The role of platelets in the pathogenesis of and immunity to helminth infections

By

Jonathan Pollock

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(Clinical Science and Immunology)

Supervisor: Assoc Prof. William Horsnell

Co-supervisor: Dr. Matthew Darby

Faculty of Health Sciences

University of Cape Town

DECLARATION

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Abstract

Background: Platelets are small, anucleate cells which circulate in blood and are often the first to respond to tissue damage and vascular inflammation caused by pathogens. Here they not only maintain tissue integrity and prevent bleeding, but also initiate and regulate a vast variety of immunologic responses. Little is known on the role of platelets in helminth infections, despite our understanding that many helminth species cause significant vascular pathology as they transfer from the circulatory system to diverse tissues as part of their life cycles. Based on previous studies showing tight association between platelets and innate immune responses during infection with other pathogens, we hypothesized that platelets significantly contribute toward acute (vascular) immunity to helminth infection.

Objectives: This project aimed to investigate the role of platelets in regulating acute innate immune responses following infection with the murine gastro-intestinal nematode *N. brasiliensis* (Nb), commonly used to model human helminthiases. Specifically, it aimed to characterized platelet-regulated responses involved in acute innate immunity during the pulmonary stage of infection, in which larvae exit the pulmonary vasculature and invade host lung tissue.

Methods: C57BL/6mice were infected with 500 L3 Nb larvae, and the association of platelets with acute innate immune responses in the circulation and the lung were established by flow cytometry and immunohistochemistry. In further experiments, mice were depleted of their platelets using antibodies prior to infection with Nb and the effect of this on pulmonary pathology and innate immune responses was inferred from flow cytometric and histologic analyses of pulmonary tissues. Lastly, antibodies were used to interfere with platelet receptors during Nb infection to gain mechanistic insight into platelet regulation of neutrophil responses.

Results: Infection with *N. brasiliensis* was associated with significant changes in the activation of platelets, their localisation into lung tissue and their interaction with innate immune cells. Additionally, platelet -immune cell interaction was associated with changes in the expression of factors known to play a role in driving the early immune response to Nb, including IFN-γ and RELM-α.
Furthermore, mice depleted of their platelets prior to infection had significantly enhanced pulmonary pathology and rapidly succumbed to infection. This was associated with significant changes in neutrophil responses, and depletion of neutrophils together with platelets significantly protected against enhanced pathology.

Finally, direct and indirect targeting of the platelet receptors CD62P and CLEC-2 did not result in significantly enhanced pulmonary pathology but was associated with altered platelet and neutrophil responses.

Conclusion: Herein, we have provided evidence that platelets tightly associate with protective host responses during acute *N. brasiliensis* infection and that their absence correlates with a dysregulated neutrophil response and enhanced helminth – associated pulmonary pathology.

These data therefore collectively show that platelets play notable roles in the acute innate immune response to *N. brasiliensis* and that future investigations into the immunological functioning of platelets during helminth infection are warranted.

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List of abbreviations

AAM	alternatively activated macrophages
ADP	adenosine di-phosphate
ALI	Acute lung injury
APC	antigen presenting cell
AR	anthelmintic resistance
ARDS	acute respiratory distress syndrome
BAL	Broncho-alveolar lavage
CD41	cluster of differentiation 41
CD62P	cluster of differentiation 62 P
CLEC-2	c type lectin-like receptor 2
CLP	chitinase-like protein
CLRs	c-type lectin receptors
CXCL4	CXC chemokine ligand 4
DMEM	Dulbecco's modified Eagle Medium
DVT	deep vein thrombosis
EETs	eosinophil extracellular traps
FACS	fluorescence – activated cell sorting
FMO	fluorescence minus one
GrzB	granzyme B
H&E	Haematoxylin & Eosin
IFN-γ	Interferon gamma
IL	interleukin
LDN	low-density neutrophils
LPS	Lipopolysaccharide
LPS	Lipopolysaccharide
Ly6G	Lymphocyte antigen 6 complex locus G6D
MDSC	myeloid-derived suppressor cells

MFI	mean fluorescence intensity
miRNAs	microRNAs
Nb	Nippostrongylus brasiliensis
NETs	neutrophil extracellular traps
NGS	next generation sequencing
Nippo	*N. brasiliensis*
Pam$_3$CSK4	Pam3CysSerLys4
PAMPs	pathogen-associated molecular patterns
PAR	Protease activated receptor
PBS	Phosphate buffered saline
PF-4	platelet factor 4
PFA	Paraformaldehyde
PRP	platelet rich plasma
RAGE	receptor for advanced glycation end products
RANTES	Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted
ROS	reactive oxygen species
SARS-CoV 2	severe acute respiratory syndrome coronavirus 2
SiglecF	Sialic-acid-binding immunoglobulin-like lectin F
TGF-βR	Transforming growth factor β receptor
T$_H$2	T helper 2
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor - α
TSLP	thymic stromal lymphopoietin
t-SNE	t-distributed stochastic neighbour embedding
vWF	von Willebrand Factor

Chapter 1: Introduction

Platelets - essential regulators of haemostasis and thrombosis

Platelets are small, anucleate cells that circulate in large numbers in blood of mammals and play important physiologic roles in haemostasis and thrombosis, i.e., in the maintenance of proper blood flow and in the prevention of haemorrhaging at vascular lesions. The precise molecular and cellular mechanisms that platelets participate in and engage with to mediate their haemostatic and thrombotic functions are complex and involve a large number of proteins and lipid mediators. However, broadly speaking, their function as haemostatic cells relies principally on their interaction with exposed sub-endothelial matrix proteins, such as collagen or von Willebrand Factor [1], and their capacity to mediate, both structurally and biochemically, the many proteolytic reactions involved in the coagulation cascade [2].

"Classical" haemostasis

The interaction of platelets with specific endothelial cell proteins results in their activation and is seminal to downstream processes involved in primary haemostasis, in other words, in the aggregation of platelets at sites of vascular injury. Platelet-endothelial cell interaction is governed by specific integrins, most notably the glycoprotein (GP) GP VI and GP Ib-V-IX complexes ,which bind many different soluble and membrane-associated ligands [3]. The engagement of platelet glycoprotein receptors with endothelial proteins elicits signal transduction pathways that mediate further processes and potentiate platelet activation[3]. In the specific context of haemostasis and thrombosis , these processes culminate in the release and secretion of a wide array of compounds stored in platelet granules , such as thromboxane A2 and ADP [3] and specific changes in the conformation of platelet integrins , notably α_{IIb}-β_3 (GP-IIa-IIIb) [4] .These events are co-ordinated by specific modifications to the plasma membrane of platelets, and heavily involve changes in the flux of calcium (Ca^{2+}) ions and other secondary messenger molecules [1].These in turn lead to significant changes in the arrangement of cytoskeletal proteins, and the formation of pseudopodia on the surfaces of activated platelets [5]. The structural and morphological changes associated with platelet activation allow them to engage in distinct functions [6] adhere strongly to a variety of different cells, notably other platelets. Morphological differences in platelets also allow them to be distinguished into nascent or old platelets [6] and allow them to be differentiated from resting platelets, which have a discoid shape [7] .

Once platelets become aggregated at lesions, they carry out pro-coagulant activities that result in, amongst other things, the generation of thrombin, which is a catalytic protein that plays key roles in the conversion of fibrinogen to fibrin. Fibrin is an insoluble scaffolding protein that, together with platelets and other cells, is an important constituent of developing thrombi [2] and is therefore crucial for haemostasis. Indeed, the dissolution of thrombi principally involves fibrinolytic processes, especially the degradation of fibrin by plasmin [7].

In short, platelets maintain haemostasis by binding to disrupted endothelial cells, predominantly via adhesive glycoproteins, then inducing autocrine and paracrine signalling pathways that elicit further platelet aggregation, and finally by facilitating the proteolytic conversion of coagulation factors, which in turn mediate the generation and deposition of fibrin. These processes are collectively termed (classical) haemostasis , and are crucial for preventing haemorrhaging in tissues and organs in response to physical damage to blood vessels [1].

Inflammatory haemostasis

In addition to preventing blood loss at vascular lesions with significant pathology, platelets also have critical function in maintaining haemostasis during inflammation (called inflammatory haemostasis), i.e. even in conditions where overt vascular damage is absent [8]. Furthermore, as recent studies demonstrate, the mechanisms underlying inflammatory haemostasis are distinct from classical haemostasis (or thrombosis), both at the cellular and molecular level [8] (Figure 1.1). For example, it has been shown that the prevention of bleeding following LPS induced lung inflammation does not rely on GP-IIa-IIIb dependent platelet aggregation, but instead depends on other receptors, including the immunoreceptor tyrosine activation motif (ITAM)–containing receptors GPVI and CLEC-2 [9]. Studies investigating the role of platelets in maintaining vascular integrity and haemostasis under disparate conditions, e.g. physical injury versus sterile inflammation, have therefore provided evidence that the role for platelets in mediating haemostatic processes is complex and that the mechanisms initiated depend on the physiological context. Furthermore, by exploring the nature of platelet as mediators of coagulatory processes under different conditions, our understanding of platelets has evolved. This has allowed us to recognise that

platelets not only perform haemostatic function, but also play significant roles in initiating and regulating inflammatory and immune processes.
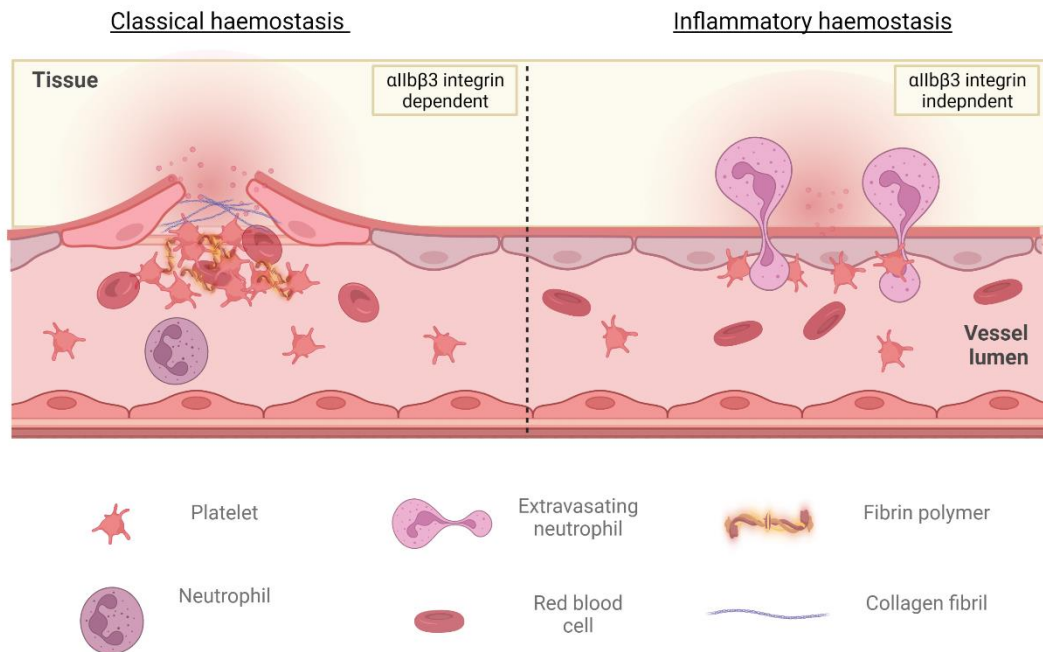
Figure 1.1: Classical haemostasis versus inflammatory haemostasis. During classical haemostasis, subendothelial matrix proteins become exposed to the intravascular lumen. Platelets aggregate at sites of damage and, together with crosslinked fibrin, form thrombi in an αIIbβ3-dependent manner. Inflammatory haemostasis entails platelets sealing breakages in vessels caused by extravasating neutrophils and other leukocytes and does not depend on the activity of αIIbβ3 . Figure created on Biorender.com.

Immunothrombosis and thrombo-inflammation : platelets bridge coagulation with inflammation and immunity

An evolutionary perspective

Haemocytes are nucleated granular cells that circulate in the blood of lower invertebrates, such as horseshoe crabs, and are thought to be the evolutionary precursors to mammalian

leukocytes [10]. Where in vertebrates and higher organisms, blood leukocytes have become specialized and are morphologically and functionally distinct from one another, in lower invertebrates, haemocytes have broad physiologic and immune function (with a lower degree of specialisation [11]) . As such, haemocytes are not only central to coagulation of the haemolymph and the sealing of breaches in the exoskeleton, but also function as bona fide immune cells and can phagocytose pathogens or produce factors that are cytotoxic to them [10]. The biology of haemocytes can therefore provide important context to recent experimental data that clearly demonstrate that platelets, similar to haemocytes, play significant roles in mediating inflammatory and immunologic processes [12].

Thrombo-inflammation

In line with our expanded understanding of platelet function, researchers have coined new terms to underscore the association of platelets with (vascular) inflammation. For example, the term "thrombo-inflammation", first used in the context of ischemia-reperfusion injury in models of stroke [13] , describes the role of platelets in mediating inflammation in a manner independent of classical platelet aggregation (e.g. that which occurs during "classical" haemostasis)[13]. Central to our reframing of platelets in stroke and ischemia-reperfusion injury, were studies that explored the effect of platelet inhibition on disease progression. These showed that disease progression (driven principally by inflammatory responses) could be modulated through the inhibition of platelets and their receptors, without affecting bleeding risk [14]. The results of these studies therefore suggested that platelets have important functions in these models , outside of their roles in haemostasis [13].

At the same time as researchers were beginning to uncover important roles for platelets in regulating inflammation during experimental stroke, others were functionally characterizing the response of platelets to direct stimulation with live pathogens or pathogen associated molecular patterns (PAMPs). An important seminal study in this regard (and which was also one of the first to use the term thrombo-inflammation), was a study by Blair et al. on the response of platelets to the TLR-2 agonist Pam$_3$CSK4 [15], in which they provided evidence that platelets functionally integrate signals received via toll-like receptors into classical inflammatory responses, including the production of reactive oxygen species and the formation of immune cell complexes [15].

It is now well established that platelets express a variety of pattern recognition receptors, including Toll-like receptors (TLRs), C-type lectin receptors(CLRs) and Sialic acid binding immunoglobulin type lectins (Siglecs), which collectively allow platelets to sense and respond to a diversity of infectious pathogens [16] and to induce (thrombo-) inflammatory responses (Figure 1.3). In accordance with this, the term thrombo-inflammation is therefore now used to describe the interaction between thrombotic and inflammatory processes in a variety of contexts, notably venous and arterial disorders and infectious disease [17].

Immunothrombosis

While the term thrombo-inflammation is typically used to describe patho-mechanisms that feature in conditions like deep-vein thrombosis (DVT) and infection [17] and that adversely impact the host, it is also clear that thrombo-inflammatory–like processes (that is, co-ordinated responses involving thrombosis and inflammation) can also have protective immunologic function. This is exemplified in the context of blood-borne infection where the formation of thrombi (particularly in micro vessels) plays important roles in the capture of invading pathogens and the prevention of systemic infection [18]. As a result, in this context, the thrombosis that is elicited in response to infection is referred to as "immunothrombosis" [12].

Immunothrombosis is now recognized as an important component of the intravascular innate response to a variety of pathogens, especially bacterial pathogens [18]. Physiologically, immunothrombosis is thought to provide four key functions : firstly, as mentioned previously, it functions to restrain pathogen dissemination; secondly, it prevents tissue invasion by keeping pathogens in the circulation; thirdly, it concentrates antimicrobial effector proteins to promote pathogen killing and fourthly, it co-ordinates downstream immune responses by providing a strong localising stimulus, which includes leukocyte recruitment and fibrin deposition [18] (Figure 1.2) .

Accordingly, immunothrombotic processes depend not only on platelets, but also the co-ordinated functioning of a variety of other immune cells, most notably granulocytes and mononuclear cells [19] . Monocytes are major drivers in stimulating coagulatory (i.e. immunothrombotic) processes by providing tissue factor on their surface and on secreted micro-vesicles [20] . In contrast, neutrophils are typically thought to invoke thrombotic

response through their production of neutrophil- extracellular traps (NETs) [21] , which are highly thrombogenic . The involvement of monocytes and neutrophils in coagulatory processes is additionally underscored by the fact that these cells also avidly interact with platelets , especially following infection [22]. Other cells, such as eosinophils, also have important function in bridging coagulation with thrombosis and are heavily implicated in regulating both fibrin deposition as well as fibrinolysis [23].

