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Investigating and imaging platelets in inflammation

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Abstract

Blood platelets are best known for their roles in hemostasis and thrombosis, but platelets also make important contributions to inflammation, immunity, and inflammatory resolution. Experiments involving depletion, genetic modification, and live imaging of platelets in animal models have increased our mechanistic understanding of platelet contributions to inflammation. In this minireview, we provide a critical overview of experimental techniques for manipulating and imaging platelets in inflammation models. We then highlight studies using innovative approaches to elucidate molecular mechanisms through which platelet subsets, platelet Fc gamma receptors, and pro-resolution platelet functions influence inflammatory responses. We also propose future technologies and research directions which might move us closer to harnessing of platelet functions for improved therapeutic modulation of inflammatory diseases.

1. Introduction: complex contributions of platelets to inflammation and immunity.

Blood platelets respond to trauma and inflammation by accumulating at sites of injury to help maintain hemostasis. Preservation of vascular barrier integrity is itself an essential component of host defense against infection, but a growing body of research indicates that platelets are much more than simple cell fragments that plug holes and occasionally dislodge to cause thrombosis.

Over the last two decades, with intensification in recent years, application of mouse genetic tools has greatly expanded our mechanistic understanding of context and time-dependent functions of platelets in inflammation. This minireview provides a summary of these advances and we refer interested readers to several broader reviews of the complex roles that platelets play in inflammation (Semple et al., 2011; Yeaman, 2014; Deppermann and Kubes, 2018; Middleton et al., 2018), and inflammatory resolution (Margraf and Zarbock, 2019; Ludwig et al., 2022).

In addition to aggregating at sites of trauma, it is now clear that platelets can roll on and become adhesive as singlets to the endothelium of inflamed blood vessels (Cleary et al., 2019; Shah et al., 2021). Platelets also interact with leukocytes, assisting in leukocyte activation and recruitment (Zarbock et al., 2006; Looney et al., 2009; Sreeramkumar et al., 2014; Pan et al., 2015; Rossaint et al., 2016; Zuchtriegel et al., 2016; Arnold et al., 2021), and promoting release of neutrophil extracellular traps (Clark et al., 2007; Caudrillier et al., 2012; Valet et al., 2022). Additionally, platelets act to seal breaches created by transmigrating leukocytes to prevent inflammatory bleeding (Gros et al., 2015; Rayes et al., 2018; Nicolai et al., 2020; Wu et al., 2021; Kaiser et al., 2022), migrate to collect and kill pathogens (Gaertner et al., 2017; Amison et al., 2018b), modulate adaptive immune responses (Amison et al.,

2018a; Wu et al., 2021), and respond to antibodies, complement activation, allergens and virulence factors (Joseph et al., 1983; Beutier et al., 2018; Cleary et al., 2020; Wu et al., 2021).

Pro-inflammatory mediators released by leukocytes can also influence the thrombotic function of platelets (Fuchs et al., 2010; Denorme et al., 2022; Joshi et al., 2022), platelet-derived extracellular vesicles can be pro-inflammatory (Boilard et al., 2010; Cloutier et al., 2013; French et al., 2020; McVey et al., 2021), and inflammation can alter megakaryocyte phenotypes and sites of platelet production (Campbell et al., 2019; Cunin et al., 2019; Pariser et al., 2021; Valet et al., 2022). Complicating matters, platelets can also promote resolution after inflammation (Rossaint et al., 2021), and mediators of inflammatory resolution can in turn affect platelet responses (Dona et al., 2008).

2. The expanding toolbox for in vivo studies of platelet contributions to inflammation.

How do we know how platelets respond to and influence complex inflammatory responses? Identification of causal roles for platelets in inflammation and the molecular mediators of these responses has been achieved through experimental depletion, reconstitution, and genetic modification of platelets predominately in mouse models of inflammation. **Table 1** provides a critical overview of these experimental approaches. In addition to these in vivo methods, approaches to alter human platelets are also useful for mechanistic studies, including recently-developed in vitro approaches to genetically or pharmacologically alter protein abundance (Suzuki et al., 2020; Trory et al., 2022).

