak e Bax, regula a meia-vida das plaquetas. Nesse sentido, com o envelhecimento plaquetário, ocorre o aumento das proteínas pró-apoptóticas iniciando a via intrínseca da apoptose bem descrita em plaquetas (LOPEZ *et al.,* 2008; MASON *et al.*, 2007). A via intrínseca cursa com a formação do poro de transição de permeabilidade mitocondrial (MPTP); despolarização da membrana mitocondrial externa ($\Delta\Psi_m$); liberação de fatores apoptogênicos, como o citocromo c, o qual por sua vez se liga à proteína adaptadora Apaf-1(fator de ativação de protease apoptótica-1) formando um "apoptossomo"; recrutamento e ativação da procaspase-9 que, por sua vez, ativa as caspases 3, 6 e 7; exposição de fosfatidilserina (PS) na membrana e formação de micropartículas plaquetárias (LEYTIN, 2012). Além do envelhecimento plaquetário, condições patológicas podem contribuir para apoptose plaquetária e conseqüentemente para a trombocitopenia observada em diversas doenças, como na dengue (HOTTZ *et al*., 2013b, 2014; LEYTIN, 2012).

Em um artigo de revisão escrito recentemente por nosso grupo, nós compilamos e descrevemos como as plaquetas através de seus PRRs e interações com leucócitos participam de diversos processos imunes e inflamatórios em doenças infecciosas e em doenças inflamatórias estéreis, influenciando fortemente na patogênese destas doenças (DIB *et al.,* 2020)(ANEXO B). Diversas evidências apontam para a participação das plaquetas em infecções virais, como na infecção por HIV (vírus da imunodeficiência humana), influenza, COVID-19 (doença por corona vírus 2019) e dengue (HOTTZ; BOZZA; BOZZA, 2018; KOUPENOVA *et al.,* 2019; MANNE *et al.,* 2020; REAL *et al.*, 2020; TRUGILHO *et al.,* 2017).Tal participação envolve, por exemplo, secreção de citocinas e quimiocinas dos grânulos plaquetários e interação plaquetas-leucócitos contribuindo para a amplificação de processos inflamatórios, desencadeando a secreção de citocinas pelos leucócitos, induzindo a liberação de NETs (do inglês *neutrophils extracellular traps*) e além disso, contribuindo para a disseminação viral, como o que ocorre no HIV a partir da transinfecção do vírus para células T CD4+ e macrófagos (BOILARD *et al.*, 2016; HOTTZ *et al.*, 2014; KOUPENOVA *et al*., 2019; REAL *et al.*, 2020).

No cenário da infecção pelo DENV ocorrem importantes alterações associadas às funções e número das plaquetas, as quais foram compiladas por nós em um artigo publicado na revista *Platelets* (ANEXO C). As plaquetas possuem a maquinaria celular necessária para a entrada do vírus e tradução do genoma viral com liberação, por exemplo, da proteína não estrutural NS1, mas não formando partículas virais maduras (KAR *et al.*, 2017; QUIRINO-TEIXEIRA *et al.*, 2020; SIMON; SUTHERLAND; PRYZDIAL, 2015). Apesar das plaquetas apresentarem um ciclo de replicação abortivo do DENV, sugere-se que elas contribuam para a patogênese da doença amplificando a resposta inflamatória.É descrito que a atividade da caspase-1 plaquetária e o aumento da expressão de IL-1β nas plaquetas e nas microvesículas (MVs) derivadas de plaquetas se correlacionam com sinais de aumento da permeabilidade vascular na dengue. De fato, experimentos *in vitro* demonstraram a ativação do inflamassoma em plaquetas infectadas por DENV e conseqüente liberação de MVs ricas em IL-1β, as quais contribuíram para a hiperpermeabilidade endotelial *in vitro* de maneira dependente de IL-1R(HOTTZ et al., 2013a). Além disso, foram observados níveis aumentados de agregados plaquetas-monócitos no sangue de pacientes com dengue, os quais foram correlacionados positivamente com o aumento da permeabilidade endotelial. Experimentos *ex vivo* demonstraram que plaquetas provenientes de pacientes com dengue interagem com monócitos induzindo a secreção de citocinas como IL-1β, IL-8, IL-10 e MCP-1 pelos monócitos (HOTTZ *et al.*, 2014). Portanto, além de células como leucócitos e células endoteliais secretarem citocinas associadas ao aumento da permeabilidade endotelial, estudos recentes têm sugerido um papel importante da atividade plaquetária na patogênese da dengue, contribuindo também para a amplificação da resposta inflamatória (HOTTZ et al., 2013a, 2014; TRUGILHO et al., 2017).

Análises proteômicas de plaquetas provenientes de pacientes infectados pelo DENV mostram um aumento significativo de proteínas relacionadas à sinalização da ativação plaquetária, além do aumento de P-selectina, um marcador de superfície de ativação plaquetária que foi correlacionado positivamente com a gravidade da dengue (TRUGILHO *et al.,* 2017). Os estímulos para ativação plaquetária na dengue não são completamente elucidados, sabe-se que as plaquetas podem ser ativadas diretamente pelo vírus através da interação do vírus,

por exemplo, com o receptor DC-SIGN (HOTTZ et al., 2013b; TRUGILHO et al., 2017). No entanto, a queda da viremia na fase crítica em que há o pico de ativação plaquetária nos indica a importância de investigar outros estímulos responsáveis por essa ativação. É descrito que a NS1 e as histonas H2A altamente presentes no plasma de pacientes com dengue são capazes de ativar as plaquetas via TLR4 (CHAO et al., 2019; QUIRINO-TEIXEIRA et al., 2020; TRUGILHO et al., 2017). Além disso, os níveis de peroxidação lipídica na DHF e na DSS são correlacionados com a trombocitopenia, conforme os níveis de MDA aumentam a contagem plaquetária diminui com uma correlação significativa (SOUNDRAVALLY *et al.,* 2008). Contudo, apesar dos mecanismos inflamatórios desencadeados por lipoproteínas de baixa densidade oxidadas (oxLDL) serem amplamente estudados em doenças cardiovasculares estéreis, a participação da oxLDL não foi até então relacionada à ativação plaquetária e aos quadros vasculares de doenças infecciosas, como na patogênese da dengue (CHYU et al., 2012; H.-C. et al., 2013; WANG et al., 2018).

2.3 Lipoproteínas de baixa densidade (LDL)

Devido à natureza hidrofóbica dos lipídios, os mesmos são transportados no plasma pelas lipoproteínas. As lipoproteínas plasmáticas são partículas esféricas formadas por uma membrana externa constituída por apolipoproteínas e lipídios anfipáticos (fosfolipídios e colesterol livre), envolvendo um núcleo hidrofóbico que contém lipídios apolares, denominados triacilglicerol (TAG) e ésteres de colesterol (ENGELKING, 2015). Existem algumas classes de lipoproteínas como: HDL (do inglês *High-density Lipoprotein)*, VLDL (do inglês *Very low-density lipoproteins*), IDL (do inglês *Intermediate-density lipoprotein*) e LDL (do inglês *Low-density lipoprotein*), as quais participam do metabolismo endógeno de lipídios e possuem algumas diferenças estruturais e funcionais, como por exemplo, os tipos de apolipoproteínas e a composição de lipídios (ORDOVAS, 2003).

A LDL é originada a partir do metabolismo endógeno de lipídios (Figura 6). A HDL é sintetizada no fígado com algumas apoproteínas, como Apo AI, Apo E e Apo CII e com a enzima LCAT (lecitina-colesterol aciltransferase) e posteriormente

liberada a partir do transportador ABCA1. Esta lipoproteína tem a função de captar colesterol dos tecidos, portanto é liberada do fígado com pouco colesterol e rapidamente adquire colesterol livre dos tecidos (ROSENSON *et al.*, 2012). À medida que os níveis de colesterol em uma célula aumentam, a formação de oxisteróis também aumenta, levando à ativação do fator de transcrição nuclear LXR e conseqüente aumento na expressão dos transportadores ABCA1 e ABCG1 com aumento do efluxo de colesterol das células para a HDL (MURTHY *et al.*, 2002; ZELCER; TONTONOZ, 2006). A HDL transforma o colesterol livre capturado dos tecidos em colesterol éster através da enzima LCAT ativada pela Apo AI e transfere o colesterol para o fígado pelo receptor scavenger classe B1 (SR-B1). Além de participar do transporte reverso de colesterol, a HDL fornece para a VLDL as apoproteínas Apo CII e Apo E(ROSENSON *et al.*, 2012).

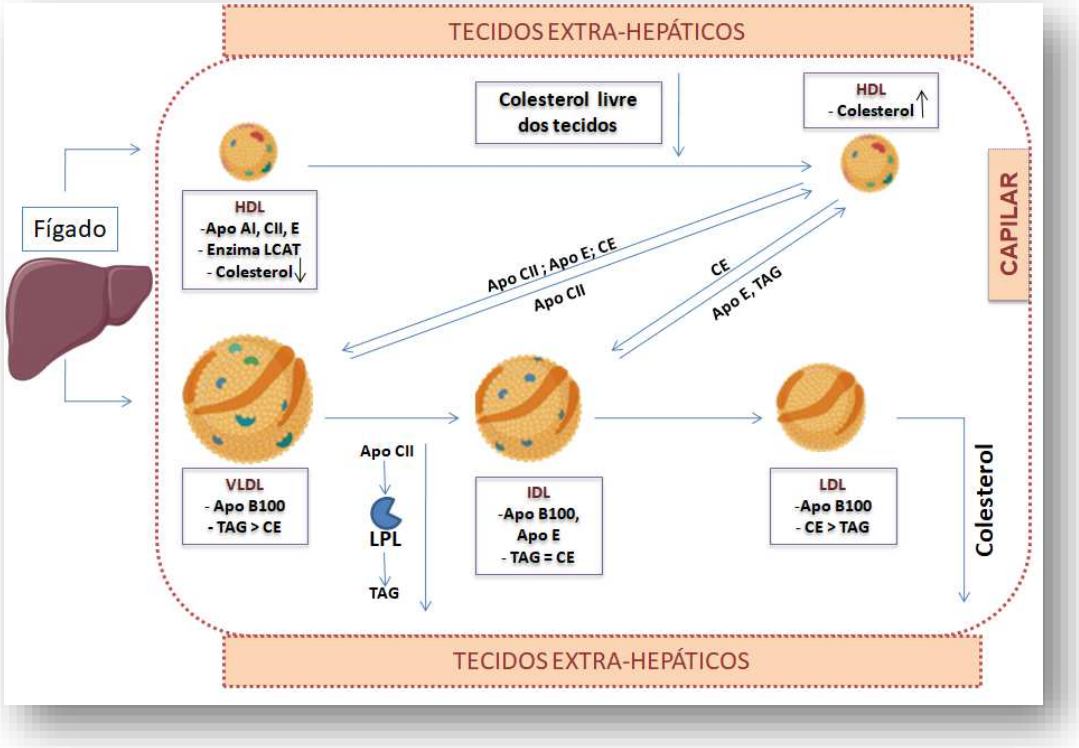

Figura 6 – Esquema representativo do metabolismo endógeno de lipoproteínas. Apo A1, Apo CII, Apo E, Apo B100: Apolipoproteínas constituintes das lipoproteínas. **LCAT:** Lecitina-colesterol aciltransferase. **TAG:** Triglicerídeos. **CE**: Colesterol esterificado. **LPL**: Lipoproteína Lipase.

A VLDL é liberada pelo fígado com mais TAG do que colesterol e com a ApoB100 (uma glicoproteína altamente anfipática que penetra repetidamente na monocamada fosfolipídica ou mesmo no interior lipídico hidrofóbico da lipoproteína,

com 4536 aminoácidos e massa molecular de ~515 kDa) (SHELNESS; SELLERS, 2001). Ao receber a Apo CII da HDL, a VLDL consegue ativar a enzima lipoproteína lipase (LPL) do endotélio capilar, liberando ácidos graxos dos TAG para os tecidos, como tecido muscular e adiposo (WOLSKA *et al.,* 2017). Dessa forma, com o conteúdo reduzido de TAG, a VLDL transfere a Apo CII para a HDL novamente e passa a ser classificada como IDL. Esta pode retornar ao fígado interagindo com receptores para Apo E ou pode interagir com a HDL, transferindo a Apo E, fornecendo TAG e recebendo colesterol a partir da enzima colesteril-éster transferase (CETP), passando a ser classificada como LDL, com mais colesterol do que TAG (Figura 7)(ROSENSON *et al.,* 2012; WANG *et al.*, 2017).

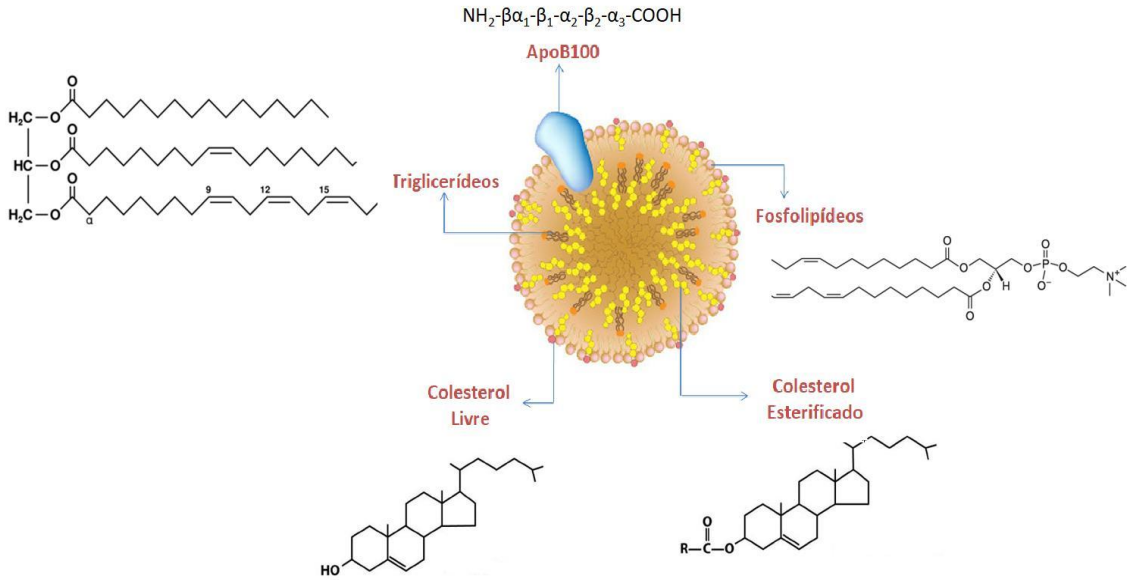

Figura 7 – Representação da lipoproteína de baixa densidade (LDL).O núcleo hidrofóbico formado pelos lipídios apolares: triglicerídeos e colesterol esterificado. A membrana externa composta por lipídios anfipáticos: monocamada de fosfolipídios e colesterol livre. A glicoproteína Apo B100 envolve a lipoproteína penetrando na monocamada fosfolipídica ou mesmo no interior lipídico hidrofóbico da lipoproteína.

A LDL consiste em uma classe de lipoproteínas com densidade entre 1,019–1,063 g/ml, com função de entregar colesterol para os tecidos extrahepáticos, possuindo em sua superfície a ApoB100, a qual é importante na manutenção da integridade estrutural e na endocitose da LDL pelo receptor (LDLR) presente no fígado e na maioria dos outros tecidos (KHOSRAVI; HOSSEINI-FARD; NAJAFI, 2018; WANG *et al.,* 2017).

A entrega de colesterol à célula diminui a atividade da HMG-CoA redutase (do inglês, *3-hydroxy-3-methyl-glutaryl-coenzyme A reductase,* uma enzima chave na biossíntese do colesterol) e diminui também a expressão dos LDLR, pois os esteróis formados do metabolismo de colesterol ativam o fator de transcrição LXR que medeia tal diminuição (ZHANG *et al.,* 2012). Por outro lado, quando os níveis de colesterol celular diminuem, o fator de transcrição SREBP (Proteínas de Ligação a Elemento Regulador de Esterol) estimula a expressão dos receptores de LDL e da enzima HMG-CoA redutase para aumentar a síntese de colesterol e endocitose da LDL (BROWN; RADHAKRISHNAN; GOLDSTEIN, 2018). As mudanças na captação de colesterol mediada pelas alterações na expressão do receptor de LDL juntas ao efluxo de colesterol mediado pelos transportadores ABCA1 e ABCG1 manterão a homeostase celular do colesterol.

A LDL pode sofrer peroxidação lipídica, esta ocorre quando espécies oxidantes adquirem hidrogênios especialmente de ácidos graxos poliinsaturados (PUFAs) livres ou que compõem os fosfolipídios e lipídios neutros presentes na LDL, formando radicais reativos lipídicos (L°). Estes radicais lipídicos reagem rapidamente com o oxigênio (O_2), formando radicais peroxi lipídicos (LOO°), os quais adquirem hidrogênios de outras moléculas lipídicas, formando hidroperóxidos lipídicos (LOOH) e mais radicais lipídicos (L°) que continuam a reação em cadeia. Ou seja, os próprios radicais lipídicos gerados por espécies oxidantes, na presença de O_2 contribuem também para a peroxidação lipídica e formação de mais radicais lipídicos (Figura 8) (AYALA; MUÑOZ; ARGÜELLES, 2014). Além dos LOOH que consistem em produtos primários da peroxidação lipídica, alguns produtos secundários reativos são formados, como os aldeídos malondialdeído (MDA) e o 4-hydroxynonenal (4-HNE) produzido pela peroxidação lipídica dePUFAs *n*-6. Estes aldeídos reativos propagam os eventos iniciais das espécies oxidantes reagindo com os aminoácidos de proteínas, modificando essas biomoléculas ao gerar adutos intermoleculares, como 4HNE-proteínas ou MDA-proteínas (AYALA; MUÑOZ; ARGÜELLES, 2014; DOMINGUES *et al.,* 2013; ESTERBAUER; SCHAUR; ZOLLNER, 1991).

Não há consenso sobre a composição específica da oxLDL e estas podem ser caracterizadas por diferentes graus de oxidação. A composição da oxLDL tem influência de diversos fatores, como: do agente oxidante; das condições nas

quais essa LDL foi submetida à oxidação; da composição da partícula de LDL antes da modificação e do status inicial de antioxidantes dessa partícula (LEVITAN; VOLKOV; SUBBAIAH, 2010). De maneira geral, diferentes concentrações de lipídios oxidados faz com que as oxLDL possam ser descritas como *"LDLs levemente oxidadas" ou "LDLs extensamente oxidadas"*. As LDLs levemente oxidadas possuem depleção antioxidante, oxidação de fosfolipídios, pouca ou nenhuma modificação de proteínas e embora quimicamente diferentes das LDLs não modificadas ainda são reconhecidas pelos receptores de LDL (LEVITAN; VOLKOV; SUBBAIAH, 2010). Já as "*LDLs extensamente oxidadas*" não são reconhecidas pelo receptor de LDL e possuem uma maior extensão de modificações na proteína ApoB100(LEVITAN; VOLKOV; SUBBAIAH, 2010; PARTHASARATHY *et al.*, 2010).

As modificações oxidativas dos componentes lipídicos da LDLoxidada (oxLDL) levam à formação de compostos bioativos que são considerados DAMPs, ativando PRRs e induzindo resposta imune e inflamação (MILLER; SHYY, 2017). A captação de oxLDL pelos macrófagos, por exemplo, leva ao acúmulo de colesterol, convertendo-os em células espumosas e contribuindo para o desenvolvimento de lesões ateroscleróticas(BERKEL *et al.,* 1995; CHISTIAKOV *et al.*, 2017). Além de servir como substrato para formação das células espumosas, foram descritas outras atividades imunopatogênicas para a oxLDL, como: recrutamento de leucócitos para locais de lesões ao induzir, por exemplo, a expressão superficial das moléculas de adesão e a liberação de quimiocinas pelas células endoteliais; transição de células musculares lisas para um fenótipo pró-inflamatório; e agregação plaquetária nas doenças cardiovasculares (CHEN *et al.*, 2001; LIU *et al.*, 2014; NAVAB *et al.*, 1991; TAKEI; HUANG; LOPES-VIRELLA, 2001). Portanto, a oxLDL pode exercer uma ampla gama de bioatividades críticas para impulsionar a imunopatogênese de doenças inflamatórias estéreis, porém o seu possível envolvimento na imunopatogênese de doenças infecciosas, como a dengue, ainda não foi descrito.

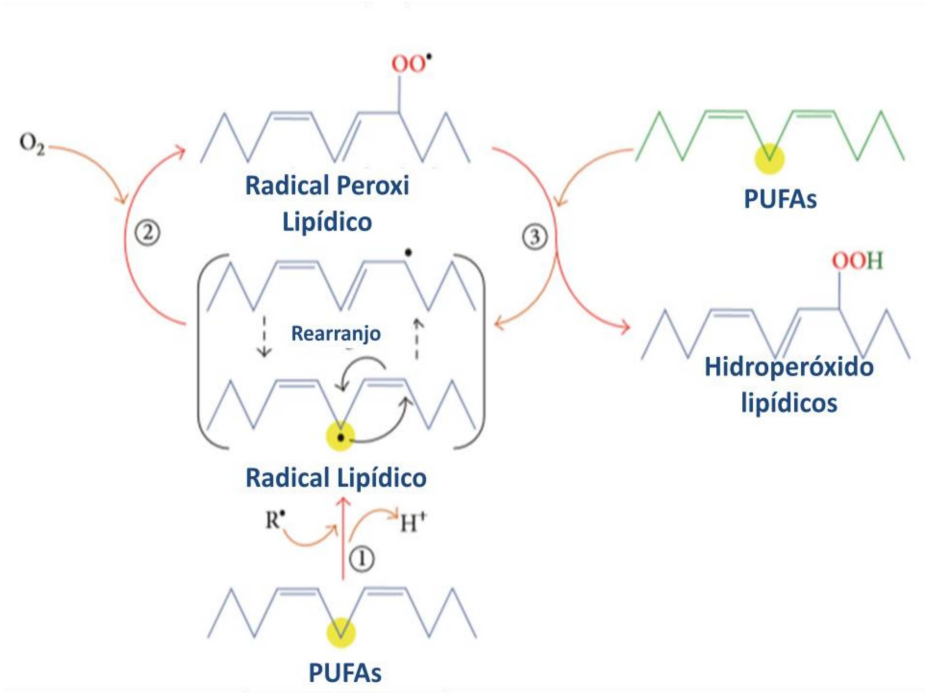

Figura 8 – Peroxidação lipídica. Na etapa 1ocorrea iniciação, com formação do radical lipídico após ação da espécie oxidante sobre os ácidos graxos poliinsaturados (PUFAs). O radical formado tende a ser estabilizado por um rearranjo molecular formando um dieno conjugado. Na etapa 2 de propagação, o radical lipídico rapidamente reage com oxigênio, formando o radical peroxi lipídico, o qual adquire hidrogênios de outras moléculas lipídicas continuando a reação em cadeia (Adaptado de AYALA; MUÑOZ; ARGÜELLES, 2014).

3 OBJETIVOS

3.1 Objetivo Geral

Investigar a participação da oxLDL na patogênese da dengue, a partir da identificação de mecanismos de ativação e apoptose plaquetária associados à resposta inflamatória na infecção.

3.2 Objetivos específicos

Isolar LDL do plasma de pacientes com dengue e caracterizar quanto à presença de lipídios oxidados;

Avaliar a habilidade das oxLDL isoladas do plasma de pacientes com dengue em promover ativação e apoptose plaquetária;

Investigar a habilidade das oxLDL isoladas do plasma de pacientes com dengue em promover secreção de mediadores inflamatórios em modelos de interação com plaquetas.

4 MATERIAL E MÉTODOS

4.1 Material biológico de pacientes com dengue e voluntários saudáveis

As amostras de plasma de pacientes com dengue e voluntários saudáveis foram obtidas de acordo com projeto multicêntrico com aprovação ética (INI # 016/2010, IOC/FIOCRUZ # 42999214.1.1001.5248 e UFJF-HU # 65064117.9.0000.5133)e foram estocadas em biorepositórios no laboratório de Imunofarmacologia (IOC/FIOCRUZ) e no laboratório de Imunotrombose (UFJF). As características clínicas e laboratoriais dos pacientes incluídos estão apresentadas na Tabela 1. A confirmação do diagnóstico foi realizada por testes sorológicos a partir da detecção de anticorpos IgM e IgG contra a proteína E do DENV e/ou do antígeno NS1, e por testes moleculares pela detecção do RNA viral por PCR, que também permitiu a determinação do sorotipo infectante (DENV-1 a 4).

Tabela 1 – Características clínicas e laboratoriais dos pacientes com dengue

	Controle (n=3)	**Dengue** (n=3)	**Valores de Referência***
Idade, anos	45 (36-53)	38 (28-44)	-
Gênero, Masculino	1 (33%)	2 (66%)	-
Plaquetometria, x 1000/mm^3	-	85 (33,6 – 111)	150-450
Contagem de leucócitos, células/mm^3	-	3646 (3340-4206)	4000-11000
Hematócrito, %	-	40 (34,5-44,42)	37-50
Albumina, g/dL	-	3,7 (3,2-4,15)	3,5-5,5
TGO/AST, U/L	-	71 (38,5-93)	10-40
TGP/ALT, U/L	-	93 (51-162)	10-40
GGT, U/L	-	69 (52-63)	8-50
Dengue Branda	-	2 (66%)	-
Dengue Branda com Sinais de Alarme[1]	-	1 (33%)	-
Manifestações hemorrágicas[2]	-	0 (0%)	-
Infecção Secundária	-	2 (66%)	-
IgM$^+$	-	3 (100%)	-
IgG$^+$	-	2 (66%)	-
NS1$^+$	-	1 (33%)	-

Dados apresentados como média (mínimo-máximo) ou número (percentual-%).
*Intervalos de referência para testes laboratoriais segundo Conselho Americano de Medicina Interna (ABIM).
[1] Dor abdominal, vômitos persistentes e aumento do hematócrito concorrente com declíniorápido da contagem de plaquetas.
[2] Sangramento de gengiva, metrorragia, hematêmese, hematúria e petéqueas.

A experimentação com plaquetas isoladas de voluntários saudáveis incluídos no Instituto de Ciências Biológicas da Universidade Federal de Juiz de Fora (ICB-UFJF) foi aprovado pelo comitê de ética (UFJF-HU # 65064117.9.0000.5133). Os critérios de inclusão para os voluntários saudáveis consistiram em: idade acima de 18 anos; ausência do consumo de alimentos contendo cafeína no dia da coleta; ausência de quadros infecciosos ou alérgicos por no mínimo 14 dias antes da coleta, assim como ausência do uso de medicações, como anti-inflamatórios, 14 dias precedentes a data da coleta. Todo material biológico foi obtido de pacientes ou voluntários saudáveis que consentiram em participar do estudo mediante assinatura de um Termo de Consentimento Livre e Esclarecido, regido de acordo com as normas da Resolução 466/12 do Conselho Nacional de Saúde.

4.2 Isolamento de LDL por gradiente de densidade e ultracentrifugação

As LDLs de voluntários saudáveis e de pacientes com dengue foram isoladas de acordo com protocolo padronizado por nosso grupo descrito em um artigo aceito para publicação na *Methods in Molecular Biology* (ANEXO D). O plasma utilizado para montagem dos gradientes foi obtido a partir do sangue periférico de voluntários saudáveis e de pacientes com dengue. O sangue foi coletado (20mL) por meio de escalpe com seringa contendo 3 ml do anticoagulante citrato-ácido-dextrose (ACD - 8 g/L de ácido cítrico, 22,4 g/L de citrato de sódio e 2 g/L de dextrose; pH 5,1). Em uma cabine de fluxo laminar, o sangue foi transferido para dois tubos cônicos estéreis de 50 mL. Os tubos foram então centrifugados a 700 x g, por 20 minutos à temperatura ambiente, com parada sem freio para obter o plasma pobre em plaquetas (PPP). Uma segunda centrifugação foi realizada a 2.500 x g, por 15 minutos à temperatura ambiente, com parada sem freio, para exclusão de corpos apoptóticos, fragmentos de membrana e restos celulares. O plasma livre de plaquetas (PLP) foi adicionado de hidroxitolueno butilado – BHT(1 µl/ml) para prevenir a oxidação *ex vivo* das LDLs plasmáticas.

Os plasmas foram submetidos ao gradiente de densidade com posterior ultracentrifugação. O gradiente foi montado em tubos de ultracentrífuga de 8 mL aos quais foram adicionados 2 mL de plasma contendo 0,5 g/mL de KBr; seguidos de 6 mL de solução de NaCl 0,9 %p/v. Os gradientes foram centrifugados a 150.000 x g por 2,5 h à temperatura de 4°C e 26 frações de 300 µL foram recuperadas. As especificações do rotor da ultracentrífuga utilizada são: rotor MLN-80, quase vertical, *Beckman Coulter*. As frações de LDL (ricas em colesterol e pobres em triglicerídeos) foram caracterizadas a partir da utilização de kits colorimétricos baseados em reações enzimáticas incluindo kit Triglicérides liquiform (87-2/250, Labtest, Brasil), kit Colesterol liquiform (76-2/250, Labtest, Brasil) e kit Albumina (19-1/250, Labtest, Brasil) (Figura 9). Além da dosagem lipídica, realizamos a pesagem de 100µL das frações para confirmar a densidade que corresponde à densidade das LDL (1,019-1,063 g/ml). Adicionou-se BHT (1 µl/ml) e Pefablock (1 µl/ml) nas amostras de LDL, as quais foram dialisadas 12 horas em 1L de solução de PBS-EDTA (tampão fosfato e salina com EDTA na concentração de 10mM), com troca da solução de PBS-EDTA para subseqüentes 6 horas de diálise, totalizando 18 horas de diálise das amostras de LDL. Acrescentou-se Pefablock (1ul/ml) nas amostras de LDL dialisadas, as quais foram armazenadas a -80ºC.

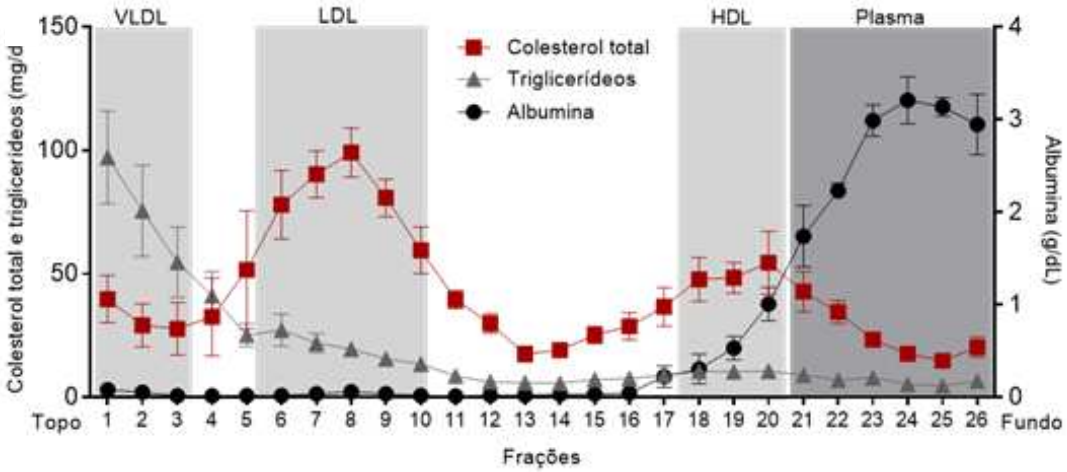

Figura 9 – Isolamento de lipoproteínas plasmáticas por ultracentrifugação-Composição das frações de plasma obtidas a partir de um gradiente de densidade bifásico para tubo de 8 mL.

4.3 Detecção de malondialdeído por teste de TBARS

Realizamos a incubação de 50 µg de amostras de LDL isoladas de pacientes com dengue ou de voluntários saudáveis com 100 µL de ácido tiobarbitúrico (TBA) 0,67% na avaliação da concentração de MDA (malondialdeído). Os volumes das amostras foram ajustados com PBS (tampão fosfato e salina). O experimento foi realizado em duplicata e as amostras foram submetidas a banho seco a 96 °C por 30 minutos. A mistura de amostra com TBA foi transferida para uma placa de 96 poços e realizou-se a leitura em espectrofotômetro a 535nm. Os valores foram representados em razão da concentração de colesterol quantificado por teste colorimétrico através do kit Colesterol liquiform (ref.: 76-2/250 Labtest, Brasil).

4.4 Detecção de 4HNE por Western blot

Amostras de LDL isoladas de voluntários saudáveis e de pacientes com dengue foram avaliadas quanto à presença da 4HNE, a qual assim como o MDA consiste em um aldeído reativo, subproduto da peroxidação lipídica. Alíquotas das amostras de LDL foram lisadas com tampão de lise (10 mM Tris; 150 mM NaCl; 0,1 mM EDTA; inibidor de proteases (ROCHE) e Triton x-100 1%). Realizou-se a dosagem de proteínas das amostras de LDL lisadas e não lisadas utilizando-se kit BCA (Thermo Scientific). Para a detecção da proteína ApoB100 (~515 kDa) no controle de carregamento do experimento foi necessário a padronização da técnica para proteínas de alto peso molecular, conforme descrevemos no artigo que submetemos para a *Methods in Molecular Biology* (ANEXO D). As amostras de LDL lisadas foram aplicadas em gel SDS-PAGE (dodecil-sulfato de sódio – poliacrilamida) a 6% para realização da eletroforese (32 mA). Realizou-se a eletrotransferência das proteínas do gel para a membrana de PVDF (Fluoreto de Polivinilideno), a 40 mA, *overnight.* As membranas foram bloqueadas com TBS-T (tampão tris salina, 0,1% Tween 20) e 5% de leite por 1 hora. Posteriormente, as membranas foram incubadas com anticorpo primário anti-4HNE policlonal (Abcam -

ab46544) produzido em cabra (1:1000), *overnight,* sob refrigeração a 4ºC. No dia seguinte, as membranas foram lavadas com TBS-T e incubadas com anticorpo secundário anti IgG de cabra (Vector, PI-9500, 1:10.000) por 1 hora. A membrana foi lavada novamente com TBS-T e revelada com os reagentes quimioluminescentes Dura (Thermo). Para o controle de carregamento do experimento, as membranas também foram incubadas com anticorpo anti Apo-B100 produzido em cabra (BioRad - AHP1267, 1:1000), *overnight,* sob refrigeração a 4ºC. No dia seguinte, as membranas foram lavadas com TBS-T e incubadas com anticorpo secundário anti IgG de cabra (Vector, PI-9500, 1:10.000) por 1 hora. A membrana foi lavada novamente com TBS-T e revelada com o reagente quimioluminescente Pico (Thermo).

4.5 Isolamento de plaquetas

A partir do sangue periférico de voluntários saudáveis coletado em seringa contendo ACD (8g/L de ácido cítrico, 22,4 g/L de citrato de sódio e 2g/L de dextrose; pH 5,1) foi realizado o protocolo de isolamento de plaquetas (HAMBURGER; MCEVER, 1990). O isolamento de plaquetas se deu com subseqüentes centrifugações de 20 minutos à temperatura ambiente e parada sem freio (1ª centrifugação para obtenção do plasma rico em plaquetas (PRP) foi realizado a 205 x *g*; e 2ª e 3ª centrifugações para obtenção do pellet de plaquetas livre de debris celulares foram realizadas a 550 x *g*). O PRP após 1ª centrifugação foi transferido para um novo tubo falcon estéril e acrescentou-se 1µL/mL de solução de prostaglandina E_1 (Cayman, 13010 - PGE_1) com PSG (5 mM de PIPES, 145 mM de NaCl, 4mM de KCl, 50 µM de Na_2HPO_4, 1mM de $MgCl_2.6H_20$, 5,5 mM de glicose; pH 6,8). O pellet formado após a 2ª centrifugação foi ressuspendido em PSG e adicionado de solução de PGE_1 novamente (1µL/mL) e o pellet formado após a 3ª centrifugação foi ressuspendido com meio 199 (M199 com EBSS, L-Glutamina e HEPES; Lonza 12-117F). A concentração plaquetária foi corrigida após contagem de plaquetas, de modo a obter $1x10^9$ plaquetas/mL.

4.6 Estímulo com LDL de pacientes com dengue e de indivíduos saudáveis*ex vivo*

As plaquetas foram estimuladas com a LDL proveniente de pacientes com dengue e de indivíduos saudáveis em quatro concentrações diferentes (5ng/mL, 10ng/mL, 100ng/mL e 1000ng/mL), com ou sem a presença da trombina (Sigma – T1063) em dose subótima (0,02U/ml), um agonista clássico plaquetário (CHEN *etal.*, 2009). Para o controle negativo dos experimentos utilizou-se plaquetas (*n*=3) não estimuladas e apenas acrescidas de M199 e para o controle positivo realizou-se estímulo plaquetário com trombina nas doses de 0,5U/mL e 0,02U/mL na ausência de estímulo com LDL. O sobrenadante das plaquetas estimuladas foi então coletado, centrifugados a 997 *x g* por 10 minutos e estocados à -80°C para posterior uso.

4.7 Análise da ativação plaquetária por citometria de fluxo

As plaquetas em diferentes condições de estímulo foram marcadas com anticorpos conjugados a fluorocromos e protegidas da luz. Após 30 minutos de estímulo, a fim de avaliar a expressão superficial da P-selectina, as plaquetas foram marcadas utilizando-se o anticorpo anti-CD62P conjugado a aloficocianina (BD Biosciences – 550888, CD62P APC, 1:100). A ativação da integrina αIIbβ3 também foi avaliada utilizando-se o anticorpo PAC-1 conjugado a isotiocianato de flureseína (BD Biosciences – 340507, PAC-1 FIT-C, 1:50). Por fim, as plaquetas também foram marcadas a fim de avaliar a expressão do receptor CD36 utilizando-se o anticorpo anti-CD36 conjugado a isotiocianato de flureseína (BD Biosciences – 555454, CD36 FIT-C, 1:100). Para a identificação das plaquetas utilizou-se o anticorpo anti-CD41 conjugado a isotiocianato de flureseína (BD Biosciences – 555466, CD41a FIT-C, 1:100) ou o anticorpo anti-CD41 conjugado a aloficocianina(BD Biosciences – 559777, CD41a APC, 1:100). Após 10 minutos, as plaquetas foram fixadas com paraformaldeído 4%. Posteriormente, as plaquetas marcadas foram adquiridas em Citômetro de Fluxo (BD FACSCanto II) através do

software FACSDiva. Os dados foram posteriormente analisados por meio do software FlowJo.