These examples illustrate the broad cellular and molecular connections that exist between coagulation and inflammation/ immunity, but, as recent studies have demonstrated, platelets not only functionally bridge these processes , but are also active participants in each [24] .
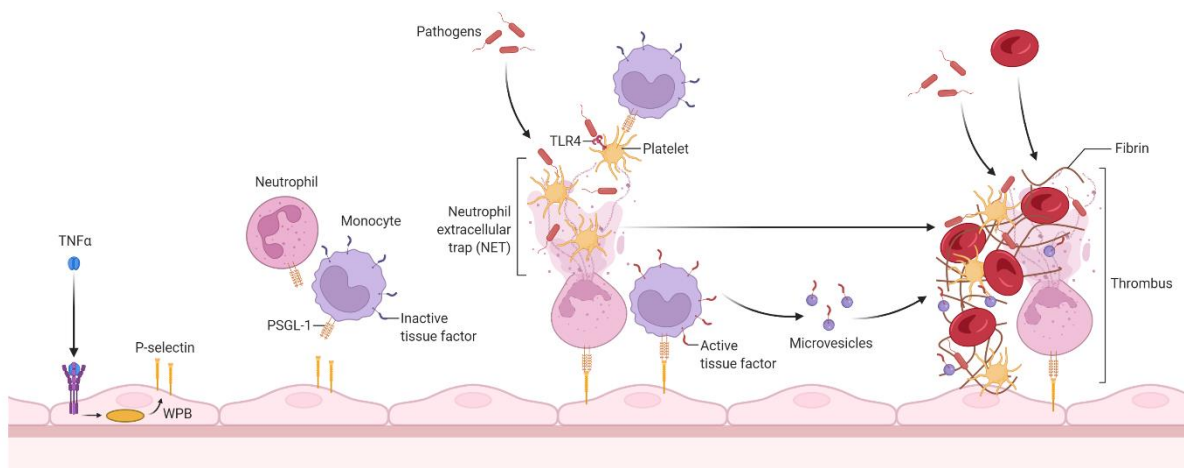
<u>Figure 1.2:</u> **Platelets coordinate with neutrophils and monocytes to induce immunothrombosis.** Major pathways involved in driving immunothrombosis, including neutrophil production of NETs and monocyte –mediated presentation of tissue factor .Figure adapted from Biorender.com. WPB = Weibel-Palade Bodies ; TNFa = Tumour Necrosis Factor α ; TLR4 = Toll-like receptor 4

Platelets : vascular patrollers with broad immune function

Platelets store and secrete an arsenal of compounds with immunologic function

The notion that platelets may play significant roles in immunity to infectious pathogens , and in sterile inflammatory conditions (some of which are now considered thrombo-inflammatory conditions) is not new, with seminal research showing platelets performing distinctly immunologic functions dating as far back as the 1970s [25] . However, only recently, with the help of novel analytic tools, such as next generation sequencing (NGS) and mass-spectrometry, have researchers been able to fully explore the immunologic repertoire of platelets. High-throughput transcriptomic and proteomic studies in particular (which until recently were lacking due to methodological constraints) have shed light on the vast variety of soluble and receptor associated molecules and proteins that platelets store in their granules and which they release into the extracellular environment upon becoming activated [26]. Although many of these, as expected, are primarily involved in stimulating classic haemostatic functions, including procoagulant factors, such as Factor V, [27] and ADP [28], many compounds that platelets store are also associated with stimulating immune processes and can have potent immunomodulatory activity [29]. Examples of immune-associated factors include cytokines such as IL-33 [30] or TGF-β [31], chemokines such as RANTES [32] or CXCL-4 (PF-4) , proteins with direct microbicidal properties, termed thrombocidins [33] and miRNAs [34] , such as miR-126-3p [35] , among many more.

Most platelet-derived compound with immune functions are stored in platelet alpha granules, with platelet dense granules primarily containing small molecular mediators such as serotonin [28] and platelet lysosomes typically containing enzymes and proteases [26] , which play a role in structuring extracellular matrix. Interestingly, recent studies have suggested that such immune factors may be packaged into distinct granules, and differentially secreted in the presence of specific environmental signals [36]. These studies help to explain how platelets can exert specific and context-dependent functions, even in the absence of a nucleus. For instance, while many studies have indicated that platelets have inherent pro-inflammatory activity, which is exemplified in many thrombo-inflammatory disorders [37] other studies have clearly demonstrated their ability to induce anti-inflammatory processes, which are of particular relevance in cancer [38].

Platelet populations are heterogenous

In line with studies showing that platelets induce context-dependent responses, evidence is emerging that the pre-cursor cells of platelets, megakaryocytes [26] , can differentiate into distinct subpopulations, e.g. in response to systemic inflammation [39]. The heterogeneity in megakaryocytes is in turn reflected in platelet populations as well. For example, studies demonstrate that specific sub-populations of platelets may be primed towards a pro-coagulant function, whereas others may be primed towards aggregation or secretion [40]

Moreover, sequencing studies, complimented by functional in vitro analyses suggest that there exists significant heterogeneity in platelet populations between different individuals , and that these differences may contribute significantly towards modulating pathophysiologic processes in a variety of diseases [40]. While some of this heterogeneity is genetically determined, as in the extreme cases of inherited platelet disorders [41] , some of it stems from disease processes. For example, platelets from septic patients often develop platelet hyperactivation, leading to thrombo-inflammatory complications, which contribute significantly towards mortality risk [42]. As a result of this, many clinical studies have now begun to use platelet-derived factors (typically platelet microparticles) as markers of inflammation or disease progression [43].

Collectively, studies investigating platelet heterogeneity have provided crucial insight into the structural and molecular diversity inherent in platelet populations which in turn, impact on their functional specificity.

Platelets as effectors of immunity

Mechanistically, platelets function as immune cells in a variety of ways, and are heavily linked to both innate as well as adaptive immune responses [44]. For instance, similar to other innate immune cells, such as macrophages or granulocytes, platelets have been shown to take up invading pathogens [45] , produce cytokines [46], release reactive oxygen species (ROS) and other compounds that have direct anti-microbial activity [45] and activate complement [47] and other innate signalling pathways (e.g. histamine release) [16].

The innate immune functions that platelets engage in are heavily dependent on the signalling of specific platelet receptors, most notably, those that detect PAMPs, e.g. TLR-4 [48], and those that sense danger associated molecular patterns (DAMPs), such as the receptor for advanced glycation end products (RAGE) [49]. Many of these receptors become exposed on the surfaces of platelets during platelet degranulation, when the membranes of alpha and dense granules fuse with platelet plasma membranes [26], thereby modulating how and when platelets can respond to stimuli. Studies that have investigated the role of platelet receptors in platelets responses to a variety of PAMPs and classic platelet agonists like thrombin, have provided evidence that platelets also adopt distinct morphology, depending on the inciting stimulus [50], giving them the ability to respond in a functionally distinct manner. In the context of infection, where platelets often function as first responders [51], their sensing of foreign pathogens and their products is associated with the initiation of an assemblage of (immunothrombotic) processes directed towards the prevention of pathogen dissemination and tissue invasion [52].

The importance of platelets as innate sensors of pathogens and first responders is made clear by the fact that thrombocytopenic hosts (that have abnormally low platelet counts) are generally characterised as having impaired acute responses and often develop severe infections, usually as a result of the systemic dissemination of pathogens [53].

By engaging in these mechanisms, platelets directly contribute towards immune functions and influence infection outcome. However platelets also have significant immunomodulatory capacity and regulate the activity of a plethora of other immune cells [54].
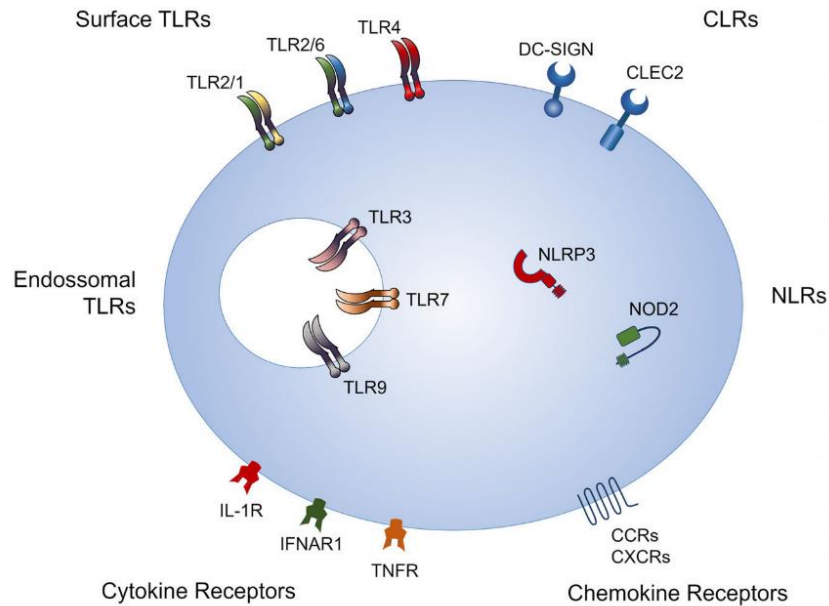
Figure 1.3: Platelets express a variety of immune-associated receptors. TLR = Toll-like receptors ; CLR = C-type lectin receptors ; NLRs = NOD-like receptors .Figure adapted from [48]

Platelets as regulators of immunity

One of the primary mechanisms through which platelets exert regulatory activity is through their direct interaction with target cells [55]. These interactions are associated with a vast assortment of effects and lead to functional changes in both platelets as well as in the cells that they bind to [56] .The main target cells that platelets interact with in the context of inflammation and immunity are endothelial cells and immune cells, especially neutrophils and monocytes [44] .

The interaction of platelets with endothelial cells supports a number of important processes involved in modulating inflammation, most notably, changes to vascular permeability [32] and the enhancement of leukocyte trafficking and infiltration into tissues [57]. In agreement with this, various studies, using a number of different models, have shown that platelet deficiencies can lead to aberrant vascular integrity [58] and impaired leukocyte recruitment [59] during condition of inflammation or infection.

Which populations are specifically recruited by platelets, and to what degree their recruitment depends on platelet receptors and soluble factors (such as P-selectin [60] and serotonin [61]) is however, dependent on the precise physiological context. In general, platelet-mediated leukocyte recruitment will either involve platelets aggregating at sites of

vascular damage, and presenting adhesive molecules (e.g. P-selectin) on their surfaces [58], or through platelet-dependent expression of ICAM-1, VCAM-1 , E-selectin and other adhesive receptors involved in facilitating leukocyte rolling and binding , on the surfaces of endothelial cells [62].

In addition to playing critical roles in the recruitment of immune populations, platelets also interact with leukocytes from both adaptive [63] and innate immune [22] branches to regulate (and initiate) their immune effector functions (Figure 1.4). Indeed the appearance of platelet-leukocyte complexes (also commonly referred to as platelet-leukocyte aggregates) is a characteristic feature of many inflammatory conditions[52]. Most interactions between platelets and leukocytes rely on engagement of platelet P-selectin (also known as CD62P) with leukocyte PSGL-1, including platelet-neutrophil [64], platelet-monocyte [65]and platelet-eosinophil[66] interaction. However, as recent studies indicate [67], other platelet and leukocyte receptors also play significant roles in facilitating interaction, including platelet GP-IIb-IIIa , GP-Ib , CD40L and CLEC–2, which associate with leukocyte SLCA42[67], CD11b [68], CD40 [69]and podoplanin[17] respectively. Under abnormal conditions (e.g. change in flow shear or during vascular inflammation) interactions between platelets and leukocytes, which typically involve only transient contact, become consolidated via the receptor–ligand pairs mentioned above to induce immune responses, for example, the expression of cytokines [48]. Platelet-regulation of cytokine expression has now been reported to occur in a variety of contexts and constitutes an important mechanism through which platelets exert regulatory function[70].

Investigations into platelet-neutrophil crosstalk have revealed that platelet-signalling can enhance neutrophil phagocytosis [71], modulate the release of ROS [72] and induce (and regulate) the formation of NETs[73]. As such, platelet-neutrophil interaction is implicated in many thrombo-inflammatory and infectious contexts, where neutrophil- mediated inflammation is a major pathophysiologic parameter. In line with this, several experimental studies have indicated that direct interference of platelet-neutrophil interaction can lead to reduced neutrophil-mediated immunopathology in a variety of conditions[62] [74]. In comparison, the inhibition of platelet-neutrophil interaction in contexts where neutrophils are known to play protective roles, has not been well studied [75].

Interactions of platelets with monocytes and macrophages are also associated with potent changes in their effector functions. Platelets and platelet-derived factors can drive monocyte expression of inflammatory cytokines such as TNF-α, IL-1β and IL-6 [76], enhance phagocytosis of pathogens and steer the polarization of macrophages towards a more pro-inflammatory phenotype [77], or a more anti-inflammatory phenotype, depending on the context. For example, platelets have also been shown to act as negative regulators of inflammation mediated by macrophages in the lung, thereby aiding in protection against excessive tissue damage [78].

While less well defined, platelets have also been shown to interact with other immune cells, including eosinophils and lymphocytes to regulate their function [63,66] and evidence is accruing that platelets may have significant roles in allergy [79] and adaptive immunity [80]. For instance, platelet-eosinophil interactions have been shown to support eosinophil recruitment during allergic airway inflammation[66] and to drive thrombosis via induction of eosinophil-extracellular traps (EETs) [81] .

The association of platelets with lymphocytes , including T and B cells on the other hand, have been demonstrated to play important roles in lymphocyte recruitment [82] and also in the differentiation of both T and B cells into specific subpopulations [83]. Platelets have even been shown to function as antigen presenting cells in certain contexts, stimulating the induction of specific T and B cell responses by presenting both antigen, and co-stimulatory molecules on their surfaces [83].

Collectively, these data suggest an important role for direct platelet interaction with leukocytes as a mechanism underlying the regulation of immune responses, especially in the context of infectious diseases. However, these interaction are also sensitive to the physiologic microenvironment in which they occur, and therefore, our understanding of platelets as immune cells is presently defined to specific anatomical and physiological settings. One of the more well-researched anatomical contexts in this regard (and which is most directly relevant to this project) is the pulmonary system.
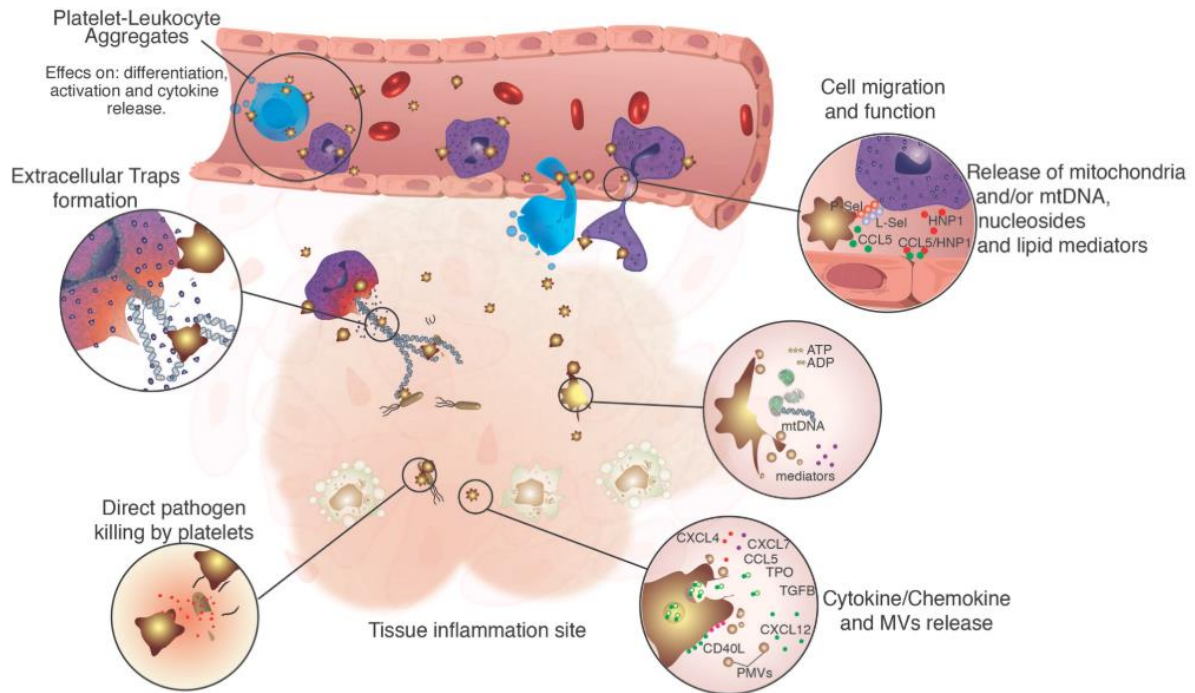
Figure 1.4: Regulation of innate immune responses by platelets. Platelets interact with immune cells such as monocytes to regulation their activation, differentiation , cytokine production and effector functions . Figure adapted from [70]

Platelets in the context of pulmonary infection and inflammation.

The lung as a hub of platelet activity

The pulmonary system, which broadly consists of the trachea, the bronchi and the alveoli is regularly exposed to foreign particles, both innocuous and pathogenic, and is an important site of immune activity. While the physiology of the lung is heavily adapted to its role in exchanging gases from the circulation with those in the environment, recent evidence is emerging that the precise (vascular) architecture of the lung also supports the formation of hematopoietic niches, that may play significant roles in driving the production of circulatory immune populations [84]. In particular, a landmark recent study provided evidence that the lung is an important of site of platelet biogenesis, and is essential to maintaining platelets

populations , both under normal , as well as abnormal conditions [85]. The findings of this study, in conjunction with the unique (highly vascularized) anatomy of the lung, means it is unsurprising that research continues to emerge on the importance of platelets in regulating lung function, especially in the context of pulmonary inflammation and infectious disease.

Platelets drive pulmonary inflammation but also protect against tissue damage

It is now well recognized that platelets have significant activity in an array of inflammatory lung conditions, ranging from acute infection to chronic sterile inflammation [86] .

Much of the evidence implicating platelets in pulmonary inflammation (and associated conditions) derive from experimental studies using animal models of acute lung injury (ALI) , pulmonary infection and allergic airway inflammation [86].These models replicate important pathological features of clinically relevant lung inflammatory syndromes, most notably acute respiratory distress syndrome (ARDS), and have been essential in establishing various features related to the immunobiology of platelets. In particular, they have revealed that platelets, in addition to mediating important physiologic functions during vascular inflammation, are heavily involved in both driving, as well as resolving [87], pulmonary inflammatory processes. They impact on these processes via the same immune mechanisms described previously: through production of cytokines and chemokines, through regulation of leukocyte trafficking and effector functions, through complement activation, through regulation of histamine release etc [22,70,88].