The ability to image platelets interacting with leukocytes and endothelial cells using in vivo models of inflammation has also helped build our understanding how platelets contribute to inflammation. Intravital imaging techniques for studying platelets are summarized in **Table 2**.

Of note, *PF4*-Cre transgenic mice crossed to fluorescent reporter lines now permit fluorescent labelling of all megakaryocytes and platelets with sufficient signal to track individual platelets over time. Imaging in these mice reveals diverse platelet responses. For example, after induction of experimental transfusion-related acute lung injury, platelets can be observed rolling on the endothelium, interacting with leukocytes and neutrophil extracellular traps, forming aggregates in response to inflammation ("immunothrombosis"), and migrating - all within one blood vessel segment (**Figure 1**) (Cleary et al., 2020).

3. Key future directions for research into platelet roles in inflammation

Harnessing platelet heterogeneity for immune-modulatory platelet transfusion therapies

The circulating platelet pool is heterogenous at baseline and after activation (Blair and Frelinger, 2019), but determining in vivo functions of different platelet subsets has been limited by the difficulty of isolating platelet populations for downstream functional assays. Valet et al., (2022) recently overcame challenges in ascertaining the ontogeny and function of a platelet subset using fluorescent reporter mice in transplantation and transfusion experiments in a model of septic peritonitis.

Spleen intravital in *PF4*-Cre:mTmG mice and flow cytometry studies enabled identification that the septic state involved increases in the number of platelet-producing splenic megakaryocytes and circulating platelets expressing high levels of surface CD40 ligand (CD40L). Splenectomy diminished the $CD40L^{high}$ platelet population and spleens transplanted from post-sepsis donors into naïve recipients were found to release $CD40L^{high}$ platelets for several days after transplantation. Together, these findings identified spleen megakaryocytes as the source of the circulating $CD40L^{high}$ platelet subset expanded in sepsis.

Platelet transfusions are under investigation as a potential therapeutic approach for treatment of sepsis (Xiang et al., 2013). Valet and colleagues hypothesized that splenic production of $CD40L^{high}$ platelets

might be an adaptive response in the context of sepsis, and so transfusions enriched for this platelet subset might have greater therapeutic value relative to platelets from healthy donors. Washed platelets isolated from post-sepsis donors, and therefore enriched for the CD40L^{high} population, were indeed found to be more protective than washed platelets from naïve mice when given as a transfusion therapy to other mice in the early stages of sepsis. Relative to platelet transfusions from naïve donors, transfusions of post-sepsis washed platelets reduced bacteremia, neutrophilia, plasma levels of TNFα and IL-6, and markers of organ injury (**Figure 2**).

The results of this study suggest that transfusions with platelet products enriched for immune-modulatory platelet subsets might be useful for treating immunopathology. Platelet products are now being produced in vitro for clinical use (Suzuki et al., 2020; Sugimoto et al., 2022). The initial focus of these efforts has been on minimizing immune interactions, but it may also be useful to develop approaches to produce platelet products enriched for immune-modulatory platelet subsets to 'tailor' lab-grown platelet products to the needs of patients.

Beneficial effects of transfusions with washed platelets from post-sepsis donors were linked to CD40L-mediated increases in neutrophil extracellular trap (NET) release (Valet et al., 2022). As NETs can be both beneficial and harmful in a context-dependent manner, it will be important to identify how this platelet subset influences pathophysiology in other disease settings and alters other cell types (Huang et al., 2012). It would also be interesting to determine whether delivery of CD40L by platelets or platelet-derived extracellular vesicles is required for protective effects in sepsis models, or whether CD40L agonism alone might be sufficient for therapeutic effects early in sepsis, as this approach has been studied in the context of cancer immunotherapy (Elgueta et al., 2009).