4.8 Ensaio de apoptose plaquetária induzida por LDL de pacientes com dengue e de indivíduos saudáveis e análise por citometria de fluxo

As plaquetas incubadas com LDL na concentração de 1000ng/mL, na presença ou ausência de trombina em dose subótima,foram marcadas para análise dos parâmetros apoptóticos de exposição de fosfatidilserina e de despolarização da membrana mitocondrial interna. Para detecção da exposição de fosfatidilserina, as plaquetas foram incubadas com um *pool* de tampão de ligação de Anexina V e Anexina V conjugada a isotiocianato de flureseína (BD Biosciences – 556419, Anexina V FIT-C, 1:20). Após 15 minutos de incubação, protegido da luz e a temperatura ambiente, as plaquetas foram diluídas em 380µL do tampão de ligação de Anexina V. Para a avaliação da despolarização da membrana mitocondrial interna, as plaquetas foram incubadas com a sonda catiônica tetrametilrodamina etil ester (TMRE)(Sigma – 879117, 100nM). Posteriormente, as plaquetas marcadas foram adquiridas em Citômetro de Fluxo (BD FACSCanto II) através do software FACSDiva. Os dados foram posteriormente analisados por meio do software FlowJo.

4.9 Dosagem de citocinas

Citocinas e quimiocinas de perfil pró-inflamatório foram quantificadas nos sobrenadantes das plaquetas estimuladas por meio de ELISA de captura (R&D systems) conforme as instruções do fabricante. Foram dosadas as citocinas IL-1α, IL-1β, MIF, PF4/CXCL4 eRANTES/CCL5. Para a dosagem das citocinas, as amostras foram diluídas, 1:2 para a dosagem de MIF, 1:200 para de PF4/CXCL4 e 1:10 para de RANTES/CCL5. Para a dosagem de IL-1α e de IL-1β não foi necessária diluição das amostras.

4.10 Análise estatística

As análises estatísticas foram realizadas utilizando-se GraphPad Prism 6.0 for Windows (GraphPadSoftware, San Diego California USA). Variáveis numéricas foram apresentadas como média ± erro padrão da média e foram avaliadas quanto à distribuição normal pelos testes de Kolmogorov-Smirnov e Shapiro-Wilk. As diferenças entre os grupos foram avaliadas por teste t de Student, sendo a significância estatística considerada sempre que $p < 0,05$.

5 RESULTADOS

5.1 Caracterização das LDL isoladas do plasma de pacientes com dengue ou de voluntários saudáveis

As LDL do plasma de pacientes com dengue, isoladas por ultracentrifugação em gradiente, foram utilizadas para a análise de peroxidação lipídica e de proteínas oxidadas. Para o controle negativo dos experimentos foram utilizadas LDL do plasma de voluntários saudáveis também isoladas por ultracentrifugação em gradiente. Sendo assim, realizamos o teste T-bars nas amostras de LDL isoladas de voluntários saudáveis e de pacientes com dengue para a avaliação da presença do malondialdeído (MDA), subproduto da peroxidação lipídica que reage com o ácido tiobarbitúrico utilizado no teste. Nossos resultados demonstram um aumento significativo da presença de MDA nas amostras de LDL provenientes dos pacientes com dengue em relação as LDL de indivíduos saudáveis, indicando uma maior peroxidação lipídica nos pacientes com dengue (Figura 10A). Além da análise do MDA, também avaliamos a presença do 4-hydroxynonenal (4-HNE), outro aldeído resultante da peroxidação lipídica. A 4HNE trata-se de um aldeído altamente reativo que forma adutos não somente com proteínas, mas também com fosfolipídios oxidados (oxPL) (DOMINGUES *et al*.,

2013). Os resultados apontam para a presença do 4HNE nas amostras de LDL de pacientes com dengue, o que não foi observado nas amostras de LDL dos voluntários saudáveis (Figura 10B).

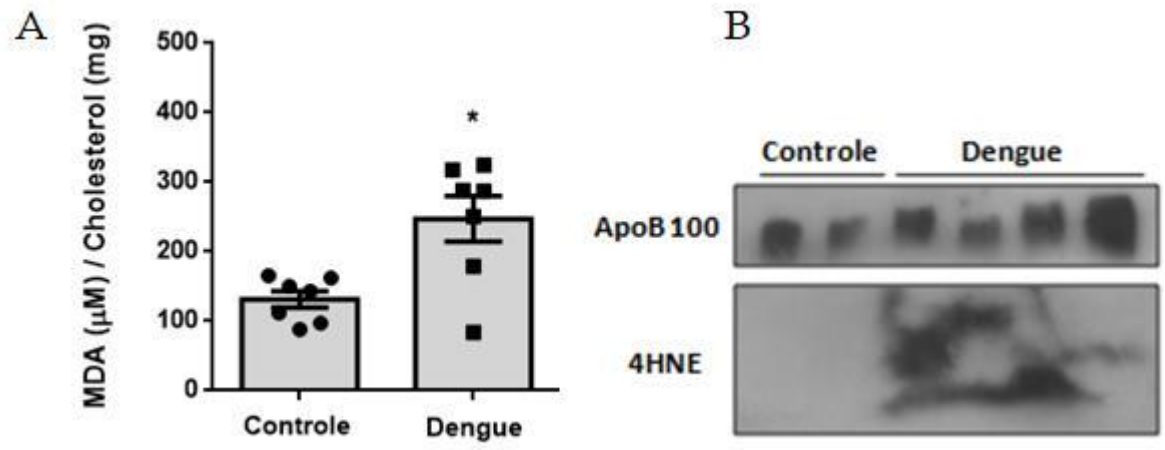

Figura 10 – Avaliação da peroxidação lipídica e de proteínas oxidadas em LDL de pacientes com dengue e voluntários saudáveis. A) Teste T-bars em amostras de LDL de voluntários saudáveis (controle) e de pacientes com dengue (dengue). A concentração de MDA foi detectada em 50 µg de proteína de cada amostra. As barras representam a média ± SEM de sete voluntários; O asterisco (*) indica a diferença estatísticaem relação ao grupo LDL de indivíduo saudável (controle) com $p < 0,05$ com base no teste t não pareado. **B)** Presença de ApoB100 e 4HNE avaliadas em amostras de LDL lisadas de voluntários saudáveis (controle) e de pacientes com dengue (dengue) através da técnica de Western Blot.

5.2 Avaliação da habilidade das oxLDL isoladas de pacientes com dengue em promover ativação e apoptose plaquetária

Objetivando avaliar se a ligação das oxLDL circulantes em pacientes com dengue promove ativação plaquetária, plaquetas isoladas de voluntários saudáveis foram estimuladas com LDL isoladas do plasma de pacientes com dengue ou de voluntários saudáveis heterólogos *ex vivo*. A ativação foi investigada pela expressão plaquetária da P-selectina (CD62P), a qual está localizada nos α-grânulos das plaquetas quiescentes e após estímulo é translocada para a membrana plaquetária (KLING *et al*., 2013). Nossos resultados demonstram que as LDL provenientes dos

pacientes com dengue nas concentrações de 5ng/mL e 1000ng/mL não foram capazes de induzir o aumento da expressão de P-selectina plaquetária quando comparadas com as LDL provenientes de indivíduos saudáveis nas mesmas concentrações (Figura 11A). Nesse cenário, Chen e colaboradores (2009) demonstraram que a combinação de LDL oxidada *in vitro* com dose subótima de trombina (0,02U/mL) evocou uma resposta de ativação plaquetária e expressão de P-selectina mais robusta do que o estímulo apenas com oxLDL ou trombina na dose subótima. Nossos resultados de ativação plaquetária por LDL oxidada *in vitro* em nosso laboratório, corroboram com os dados da literatura, em que a LDL oxidada *in vitro* na concentração de 5ug/mL induz ativação plaquetária na presença de dose subótima de trombina. Sendo assim, nós incubamos as plaquetas com as LDL oxidadas dos pacientes com dengue na presença de dose subótima de trombina a fim de avaliar o sinergismo entre os estímulos. Para o controle do experimento e das marcações com anticorpos, nós avaliamos a expressão da P-selectina em plaquetas não estimuladas apenas acrescidas de M199 e em plaquetas estimuladas com a trombina nas doses de 0,5 e 0,02U/mL. Não houve uma resposta plaquetária significativa à dose subótima de trombina (0,02U/mL) comparado com as plaquetas não estimuladas, enquanto que a dose ótima de 0,5U/mL foi capaz de induzir uma ativação plaquetária significativa (Figura 11B). No entanto, nossos resultados demonstram que a LDL proveniente dos pacientes com dengue nas concentrações de 5ng/mL, 10ng/mL, 100ng/mL e 1000ng/mL também não foram capazes de potencializar a ativação de plaquetas estimuladas com a trombina na dose subótima (0,02U/mL), não induzindo o aumento da expressão de P-selectina plaquetária (Figura 11 C-F).

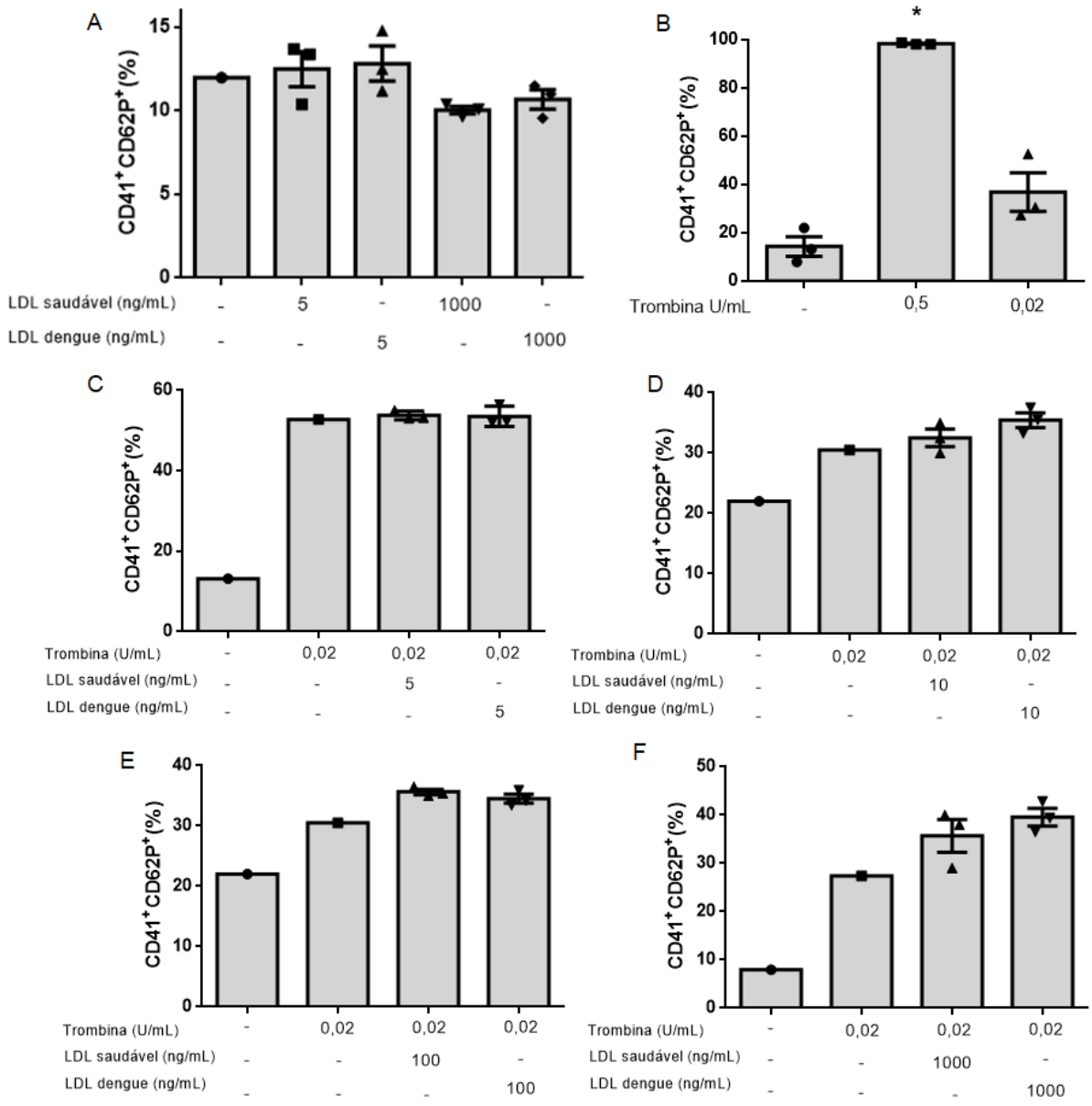

Figura 11 – Avaliação da expressão de P-selectina em plaquetas estimuladaspor LDL de indivíduo saudável (LDL saudável) ou LDL proveniente de paciente com dengue (LDL dengue). A expressão de P-selectina foi quantificada (% $CD41^+CD62P^+$) em plaquetas estimuladas por 3 LDL provenientes de três pacientes com dengue ou três voluntários saudáveis, a 5 ou 1000ng/ml **(A)**; em plaquetas de três voluntários em experimentos independentes estimuladas com trombina nas doses de 0, 0,5 e 0,02 U/mL para controle dos experimentos **(B)**; e em plaquetas estimuladas por dose subótima de trombina após estímulo com LDL de 3 pacientes com dengue ou 3 voluntários saudáveis nas concentrações de 5ng/mL **(B)**; 10ng/mL **(C)**; 100ng/mL **(D)** e 1000ng/mL **(E)**. Nos gráficos estão apresentados a média +/- erro padrão da média do percentual de plaquetas positivas para a expressão da P-selectina.

Além da análise da expressão da P-selectina plaquetária, também avaliamos se o estímulo com LDL de pacientes com dengue induz ativação da integrina αIIbβ3. Esta integrina é altamente expressa em plaquetas e adota uma conformação inativa em plaquetas em repouso. Após estimulação com agonista, a transducação de sinais induz a conformação ativa dessa integrina, mudando de um estado de baixa afinidade para alta afinidade para fibrinogênio e outros ligantes (BLEDZKA; SMYTH; PLOW, 2013). A alteração da expressão de CD36 também foi investigada, um receptor *scavenger* constitutivamente expresso em plaquetas e descrito estar envolvido no reconhecimento de lipídeos oxidados (PODREZ et al., 2007). Nossos resultados demonstram que os estímulos com LDL provenientes de pacientes com dengue nas concentrações de 5 e 1000ng/mL não induziram ativação da integrina αIIbβ3 (Figura 12A) e nem alteração na expressão do receptor CD36 (Figura 13A).

A fim de avaliar se há sinergismo entre os estímulos com LDL e dose subótima de trombina para a ativação da integrina αIIbβ3 e da expressão do receptor CD36, a análise também foi realizada em plaquetas estimuladas pelas LDL na presença de 0,02U/mL de trombina. Para o controle do experimento e das marcações com anticorpos, nós avaliamos a ativação da integrina αIIbβ3 e a expressão do CD36 em plaquetas não estimuladas apenas acrescidas de M199 e em plaquetas estimuladas com a trombina nas doses de 0,5 e 0,02U/mL. Não houve uma ativação significativa da integrina αIIbβ3 em resposta à dose subótima de trombina (0,02U/mL) comparado com as plaquetas não estimuladas, enquanto que a dose ótima de 0,5U/mL foi capaz de induzir uma ativação da integrina αIIbβ3 significativa (Figura 12B). Em relação ao receptor CD36, a expressão não foi alterada nas plaquetas estimuladas com 0,5U/mL ou 0,02U/mL de trombina em comparação às plaquetas não estimuladas (Figura 13B). Além disso, nossos resultados demonstram que a LDL proveniente dos pacientes com dengue nas concentrações de 5ng/mL, 10ng/mL, 100ng/mL e 1000ng/mL também não foram capazes de potencializar a ativação de plaquetas estimuladas com a trombina na dose subótima (0,02U/mL) em relação ao aumento da ativação da integrina αIIbβ3 (Figura 12 C-F) e a alteração da expressão do receptor CD36 (Figura 13 C-F).

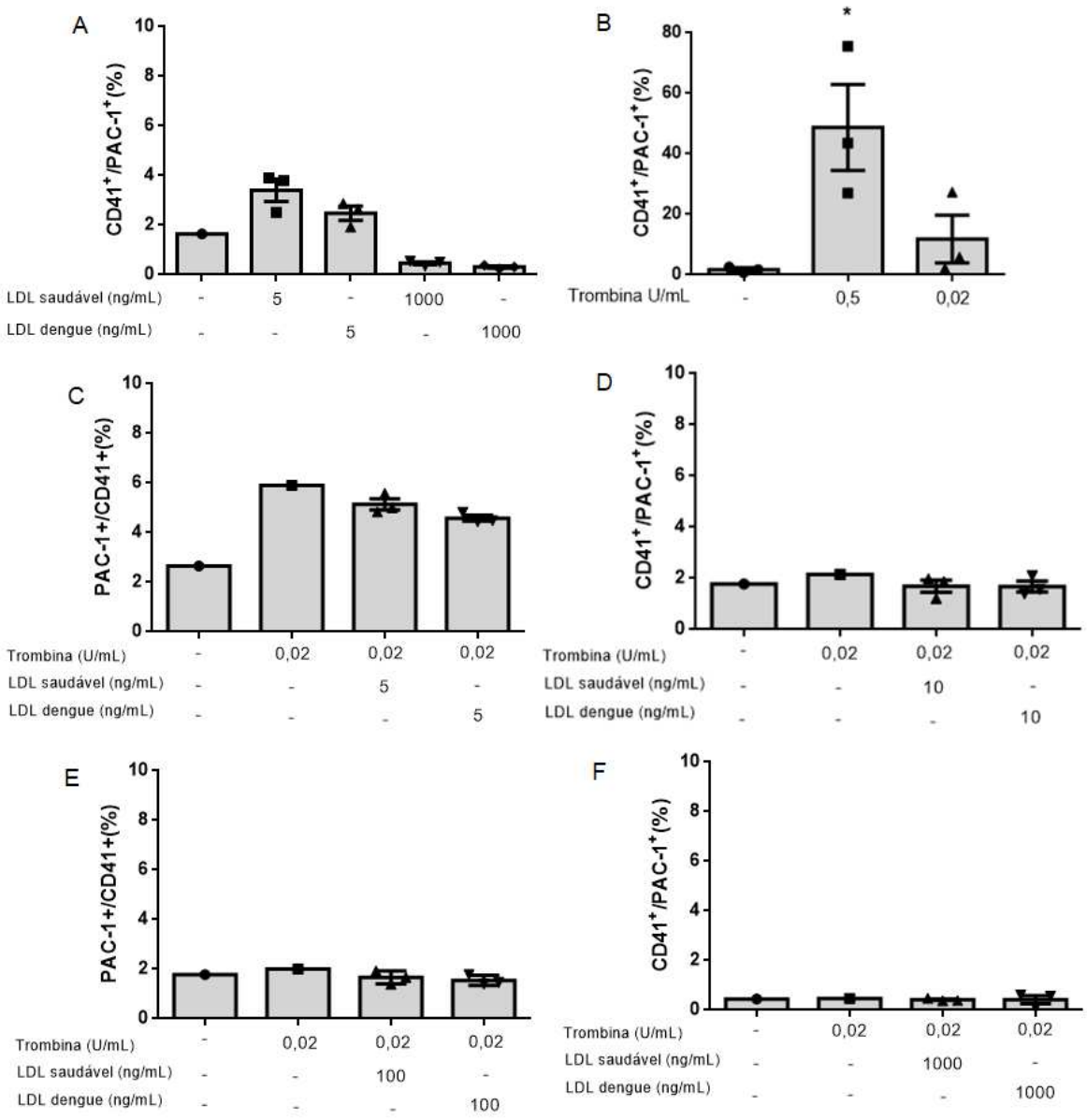

Figura 12 – Avaliação da ativação da integrina αIIbβ3 plaquetária por LDL de indivíduo saudável (LDL saudável) ou LDL proveniente de paciente com dengue (LDL dengue). A ativação da integrina αIIbβ3 foi quantificada (% $CD41^+PAC\text{-}1^+$) em plaquetas estimuladas por 3 LDL provenientes de três pacientes com dengue ou três voluntários saudáveis, a 5 ou 1000ng/ml **(A)**; em plaquetas de três voluntários em experimentos independentes estimuladas com trombina nas doses de 0, 0,5 e 0,02 U/mL para controle dos experimentos **(B)**; e em plaquetas estimuladas por dose subótima de trombina após estímulo com LDL de 3 pacientes com dengue ou LDL de 3 voluntários saudáveis nas concentrações de 5ng/mL **(C)**; 10ng/mL **(D)**; 100ng/mL **(E)** e 1000ng/mL **(F)**. Nos gráficos estão apresentados a média +/- erro padrão da média do percentual de plaquetas positivas para a ativação da integrina αIIbβ3.

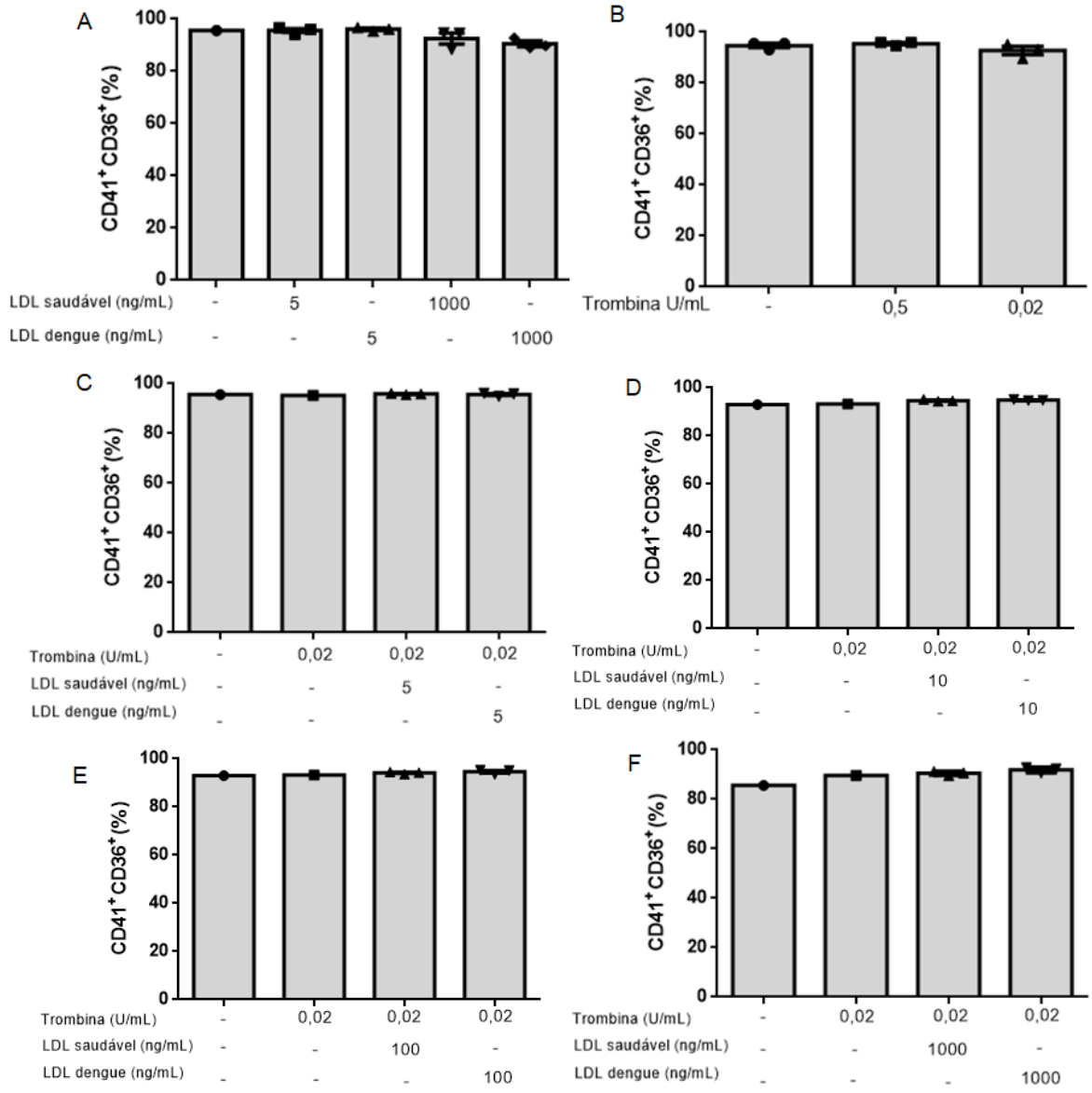

Figura 13 – Avaliação da expressão do receptor CD36 plaquetário frente estímulo por LDL de indivíduo saudável (LDL saudável) ou LDL proveniente de paciente com dengue (LDL dengue). A expressão do receptor CD36 foi quantificada (% $CD41^{+}CD36^{+}$) em plaquetas estimuladas por 3 LDL provenientes de três pacientes com dengue ou três voluntários saudáveis, a 5 ou 1000ng/ml **(A)**; em plaquetas de três voluntários em experimentos independentes estimuladas com trombina nas doses de 0, 0,5 e 0,02 U/mL para controle dos experimentos **(B)**; e em plaquetas estimuladas por dose subótima de trombina após estímulo com LDL de 3 pacientes com dengue ou LDL de 3 voluntários saudáveis nas concentrações de 5ng/mL **(C)**; 10ng/mL **(D)**; 100ng/mL **(E)** e 1000ng/mL **(F)**. Nos gráficos estão apresentados a média +/- erro padrão da média do percentual de plaquetas positivas para a expressão do receptor CD36.

Além de avaliar a ativação plaquetária, as plaquetas isoladas de voluntários saudáveis foram estimuladas com LDL isoladas do plasma de pacientes com dengue ou de voluntários saudáveis heterólogos *ex vivo*, e caracterizadas quanto à presença de parâmetros apoptóticos. As células apoptóticas apresentam despolarização mitocondrial e exposição aumentada de fosfatidilserina (ELMORE, 2007). Portanto, para identificação da população apoptótica realizou-se a avaliação das células duplo-marcadas (%$TMRE^{-}$/Anexina-V^{+}) conforme apresentado em nossa estratégia de *gate* (Figura 14 A). Para o controle negativo dos experimentos utilizou-se plaquetas não estimuladas e apenas acrescidas de M199 e para o controle positivo realizou-se estímulo plaquetário com heme sintético (20μM). O heme sintético consiste em um grupo prostético, com propriedades tóxicas quando em estado livre de hemoproteínas (KUMAR; BANDYOPADHYAY, 2005). Em ensaios realizados por nosso grupo, o heme se demonstrou um bom indutor de apoptose plaquetária (dados ainda não publicados). No entanto, as plaquetas estimuladas com LDL de pacientes com dengue na concentração de 1000ng/mL, na presença ou ausência da dose subótima de trombina, não apresentaram fenótipo apoptótico em comparação as plaquetas estimuladas com LDL de voluntários saudáveis nas mesmas condições (Figura 14B).

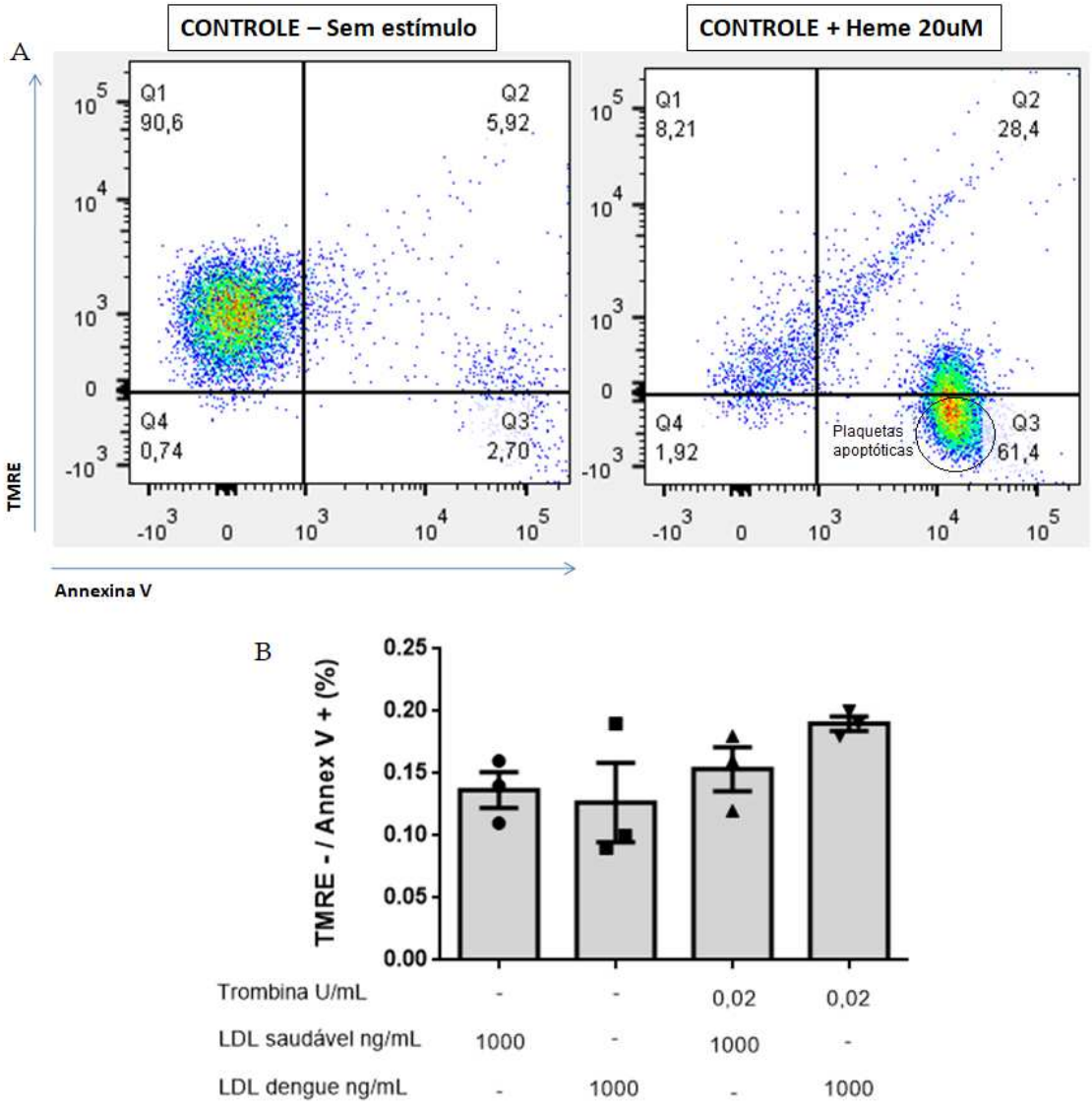

Figura 14 – Avaliação da apoptose plaquetária frente estímulo por LDL de indivíduo saudável ou LDL proveniente de paciente com dengue. A estratégia de *gate* utilizada para caracterização das células apoptóticas se deu pela avaliação das células duplo-marcadas (% TMRE $^{-}$/Annex V^{+}): negativas para a sonda TMRE e, portanto, com despolarização mitocondrial e positivas para a marcação com anexina V e, portanto, com exposição de fosfatidilserina **(A)**. A apoptose plaquetária (% TMRE^{-}/Annex V^{+}) foi então quantificada em plaquetas estimuladas por 3 LDL de indivíduos saudáveis (LDL saudável) e de 3 pacientes com dengue (LDL dengue) na concentração de 1000ng/ml, com ou sem a presença de trombina na concentração subótima de 0,02U/ml **(B)**. As barras representam a média ± erro padrão da média do percentual de plaquetas TMRE-/Annex V+.

5.3 Avaliação da habilidade das oxLDL isoladas de pacientes com dengue em promover secreção de mediadores inflamatórios plaquetários

A secreção de mediadores inflamatórios estocados em grânulos plaquetários já é bem descrita (FITCH-TEWFIK; FLAUMENHAFT, 2013). Portanto, nós investigamos a habilidade das LDL provenientes de pacientes com dengue em estimularem as plaquetas a secretarem quimiocinas e citocinas. A dosagem das quimiocinas PF4/CXCL4 e RANTES/CCL5, assim como das citocinas IL-1α, IL-1β e MIF foi realizada no sobrenadante coletado após estímulo plaquetário com as LDL. Nossos resultados demonstram que a LDL dengue nas concentrações de 5ng/mL, 10ng/mL, 100ng/mL e 1000ng/mL não foram capazes de induzir a secreção das quimiocinas PF4/CXCL4 (Figura 15 A) e RANTES/CCL5 (Figura 16 A), nem da citocina MIF (Figura 17 A). Além disso, a LDL dengue nas concentrações de 5ng/mL, 10ng/mL, 100ng/mL e 1000ng/mL na presença de dose subótima de trombina (0,02U/mL) também não foram capazes de potencializar a secreção das quimiocinas PF4/CXCL4 (Figura 15 B-E) e RANTES/CCL5 (Figura 16 B-E), nem da citocina MIF (Figura 17 B-E). As citocinas IL-1α e IL-1β não foram detectadas no ensaio imunoenzimático, ficando abaixo da curva padrão para todas as amostras.

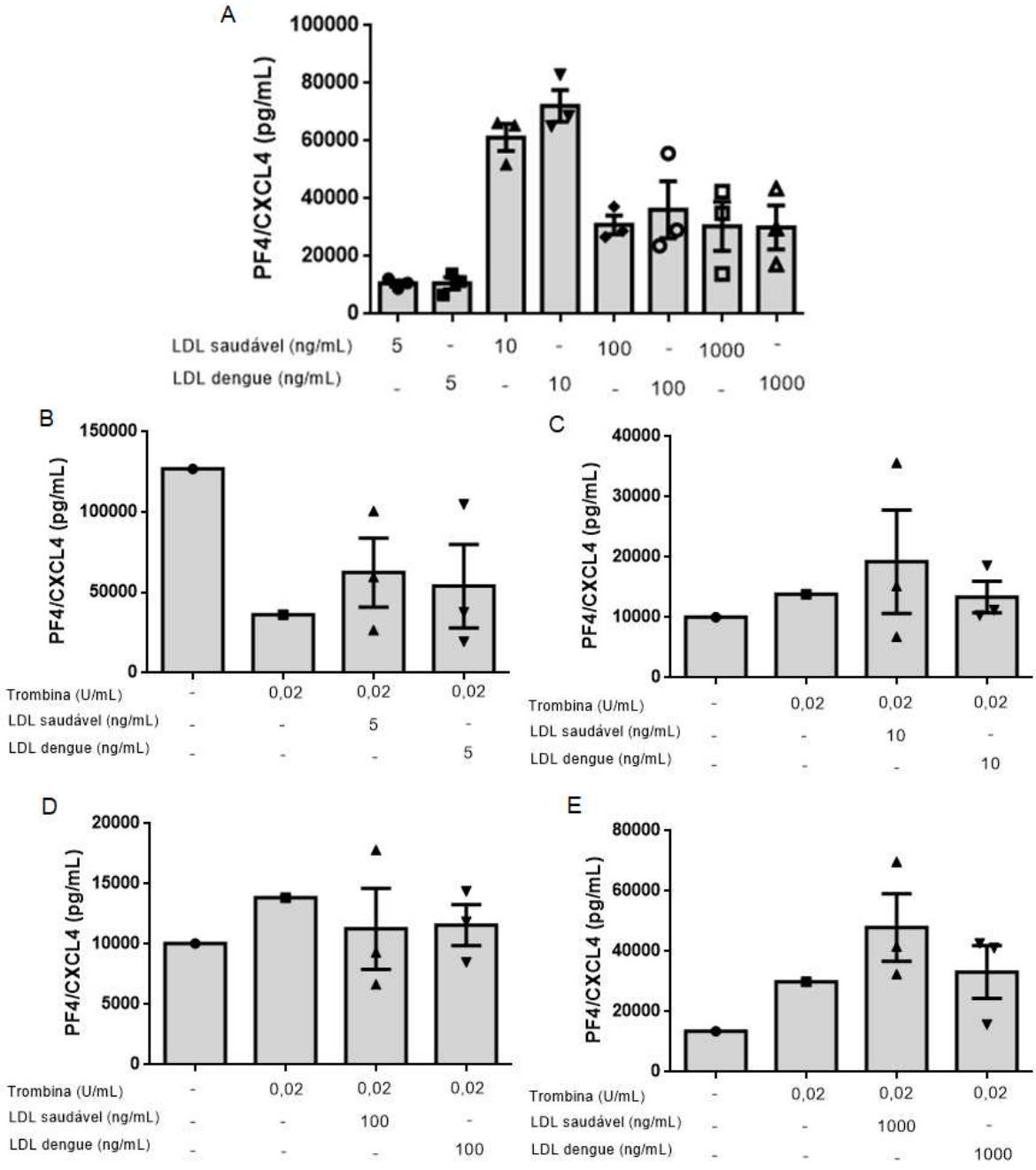

Figura 15 – Avaliação da secreção de PF4/CXCL4 frente estímulo plaquetário por LDL de indivíduo saudável ou LDL proveniente de paciente com dengue. A secreção de PF4/CXCL4 foi quantificada (pg/mL) no sobrenadante de plaquetas estimuladas por 3 LDL provenientes de três pacientes com dengue ou três voluntários saudáveis, a 5, 10, 100 ou 1000ng/ml **(A)**; e no sobrenadante de plaquetas estimuladas por dose subótima de trombina (0,02U/mL) após estímulo com LDL de 3 pacientes com dengue ou LDL de 3 voluntários saudáveis, nas concentrações de 5ng/mL **(B)**; 10ng/mL **(C)**; 100ng/mL **(D)** e 1000ng/mL **(E)**. Nos gráficos estão apresentados a média +/- erro padrão da média do percentual de plaquetas positivas para a secreção de PF4/CXCL4.