Whether the outcome of platelets performing these immune functions is ultimately beneficial or detrimental to the host seems to depend on the precise context. For instance, within studies looking at the role of platelets in pulmonary infection, platelets are generally characterised as playing protective roles, either aiding in maintaining lung integrity [89] or directly contributing towards antimicrobial responses that prevent systemic dissemination [53].

On the other hand, in the context of ALI and other conditions that predispose to ARDS , such as in aspiration of acid or hypoxia, platelets are typified as pro-inflammatory cells that become sequestered in lung tissue and which contribute towards diffuse alveolar damage [62,90] . The pathological roles of platelets in ALI are suggested by studies that demonstrate

that depletion of platelets (with the use of antibodies or through genetic means) or their pharmacological inhibition is associated with improved indices of lung pathology, and this is thought to be due to platelet regulation of neutrophil responses [91]. Platelets are also thought to play detrimental roles in allergic airway inflammation and asthma [92], in particular, by inducing and regulating eosinophil function [66].

In aggregate, these findings suggest that platelets, similar to other innate immune cells such as neutrophils, play crucial roles in containing pathogens in the lung, but may also contribute significantly towards tissue destruction, thereby facilitating the progression to severe pulmonary inflammation such as that which occurs during ARDS. Platelets can therefore be considered in many instances to function as a double-edged sword. This is particularly evident in pulmonary viral infection, including with SARS-CoV 2, where platelets both contribute towards host control of viral replication, as well as drive coagulopathy and pathologic inflammation [93].

This once again highlights the importance of scrutinising the pathophysiologic context in which platelets are performing their function , especially in light of recent studies which have demonstrated that platelets may also have significant anti-inflammatory (and protective) activity in the lung, particularly through their regulation of resident macrophages responses[94]. These recent findings emphasise the value of investigating platelets in a wide variety of infectious and non-infectious contexts to further clarify their immunologic function. In this regard, it is noteworthy, that much of the focus of platelet research, specifically in the realm of pulmonary infection, has centred on bacterial and viral infections, with few studies explicitly investigating the role of platelets in mediating pulmonary immunity to parasitic infections [95]. Helminthiasis in particular, many of which involve the lung an important site of host-pathogen interaction, have not been studied with regard to platelet-mediated immunity[95] .

Helminthiases – a collection of neglected tropical diseases with global impact

Helminths are parasitic worms that are an extremely common source of infection globally, causing widespread morbidity and contributing significantly towards the loss of healthy life years (HALYs) worldwide [96]. Recent estimates suggest that as many as 1.5 billion individuals carry helminth parasites, with the highest prevalence of infection occurring within sub-

Saharan Africa, Latin -and South America and the pacific islands of Asia [97]. As such, helminth infections are generally associated with developing nations, although areas with low socio-economic standing in developed countries may also be significantly affected[98].This association is thought to be driven primarily by poor access to adequate water, sanitation, and hygiene in these areas. This is especially true for soil-transmitted helminths (STH) infections, where exposure typically entails either peroral or percutaneous infection[99] . STHs comprises mainly 4 types of helminth parasites, with broad global distribution and relevance, namely roundworms (especially *A. lumbricoides*), whipworms (most notably *T. trichuris)*, hookworms (including *A. duodenale* and *N. americanus)* and threadworms (most commonly *S. stercoralis*). Collectively , these helminth parasites are involved in the majority of human helminthiases and have therefore received the most attention with regard to epidemiological and clinical research efforts and public health interventions [99]. Outside of improving public infrastructure and bettering access to clean water and bathrooms, public health control measures have centred on the mass administration of anthelmintics to at-risk populations living in helminth endemic areas. While anthelmintics have been effective in curbing the prevalence and severity of infections caused by STH , they do not offer long lasting protection and due to the small number of classes of anthelminthics, are prone to emerging resistance[100]. Indeed, widespread anthelmintic resistance (AR) is already common for helminth species that parasitise livestock and other economically important animals[100],which has resulted in veterinarians and parasitologists adopting a more targeted approach with regard to the use of anthelmintics for veterinary purposes [100]. While these have proven to be successful, they have yet to be widely incorporated into treatment strategies used to combat human helminthiases [100]. Facing this, vaccines against helminths will likely be essential for future management of helminth infections. Presently, only veterinary helminth vaccines, which target specific species have been successfully deployed, with human vaccines , while under clinical development, currently lacking [101]. This is in part due to the complex biology and life cycle of helminth parasites, which in most cases consist of several larval and adult stages , each with their own proteome and metabolome, and the complex migratory patterns involved in many helminthiases. A further hindrance is our incomplete understanding of the molecular and cellular interactions that govern host-pathogen interplay, especially at very early stages of infection [102].

Experimental models of helminth infection and an overview of the life cycle and clinical features of hookworms

Animal models of helminth infection, most notably rodent models, have been indispensable for furthering our understanding of these complex pathogens as they parasitise their hosts. Three nematode species are commonly used as models for human intestinal-nematode infections in mice as they have broadly similar genetic and antigenic characteristics [103] : *Nippostrongylus brasiliensis* (the helminth used within this project) *, Heligmosomoides bakeri* and *Trichuris muris*. Each model is useful for replicating the pathology and immunity induced by important human parasites [104]. For example*, N.brasiliensis* has a very similar life cycle to the prevalent human hookworms *A. duodenale* and *N. americanus*, which broadly involves the following : firstly, the penetration of infective-stage larvae through primary barriers such as skin, and invasion of the hosts circulatory system; secondly, the transport of larvae to pulmonary capillaries and the migration of larvae out of the circulation and into lung tissue ; thirdly, the crawling of larvae up the trachea and into the larynx and finally, the swallowing of larvae into the small intestine , where final maturation and mating occurs [105].
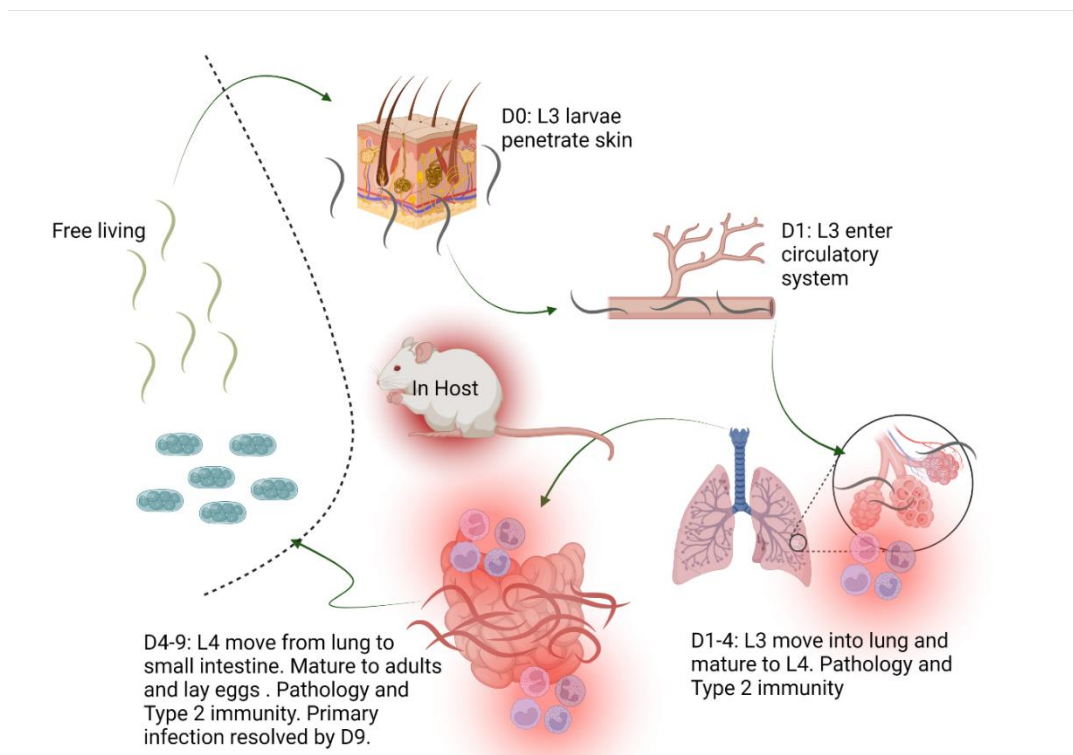
Figure 1.5. : The life cycle of hookworms such as N.brasiliensis**.** Figure created on Biorender.com.

As such, hookworm parasites (and other STH) encounter different physiological and anatomical environments as they infect their hosts. Owing to these differences, the separate stages of helminth infection may have either broadly similar or specific pathophysiologic features, which associate with distinct clinical sequelae [99] .For instance, patients infected with hookworms may present with eosinophilic pneumonia (also called Löffler's syndrome, which results from larval migration through the lung) and symptoms such as cough, dyspnoea and haemoptysis, or (more commonly) they may present with gastrointestinal symptoms, including abdominal pain, diarrhoea and melaena [106], which results from parasite-mediated intestinal inflammation and pathology. Severely infected individuals and those that are more susceptible to infection, including children, may also develop anaemia and nutritional deficiencies [107]. These, in turn, have been associated with significant developmental and cognitive impairment , and carry significant socioeconomic costs [108]. However, it is important to mention that most hookworm infections are asymptomatic [109].This is believed to be a result of many factors, notably the kinetics of infection (in other words the number of parasites that infect humans in a natural environment), the extended co-evolution of helminths with humans (which has promoted the emergence of tolerogenic mechanisms) and the tightly co-ordinated immune responses that are elicited in infected hosts and which are heavily modulated by helminths and their products [110] .

Immunology of hookworm infections

While the specific responses associated with immunity to hookworms such as *N.brasiliensis* depend in part on the stage of the infectious cycle and the anatomical and physiologic backdrop in which responses are occurring , they are largely driven and governed by type 2 associated cells, including group 2 innate lymphoid cells (ILC2s), eosinophils, T_H2 cells and alternatively activated macrophages (AAM), among others[111]. These cells , which either reside in infected tissues or rapidly recruit to sites of parasite invasion through the sensing of innate cytokines and other alarmins produced from damaged epithelial and mucosal cells , notably IL-33, IL-25 and TSLP [112], are the primary effectors of classical type 2 responses. These responses broadly centre around the type 2 associated cytokines IL-4 and IL-13 and

the downstream processes they elicit when they signal through their shared co-receptor IL-4Rα [113] (though IL-4 and IL-13 independent responses are also crucial in many instances[114]) (Figure 1.6). Of major relevance for the expulsion of parasites from the intestines are epithelial cell responses, including goblet and tuft cell hyperplasia [115] and the production of mucins and surfactants [116], which, together with smooth muscle contractions, function to dislodge parasites from the intestines and from other sites such as the lung [117] . These responses also typically associate with the induction of adaptive immune responses, such as expansion of B cells [118] and CD4+ T cells [119] which are crucial for optimal type 2 immunity. As such , immunity to hookworm infections entails the co-ordinated interaction of a diversity of cells and their effector molecules and heavily involves both innate [102] and adaptive immune cell –mediated responses. These not only serve to elicit protection to invading helminth parasites but also to induce wound healing and tolerogenic [120] responses that are crucial for preventing excessive helminth-associated tissue destruction [121] . This is especially true in the lung , where dysfunctional type 2 responses are known to associate with markedly enhanced pathology [121].
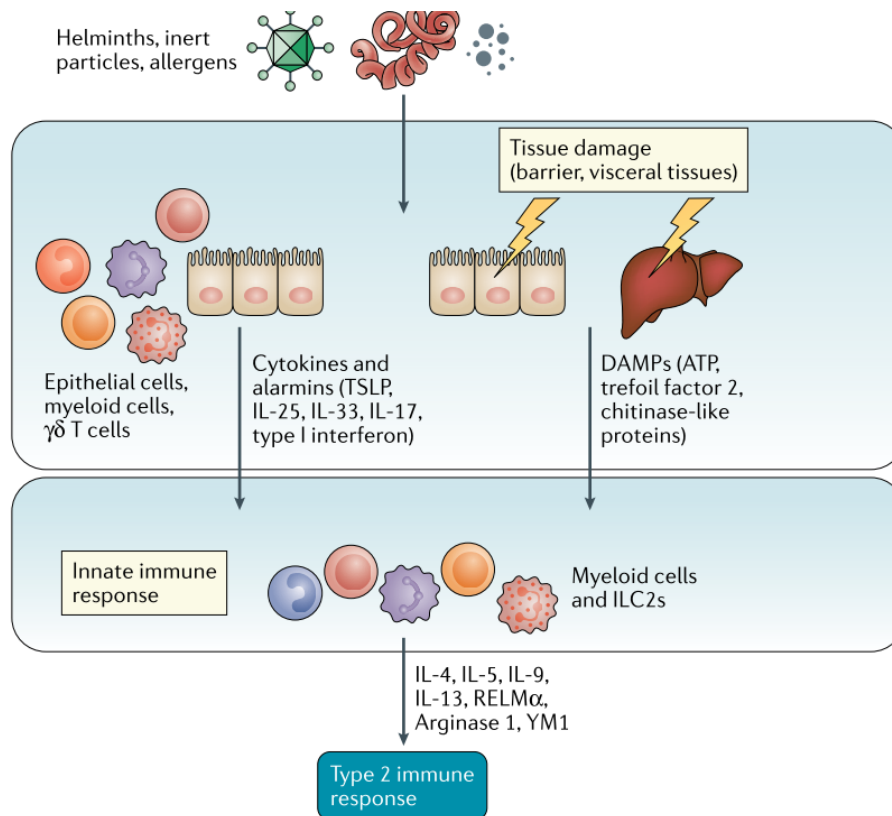
<u>Figure 1.6</u>: The prototypical initiation of type 2 immune responses . Helminths encounter host barrier surface and cause tissue damage. This leads to the release of alarmins and cytokines that signal to barrier-associated cells such as ILC2s and γδ T cells to produce factors that induce further innate responses, ultimately leading to the expression of type 2 cytokines and effector molecules which propagate the type 2 immune response. Figure adapted from [122].

Innate immune responses in the lung: a fine-tuned balance between parasite control and host immunopathology

At this stage many of the major features of immunity to hookworms, outlined above, are mechanistically well understood. However, an area in our understanding of hookworm infections that requires further attention revolves around the acute innate immune response that develops in the lungs during the pulmonary stage of infection. In humans, often children, pulmonary migration of larvae can result in significant eosinophilic pneumonia leading a clinical diagnosis of Löffler's Syndrome [123] , where severity correlates with infection intensity[124]. While epidemiological investigations into helminth-associated pulmonary disease are sparse, a recent study by Jõgi et al. [125] demonstrated that exposure to *Ascaris lumbricoides* (which, similar to hookworms, also transits the lung) was associated with pulmonary dysfunction [125]. Importantly, the participants of this study were from regions where prevalence of helminth parasites is relatively low [126], and where transmission events are confined. This has significant implications for predicting the burden of pulmonary disease in helminth-endemic populations, where repeated exposure is common and infection intensity may be higher [127]. In conjunction with the fact that the lung also functions as an important site of activity during recall immunity [128], an understanding of the factors and cells involved in driving host-pathogen interaction in this setting is crucial.

Within mice, the migration of helminth parasites out of the vasculature and into the lung parenchyma is accompanied by significant damage to lung capillaries and epithelium and results in allergy-like pathology [129] and emphysema [130]. The initial inflammatory response that is elicited in the lung is driven by the production of alarmin cytokines and chitinase-like proteins (CLPs) from damaged epithelial cells [131]. These in turn mediate the production of IL-

17a, primarily by γδ T cells , which provides a strong chemotactic stimulus for innate immune cells, especially neutrophils [132]. These rapidly begin to recruit to the lung and are present in significant numbers within 2 days post infection [132]. While neutrophils play important roles in limiting larval migration, their influx into the lung has been shown to also drive immunopathology and is associated with significant pulmonary haemorrhaging [121] . In line with this, studies have revealed that the blockade of neutrophil chemotaxis to the lung during Nb infection , for instance , through blockade of IL-17a [132] , is associated with reduced pulmonary pathology and bleeding. Interestingly , reducing neutrophil counts only protects against acute pathology , with mice exhibiting enhanced pulmonary pathology at later stages of infection [132]. This is consistent with neutrophils also playing protective roles in the lung through their induction of type 2 associated responses , notably , the priming and expansion of (M2) macrophages [133]. M2 macrophages, which respond to IL-4 and IL-13 [133] and are typically characterized by their expression of arginase-1 [134] , resistin-like molecule α (RELM-α) [135] and Ym1 [136], are crucial effectors of immunity during pulmonary *N.brasiliensis* infection. They not only tightly co-ordinate with neutrophils to mount rapid anti-parasitic responses [133], but are also indispensable for stimulating wound healing processes and for protection from excessive lung damage [137,138]. And together with IL-4Rα signalling [121], M2 macrophages also serve to limit excessive inflammation [139]. In many ways they can therefore be considered as counterbalancing the inflammatory response elicited to tissue damage caused by migrating larvae and infiltrating neutrophils [102] . This is especially evident in their uptake of apoptotic neutrophils [140] and in their ability to integrate a diversity of signals from the microenvironment into protective responses [141] . M2 macrophages, together with dendritic cells also markedly impact on type 2 cell-mediated immunity at stages immediately downstream of acute helminth infection [142] and also play, together with CD4+ T cells [143] and B cells [118], crucial roles in recall immunity [133].