Testing therapeutics for antibody and platelet-mediated inflammatory diseases using humanized mice

A major limitation of using mouse models to study platelet responses in human inflammatory diseases is that human platelets express functional FcγRIIA (FCGR2A, CD32A) whereas mouse platelets lack Fcγ receptors. This difference makes mouse platelets weakly responsive to antigen-bound IgG relative to human platelets. The absence of Fcγ receptors on mouse platelets has had major consequences for clinical translation. For example, anti-CD40L blocking antibodies had powerful immunosuppressive effects in mice, but trials which were discontinued when anti-CD40L treatment was associated with Fcγ receptor-dependent thromboembolism (Kawai et al., 2000).

To better model human biology, transgenic mice expressing human FcγRIIA on platelets and myeloid cells were created (McKenzie et al., 1999). These mice display increased susceptibility to pathophysiology in models of immune thrombocytopenia (McKenzie et al., 1999), immune complex-mediated shock and thrombosis (Robles-Carrillo et al., 2010; Beutier et al., 2018; Cloutier et al., 2018; Laroche et al., 2022), rheumatoid arthritis (Mkaddem et al., 2014; Duchez et al., 2015), and systemic lupus erythematosus (Melki et al., 2020).

A recent study from el Mdawar et al., (2021) used FcγRIIA-humanized mice to explore potential therapeutic approaches to limit immunopathology driven by platelet FcγRIIA. Acute lung injury was induced in mice through passive intravenous transfer of an MHC class I monoclonal alloantibody. Mice expressing FcγRIIA developed worse antibody-mediated acute lung injury compared to littermate controls lacking FcγRIIA expression (**Figure 2**). Alloantibodies were found to trigger platelet serotonin release through FcγRIIA, and the 5HT2A/5HT2BA serotonin receptor antagonist sarpogrelate showed efficacy against antibody-mediated acute lung injury.

Modulation of platelet Fcγ receptor responses will be an area of future interest as Fcγ receptors have been implicated as mediators of platelet and immune cell pathophysiology in severe COVID-19 (Althaus et al., 2021; Combes et al., 2021; Apostolidis et al., 2022; Junqueira et al., 2022). Biologic

approaches to block FcγRs have been developed (Mkaddem et al., 2014), but it is currently unclear whether these therapeutic candidates inhibit platelet FcγRIIA responses in vivo. In addition to serotonin, platelets also release its metabolite 5-HIAA which can act as a neutrophil chemoattractant via GPR35 receptors (de Giovanni et al., 2022), so it would be of interest to determine whether approaches to block GPR35 affect antibody-mediated inflammation.

Identifying platelet-derived pro-resolution mediators in neutrophilic inflammation

Inflammatory platelet responses can occur within seconds (**Figure 1**). Many studies have therefore focused on the role of platelets in initiating inflammation and immunity. However, most patients requiring treatment for immune-mediated diseases present after acute inflammatory onset and have already manifested collateral tissue damage. From a translational perspective, mechanisms driving inflammatory resolution and tissue repair are therefore of great interest for therapeutic development.

A recent study provides useful examples of strategies for examining roles of platelets in inflammatory resolution and compelling findings as to why studying inflammatory resolution is important. Previous reports demonstrated that platelet depletion before bacterial lung infections results in decreased lung neutrophilia and impaired bacterial clearance (de Stoppelaar et al., 2015; Rossaint et al., 2016; Amison et al., 2018b). Rossaint and colleagues (2021) took a different approach of assessing the effects of platelet depletion two days after lung infection with *Klebsiella pneumoniae* – a time point when infections have typically cleared but immunopathology continues to cause acute lung injury.