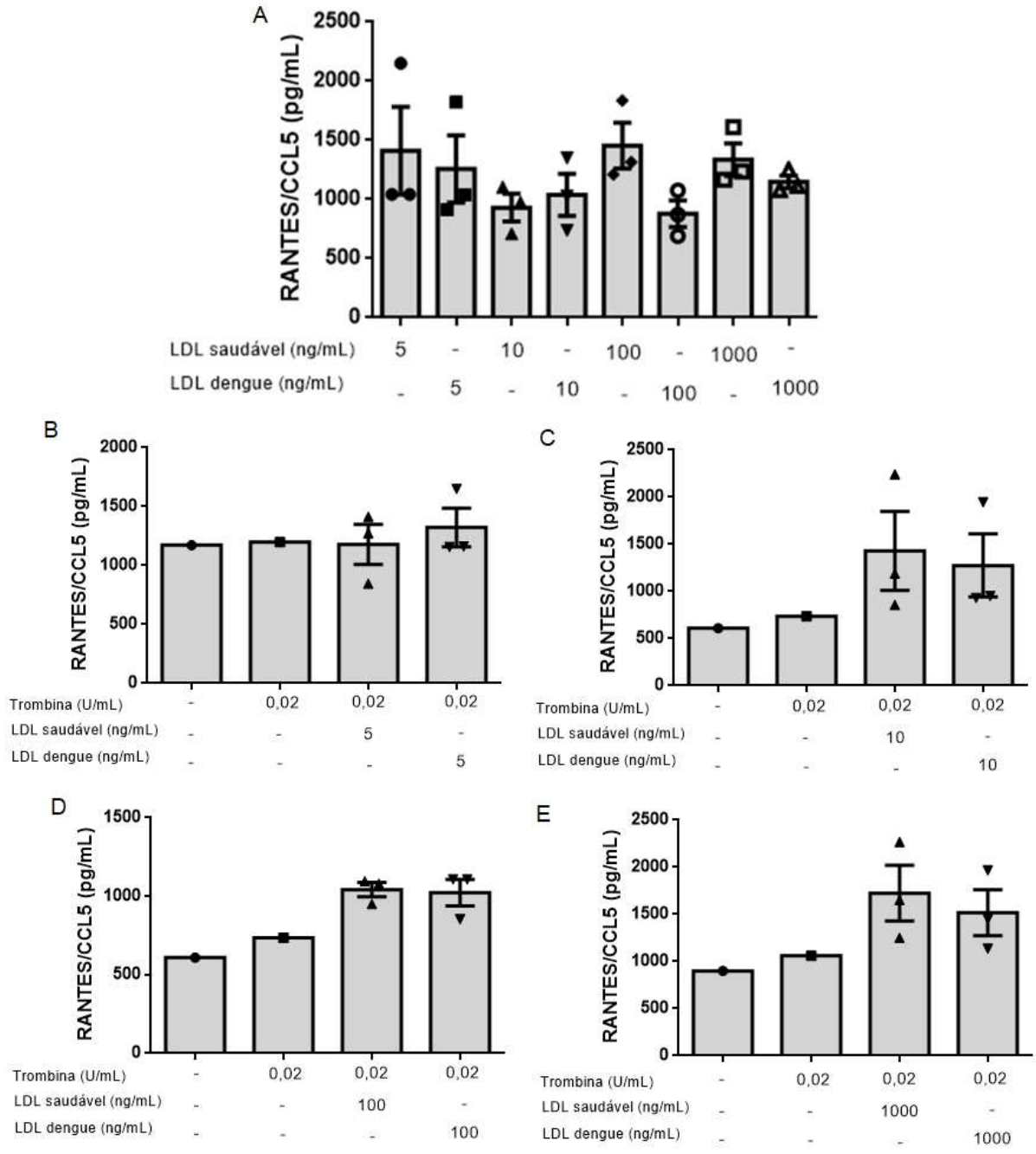

Figura 16 – Avaliação da secreção de RANTES/CCL5 frente estímulo plaquetário por LDL de indivíduo saudável ou LDL proveniente de paciente com dengue. A secreção de RANTES/CCL5 foi quantificada (pg/mL) no sobrenadante de plaquetas estimuladas por 3 LDL provenientes de três pacientes com dengue ou três voluntários saudáveis, a 5, 10, 100 ou 1000ng/ml **(A)**; e no sobrenadante de plaquetas estimuladas por dose subótima de trombina (0,02U/mL) após estímulo com LDL de 3 pacientes com dengue ou LDL de 3 voluntários saudáveis, nas concentrações de 5ng/mL **(B)**; 10ng/mL **(C)**; 100ng/mL **(D)** e 1000ng/mL **(E)**. Nos gráficos estão apresentados a média +/- erro padrão da média do percentual de plaquetas positivas para a secreção de RANTES/CCL5.

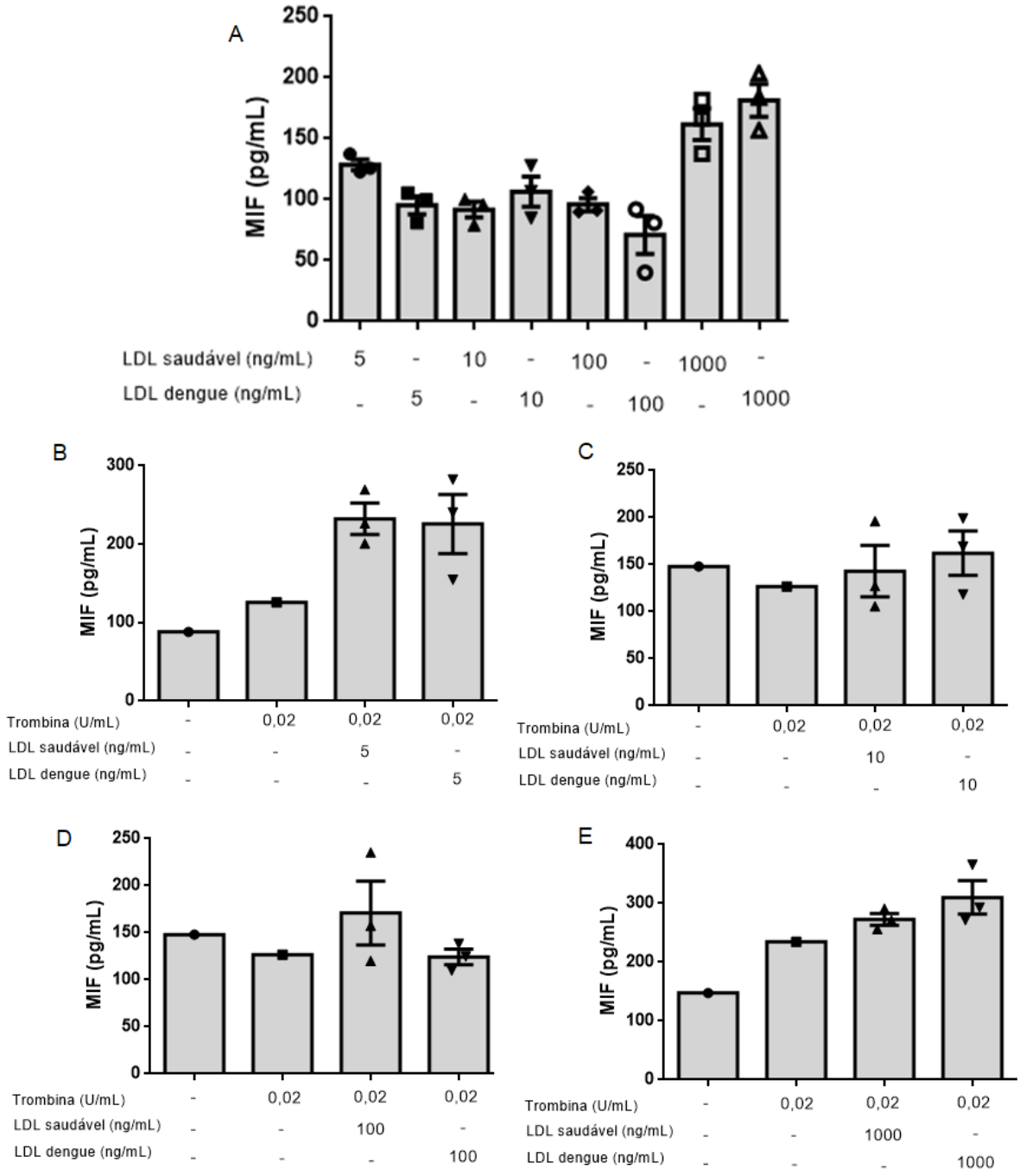

Figura 17 – Avaliação da secreção de MIF frente estímulo plaquetário por LDL de indivíduo saudável ou LDL proveniente de paciente com dengue. A secreção de MIF foi quantificada (pg/mL) no sobrenadante de plaquetas estimuladas por 3 LDL provenientes de três pacientes com dengue ou três voluntários saudáveis, a 5, 10, 100 ou 1000ng/ml **(A)**; e no sobrenadante de plaquetas estimuladas por dose subótima de trombina (0,02U/mL) após estímulo com LDL de 3 pacientes com dengue ou LDL de 3 voluntários saudáveis, nas concentrações de 5ng/mL **(B)**; 10ng/mL **(C)**; 100ng/mL **(D)** e 1000ng/mL **(E)**. Nos gráficos estão apresentados a média +/- erro padrão da média do percentual de plaquetas positivas para a secreção de MIF.

6 DISCUSSÃO

Alterações significativas dos lipídios séricos foram descritas na dengue grave, havendo uma ligação direta entre a progressão da infecção pela dengue e as alterações do perfil lipídico (CUI *et al.*, 2013; DURÁN *et al.*, 2015; VOGE *et al.*, 2016). Durán e colaboradores (2015) descreveram as seguintes alterações no curso da dengue: aumento de HDL na dengue com sinais de alarme; aumento de triglicerídeos, HDL e VLDL e diminuição de LDL na dengue grave; e a diminuição do colesterol total foi encontrada em todos os quadros de dengue. Além disso, análises metabolômicas de amostras séricas de pacientes com dengue apontaram alterações significativas de metabólitos lipídicos no curso da dengue (CUI *et al.*, 2013; VOGE *et al.*, 2016). Foi observada uma diminuição de fosfolipídios e um aumento de ácidos graxos poliinsaturados (PUFAs, do inglês *poli-unsaturated fatty acids*), indicando um possível aumento do catabolismo fosfolipídico nas fases febril e de defervescência da dengue comparadas com a fase de convalescença e controle. É descrito também um aumento de substratos associados à biossíntese e β-oxidação de ácidos graxos e à via metabólica da lipólise no curso da dengue comparado ao controle (CUI *et al.*, 2013). Além disso, metabólitos fosfolipídicos como lisofosfatidilcolina e fosfatidilcolina, e ácidos graxos poliinsaturados como ácido araquidônico foram significativamente associados à DHF/DSS em relação à DF (VOGE *et al.,* 2016). Essas alterações lipídicas observadas na dengue corroboram com o aumento da demanda lipídica e conseqüente catabolismo lipídico das células infectadas, tendo em vista que é descrito que os lipídios são essenciais para a entrada e replicação do DENV (CEBALLOS-OLVERA et al., 2010; HEATON; RANDALL, 2010; OSUNA-RAMOS; REYES-RUIZ; DEL ÁNGEL, 2018; SAMSA et al., 2009; SOTO-ACOSTA et al., 2013; TONGLUAN et al., 2017). Em células infectadas pelo DENV observa-se um aumento da formação e consumo de corpúsculos lipídicos (organelas formadas por uma monocamada de fosfolipídios que englobam lipídios neutros) e de *lipids rafts* (microdomínios membranares ricos em colesterol e esfingolipídios), além do aumento de LDLR na superfície celular e da atividade da HMG-CoA redutase envolvidos na biossíntese do colesterol (HEATON; RANDALL, 2010; SAMSA *et al.*, 2009; SOTO-ACOSTA *et al.,* 2013). Nesse contexto, podemos imaginar um cenário

em que a maior requisição celular de lipídios por células infectadas pelo DENV poderia contribuir para os menores valores observados de LDL no plasma, que estaria sendo mais consumida. Também podemos propor paralelamente que a elevada demanda de lipídios nas células infectadas poderia contribuir para o aumento da exposição dessas partículas à oxidação.

É consensual que a resposta inflamatória decorrente, por exemplo, de infecções virais, está associada a um ambiente pró-oxidante, com presença de enzimas leucocitárias e vasculares envolvidas na produção de espécies reativas de oxigênio (ROS) e de nitrogênio (RNS) no local da infecção, contribuindo para o estresse oxidativo. Este estresse oxidativo torna-se um desafio sistêmico em respostas inflamatórias exacerbadas com disfunção endotelial e dano vascular (AVIRAM *et al.*, 1996; FENSTER; TSAO; ROCKSON, 2003; HAZEN; HEINECKE, 1997; VALACCHI *et al.,* 2018). Os mecanismos pelos quais o estresse oxidativo alimenta a resposta inflamatória e contribui para o dano vascular envolvem processos de peroxidação lipídica. Portanto, nesse cenário de aumento da peroxidação lipídica frente à inflamação e estresse oxidativo, sugerimos um possível aumento de oxLDL no curso da dengue, que poderia estar influenciando fortemente os baixos níveis de LDL na dengue grave. De fato, essa hipótese corrobora com os resultados de Soundravally e colaboradores (2008) que descreveram um aumento significativo de MDA sérico e proteínas carboniladas séricas (estas podem ser formadas por MDA e 4HNEque formam adutos com as proteínas) em pacientes com dengue (DF, DHF e DSS) em relação ao controle. Na DHF e DSS houve um aumento significativo de MDA e de proteínas carboniladas séricas no curso da doença e uma diminuição significativa do status antioxidante total do plasma, o que não ocorreu na DF. Nossos dados corroboram com esses resultados e demonstram um aumento significativo de marcadores de peroxidação lipídica (MDA e 4HNE) nas LDLs provenientes de pacientes com dengue em relação às LDLs provenientes de indivíduos saudáveis.

É reconhecido que a HDL possui atividade antioxidante ao remover lipídios oxidados da partícula de LDL e dos tecidos. Esses lipídios podem ser hidrolisados por enzimas como paraoxonase (PON1) presente na HDL, reduzidos pela apolipoproteína Apo-AI e seletivamente removidos pelos hepatócitos, saindo de circulação (CHRISTISON *et al.*, 1996; NAVAB *et al.,* 2000a, 2000b; WATSON *et al*.,

1995). Apesar da atividade antioxidante proposta, é descrito que em respostas agudas, como na influenza e em respostas inflamatórias crônicas, como na aterosclerose e síndrome metabólica, o estresse oxidativo contribui para a disfunção da atividade antioxidante da HDL, com aumento de lipídios oxidados nessas lipoproteínas e com falhas na inibição da oxidação de LDL (HANSEL et al., 2004; LENTEN et al., 1995; MCMAHON et al., 2006; MORGANTINI et al., 2011; NAVAB et al., 2000a, 2000b; VAN LENTEN et al., 2001). Portanto, algumas vias poderiam ser propostas de mecanismos envolvidos no aumento de oxLDL no plasma de pacientes com dengue frente ao aumento do consumo lipídico e resposta inflamatória exacerbada. A HDL poderia, por exemplo, captar lipídios oxidados perifericamente no efluxo de colesterol e trocar tais lipídios com a LDL sérica. Na camada subendotelial, a LDL por sua vez, também poderia sofrer oxidação dos seus próprios compostos lipídicos, contribuindo para o aumento de oxLDL na dengue (NAVAB *et al.,* 2000b). Além disso, sugerimos também que a transferência dos lipídios oxidados da HDL para os hepatócitos e a infecção de hepatócitos pelo DENV poderiam contribuir para que o fígado liberasse partículas de VLDL já contendo lipídios oxidados, dando origem às oxLDLs na circulação. Apesar de todas essas hipóteses, as possíveis origens e vias da oxLDL na dengue ainda precisam ser elucidadas.

É descrito que oxLDLs possuem fosfolipídios semelhantes ao Fator de Ativação Plaquetário (PAF, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) gerados pela oxidação não enzimática de PUFAs. O PAF é um reconhecido mediador lipídico pró-inflamatório ligante de um GPCR, denominado PAFR(MARATHE *et al.,* 1999; ZIMMERMAN *et al.*, 2002). Segundo Marathe e colaboradores (1999) o processo de oxidação das LDLs leva à oxidação de alguns fosfolipídios na posição *sn-2,* fazendo com que se assemelhem estruturalmente ao PAF. É descrito que alguns fosfolipídios oxidados *in vitro* ou provenientes da oxLDL que se assemelham à PAF levam ao aumento de cálcio citosólico em plaquetas assim como o PAF, através do PAFR plaquetário (CHEN *et al.*, 2009; MARATHE *et al.*, 2002). Além disso, partículas inteiras de oxLDL em baixas doses (5μg/ml) demonstraram estimular plaquetas através do PAFR em sinergismo com doses subótimas de trombina (0,02U/ml), ADP (1 μM) e colágeno (0,1 μg/ml) aumentando significativamente o cálcio intracelular, assim como aumentando a expressão de P-selectina no sinergismo com a trombina (0,02U/ml)(CHEN *etal.*, 2009). Em contrapartida a esses resultados, foi descrito que

LDLs extensamente oxidadas provenientes de pacientes com infarto agudo do miocárdio induziram a agregação plaquetária em sinergismo com ADP somente em doses maiores (25 ou 50 µg/ml), o que não ocorreu com a menor dose de 5 µg/ml de LDL extensamente oxidada associada ao ADP(H.-C. *et al.,* 2013). Além disso, 25 µg/ml dessa LDL extensamente oxidada foi capaz de aumentar a expressão de P-selectina e ativação de GPIIb/IIIa sem a presença de agonistas plaquetários (H.-C. *et al.,* 2013).

As diferenças de respostas plaquetárias à diferentes doses de LDL e na presença ou não de agonistas podem estar associadas às diferenças possíveis nos níveis de oxidação e vias de sinalização envolvidas em diferentes condições. No estudo de Chan e colaboradores (2013), por exemplo, os autores consideraram que as LDLs extensamente oxidadas são LDLs mais eletronegativas. O aumento da carga negativa (L5) permite com que ocorram interações dessa LDL com resíduos carregados positivamente do receptor *scavenger* LOX1 (do inglês, *Lectin-like oxidized low-density lipoprotein receptor-1*) (L.-Y. *et al.*, 2011). De fato, Chan e colaboradores (2013) demonstraram que as vias envolvidas na ativação plaquetária por L5 parecem envolver PAFR, LOX-1 e as proteínas de sinalização PKC, Akt e PI3K.Caso o LOX-1 seja um importante receptor mediando a ativação plaquetária no estudo de Chan *et al* (2013), as menores doses de oxLDL associadas ao ADP poderiam não estar sendo capazes de induzir a expressão de LOX-1. É descrito que esse receptor só é expresso após ativação plaquetária, não mediando a ligação das oxLDLs em plaquetas não estimuladas, diferentemente do CD36, um receptor *scavenger* constitutivamente expresso em plaquetas que medeia a interação de oxLDL com plaquetas não estimuladas (CHEN *et al.,* 2001).

No estudo realizado por Podrez e colaboradores (2007) foi demonstrado que a oxidação de fosfolipídios encontrados na LDL (PAPL, 1-hexadecanoil-2-eicosatetra-5′,8′,11′,14′-enoil-sn-glicero-3-fosfocolina e PLPC, 1-hexadecanoil-2-octadecadi-9′,12′-enoil-sn-glicero-3-fosfocolina*)* e da LDL pela enzima mieloperoxidase leva à interação destes fosfolipídios e da LDL oxidada com o receptor CD36 nas plaquetas. O fosfolipídio KODA-PC (do inglês, *9-keto-12-oxo-10-dodecenoic acid ester of 2-lyso-phosphocholine*) proveniente da oxidação de PLPC, assim como oxPAPL e oxLDL (6 µg/mL) induziram através do CD36 a ativação da integrina GPIIbIIIa e expressão de P-selectina plaquetária (PODREZ *et al.,* 2007).

Além disso, um estudo recente demonstrou, ao bloquear os receptores plaquetários TLR2 e TLR6 *in vitro* e ao utilizar plaquetas provenientes de camundongos com deleção genética de TLRs e MyD88, que a ativação plaquetária por KODA-PC envolve a sinalização de TLR2 e TLR6 via formação de complexo CD36/TLR2/TLR6, induzindo a ativação da integrina GPIIbIIIa e expressão de P-selectina nas plaquetas (BISWAS S., ZIMMAN A., GAO D., BYZOVA T., 2017). Em contrapartida a esses resultados, nossos dados demonstram que a LDL oxidada proveniente dos pacientes com dengue não induziu alteração da expressão do CD36, ativação da integrina GPIIbIIIa e expressão de P-selectina plaquetária.

As diferenças associadas às composições lipídicas das oxLDL, assim como a extensão dessa oxidação podem explicar as diferenças nas respostas plaquetárias frente ao estímulo por LDL oxidadas em diferentes condições. A oxidação das LDL na dengue poderia, por exemplo, não estar associada com a formação de $oxPC_{CD36}$ (família de glicerofosfolipídios de colina oxidados que servem como ligantes de alta afinidade para o receptor CD36, como o KODA-PC) e sim com a formação de fosfolipídios oxidados que não demonstram alta afinidade de interação com CD36, como o OV-PC livre (1-palmitoil-2- (5-oxovaleroil) -sn-glicero-3-fosfatidilcolina) também formado em LDL oxidadas (BOULLIER *et al*., 2005; PODREZ *et al.*, 2002; WATSON *et al*., 1997). Além da hipótese qualitativa para nossos resultados, que diz respeito à composição lipídica, também podemos propor uma hipótese quantitativa, na qual as LDL oxidadas na dengue podem até formar lipídios oxidados associados à respostas plaquetárias, mas em concentrações não suficientes para tal. Segundo Levitan e colaboradores (2010), a composição reportada de malondialdeído em LDL minimamente oxidadas fica abaixo de 30 nmol/mg ApoB100 e ao avaliarmos a concentração de MDA em LDL de pacientes com dengue, estas segundo esta classificação poderiam ser categorizadas como LDL minimamente oxidadas. Nesse cenário, a extensão da oxidação da LDL poderia depender das apresentações clínicas da doença, em que casos de dengue branda podem não favorecer a oxidação severa das LDL, enquanto que nos casos que evoluem para dengue grave, o ambiente seria mais propício para a extensa oxidação das mesmas. Na coorte utilizada em nosso estudo (Tabela 1), tendo em vista que as amostras de LDL foram provenientes de pacientes com dengue branda ou com dengue branda com sinais de alarme, um menor grau de oxidação das

mesmas poderia ser esperado em comparação ao que ocorre em casos que evoluem para dengue grave. Nesse sentido, a extensa oxidação das LDL poderia ter um papel relevante na amplificação dos eventos imunopatogênicos e evolução para a dengue grave.

Em relação às vias de sinalização da ativação do CD36, foi demonstrado que a ativação de CD36 plaquetário com 50 µg/ml de oxLDL leva a fosforilação de Src quinase e síntese pela enzima NOX de espécies reativas, como ânion superóxido ($O_2^{\bullet -}$) e superóxido dismutase (H_2O_2). As espécies reativas de oxigênio modulam a sinalização da MAPK ERK5 nas plaquetas, resultando em agregação plaquetária (YANG *et al.*, 2017). Nesse cenário, é sabido que radicais livres podem induzir a via intrínseca de apoptose e, eventos apoptóticos plaquetários poderiam contribuir fortemente para a trombocitopenia característica da patogênese da dengue. Sendo assim,em nossos experimentos com marcação de fosfatidilserina e análise da polarização mitocondrial, exploramos o efeito das LDLs de pacientes com dengue sobre a apoptose, um processo de morte celular que cursa sem perda da integridade de membrana plasmática (ELMORE, 2007). No entanto, assim como as LDL de pacientes com dengue não induziram ativação plaquetária, também não foram capazes de induzir apoptose plaquetária.

O CD36 plaquetário também foi sugerido como um importante receptor envolvido na captação de oxLDL por monócitos mediada por plaquetas (BADRNYA *et al.,* 2014). Badrnya *et al* (2014) demonstraram que as plaquetas formam complexos com monócitos (PMA) em resposta ao estímulo com 50 µg/ml de oxLDL *in vitro* de forma dependente de P-selectina, assim como a formação de PMA ocorre *in vivo* em camundongos C57BL/6J em resposta a infusão de oxLDL. Além disso, as plaquetas quando associadas à oxLDL contribuem para ativação de monócitos e aumentam significativamente a transmigração de monócitos através de uma monocamada de células endoteliais da veia umbilical humana (BADRNYA *et al.*, 2014). Corroborando com esses dados, a ativação plaquetária mediada por LOX-1 e oxLDL (25, 50 e 100 µg/ml) *in vitro* aumentou significativamente a expressão de CD147 nas plaquetas, um indutor de metaloproteinases da matriz extracelular, o qual por sua vez também foi apontado como um possível mediador da interação plaquetas-monócitos *in vitro* (SCHULZ *et al.*, 2011; YANG; LI; DU, 2013). Nesse cenário, vale ressaltar os níveis aumentados de agregados plaquetas-monócitos no

sangue de pacientes com dengue, os quais conforme já citamos foram correlacionados positivamente com o aumento da permeabilidade endotelial (HOTTZ *et al.,* 2014). Como demonstramos que a oxidação das LDL de pacientes com dengue não foram suficientes para ativação plaquetária, não podemos afirmar que os agregados plaquetas-monócitos poderiam ter influência da ativação plaquetária por oxLDL. No entanto, a interação de LDL minimamente oxidadas com monócitos é bem descrita e, portanto, podemos sugerir a possibilidade desse evento configurar uma via indireta de ativação plaquetária por oxLDL e interação plaqueta-monócitos na dengue (LEE et al., 1999; WATSON et al., 1997).

É sugerido que as plaquetas na dengue liberem *in vivo* PF4/CXCL4 e RANTES/CCL5 de seus grânulos α, devido à redução dessas quimiocinas nessas plaquetas *ex vivo*, contribuindo assim para os níveis plasmáticos aumentados de PF4/CXCL4 e RANTES/CCL5 em pacientes infectados com dengue em comparação a voluntários saudáveis (TRUGILHO *et al.*, 2017). A ativação plaquetária com diferentes concentrações de oxLDL levou à liberação de CXCL4 pelas plaquetas de forma dose-dependente *in vitro* (BADRNYA *et al.*, 2014). No entanto, ainda não havia sido investigado se a ativação plaquetária por LDL de pacientes com dengue poderia induzir a secreção dessas quimiocinas ou outras citocinas contribuindo para a patogênese da dengue. Nesse sentido, em nossos experimentos de análise da secreção de mediadores inflamatórios plaquetários frente estímulo por LDL de pacientes com dengue, nós demonstramos que não houve secreção de quimiocinas (PF4/CXCL4 e RANTES/CCL5) e nem de citocinas (IL-1α, IL-1β e MIF).

Além dos fosfolipídios oxidados semelhantes ou não à PAF, produtos fosfolipídicos decorrentes da oxidação dos mesmos são gerados nas oxLDL. Um produto fosfolipídico da oxidação da LDL, denominado lisofosfatidilcolina (LPC, 1-acila-sn-glicerol-3-fosfocolina), é descrito ser produzido a partir da ação da enzima PAF-acetil hidrolase (PAF-AH) (CHAPMAN *et al.*, 2020; LEVITAN; VOLKOV; SUBBAIAH, 2010; MAHFOUZ; KUMMEROW, 2000; MATSUMOTO; KOBAYASHI; KAMATA, 2007). Esta consiste em uma fosfolipase que atua na fosfatidilcolina oxidada que se assemelha ao PAF removendo ácidos graxos da posição *sn*-2 (CHAPMAN *et al*., 2020; LEVITAN; VOLKOV; SUBBAIAH, 2010; MATSUMOTO; KOBAYASHI; KAMATA, 2007). As plaquetas expressam o receptor de LPC, denominado receptor G2A ou GPR132 (do inglês *G protein coupled receptor*

132)(DIEHL *et al.*, 2019). É descrito que efeitos plaquetários decorrentes do estímulo por LPC dependem das concentrações presentes na oxLDL. Em doses de 60 ug/mL ou 130µg/ml este produto fosfolipídico demonstrou contribuir para a ativação plaquetária e em altas doses (300 µg/ml) esteve associado à inibição da ativação plaquetária na presença de agonistas (DIEHL *et al.*, 2019; KOROTAEVA; CHEGLAKOV; PROKAZOVA, 1997; MAHFOUZ; KUMMEROW, 2000). No trabalho de Mahfouz e Kummerow (2000) também foi demonstrado que quando a concentração de LPC foi aumentada de 15 µM para 30 µM ou mais, houve um aumento repentino da perda de lactato desidrogenase(LDH) pelas plaquetas demonstrando a possibilidade de perturbação da membrana e/ou lise plaquetária.

Além do LPC, outro lisofosfolipídio bioativo descrito estar presente em LDLs levemente oxidadas é o ácido lisofosfatídico (LPA, mono acilglicerol-3-fosfato). O LPA pode ser sintetizado a partir da ação da PAF-AH que lisa o ácido fosfatídico (diacil-glicerolfosfolipídeo) na posição *sn*-2 ou a partir da lisofosfolipase D que cliva a LPC, removendo o grupo colina (LEVITAN; VOLKOV; SUBBAIAH, 2010; SIESS *et al.*, 1999). O LPA em baixas doses (18nM) já foi capaz de induzir mudança conformacional nas plaquetas *in vitro* e em doses maiores (10-100 µM) induziu secreção de serotonina (grânulo denso) e agregação plaquetária irreversível no sangue total, no PRP e em plaquetas isoladas na presença de fibrinogênio em sinergismo com ADP plaquetário (HASERÜCK et al., 2004; MASCHBERGER et al., 2000; SIESS et al., 1999). Em altas doses também levou à exposição de P-selectina em plaquetas isoladas e induziu a agregação plaquetas-monócitos no sangue total de forma dependente de LPA, dependente de P-selectina e independente de fibrinogênio (HASERÜCK *et al.*, 2004). É importante ressaltar, portanto, que os estudos avaliando a ação de lisofosfolipídios formados em oxLDL como estímulos plaquetários demonstram que as concentrações dessas partículas influenciam diretamente nas respostas plaquetárias, estando na maioria das vezes, doses maiores associadas a efeitos mais significativos. Sendo assim, esses dados contribuem para o melhor entendimento dos nossos resultados, pois o fato das LDL provenientes dos pacientes com dengue não induzirem ativação plaquetária pode estar associado à extensão da oxidação dessas partículas não ser suficiente para tais efeitos biológicos.

Nesse cenário, em que o grau de oxidação das LDL importa, é válido

considerar também que as condições metabólicas pré-infecção com o vírus da dengue poderiam ter forte influência na extensão de oxidação das LDL na dengue e possivelmente nos efeitos destas sobre a ativação plaquetária ou em outros processos inflamatórios. Diversos estudos demonstram um aumento de oxLDL circulante em pacientes com diabetes mellitus e com resistência a insulina (ISHIGAKI; OKA; KATAGIRI, 2009; LINNA *et al.*, 2015; NAKHJAVANI *et al*., 2010). Além disso, a perda de peso está associada com a diminuição dos níveis de oxLDL em indivíduos obesos e altos níveis de oxLDL foram associados ao aumento da incidência de síndrome metabólica geral, bem como de seus componentes de obesidade abdominal, hiperglicemia e hipertrigliceridemia (HOLVOET *et al*., 2008; UZUN *et al.,* 2004; VASANKARI *et al.*, 2001). Nesse sentido, a presença de oxLDL em doenças crônicas pode ser apontada como um importante link entre tais doenças e o risco aumentado de doenças cardiovasculares, tendo em vista que as oxLDL são consideradas importantes fatores patogênicos na formação de placas ateroscleróticas (GAO *et al.,* 2017; TRPKOVIC *et al.*, 2015). Sendo assim, as conseqüências cardiovasculares e imunopatogênicas das oxLDL poderiam ter impacto no prognóstico de doenças infecciosas, como a dengue. Em nosso artigo publicado recentemente, a oxidação da LDL é apontada como um fator possivelmente envolvido no aumento do risco cardiovascular e pior prognóstico de obesos com influenza ou doença por corona vírus 2019 (ANEXO A). No entanto, a utilização dos dados clínicos metabólicos pré-infecção como biomarcadores do pior prognóstico em doenças infecciosas ainda requer maiores elucidações. Nesse sentido, seria interessante em estudos futuros a caracterização da oxidação das LDL em associação com dados clínicos desses pacientes pré-infecção, assim como com os desfechos clínicos.

7 CONSIDERAÇÕES FINAIS

A trombocitopenia é um importante parâmetro associado à evolução para o quadro grave da dengue e, portanto, é de extrema importância investigar a participação das plaquetas na doença. Evidências emergentes apontam para a

participação das plaquetas em processos inflamatórios, atuando não somente com ações hemostáticas, mas também secretando mediadores inflamatórios, expressando moléculas de interação com outras células imunológicas e amplificando a resposta inflamatória. Sendo assim, sugerimos que a ativação plaquetária possa estar envolvida na resposta inflamatória exacerbada e tempestade de citocinas descritas nos quadros graves da dengue (DIB et al., 2020; FITCH-TEWFIK; FLAUMENHAFT, 2013). Os estímulos para ativação plaquetária ainda precisam ser avaliados e neste trabalho sugerimos que as oxLDL poderiam ser potenciais alvos nesse sentido.

Evidências corroboram com essa hipótese demonstrando que os parâmetros lipídicos são alterados no curso da doença e identificando que a peroxidação lipídica está elevada nesses pacientes. Além disso, a peroxidação lipídica se correlaciona negativamente com a contagem de plaquetas, enquanto a quantificação da LDL sérica se correlaciona positivamente (CUI et al., 2013; DURÁN et al., 2015; SOUNDRAVALLY et al., 2008). Nossos dados de análise das LDLs provenientes de pacientes com dengue demonstraram um aumento da oxidação nessas lipoproteínas. No entanto, devido à grande variabilidade em relação aos componentes lipídicos oxidados presentes na LDL, assim como diferenças no grau e extensão dessa oxidação, apesar do maior nível de oxidação das LDLs de pacientes com dengue branda em relação as LDL de indivíduos saudáveis, estas não foram capazes de induzir ativação, apoptose e secreção de mediadores inflamatórios plaquetários, não podendo ser apontadas, portanto, como contribuintes para amplificação inflamatória plaquetária da infecção.

Outros estímulos envolvidos na ativação plaquetária durante a dengue devem ser elucidados, incluindo aqueles que podem atuar em sinergismo com as LDL oxidadas dos pacientes. Além disso, também sugerimos que condições metabólicas pré-existentes que cursam com inflamação crônica e estresse oxidativo, como obesidade, possam influenciar na extensão da oxidação das partículas de LDL na dengue e na gravidade da doença. A gravidade da doença por sua vez também poderia contribuir para a maior extensão de oxidação das LDLs. Em estudos futuros, a associação da caracterização das LDL com informações clínicas metabólicas e com os desfechos clínicos, podem ser interessantes para maiores elucidações do envolvimento da oxLDL no curso clínico da dengue.

8 CONCLUSÃO

- Nós observamos um aumento significativo da oxidação nas LDL provenientes de pacientes com dengue branda com ou sem sinais de alarme em comparação com as LDL provenientes de voluntários saudáveis.

- As LDL oxidadas dos pacientes com dengue branda com ou sem sinais de alarme não induzem um aumento da expressão da P-selectina, ativação da integrina αIIbβ3 ou alteração da expressão do receptor CD36 nas plaquetas, quando comparadas com as LDLs de voluntários saudáveis. Assim como não potencializaram a ativação plaquetária na presença de dose subótima de trombina.

- As LDL oxidadas dos pacientes com dengue branda com ou sem sinais de alarme também não induzem um aumento da apoptose plaquetária, quando comparadas com as LDLs de voluntários saudáveis na ausência ou na presença de dose subótima de trombina.

- As LDL oxidadas dos pacientes com dengue branda com ou sem sinais de alarme não induzem um aumento da secreção de mediadores inflamatórios (MIF, RANTES, PF4, IL-1β e IL-1α) pelas plaquetas, quando comparadas com as LDLs de voluntários saudáveis. Assim como também não potencializaram a secreção de mediadores inflamatórios plaquetários na presença de dose subótima de trombina.

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REVIEW
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The Weight of Obesity in Immunity from Influenza to COVID-19

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The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has emerged in December 2019 and rapidly outspread worldwide endangering human health. The coronavirus disease 2019 (COVID-19) manifests itself through a wide spectrum of symptoms that can evolve to severe presentations as pneumonia and several non-respiratory complications. Increased susceptibility to COVID-19 hospitalization and mortality have been linked to associated comorbidities as diabetes, hypertension, cardiovascular diseases and, recently, to obesity. Similarly, individuals living with obesity are at greater risk to develop clinical complications and to have poor prognosis in severe influenza pneumonia. Immune and metabolic dysfunctions associated with the increased susceptibility to influenza infection are linked to obesity-associated low-grade inflammation, compromised immune and endocrine systems, and to high cardiovascular risk. These preexisting conditions may favor virological persistence, amplify immunopathological responses and worsen hemodynamic instability in severe COVID-19 as well. In this review we highlight the main factors and the current state of the art on obesity as risk factor for influenza and COVID-19 hospitalization, severe respiratory manifestations, extrapulmonary complications and even death. Finally, immunoregulatory mechanisms of severe influenza pneumonia in individuals with obesity are addressed as likely factors involved in COVID-19 pathophysiology.

Keywords: obesity, COVID-19, severe influenza, immunity, immunopathology

INTRODUCTION

In December 2019, several cases of acute pneumonia of unknown etiology emerged in Wuhan, China. The new pathogen was readily sequenced and phylogenetically related to the previous severe acute respiratory syndrome coronavirus (SARS-CoV). The new coronavirus was therefore termed SARS-CoV-2, the etiological agent of the coronavirus disease 2019 (COVID-19) (Li et al., 2020; Zhou P. et al., 2020). By march 2020 the coronavirus outbreak had emerged to the status of pandemic, significantly challenging human health worldwide (Li et al., 2020; Zhou P. et al., 2020). Clinical manifestations of SARS-CoV-2 infection may vary from asymptomatic infection to mild or severe respiratory syndromes. Approximately 15% of infected individuals develop severe diseases that require hospitalization and ventilation support, whereas 5% of them need admission in intensive care units

(ICU) due to complications such as acute respiratory distress syndrome (ARDS), sepsis, thromboembolism, and/or multiorgan failure (Feng et al., 2020).

There are no specific clinical signs to anticipate the progression of mild COVID-19 to severe presentations. However, some pre-established conditions including older age and the presence of comorbidities, such as hypertension, diabetes and obesity were identified as risk factors for severe COVID-19, as previously shown for severe influenza pneumonia as well (Guo T. et al., 2020; Hartshorn, 2020; Shi et al., 2020; Zhou F. et al., 2020). Obesity is a high prevalent disease that has major impact on global health. According to the WHO (2020), overweight and obesity almost tripled from 1975 to 2016, with more than 1.9 billion overweight adults and 650 million ones with obesity. Data from 2016 have shown that almost 40% of the world population were overweight and approximately 13% were obese (Chooi et al., 2018; World Health Organization, 2020). The countries leading the ranking of obesity prevalence in the world are: China, USA, Brazil, India, Russia, Mexico, Germany, UK, Italy and France; and the countries leading the rank of severe obesity are: USA, China, Russia, Brazil, Mexico, Egypt, UK, Germany, Turkey and France (NCD Risk Factor, 2016). In addition, obesity estimates for 2030 antecipates an increse towards 42.1% of the populationin in the African continent, 45.5% in Asia, 36% in Europe, 44.5% in North America, 35.2% in South America and 65.8% in Oceania (Ampofo and Boateng, 2020).