Taken together, it is clear that the interaction between macrophages and neutrophils has profound consequences for immunity to helminth parasites and for recovery from the damage that they cause as they migrate through the lung. Many of the factors that mediate this interaction, and therefore impact on balancing anti-parasitic responses with tolerogenic responses, are now known (Figure 1.7). However, a notable gap in our understanding, which

we partially address within this project, are the role that platelets play in this immunologic landscape.
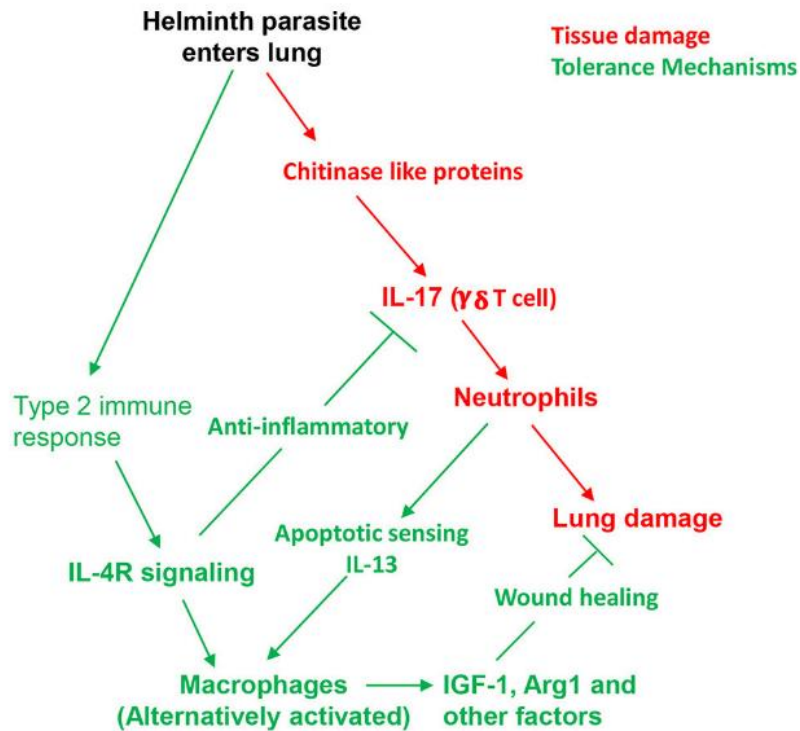
Figure 1.7: Host-tolerance mechanisms balance the neutrophil-mediated inflammation during pulmonary helminth infection and contribute towards type 2 responses. Factors released during pulmonary helminth infection can be principally characterised based on their association with either tissue damage or tolerance mechanisms. IGF-1 = Insulin-like growth factor 1 ; Arg1 = Arginase 1 .Figure adapted from [120]

Platelets and helminth parasites

Our understanding of the role of platelets in helminth infection, as in many other parasitic infections, is very limited. Although it has long been appreciated that many helminth parasites, including diverse species of hookworms [144,145] and flatworms [146], produce factors that interfere with platelet function, few studies have empirically investigated how platelets contribute towards immunity to helminth infections. This is despite an understanding that the interference with haemostasis is an immune evasion strategy that is represented in

many other types of infections , and likely contributes to allowing helminths to parasitise their hosts for extended periods of time [146]. This is underscored by the observation that many helminths, especially schistosomes, have evolved multiple distinct mechanisms to interfere with platelet (haemostatic) function[146] (Figure 1.8).
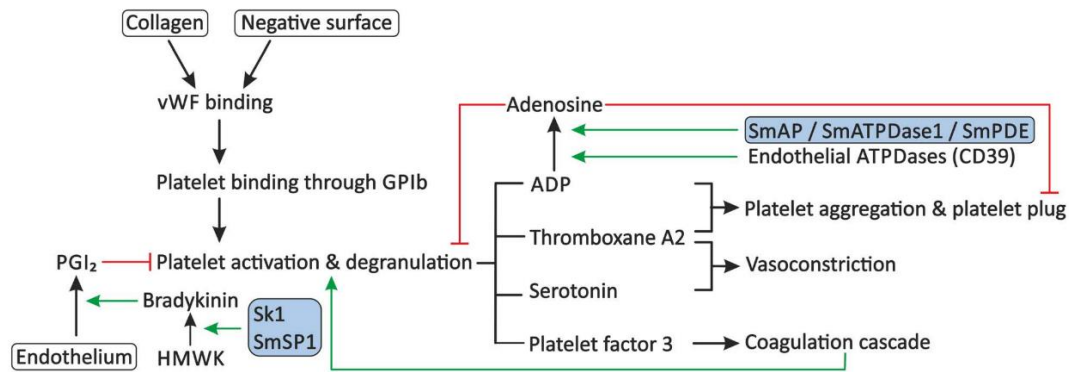
Figure 1.8: Proposed mechanism underlying the interference of primary haemostasis by schistosomes. Schistosome-derived regulatory factors are outlined in the blue boxes. Stimulation is indicated by green arrows and inhibition by red lines. GPIb= glycoprotein Ib; HMWK = High-molecular-weight kininogen, PGI2 = prostacyclin ; SmAP = Schistosoma mansoni alkalinephosphatase, SmATPDase1= S.mansoni ATP-diphosphohydrolase-1, SmPDE = Schistosoma mansoni phosphodiesterase ; VWF = von Willebrand Factor. Figure adapted from Mebius et al., [146]

In agreement with this, animal studies have also suggested that platelets may also have direct cytotoxic effects on helminth larvae. For instance , seminal experiments carried out by Joseph et al. [147] demonstrated that the culturing of platelets with schistosomula (schistosome larvae) lead to significant declines in parasite viability and that this effect was enhanced when the larvae were cultured with platelets from mice that had robust anti-schistosome immunity. While the precise mechanism underlying platelet-mediated cytotoxicity requires further clarification, inflammatory cytokines , including IFN-γ and IL-6, and plasma proteins , such as C-reactive protein (CRP) are thought to be involved, especially in the priming of platelets [148]. In vivo cytotoxicity of platelets towards schistosomula was also demonstrated in subsequent studies, in which both the heterologous transfer of

primed platelets [149] as well as the depletion of platelets using anti-platelet serum [150] resulted in marked differences in worm burdens in *S. mansoni* infected mice.

Taken together, these studies have provided evidence that platelets constitute an important barrier to schistosome infection, however, mechanistic insight is lacking, especially in the in vivo setting. Moreover, given the distinct pathophysiologic features involved in schistosomiasis and the fact that schistosome larvae have a different life cycle relative to many other helminth species, it remains unclear whether platelets also play significant roles in other helminth infections. As such, it is clear that more research is required into the roles that platelets play in helminth infection, particularly with regard to STH infections.

To the best of our knowledge, no other studies have addressed the role of platelets in the context of a gastrointestinal nematode infection.

The aim of this project is therefore three-fold:

I) To establish if platelets are a feature of immunity to *N. brasiliensis* and to determine how platelets associate with acute immune responses.

II) To ascertain whether the depletion of platelets with antibodies significantly disrupts platelet- dependent immune processes.

III) To identify receptors and platelet-derived factors through which platelets may be exerting their immunologic function.

To infer a role for platelets in helminth infection, a range of experimental techniques including complex flow cytometry, immunohistochemistry and protein and DNA spectrophotometry were used.

Chapter 2: Material and Methods

2.1) Mice and experiments

All work with animals was performed as per the approved protocol (#018/041) and in accordance with guidelines laid down by the South African bureau of Standards and the South African Veterinary Council (SAVC). All para-veterinary procedures and animal handling were carried out by certified persons, and the welfare of the animals assured by animal technicians employed at the University of Cape Town's research animal facility (RAF).

All mice were procured through UCT RAF, were of the C57BL/6J genetic background, weighed 20+ grams, were between 8 and12 weeks old and male. Animals were housed in specific pathogen-free conditions, at a temperature of 22± 2 degrees Celsius and a 12:12 light: dark cycle and provided with food and water *ad libitum.*

Experimental groups were as follows:

1) Naïve C57BL/6J mice

2) C57BL/6J mice infected with *N. brasiliensis* (Nb)

3) C57BL/6J mice treated with anti-CD41 antibody

4) C57BL/6J mice infected with Nb and treated with anti-CD41 antibody

5) C57BL/6J mice infected with Nb and treated with anti-CD62P antibody

6) C57BL/6J mice infected with Nb and treated with anti-podoplanin antibody

7) C57BL/6J mice infected with Nb and treated with anti-CD62P and anti-podoplanin antibodies

Experiments that were performed on these mice include**: I)** A platelet kinetic experiment, in which mice infected with *N. brasiliensis* (Nb) and naïve mice (uninfected) were tail bled several times over the course of ten days. Platelet levels were then determined using flow cytometry). **II)** Several experiments in which mice were infected with Nb and then euthanised at either 18 hours, 2 days or 3 days post infection (where each of these time

points constitutes a separate experiment). Cardiac blood, broncho-alveolar lavage (BAL) fluid and lung was collected and analysed as described in 2.1.2). **III)** Several experiments in which mice were treated with (or weren't treated with) anti-CD41 antibody prior to being infected with Nb and then euthanised at 18 post infection. Cardiac blood, BALF and lung were collected and analysed as described in 2.1.2. **IV)** A set of experiments in which mice were treated with either anti-CD41, anti-Ly6G or a combination of both prior to infection with Nb. Cardiac blood, BALF and lung were collected and analysed as in 2.1.2. **V)** A series of experiments in which mice were treated with either anti-CD62P, anti-podoplanin or a combination of both antibodies prior to infection with Nb. Cardiac blood, BALF and lung were collected and analysed as described previously.

2.1.1) Overview of major experiments
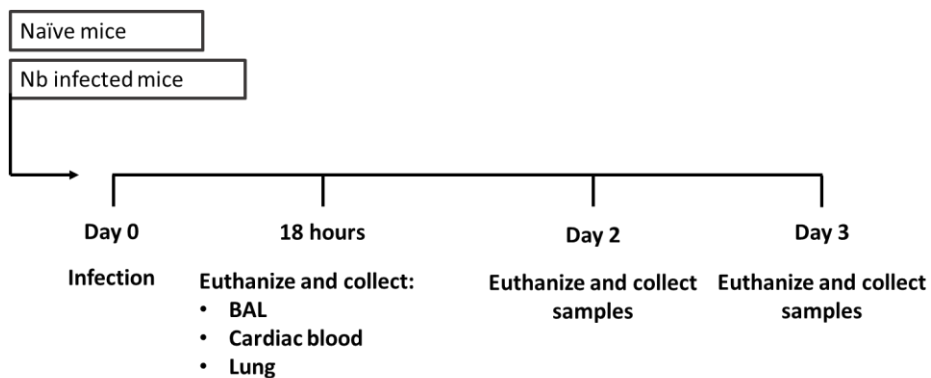
Figure 2.1: Layout of experiments comparing naïve and Nb infected mice in terms of platelet-associated immune responses at various time points. Note, each time point constitutes a separate experiment.
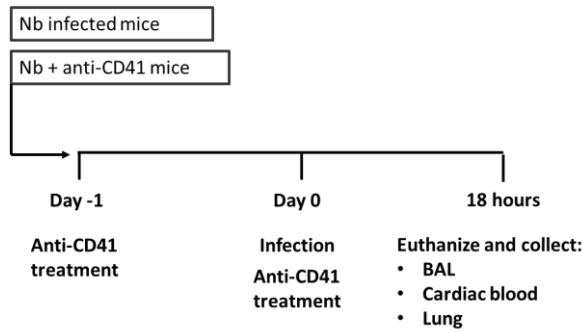
Figure 2.2: Layout of experiments comparing acute innate immune responses to Nb in platelet depleted and non-depleted mice. Anti-CD41 antibodies were administered intraperitonially.
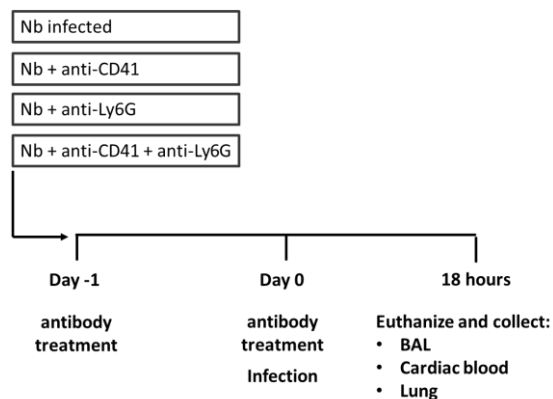
Figure 2.3: Layout of experiments comparing acute innate immune responses to Nb in platelet depleted, neutrophil depleted and co-depleted mice. Depleting antibodies were administered intraperitoneally.
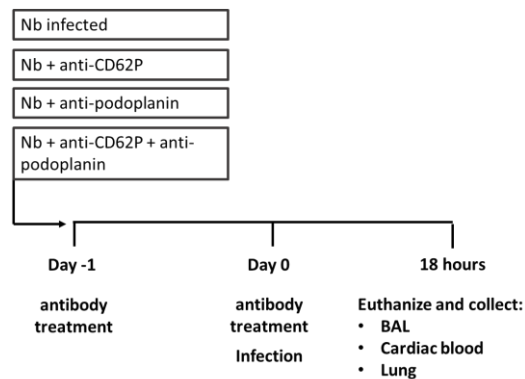
Figure 2.4: Layout of experiments comparing acute innate immune responses to Nb in mice treated with antibodies against platelet receptors. Note: each antibody treatment constitutes a separate experiment. All antibodies were administered intraperitoneally.

2.1.2) Analyses performed on collected tissues and serum

As outlined in figures 2.1 – 2.4, following killing, cardiac blood, BAL fluid and whole lungs were collected. An overview of sampling methodology and analyses performed are outlined in figure 2.5. Cardiac blood was collected, processed, and analysed as outlined in 2.2.2. BAL was collected, processed, and analysed as outlined in 2.5. Lung was collected and processed as outlined in 2.2.1.
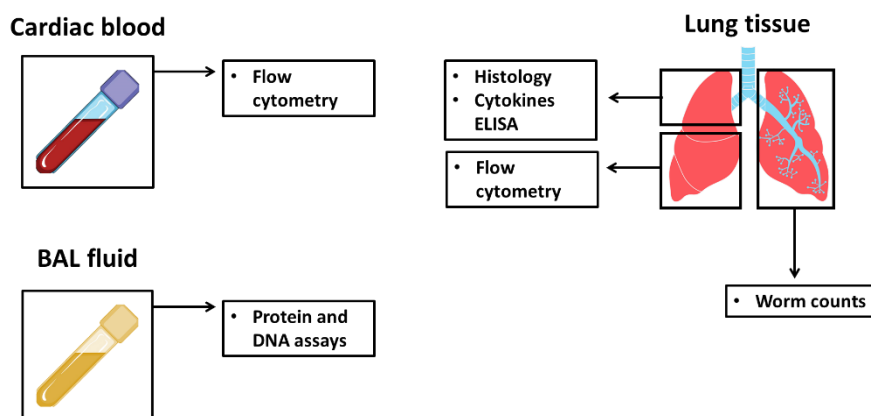
Figure 2.5: Overview of the analyses performed on cardiac blood, BAL fluid and lung tissues samples.

2.1.3) Antibody treatments

To deplete mice of their platelets, select groups were treated with anti-CD41 antibody (Biolegend, clone eBioMWReg-30). An outline of the treatment strategy is given in figure 2.2. 5 μg of anti- CD41 antibody were diluted in 100 ul of sterile PBS and injected intra-peritoneally at 24 hours prior to- and then again shortly before - Nb infection. This dosing and treatment strategy lead to a more than 90 % decrease in circulating platelet levels (Appendix , figure 5.3) and is similar to doses used in previous studies [151].

This same treatment regimen was also used to deplete mice of their neutrophils (outlined in figure 2.3). In the case of these experiments, mice received 25 ug of purified anti-Ly6G (Biolegend, clone 1A8) at each treatment.

In further experiments mice were treated with antibodies to interfere with platelet receptor signalling – again, the same treatment regimen was used as in previous experiments (figure 2.4) . Anti-CD62P antibodies (BD biosciences, clone RB40.34) were administered at a dose of 25 ug per treatment. Anti-podoplanin antibodies (courtesy of Julie Rayes at the University of Birmingham, clone 8.1.1 RF10) were administered at a dose of 50 ug per treatment. These concentrations were also used for mice treated with a combination of anti-podoplanin and anti-CD62P. Isotype antibodies were used as controls in experiments assessing the effects of anti-CD62P and anti-podoplanin treatments.

2.1.4) *N. brasiliensis* infection

N. brasiliensis L3 (infective stage) larvae were collected from the faeces of Wister rats used for stock maintenance. Larvae were washed twice in sterile PBS and suspended to a final concentration of 2500 L3 larvae per 1 ml. Each mouse then received 500 L3 larvae through sub-cutaneous injection of 200 μl of the prepared suspension.

2.2) Flow cytometry

Multicolour flow-cytometry was performed on lung and blood samples. After processing of samples, cells were treated with a cocktail of fluorophore- conjugated antibodies (tables 2.1, 2.2 and 2.3), stained and analysed. An outline of the processing and staining methodology is given in 2.2.1 (for lung cells) and 2.2.2 (for blood cells).

Flow cytometry was performed on a BD LSR Fortessa and acquisition analysis performed using FACS Diva software. For spectral unmixing, compensation beads were used (BD biosciences). Supervised and non-supervised (t-SNE) analyses were then conducted using FlowJo version 10 (Becton, Dickinson & Company). In the case of supervised analysis, gating was adjusted based on unstained and appropriate fluorescent – minus one (FMO) control.