Studying outcomes of mice depleted of platelets using two different approaches revealed that platelet depletion two days after infection led to failed resolution of lung neutrophilia. Delayed neutrophilic inflammation in thrombocytopenic mice could be reversed with intravenous or intratracheal platelet transfusions. Mechanistically, induction of platelet-dependent inflammatory resolution was linked to action of CD40L and ADAM8, mediators associated with pro-inflammatory responses at initiation of inflammation (Conrad et al., 2022; Valet et al., 2022), promoting increased interactions of platelets with T regulatory lymphocytes involving P-selectin, PSGL-1, and CD40 leading to altered production of cytokines and pro-resolving lipid mediators (**Figure 2**).

Based on these results, failed resolution of neutrophilic inflammation following bacterial pneumonia might have negative effects on the health of patients who are thrombocytopenic, perhaps explaining some of the association of thrombocytopenia with poor outcomes in critically ill patients (Vanderschueren et al., 2000; Wang et al., 2014). It could therefore be interesting to test whether washed platelet transfusions improve clinical outcomes in the context of thrombocytopenia and neutrophilic acute lung injury. The results of this study suggest that failed resolution of neutrophilic lung inflammation may also warrant investigation as a potential adverse effect of therapeutics designed to inhibit inflammation, as mouse experiments indicate that P-selectin blockers (e.g. crizanlizumab) or inhibitors of CD40L (e.g. dapirolizumab pegol) might delay resolution of neutrophilic inflammation.

4. Concluding remarks

Advances in molecular biology have provided a wealth of tools for interrogating the contribution of platelets to inflammatory responses in vivo. Translating research findings from these models into improved health outcomes will require careful dissection of the roles of various platelet subsets across the complete time course of inflammatory responses in models designed to reflect human immune systems. In **Table 3** we suggest potential future experimental approaches which could be useful for advancing research aimed at therapeutic harnessing of the roles of platelets in inflammation.

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421 **Table 1. Critical overview of experimental approaches to genetically modify or deplete platelets in animal models.**

Megakaryocyte/platelet-restricted genetic modification strategies			
Strategy	**Strengths**	**Limitations**	**References**
PF4-Cre	• Efficient Cre-lox recombination in all megakaryocytes and platelets. Co-expression of Cre and loxP-flanked exons can be used for lineage restricted gene knockout, and Cre lines can also be combined with lox-stop-lox reporters (e.g. Ai14, mTmG lines) for imaging and lineage tracing (see **Table 2**).	• Can induce recombination in some non-megakaryocyte hematopoietic cells as well as non-hematopoietic lineages. • Potential for immunological abnormalities due to copies of CXC chemokine genes in Cre construct. • Genomic location of transgene insertion unknown. • Carriers show subtle changes in platelet count and volume.	(Tiedt et al., 2007; Pertuy et al., 2015; Beckers et al., 2017; Nagy et al., 2019)
Gp1ba-Cre	• Cre-lox recombination exclusive to mature megakaryocytes and platelets without affecting leukocytes.	• Does not induce recombination in all megakaryocytes or platelets. • Cre knock-in replaces endogenous *Gp1ba* gene resulting in the subtle platelet phenotype of heterozygous loss of Gp1b-α function.	(Nagy et al., 2019)
Platelet depletion strategies			
Strategy	**Strengths**	**Limitations**	**References**
Busulfan	• Has been used in rat, rabbit and guinea pig models as well as in mice. • 'Add-back' experiments possible using platelet transfusions.	• Platelet depletion only partial. • Although dosing can be titrated to have some selectivity for megakaryocytes, busulfan likely affects all proliferating cells. • Repeated injections required. • Effects of busulfan on inflammatory responses may be due to widespread platelet/cell apoptosis rather than due to low blood platelet counts.	(Kuter and Rosenberg, 1995; Sirois et al., 1997; Keir et al., 2015; Pan et al., 2015; Qiao et al., 2016)
Anti-platelet antibodies	• Monoclonal antibodies can be highly specific to platelet antigens e.g. CD41 (Itga2b) or CD42b (Gp1ba). • Polyclonal antisera-mediated depletion approaches have been used in non-mouse animal models. • Depletion can be near-complete with rapid onset (within hours) and sustained reduction.	• Effects of antibodies on inflammatory responses can be due to Fcγ receptor ligation, mononuclear phagocyte system overload or platelet clumping/activation rather than low blood platelet counts. • 'Add-back' platelet transfusions only possible with transfusions of platelets lacking antigen of platelet-depleting antibody.	(Lefort and Vargaftig, 1978; Nieswandt et al., 2000; Looney et al., 2009; Strait et al., 2011; Hechler et al., 2016)
PF4-Cre: *Rosa26*^{iDTR}	• Depletion can be near-complete. • Depletion relatively fast (within days of treatment with diptheria toxin (DT)), sustained reduction. • 'Add-back' experiments possible using platelet transfusions.	• Mean platelet volume slightly increased before DT injections. • Repeated DT injections required, with antibody responses typically generated against DT which may alter immune responses. • Breeding and genotyping of mice required.	(Hechler et al., 2016; Salzmann et al., 2020)
c-mpl$^{-/-}$ (*Mpl*$^{-/-}$)	• Constitutively thrombocytopenic due to knockout of the receptor for thrombopoietin (Tpo). • 'Add-back' experiments possible using platelet transfusions.	• Thrombocytopenia only partial. • Mice also have altered hematopoietic stem cell phenotype.	(Gurney et al., 1994; Kimura et al., 1998)
Tpo-targeted ASO	• Inducible approach to mimic c-mpl$^{-/-}$ thrombocytopenia phenotype. • 'Add-back' experiments possible using platelet transfusions.	• Depletion only partial. • Repeated injections required. • Potential off-target effects of exogenous RNA and loss of Tpo expression.	(Barrett et al., 2020)