Epidemiological evidences have shown obese COVID-19 patients at increased risk to require hospitalization, ICU admission and to evolve to death (Popkin et al., 2020). This increased susceptibility is positively correlated to the body mass index (BMI) with increasing risk of severity in overweight individuals, stage I obesity and stage II obesity. The BMI is currently used to stratify subjects as overweight (BMI 25 - 30 kg/m^2) obesity stage I (BMI 30 - 35 kg/m^2), obesity stage II (BMI 35 - 40 kg/m^2) and obesity stage III (BMI > 40 kg/m^2) (Bhaskaran et al., 2018). Obesity has been highlighted as significant risk factor for COVID-19 severity even at younger ages (Busetto et al., 2020; Malik et al., 2020). Accordingly, overweight and obesity were independent risk factors for severe COVID-19 in patients with type 2 diabetes, a risk that was no longer observed in elderly patients (above 75 years) (Smati et al., 2020). A similar risk has been observed for severe influenza pneumonia among people living with obesity. Epidemiological studies reveal that obesity significantly increases the risk of critical and fatal complications of influenza virus infection (van Kerkhove et al., 2011; Sun et al., 2016). In addition, individuals with obesity have less protection against influenza, and are 2 to 3 times more likely to develop disease than non-obese individuals (Neidich et al., 2017). Obese patients with influenza also take longer to eliminate the virus, which may increase the risk of transmission (Maier et al., 2018). Similar immune processes responsible for the increased susceptibility of obese individuals to influenza, including dysregulated innate immunity, are very likely involved in the increased risk of obese individuals to develop severe COVID-19 (Hartshorn, 2020).

People with obesity are known to present changes at different innate and adaptive immune responses due to chronic low-grade inflammation (Karczewski et al., 2018). These preexisting responses may contribute to increased morbidity and mortality in severe respiratory diseases, since overwhelming cytokine production – known as cytokine storm – is a key pathological phenomenon in severe influenza pneumonia and COVID-19 (Gu et al., 2019; Coperchini et al., 2020; Ye et al., 2020). Metabolic and immunological changes in obesity constitute a favorable environment for the development of cardiovascular disorders, which are strongly associated with COVID-19 and influenza severity and mortality (Mamas et al., 2008; Csige et al., 2018; Fountoulaki et al., 2018; Graupera and Claret, 2018; Guo T. et al., 2020; Nishiga et al., 2020; Shi et al., 2020). Thus, this review aims to address metabolic, immunological and inflammatory disorders in obesity that may contribute to pathophysiological mechanisms involved in severe influenza pneumonia and COVID-19.

IMMUNE RESPONSE IN OBESITY

Obesity is a non-transmissible chronic disease that has outspread and became highly prevalent in humans worldwide. It is described as a multifactorial and endocrine-metabolic disease that involves interactions among hormonal, genetic and environmental factors triggering adiposity in excess (McCafferty et al., 2020). The adipose tissue is a metabolically active organ, and therefore, featuring obesity just as fat accumulation is an oversimplistic view (Vegiopoulos et al., 2017). The loss of fat storage capacity in dysfunctional white adipose tissue during obesity impairs metabolic and endocrine processes leading to systemic low-grade inflammation in obese individuals. These alterations set the stage for metabolic and cardiovascular complications such as insulin resistance, diabetes, atherosclerosis, hypertension and many cancer types (Karczewski et al., 2018).

The Dysfunctional Adipose Tissue in Obesity

Several factors contribute as potential triggers of systemic and adipose tissue inflammation in obesity. First, adipocyte hypertrophy and fat storage capacity loss *per se* is linked to inflammation, as the flow of lipids to non-adipose organs and ectopic fat accumulation lead to lipotoxic effects, tissue inflammation and metabolic dysfunction (**Figures 1A, B**) (Vegiopoulos et al., 2017). In this regard, although the hypertrophy of subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) are both positively correlated to metabolic risk factors, VAT expansion is closely associated with adverse metabolic complications (Fox et al., 2007). It has been observed that VAT and perivascular adipose tissue (PVAT) have a twice higher macrophage infiltration than SAT (Kralova Lesna et al., 2016). Macrophages are activated by saturated fatty acids (SFA) through the interaction with pattern recognition receptors (PRRs) of the innate immunity, including Toll Like receptor-4

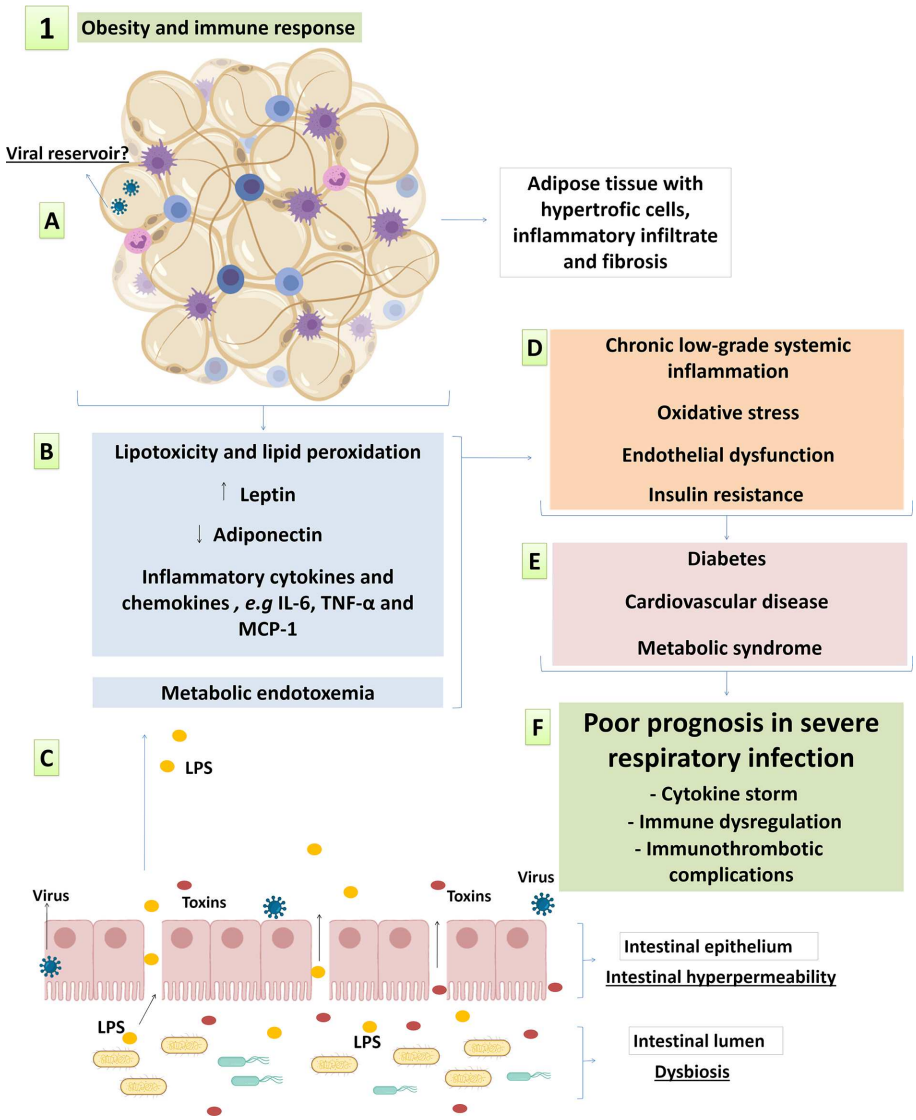

FIGURE 1 | Main factors of obesity-related inflammation and metabolic syndrome possibly involved in pathophysiology of respiratory viral infections. **(A)** changes in adipose tissue architecture with hypertrophic adipocytes, inflammatory infiltrate and fibrosis; **(B)** the loss of fat storage capacity and altered adipokinesecretionin dysfunctional white adipose tissue; and **(C)** metabolic endotoxemia resulting from the intestinal endothelial hyperpermeability also contribute to systemic metabolic and immunological complications; leading to **(D)** systemic low-grade inflammation, metabolic syndrome and increased cardiovascular risk. This syndrome evolves with **(E)** obesity-associated comorbidities and **(F)** increased susceptibility to infectious diseases, including Influenza and COVID-19. See the text for details and references.

(TLR-4), TLR-2/1 and TLR-2/6 (Lee et al., 2004; Nguyen et al., 2007). In addition, the pro-inflammatory and pro-oxidant environment of obesity allows oxidation of polyunsaturated fatty acids (PUFAs). Increase lipid peroxidation and circulation of oxidized low-density lipoproteins (oxLDL) are often observed in people living with obesity, and are associated with increased risk for cardiovascular diseases (Njajou et al., 2009; Ayala et al., 2014; Caimi et al., 2019). Lipid peroxidation culminates in the formation of damage-associated molecular patterns (DAMPs) that activate many PRRs present in innate immune cells and other cell types, including in vascular and stromal cells (Miller and Shyy, 2017).

Inflammatory Infiltrate in Adipose Tissue

Macrophage infiltration is a characteristic of obesity that is positively associated with the BMI and adipocytes size (Weisberg et al., 2003). Macrophages' infiltration in adipose tissue comes along with the switch from alternatively activated anti-inflammatory (M2) to classically activated pro-inflammatory (M1) macrophages (Lumeng et al., 2007). These two main macrophage phenotypes are the extremes of a broad

spectrum of functions that range from tolerogenic to pro-inflammatory properties. Macrophages are polarized to the M1 profile when activated by PAMPS as LPS or proinflammatory cytokines as IFN-γ and TNF-α. The secretion of TNF-α, IL-1β, IL-6 and CCL2 and the increased expression of iNOS are classical M1 profile markers (Murray et al., 2014). M2 polarization is achieved by stimulation with anti-inflammatory cytokines and its hallmarks are the expression of arginase-1 and production of CCL22, CCL17 and IL-10 (Murray et al., 2014). Therefore, M1 polarization in adipose tissue macrophages strongly contributes to the proinflammatory environment in obesity (Lumeng et al., 2007). Alongside macrophages, both B and T cells, from CD4+ and CD8+ subsets, infiltrate the hypertrophic adipose tissue, whereas regulatory T cells reduction is observed, contributing to increased cytokine production, mainly in VAT (Duffaut et al., 2009; Feuerer et al., 2009; Yang et al., 2010; Mclaughlin et al., 2014).

Adipokines

Besides ectopic fat deposition and lipotoxicity, other adipose-derived mediators are important triggers of inflammation. It is well documented that adipocytes release hormones and cytokines that are collectively classified as adipokines, including leptin and adiponectin, which have pro- and anti-inflammatory effects respectively (**Figure 1B**) (Giralt et al., 2015). Leptin is produced by adipocytes and hyperleptinemia is observed during obesity in parallel to central resistance to its anorexigenic action (Myers et al., 2010). In addition, leptin has several effects on a variety of immune cells, including: 1) increased secretion of proinflammatory cytokines and increased phagocytosis by monocytes, dendritic cells and macrophages (Mancuso et al., 2012; Tsiotra et al., 2012; Moraes-Vieira et al., 2014); 2) increased neutrophil migration (Souza-Almeida et al., 2018); 3) improved cytotoxic activity and IFN-γ secretion in NK cells, but reduced NK cell proliferation and function after long-term exposure (Wrann et al., 2011); and 4) immunometabolic reprogramming of T cells promoting Th1 and Th17 polarization alongside immune tolerance inhibition (Liu et al., 2012; Saucillo et al., 2014; Reis et al., 2015). Adiponectin, on the other hand, is reduced in individuals with obesity. The levels of adiponectin are negatively correlated with visceral fat, insulin resistance, type 2 diabetes and cardiovascular complications (Hoffstedt et al., 2004; Nakamura et al., 2004; Li et al., 2009; Ye and Scherer, 2013). The correlation between low adiponectin levels and obesity-associated comorbidities may be explained by adiponectin's anti-inflammatory effects, including: 1) macrophage reprogramming from M1 to M2 polarization (Ohashi et al., 2010); 2) TLR-mediated signaling inhibition (Yamaguchi et al., 2005); 3) Suppression of class A scavenger receptor-mediated foam cell formation (Ouchi et al., 2001); and 4) improved endothelial function by increased NO generation and reduced superoxide production (Deng et al., 2010).

Adipocytes also secrete inflammatory cytokines and chemokines as IL-8, IL-6, TNF-α and MCP-1 (**Figure 1B**) (Skurk et al., 2007). Pro-inflammatory cytokines secretion, as well as leptin secretion, are significantly higher in adipocytes presenting greater volume (Skurk et al., 2007). Moreover, it is reported that leptin induces pro-inflammatory cytokine profile in adipocytes, including IL-6 and TNF-α secretion (Palhinha et al., 2019). In addition to increased inflammatory cytokines, the expansion of adipose tissue with hypertrophic adipocytes contributes to impaired vascularization, local hypoxia, fibrosis and cell death (Rausch et al., 2008; Halberg et al., 2009; Spencer et al., 2011). Collectively, these changes in adipose tissue architecture alongside high infiltration of immune cells contribute to chronic low-grade systemic inflammation, metabolic syndrome, oxidative stress and endothelial dysfunction (**Figures 1A, B**) (Weisberg et al., 2003; Curat et al., 2004; Cancello et al., 2005; Vegiopoulos et al., 2017; Graupera and Claret, 2018; Karczewski et al., 2018).

Intestinal Hyperpermeability and Metabolic Endotoxemia

Intestinal hyperpermeability in individuals with obesity leads to metabolic endotoxemia by increasing the amount of circulating LPS, which is a powerful innate immunity trigger (Damms-Machado et al., 2016). LPS triggers inflammation in TLR4-bearing cells, including macrophages and adipocytes (Song et al., 2006; Medzhitov and Horng, 2009) and may contribute to systemic and adipose tissue inflammation in obese individuals. Metabolic endotoxemia is positively correlated to inflammation, oxidative stress, and macrophage infiltration in the adipose tissue, which were all reduced by antibiotic therapy in high-fat diet (HFD)-fed mice (**Figure 1C**) (Cani et al., 2008a; Cani et al., 2008b). These findings are in line with the "infectobesity" concept, according to which in addition to intestinal microbial agents, viral infections are also associated with adipogenesis and inflammation (Voss and Dhurandhar, 2017; Tian et al., 2019). Even though the cause and effect relationship is not well established, a reciprocal causality may be proposed in which chronic low-grade inflammation in obesity leads to an impaired immunity to pathogens, whereas infections contributes to systemic inflammation and unbalanced lipolysis and adiposity (Voss and Dhurandhar, 2017; Honce and Schultz-Cherry, 2019).

Metainflammation

It is well established that pro-inflammatory cytokines impair the insulin signaling pathway and consequently lead to insulin resistance (Lackey and Olefsky, 2016). In the absence of insulin orchestration, metabolic impairment advances with increased lipolysis and low glucose uptake. This process leads to hyperglycemia and lipotoxicity, and reciprocally feeds inflammation and oxidative stress (Perry et al., 2015) (**Figure 1D**). This metabolic attachment to the immune response in obesity is called "metainflammation" and is the basis for metabolic and cardiovascular complications, which turns obesity into strong risk factor for chronic diseases such as diabetes, atherosclerosis and hypertension (**Figure 1E**) (Lackey and Olefsky, 2016). It is important highlighting that although inflammatory and metabolic dysfunctions prevail in elderly individuals, they increasingly affect youngsters, who have been

more often obese nowadays than in the past. Thus, factors associated with life style, including the ones accounting for weight gain, are causing earlier hyperinflammatory reactions in the general population (Wärnberg et al., 2007). This host homeostasis reprogramming process changes the immune response regulation and the inflammatory environment during infections, including the ones caused by influenza and SARS-CoV-2, over which obesity and metabolic syndrome have major consequences to disease progression, severity and mortality (**Figure 1F**) (Nishiga et al., 2020; Richardson et al., 2020; Wang et al., 2020a; Yang J. et al., 2020).

IMMUNE RESPONSE TO INFLUENZA AND COVID-19 IN OBESITY

Epidemiological analyses of COVID-19 have highlighted obesity as a risk factor for severe disease complications. Based on these analysis, the likelihood of individuals with obesity to develop severe COVID-19 syndrome is higher regardless of sex, age and existence of associated comorbidities (Busetto et al., 2020; Malik et al., 2020; Simonnet et al., 2020). Individuals who have obesity are at higher risk of progressing to critical COVID-19 and of requiring admission to intensive care units (ICU) (Sales-Peres et al., 2020), as well as of presenting non-respiratory-related mortality such as shock and acute renal failure (Onder et al., 2020). Recent studies highlight that presenting with overweight is sufficient to increase the risk of hospitalization and even mechanical ventilation during COVID-19, even though the risk is further increased by mild and severe obesity (Lighter et al., 2020; Simonnet et al., 2020; Wang et al., 2020b). In agreement, COVID-19 morbidity and mortality are further increased in obese individuals who present visceral adiposity and ectopic fat deposition (Yang Y. et al., 2020). Regardless the risk of severity, people with overweight and obesity have greater chances of a positive result in SARS-CoV-2 diagnosis, suggesting higher risk of infection and/or symptomatic illness (Eastment et al., 2020; Kim et al., 2020; Wang et al., 2020b; Breland et al., 2021). Similarly, overweight and obesity also increase the risk of having a positive diagnosis for influenza and increased risk of hospitalization for influenza pneumonia, with even higher risk identified among people with severe obesity (Karki et al., 2018).

Immunopathogenesis

The COVID-19 immunopathological processes are not completely understood, but they may involve similar systemic and airway inflammation processes observed in influenza pneumonia. Severe COVID-19 presentations are associated with imbalance between protective antiviral response and exacerbated pro-inflammatory injury, leading to viral persistence and tissue damage (Felsenstein et al., 2020). The virus can evade innate immunity allowing replication in the initially infected tissues. It is suggested that SARS-CoV-2 infection reduces type I IFN secretion and signaling, pointing to a critical role of IFN-induced anti-viral response in pathogen control (Blanco-Melo et al., 2020; Hadjadj et al., 2020). The lack of virological control may contribute to overwhelmed inflammation and cytokine storm (Tang Y. et al., 2020), which have been associated with COVID-19 and influenza pneumonia main clinical and pathological presentations as ARDS and multiple organ failure (Huang et al., 2020). Along with the cytokine storm, one can observe the disruption of the immune homeostasis with an exacerbated response from the innate immunity, like neutrophils and macrophages, and the impairment of natural killer (NK) and CD8+ T cells, both cytotoxic cells directly involved in viral elimination (Giamarellos-Bourboulis et al., 2020). Thus, patients with severe COVID-19 and influenza pneumonia have irregular immune response that leads to lung injury and increased mortality (Lin et al., 2020; Xu et al., 2020; Zhang et al., 2020a). In this sense, chronic inflammation during obesity may contribute to dysregulated immune homeostasis associated with severe lung injury in SARS-CoV-2 infection, similarly to what is observed in patients with influenza (Luzi and Radaelli, 2020).

Obesity-associated chronic low-grade inflammation sets a preexisting release of inflammatory factors and impaired immune system, which increases the susceptibility of individuals with obesity to infections (De Heredia et al., 2012; Hegde and Dhurandhar, 2013). Given the high prevalence of people with obesity among severe COVID-19 patients (Sales-Peres et al., 2020), it is of paramount importance to clarify the immunoregulatory mechanisms of COVID-19 in people with obesity. It is noteworthy that inflammatory cytokines are already increased in people with overweight, including inflammatory cytokines as IL-6, IL-4, MCP-1 and TNF-α. Nevertheless, the levels of the pro-inflammatory cytokines show greater increment in obese individuals and are even higher in severe obesity, since the white adipose tissue itself is a source of these cytokines (Calder et al., 2011; Fan et al., 2019; Pedersen et al., 2019). TNF-α, IL-1 and IL-6 are the main inflammatory cytokines derived from adipose tissue (Skurk et al., 2007) and increased IL-6 levels are predictive of COVID-19 severity and mortality (Ulhaq and Soraya, 2020). Therefore, pro-inflammatory environment prior to SARS-CoV-2 infection as observed in obesity could contribute to amplify the cytokine storm and COVID-19 severity (**Figure 2A**). However, further studies are needed to clarify the role played by IL-6 and other cytokines in individuals with obesity and overweight presenting with SARS-CoV-2 infection.

The Impact of Metainflammation in COVID-19 and Influenza

Several cytokines and hormones may mediate obesity effects on metabolic reprogramming and impaired effector functions of immune cells. Thus, leptin has several actions in innate and adaptive immunity that can contribute to exacerbate the inflammatory response during respiratory infections. From this viewpoint, leptin is increased in human and murine lungs after severe lung injury due to bacterial sepsis or influenza A (H1N1) pneumonia (Ubags et al., 2015). When it comes to the possible

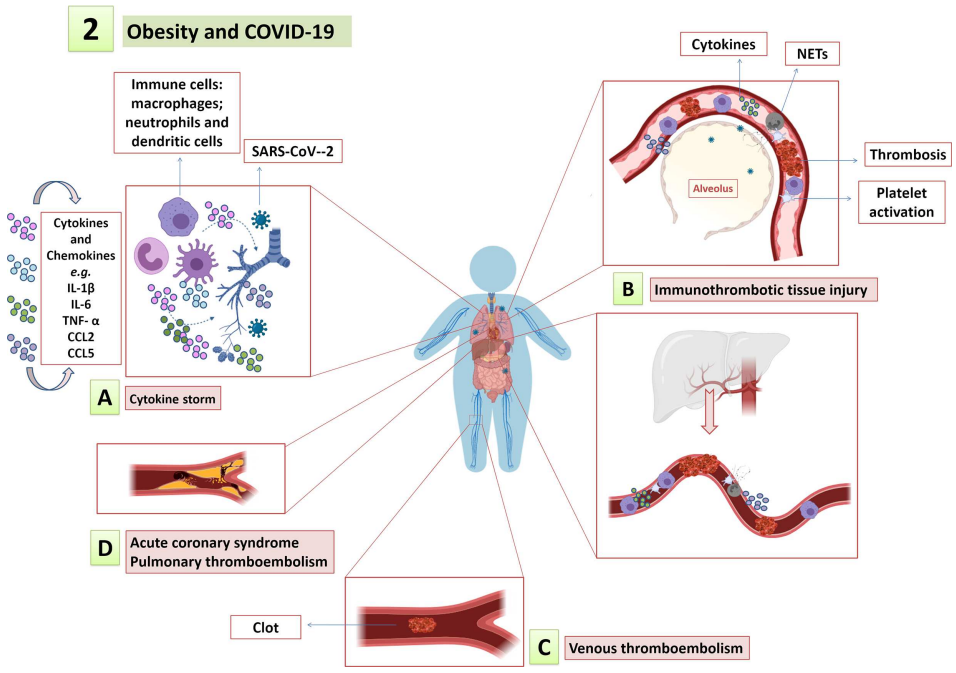

FIGURE 2 | Obesity and COVID-19. The low-grade systemic inflammation, endothelial dysfunction and procoagulant state in obesity amplify and contribute to COVID-19 immunopathology, such as **(A)** cytokine storm; **(B)** immunothrombotic tissue injury; and **(C)** increased risk for thromboembolic events, including **(D)** myocardial infarction, pulmonary thromboembolism, among others.

immunoregulatory effects of leptin observed in lung injury models, one can state that leptin plays important roles in recruiting neutrophils to the airways in pneumonia models *in vivo*. This finding corroborates leptin's likely participation in inflammatory amplification and tissue damage in COVID -19 (Kordonowy et al., 2012; Ubags et al., 2015). Severe COVID-19 patients have increased neutrophil count and activation compared to mild infection, it is also possible observing neutrophil infiltration and extracellular trap (NETs) extrusion in the lungs of critically ill patients (Middleton et al., 2020; Nicolai et al., 2020a). Therefore, we can hypothesize that leptin can also contribute to neutrophils' infiltration in airways and in other tissues during COVID-19, which also contribute to worsen prognosis in obese individuals. Thus, leptin is an interesting target for future mechanistic research on factors involved in obesity immune dysregulations affecting influenza and COVID-19 pathogenesis.

Chronic inflammation in obesity is followed by immune dysregulation and dysfunctional NK and CD8+ T cells, which also have their function impaired in severe COVID-19 and influenza patients (Sheridan et al., 2012; Zheng et al., 2020). The lipotoxic environment in obesity reduces NK cell counts and impairs NK cells cytotoxic activity, leading to immune paralysis (Michelet et al., 2018). In addition to lipotoxicity, some adipokines as IL-6 and leptin that get higher in obesity also impair the cytotoxic activity of NK cells (Lamas et al., 2013; Cifaldi et al., 2015; Bähr et al., 2017; Bähr et al., 2020). Actually, weight loss in people with obesity increases the secretion of IFN-γ by NK cells (Jahn et al., 2015). In addition, obesity negatively regulates the number of T cell progenitors in the thymus and bone marrow, limits TCR diversification and impairs the metabolic reprogramming of effector CD8+ T cells (Rebeles et al., 2019). Peripheral blood mononuclear cells (PBMC) from people with obesity and overweight show less CD8+ T cell activation and cytotoxic granule release when they are challenged by the influenza virus than cells from eutrophic individuals (Sheridan et al., 2012; Paich et al., 2013). Experimental influenza in HFD-fed mice has been associated with reduced IFN-γ secretion by memory CD8+ T cells in the lungs of mice during secondary influenza infection (Rebeles et al., 2019). Based on the aforementioned processes, obesity-associated inability to produce type I and II interferons during influenza infection allows viral replication and persistence (Honce and Schultz-Cherry, 2019). In order to clarify the association between obesity and influenza virus transmission, some studies have shown that obesity significantly increased the duration of virus shedding by obese adults when compared to eutrophic individuals (Maier et al., 2018), and viral quantification in air samples exhaled by people with influenza showed a positive correlation between obesity and the amount of virus released (Yan et al., 2018). Thus, since immunological changes in obesity during influenza infection increase the risk of severity and potential for transmission, we hypothesize that obesity in individuals with COVID-19 may behave in a similar

fashion, which requires new studies. If this hypothesis becomes to be true, it has major implications for social distancing measures, masking and vaccination among people with obesity.

Is Adipose Tissue a Target for Viruses?

Viruses often take several routes to maximize their infectious potential. SARS-CoV-2 attachment into host cells is known to occur through ACE2 (angiotensin-converting enzyme 2) (Shang et al., 2020). Patients with COVID-19 who present comorbidities such as hypertension and respiratory diseases have higher pulmonary expression of ACE2 (Pinto et al., 2020). Such a fact likely explains how these comorbidities, which are closely related to obesity, contribute to COVID-19 severity (Richardson et al., 2020; Yang X. et al., 2020). Overloading transcription, expression and enzymatic activity of ACE2 have been observed in adipocytes in murine model of diet-induced obesity (Gupte et al., 2008). In addition, a study based on a large cohort of obese patients with COVID-19 showed increased serum ACE2 levels in this population (Emilsson et al., 2020). Individuals with obesity have larger adipose tissue volume and a larger number of cells expressing ACE2, which may favor viral replication. Dipeptidil Peptidase 4 (DPP4) is another receptor involved in SARS-CoV-2 entry found in adipose tissue and in epithelial cells of various organs besides the lungs (Bassendine et al., 2020). Considered an adipokine (Lamers et al., 2011), DPP4 is involved in glucose homeostasis, inflammation and immunity. Experimental obesity in mice showed greater DPP4 release compared to lean. If one takes in consideration DPP4 overloading in obesity (Lamers et al., 2011), increased DPP4 expression may favor SARS-CoV-2 entry in host cells in addition to altering the immunological and metabolic processes involved in COVID- 19 pathogenesis. In addition, studies have shown that SARS-CoV-2 is capable of binding the receptor neuropilin-1 (NRP1), which is abundantly expressed in the respiratory and olfactory epithelium. However, despite satisfactory *in vitro* results, further studies are still needed to clarify the mechanisms of SARS-CoV-2 attachment to NRP1, especially in adipose tissue and neuro-adipose connections (Cantuti-Castelvetri et al., 2020; Daly et al., 2020). The impact of the adipocyte expression of SARS-CoV-2 receptors on the pathogenesis of COVID-19 and the virus likelihood of replicating in adipose tissue are topics that still deserve in-depth investigation.

OBESITY AND INCREASED CARDIOVASCULAR RISK IN INFLUENZA AND COVID-19

As already mentioned, chronic inflammation and oxidative stress in obesity set up a fertile ground for insulin resistance and endothelial injury, increasing the risk of diabetes and cardiovascular complications (Engin, 2017). Accordingly, the high cardiovascular risk (HCR) posed to individuals with obesity could be related to COVID-19 severity. Likewise, HCR can also be involved in increased risk for obesity-associated comorbidities such as hypertension and diabetes (Nishiga et al., 2020; Richardson et al., 2020; Yang J. et al., 2020; Zhou F. et al., 2020). This pre-existing cardiovascular risk may contribute to COVID-19 severity as SARS-CoV-2 infection *per se* can cause complications as acute coronary syndrome and venous and arterial thromboembolism, which are leading causes of mortality (Klok et al., 2020; Nishiga et al., 2020). Accordingly, laboratory analyses point towards significant increase in cardiac injury and coagulation activation markers as predictive of mortality in severe COVID-19 (Tang N. et al., 2020; Zhou F. et al., 2020). The risk of thromboembolic complications as acute myocardial infarction is also higher in influenza virus infection (Kwong et al., 2018; Musher et al., 2019). Nevertheless, alveolar-capillary microvascular thrombosis is almost ten times more frequent in autopsies from COVID-19 fatalities than in those from H1N1 influenza pneumonia (Ackermann et al., 2020). This finding highlights differences in pathophysiological mechanisms linked to the risk of thrombosis between these diseases.

Vascular Disorders in Influenza and COVID-19

Increased cardiovascular complications of acute influenza pneumonia involve endothelial damage, inflammation and coagulation activation (Fountoulaki et al., 2018; Peretz et al., 2019). Assumingly, cytokine storm in influenza leads to endothelial barrier rupture and vascular hyperpermeability (Wang et al., 2010). Studies conducted with proatherogenic animal models have evidenced that the influenza virus, besides leading to systemic inflammation, also infects and resides in arterial walls and atherosclerotic plaques. Such a process may contribute to mechanisms of cardiovascular complication and thromboembolic events in influenza (Naghavi et al., 2003; Haidari et al., 2010). Extrapolating this process to COVID-19, it is not yet known whether vascular disorders result from a direct effect of the virus on atherosclerotic or normal arteries. However, some findings point towards the presence of the virus in human endothelial cells, and it possibly contributes to the endothelial damage observed in some tissues (lung, kidneys, small bowel, skin) and to multiorgan impairment (Colmenero et al., 2020; Pons et al., 2020; Varga et al., 2020). Endothelial inflammation is a potential trigger for atherosclerosis and represents an initial pathological event of cardiovascular diseases, such as myocardial infarction and other thromboembolic events described in COVID-19 (Gimbrone and García-Cardeña, 2016; Nishiga et al., 2020). Thus, endothelial inflammation could play important roles in cardiovascular disorders caused by COVID-19 and this hypothesis should be strongly considered as pathogenic mechanism.

The mechanisms involved in cardiovascular complications of COVID-19 still need further clarification. Assumingly, the development of cardiovascular and thromboembolic complications due to SARS-CoV-2 infection also involves the direct cytopathic effect of the virus, systemic inflammation and cytokine storm (Sokolowska et al., 2020), which contributes to platelet activation, neutrophils recruitment and endothelial activation/injury, mainly in severe cases (Hottz et al., 2020;

Huertas et al., 2020; Middleton et al., 2020). On this regard, increased platelet activation and platelet-monocyte interaction leading to pathogenic tissue factor expression has been associated with hypercoagulability, severity and mortality in severe COVID-19 patients (Hottz et al., 2020). Platelet-neutrophil interactions have been also reported alongside NET extrusion in pulmonary microvascular thrombi from COVID-19 autopsies (Middleton et al., 2020), as well as in other organs (Nicolai et al., 2020a). A recent report have shown higher levels of NET-containing pulmonary vascular occlusive thrombi in COVID-19 than in H1N1 influenza pneumonia (Nicolai et al., 2020b), and this finding suggests differences in the mechanisms of thromboinflammation between influenza and COVID-19.

Obesity: A Breeding Ground for Cardiovascular Risk During COVID-19 and Influenza

If one takes into consideration that the factors associated with cardiovascular complications in viral infections involve endothelial dysfunction linked to inflammatory and pro-coagulant responses, some pre-existing conditions such as obesity could contribute to cardiovascular complications of the infection. Accordingly, it is well described that chronic inflammation in obesity disrupts endothelial homeostasis (Kwaifa et al., 2020). The endothelial and vascular dysfunction observed in multiple tissues in overweight and obese individuals is closely associated with increased risk for metabolic and cardiovascular disorders (Csige et al., 2018; Graupera and Claret, 2018). Endothelial homeostatic imbalance under these conditions involves reduced availability of vasoprotective and vasodilator molecules, including NO (Förstermann and Sessa, 2012; Xia and Li, 2017), also contributing to inflammatory infiltration (Tabit et al., 2010; Sansbury et al., 2012; Savini et al., 2013; Yao et al., 2017).

Aligned with endothelial dysfunction in obesity, high oxLDL and adipokines levels support the development of atherosclerosis (Berkel et al., 1995; Morange and Alessi, 2013; Chistiakov et al., 2017). Some leptin effects support atherogenesis by increasing oxidative stress, proliferation and matrix remodeling in vascular endothelial and smooth muscle cells, which are linked to plaque vulnerability (Konstantinides et al., 2001; Yamagishi et al., 2001; Li et al., 2005). On the other hand, adiponectin reduction in obesity contributes to increased cardiovascular risk as its protective roles in atherogenesis oppose that of leptin (Okamoto et al., 2002; Yamauchi et al., 2002). Therefore, the association of atherosclerotic process in individuals who have obesity with vascular stress induced by SARS-CoV-2 infection may trigger the rupture of already vulnerable atherosclerotic plaques, leading to arterial thromboembolic events and tissue ischemia. Consistently, platelet activation, which has been associated with COVID-19 severity and mortality (Hottz et al., 2020), is also described in individuals with obesity and is positively correlated with the BMI (Koupenova et al., 2015; Barrachina et al., 2019). Thus, low-grade systemic inflammation, endothelial dysfunction and procoagulant state in obesity could amplify immunothrombotic tissue injury and contribute to thromboembolic events described in COVID-19 (**Figures 2B–D**). A recent study pointed out obesity as strong risk factor for venous thromboembolism in patients with COVID-19 (Hendren et al., 2021). However, BMI effect on cardiovascular outcomes in patients with COVID-19 or influenza remains uncertain and needs to be evaluated in studies with larger and more diverse populations. Besides, experimental animal models could be used to assess the possible cardiovascular outcomes in obese animals with influenza or COVID-19 infection when compared to eutrophic infected animals.

Metabolic Syndrome, Hyperglycemia and Cardiovascular Risk in COVID-19

Obesity-associated inflammatory and metabolic changes contribute to insulin resistance and increase the risk for diabetes, which, in turns, is a risk factor for COVID-19 severity and mortality (Zhang et al., 2020b; Zhu et al., 2020). Since diabetes has been associated with poor prognosis in COVID-19, the glycemic control of COVID-19 patients has gained great attention. Improved clinical outcomes are observed after better glycemic control of COVID-19 patients with pre-existing type 2 diabetes (Zhu et al., 2020). Although the potential pathogenic link between COVID-19 severity and hyperglycemia are not yet fully clarified, it assumingly involves the HCR associated with inflammation, oxidative stress and endothelial dysfunction under hyperglycemic conditions (Lim et al., 2021). It is well described that hyperglycemia is an independent risk factor for cardiovascular complications (Stratton et al., 2000; Elley et al., 2008). The induction of hyperglycemia acts directly on endothelial dysfunction through pro-inflammatory and pro-oxidative processes described in both diabetes and obesity/overweight (Ceriello et al., 2002; Perkins et al., 2015; Low Wang et al., 2016). The pro-inflammatory environment associated with acute hyperglycemia also impairs the innate immune response, which may harm the fight against SARS-CoV-2 and possibly contribute to COVID-19 poor prognosis (Jafar et al., 2016; Guo W. et al., 2020; Pal and Bhadada, 2020). Some studies suggest that SARS-CoV-2 infection contributes to hyperglycemia and worsens dysglycemia in patients with this pre-existing condition. This finding points to likely bidirectional relationship between COVID-19 and comorbidities as diabetes and obesity/overweight (Apicella et al., 2020; Pal and Bhadada, 2020).

In addition to the physiological vascular consequences of hyperglycemia, a recent study indicates that increased glucose availability contributes for viral replication and leukocyte inflammation in SARS-CoV-2 infection (Codo et al., 2020). Thus, the metabolic reprogramming of monocytes infected by SARS-CoV2 requires the glycolytic metabolism supporting increased inflammatory response, viral replication and pulmonary epithelial cell death (Codo et al., 2020). These data suggest the important participation of glucose metabolism in monocyte-driven exacerbated inflammation, immune dysfunction and lung injury in SARS-CoV-2 infection. Altogether, the abovementioned evidences highlight the well-

established contribution of insulin resistance and hyperglycemia to inflammation, oxidative stress and endothelial dysfunction, which breeds the ground for cardiovascular diseases (Petrie et al., 2018). Diabetes involvement in worsening COVID-19 prognosis assumingly involves similar mechanisms as obesity, such as metabolic impairment, pre-existing inflammation, immune dysregulation and cardiovascular disorders (Muniyappa and Gubbi, 2020).

Overall, it is possible suggesting that immune dysregulation, exacerbated inflammation, and high cardiovascular risk could be powerful links between obesity and the severity of influenza and SARS-CoV-2 infection. However, it is essential to better understanding the obesity-related pathophysiological mechanisms exclusively involved in the pathogenesis of severe COVID-19 and the ones shared with influenza virus infection, since it can contribute to improved clinical management and therapeutic strategies applied to individuals with obesity.

CONCLUSION

Obesity emerged as important risk factor during viral infections throughout the years, not just because of its persistent proinflammatory condition but also due to its associated metabolic complications and comorbidities, such as diabetes, hypertension, endocrine dysfunctions and immune dysregulation, among others. Altogether, these factors play important roles in worsening viral infections. Better understanding how these conditions contribute to COVID-19 and influenza severity may help decision-making processes at patient screening and management, since early intervention is key to prevent mortality. Besides, it may support the formulation of health policies and hospital beds projections in ICUs and wards. Based on the recent pandemic, it will be essential to stablish stricter surveillance for viral detection and disease morbidity and mortality, by developing therapeutic and clinical strategies for obesity-related mechanisms of pathology in COVID-19 and influenza pneumonia, as well in other infections.