2.2.1) unsupervised analysis using t-SNE

To gain oversight on broad qualitative differences in immune populations between experimental groups, t-SNE analysis was conducted. First, populations of interest (e.g., neutrophils) were manually gated out based on their expression of traditional immunophenotypic markers (see gating strategies in 2.2.6). Populations of interest were then downsampled using the in-built plugin in FlowJo v.10. Downsampling was performed to make uniform population sizes. The number of cells that were selected as downsampling parameters were specific to experiments and are outlined in figure 2.10 and 2.11. Following downsampling, the fcs files of individual populations were concatenated (i.e. combined) into a composite fcs file, on which t-SNE analysis was performed. T-SNE analysis was performed within FlowJo v.10 using the in-built t-SNE plugin. The default parameters were used for analysis. These included the following: 1000 iterations, a perplexity of 30 and a learning rate of 700. The markers used for t-SNE analysis are given in figure 2.10 and 2.11.

2.2.2) Flow cytometry of lung cells

Lungs were removed and sectioned into several parts, as outlined in figure 2.5. 2/3rd of the right lobes was stored in 2ml of complete media (Dulbecco's Modified Eagle Medium (DMEM) solution supplemented with 5% foetal calf serum and 1X Penicillin/Streptomycin) (all purchased through Thermofischer). Single cell suspensions were then prepared by pressing lung sections through a 70 um cell strainer. Single cell suspensions were then centrifuged at 400 XG for 5 minutes. After removal of the supernatant, the remaining pellets were suspended in 1000 ul of ACK lysing buffer (Thermofischer) for 2 minutes before being centrifuged again for 5 minutes at 400 XG. Cells were then washed in PBS before being resuspended in 1 ml of complete media. For cell counting, 10ul of this suspension was mixed with 20 ul of Trypan blue and placed on an improved Neubauer counting chamber (Sigma-Aldrich). For flow cytometry, $1*10^6$ cells were incubated with a cocktail of antibodies (Table 2.1) for 1 hour, in dark conditions. After staining, cells were washed, resuspended in 150 ul of MACS buffer (1X PBS, 0.5% BSA and 2mM EDTA) and analysed.

For intracellular cytokine staining (RELM-α) cells were first fixed and permeabilized in BD Cytofix/Cytoperm™ (BD Biosciences) for 20 minutes and washed twice in MACS buffer. Cells were then stained and analysed as outlined above. For intracellular staining of IFN-y (figure 3.2.6), washed blood cells were incubated in complete DMEM, containing 50ng/ml Phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich®) and 1 μg/ml Ionomycin (Sigma-Aldrich®) for 4 hrs at 37°C, in the presence of 10 μg/ml brefeldin A (BFA; Sigma- Aldrich®). After stimulation, cells were washed at 400 XG in PBS and then fixed and permeabilized as detailed previously. Once fixed and permeabilized, cells were then washed twice in MACS buffer and finally, stained and analysed.

Table 2.1: Antibody cocktail used to analyse innate myeloid populations and platelet-leukocyte complexes in single cell suspension of lung samples.

Marker	Fluorophore	Manufacturer	Clone
CD45	Alexa Fluor 700	Biolegend	30 - F11
CD41	PercP	Thermofischer	eBioMWReg30
CD11b	Brilliant Violet 421	Biolegend	M1/70
Ly6G	APC-Cy7	Biolegend	1A8
RELM-alpha	FITC	Santa Cruz Biotechnology	E-19
SiglecF	PE	Biolegend	S17007L
CD11c	APC	BD Biosciences	HL3
IFN-gamma	APC	Biolegend	XMG1.2

2.2.3) Flow cytometry of cardiac blood

To analyse circulatory innate immune responses 600-800 ml cardiac blood was taken from mice shortly after death. Blood was stored in 75ul (~10%) EDTA to prevent coagulation and kept at room temperature. Then, 150 ul of whole blood was aliquoted into new tubes for further processing. The rest of the blood was centrifuged at 180 XG for 10 minutes and then left to settle. This produces three distinct layers, with the top layer containing platelet -rich plasma (PRP). 50 ul of PRP of each sample was then transferred into a 96 well plate for further processing and the rest was stored at -20 degrees Celsius for downstream analysis.

To prepare for staining, whole blood aliquots were next treated with 850 ul of ACK lysis buffer, left to incubate for 2 minutes and then centrifuged at 400 XG for 5 minutes. After discarding of the supernatant, pellets were washed twice in 500 ul PBS, stained with antibodies (table 2.2) and suspended in 100 ul of MACS buffer. Finally, 50 ml of Precision Count Beads solution (Biolegend) were added to each sample before flow cytometric analysis and the number of leukocytes were analysed as per the manufacturer's instructions. For experiments in which intracellular cytokines were analysed (IFN-y and granzyme B), blood leukocytes were permeabilized and fixed as outlined in 2.2.1.

Similarly, to prepare PRP for staining , 250 ul of ACK lysing buffer were added to each well and left to incubate for 2 minutes, after which, cells were spun at 1000 XG for 5 minutes. After removal of the supernatant, cells were stained with 25 ul of an antibody cocktail (table 2.3) for 1 hour in the dark. After staining, cells were resuspended in 150 ul of MACS buffer. Finally, 50 ul of Precision Count Beads (Biolegend) solution were added to each sample and the samples were analysed and used to determine the number of platelets in each sample, as outlined above.

Table 2.2: Antibody cocktail used to analyse myeloid innate cells and platelet-leukocytes complexes in washed cardiac blood samples.

Marker	Fluorophore	Manufacturer	Clone
CD45	Alexa Fluor 700	Biolegend	30 - F11
CD41	PercP	Thermofischer	eBioMWReg30
CD11b	Brilliant Violet 421	Biolegend	M1/70
Ly6G	APC-Cy7	Biolegend	1A8
Ly6C	FITC	Biolegend	HK1.4
IFN-gamma	APC	Biolegend	XMG1.2
Granzyme-B	PE	Biolegend	QA16A02

Table 2.3: Antibody cocktail used to analyse platelets in PRP

Marker	Fluorophore	Manufacturer	Clone
CD41	PercP	Biolegend	eBioMWReg30
CD62P	PE	Biolegend	RMP1

2.2.4) Gating strategies
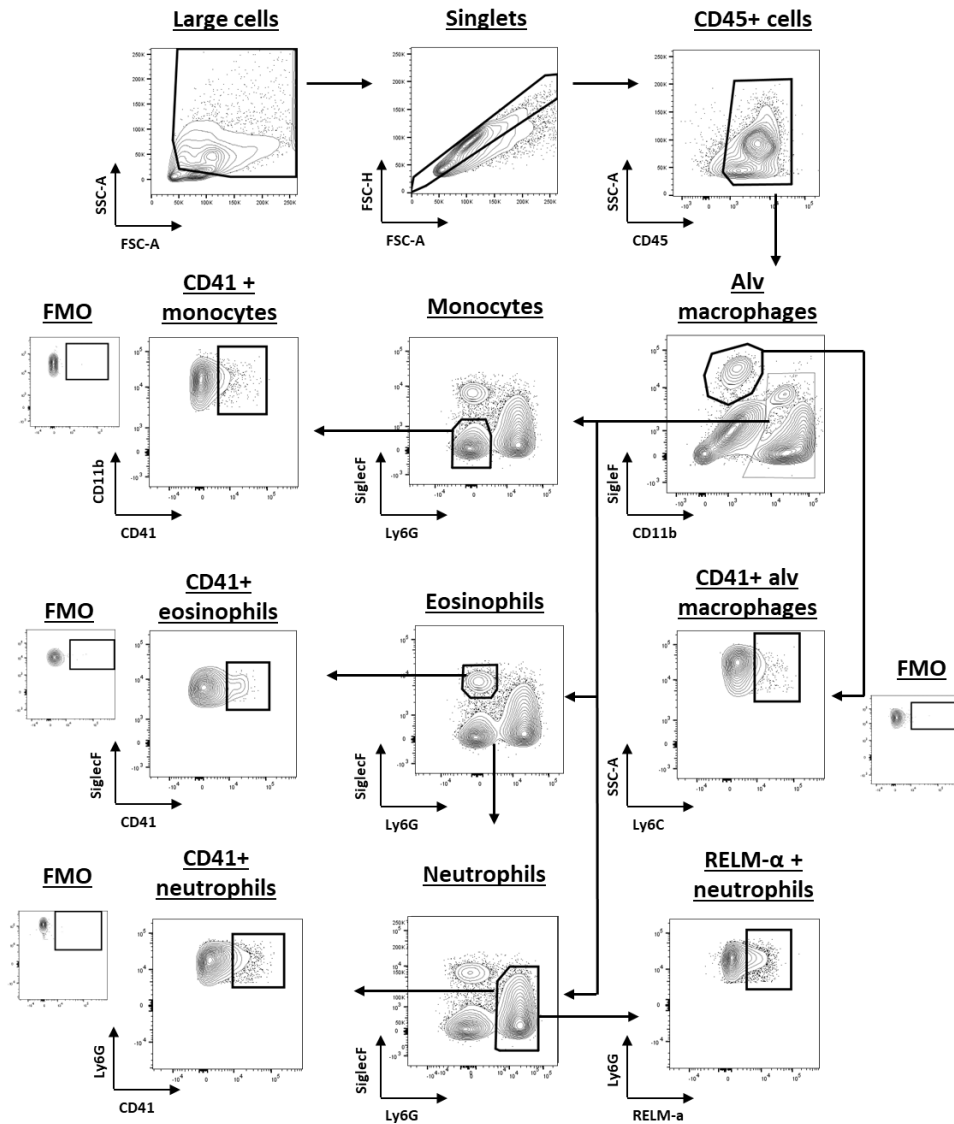
<u>Figure 2.6:</u> Gating strategy used for the determination of myeloid cell populations, and myeloid cell-platelet complexes in lung samples. Eosinophils were identified as being (CD45+, SiglecF+, CD11b+). Alveolar macrophages were identified as being (CD45+, SiglecF+, CD11b^{lo}). Monocytes were identified as being (CD45+, CD11b^{Hi}, SiglecF -, Ly6G -) or (CD45+, CD11b+ , Ly6C+, Ly6G -). Neutrophils were identified as being (CD45+, CD11b^{Hi}, Ly6G +). Association of these immune populations with platelets was determined by CD41 positivity.
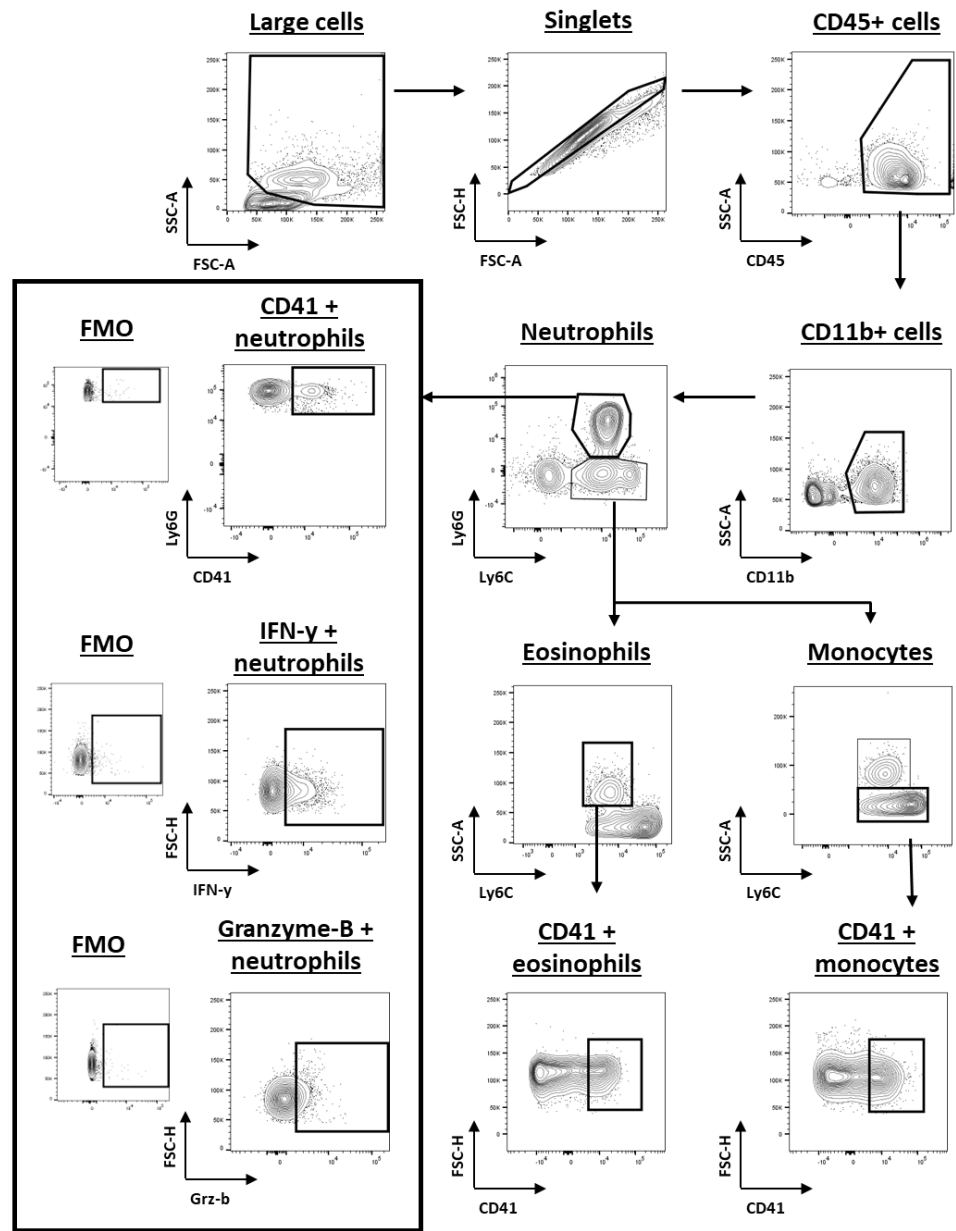
Figure 2.7: Gating strategy used for determination of myeloid cell populations and their association with platelets in cardiac blood samples. Eosinophils were identified as being (CD45+, CD11b+, Ly6G -, Ly6C^{Low}, SSC-A ^{Hi}) . Monocytes were identified as being (CD45+, CD11b+, Ly6G - , Ly6C^{variable} , SSC-A ^{Low}). Neutrophils were identified as being (CD45+, CD11b+, Ly6C ^{int}, Ly6G^{Hi}). Association of these immune populations with platelets was determined by CD41 positivity.
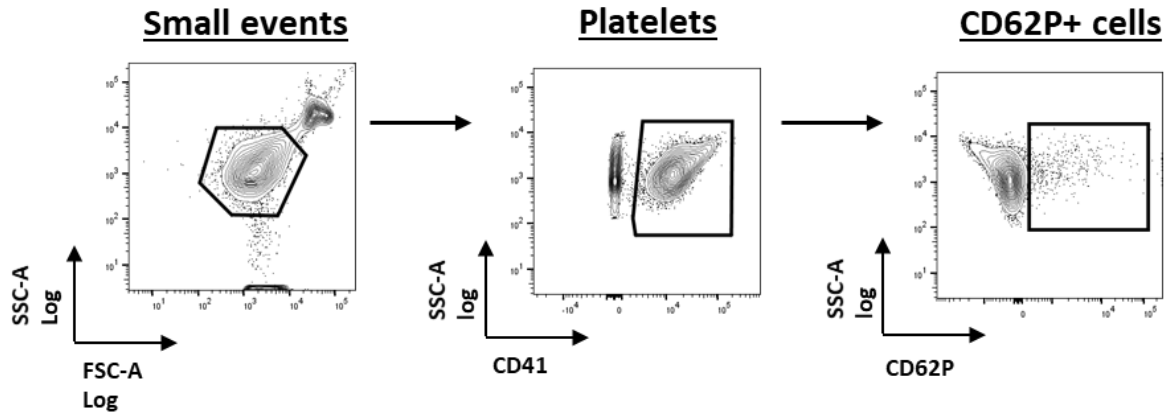
Figure 2.8: Gating strategy used for the determination platelets in PRP. Platelets were gated as (SSC-A ^{Low,} FSC-A^{Low}, CD41 +) . Platelet activation was determined by CD62P positivity.
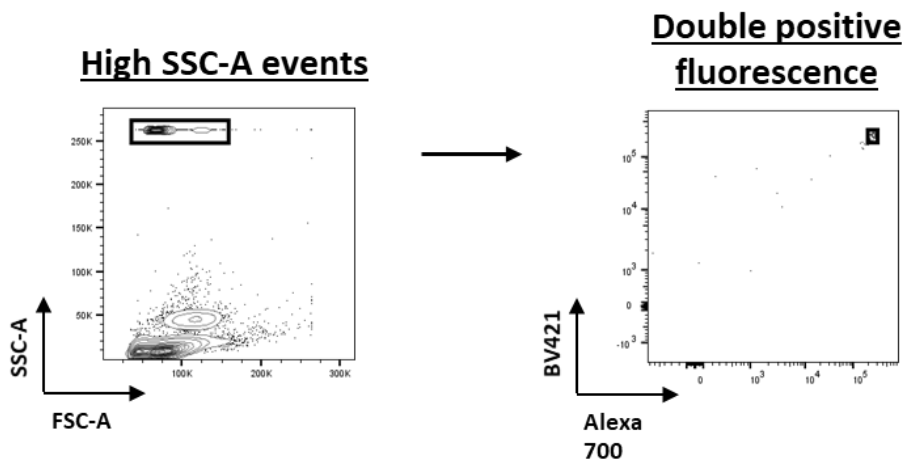
Figure 2.9: Gating strategy used for the determination of precision counting beads in blood samples. Counting beads were gated as (SSC-A $^{very\,high}$, BV421 ^{High}, Alexa Fluor 700 ^{High}).
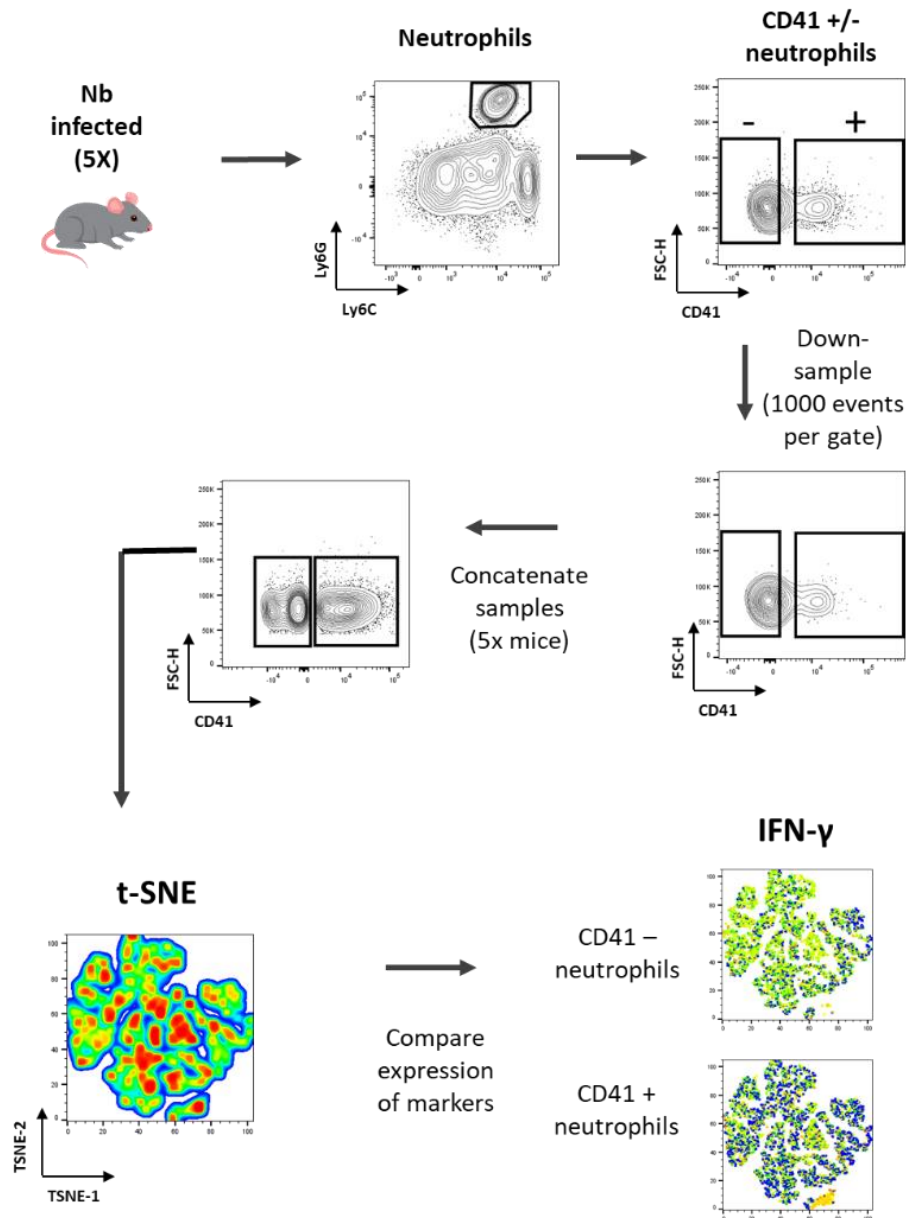
Figure 2.10: Overview of the workflow used for unsupervised (t-SNE) analysis of CD41 + and CD41 − neutrophils from Nb infected mice. Neutrophils were gated out using the gating strategy in figure 2.7 and separated into CD41(+) and CD41- populations. These populations were then down sampled to 1000 events per gate and subsequently concatenated into a composite population on which t-SNE analysis was performed. The markers used for t-SNE analysis were: CD45, CD11b, Ly6G, Ly6C, IFN-y, granzyme B. The composite population was then separated back into CD41+ and CD41- populations and these were visualized on the t-SNE plane. Lastly, expression of markers was compared.
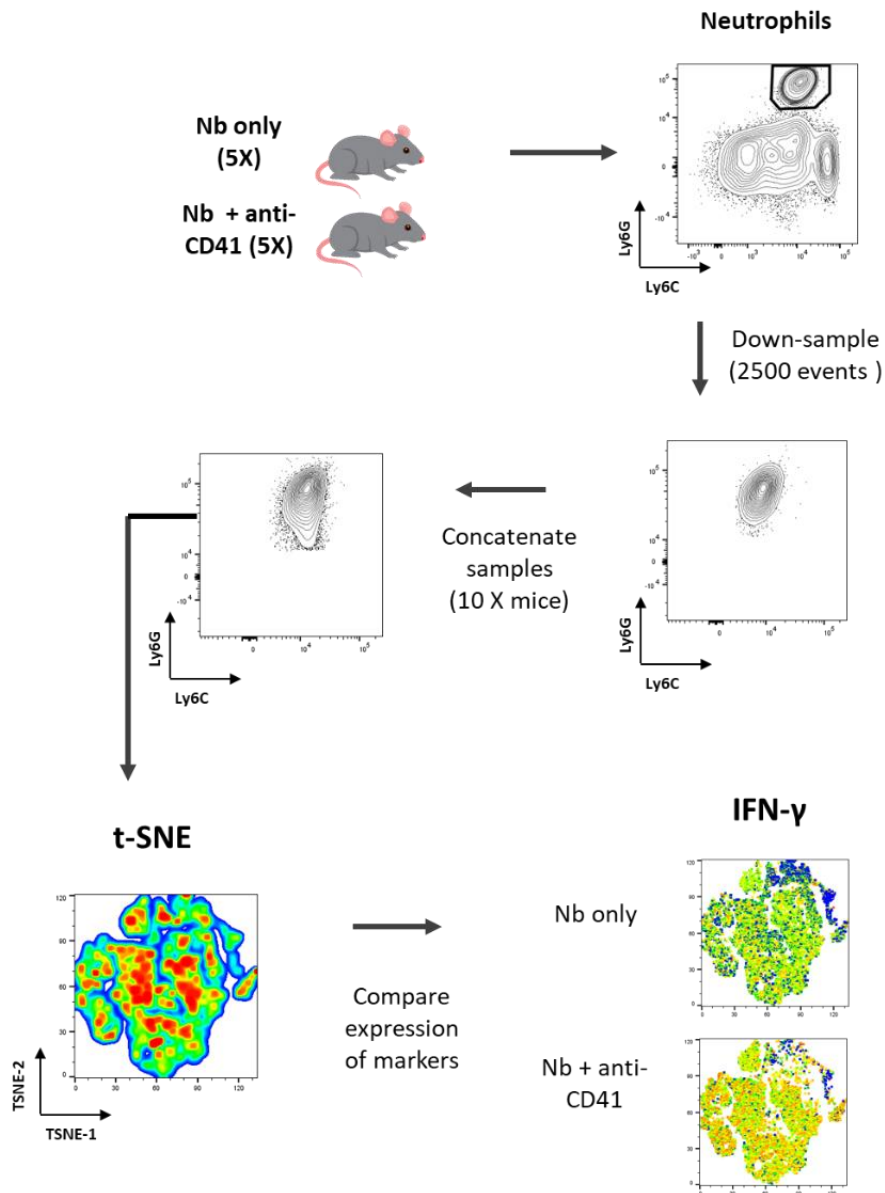
Figure 2.11: Overview of the workflow used for unsupervised (t-SNE) analysis of neutrophils from Nb only and Nb + anti-CD41 mice. Neutrophils were gated out using the gating strategy in figure 2.7. Neutrophil populations were then down sampled to 2500 events per sample and subsequently concatenated into a composite population on which t-SNE analysis was performed. The markers used for t-SNE analysis were: CD45, CD11b, Ly6G, Ly6C, IFN-y, granzyme B, CD41. The composite population was then separated back into Nb only and Nb + anti-CD41 populations and these were visualized on the t-SNE plane. Lastly, expression of markers was compared.