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Table 2. Strengths and limitations of intravital microscopy approaches to image platelet responses in inflammation.

Platelet imaging approach	Strengths	Limitations	References/example(s)
Phase contrast microscopy	• Label-free.	• Only rolling/adhesive platelets visible. • Not always clear whether small objects are platelets.	(Frenette et al., 1995)
Injections with dyes e.g. Rhodamine 6G	• Simple to use.	• Also labels leukocytes • Not useful for imaging megakaryocytes.	(Herr et al., 2015)
Transfusions with ex-vivo labelled platelets	• Bright and specific platelet labeling.	• Potential for altered function of platelets. • Time taken for platelet isolation and labeling. • Only a subset of platelets labelled. • Not useful for imaging megakaryocytes.	(Frenette et al., 1995)
Fluorophore-conjugated antibodies targeting platelet markers e.g. CD41 or CD49b	• Simple to use.	• Not useful for imaging megakaryocytes. • Potential for antibodies to affect platelet function/lifespan.	(Rauova et al., 2010; Jenne et al., 2011; Wong et al., 2013; Shah et al., 2021)
CD41-YFP transgenics.	• Bright platelet labelling.	• Only a subset of megakaryocytes and platelets labelled. • Basophils also labelled.	(Zhang et al., 2007; Jenne et al., 2011)
PF4-Cre combined with fluorescent reporter alleles. .	• All megakaryocytes and platelets can be labeled. • Possible to combine with other genetic/antibody labels. • Different reporters provide options to label membranes (mTmG), cytoplasm (Ai14) or stochastic labelling with 1 of 4 fluorophores (Confetti).	• *PF4*-Cre can label some cells outside of the megakaryocyte/platelet lineage. • Potential for recombination or fluorescent proteins to affect cellular function.	(Tiedt et al., 2007; Ortiz-Muñoz et al., 2014; Gaertner et al., 2017; Lefrançais et al., 2017; Cleary et al., 2019, 2020; Nicolai et al., 2020; Bornert et al., 2021; Pariser et al., 2021)