Understanding the mechanisms underlying overwhelming inflammation, cytokine storm and immune dysfunction will be of paramount importance to determine life-style and environmental interventions to mitigate the risk of individuals with obesity to severe respiratory infections. Efforts on future research will solidify the scientific basis and guide health professionals in the clinical management of patients with obesity. While specific therapies and vaccine coverage to control the pandemic are yet to emerge, special care must be provided mainly to the most vulnerable populations, among them elderlies, immunosuppressed patients and people with obesity.

AUTHOR CONTRIBUTIONS

FA, AG, and CR wrote the manuscript draft. NP, JG, and EH edited and revised the manuscript. All authors contributed to the article and approved the submitted version.

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ANEXO B

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JLB JOURNAL OF LEUKOCYTE BIOLOGY

REVIEW

Innate immune receptors in platelets and platelet-leukocyte interactions

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Abstract

Platelets are chief cells in hemostasis. Apart from their hemostatic roles, platelets are major inflammatory effector cells that can influence both innate and adaptive immune responses. Activated platelets have thromboinflammatory functions linking hemostatic and immune responses in several physiological and pathological conditions. Among many ways in which platelets exert these functions, platelet expression of pattern recognition receptors (PRRs), including TLR, Nod-like receptor, and C-type lectin receptor families, plays major roles in sensing and responding to pathogen-associated or damage-associated molecular patterns (PAMPs and DAMPs, respectively). In this review, an increasing body of evidence is compiled showing the participation of platelet innate immune receptors, including PRRs, in infectious diseases, sterile inflammation, and cancer. How platelet recognition of endogenous DAMPs participates in sterile inflammatory diseases and thrombosis is discussed. In addition, platelet recognition of both PAMPs and DAMPs initiates platelet-mediated inflammation and vascular thrombosis in infectious diseases, including viral, bacterial, and parasite infections. The study also focuses on the involvement of innate immune receptors in platelet activation during cancer, and their contribution to tumor microenvironment development and metastasis. Finally, how innate immune receptors participate in platelet communication with leukocytes, modulating leukocyte-mediated inflammation and immune functions, is highlighted. These cell communication processes, including platelet-induced release of neutrophil extracellular traps, platelet Ag presentation to T-cells and platelet modulation of monocyte cytokine secretion are discussed in the context of infectious and sterile diseases of major concern in human health, including cardiovascular diseases, dengue, HIV infection, sepsis, and cancer.

KEYWORDS

innate immunity, pattern recognition receptors, platelet activation, thromboinflammation

Abbreviations: ADP, adenosine diphosphate; AIDS, acquired immunodeficiency syndrome; ApoE, apolipoprotein E; ART, antiretroviral therapy; BAD, BCL2 antagonist of cell death; C1q, complement component 1q; C3, complement factor 3; CAPs, carboxy(alkylpyrrole) protein adducts; cGMP, cyclic guanosine monophosphate; CLEC, C-type lectin-like receptor; CLR, C-type lectin receptors family; CRP, C reactive protein; DAMP, damage-associated molecular patterns; DC, dendritic cell; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; DENV, dengue virus; ERK, extracellular signal-regulated kinase; FcγR, receptor for Fc-portion of immunoglobulin-G; FcεR, receptor for the Fc region of immunoglobulin-E; GP, glycoprotein; HETE, Dihydroxyicosatetraenoic acid; HFD, high fat diet; HLA, human leukocyte Ags; IAV, influenza A virus; ICOSL, inducible T cell costimulator ligand; IFITM3, IFN-induced transmembrane protein 3; IL-1R, IL-1 receptor; IL-2R, IL-2 receptor; ITAM, immune receptor tyrosine-based activation motif; LOX-1, lectin-like oxidized low-density lipoprotein receptor-1; Mac-1, Mϕ-1 Ag; MCP-1, monocyte chemoattractant protein-1; MDC, Mϕ-derived chemokine; MDP, muramyl dipeptide; MIF, Mϕ migration inhibitory factor; MVs, microvesicles; NET, neutrophil extracellular trap ; NKG2D, natural killer group 2D receptor; NLR, Nod like receptor; NLRP, nucleotide-binding domain leucine-rich repeat containing pyrin; NO, nitric oxide; NOD, nucleotide-binding oligomerization domain; NS1, nonstructural protein 1; ObR, leptin receptor; oxLDL, oxidized low-density lipoprotein; PAF, platelet-activating factor; PAFR, platelet-activating factor receptor; PAMP, pathogen-associated molecular patterns; PAR, protease-activated receptor; PDGF, platelet-derived growth factor; PF4, platelet factor 4; PRR, pattern recognition receptor; PSGL, P-selectin glycoprotein ligand; RA, rheumatoid arthritis RAGE; RANTES, regulated upon activation in normal T cells expressed and secreted; RAGE, receptor for advanced glycation end products; RBC, red blood cells; ROS, reactive oxygen species; SCD, sickle cell disease; sCD40L, soluble CD40 ligand; SDF-1α, stromal cell-derived factor 1α; TARC, thymus- and activation-regulated chemokine; Tat, HIV-1 transactivator of transcription; TNFR, TNF receptor; TRIF, TIR-domain containing adapter-inducing interferon-β; TXA_2, thromboxane A_2; VEGF, vascular endothelial growth factor.

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1 | INTRODUCTION

Platelets are chief cells of hemostasis and pathological thrombosis. Considering their small and anucleate status, platelet activities described in the literature were, for a long time, limited to rapid activation-dependent responses involved in hemostasis and wound repair.[1,2] However, new studies have shown platelets as important effectors of innate and adaptive immunity through many functions that last hours after pro-hemostatic adhesion and aggregation.[3–5]

Platelets circulate at high-shear rates and are activated following binding to subendothelial matrix proteins exposed during vascular injury or inflammation.[6–9] Stable adhesion to endothelium promotes platelet release of several stored mediators, as coagulation and growth factors from α-granules or serotonin and nucleotides from dense-granules, and newly synthesized eicosanoids, especially thromboxane A_2 (TXA_2). These secreted mediators amplify platelet activation and hemostatic responses.[10,11] These classical platelet activation pathways generally involve activation of G protein-coupled receptors and are regulated by rapid responses involving intracellular calcium signaling and platelet adhesion through integrins and selectins.[4,12]

Beyond classical pro-hemostatic activities, platelets are capable of recognizing damage- and pathogen-associated molecular patterns (DAMPs and PAMPs, respectively) by several pattern recognition receptors (PRRs), initiating inflammatory and immune responses.[13–15] Aside from pathogen sensing, many recently identified platelet functions participate in both coagulation and inflammation. Platelets contain RNA molecules and activation-dependent post-transcriptional mechanisms for process intronic RNA enabling the synthesis of immunoregulatory proteins such as IL-1β and antimicrobial peptides such as β-defensins, among others.[16–20] Activated platelets also release several inflammatory molecules from α-granules, including chemokines and cytokines, critical for the assembly of immune response.[21–24] Platelets have been also demonstrated at the extravascular space, influencing local immune responses in different tissues,[25–27] including the synovia during rheumatoid arthritis,[28,29] lungs in influenza,[26] and in solid tumors.[30] Thus, platelets are recognized as sentinels with surveillance activities in the vasculature and at the extravascular milieu. Platelet activities initiated by PAMPs and DAMPs engagement to innate immune receptors will be the subject of this review, and the participation of platelet PRRs and platelet-leukocyte interactions in the pathogenesis of sterile inflammation and infectious diseases will be addressed in the following sections.

2 | PATTERN RECOGNITION RECEPTORS IN PLATELETS

Platelets express different families of PRRs, which can be encountered on platelet surface or intracellularly, in their cytoplasm and endosomes (Fig. 1). The PRR families described in platelets are TLRs, NOD-like receptors (NLRs), and C-type lectin receptors (CLRs).[13–15,31] Platelets express several TLRs including TLR1, 2, 4, and 6 on surface, and TLR3, 7, and 9 in endosomes.[14,32–34] TLR4 is the best described in the literature, especially in response to LPS.[35–40] Although platelets lack membrane-bound CD14 as co-receptor for TLR4, soluble CD14 enhances platelet response to LPS.[38–40] Upon LPS stimulation in vitro, platelets respond with P-selectin surface expression, secretion of soluble CD40 ligand (sCD40L), and other inflammatory mediators as cytokines and chemokines.[35,36,39] Platelet TLR4 activation by LPS occurs through MyD88 or TRIF-dependent pathways, leading to NF-κB activation.[36,39] Since NF-κB and other transcription factors regulate platelet functions despite the absence of nucleus, transcription factors have non-transcriptional activities in platelets (and nucleated cells) that remain under investigation.[41–44]

Besides LPS, other PAMPs and DAMPs induce platelet TLR4 activation. Cell-free histones activate platelet TLR2 and TLR4 leading to P-selectin translocation, aggregation, chemokine secretion, and thrombin generation.[45–47] In vitro and in vivo experiments using platelet-specific TLR4 knockout demonstrated that high-mobility group box 1 (HMGB1) induces platelet activation and thrombosis via TLR4 and cGMP signaling.[48,49] Recent studies have shown that nonstructural protein 1 (NS1), a viral PAMP secreted by dengue virus (DENV)-infected cells, stimulates platelet activation depending on TLR4[31,50] (see Section 6.1).

Another TLR widely studied in platelets is TLR2, which forms heterodimers with TLR1 or 6.[51] Stimulation with the TLR2/1 synthetic agonist Pam_3CSK_4 induces platelet aggregation, adhesion, and release of chemokines.[35,52,53] Opposed to its signaling in nucleated cells, platelet TLR2/1 activation requires glycoprotein (GP)VI signaling cascade.[54,55] Besides, platelet TLR2/6, but not TLR2/1, cooperates with CD36 scavenger receptor in response to oxidized phospholipids found in dyslipidemia[56,57] (see Section 5.1). Regarding full pathogens, platelet TLR2 triggers platelet activation in response to human cytomegalovirus[58] and strains of group B streptococci[59] (see Section 6).

Endosomal TLRs are specialized in recognizing genetic material from pathogens, especially viruses.[60] TLR7 is an important endosomal receptor for single-stranded viral RNA,[61] while TLR3 and 9 recognize double-stranded RNA and unmethylated DNA, respectively.[62,63] Platelet stimulation with the TLR7 synthetic agonist Loxoribine or infection with RNA viruses increase platelet-neutrophil aggregation.[14,64] This platelet response leads to thrombocytopenia in mice as platelets become activated and aggregate with neutrophils (details in Section 6.1).[14,64] While nucleated cells express TLR9 exclusively within the endosomes, platelets translocate TLR9 to the surface under activation.[65] Classical platelet agonists as thrombin, ADP, and collagen induce TLR9 translocation.[66,67] Platelet TLR9 stimulation using its synthetic agonist oligodeoxynucleotide induces α- and dense-granules release.[68] TLR9 activation by carboxy(alkylpyrrole) protein adducts (CAPs), an endogenous DAMP, also leads to platelet activation and aggregation in vitro and participates in thrombosis in atherosclerotic mice[69] (see Section 5.1).

CLRs recognize PAMPs and DAMPs containing glycoproteins and glycolipids.[70] C-type lectin-like receptor 2 (CLEC2) is a transmembrane receptor expressed as a dimer and activated by its endogenous glycoprotein ligand podoplanin, expressed on the surface of several

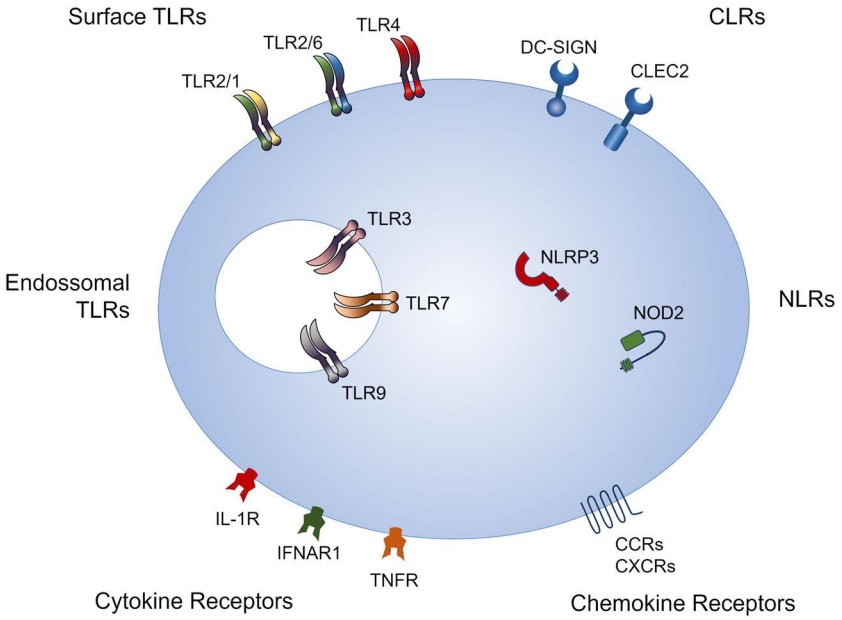

FIGURE 1 Pattern recognition receptors, cytokine receptors and chemokine receptors are present in platelets. Platelets possess the main families of pattern recognition receptors including TLRs, which can be expressed on platelet surface (TLR1, 2, 4, and 6) or in endosomal vesicles (TLR3, 7, and 9); C-type lectin receptors (CLR) expressed on cell surface (CLEC-2 and DC-SIGN); and NOD-like receptors (NLR) at the cytosol (NOD2 and NLRP3). Platelets also express major type I and II cytokine receptors as IL-1 receptor (IL-1R), type I IFN receptor (IFNARI), and TNF-α receptor (TNFRI and TNFRII), as well as CC motif-containing and CXC motif-containing chemokine receptors (CCRs and CXCRs). See the text for details and references

cells.[71] Physiologically, CLEC2-podoplanin engagement maintains the separation between blood and lymphatic vessels,[72,73] but also plays an indispensable role in pathological thrombosis.[74] CLEC2 is also involved in platelet activation by pathogens as DENV and HIV, leading to α- and dense-granules release and shedding of microvesicles (MVs).[75,76] Both DENV and HIV-1 can be recognized by another CLR, namely dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), also leading to platelet activation[13,77,78] (see Section 6.1). Apart from pathogens, platelet DC-SIGN recognizes extracellular mitochondrial DNA, inducing platelet activation and thrombus formation.[79,80]

Regarding platelet cytosolic sensors the main family is NLRs, including nucleotide-binding oligomerization domain 2 (NOD2) and nucleotide-binding domain leucine-rich repeat-containing pyrin 3 (NLRP3).[81–83] NLRP3 is an important mediator of inflammasome assembly, which activates caspase-1 to cleave the pro-inflammatory cytokines pro-IL-1β and pro-IL-18 into active cytokines.[84,85] Inflammasome assembly and cytokine processing in nucleated cells require 2 signals: the first to induce transcription of the cytokines and inflammasome components and the second to trigger inflammasome activation.[83] Recent reports using different models have shown that platelet inflammasome activation requires TLR4-mediated signaling,[15,86,87] suggesting a similar 2-signals response in platelets. The involvement of ATP from dense-granules in platelet NLRP3 activation is shown to mediate platelet prothrombotic responses through IL-1β secretion.[88] During dengue infection, platelet inflammasome activation requires reactive oxygen species generated in mitochondria, but not ATP release from granules[50,83] (see Section 6.1). Hence, other evidence regarding NLRP3 and IL-1β participation in platelet prothrombotic responses have emerged.[15,86–90]

NOD2 receptor is characterized by 2 caspase activation and recruitment N-terminal domains and recognizes muramyl dipeptide (MDP) from Gram-positive and Gram-negative bacteria.[91,92] Platelet NOD2 activation with MDP potentiates platelet aggregation, ATP release, and clot retraction in response to prothrombotic stimuli.[81] Accordingly, transfusion of MDP-treated platelets accelerates thrombosis in $FeCl_3$-injured artery depending on platelet NOD2 expression.[81] Furthermore, MDP-stimulated platelets release mature IL-1β depending on NOD2,[81] suggesting a link between NOD2 and inflammasome activation in platelets.

3 | CYTOKINE AND CHEMOKINE RECEPTORS ON PLATELETS

Cytokines are a group of small proteins secreted by immune cells with anti- and pro-inflammatory effects when signaling through their receptors.[93] There are evidences of cytokine receptors on platelets and megakaryocytes (Fig. 1) influencing either thrombopoiesis or peripheral platelet activities.[17,94–99] IL-1 receptor (IL-1R) is a receptor of type 1 ILs expressed on platelets amongst other immune cells.[17,98] Platelet IL-1R stimulation potentiates thrombin-induced aggregation and adhesion to collagen and fibrinogen.[98] This receptor is activated by platelet-derived IL-1β through an autocrine loop after inflammatory and prothrombotic stimuli as LPS and thrombin.[17,88] Blood stimulation with IL-1β, among other cytokines as IL-6 and IL-8, induces platelet hyperactivation and unstable clot formation, but whether this depends on platelet responses directly to the cytokines is unknown.[100]

TNF receptors, TNFRI and TNFRII, are also expressed by platelets.[95] Increased TNFR expression and TNF-α binding on platelets are observed in diabetes mellitus,[95] but whether platelet TNFR engagement participates in diabetes-associated thrombosis remains to be demonstrated. In a mouse model of aging, enhanced TNF-α levels increase platelet activation and adhesion.[96] These peripheral platelet responses depend on TNF-α-induced changes of megakaryocyte transcriptome and proteome, leading to the production of hyperactive platelets.[96] Similarly, RNA for IFN receptor

subunits is present in megakaryocytes and platelets, although the protein is expressed and functional only in megakaryocytes.[101,102] The type I IFNs, IFN-α and -β, are produced mainly by virus-infected cells exhibiting potent antiviral activity by inducing IFN-stimulated genes (ISGs).[102] Type I IFN signaling in megakaryocytes inhibits thrombopoiesis, causing thrombocytopenia in viral infections.[101,103] A recent study demonstrated up-regulated expression of the ISG IFN-induced transmembrane protein 3 (IFITM3) in platelets from dengue and influenza patients.[94] This may occur through IFN signaling in megakaryocytes, once IFN-stimulated or DENV-infected megakaryocytes increase IFITM3 expression, protecting these cells from subsequent viral challenge.[94]

Leptin is a hormone produced by adipocytes, classically described as a regulator of food intake and energy balance.[97] Recent studies have shown leptin signaling in immune cells, characterizing it as a cytokine.[104,105] Leptin signals through its membrane receptor (ObR) and the long-form ObRb is expressed by platelets.[99] Leptin induces phosphorylation of JAK2-STAT3/AKT pathway leading to TXA_2 synthesis in platelets, which potentiates platelet adhesion and aggregation in response to ADP.[99,106,107] Leptin involvement in platelet activation and thrombosis in obesity is discussed in Section 5.2.

Fewer studies have investigated anti-inflammatory cytokine receptors on platelets.[108,109] Platelets are known to express the surface receptor glycoprotein-A repetitions predominant for TGF-β, also reported on regulatory T cells.[108] TGF-β is an abundant cytokine from megakaryocytes and platelets that mediate anti-inflammatory responses.[109] More studies are necessary to investigate platelet receptors for classical anti-inflammatory cytokines such as IL-10 and IL-4.

Regarding chemokines, platelets express both CC and CXC motif-containing chemokine receptors (CCR and CXCR) including CCR1, 3 and 4, and CXCR1, 2, 3, 4, 6 and 7.[110–115] PI3K-Akt signaling is initiated in platelets after CXCR6 and CXCR7 activation by CXCL16 or Mϕ migration inhibitory factor (MIF), respectively.[111,112] These pathways may either enhance platelet prothrombotic responses following CXCL16,[111] or inhibit BAD-mediated apoptosis after MIF-CXCR7 engagement.[112] Likewise, stromal cell-derived factor 1α (SDF-1α/CXCL12) engages CXCR4 and CXCR7 on platelets rescuing them from apoptosis.[113,114] MDC/CCL22 or TARC/CCL17 binding to platelet CCR4 and eotaxin/CCL11 or RANTES/CCL5 engagement to CCR3 lead to Ca^{++} influx-mediated platelet aggregation and degranulation.[110,115] These evidence indicate the participation of cytokine and chemokine receptors in platelet prothrombotic and inflammatory responses.

4 | INNATE IMMUNE RECEPTORS IN PLATELET-LEUKOCYTE INTERACTIONS

Besides inducing platelet aggregation, adhesion, and secretion, prothrombotic or inflammatory stimuli (including PAMPs, DAMPs, and cytokines) also induce platelet-leukocyte aggregation[116] (Fig. 2). These aggregates involve regulated protein interactions that signal to leukocyte oxidative burst, extravasation, phagocytosis, NF-κB activation, transcription and release of cytokines, and neutrophil extracellular traps (NET).[117–121] Platelets induce IL-1β and NLRP3 transcription in leukocytes, potentiating their response to classical inflammasome triggers as ATP and nigericin.[122] Thus, platelets provide the first signal for leukocyte inflammasome. It is well established that platelet-leukocyte aggregation is mediated by the interaction of P-selectin on activated platelets with P-selectin glycoprotein ligand (PSGL)-1 on leukocytes.[123,124] P-selectin, stored in α-granules, is translocated to the surface under stimulation allowing PSGL-1 adhesion.[23] Integrin αIIbβ3 and Mac-1 fibrinogen receptors also play important roles in platelet-leukocyte aggregation,[125–129] hence fibrinogen bridges platelet and leukocyte integrins.[125–129] Besides contact-dependent signaling, platelet soluble factors participate in leukocyte reprogramming during platelet-leukocyte interactions.[122,130,131]

The interaction between platelets and neutrophils is well established (Fig. 2A). NET extrusion by platelet-neutrophil aggregates depends on P-selectin-PSGL-1 binding[23] and platelet GPIb interaction with neutrophil Mac-1.[132] Beyond classical platelet agonists,[23,132] platelet TLRs engagement also induce the release of NET.[64,118] This biological feature has been shown in platelet TLR4 stimulation by LPS,[118] platelet TLR2 in response to Pam3CSK4,[132] and through TLR7 in platelets infected with influenza A virus (IAV).[64] CLEC2 activation by podoplanin or DENV signals NET extrusion through neutrophil CLEC5 and TLR2.[75] P-selectin-mediated adhesion also induces the synthesis of the eicosanoid 12(S),20-Dihydroxyicosatetraenoic acid (12,20-HETE) by neutrophils through the transfer of 12-HETE from platelets.[133–135] Furthermore, neutrophils secrete matrix metalloproteinase 9, myeloperoxidase, and translocate CD11b upon interaction with activated platelets.[64,136]

Platelets also interact with and reprogram monocyte responses (Fig. 2B). Thrombin-activated platelets induce PSGL1-mediated NF-κB translocation, increasing IL-1β, TNF-α, IL-8, and monocyte chemoattractant protein 1 (MCP-1) secretion, as well as cyclooxygenase 2 expression by monocytes.[119,137–139] Beyond P-selectin-PSGL1 adhesion, monocyte cyclooxygenase 2 expression depends on IL-1β signaling, and MCP-1 secretion depends on RANTES from activated platelets.[137,139] Stimulation with LPS increases platelet-monocyte aggregation and monocyte IL-10 expression through CD40-CD40L binding.[22] TLR4 also participates in platelet-monocyte interaction in sterile inflammation. TLR4 deficiency impairs P-selectin-mediated platelet-monocyte aggregation and TNF-α expression in experimental liver disease.[140] Platelet CD36 activation by oxidized low-density lipoprotein (oxLDL) also induces platelet-monocyte aggregates through P-selectin-PSGL1 binding, increasing foam-cell formation, CD11b expression, and monocyte extravasation in vitro and in vivo.[141]

Regarding platelet-Mϕ interactions, platelets are phagocytosed by Mϕs through GPIbα-mediated adhesion and phosphatidylserine recognition, triggering proinflammatory cytokine secretion.[142,143] Under subthreshold LPS stimulation, platelets induce Mϕ polarization into pro-inflammatory (M1-like) profile depending on GPIb-CD11b

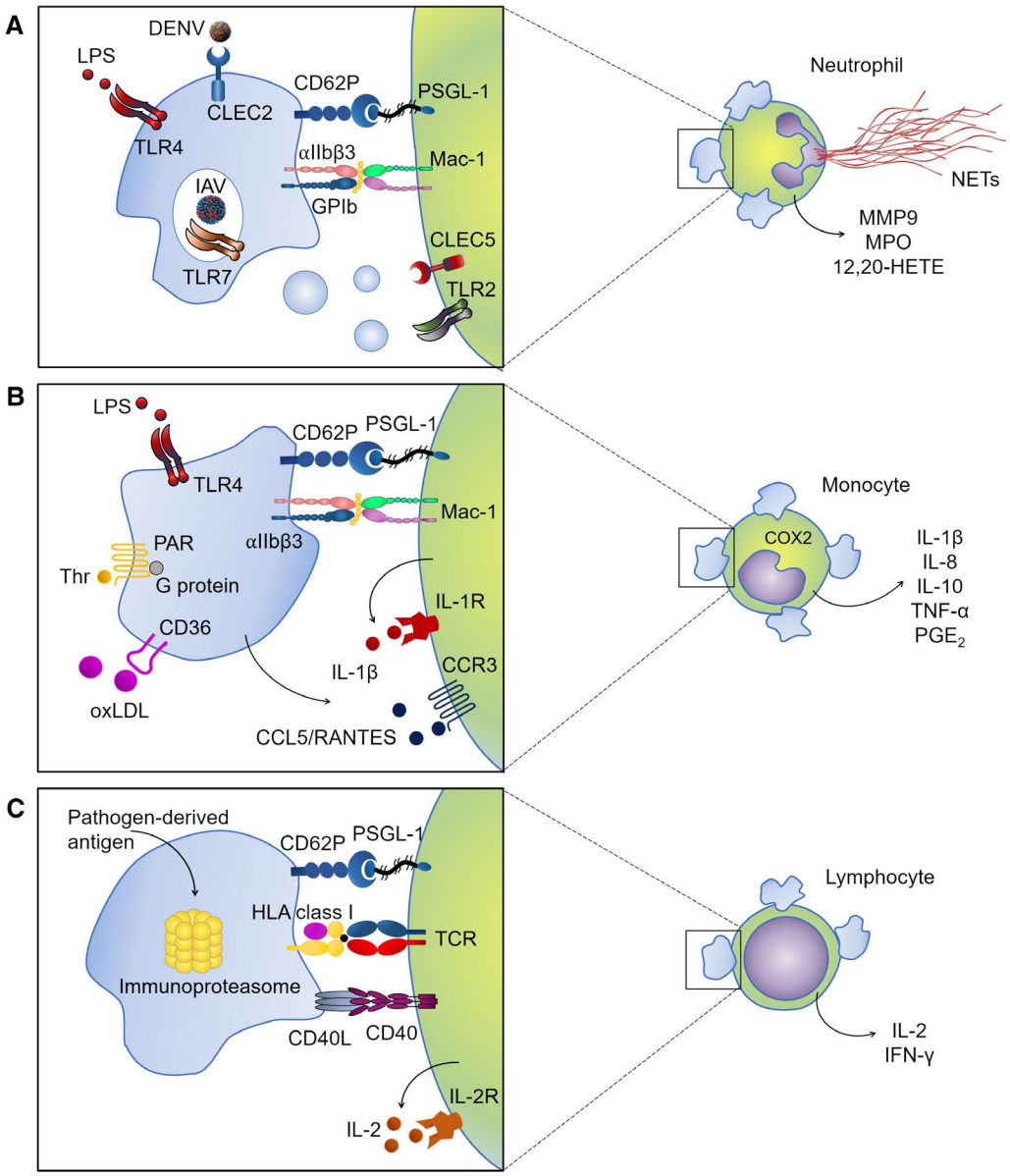

FIGURE 2 **Innate immune receptors participate in platelet-leukocyte interactions.** Schematic representation of the main pattern recognition receptors involved in recognition of PAMPs and DAMPs by platelets, and their consequence to platelet interactions with **(A)** neutrophils, **(B)** monocytes, and **(C)** lymphocytes. The molecular interactions (receptors and adhesion molecules) involved in platelet interaction with leukocytes are represented at the left; leukocytes responses modulated by these interactions, including neutrophil extracellular traps (NET) release and secretion of cytokines and eicosanoids, are represented at the right. See the text for details and references

interaction[144] (see Section 6.2). Platelet-Mϕ interaction in an arthritis model by zymosan-TLR2 also leads to M1-like polarization through GPVI-mediated activation and TXA_2 release.[145] However, long-term co-culture with platelets during monocyte differentiation into Mϕs have been shown to induce polarization toward anti-inflammatory (M2-like) profile.[146,147] New studies are still necessary to address how platelets under different stimulation and co-culture conditions distinctly interfere in Mϕs polarization.

The interaction of platelets with dendritic cells (DC) has been demonstrated. Platelet-DC aggregation leads to maturation of DC and platelet phagocytosis in vitro.[148] Platelets interaction with bone marrow-derived DC through CD40-CD40L induces IL-6 and IL-12 secretion.[149] Besides, platelet-plasmacytoid DC aggregation increases IFN-α release in systemic lupus erythematosus.[150]

Fewer studies have reported the interaction between platelets and lymphocytes (Fig. 2C). Platelets interact with B-cells through CD40L-CD40 participating in Ab isotype switch, and with T cells increasing the frequency of IFN-γ-expressing Th1 subset.[149] Importantly, platelets have the necessary machinery for Ag presentation, including immunoproteasome, β2-microglobulin, and all human leukocyte Ags (HLA) (or MHC in mice) class I subunits.[151–153] Platelets also express the co-stimulatory molecules CD86, CD40, and ICOSL, being able to present Ags to T lymphocytes inducing both IL-2 and IFN-γ secretion and CD69 and IL-2R expression.[154]

5 | PLATELET INNATE IMMUNE RECEPTORS IN STERILE INFLAMMATION

Sterile inflammation consists of an immune response in the absence of infection, in which inflammatory triggers can be broadly divided between intracellular components or extracellular matrix molecules.[155] In these cases, intracellular and extracellular DAMPs are recognized by PRRs and non-PRRs on both immune and non-immune cells.[156] Depending on the inflammatory trigger, the receptors involved in sterile inflammation may be shared with the response to pathogens.[155] The participation of platelet innate immune receptors in chronic conditions that trigger sterile inflammation including atherosclerosis,[157] obesity,[158] sickle cell disease (SCD),[159] and rheumatoid arthritis[160] are summarized in Fig. 3 and will be discussed in this section.

5.1 | Platelet innate immune receptors in atherosclerosis and cardiovascular diseases

Atherosclerosis is a low-grade chronic inflammatory disease that involves complex vascular injury and represents initial pathological events of cardiovascular diseases.[157] Genetic and environmental risk factors are associated with atherosclerosis pathogenesis involving 2 fundamental characteristics: inflammation and dyslipidemia.[157,161] In this inflammatory environment, circulating lipoproteins are oxidized and captured by Mϕs forming the foam cells, which support smooth muscle proliferation, angiogenesis, and plaque formation.[162] Finally, rupture or erosion of vulnerable atherosclerotic plaques leads to arterial thromboembolic events, which may lead to skeletal muscle ischemia or fatal outcomes as myocardial infarction and stroke.[2]??

Platelets are recruited into atherosclerotic plaques for their well-described functions in thrombosis and emerging roles in vascular inflammation.[157] Platelet adhesion to the carotid artery endothelium temporally precedes leukocytes recruitment.[163] Blocking platelet adhesion through anti-GPIbα Abs or P-selectin genetic deletion reduces leukocyte recruitment to atherosclerotic lesions and plaque development.[163,164] Platelet-leukocyte aggregation through CD40L-CD40 also facilitates leukocyte arrest on inflamed endothelium, accelerating atherosclerosis.[165,166] Platelet-derived RANTES and PF4 are chief chemokines for leukocyte recruitment into atherosclerotic plaques in ApoE$^{-/-}$ (proatherogenic) mice models.[21,164,167–169] Additionally, PF4 facilitates the esterification and uptake of oxLDL by Mϕs leading to foam cell development,[170] a key feature in atherosclerosis. Therefore, platelet-leukocyte interactions contribute to thromboinflammation and vascular remodeling in atherosclerosis.[2]

In atherosclerotic plaques, DAMPs as oxLDL,[171] CAPs,[69] podoplanin,[172] and HMGB1[173] serve as triggers of platelet activation (Fig. 3A). OxLDL can activate platelets through diverse pathways, including scavenger receptors and platelet-activating factor (PAF) receptor (PAFR).[171,174,175] OxLDL contains PAF-like oxidized phospholipids, serving as agonists for PAFR-expressing cells.[176,177] Platelet PAFR activation by oxLDL occurs in synergism with classical agonists as thrombin and ADP initiating prothrombotic responses.[171] Platelet LOX-1, a scavenger receptor, recognizes oxLDL in atherogenic environment inducing platelet activation and aggregation.[174] In addition, oxLDL engages platelet CD36 inducing platelet activation, adhesion, and aggregation through ROS-dependent activation of ERK5 redox sensor.[175] Accordingly, platelet-specific ERK5 deficiency decreases arterial thrombosis in high-fat diet (HFD)-fed proatherogenic mice,[175] highlighting the importance of this pathway in atherosclerosis.

TLRs may be involved in platelet activation during cardiovascular diseases. TLR2 and 4 are enriched in platelets from patients with acute coronary syndrome, stable angina pectoris, and atrial fibrillation.[178–180] Platelet transcripts for both, TLR2 and 4, positively correlate with circulating inflammatory markers as C reactive protein (CRP) and IL-6 in patients with high-risk factors for cardiovascular diseases.[181] Similarly, TLR9 expression is elevated on platelets from acute coronary syndrome patients,[68] and TLR1 and 4 are up-regulated in patients that had suffered an acute myocardial infarction.[182] Although TLR-mediated platelet activation was not shown, these reports suggest that platelet TLRs may play a role in cardiovascular diseases.

As aforementioned, CAPs are endogenous protein adducts from phospholipid oxidation. Increased CAPs levels in plasma and atherosclerotic plaques, as well CAPS sequestration by platelets, are observed in HFD-fed proatherogenic mice.[69] CAPs engage to platelet TLR9 inducing platelet activation in vitro and accelerating arterial thrombosis in vivo.[69] Also, phosphatidylethanolamine (PE) oxidative modification generate CAP-PE derivatives that activate platelets through TLR2/1.[56] HFD-fed proatherogenic mice have increased plasma levels of CAP-PE, which accelerates thrombosis through mechanisms depending on TLR2/1 and CD36 independently.[56] TLR2/6, on the other hand, cooperates with CD36 in platelet activation, contributing to in vivo thrombosis in hyperlipidemic mice.[57] It was reported that TLR2, TLR6, and/or CD36 deficiency similarly protect atherosclerotic mice from platelet aggregation and thrombosis.[57] Besides, oxidized phospholipids activate platelets in vitro through CD36/TLR2/TLR6 supercomplex,[57] confirming the cooperation of these receptors for platelet hyperreactivity in hyperlipidemia.[57]

Besides DAMPs from lipid oxidation abovementioned, HMGB1 is present in coronary artery thrombi[183] and platelets[180] from patients with cardiovascular diseases. HFD-fed proatherogenic mice treated with anti-HMGB1 Abs show reduced plaque infiltration and slower atherosclerotic development.[184] HMGB1 is expressed by platelets along NETs in coronary thrombi of patients with acute myocardial infarction.[185] Complementary in vitro experiments using pharmacologic and genetic models show that platelet HMGB1 induces NET via the receptor for advanced glycation end products (RAGE) on neutrophils.[185] Besides, platelet RAGE expression is up-regulated in patients with acute ST-elevation myocardial infarction.[183] These evidence suggest that platelet HMGB1 and RAGE have crucial roles in cardiovascular diseases. Even though serum HMGB1 and platelet TLR4 are increased in atrial fibrillation patients,[180] HMGB1-mediated platelet TLR4 activation in cardiovascular diseases remains elusive.[183]

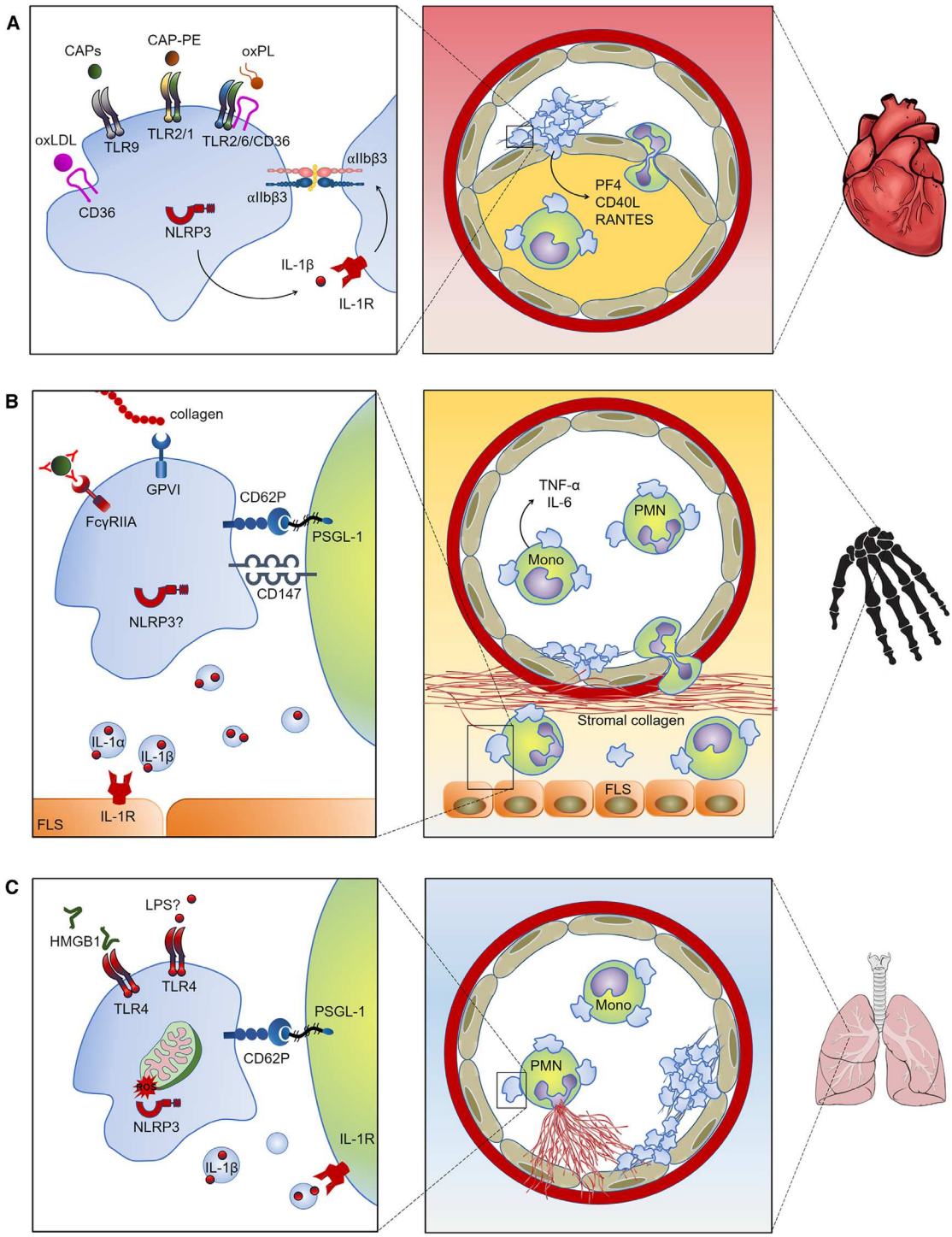

FIGURE 3 **Innate immune receptors in platelets and platelet-leukocyte aggregates participate in pathogenesis of sterile inflammatory diseases. (A)** DAMPs generated by hyperlipidemia and oxidative stress activate platelet PRRs triggering platelet activation, aggregation and thrombosis in atherosclerosis and cardiovascular diseases. Platelet aggregation and thrombosis require NLRP3-dependent IL-1β signaling to inside-out activation of integrin αIIbβ3. **(B)** FcγRIIA and GPVI are the main triggers of platelet activation in rheumatoid arthritis. Platelet activation by local exposure to collagen at inflamed joints induces the release of IL-1-containing microvesicles that amplify inflammation by activating fibroblast-like synoviocytes (FLS). **(C)** Platelet TLR4 activation by HMGB1 or exogenous LPS leads to NLRP-3 dependent IL-1β-mediated neutrophil extracellular traps (NET) release and pulmonary vaso-occlusion in sickle cell disease. See the text for details and references

TLR4 and NLRP3 signaling in platelets participate in muscle ischemia-reperfusion in mice.[86] Increased caspase-1 activation and IL-1β processing in platelets from ischemic mice depend on NLRP3 activation, and are inhibited in mice whose platelets lack TLR4, indicating TLR4-mediated inflammasome activation.[86] NLRP3 or platelet TLR4 deficiency impairs platelet hyperaggregability and improves ischemic muscle reperfusion over time,[86] suggesting involvement of these receptors in prothrombotic and pro-angiogenic responses. Accordingly, platelet NLRP3 and IL-1β participate in arterial thrombosis.[17,88] IL-1β from platelet translation accumulates in carotid artery occlusive thrombus in vivo[17] and in platelet-fibrin clump in vitro.[186] Transfusion of NLRP3$^{-/-}$ platelets in wild-type mice is sufficient to reduce thrombi size and shorten the time to occlusion after arterial injury.[88] Complementary platelet functional assays show that aggregation, adhesion, and clot retraction depend on platelet NLRP3 and IL-1R signaling.[88,90] Mechanistically, NLRP3-dependent platelet IL-1β secretion sustains integrin αIIbβ3 outside-in signaling, supporting platelet aggregation and thrombosis[88] (Fig. 3A).