2.4) Microscopy

2.4.1) Stereomicroscopy

For analysis of worm burdens in the lung of Nb infected mice, the left lobes (outlined in figure 2.5) were removed, cut into small pieces , wrapped in gauze and suspended in falcon tubes containing a solution of 0.9 % NaCl. After overnight incubation at 37° C worms, which collect at the bottom of the tube, were carefully resuspended in a small volume of NaCl and pipetted onto a well of a gridded 12-well plate. Using a brightfield microscope the total number of larvae were then enumerated.

2.4.2) Histology and immunohistochemistry

As outlined in 2.1.2, one section of lung was removed and used for histological analyses. This first entailed fixing of lung samples in 4% paraformaldehyde, after which they were embedded in paraffin and sectioned.

For Haematoxylin & Eosin (H&E) staining, lung sections were processed and stained using a standard protocol. Briefly, this involved suspending lung sections in the following reagents (in order): Xylene, Ethanol, H_2O, Hematoxylin, H_2O, Differentiator, H_2O , Ethanol, Eosin, Ethanol, and finally, Xylene.

For CD41 IHC staining, paraffin embedded lung sections were dewaxed and rehydrated before being subjected to heat-mediated antigen retrieval in TRIS-EDTA buffer (pH 9). After endogenous peroxidase blocking, lung sections were then washed and stained with 100 ul of a rabbit monoclonal anti-CD41 primary antibody (Abcam, clone EPR17876) (1:250 dilution) for 90 minutes. After staining, sections were rinsed with PBS and visualized using a DAB chromogen anti-rabbit system (Dako K4003) and counterstained with Haematoxylin .

Slides were then further rinsed, dehydrated and finally, covered and imaged using an upright light microscope (Zeiss) with Zenmax software at 40X, 100X and 400X magnifications.

2.5) BAL assays

To assess pulmonary pathology, the concentration of protein and DNA in BAL fluid from infected mice was quantified. BAL fluid was collected post cannulation of the trachea and lavage of the lungs with 1ml sterile PBS (supplemented with 2% BSA and 0.25 M EDTA). BAL samples were centrifuged at 400 XG for 5 minutes and the supernatant (containing the protein and DNA fraction) was transferred to new tubes and analysed using a Nanodrop 1000C spectrophotometer (Thermofischer). For blanking purposes, the PBS solution used for the lavages was used.

Additionally, for analyses of BAL haemorrhaging, the optical density (OD) at 540 nm of BAL samples was analysed. The absorption of light at this wavelength correlates strongly with the concentration of haemoglobin and is commonly used to analyse pulmonary haemorrhaging [152].

2.6) Statistics

Flow cytometry data, BAL protein, BAL DNA and BAL OD_{540} are expressed as mean $\pm$ standard error of the mean (SEM). For statistical analysis of data sets with two groups, non-parametric two-tailed t-tests (Mann-Whitney U-test) or paired t-test were used. In experiments in which three or more groups were compared, differences were assessed using one – way ANOVA, followed by Tukey's multiple comparison post-hoc test. A p-value less than 0.05 was considered statistically significant. All data were analysed using GraphPad Prism version 8 (GraphPad Prism software; La Jolla, CA).

Chapter 3: Results

Chapter 3.1:

Establishing platelets as a component of the acute innate immune response to N. brasiliensis

3.1.1 Platelets become activated following Nb infection and localise to extravascular lung compartments

An important requisite for platelets performing immunological functions is that they become activated [7]. Similar to other immune cells , their activation can be tracked and analysed using expression profiles of immune receptors (most commonly the receptor CD62P) and soluble factors [7] . Additionally, an increasing number of studies point to the formation of circulatory platelet-leukocyte complexes as a sensitive and durable marker of platelet activation, and it is now well established that these complexes form in response to a variety of infections [48]. However, whether these complexes also form (i.e., whether platelets become activated) following a helminth infection is, to the best of our knowledge, not known.

To test this, we collected cardiac blood from naïve and Nb infected mice (taken at 18 hours post infection) and compared them on the basis of these known indices of platelet activation. We found that Nb infected mice exhibited significant circulatory platelet activation following infection. This included increased levels of platelet-leukocyte (CD45+ cells) complexes **(figure 3.1.1, A**), increased platelet expression (MFI) of CD41 **(figure 3.1.1, B)**, increased levels of CD62P + platelets (figure 3.1.1., **C**) and increased platelet expression of CD62P **(figure 3.1.1, D**).

Furthermore, given previous reports that platelets leave the vascular space, often in conjunction with other immune cells during conditions of pulmonary inflammation [153], we examined whether Nb infection was also associated with infiltration of platelets into extravascular compartments . Using immunohistochemistry, we identified CD41+ cells aggregating at vessel barriers in both naïve and Nb infected mice but were only able to find extravascular platelets in infected mice. Notably, extravascular platelets were often in close proximity to infiltrating immune cells **(figure 3.1.1, E).**

Taken together, our data indicates that platelets become rapidly activated following Nb infection and localise to extravascular lung compartments.
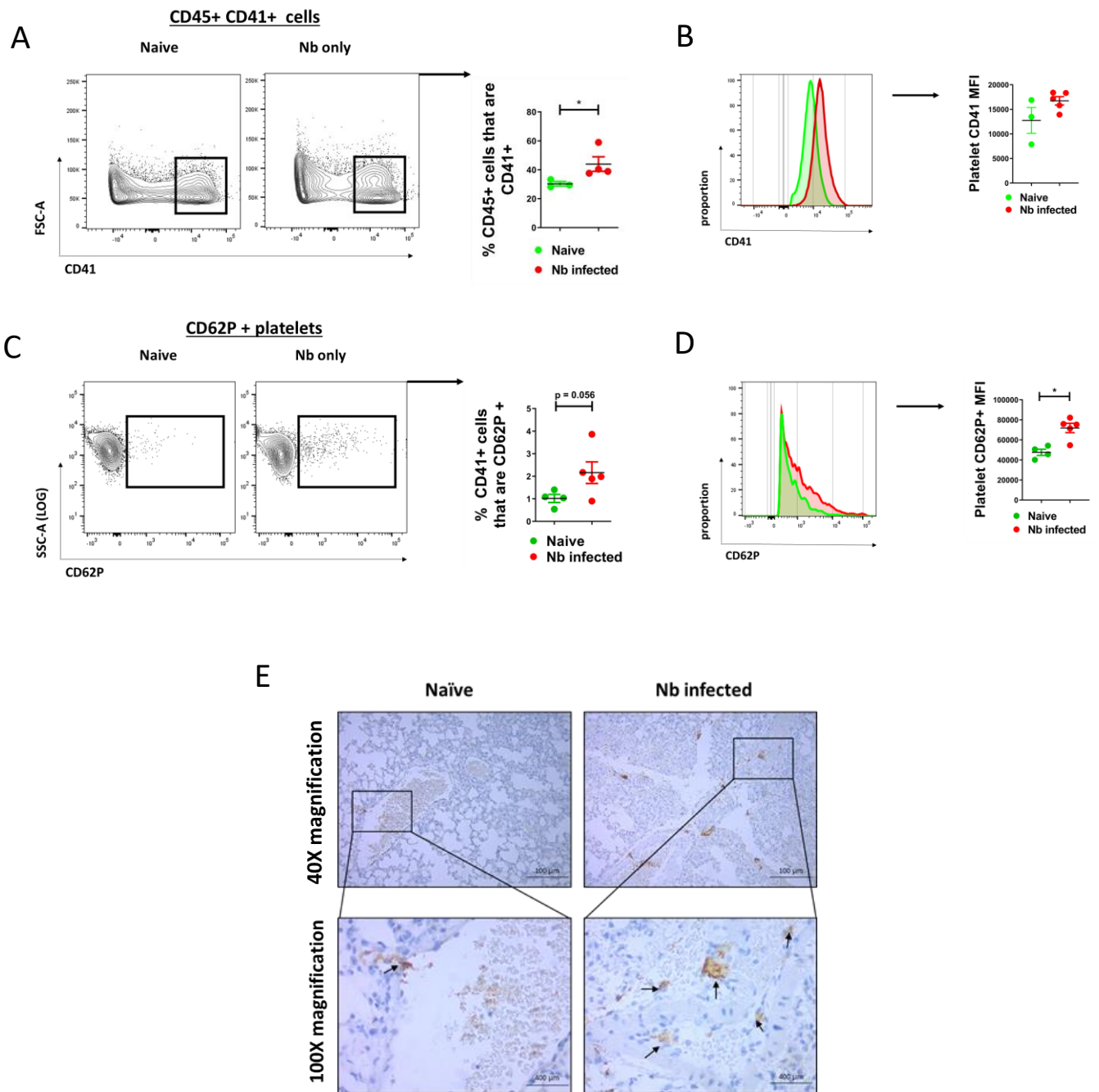
Figure 3.1.1: Platelets become activated following Nb infection and localise to extravascular lung compartments. Platelet circulatory responses were analysed with flow cytometry. Markers of platelet activation including their association with immune cells **A)** and their expression of CD62P **C)** at 18 hours post infection (p.i.)was assessed. Additionally, the expression (mean fluorescence intensity) of CD41 **B)** and CD62P **D)** was compared in platelets from naïve and Nb infected mice. Lastly, immunohistochemical analyses was used to investigate platelet infiltration into extravascular spaces **E)**. Arrows indicated CD41+ events. * represents a p -val < 0.05 (Mann-Whitney U-test). Data is representative of 2 experiments, n= 4-5 mice.