427 **Table 3: Potential future additions to the platelet research toolbox.**

Tools that could be useful for platelet research	Why?
Platelet/megakaryocyte-restricted Dre lines.	Expanding experimental/imaging study possibilities by allowing combined use with other Cre reporters/knockouts.
Inducible platelet/megakaryocyte-restricted Cre/Dre/rtTA lines.	Experimental genetic alteration of megakaryocytes/platelets with temporal and potentially spatial control.
Transgenic lines and reagents for use in studying platelets in model organisms other than mice.	Allowing study of platelets in context where differences between humans and mice make studies in non-mouse model organisms desirable.
Improved fluorescent reporters and microscopy approaches.	Current intravital imaging approaches lack spatiotemporal resolution needed to live-image platelet microparticles.
Approaches to sort/enrich large numbers of functional platelets without functional disturbances.	Low throughput and functional disturbances mean that current flow-assisted cell sorting approaches are not appropriate for most transfusion/ex vivo studies comparing function of platelet subsets.
Additional platelet-megakaryocyte-restricted Cre lines.	Existing Cre lines have specificity issues (*PF4*-Cre) or suffer from low penetrance and effects from replacing endogenous targeting locus (*Gp1ba*-Cre).
New humanized mouse lines.	Mouse model validity is limited by differences between mice and human platelets which have not been successfully addressed by humanization, for example differences in proteinase-activated receptor repertoire.
New imaging agents for labelling platelets and platelet activation markers.	Commercially available antibodies can activate platelets or accelerate their clearance. New molecular imaging approaches are needed for non-invasive detection of platelets responses in microvasculature using microscopy and whole-body imaging.