These reports show activation of platelet innate immune receptors during cardiovascular diseases in the interplay of hemostasis and inflammation, promoting atherogenesis in early stages and affecting cardiovascular dysfunction at the late stages of these diseases.

5.2 | Platelet innate immune receptors in obesity

Obesity is a metabolic and inflammatory disease characterized by adipose tissue accumulation as a consequence of adipocyte hypertrophy and hyperplasia.[187] Impaired capacity to further store fat alongside a pro-inflammatory and lipolytic environment leads to free fatty acids circulation and ectopic fat deposition, inducing tissue inflammation, and metabolic syndrome.[188] Dyslipidemia and increased risk of thromboembolic events are among the features of obesity-associated metabolic syndrome.[189] Platelet activation and hyperaggregability are increased in obese compared to eutrophic individuals, which may be associated with impaired L-arginine-NO pathway.[190,191]

Large platelet transcriptome studies demonstrated an association of higher body mass index with the up-regulation of inflammatory genes, particularly those in NF-kB pathway such as IL-1R and TLRs.[192,193] Platelet mRNA sequencing also demonstrated the up-regulation of CLEC2 transcript in platelets from morbid obese individuals.[194] These reports suggest that PRRs and cytokine receptors may be involved in platelet activation in obesity-related inflammation and procoagulant state.

Hypercholesterolemia alongside systemic inflammation generates high levels of oxLDL in obesity.[195] OxLDL from obese individuals increases TXA_2 secretion and platelet hyperaggregability through p38-MAPK signaling.[195] However, the receptors mediating oxLDL-induced platelet hyperactivity in obesity remain unknown. Another feature of obesity is hyperleptinemia.[196] The participation of leptin in thrombosis is evidenced by unstable non-occlusive thrombus formed in leptin-deficient (ob/ob) mice after carotid injury, which is reversed by exogenous leptin injection.[197] Leptin induces hyperaggregability of wild-type, but not ObR deficient (*db/db*) platelets, evidencing specific receptor-mediated activity.[197] ObR-dependent leptin-induced hyperaggregability is also observed in platelets from obese and overweight individuals, although platelets may represent a site of leptin resistance in obesity.[97]

5.3 | Platelet innate immune receptors in rheumatoid arthritis

Rheumatoid arthritis (RA) is characterized by a chronic inflammation of the synovia resulting in painful joint deformity and immobility.[25,198,199] RA evolves with an elevated risk of cardiovascular diseases and thromboembolic events, the main mortality cause in the disease.[200–202] Exposure of extracellular matrix in the inflamed joint and the inflammatory milieu of RA represent relevant agonistic stimuli for platelet activation.[198,199,203] Increased platelet counts and activation markers such as P-selectin, sCD40L, and platelet-leukocyte aggregation associate with disease activity score and with cardiovascular complications in arthritic patients.[200–204]

Platelet activation by collagen in exposed extracellular matrix plays major roles in RA pathogenesis, contributing to a prothrombotic and proinflammatory state.[198,199] Platelet immune receptor tyrosine-based activation motif (ITAM) GPVI and FcγRIIa are directly involved in these processes (Fig. 3B).[199,202] Studies using pharmacologic and genetic models demonstrated that the collagen receptor GPVI is a key trigger for platelet MVs, amplifying joint inflammation through IL-1-mediated activation of synoviocytes.[199] GPVI and FcγR genetic deficiency both decrease platelet MVs production.[199] Platelet activation via FcγRIIa engagement occurs mostly by anti-citrullinated protein Abs,[202] which are highly specific for RA and predictive of the onset of symptoms.[205] Platelet activation is increased in RA patients presenting these Abs.[202,206] Exposure to plasma from these patients activate platelets ex vivo, which is prevented by neutralizing Abs against FcγRIIa,[202] supporting participation of immune complexes and FcγRIIa in platelet activation during RA.[202]

Platelet-leukocyte interactions are commonly observed in arthritic patients.[200–204] Direct platelet-monocyte interaction during RA induces a decrease of the classical and expansion of the intermediate and inflammatory monocyte subsets, with increased expression of TNF-α and IL-6.[204] In co-culture experiments, CD147 neutralization prevents platelet-monocyte aggregation and partially reverses the alterations of monocyte subpopulations,[204] suggesting a role in monocyte reprogramming in RA. In Ag-induced arthritis in mice, P-selectin-dependent platelet-leukocyte interaction facilitates leukocyte recruitment into the inflamed synovium,[207,208] worsening clinical and histological indices.[208] Therefore, platelet activation and platelet-mediated leukocyte reprogramming contribute to inflammation and joint pathology in arthritis.

5.4 | Platelet innate immune receptors in sickle cell disease

SCD is a genetic mutation in hemoglobin β subunit that results in altered RBC shape.[209,210] Sickle RBCs interact with multiple immune

cells promoting inflammation, endothelial damage, vascular obstruction, pain crisis, and may lead to death.[209,211] Platelet activation is elevated in patients under steady-state conditions and can be further enhanced during acute vaso-occlusive crisis.[212] High platelet activation is mainly related with the hemolysis of sickle RBCs that release classical platelet agonists as ADP and ATP and erythrocyte-derived DAMPs such as hemoglobin, which blocks the inhibitory effects of NO.[87,212,213] In addition, free heme is a known DAMP that activates TLR4 in nucleated cells,[214] but the role of heme in platelet TLR4 activation remains unknown.

SCD patients exhibit high levels of tissue factor, thrombin, and fibrin degradation products, characterizing an hypercoagulability state.[215–218] Thrombin activates protease-activated receptors (PAR) in different cell types, including platelets, inducing inflammatory cytokines and platelet-leukocyte aggregation in SCD.[219–221] Platelets from patients or animal models of SCD are more sensitive to PAR4 agonists,[212,213] and PAR4 hyperstimulation is enhanced in patients presenting pulmonary hypertension.[212] Activated platelets promote sickle RBC adhesion to endothelium and multicellular aggregation with leukocytes, supporting vascular occlusion, pulmonary hypertension, and lung injury.[212,222–226] Platelet-neutrophil aggregation occurs mostly in a P-selectin-dependent manner, contributing to vaso-occlusion and acute lung injury by amplifying the inflammatory process.[225–227]

This inflammatory amplification may occur by increased platelet production of cytokines as IL-1β, in which secretion by platelets correlates with allo or autoantibodies.[228] In addition to secreting free IL-1β, platelets in SCD patients also release MVs,[228,229] which have been shown to carry active IL-1β and caspase-1 in SCD patients and animal models.[87] Platelet inflammasome activation in patients has been demonstrated by NLRP3 and ASC colocalization and increased caspase-1 activity,[15,87] which is higher at the onset of pain crisis.[15] A main trigger of platelet NLRP3 in SCD is HMGB1, which is increased in plasma from SCD patients.[15,230] HMGB1 in SCD plasma activates caspase 1 in platelets from healthy volunteers depending on NLRP3 and TLR4.[15] Platelet-neutrophil aggregation in blood from SCD patients is triggered by a subthreshold LPS stimuli depending on caspase-1 and mitochondrial ROS signaling.[87] In an SCD murine model, challenging with LPS or MVs from LPS-treated SCD mice increases platelet-neutrophil aggregation in the lungs, leading to vaso-occlusion and lung injury through IL-1β-dependent NET release.[87] These translational data from SCD patients and animal models show HMGB1 and NLRP3 participation in platelet-neutrophil pathogenic interactions inducing acute chest syndrome, a major complication of SCD[87] (Fig. 3C).

Translocation of LPS from the gut into the bloodstream contribute to vaso-occlusion in SCD mice through neutrophil activation.[87,231,232] However, the role of LPS from a microbial translocation in platelet activation during SCD has not been formally shown. Indeed, oral or gut microbial translocation has been also described in other sterile inflammatory diseases such as obesity, atherosclerosis, and cardiovascular diseases.[233,234] Hence, the "sterile" condition may be questioned in the majority of the diseases discussed in this section. Although the onset of the immune process is not infectious, inflammation perpetuation generates microbial translocation and, therefore, LPS among other PAMPs amplifies the inflammatory process.[232,234] Nonetheless, platelet activation by PAMPs in sterile inflammatory diseases has not been investigated.

6 | PLATELET INNATE IMMUNE RECEPTORS IN INFECTIOUS DISEASES

Infectious diseases are an important cause of mortality worldwide. Infectious diseases increase the risk of thrombosis, and thrombocytopenia is a common feature during infections.[235–238] It is noticeable that platelets play a key role during infections, participating as procoagulant or inflammatory agents in protective or damaging responses.[26,239,240] Among many ways platelets may be involved in pathogenesis of infectious diseases, recognition of PAMPs by innate immune receptors has critical roles in platelet-mediated responses.[31,64,118] Platelet activities triggered by innate immune receptors participating in protective or pathogenic responses to bacterial, viral, and parasite infections will be discussed in this chapter.

6.1 | Platelet innate immune receptors in viral infections

Viruses rely on viral particles engulfment and viral genome replication by target cells to their dissemination in the host. Many PRRs are exploited by viruses to attachment and entry in host cells, especially those from the CLR family.[70] Platelets possess the cellular machinery for viral attachment, entry, and replication.[78,82] They also sense and respond to viral components through PRRs, especially surface TLRs and CLRs for viral binding, and endosomal TLRs for viral genome recognition.[14,58,64,70,76] Human cytomegalovirus, for example, engages platelet TLR2 leading to platelet-neutrophil interaction and pro-inflammatory cytokine release.[58] Platelet neutrophil-aggregates are also observed after platelet recognition of encephalomyocarditis virus through TLR7, inducing thrombocytopenia but also protective immune response in mice.[14] Broader discussions on viruses attachment to a diversity of platelet receptors have been reviewed elsewhere.[82,241–244] In this section, we will focus on the participation of platelet innate immune receptors in 3 viral infections of global impact in human health: dengue, HIV, and influenza (Fig. 4).

Endocytosed viral particles and the presence of viral genome have been evidenced in circulating platelets from dengue, HIV, and influenza-infected patients, as well in platelets infected with these viruses in vitro.[64,77,245–249] Image tracking analysis of platelets infected with an HIV-1 reporter virus revealed virus trafficking from early to late endosomes,[250] which led to platelet activation through TLRs.[250] Endocytosed influenza A virus (IAV) also induces platelet activation through TLR7, increasing platelet-neutrophil aggregation and NET release in vitro and in vivo.[64] These data highlight platelets' ability to endocytose viral particles and employ endosomal TLRs, a classical viral recognition pathway, to respond to viruses of different etiologies, including airborne and blood-borne viruses (Fig. 4).

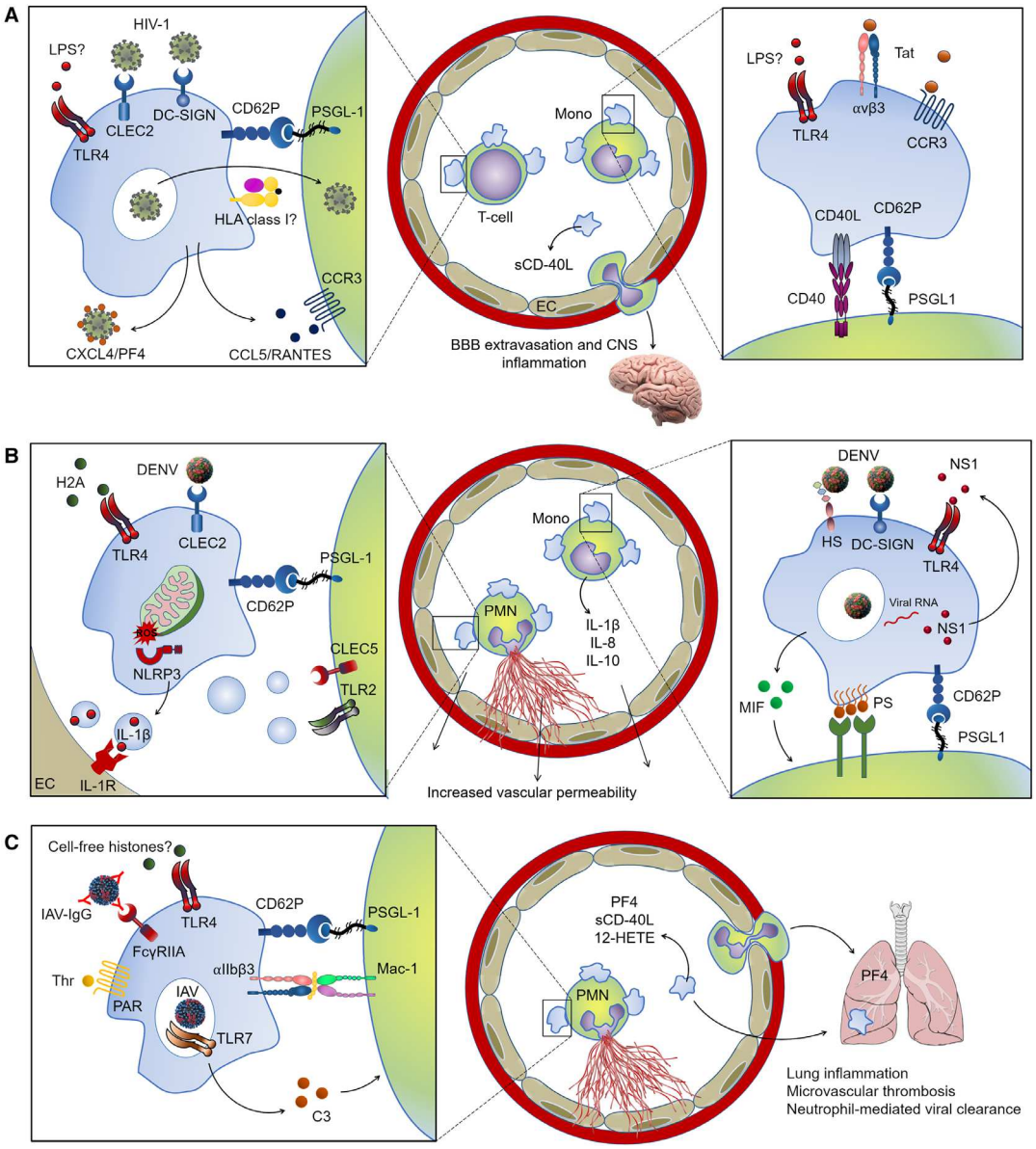

FIGURE 4 **Innate immune receptors in platelets and platelet-leukocyte interactions during viral infections.** (A, left panel) HIV-1 internalization in platelets through DC-SIGN and CLEC2 supports HIV-1 transinfection to susceptible T-cells in vitro. Activated platelets can also inhibit HIV-1 infection through the secretion of the HIV suppressive chemokines PF4/CXCL4 and RANTES/CCL5. (A, right panel) Platelet activation by HIV-1 transactivator of transcription (Tat) and platelet expression of CD40L play a major role in HIV-associated neuroinflammation by increasing blood-brain barrier (BBB) permeability and monocyte adhesion and migration to the central nervous system (CNS). (B, left panel) Platelet PRRs including TLR4, CLEC2, and NLRP3 participate in dengue-associated vascular dysfunction through mechanisms involving secretion of IL-1β-containing microvesicles and induction of neutrophil extracellular traps (NET). (B, right panel) Platelets internalize DENV through DC-SIGN and heparan sulphate proteoglycans (HS) and support DENV genome translation and replication, which amplify platelet activation through NS1-TLR4 engagement. DENV-infected platelets expose P-selectin (CD62P) and phosphatidylserine (PS) modulating monocyte cytokine secretion in platelet-monocyte interactions. (C) Platelet TR7 recognizes endocytosed IAV genome and platelet FcγRIIA recognizes IAV-IgG immunocomplexes leading to platelet activation, platelet-neutrophil aggregates and NET release. Platelet-factor 4 (PF4/CXCL4) also participates in neutrophil migration to the lungs. See the text for details and references

Even though the emergence of antiretroviral therapy (ART) has changed HIV-1 epidemiology from life-threatening AIDS into a chronic manageable disease,[251] people living with HIV still experience earlier mortality by an increased incidence of non-infectious comorbidities, especially cardiovascular diseases.[252–254] HIV-1 enters target T-cells through the interaction of viral glycoprotein 120 (GP120) with the cellular receptor CD4 and the co-receptors CCR5 or CXCR4.[255] In addition, HIV-1 enter non-target cells as DCs and platelets through DC-SIGN or other CLRs.[76,77,256] Through this pathway, HIV-1 is internalized without membrane fusion and stored in endosomes as infective viral particles that can be transferred to T-cells during Ag presentation.[256,257] This intercellular transmission is called HIV-1

transinfection.[76,257,258] Platelets interact with and internalize HIV-1 through DC-SIGN and CLEC2.[76,77,246] Platelet ability to preserve HIV-1 infectivity and transfer it to T-cells has been evidenced in vitro[76] and, recently, in platelets from HIV-infected subjects, especially those with poor immunological recovery after ART.[247] However, the participation of HLA class I Ag presentation in platelet HIV-1 transinfection remains unknown (Fig. 4A, left panel).

Ultrastructural studies show activation-associated morphology in platelets that endocytosed GP120-expressing transgenic lentiviruses, and evidence fusion of virus-containing endosomal vesicles with α-granules allowing viral degradation.[77] PF4 was identified as the main viral-restrictive factor in platelet granules.[259] PF4 dose-dependently inhibits HIV-1 infectivity to T-cells and Mϕs through direct interacting with GP120.[260] RANTES, another chemokine abundantly expressed in platelet granules, inhibits HIV-1 entry by binding the R5 co-receptors.[261] In platelet-T-cell co-cultures, platelets were able to inhibit HIV-1 spread in a concentration- and activation-dependent manner.[259] Exhausted platelets lost their inhibitory capacity while activated platelet supernatants conserved the inhibitory activity depending on PF4.[259] However, a recent study showed PF4 restrictive effects in a narrow concentration range, while physiologically relevant concentrations actually intensified virus attachment by bridging GP120 to cell surface glycosaminoglycans.[262] These evidence indicate that platelets may have a dual role in HIV spreading, being able to inactivate or shelter endocytosed viruses depending on platelet α-granules content (Fig. 4A). Of note, increased platelet activation with the exhaustion of α-granule chemokines is associated with high viral load and immune suppression in AIDS patients,[263] and persists in HIV-infected subjects under virologic control.[264,265]

Increased levels of platelet-derived sCD40L participate in HIV-associated neuroinflammation by inducing platelet-monocyte aggregation and infiltration through the blood-brain barrier (Fig. 4A).[266–268] HIV-1 transactivator of transcription (Tat), a viral protein found in the circulation of infected individuals, activates platelets through β3 integrin and CCR3 chemokine receptor.[269] In Tat injected mice, platelet-derived CD40L mediates leukocyte adhesion, rolling, and transmigration through the blood-brain barrier.[267] Consistently, postmortem brain tissue sections from patients who died of HIV-associated encephalopathy show increased platelet-monocytes aggregates attached to the brain microvasculature, suggesting an association with neuroinflammation.[268] Tat synergizes with CD40L inducing the secretion of TNF-α by the microglia, thereby amplifying inflammatory processes within the central nervous system and influencing neuronal survival.[266]

The most characteristic feature of platelets in viral infections is thrombocytopenia. Lower platelet counts in AIDS patients are usually reversed by ART,[235] but persists in average 20% of HIV-infected subjects.[270] Thrombocytopenia is also a hallmark of dengue, being associated with increased vascular instability and the severity of dengue syndrome.[82,236,271] Combined factors may contribute to thrombocytopenia in both infections.[235,272,273] Increased platelet activation and apoptosis have been reported in both dengue and HIV[13,245,264] and associate with the degree of thrombocytopenia in dengue.[13,245] Decreased platelet counts also correlate with increased platelet-monocyte aggregation in both diseases.[138,274,275] On this regard, apoptotic platelets from patients with dengue or from in vitro infection are phagocytosed when co-cultured with monocytes depending on phosphatidylserine recognition.[245,276]

Severe dengue is characterized by increased vascular leakage, hemorrhage, and organ failure due to systemic inflammation.[271,277–280] Increased platelet activation contributes to thromboinflammatory responses in dengue by secreting inflammatory mediators and by reprogramming leukocyte functions.[45,83,138,281] These platelet responses involve platelet recognition of PAMPs and DAMPs through specialized receptors (Fig. 4B). Platelets from dengue patients or platelets infected with DENV in vitro secrete stored chemokines as PF4 and RANTES, small molecules as NO and serotonin, and newly synthesized cytokines as IL-1β.[45,50,83,138,281–283] Such mediators are potentially involved in dengue-associated vasculopathy. Activation of the NLRP3 inflammasome and increased caspase-1 activity supports the shedding of IL-1β-containing MVs in dengue.[83] Platelet caspase-1 activity and IL-1β expression in platelets and platelet-derived MVs correlate with increased vascular permeability in dengue patients, and MVs from infected platelets increase the permeability of endothelial cell cultures through IL-1R.[83] Therefore, platelet NLRP3 and IL-1β may contribute to vascular leakage during dengue infection.

Platelets internalize DENV after viral attachment to DC-SIGN and heparan sulfate proteoglicans.[78] DENV-infected platelets sustain viral genome replication and viral protein translation, as evidenced by accumulation of viral RNA and DENV NS1.[50,78,284] However, platelets do not sustain the secretion of infective viral particles to the extracellular milieu,[50,78,284] indicating an abortive DENV replication in platelets. DENV attachment to DC-SIGN also increases platelet activation and apoptosis, which has been observed in vitro and in platelets from patients.[13,245] This activation signaling may involve viral genome replication and recognition, since the levels of DENV RNA in platelets from patients correlate with platelet activation.[245] Interestingly, DC-SIGN expression is decreased on platelets from severe dengue patients,[285] which may be a protective platelet response against infection or a consequence of activated and apoptotic platelet clearance. Aside from DC-SIGN, DENV activates platelet CLEC2, resulting in α- and dense-granules secretion, MVs release and platelet-neutrophil aggregation.[75]

TLR4 participates in platelet activation in dengue. Recent studies demonstrated that NS1, a viral PAMP secreted by DENV-infected cells, activates platelet TLR4 inducing platelet activation, aggregation with monocytes and endothelial cells, and secretion of inflammatory cytokines.[31,50] In DENV-infected platelets, an NS1-TLR4 autocrine loop contributes to platelet activation.[50] DENV-induced thrombocytopenia and hemorrhage in mice are diminished under TLR4 deficiency or in mice infected with NS1-depleted inoculum,[31] indicating the participation of NS1 in dengue pathogenesis. In a work from our group, higher levels of histones H2A were found in plasma from severe dengue.[45] Histone H2A in plasma from patients activates platelets from healthy volunteers ex vivo depending on TLR4.[45] These data show TLR4 participation in platelet activation during dengue infection (Fig. 4B).

Dengue induces the expression of P-selectin, CD40L, and HLA class I on the platelet surface,[13,45,286] all involved in platelet-leukocyte interactions (Fig. 2). Platelet-leukocyte aggregates are observed among lymphocytes, monocytes, and neutrophils in dengue.[138,287] Platelet-monocyte aggregation correlates with increased vascular permeability in dengue patients.[138,287] Platelets activated by dengue infection reprograms monocyte responses ex vivo inducing the secretion of IL-1β, IL-10, and IL-8.[138] Complementary in vitro experiments show that cytokine secretion by platelet-monocyte interaction is regulated by P-selectin-mediated adhesion and phosphatidylserine recognition on activated and apoptotic platelets.[138] In addition, combined signaling of platelet adhesion plus MIF from DENV-infected platelets induces lipid droplets biogenesis and PGE_2 synthesis in monocytes.[131] During platelet-neutrophil interactions, platelet MVs from CLEC2 activation act upon neutrophil CLEC5 and TLR2 inducing NET release.[75] Platelet-induced CLEC5 and TLR2-dependent NETosis increase endothelial permeability in co-culture with endothelial cells and in experimental DENV infection in mice.[75] Hence, platelet and leukocyte innate immune receptors participate in pathogenic cellular interactions during dengue, inducing hyperinflammation and vasculopathy (Fig. 4B).

Platelet-induced intravascular NETosis is also observed in influenza infection (Fig. 4C). TRL7-dependent platelet activation increases C3 release, which mediates NET and myeloperoxidase extrusion by neutrophils in vitro and in vivo. C3 levels and NETosis are both increased in patients with influenza, and platelets are the main source of C3 in IAV-infected mice.[64] In another study, extracellular histones (which may or may not come from NETosis) are highly present in nasal wash samples from influenza A patients and in bronchoalveolar wash from infected mice.[288] Experimental influenza revealed extracellular histones in association with platelets, CD40L, and fibrin mesh in the pulmonary vasculature, indicating their role in pulmonary microvascular thrombosis.[288] Potential receptors mediating this interaction include TLR2 and 4,[46,47] but their participation in platelet activation during influenza pneumonia remains to be investigated.

Pulmonary microvascular thrombosis alongside exacerbated inflammation of the airways are chief pathological features in influenza pneumonia.[289–291] Platelet activation in the lungs participates in both prothrombotic and inflammatory features of lung injury in influenza.[26,292] Platelets also participate in innate mechanisms of viral clearance and survival during experimental influenza through PF4-dependent neutrophils recruitment to the lungs (Fig. 4C).[239] Beyond the recognition of PAMPs and DAMPs mentioned above, immune complexes formed by IAV with specific (H1N1) or cross-reactive (H3N2) Abs also activate platelets promoting degranulation, 12-HETE synthesis, and MVs release through FcγRIIA signaling.[292] Highlighting the thromboinflammatory overlap in IAV infections, a synergism between FcγRIIA and thrombin amplifies platelet responses in influenza.[292]

Secondary bacterial infection is a major complication and cause of mortality in severe influenza pneumonia.[293] In addition, microbial translocation is observed in HIV-1 and severe dengue, with increased levels of LPS in circulation setting systemic immune activation and thromboinflammation.[294–296] Nevertheless, whether LPS or other bacterial PAMPs contribute to platelet activation in these viral infections remains unknown. Platelet innate immune receptors involved in thrombotic and inflammatory responses to bacteria are discussed below.

6.2 | Platelet innate immune receptors in bacterial infections and sepsis

Sepsis and septic shock represent a dysregulated systemic inflammatory response to infection driving life-threatening organ dysfunction.[297,298] Septic shock is a severe manifestation of sepsis with the circulatory and metabolic collapse that substantially increase the mortality risk.[298] Cardiovascular system, lungs, and kidneys are the most affected organs due to macro and microcirculatory failure.[299,300] Hypercoagulability and thrombocytopenia are associated with a poor prognosis in sepsis.[301–303] Early during infection, platelet activation contributes to host defense and bacterial clearance.[144,304,305] However, uncontrolled platelet activation can contribute to sepsis pathogenesis through hyperinflammation and disseminated intravascular coagulation (DIC).[237,303,306] Platelet activities mediated by innate immune receptors in sepsis are summarized in Fig. 5A and will be discussed below.

Platelet TLRs have major relevance during bacterial infection and sepsis.[118,307–309] Specific deletion of MyD88 in platelets demonstrates TLR-induced platelet activation during *Klebsiella pneumonia* infection, inducing high levels of TNF-α, CXCL1, and MCP-1.[307] Since these cytokines are absent in platelets, this report highlights the importance of platelet TLRs to leukocyte inflammatory response during infection.[307] Platelet TLR4 is the main responsible for microvascular thrombosis in endotoxemia. Transfusing wild-type platelets into TLR4-deficient mice is sufficient to restore thromboembolic vascular occlusion after LPS injection.[310] Platelet stimulation with LPS or plasma from septic patients induces platelet-neutrophil aggregation and NET release depending on TRL4 signaling.[118] LPS-induced platelet-neutrophil aggregates increase *E. coli* trapping by neutrophils in vivo, but also results in endothelial and liver damage in infected mice.[118,311] Similarly, platelet-Mϕ interaction reprogram Mϕs toward a pro-inflammatory profile with higher bacterial phagocytosis capacity.[144] This Mϕ phenotype is observed in mice receiving early platelet transfusion during polymicrobial sepsis, impacting bacterial clearance and survival[144] (Fig. 5A, right panel).

TLR2 also participates in platelet activation in sepsis. Platelet stimulation with Group B *Streptococci* isolated from septic patients enhances TLR2 expression and induces TLR2-dependent hyperaggregability.[59] DAMPs generated from tissue damage also induce platelet TLR activation in sepsis. Platelets were identified as the main source of circulating HMGB1 in sepsis.[309] Platelet-specific HMGB1 deficiency impairs platelet activation, platelet-neutrophil aggregation, and neutrophil chemotaxis, worsening bacterial clearance and survival in polymicrobial sepsis.[312] Other DAMPs potentially involved in platelet TLR activation during sepsis are the extracellular histones. The degree of thrombocytopenia and DIC correlates with plasma H3 histones in sep-

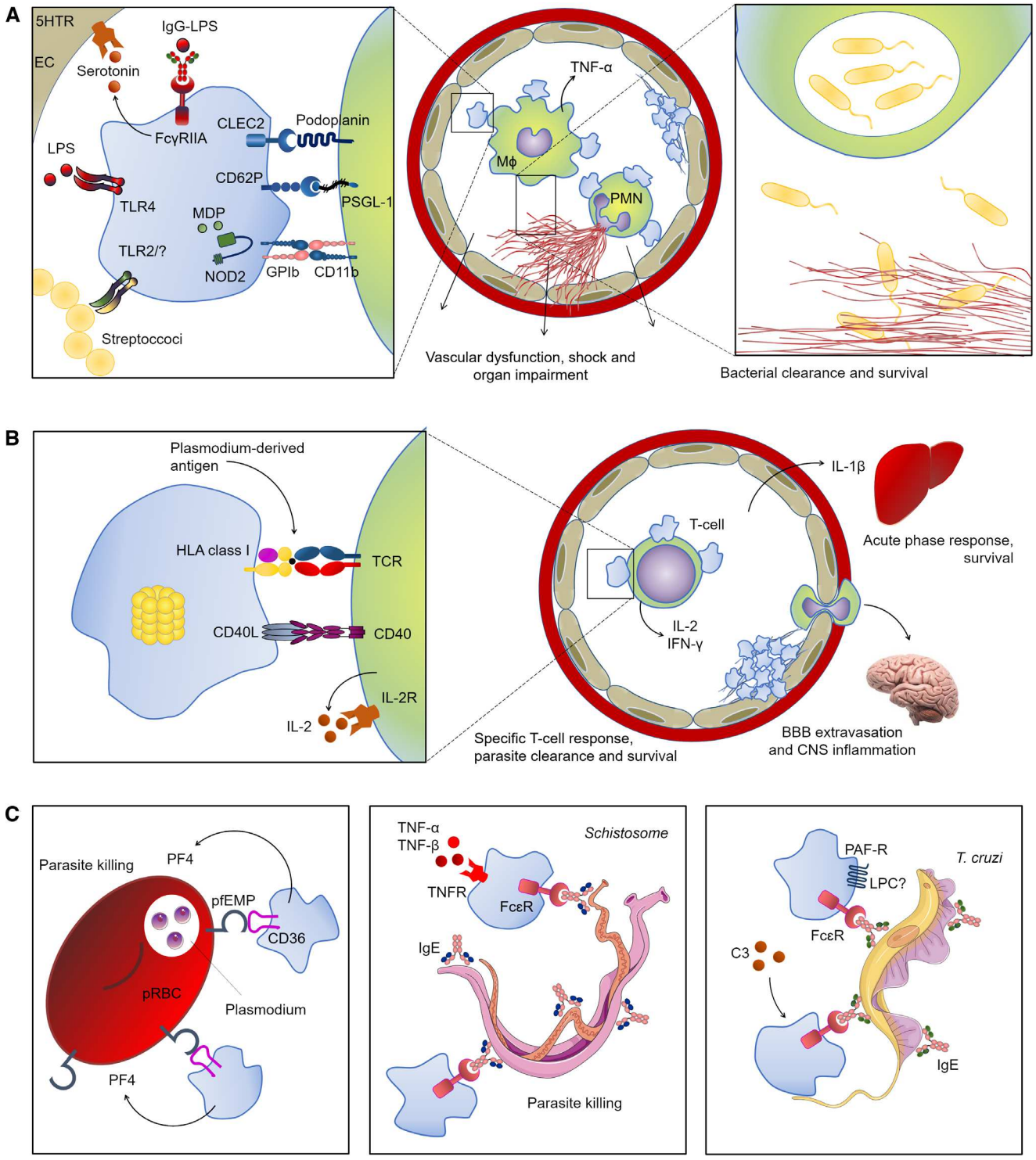

FIGURE 5 **Innate immune receptors in platelets and platelet-leukocyte interactions during bacterial and parasite infections.** (A, left panel) Platelet innate immune receptors are activated by immunocomplexes and bacterial-derived PAMPs, such as LPS and MDP, mediating immunopathogenic responses in sepsis. Platelet-derived serotonin, platelet-dependent NET extrusion, and platelet CLEC2 activation by podoplanin-expressing Mϕs play major roles in tissue damage and shock in sepsis. (A, right panel) Platelet-dependent bacterial clearance by neutrophils and Mϕs. Platelet-Mϕ interaction induces polarization toward a pro-inflammatory (M1-like) phenotype with increased bacterial phagocytosis and clearance. Platelet-dependent NET extrusion also induces bacterial trapping in sepsis. (B, left panel) Platelets present *Plasmodium*-derived Ags to $CD4^+$ and $CD8^+$ T-cells inducing Ag-specific immune response in malaria. (B, right panel) Platelet-mediated clumping and cytoadherence are pathological, while platelet-derived IL-1β has protective roles in cerebral malaria. (C) Mechanisms of platelet-mediated parasite killing involve the receptor for Fc portion of IgE (FcεR), TNF-α and β receptor (TNFR) and CD36 scavenger receptor. Platelet adhesion to *Plasmodium* parasitized red blood cells (pRBC) depends on CD36 binding to *Plasmodium falciparum* erythrocyte membrane protein (pfEMP), and induce PF4/CXCL4-mediated parasite killing. See the text for details and references

tic patients.[313] Nevertheless, new studies are still necessary to establish platelet activation by cell-free histones in sepsis.

Platelets ITAM receptors GPVI, CLEC2, and FcγRIIA also participate in platelet responses in sepsis.[314–316] In *K. pneumoniae* infection, platelet GPVI is indispensable for local host defense and bacterial clearance from the lungs.[314] To reaffirm GPVI importance, the addition of GPVI agonist in *K. pneumoniae*-infected blood increased platelet-leukocyte aggregation and bacteria phagocytosis by monocytes and neutrophils.[314] Regarding CLEC2, platelet activation by podoplanin-expressing liver Mϕs causes thrombocytopenia and liver vascular thrombosis in systemic *Salmonella typhimurium* infection.[317] In another study, however, platelet CLEC2a has a protective response in sepsis.[315] Platelet CLEC2 deletion increased organ damage and pro-inflammatory cytokines production in mice subjected to endotoxemia or polymicrobial sepsis.[315] Both protective and pathological platelet responses depended on podoplanin expression by leukocytes, suggesting a role for CLEC2 in platelet-leukocyte interactions.[315,317] Finally, FcγRIIA has been implicated in the onset of shock in a model of endotoxemia. In transgenic mice expressing FcγRIIA, LPS-immunization led to anti-LPS immunocomplexes during endotoxemia, which activates FcγRIIA inducing transient platelet aggregation and thrombocytopenia. Aggregated platelets secrete high levels of serotonin, which was the major driver of shock in this model[316] (Fig. 5A, left panel).