3.1.2 : Early Nb infection is characterised by a circulatory neutrophil response

Most studies investigating acute innate immunity to Nb infection have focused either on the immune response at the site of inoculation (i.e., in the skin) or the immune response in the lungs, with very few studies examining the circulatory response shortly after (within 24 hours) of Nb infection. These studies have clearly implicated myeloid innate immune cells, such as neutrophils, as crucial effectors of early immunity [154], however , how circulatory neutrophils and other myeloid derived innate cells are acutely affected following Nb infection remains ill-defined.

To characterize the early circulatory innate immune response to Nb infection, using flow cytometry, we compared the levels and number of circulating neutrophils, monocytes, and eosinophils in naïve and Nb infected mice at 18 hours post infection. We found that Nb infected mice had significantly raised levels (i.e. proportion CD45+ cells) and numbers of neutrophils (CD45+, CD11b+, $Ly6C^{int}$ $Ly6G^{Hl}$ cells) (**figure 3.1.2, A)**, but similar levels circulatory monocytes (CD45+, CD11b +, Ly6C +, SSC-A $^{Low)}$ (**figure 3.1.2, B**) compared to naïve mice. While we did observe a trend for increased proportions and numbers of eosinophils (CD45+, CD11b +, Ly6C ^{int} , SSC-A ^{Hi}) [155] (**figure 3.1.2, C**) in Nb infected mice, this was not statistically significant. These data suggest that , in line with previous studies [154] , neutrophils dominate the early response to Nb infection.
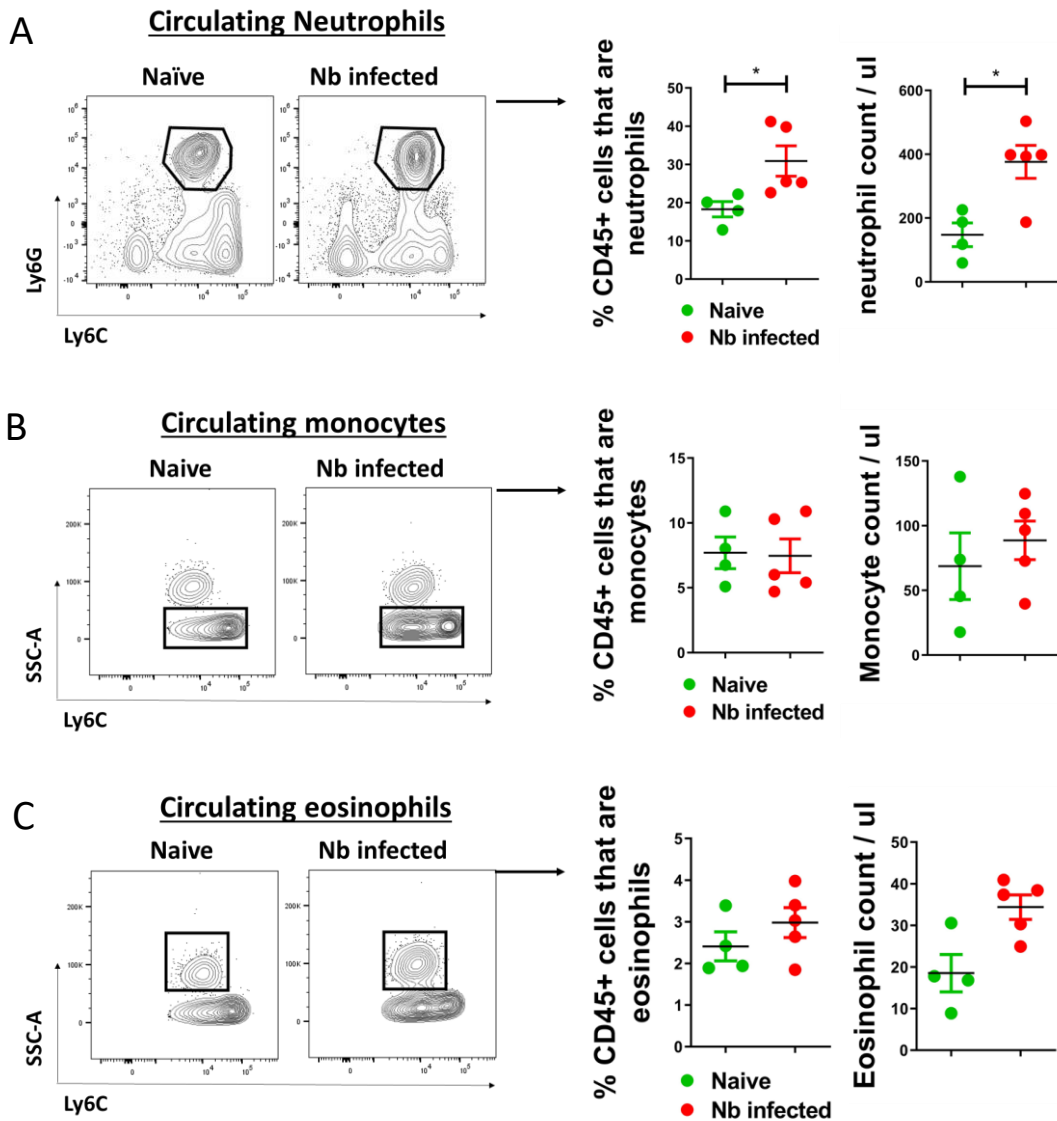
Figure 3.1.2: Early Nb infection is characterised by a circulatory neutrophil response. Flow cytometric analysis was performed on cardiac blood of naïve and Nb infected mice at 18 hours p.i. and acute circulatory myeloid cell responses were compared. In particular, changes in neutrophils **A**) , monocytes **B**) and eosinophils **C**) proportion and number were determined. Data is representative of 2 experiments, n = 4-5 mice. (Mann-Whitney U-test).

3.1.3: Early Nb infection involves recruitment of neutrophils and eosinophils to the lung

Previous studies have indicated that the number of Nb larvae that reach the lung peaks at around 2 days post infection [156]. Similarly, many studies investigating acute pulmonary immune responses to Nb have concentrated on this time point, as this is also when peak neutrophil responses are observed [157]. Comparatively little is known about how pulmonary responses develop at an earlier time point, (e.g. 18 hours post infection), despite evidence that significant numbers of larvae begin to reach the lung within 24 hours of inoculation [158] .We therefore next sought out to characterise the early recruitment of myeloid innate cells in the lung at 18 hours post infection.

As with our analyses of the circulatory response in cardiac blood, flow cytometry was used to compare the levels and number of neutrophils, monocytes and eosinophils in the lung of naïve and Nb infected mice.

In line with expectation, we found that Nb infected mice had significantly raised levels and numbers of neutrophils (CD45+, CD11b +, Ly6G ^{Hi} cells) in their lungs relative to naïve mice **(figure 3.1.3, A)**. Interestingly, infected mice also had moderately (but significantly) increased levels and numbers of eosinophils (CD45+, CD11b+, Ly6G -, SiglecF ^{Hi} cells) in their lungs at this time point **(figure 3.1.3, C).** However, as with our analyses on cardiac blood samples, monocyte (CD45+, CD11b, Ly6G -, SiglecF -, Ly6C + cells) numbers were not significantly different, although their proportion in the CD45+ population was decreased **(figure 3.1.3, B)**, likely as a result of the increased proportions of neutrophils and eosinophils.
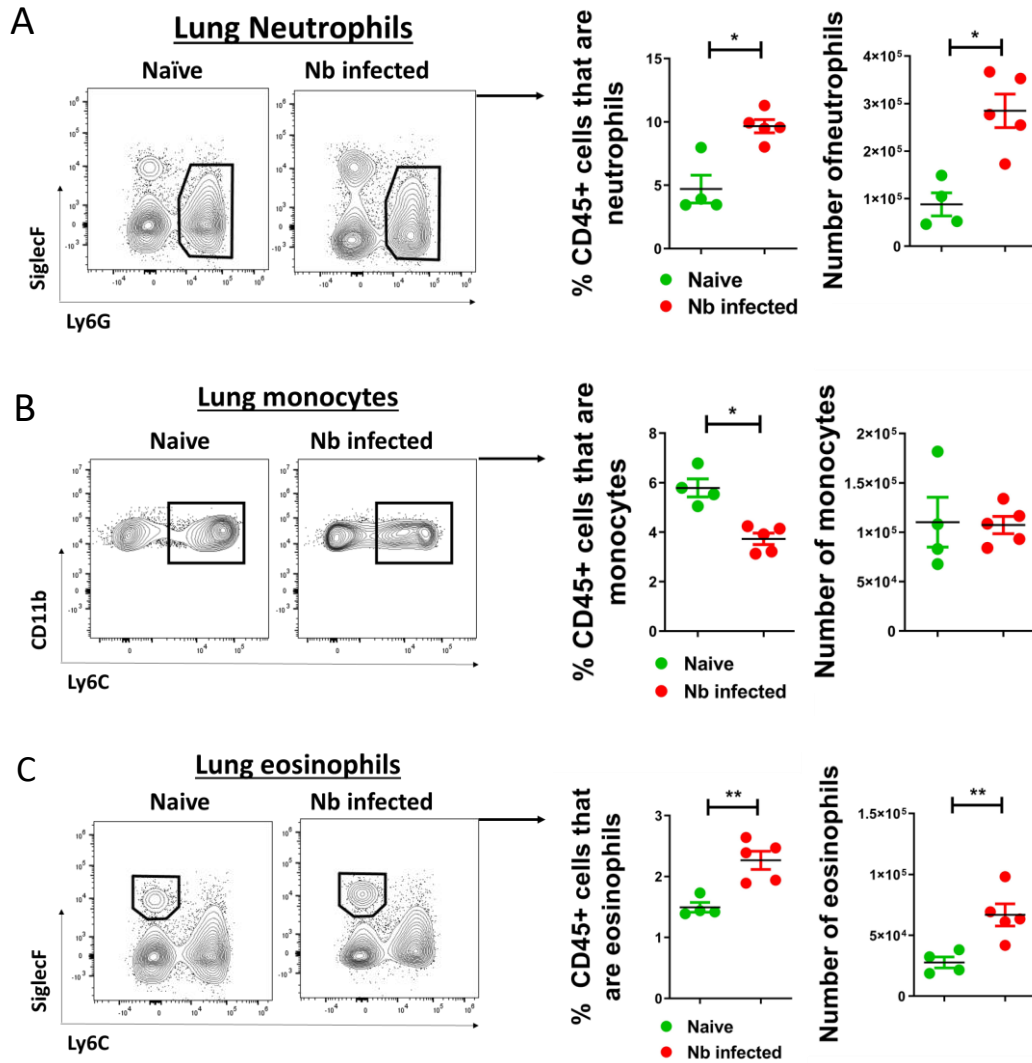
Figure 3.1.3: Early Nb infection involves recruitment of neutrophils and eosinophils to the lung. Flow cytometry of lung samples taken at 18 hours p.i. was performed to analyse acute pulmonary immune responses. The proportion and number of neutrophils **A)**, monocytes **B)** and eosinophils **C)** were compared. Data is representative of 2 experiments, n = 4-5 mice. ** represents a p-value < 0.01 (Mann Whitney U- test).

3.1.4: Platelets interact with immune cells in the lung during pulmonary Nb infection

Our previous findings indicated that acute pulmonary immunity to Nb invasion at 18 hours p.i. involved the recruitment of granulocytes, including neutrophils and eosinophils to the lung. These cells, especially neutrophils, are known to play important roles in mediating the early immune response [102]. However, resident immune cells , such as alveolar macrophages are also thought to play crucial roles [159].

Previous studies indicate that the association of platelets with immune cells is an important regulatory mechanism underlying their immune function. To establish whether platelets are a feature of the early pulmonary immune response, we next analysed the association of important innate effector cells, including neutrophils , alveolar macrophages and eosinophils with platelets, which we determined through their respective expression of the platelet marker CD41.

Flow cytometric analyses revealed increased association of neutrophils and alveolar macrophages with platelets **(figure 3.1.4, A and B)** following Nb infection. Furthermore, we also found increased numbers of CD41 + neutrophils and CD41+ alveolar macrophages (CD45+, CD11b ^{Low}, SiglecF ^{High} cells) in infected mice relative to naïve mice. There were also increased numbers of CD41+ eosinophils in Nb infected mice compared to naïve mice, although the proportion of eosinophils staining CD41+ was similar **(figure 3.1.4, C).**

These data, in combination with our previous findings showing localisation of platelets in extravascular spaces **(figure 3.1.1)** , further implicate platelets in the acute innate response to Nb infection.
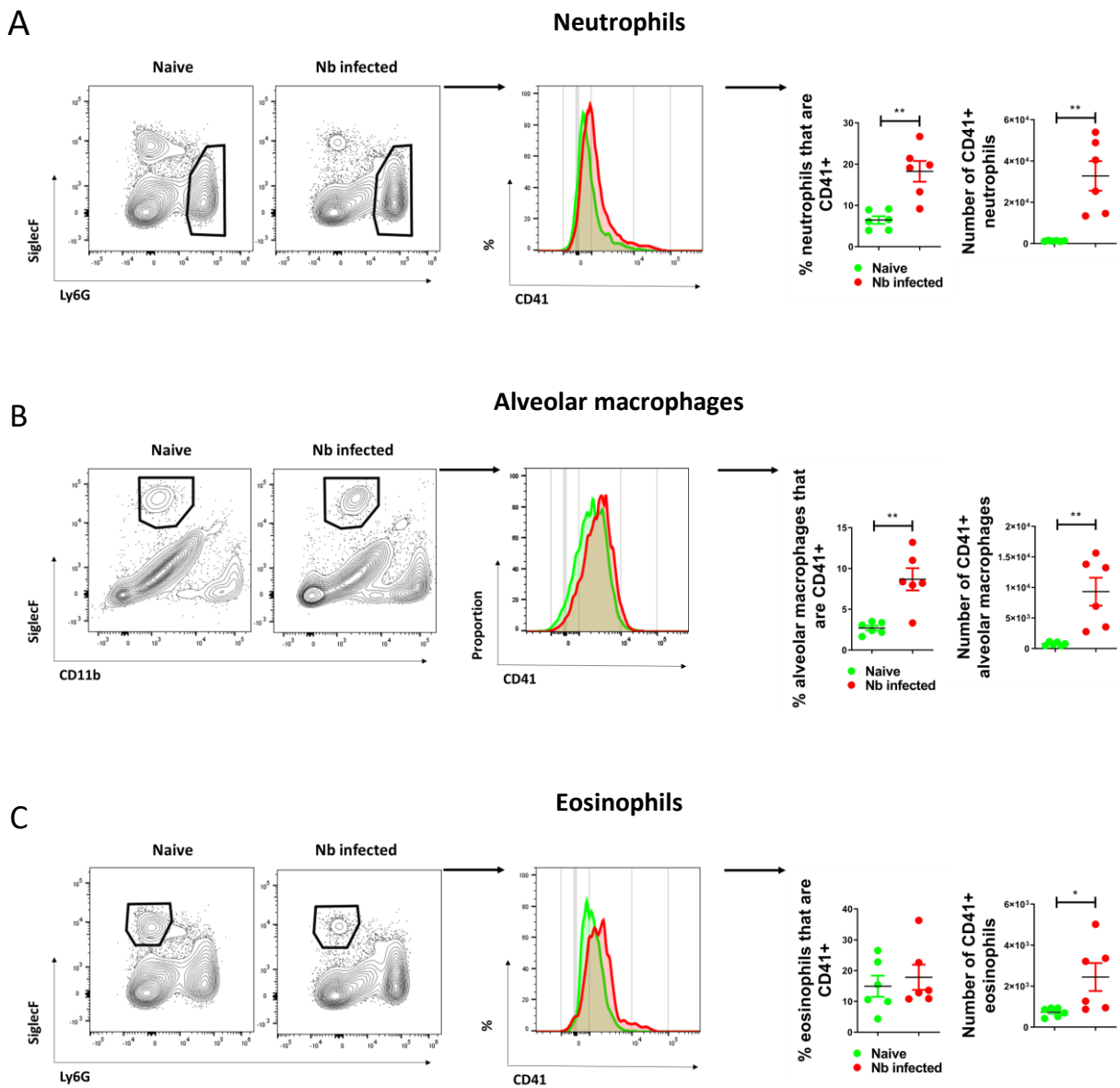
Figure 3.1.4: Platelets interact with immune cells in the lung during pulmonary Nb infection. Flow cytometric analysis of neutrophils A), alveolar macrophages B) and eosinophils C) in single cell suspensions was performed. Interaction of these immune populations with platelets was determined using CD41 expression (FMO were used to guide gating). The data show representative flow plots and histograms of CD41 expression. Data is representative of two experiments: n= 5-6 mice. (Mann-Whitney U- test).

3.1.5: Association of immune cells with platelets increases as the infection progresses in the lung

Having established that platelets interact with recruited and lung resident immune cells in the lung early on (18 hours) post infection with Nb, we next investigated whether platelets also associate with immune cells in the lung at other time points. We chose to analyse platelet -association with immune cells at day 2 and day 3 post infection, to give insight into how platelets respond during peak larval burden (day 2) and at stages just following this (day 3) [158].