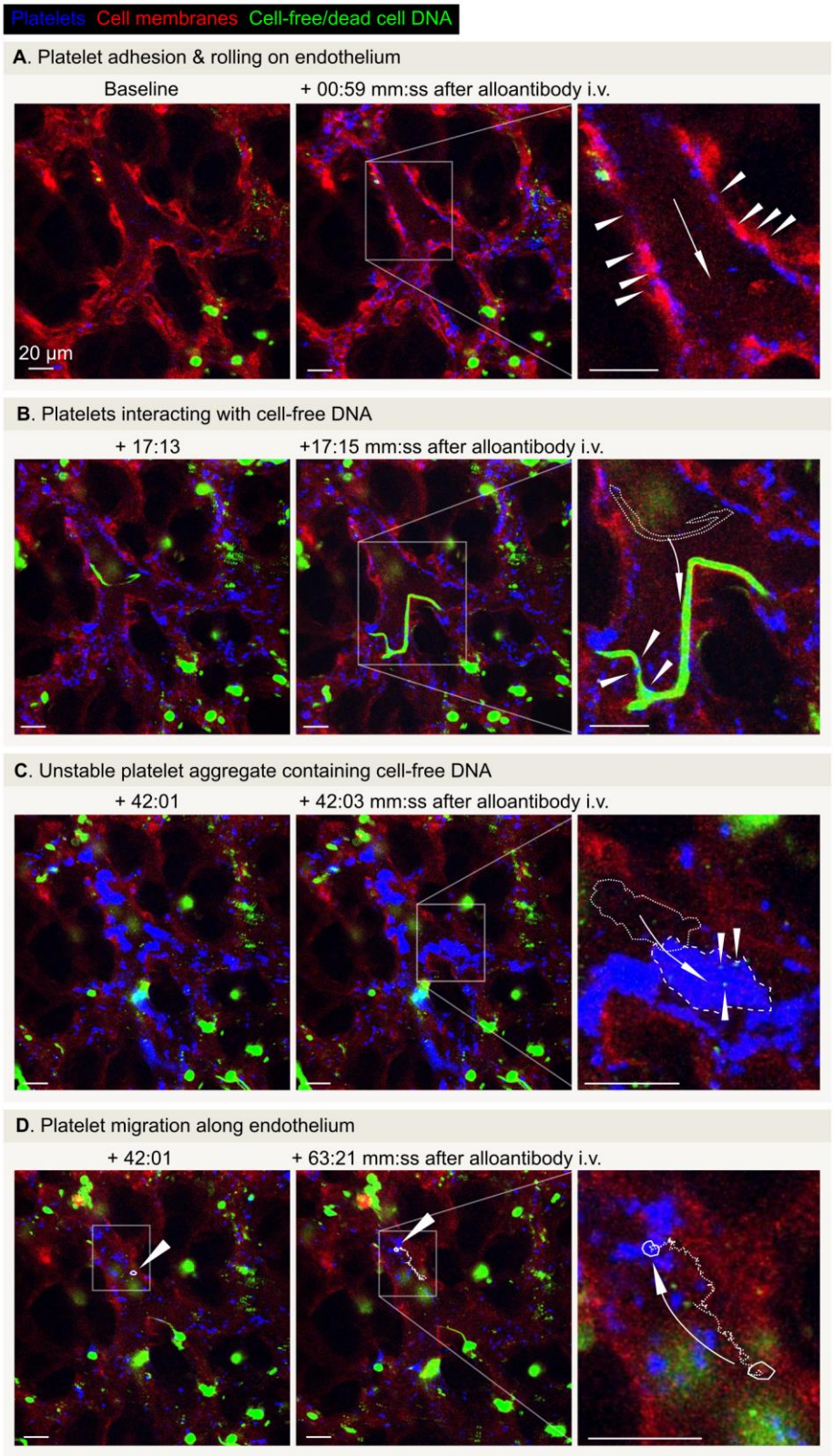

Figure 1. **Diverse platelet responses during initiation of acute lung inflammation.**
A. Intravital imaging of platelets (blue, *PF4*-Cre:*Rosa26*^{mTmG} reporter mouse), cell membranes (red) and cell-free/dead cell DNA (green, intravenous (i.v.) labeling with SYTOX Green dye). Images show changes in a pulmonary arteriole and surrounding alveolar capillaries following induction of inflammation with an i.v. injection of MHC class I alloantibody. Arrow: Direction of blood flow. Arrowheads: Platelets rolling on arteriolar endothelium.
B. Single platelets interacting with a cell-free DNA fiber, likely from neutrophil extracellular trap (NET) release. Arrow: Movement of DNA fiber from bloodstream to arteriolar branch. Arrowheads: Platelets associated with DNA fiber.
C. Unstable platelet aggregate in inflamed lung. Arrow: Movement of platelet aggregate from endothelium into bloodstream. Arrowheads: Cell-free DNA within platelet aggregate.
D. Platelet migration against flow of blood towards a platelet aggregate. Arrowheads: Migratory platelet. Arrow: Direction of platelet movement of platelet movement. Dotted line: Platelet migration track.
Adapted from movie available in supplement of Cleary et al. (2020). All scale bars are 20 µm.