Among NLRs, platelets NOD2 and NLRP3 are both involved in pathogenic mechanisms of sepsis.[81,318] Plasma from septic patients induces platelet hyperaggregability depending on NOD2 expression, indicating platelet NOD2 activation by MDP or other agonists produced in sepsis.[81] Moreover, platelets from CLP rats have shown increased colocalization of NLRP3 and ASC, as well as caspase-1 activation and IL-1β secretion, suggesting NLRP3 activation during experimental sepsis in mice. However, new studies are still necessary to investigate the role of NLRs in systemic inflammation and organ dysfunction in sepsis.[318]

6.3 | Platelet innate immune receptors in parasite infections

Parasites are still important causes of human diseases worldwide.[319] Increased platelet activation is observed in protozoa and helminthic infections[320–323] and platelets participate in immune response against parasites.[324–327] In this section, we will focus on platelet receptors and molecular mechanisms involved in malaria, Chagas disease, and schistosomiasis (Fig. 5B and C). A common feature of these diseases is thrombocytopenia,[238,328–331] which associates with the severity of each disease.[321,329–332] Thrombocytopenia may be related with increased platelet activation in these infections.[333,334] Higher levels of sP-selectin associated with the onset of cardiac disease in chronic chagasic patients.[322] There is evidence of hemostatic dysfunctions in malaria, Chagas disease, and schistosomiasis leading to hypercoagulability,[322,329,333,335] even though hypocoagulability and hyperfibrinolysis may be also present.[329,333]

Evidence from integrated metabolomics and transcriptomics revealed platelet activation as one of the most changed processes in *Plasmodium vivax* malaria.[336] Platelet accumulation in brain microvasculature[337,338] contributes to the cytoadherence and clustering of parasitized RBC (pRBC). This cell clustering involves platelet CD36 and C1q receptors and supports cerebrovascular obstruction and injury.[339–341] Besides, platelet degranulation with increased levels of PF4 in both brain and plasma is involved in blood-brain barrier permeability and T cell infiltration to CNS, contributing to higher mortality in experimental cerebral malaria[337,342] (Fig. 5B).

Despite the pathological consequences of platelet activation at the late stages of infection, early platelet activation has a protective role in experimental cerebral malaria, which depends on platelet-derived IL-1β.[343] Platelet depletion completely blunts IL-1β levels in infected mice, highlighting platelets and platelet-leukocyte interactions as main sources of IL-1β.[343] Platelet-dependent IL-1β secretion triggers the acute phase response in the liver, limiting parasite burden and improving survival.[343] Platelet depletion also has major impacts in T-cell activation, as platelets present plasmodium-derived Ag on HLA class I.[154] Accordingly, vaccination with Ag-pulsed platelets enhance specific T-cell activation and memory, reducing parasitemia and mortality in a subsequent malaria chalange[154] (Fig. 5B). Thus, platelets bridge innate and acquired immunity in malaria. Finally, platelets induce intraerythrocytic parasite killing of all major *Plasmodium* species.[324,344] Platelet CD36 binds to pRBC through *P. falciparum* erythrocyte membrane protein.[324,337,345] In this interaction, PF4 through the erythrocyte Duffy-Ag receptor for chemokines induces the disruption of the parasite digestive vacuoles and plasmodial killing without RBC lysis[324,344,346,347] (Fig. 5C, left panel).

Platelets also have antiparasitic effects against *T. cruzi* and schistosomes evidenced by increased parasitemia in platelet-depleted mice.[348,349] Specific IgE Abs and platelet FcεR participate in platelet-mediated cytotoxicity against these parasites[325,326] (Fig. 5C, middle and right panel). Platelets exposed to serum from chronic chagasic patients had increased cytotoxic activity against *T. cruzi* trypomastigotes in vitro.[350] Besides, anti-*T. cruzi* immune serum or purified IgG from *T. cruzi*-infected mice induce platelet clumping with consequent parasite lysis, which was impaired in the absence of C3.[350] Therefore, Ab-mediated platelet antiparasitic effects may involve complement activation. Other serum components implicated in platelet-mediated parasite killing are CRP,[327] TNF-α, and TNF-β.[351] There is a significant increase in serum CRP in rats infected with *S. mansoni*.[327] Platelets activated with CRP-containing serum have greater cytotoxic activity than with CRP-depleted serum, and transfusing CRP-stimulated platelets protect rats from subsequent schistosome infection.[327] Platelets also exhibit enhanced cytotoxicity to schistosomula following exposure to recombinant TNF-α or -β, and passive transfer of TNF-stimulated platelets reduces the worm burden up to 65%.[351]

Despite their participation in defense, platelet adhesion to schistosome eggs contributes to their attachment to the endothelium, facilitating their extravasation and liver pathology.[352] Moreover, adhesive properties of schistosome outer surfaces differ dramatically in the different life-cycle stages. Mature adult worms are unable to support platelet adhesion and have evasion mechanisms that inhibit platelet activation.[352] The schistosome tegumental ATP diphospho-

hydrolase, for example, inhibit platelet-mediated killing by degrading host inflammatory and prothrombotic signals as ATP and ADP.[353] Adversely, a scape mechanism of *T. cruzi* involves the synthesis of a parasite lysophosphatidylcholine ($_{TC}$LPC) with PAF-like activity.[354,355] This lipid stimulates epimastigotes differentiation into metacyclic trypomastigotes and induces platelet aggregation through PAFR.[354,355]

These evidence demonstrate platelets' participation in beneficial and harmful responses in parasite infections. The contribution of $_{TC}$LPC in platelet-mediated inflammation during Chagas disease, as well as the participation of RBC-derived DAMPs and classical malarial PAMPs as hemozoin,[356] glycosylphosphatidylinositol anchors,[357] and plasmodial DNA[358] in platelet activation during malaria remain to be determined. New studies are still necessary to understand the signaling pathways and effector responses induced by platelet innate immune receptors in parasite infections.

7 | PLATELET INNATE IMMUNE RECEPTORS IN CANCER

Cancers are complex microenvironments composed of different cell types, including vascular cells, leukocytes, and matrix-producing fibroblasts.[359,360] Platelets have major roles in tumor microenvironment development and tumor progression.[361,362] Cancer patients present a hypercoagulability state with elevated risk for thromboembolic events.[363,364] While increased platelet counts are associated with poor prognosis, thrombocytopenia has been related to protection against metastasis and death.[365–367] These evidence suggest that platelets play crucial roles in cancer pathogenesis. In this section, we focus on platelet immune receptors in direct interactions with tumor cells and their contributions to tumor microenvironment development (Fig. 6A).

Tumor cells interact with and influence platelet physiology, while activated platelets reciprocally participate in tumor growth and invasion.[368–370] Diverse human carcinoma cell lines promote platelet aggregation in co-culture.[371–373] Beyond platelet aggregation, platelet-tumor cell interaction induces thrombin and TXA_2 generation[374–376] and secretion of granule-stored mediators, including growth factors of major importance for tumor development.[377,378] Accordingly, platelet depletion reduces vessel density and tumor perfusion, increasing primary tumor hemorrhage and preventing metastasis in a melanoma model.[379,380] Tumoral hemorrhage in thrombocytopenic mice is reversed by transfusion of resting, but not degranulated platelets, indicating that platelet stored factors contribute to tumor vascular stability.[380] Thus, platelet activation in tumor stroma may support tumor development by maintaining vascular stability and inducing angiogenesis (Fig. 6A).

Platelets are a rich source of angiogenic factors as platelet-derived growth factor (PDGF), TGF-β, and vascular endothelial growth factor (VEGF).[378,381,382] In healthy individuals and cancer patients, platelets are the major source of VEGF, contributing to >80% of the circulating levels.[383,384] Platelet-derived VEGF stimulates new blood vessel formation and supports oxygen and nutrients diffusion for tumor growth.[378] Platelets are also the largest storage of TGF-β, a critical cytokine for tumor development.[385,386] Platelet-specific TGF-β knockout damps the invasiveness of mesenchymal-like tumor cells and lung metastasis in mice.[386] This epithelial-mesenchymal transition is characteristic of early tumor migration and is supported by platelet-derived TGF-β in many metastatic murine models.[386–388] Platelets also have crucial roles in leukocyte recruitment to metastatic sites.[389,390] Platelet-tumor cell interaction triggers platelet chemokines secretion, including SDF-1α, a critical factor in leukocyte recruitment into tumor microenvironment.[390,391] Platelet-derived SDF-1α is also critical for migration of $CXCR4^+$ tumor cells and directly stimulates their growth.[391] However, the mechanisms of platelet activation in tumor stroma remain poorly characterized, even though innate immune receptors are involved (Fig. 6A).

TLR4 directly mediates platelet interaction with HMGB1-expressing tumor cells.[392] TLR4-deficient platelets have reduced adhesion to carcinoma cells compared to wild-type. Interaction with TLR4-deficient platelets induces lower TGF-β secretion and reduced invasiveness of carcinoma cell lines.[392] These TLR4-dependent platelet responses were dumped by HMGB1 neutralization in vitro and in vivo.[392] Studies regarding the participation of other platelet TLRs in cancer are still needed. Another PRR involved in tumor development and cancer-associated mortality is NLRP3. In a murine model of pancreatic cancer, platelet NLRP3 signaling is up-regulated promoting platelet activation and aggregation.[89] Transfusion of NLRP3-deficient platelets after platelet depletion improved the survival of wild-type mice to pancreatic cancer compared to animals receiving wild-type platelets.[89] Nevertheless, the triggers of NLRP3-induced platelet responses in this model remain unknown.

Platelet activation through the ITAM receptors CLEC2 and GPVI also participate in cancer pathogenesis.[393,394] Podoplanin-positive tumor cells induce platelet aggregation and secretion of PDGF and TGF-β through CLEC2 activation,[387,395] supporting tumor cell proliferation and epithelial-mesenchymal transition. In a melanoma murine model, platelet-fibrin(ogen) microthrombi and platelet adhesion to tumor microvasculature are abrogated by anti-CLEC2 Abs, demonstrating CLEC2-mediated intratumoral thrombosis.[395] These podoplanin-expressing tumor cells have increased capacity of lung metastasis, which is inhibited in CLEC2-deficient mice.[387,394,395] Thus, podoplanin-CLEC2-mediated aggregation facilitates tumor embolization, tumor cells extravasation, and metastasis. Similarly, GPVI deficiency reduces tumor metastasis in the lungs during melanoma, colon, breast, and Lewis lung carcinoma models.[396,397] Anti-GPVI Abs inducing either platelet depletion or GPVI functional inhibition induce tumor hemorrhage and decrease primary tumor growth.[393] Accordingly, traditional chemotherapy combined with GPVI inhibition increase intratumoral deposition of the chemotherapeutic agents and reduced prostate and mammary tumors in mice.[393] Thus, platelets emerge as a potential strategy of antitumor co-therapy.

Aside from their roles in chemotaxis and angiogenesis, platelets shield circulating tumor cells from blood shear stress and immunosurveillance, supporting tumor metastasis[398–400] (Fig. 6B). Adhered platelets form a physical shield around circulating tumor cells that

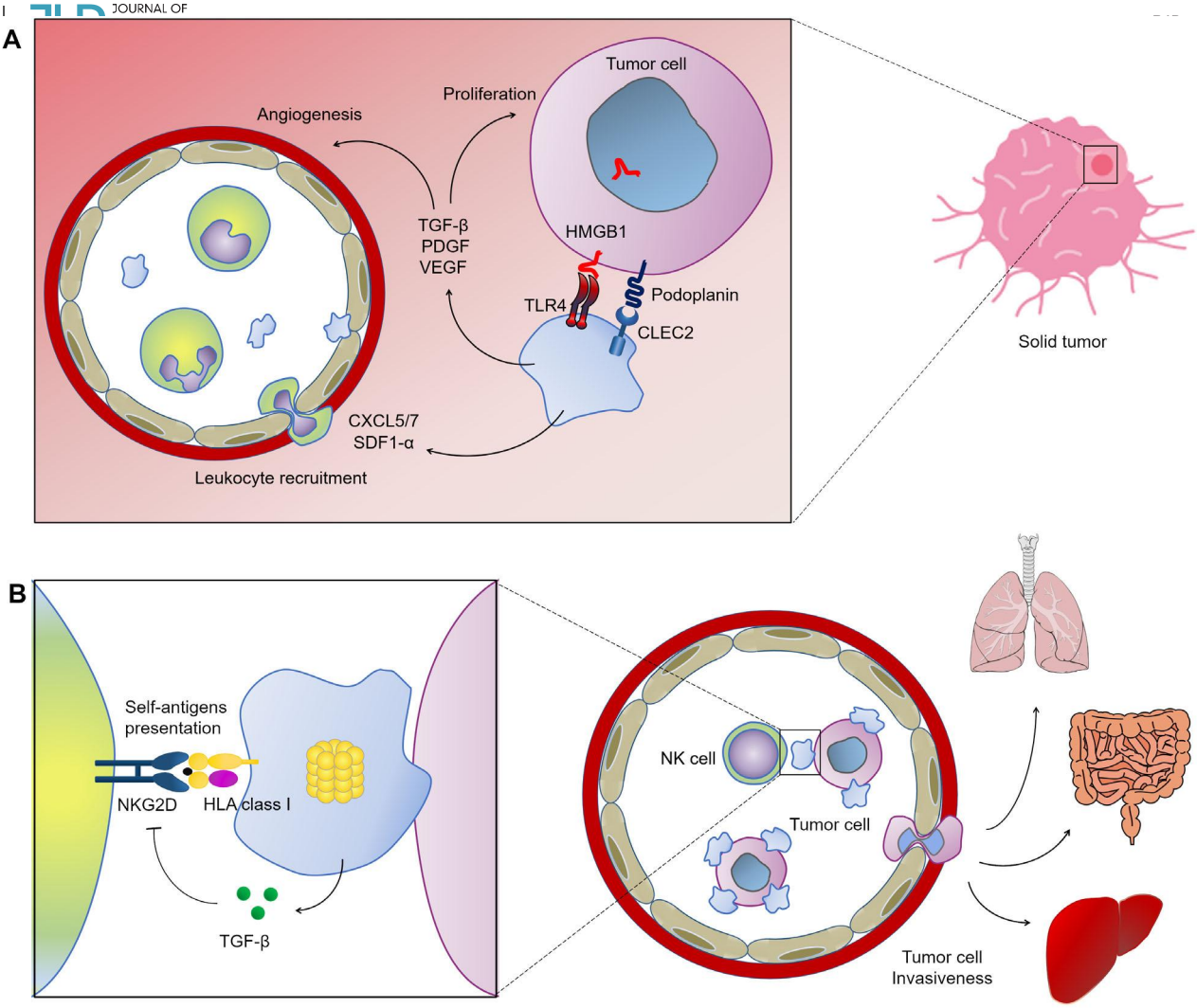

FIGURE 6 **Innate immune receptors in platelets and platelet-leukocyte aggregates participate in tumor development and metastasis. (A)** Platelets in contact with solid tumors become activated through TLR4 engagement of tumor-derived HMGB1, and CLEC2 interaction with podoplanin-expressing tumor cells. Activated platelets secrete chemokines and growth factors that support tumor microenvironment development by inducing proliferation, angiogenesis and leukocyte infiltration. **(B)** Platelets also aggregate with circulating tumor cells and protect them from immunosurveillance by presenting self-Ags to NK cells, which confers a pseudonormal phenotype to platelet-tumor cell aggregates and support metastasis

impair NK cell cytotoxic activity.[398,399] Platelet-tumor cell interaction confers platelet HLA class I molecules to circulating tumor cells, thereby preventing NK cell-mediated destruction.[400] Furthermore, platelet-derived TGF-β1 down-regulates NKG2D on NK cells, leading to decreased NK cell cytotoxicity and IFN-γ production, important antitumor effector functions.[401] Altogether, these evidence show platelet participation in cancer pathophysiology by supporting both tumor microenvironment development and metastasis.

8 | CONCLUSION

Platelets are dynamic and crucial cells for prothrombotic and proinflammatory processes in different pathological conditions. We compiled reports of platelet responses to infectious and immunoinflammatory stimuli through several mechanisms involving innate immune receptors. As discussed above, platelet activation through PRRs influences the secretion of stored and newly synthesized mediators and complex interactions with leukocytes either in the vasculature or the extravascular space. These platelet features may have beneficial or harmful immune functions and directly affect infectious diseases, sterile inflammation, thrombosis, and cancer. Therefore, a better understanding of immunoregulatory abilities of innate immune receptors on platelets will improve the knowledge on platelet contributions to a wide range of pathologies, as well as therapeutic strategies and clinical management.

AUTHORSHIP

P.R.B.D., A.C.Q.-T., L.B.M., M.B.M.P., S.V.R., and F.B.A. wrote the manuscript draft. P.R.B.D. and E.D.H. drew the figures. E.D.H. designed

the review article, edited, and revised the manuscript. All authors discussed the concepts.

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DISCLOSURE

The authors declare no conflict of interest.

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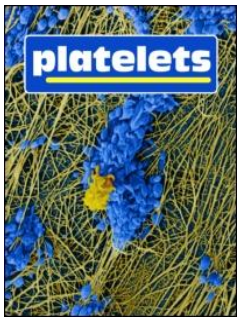

Platelets

ANEXO C

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Platelets in dengue infection: more than a numbers game

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Platelets in dengue infection: more than a numbers game

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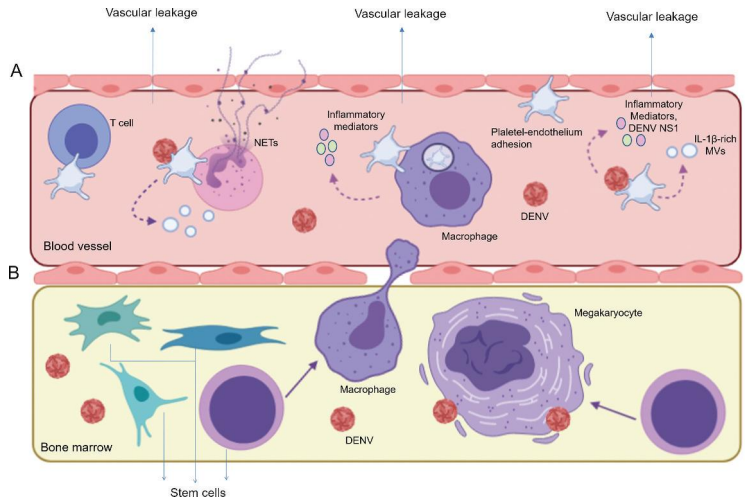
Abstract

Dengue virus (DENV) infection is responsible for the development of dengue illness, which can be either asymptomatic, present mild manifestations or evolve to severe dengue. Thrombocytopenia is an important characteristic during DENV infection, being observed both in mild and severe dengue, although the lowest platelet counts are encountered during severe cases. This review gathers information regarding several mechanisms that have been related to alterations in platelet number and function, leading to thrombocytopenia but also platelet-mediated immune and inflammatory response. On this regard, we highlight that the decrease in platelet counts may be due to bone marrow suppression or consumption of platelets at the periphery. We discuss the infection of hematopoietic progenitors and stromal cells as mechanisms involved in bone marrow suppression. Concerning peripheral consumption of platelets, we addressed the direct infection of platelets by DENV, adhesion of platelets to leukocytes and vascular endothelium and platelet clearance mediated by anti-platelet antibodies. We also focused on platelet involvement on the dengue immunity and pathogenesis through translation and secretion of viral and host factors and through platelet-leukocyte aggregates formation. Hence, the present review highlights important findings related to platelet activation and thrombocytopenia during dengue infection, and also exhibits different mechanisms associated with decreased platelet counts.

Graphical abstract:
Schematic mechanistic representation of platelet-mediated immune responses and thrombocytopenia during dengue infection. (A) DENV-infected platelets secrete cytokines and chemokines and also adhere to activated vascular endothelium. Platelets aggregate with leukocytes, inducing the secretion of NETs and inflammatory mediators by neutrophils and monocytes, respectively. (B) DENV directly infects stromal cells and hematopoietic precursors, including megakaryocytes, which compromises megakaryopoiesis. Both central and peripheric mechanisms contribute to DENV-associated thrombocytopenia.



Keywords

DENV, inflammation, platelet, platelet-leukocyte adhesion, thrombocytopenia

History

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Introduction

Dengue is an arboviral disease caused by Dengue virus (DENV) and transmitted by *Aedes* mosquitos. DENV infection is a serious worldwide problem due to its severity, high incidence, and rapid dissemination [1]. Furthermore, dengue presents a wide spectrum of clinical manifestations ranging from asymptomatic cases to severe dengue, characterized by increased vascular leakage, hemorrhage and organ impairment [1]. Recent evidences demonstrate that platelets are important players in disease progression, modulating immune and inflammatory responses through several mechanisms [2–5].

DENV-infected platelets secrete stored and newly synthesized inflammatory mediators as RANTES, MIF and IL-1β, highlighting platelet participation in dengue pathogenesis [2,3]. These inflammatory cytokines and chemokines are elevated in the plasma of severe dengue patients [6,7] contributing to vascular leakage [8,9]. Furthermore, platelets also interact with leukocytes forming platelet-leukocyte aggregates, especially in blood of patients with increased vascular leakage [4,10]. Platelets then induce inflammatory responses by the leukocytes [4,5,11], worsening the vascular leakage.

Platelet-leukocyte aggregates also contribute to decreased platelet counts, which is in agreement with augmented aggregation in thrombocytopenic patients [4]. Thrombocytopenia is a critical feature found both in mild and severe dengue, although severe dengue patients present the lowest platelet counts [12,13]. Furthermore, severe dengue patients present elevated levels of activated platelets in comparison with mild dengue [14]. Our group and others have demonstrated that activated and apoptotic platelets are phagocytosed by monocytes, evidencing an association between activated platelets and thrombocytopenia [4,15]. Considering the association of thrombocytopenia and disease severity during dengue infection, the present review will address the distinct mechanisms underlying the decrease in platelet counts and platelet-mediated inflammatory response in dengue, and the influence of platelets on the development of the disease pathogenesis.

Clinical Aspects

DENV infection has a large spectrum of clinical manifestations that range from asymptomatic to symptomatic disease, initiating as mild dengue with or without warning signs. The warning signs may indicate an evolution to severe dengue, although patients without warning signs may also progress to severe dengue syndrome[1]. The incubation period lasts 4–10 days and the symptoms begin abruptly with fever, malaise, headaches, muscle pain, and vomiting at the febrile phase, when high viremia is detected [1,16]. Usually, fever settles after 3 days, the viremia decreases and around this time of defervescence the critical phase begins [1]. At this point, there is systemic inflammatory response [17], decreased platelet counts, and leukopenia [1]. Plasma leakage also increases, leading to hypotension and hemoconcentration[1]. Following the critical phase, the patient may improve and recover with a gradual reabsorption of extravascular fluid and hemodynamic status stabilization [1]. However, if the inflammatory response is not controlled, the patient can evolve to severe dengue, mostly characterized by increased plasma leakage, severe hemorrhage, and organic failure, which may evolve to death [1,16].

Replication Cycle

DENV targets several cell types, including monocytes, macrophages, lymphocytes, endothelial cells and dendritic cells[18]. Recently, studies have shown that DENV also binds and enters platelets [19,20] through the receptors dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) and heparan sulfate proteoglycan (Figure 2A-B) [20,21]. However, other receptors are described in other cell types, including mannose receptors and CD14 on monocytes/macrophages, and DC-SIGN itself in dendritic cells[18].

DENV entry into the host cell is mediated by the viral envelope (E) glycoprotein binding to permissive cell receptors[18]. Following attachment, DENV is endocytosed, which occurs primarily through clathrin-dependent mechanisms[22]. After internalization, endosome acidification leads to conformational changes in the E protein, catalyzing homotrimerization, and resulting in endosomal-viral membrane fusion[22]. Thus, the nucleocapsid is released into the cytoplasm, the virus is uncoated and the single-stranded positive-sense viral RNA is translated into a polyprotein into the endoplasmic reticulum. The viral polyprotein is cleaved by host and viral proteases into three structural proteins – capsid protein (C), pre-membrane protein (prM), and E protein – and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5)[22]. Viral NS proteins form the replication complex, which synthesizes a complementary negative-sense RNA from the positive-sense RNA serving as a mold for viral genome replication [22,23]. The encapsidation occurs after translation and replication of the newly synthesized RNA, in which viral RNA is joined by C protein forming nucleocapsids[22]. The nucleocapsids are enveloped by heterodimer prM-E anchored in the endoplasmic reticulum to build immature viral particles[22]. The immature particles transit through the secretory pathway and reach Golgi network, in which viral membrane proteins undergo glycosylation [23]. The trans-Golgi network triggers prM cleavage into M by host furin protease, followed by E protein homodimerization, resulting in the formation of mature virus [22,23]. The infectious virion is then exocytosed [22,23].

Analysis of infected platelets demonstrate increasing detection of DENV RNA copies overtime, indicating that platelets are permissive to viral binding and sustain viral genome replication [2,20]. Intracellular and extracellular NS1 accumulation in infected platelets indicates viral proteins translation, which is indispensable for viral genome replication [2,19]. Nevertheless, DENV genome and infectious particles were not detected in platelet supernatant, indicating that platelets cannot secret newly assembled virions [2,19]. This is consistent with the fact that infectious DENV does not accumulate in platelet supernatant overtime [20]. Altogether, these data indicate that infected platelets produce an abortive DENV replication cycle (Figure 2A-B).

Mechanisms of Thrombocytopenia

Thrombocytopenia is an important hematological change in dengue, with lower platelet counts described in severe dengue cases in association with increased vascular leakage [24,25]. The suggested mechanisms that contribute to thrombocytopenia in dengue include reduced thrombopoiesis through bone marrow (BM) suppression, and increased platelet clearance in peripheral blood (Figure 1)[25]. Regarding the reduced thrombopoiesis, hematopoietic suppression is a well-known phenomenon during DENV infection and probably begins before infection is clinically overt, in the incubation period around 4 ± 5 days after the inoculum [26]. A marked BM hypocellularity is described at onset of the febrile phase with pancytopenia, including megakaryocytes[27]. In fact, DENV infection of humanized mice, elaborated by adoptive transfer of human CD34+ hematopoietic stem cells into immunodeficient mice, leads to decreased platelet production and identifies the depletion of human progenitor cells and

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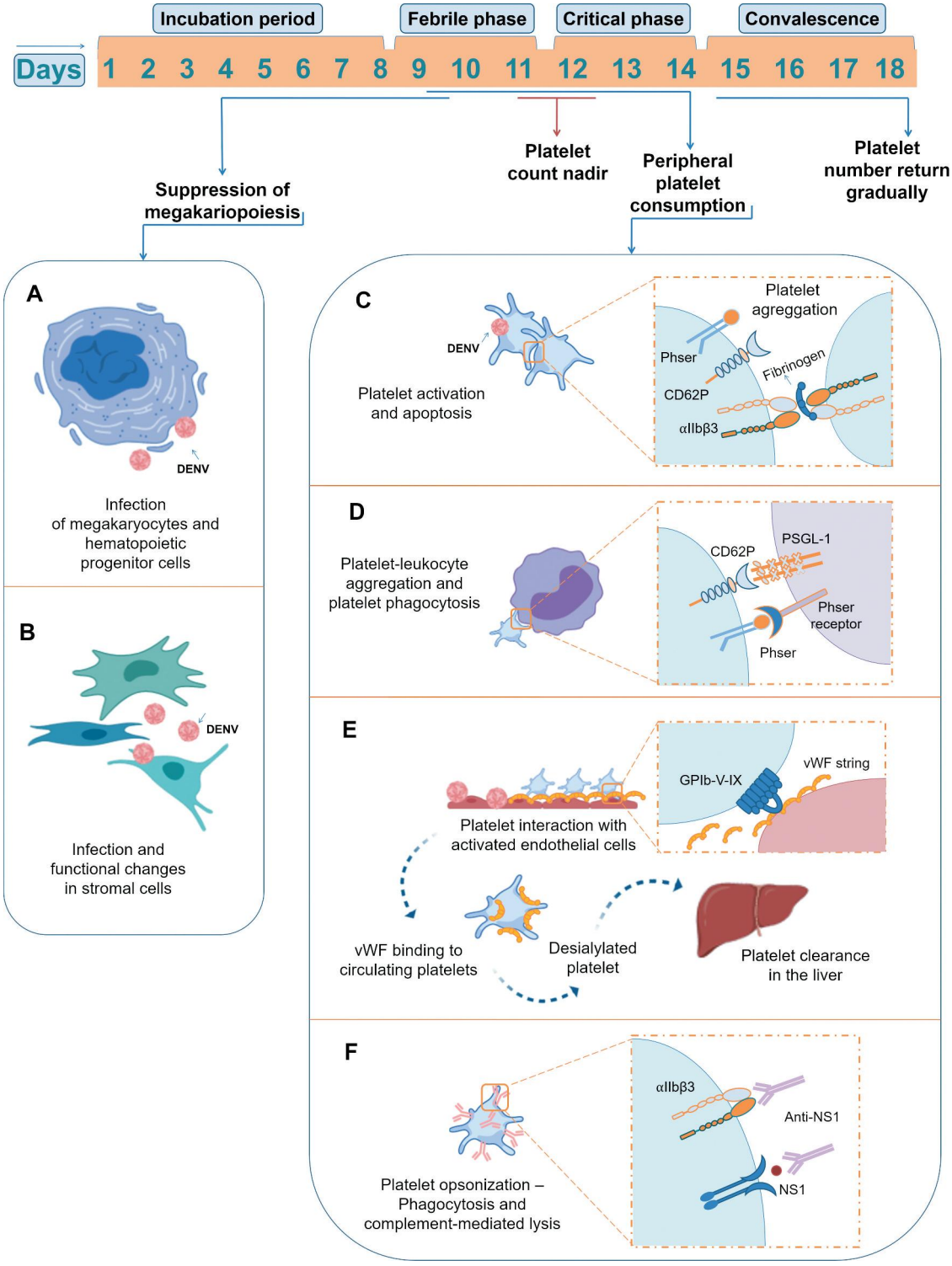

Figure 1. Mechanisms of thrombocytopenia in dengue. Thrombocytopenia may occur due to a reduction in megakaryopoiesis or peripherical consumption of platelets. The suppression of megakaryopoiesis may be caused by (A) the direct infection of megakaryocytes or by (B) the infection of stromal cells. The peripherical platelet consumption may be due to (C) platelet apoptosis, (D-E) platelet heterologous aggregation with (D) leukocytes or (E) endothelial cells, or (F) platelet opsonization by anti-platelets antibodies and complement system.

megakaryocytes as a fundamental mechanism underlying thrombocytopenia[28].

Megakaryopoiesis suppression may occur due to DENV infection of hematopoietic cells, considering that megakaryocyte progenitor cells are permissive to DENV infection[29]. Besides, megakaryocytes from healthy human BM or from *in vitro* differentiated CD34^{+} stem cells are also susceptible and permissive to DENV infection, as well as human megakaryocytes in experimentally infected humanized mice [29,30]. Although *in vitro* experiments demonstrate a reduction in DENV-infected megakaryocytic progenitors' differentiation and possible cell death by apoptosis, the role played by hematopoietic cell infection to thrombocytopenia needs further elucidation[31]. Paradoxically, Sridharan et al. [28] identified megakaryocytes depletion and thrombocytopenia in DENV-infected humanized mice in the absence of DENV-infected hematopoietic cells. On the other

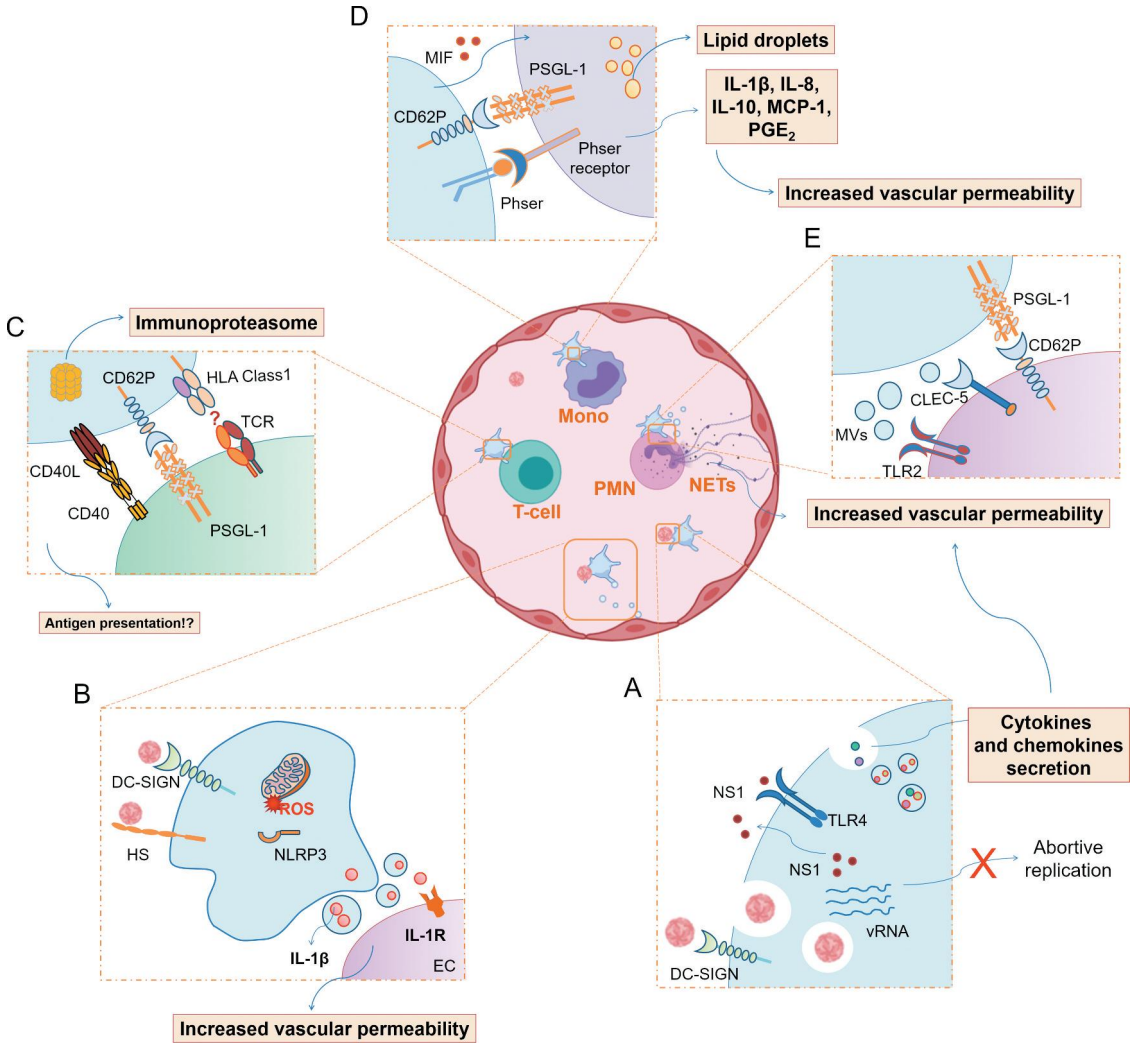

Figure 2. Platelets are major players in the inflammatory response in Dengue. (A) DENV interacts with platelets through DC-SIGN, DENV genome is translated and replicated, leading to the accumulation of viral proteins and new viral RNA copies. DENV-infected platelets secrete cytokines, chemokines and newly synthesized NS1, but do not produce new DENV particles. Platelet-derived NS1 contribute to platelet activation by engaging TLR4 autocrinally. (B) DENV-infected platelets assemble NLRP3 inflammasome and secrete IL-1β-containing EVs, which engage to IL-1R on endothelial cells contributing to increased endothelial permeability. Platelets interact with (C) lymphocytes, (D) monocytes and (E) neutrophils, through CD40-CD40L, CD62P-PSGL-1 and by secreting EVs to bind CLEC-5 and TLR2, respectively. All these interactions contribute to leukocyte activation and induce the release of cytokines, chemokines, eicosanoids and NETosis.

hand, Vogt et al. [30] identified the depletion of mature megakaryocytes alongside megakaryocytes infection in humanized mice regardless the development of thrombocytopenia. Besides, DENV infection did not affect megakaryocyte viability *in vitro* [28,30].

The mechanisms possibly underlying BM suppression and thrombocytopenia may also be associated with functional changes on stromal cells, that regulate cytokine secretion and hematopoiesis, altering platelet production quantitatively and qualitatively [28,29]. Clark et al. [29] identified the presence of DENV antigen in plasmocytoid dendritic cells and monocytes in non-human primates BM, and detected phagocytic cells that have engulfed DENV-positive vesicles from megakaryocytes. Sridharan et al. [28] also identified a significant reduction in $CD4^+/CD8^+$ T cells ratio in the BM of DENV-infected humanized mice, indicating BM microenvironment changes. However, the BM suppression mechanisms underlying thrombocytopenia need further clarification.

Despite BM acute suppression recovery with return of hematopoiesis preceding the critical phase, platelet counts nadir coincides with defervescence and progression to severity, and platelet count recovery only occurs at the convalescence [27,32]. Hence, peripheral platelet clearance mechanisms may play major roles in dengue-associated thrombocytopenia [15,21]. Platelet activation and apoptosis markers as *P*-selectin and CD63 translocation, integrin αIIbβ3 activation, phosphatidylserine exposure, and mitochondrial disfunction are associated to thrombocytopenia during the infection [15,21]. Hence, it is reported that *in vitro* DENV infection induces platelet activation and apoptosis [15,21]. The frequency of apoptotic platelets is significantly increased in severe dengue patients compared to mild dengue. Also, platelet apoptosis during infection correlates with increased monocyte-mediated platelet phagocytosis [32]. Accordingly, apoptotic platelets from patients, or following *in vitro* infection, are phagocytosed by macrophages in phosphatidylserine recognition-dependent manner [32]. *In vitro* platelet phagocytosis involves both phosphatidylserine recognition and platelet activation with surface *P*-selectin contributing to platelet–monocyte interactions [4,15].