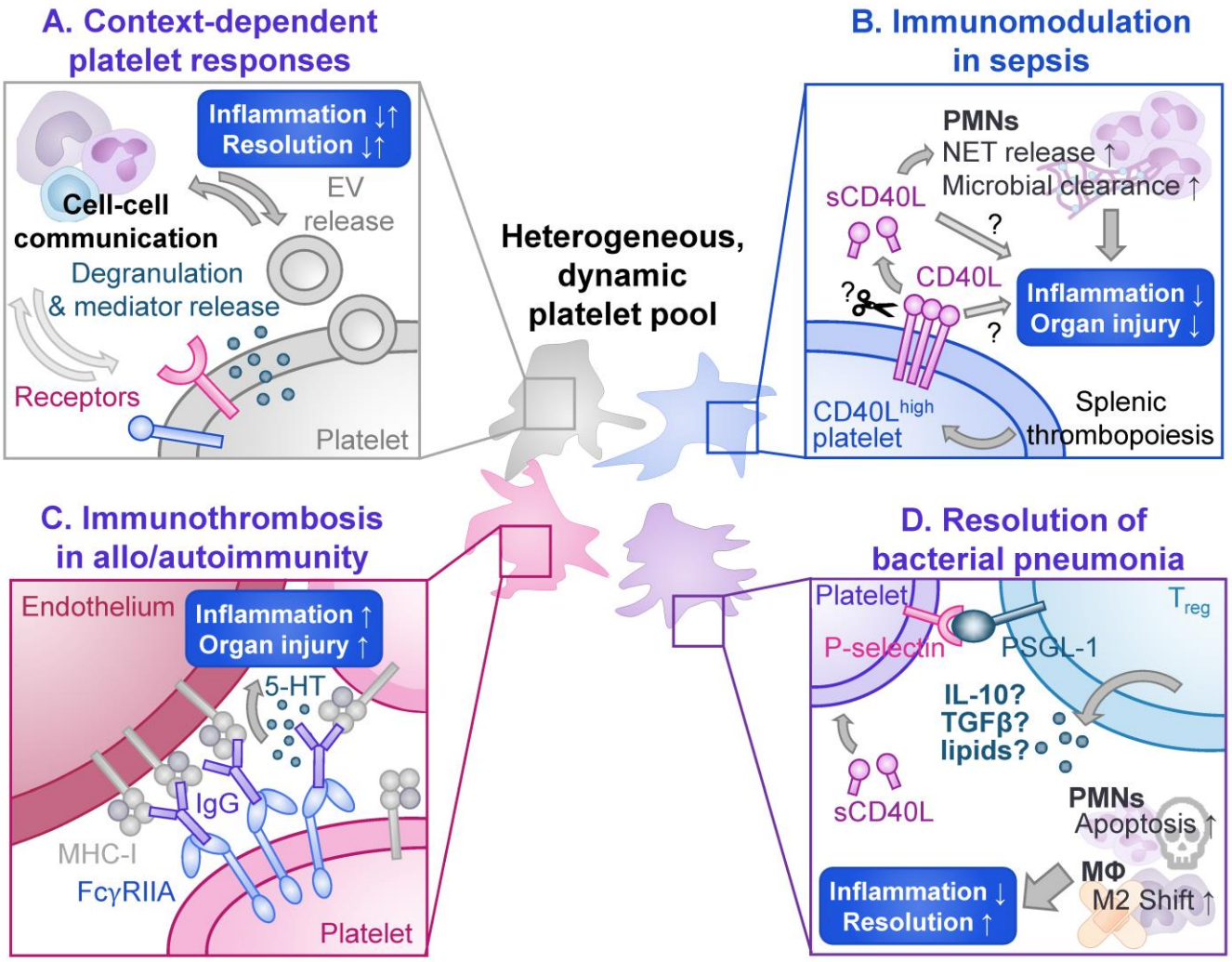

Figure 2. **Recent mechanistic insights into platelet roles in inflammation.**
A. The circulating platelet pool is heterogeneous, dynamic and can contribute to initiation, propagation and resolution of inflammation depending on context and timing. Platelets communicate with other cells through reciprocal exchange of extracellular vesicles, granule contents and various mediators, with adhesion and signaling mediated through surface receptors.
B. In models of sepsis, $CD40L^{high}$ platelets are produced in the spleen. This platelet subset promotes release of neutrophil extracellular traps (NETs) and microbial clearance, reducing inflammation and injury (Valet et al., 2022).
C. IgG alloantibodies targeting MHC class I (MHC-I) are bound by FcγRIIA on platelets resulting in serotonin (5-HT) release which exacerbates inflammation and injury (el Mdawar et al., 2021).
D. Circulating soluble CD40L (sCD40L) increases after bacterial lung infection, promoting platelet-T regulatory cell (T_{reg}) interactions through mediated by P-selectin and PSGL-1. After infection and inflammation are established, platelets are important for increases the number of T_{regs} in lungs, apoptosis of neutrophils (PMNs) and switching of macrophages (MΦ) to pro-reparative 'M2' phenotypes to resolve inflammation (Rossaint et al., 2021).