In fact, increased platelet-monocyte aggregates formation in dengue patients is associated with thrombocytopenia [4]. During dengue infection, interactions of platelet with leukocytes,

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including lymphocytes and granulocytes, are also upregulated in peripheral blood [10]. Similarly, a non-human primate model of DENV-2 infection showed increased platelet-monocytes and -neutrophils aggregates [33]. Besides platelet–leukocyte interaction, platelet–endothelial cell interaction and platelet deposition in the microvascular bed are also encountered, and platelets that interact with *in vitro* infected endothelial cells become activated [4,34]. Accordingly, histopathological and electron microscopy analysis identified platelet adhesion to splenic and hepatic endothelium, and adherent platelets in heart, liver and spleen endothelial cells [35]. Perfusion of DENV-infected whole blood on histamine-activated endothelial cells showed increased thrombus area and platelet-von Willebrand factor (vWF) strings formation compared to uninfected control [15]. Furthermore, endothelial cells can also contribute to platelet clearance by secreting vWF. DENV infection induces a marked increase in vWF binding to circulating platelets, which is inversely correlated to platelet counts [36]. *In vitro* experiments have shown that increased vWF binding to platelet GPIbα is the fundamental mechanism underlying platelet desialylation, observed in circulating platelets from dengue patients [36]. As sialic acid removal exposes terminal β-galactose and accelerates platelet clearance by liver macrophages and hepatocytes, it may contribute to thrombocytopenia in dengue [37].

Dengue-associated thrombocytopenia may also involve antibody-mediated platelet clearance. In patients with secondary dengue infection, inverse correlations between platelet count and platelet-associated immunoglobulins G (IgG) and M (IgM) were observed [38,39]. Platelet-associated immunoglobulins were significantly increased in severe dengue and showed anti-DENV activity, suggesting a cross-reactivity of antibodies against viral proteins to platelet antigens [38]. It is known that anti-NS1 immunoglobulins can opsonize human platelets, leading to complement-mediated lysis and increased platelet–macrophage interactions [40]. This is possible because homologous sequences between NS1 and integrins, such as GPIIb/IIIa in activated platelets, allow a cross-reactivity between anti-NS1 and integrins causing platelet-antibody binding[41]. Hence, *in vitro* DENV-induced platelet activation increased platelet binding to IgG and to complement factor C3 on the plasma of DENV-infected patients [15]. Hence, platelet activation proved to be necessary since in the presence of the platelet inhibitor prostacyclin IgG and C3 binding were abrogated[15]. However, platelet opsonization by cross-reactive anti-DENV antibodies needs further clarification.

Recently, our group has demonstrated that DENV NS1 directly binds to platelets [2]. In addition, it has been shown that soluble NS1 activates the complement complex in the absence of antibodies or in an antibody-dependent manner when associated within cell membrane [42]. Thus, NS1 binding to platelets may be one of the factors contributing to the observations that C3 levels are directly correlated with platelet levels and inversely correlated with C3a and C5a, indicating that the greater the activity of the complement system the lower the platelet count during infection [43]. Through mechanisms as opsonization and complement activation, the binding of antibodies and/or immune complexes to platelets may contribute to platelet activation and thrombocytopenia, supporting pathogenic mechanisms as described below [44].

Several processes associated with BM suppression and platelet activation at the periphery, such as interactions with leukocytes and endothelial cells, phagocytosis, complement-mediated lysis, aggregation and clot formation, may act in succession or in combination and contribute to thrombocytopenia in infected patients [15].

Platelets in the Pathogenesis of Dengue

Until recently, platelets were considered solely as effectors of the coagulation, aiding hemostatic responses through adhesion and secretion of pro-coagulant factors. However, recent studies have demonstrated that platelets are more than just fragments from megakaryocytes, being considered as cells that participate in inflammatory and immunological processes [45,46]. A recent review from our group has summarized platelet participation in several sterile and infectious diseases, gathering information regarding the mechanisms and receptors involved in platelet-mediated inflammatory responses of such conditions, including dengue [46]. Platelets from DENV-infected patients are highly activated and increased platelet activation associates with severe dengue, as demonstrated by increased surface expression of *P*-selectin, CD63, and phosphatidylserine [14,21,47]. Activated platelets have been shown to participate in inflammatory amplification in dengue by interacting with leukocytes and also secreting inflammatory mediators as cytokines and chemokines (Figure 2) [2–4,46].

It is well established that the levels of inflammatory mediators are elevated in DENV-infected patients, in which pro-inflammatory factors play major roles in hemodynamic instability [6,48]. It has already been demonstrated that DENV-infected platelets *in vitro* or platelets from patients with dengue highly secrete stored cytokines and chemokines, as PF4/CXCL4 and RANTES/CCL5 (Figure 2A) [2,14]. Small molecules, such as nitric oxide and intra-platelet serotonin are also increased during dengue infection [49,50]. Platelets have been shown to be an important source of serotonin following dengue infection, and serotonin, in its turn, induces platelet activation[51]. Furthermore, studies have demonstrated that vascular endothelial growth factor (VEGF), which is mainly secreted by platelets [52], is also elevated in DENV-infected patients[53]. Interestingly, elevated VEGF concentrations have been associated with disease severity, once the plasma levels of VEGF positively correlate with markers of liver damage, increased plasma leakage [54] and negatively correlates to platelet counts [53,54].

Despite being anucleated cells, platelets are also capable of synthesizing new proteins through processing of stored RNA [55]. Previously, our group has shown that DENV induces both platelet synthesis of IL-1β and the assemble of nucleotide-binding domain leucine-rich repeat-containing pyrin-3 (NLRP-3) inflammasome, leading to processing and secretion of IL-1β in extracellular vesicles (EVs) (Figure 2B) [3]. This study also demonstrated that platelet IL-1β secretion is associated with increased vascular permeability *in vivo* and increases endothelial permeability *in vitro*. Hence, platelets contribute to vascular leakage through secretion of IL-1β-containing EVs.

In addition to the synthesis of inflammatory mediators through stored RNA processing, infected platelets also translate the DENV genome, leading to the synthesis and secretion of NS1 [2]. Platelet activation by NS1 is involved in the development of hemorrhagic and inflammatory events *in vivo* and *in vitro* [2,56]. A recent study from our group has demonstrated that NS1 secreted by DENV-infected platelets engages platelet toll-like receptor-4 (TLR4) inducing platelet activation and secretion of stored and newly synthesized inflammatory mediators through an autocrine loop [2]. Moreover, platelet-derived NS1 may also be involved in the activation of TLR4 on other cells [2,57], potentially contributing to inflammatory amplification in dengue.

Beyond its direct influence on the elevated levels of cytokines and chemokines, platelets also communicate with other immune cells, amplifying inflammatory response. DENV infection has been shown to induce platelet expression of *P*-selectin, CD40L and human leukocyte antigen (HLA) class I [14,21,58], which are

involved in interactions with leukocytes. Also, platelets are able to present antigens to T-cells [59]. Increased levels of platelet-lymphocyte aggregates have been observed in patients with dengue (Figure 2C) [10]. Trugilho and Hottz et al. have demonstrated that DENV-infection *in vitro* increased HLA class I surface expression on platelets, which depended on proteasome activity [14]. Furthermore, platelets from DENV-infected patients presented elevated levels of HLA class I [14]. Nonetheless, new studies are still necessary to investigate whether platelets are able to present DENV antigens to lymphocytes, which might contribute to both specific immune response against DENV or worsening the inflammatory response during infection.

Studies from our group and others have demonstrated increased platelet-monocyte aggregates formation in blood from dengue patients, especially those with thrombocytopenia and increased vascular permeability (Figure 2D) [4,10,33]. Platelet-monocyte aggregates formed by *ex vivo* interaction of platelets from dengue patients and monocytes from healthy volunteers induced the secretion of IL-1β, CXCL8/IL-8 and IL-10, which relies on the platelet expression of *P*-selectin and phosphatidylserine [4,5]. Platelets also induce MCP-1, MMP-9 and COX-2 expression by monocytes [55,60]. DENV-infected platelets have been recently shown to stimulate COX-2 expression alongside the biogenesis of lipid droplets in monocytes, consequently inducing the secretion of PGE_2 [5]. Furthermore, MCP-1 and MMP-9 have been associated with increased vascular leakage during dengue [61,62], whilst COX-2 has proved important for viral replication and chemotaxis [63,64]. Altogether, these findings suggest that platelet-monocyte aggregation are major mechanism of immune regulation and inflammatory amplification in dengue.

DENV-infected platelets also interact with neutrophils (Figure 2E), as shown by studies using macaques and *in vitro* infection of human platelets [10,33]. Such interaction occurs through the release of platelet EVs, which interacts with CLEC5 and TLR2 on neutrophils, leading to the release of neutrophil extracellular traps (NETs) [11]. Platelet-induced NETs extrusion was shown to participate in the increased endothelial permeability in an *in vitro* co-culture model [11]. These findings were confirmed *in vivo* by infection of STAT1-/- mice [11]. Since immunocompetent mice are resistant to DENV infection, STAT1-/- is a widely used experimental dengue model once it does not develop IFN-mediated anti-viral responses. STAT1-/-CLEC5-/- mice showed reduced NET deposition in the spleen, lower vascular permeability and increased survival in comparison to STAT1-/- mice, confirming CLEC5 participation on these events [11]. Further impairment in NETosis, plasma leakage and mortality were achieved in STAT1-/-CLEC5-/- mice treated with anti-TLR2 antibodies [11]. Hence, CLEC5- and TLR2-mediated NET induced by CLEC2-dependent platelet-derived EVs play a pathogenic role in experimental DENV infection.

Besides participating in dengue pathogenesis through the diverse mechanisms abovementioned, platelets may also have beneficial effects during dengue infection. Platelets from patients with dengue, as well as from influenza pneumonia patients, highly express interferon-induced transmembrane protein 3 (IFITM3), which was associated with milder disease and increased survival [65]. Complementary *in vitro* experiments with IFITM3 overexpression or loss-of-function mutation demonstrated IFITM3 as a major restriction factor of DENV replication in megakaryocytes [65]. Furthermore, platelets are important to vascular integrity maintenance by secreting factors as lysophosphatidic acid and sphingosine-1-phosphate, among others, that stabilize vascular integrity [66]. Therefore, platelet-mediated immune response in dengue presents a dual aspect that may contribute to protective and pathologic responses, which must be balanced.

Conclusion

Platelets play important roles in dengue pathogenesis through diverse mechanisms. Although not being able to release new viral particles, DENV-infected platelets can secrete a diversity of inflammatory mediators and the viral toxin NS1, which modulates immune response in leukocytes, endothelial cells and platelets themselves. Moreover, thrombocytopenia is an important hematological alteration encountered both in mild and severe cases. Such alteration may be due to several mechanisms that lead to a decreased hematopoiesis or an increased platelet clearance from the blood, as discussed above. Altogether, the information we gathered in this review demonstrates that platelets aid the potentially detrimental immune response during dengue, participating in dengue pathogenesis. Hence, elucidating all the mechanisms underlying platelet-mediated response are crucial for the better understanding of dengue pathogenesis and clinical aspects.

Authorship

ACQT, FBA, MBMP and SVF wrote the chapters draft. ACQT integrated the manuscript. EDH edited and revised the manuscript. All authors have critically discussed the concepts, participated in manuscript writing and approved the final submitted version.

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Conflicts of Interest Disclosure

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Abstract

Dengue is an infectious disease caused by Dengue Virus, mainly transmitted by *Aedes aegypti* mosquitoes. Severe dengue is a potentially fatal syndrome in consequence of overwhelmed inflammation, in which thrombocytopenia and increased vascular permeability are frequently observed. Several experimental evidences point to the participation of both microvesicles (MVs) and circulating lipoproteins in inflammatory amplification in dengue pathogenesis. On this regard, many protocols for isolating plasma MVs have shown lipoproteins as the main contaminant. This is a limitation to studies aiming at the functional characterization of MVs, since both MVs and lipoproteins can modulate inflammatory responses. Here, we describe a biphasic density-based gradient ultracentrifugation as a tool for concomitant isolation of MVs and lipoproteins without cross-contamination. Flow cytometry for MVs quantification and western blot for detection of apoB100 may be used to confirm the isolation and purity of the MVs.

Keywords (separated by '-') Extracellular vesicles - Microvesicles - Lipoproteins - Isolation protocols - Gradient ultracentrifugation

Chapter 17

Isolation of Microvesicles from Plasma Samples Avoiding Lipoprotein Contamination

Laura B. Merij, Fernanda B. Andrade, Adriana R. Silva, and Eugenio D. Hottz

Abstract

Dengue is an infectious disease caused by Dengue Virus, mainly transmitted by *Aedes aegypti* mosquitoes. Severe dengue is a potentially fatal syndrome in consequence of overwhelmed inflammation, in which thrombocytopenia and increased vascular permeability are frequently observed. Several experimental evidences point to the participation of both microvesicles (MVs) and circulating lipoproteins in inflammatory amplification in dengue pathogenesis. On this regard, many protocols for isolating plasma MVs have shown lipoproteins as the main contaminant. This is a limitation to studies aiming at the functional characterization of MVs, since both MVs and lipoproteins can modulate inflammatory responses. Here, we describe a biphasic density-based gradient ultracentrifugation as a tool for concomitant isolation of MVs and lipoproteins without cross-contamination. Flow cytometry for MVs quantification and western blot for detection of apoB100 may be used to confirm the isolation and purity of the MVs.

Keywords Extracellular vesicles, Microvesicles, Lipoproteins, Isolation protocols, Gradient ultracentrifugation

1 Introduction

Dengue is an infectious disease caused by Dengue Virus (DENV), mainly transmitted by *Aedes aegypti* mosquitoes. A wide spectrum of clinical manifestations is observed in DENV infection, which may characterize a mild dengue syndrome with self-limited clinical course or progress to severe disease, with complications involving thrombocytopenia and increased vascular permeability [1]. Even though the pathophysiological mechanisms of severe dengue are not completely understood, a strong inflammatory response is known to mediate vasculopathy, tissue damage, and organ impairment [2]. In this context, increased levels of circulating inflammatory microvesicles (MVs) are described in patients with severe dengue [3]. DENV infection alters the levels and

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composition of serum MVs, which are potential biomarkers for dengue prognosis [3, 4] as well as being involved in pathophysiological mechanisms [5].

MVs, or microparticles, are membrane fragments released into the extracellular space by virtually all cells under activation/stress conditions, contributing to pathological processes [6]. They act by protecting their content from the extracellular milieu and delivering bioactive molecules to target cells via surface signaling or membrane fusion [6]. MVs generated from erythrocytes and platelets comprised two major populations in the circulation of dengue patients [3] and platelet-derived microvesicles have been shown to contribute to endothelial hyperpermeability, a signal associated with progression to severe dengue [5]. However, previous attempts of isolating circulating MVs have shown contamination with lipoproteins, mainly low-density lipoprotein (LDL) [7]. This contamination is a setback on MVs functional studies once lipoproteins can also modulate inflammatory responses [8].

In this chapter, we present an optimized protocol for the isolation of MVs samples avoiding contamination by LDL, and also allowing the isolation of lipoproteins in parallel. The protocol includes a biphasic density-based gradient ultracentrifugation with subsequent dialysis followed by centrifugation to pellet the purified MVs. Isolated MVs are further analyzed through flow cytometry and their purity confirmed by detection of ApoB100 (the apolipoprotein present in LDL [9]) by Western Blot.

2 Materials

1. Conical polypropylene sterile tubes (50 mL and 1.5 mL).
2. Tube racks.
3. Beakers.
4. Centrifuges.
5. Laminar flow cabinet.
6. Analytical scale.
7. Bunsen burner.
8. Agitator.
9. Vortex.
10. Laboratory water bath.
11. Plate spectrophotometer.
12. Ultrasonic bath.
13. Cytometer.

2.1 Isolation of Plasma

1. Blood collection materials.
2. Acid-citrate-dextrose (ACD): 22.4 g/L sodium citrate, 8 g/L citric acid, 2 g/L dextrose, pH 5.1, in ultrapure water (Milli-Q quality), 0.22 μm vacuum filtered.
3. Butylated hydroxytoluene (BHT) diluted in ethanol (2 mg/mL).

2.2 Gradient Assembly and Handling

1. 8 mL polypropylene round-top ultracentrifuge tubes with heat-sealing.
2. Fixed-angle vertical or near-vertical ultracentrifuge rotor.
3. Sterile glass Pasteur pipette with rubber bulb.
4. Saline solution: 0.9% NaCl w/v.
5. Potassium bromide.
6. Quick-Seal Cordless Tube Topper kit (Beckman Coulter).
7. Sterile needles (18G, 1.2 × 40 mm).

2.3 Dialysis and MVs Precipitation

1. Pore membranes: 1 kDa *cut-off*, 2 mL.
2. Phosphate-Buffer Saline (PBS): 0.137 M NaCl, 0.0027 M KCl, 0.01 M Na_2HPO_4, 0.0015 M KH_2PO_4 diluted in Ultrapure water (Milli-Q quality), pH 7.4.

2.4 Colorimetric Essays

1. Triacylglycerol liquiform kit.
2. Cholesterol liquiform kit.
3. BCA Protein Assay kit.
4. Albumin quantification kit.

2.5 Flow Cytometry

1. Annexin V-PE conjugate probe.
2. 10× Annexin V Binding Buffer (AVBB): 0.1 M HEPES (pH 7.4), 1.4 M NaCl, and 25 mM $CaCl_2$, filtered in 0.2-μm syringe filter.
3. 1 μm latex beads (4.55×10^{10} particles/mL).
4. 5 mL round bottom polystyrene test tube.

2.6 Western Blot

1. Vertical mini gel electrophoresis system.
2. Mini transfer system including gel holder cassettes, foam pads, electrode assembly plus thick blot filter paper.
3. Basic power supply with power pack adaptor cords.
4. Sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE):
   (a) Polyacrylamide separating gel: 6% Acrylamide/bis Acrylamide Solution (ratio: 37.5:1), 0.375 M Tris–HCl pH 8.8; 0.1% SDS (m/v), 0.2% APS—Ammonium persulfate (m/v), 0.08% tetramethylethylenediamine (TEMED) in ultrapure water (Milli-Q quality).

(b) Polyacrylamide Stacking gel: 4% Acrylamide/bis Acrylamide Solution (ratio: 37.5:1), 0.25 M Tris–HCl pH 6.8, 0.1% SDS (m/v), 0.1% APS—Ammonium persulfate (m/v), 0.01% Bromophenol Blue (BPB), 0.16% TEMED, in ultrapure water (Milli-Q quality).

5. 5× concentrated lysis buffer: 750 mM NaCl, 50 mM Tris–HCl, pH 8.0, 0.5 mM EDTA, 2.5% Triton X-100 (v/v), 5% Protease inhibitor in ultrapure water (Milli-Q quality).
6. 5× concentrated sample buffer: 5% SDS (m/v), 12.5% β-mercaptoethanol (BME) (v/v), 25% Glycerol (v/v), 156.25 mM Tris–HCl, 0.01% BPB (m/v), pH 6.8.
7. High molecular weight (MW) range protein ladders.
8. Running buffer: 25 mM Tris-base, 190 mM Glycine, 0.1% SDS (m/v) in ultrapure water (Milli-Q quality).
9. Transfer buffer: 25 mM Tris-base, 190 mM Glycine, 0.1% SDS (m/v), 5% methanol (v/v) in ultrapure water (Milli-Q quality).
10. Polyvinylidene fluoride (PVDF) membrane.
11. Tris-buffered saline with Tween-20 (TBS-T) buffer: 20 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20 (v/v).
12. Blocking solution: 5% non-fat dried milk in TBS-T.
13. Primary antibody against Apo B100.
14. Secondary antibody conjugated to horseradish peroxidase (HRP) generated against host species of primary antibody.
15. Super Signal enhanced chemiluminescent substrate.
16. Hyperfilm ECL-high performance chemiluminescent film 18 × 24 cm and X-ray film cassette.

3 Methods

3.1 Plasma Isolation

1. Centrifuge the blood tubes at 700 × *g* for 20 min at room temperature without applying the centrifuge brake to obtain platelet-poor plasma (PPP). Collect the PPP in a new centrifuge tube.
2. Perform a second centrifugation at 2500 × *g* for 15 min, at room temperature, without brake, to exclude apoptotic bodies, membrane fragments, and cell debris obtaining the platelet-free plasma (PFP) (*see* **Notes 1** and **2**).
3. Collect the PFP and add BHT (1 μL/mL) to prevent ex vivo oxidation of plasma lipoproteins (*see* **Note 3**).
4. Separate a fraction of PFP plasma to be used as a control of MVs isolated without lipoprotein elimination later.

3.2 Gradient Assembly

1. Dilute 0.5 g/mL of KBr in PFP and thoroughly homogenize, avoiding foam formation according to Fig. 1.
2. Add 2 mL of PFP containing 0.5 g/mL of KBr in the polypropylene round-top quick-seal centrifuge tubes using a sterile glass Pasteur pipette with rubber bulb (Fig. 1a) (*see* **Note 4**).
3. Using another sterile glass Pasteur pipette with rubber bulb, gently add sterile saline solution (NaCl 0.9% w/v) above the PFP-KBr mixture in the same tube until it is completely filled (Fig. 1a).
4. Check the weight of the tubes making sure opposite tubes have the same weight to ensure proper balance at the time of ultracentrifugation (*see* **Note 5**).
5. Seal the tube for ultracentrifugation.
6. Ultra-centrifugate the tubes at 150,000 × *g* for 2 h and 40 min at 4 °C with medium acceleration and deceleration.
7. Warm the needle in the Bunsen burner and pierce two holes in each tube, one on the bottom and the other at the top (Fig. 1b). Be careful, once you pierce the second hole the drops will start to fall.
8. Collect 26 fractions from the bottom to the top of the gradient in individual 1.5 mL conical tubes (Fig. 1b) (*see* **Note 6**).

3.3 Quantification of Plasma Lipoproteins and Albumin

1. Locate the plasm lipoproteins and albumin in each fraction. For this you must quantify cholesterol, triacylglycerol (TAG), and albumin in all fractions obtained after ultracentrifugation using specific colorimetric essay kits as per manufacturer's instructions. Through this step, you will be able to identify the fractions containing very low-density lipoprotein (VLDL—rich in TAG at the top of the gradient), low-density lipoprotein (LDL—rich in cholesterol below VLDL), high-density lipoprotein (HDL—rich in cholesterol below LDL), and plasma (rich in albumin at the bottom) (Fig. 1c).
2. For further confirmation of the LDL-containing fractions, weigh 100 μL of the fractions to check the density (LDL density is between 1.019 and 1.063 g/mL).

3.4 Quantification of MVs in Plasma Through Flow Cytometry

1. Prepare 1× Annexin V Binding Buffer (AVBB) by diluting the 10x stock solution in ultrapure water (Milli-Q quality).
2. Prepare an intermediate suspension of latex beads by diluting the beads stock at 1:1000 ratio in AVBB (1×). Place the beads suspension in the ultrasonic bath. Beads must remain in ultrasound until the moment of use to prevent aggregation (*see* **Note 7**).
3. Separate a sample of each fraction of the gradient in 1.5 mL conical tubes and add the 10× concentrated AVBB and

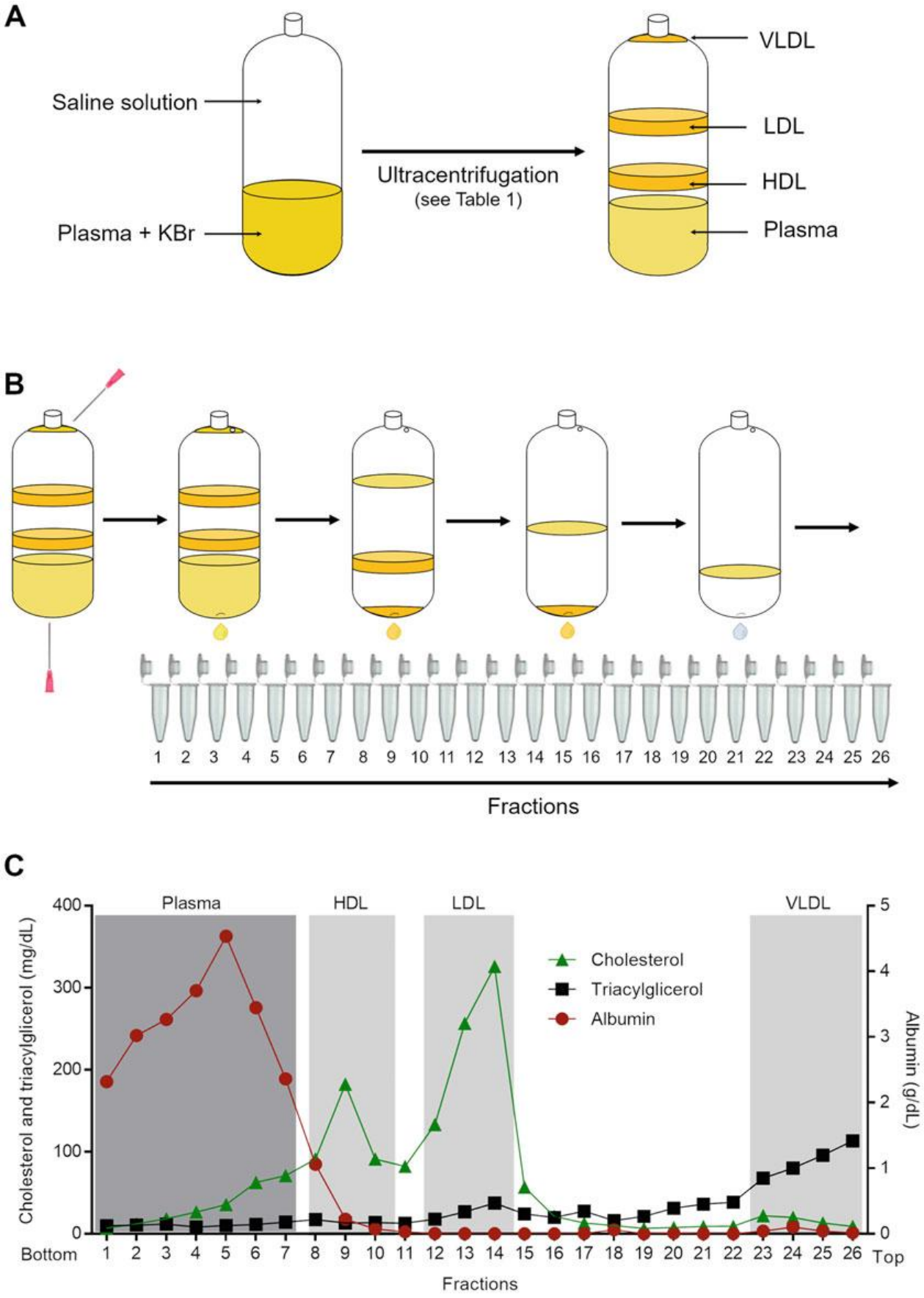

Fig. 1 Schematic representation of the biphasic gradient assembly and fractionation. (**a**) The layout of the solutions used to assemble the gradient (left) and the expected distribution of the lipoproteins and plasma fractions after ultracentrifugation (right). (**b**) Schematic representation of suitable locations for needle drilling

PE-labeled Annexin V at 1:10 dilution (for 40 μL of sample, add 5 μL of AVBB 10× and 5 μL of Annexin V).

4. Let it rest for 30 min protected from light and then complete de volume with AVBB 1× so that it is ten times diluted.
5. To set the flow cytometer, prepare a tube containing beads only by diluting the intermediate bead suspension at 1:100 ratio in AVBB (1×). Acquire the bead sample to set a gate including events of 1 μm or smaller (<beads population) and excluding the electronical noise (Fig. 2a left panel).
6. Right before the acquisition, add the intermediate latex beads suspension to the sample at 1:100 dilution (5 μL in 500 μL of sample). Beads should not be added to all samples at once, only when they are about to be acquired, avoiding aggregation.
7. Among events smaller than 1 μm, set one gate in the population of beads and one gate in the population of MVs (annexin V-positive events) (Fig. 2a right panel).
8. To estimate the concentration of MVs in each sample, calculate the MVs/beads ratio and multiply for the bead concentration in the acquired sample. MVs will be found in the plasma fraction together with albumin (Fig. 2b).

3.5 Sample Dialysis

1. Pool the gradient fractions containing the lipoprotein of interest and the fractions containing plasma in individual tubes.
2. Deposit each sample type (plasma, VLDL, LDL, and/or HDL) into a pore membrane dialysis system.
3. Dialyze against PBS for 18 h at 4 °C under slow homogenization using a magnetic agitator. Replace the PBS at least once.
4. Recover the dialyzed samples, fractionate into smaller aliquots, and freeze until use.

3.6 MVs Isolation from Plasma

1. Centrifuge the dialyzed plasma samples obtained after lipoprotein elimination and the control plasma sample in 1.5 mL conical tubes at 19,000 × *g* for 2 h at 4 °C to pellet the MVs.
2. Carefully discard the supernatant with a pipette leaving only 50 μL in each tube.
3. Resuspend the MVs in 1450 μL of 0.22 filtered sterile PBS and homogenize the sample.
4. Repeat **steps 1** and **2**. Resuspend the MVs in the remaining 100 μL.

Fig. 1 (continued) and the method used to collect the gradient fractions. (**c**) The concentration of total cholesterol, triacylglycerol, and albumin in each fraction obtained after gradient ultracentrifugation. The gray rectangles highlight the fractions containing plasma, high-density lipoproteins (HDL), low-density lipoproteins (LDL), and very low-density lipoproteins (VLDL)

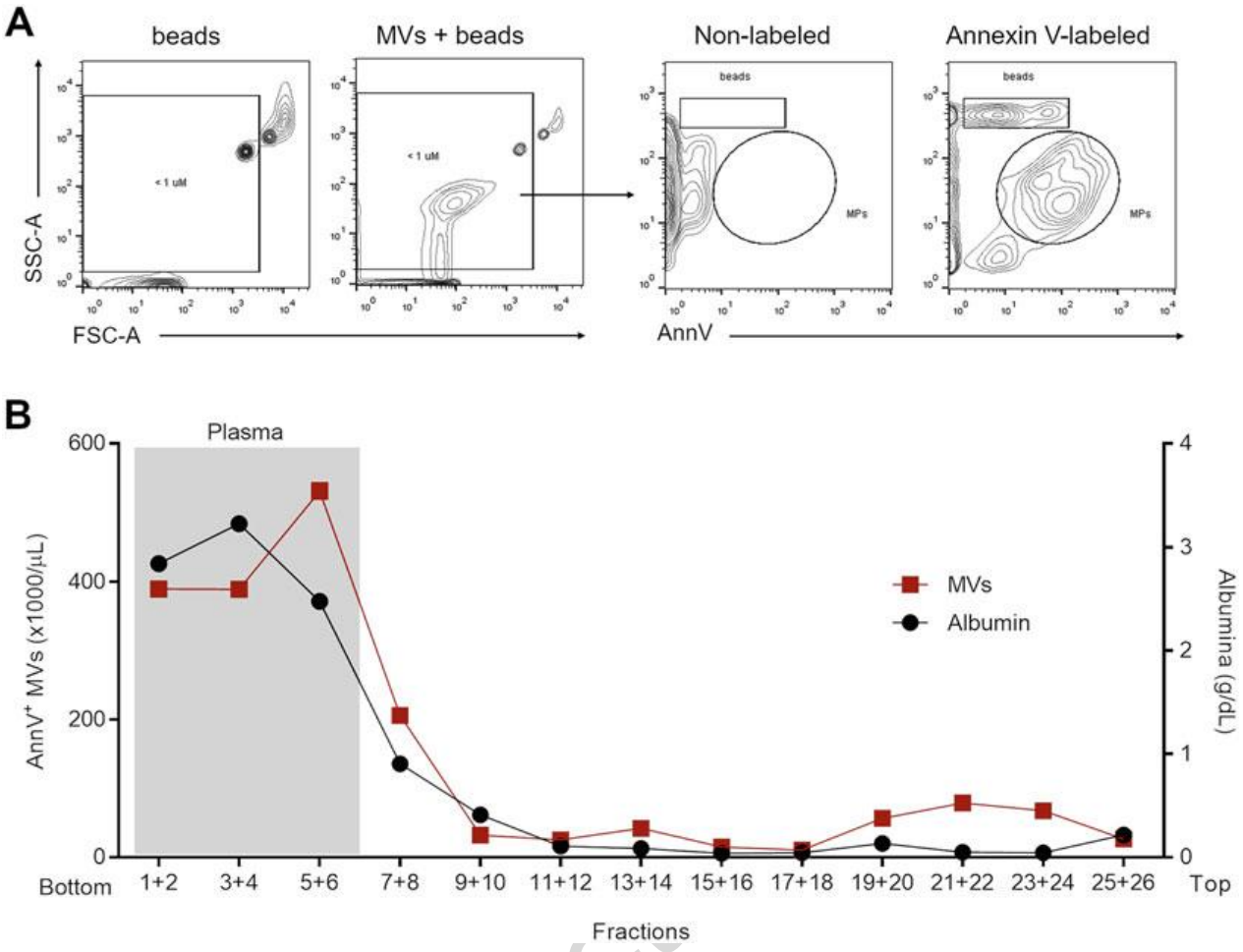

Fig. 2 Microvesicles identification and quantification through flow cytometry. (**a**) Gating strategy for flow cytometric analysis of microvesicles (MVs). Latex beads and MVs samples were acquired in a FACSCanto (Becton Dickson) cytometer. On the left, gating strategy to analysis of particles smaller than 1 μm (known size of the latex beads). On the right, gating strategy for counting beads and MVs (<1 μm and Annexin V^+ events). (**b**) The concentration of MVs and albumin in each two fractions of the gradient. The gray rectangle highlights the fractions containing plasma and MVs AU7

5. The recovery of MVs from control plasma and from lipoprotein-depleted plasma may be assessed through flow cytometry as described in Subheading 3.4. 239 240 241
6. Freeze the MVs samples until use. 242

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3.7 Detection of Apo B100 Through Western Blot

1. In order to ensure that the MVs samples are not contaminated by LDL, a Western blot can be performed targeting Apo B100. The transfer of high molecular weight proteins (i.e., >200 kDa) is challenging [12]. Therefore, due to the Apo B100 high MW (~513 kDa), some specific practices will be addressed in this section aiming at the efficient transfer of the proteins, including: 244 245 246 247 248 249 250
   (a) Low percentage of polyacrylamide separating gel (6%). 251
   (b) Transfer buffer with low methanol concentration (5%) containing 0.1% SDS. 252 253

(c) Transfer into PVDF instead of nitrocellulose membrane.

(d) Prolonged electro-transferring (16 h) at 40 mA, 4 °C.

3.7.1 MVs Samples Preparation

1. Unfreeze samples of isolated MVs from control or lipoprotein-depleted plasma (obtained in Subheading 3.6) and keep them on ice until use.
2. Homogenize the samples and remove a small volume (10 μL) of samples to quantify the protein concentration.
3. Separate 16 μL of the sample with the lowest protein concentration and a smaller volume of the more concentrated ones to have the same protein amount of each sample. Normalize the volume of all samples to 16 μL with distillate water. Add 4 μL of the 5× concentrated lysis buffer stock solution to obtain 20 μL of each sample. Briefly vortex and place the samples in the ultrasonic bath for 10 min.
4. Add the sample buffer by diluting the 5× concentrated stock into each sample (5 μL in 20 μL of sample). Boil at 95 °C for 5 min.

3.7.2 Protein Separation by Gel Electrophoresis

1. Prepare the separating 6% polyacrylamide gel and stacking gel by mixing the items described in Subheading 2.6, **item 4** (*see* **Note 8**).
2. Assemble the electrophoresis apparatus and set the gel; fill the gel box chamber with the running buffer.
3. Load 25 μL of each sample in different wells of the gel along with MW marker.
4. Turn on power supply and run the samples at 32 mA for about 120 min (*see* **Note 9**).

3.7.3 Protein Transfer to PVDF Membrane

1. Treat the PVDF membrane with 100% methanol for 2 min to activate the membrane before use because PVDF membrane is water repellent.
2. Assemble a transfer sandwich on the gel support cassette, which must be placed in the tank with the transfer buffer.
3. Transfer proteins from the gel to the membrane at 4 °C overnight at 40 mA (*see* **Note 10**).

3.7.4 Membrane Blocking and Immuno-Detection

1. After electroblotting, block the membrane with 5% low-fat dried milk in TBS-T for 1 h under continuous gentle shaking at room temperature.
2. Incubate the membrane with the primary antibody against Apo B100 solution (1:2000 in 5% low-fat dried milk in TBS-T) overnight under continuous gentle shaking at 4 °C.

3. Wash the membrane five times with TBS-T and incubate with the secondary antibody solution (1:10,000 in 5% low-fat dried milk in TBS-T) for 1 h under continuous gentle shaking at room temperature.
4. Wash the membrane five times with TBS-T, soak the membrane in the pico ECL substrate, and wrap the membrane in a plastic clear sheet protector. In a dark room, expose the hyperfilm ECL to the wrapped membrane in an X-ray film cassette (*see* **Note 11**).
5. ApoB100 must be detected only in MVs from plasma that was not subjected through gradient ultracentrifugation.

4 Notes

1. The quantification of MVs and other analysis such as procoagulant activity are described to be strongly influenced by pre-analytical variables in the MV isolation protocol [11, 12]. Some critical variables must be standardized in the plasma preparation stage, which are: do not use vacuum blood collection systems; handle blood carefully; use specific centrifugation conditions mentioned below; use PFP instead of PPP; use fresh samples.
2. Biosafety practices must be followed by a trained blood collection team and blood should be collected in the presence of anticoagulants (15% v/v of sterile ACD). Thoroughly homogenize the blood in the syringe and transfer it to conical polypropylene sterile centrifuge tube. To avoid unnecessary agitation, transport of the tubes must be carried out with care.
3. Store samples of PFP plus BHT in refrigeration if ultracentrifugation occurs the next day or freeze at −20 °C.
4. For 8 mL ultracentrifuge tube, add 2 mL of PFP-KBR mixture plus 6 mL of 0.9% saline solution. When using clinical samples, smaller sample volumes can be adapted for other tubes and ultracentrifuge rotors.
5. Equalize the weight by carefully removing the saline from the tube with more solution using the glass Pasteur pipette. Unbalanced tubes can damage your equipment.
6. Each drop contains approximately 50 μL, so to get 300 μL you must collect 6 drops for each fraction.
7. Preventing latex beads aggregation is important since beads counting in flow cytometry will be used to quantify the MVs concentration.
8. The glass sandwich with gels can be stored at 4 °C in running buffer for about 3 days.

9. You should see bubbles in the tank if the current is flowing. Since a low percentage polyacrylamide gel (6%) is used, low MW protein markers are expected to process faster than high MW proteins markers.
10. Place cooling ice packs in the tank around the transfer case. It is important that the system supports cooling overnight.
11. In samples where LDL is present, APOB-100 is abundant. Therefore, after 5 min of exposure there is a good detection of the protein.

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