As before, flow cytometry was used to analyse CD41 expression on select immune populations in the lung, and levels of expression were compared between different time points. Our analyses revealed that a significantly greater proportion of neutrophils (**figure 3.1.5, A),** alveolar macrophages **(figure 3.1.5, B)** and eosinophils **(figure 3.1.5, C)** stained positive for CD41 at day 2 relative to 18 hours p.i. Furthermore, CD41 detection remained elevated at day 3 p.i..

Similarly, our analysis of the MFI of CD41 on these immune populations produced similar findings: all three immune populations demonstrated increased MFI of CD41 on day 2 and day 3 relative to 18 hours post infection. However, with the exception of alveolar macrophages, where significant differences were found between both day 2 and 18 hours and day 3 and 18 hours post infection **(figure 3.1.4, B)**, statistical significance was only reached when comparing day 3 and 18 hours post infection **(figure 3.1.4, A and C)**.
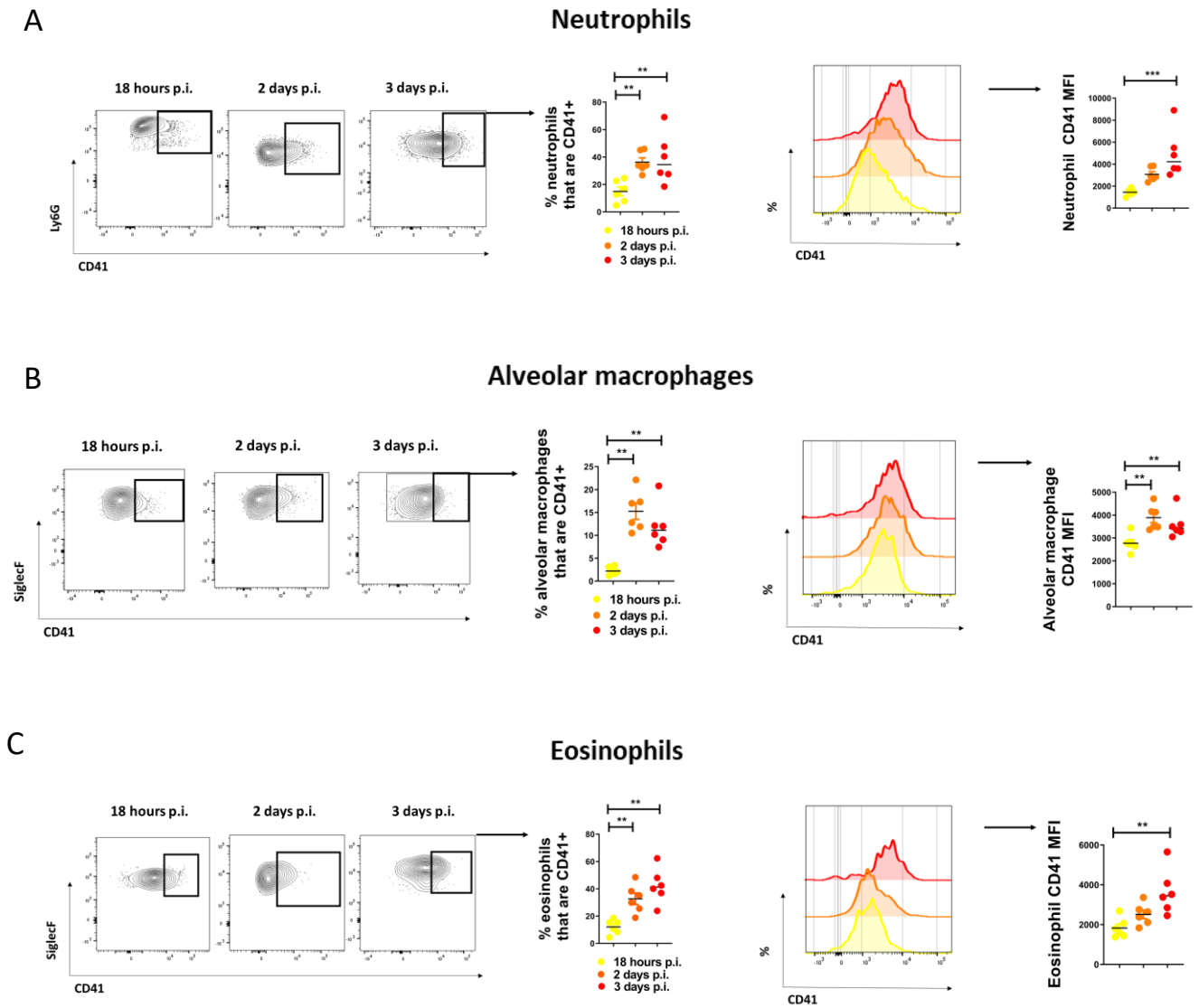
Figure 3.1.4: Platelets interact with immune cells in the lung during pulmonary Nb infection. Flow cytometric analysis was performed to determine differences in the association neutrophils A), alveolar macrophages B) and eosinophils C) with platelets in the lung at 18 hours p.i..CD41 + gates are based on unstained controls. Representative flow plots of cell populations and histograms showing CD41 expression are presented. ** indicates a p-value of < 0.01 (one -way ANOVA), n = 5 mice.

3.1.6: CD41+ and CD41- circulatory neutrophils represent distinct subsets with altered phenotype

Given our previous findings showing tight association of platelets with immune cells in the circulation and the lung during acute Nb infection, we next tested whether platelet associated and non-associated cells were phenotypically distinct, as has been shown in other studies [87]. To investigate this, we used both supervised and unsupervised (t-SNE) flow cytometric data analysis to probe for differences between circulatory CD41+ and CD41 – neutrophils in Nb infected mice. We chose to focus our analysis on neutrophils considering our previous findings showing that acute Nb infection is characterized by a circulatory neutrophil response (**figure 3.1.2**).

The results of our t-SNE analyses, as outlined in **figure 3.1.6, A** demonstrated that CD41+ and CD41- neutrophils displayed similar patterns of expression of the neutrophil markers Ly6G, Ly6C and CD11b, but qualitatively distinct expression of IFN-y and granzyme -B .

To investigate this further we quantified the mean fluorescence intensity (MFI) of the same receptor and effector molecules visualized with t-SNE in **figure 3.1.6, A** . Our results indicated that, as predicted by the t-SNE analyses, CD41+ and CD41- circulatory neutrophils had similar expression of Ly6G , Ly6C and CD11b **(figure 3.1.6, Bi – Biii)**, but altered expression of IFN-y and granzyme-B. More specifically, we observed reduced expression of IFN-y and significantly higher expression of granzyme- B on CD41+ neutrophils relative to CD41 - neutrophils **(figure 3.1.6, Bv -Bvi).**

To further test the association of CD41+ expression (i.e. platelet association) with differences in IFN-y and granzyme-B expression, CD41+ neutrophils from Nb infected mice were divided into CD41 intermediate (int) and CD41 high expressing populations and the expression of IFN-y and granzyme -B compared. In line with the results of our analyses comparing CD41+ and CD41 – neutrophils, we observed decreased expression of IFN-y and increased expression of granzyme-B on CD41 high cells relative to CD41 int cells.

These data suggest that CD41+ and CD41- neutrophils are distinct from one another and that CD41+ expression negatively associates with IFN-y expression and positively with granzyme-B expression.
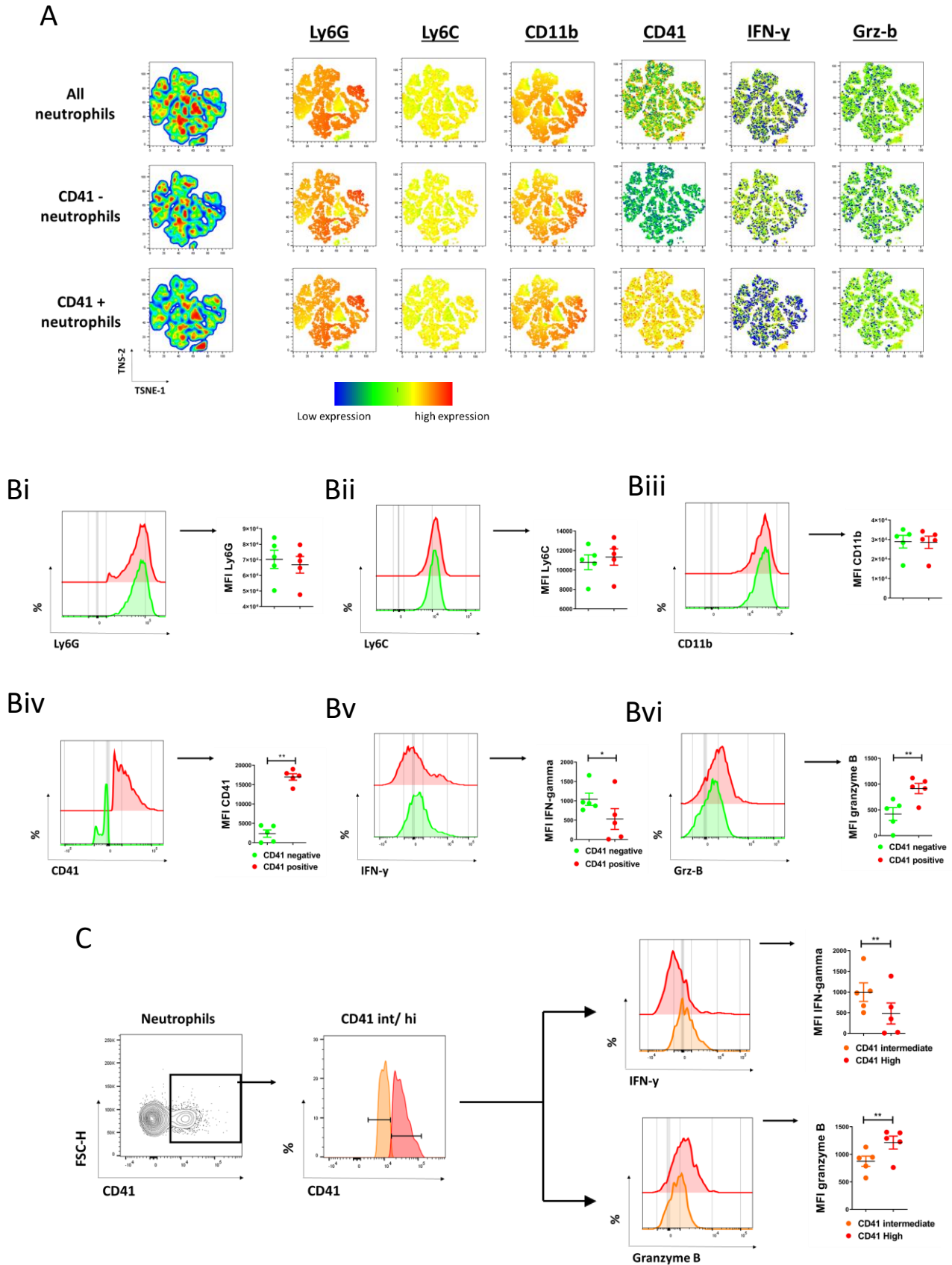
Figure 3.1.6 CD41 positive and negative circulatory neutrophils represent distinct subsets with altered phenotype. T-SNE analysis was performed on CD41+ and CD41 – neutrophils. Their visualization on the t-SNE landscape and expression patterns of neutrophil receptors and effector molecules are shown **A**). Additionally, MFI of individual receptors and effector molecules are shown **B.i – Bvi**). Finally, CD41+ were gated into CD41 High and CD41 int populations and the expression of IFN-γ and granzyme-B was compared **C**). Data is representative of 2 experiments, n = 5. Paired t-test was used for statistical analysis**.**

3.1.7: CD41 positive and negative lung neutrophils differentially express RELM-alpha.

In light of our previous results, demonstrating close assoiation of platelets with immune effector cells, including neutrophils in the lung following Nb infection, we next decided to investigate whether platelet associated (CD41 +) and CD41 − lung neutrophils would , similar to circulatory neutrophils , show differences in their phenotpe. To do this we again made use of t-SNE analysis and compared the expression patterns of neutrophil receptor and effector molecules in CD41 + and CD41 − lung neutrophils.

Our results indicate that, in contrast to circulatory neutrophils, CD41 + and CD41 − lung neutrophils displayed qualitatively different expression of the neutrophil markers Ly6G and CD11b **(figure 3.1.7 , A)** . Furthermore, CD41 + and CD41 − neutrophils significantly differed in terms of their expression of RELM-α - a type 2 associated molecule that plays important roles in mediating early protective responses against Nb in the lung [137].

As before, to quantify differences between CD41 + and CD41 − lung neutrophils, analyses of their respective MFIs of neutrophil Ly6G, CD11b and RELM-α was perfomed . In agreement with our t-SNE analysi, CD41 + neutrophils demonstrated higher expression of Ly6G and RELM-α and reduced expression of CD11b **(figure 3.1.7, Bi − Bii).**

Moreover, upon separation of the CD41+ lung neutrophil population into CD41 intermediate (int) an CD41 high populations it was observerd that CD41 high populations had increased MFI of RELM-α relative to CD41 int populations **(figure 3.1.7 , C)** .

Taken together, our analysis indicates that, similar to circulatory CD41+ and CD41- neutrophils, lung neutrophils that are platelet-assoicated are distinct from non-associated populations and differentially express RELM-α
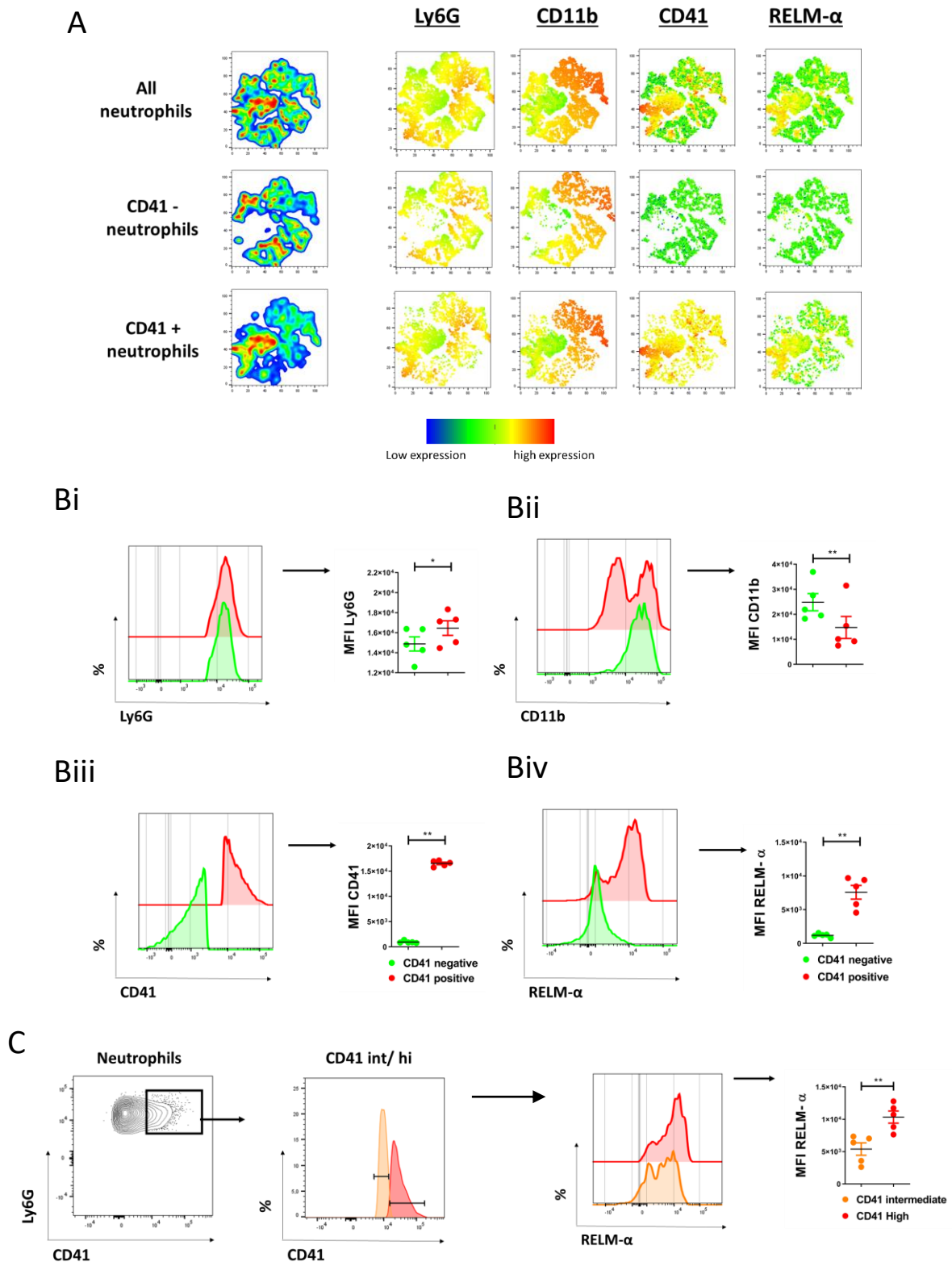
Figure 3.1.7: CD41 positive and negative lung neutrophils differentially express RELM-alpha. T-SNE analysis was performed on CD41+ and CD41 – neutrophils. Their visualization on the t-SNE landscape and expression patterns of neutrophil receptors and effector molecules are shown A). Additionally, MFI of individual neutrophil receptors B.i – B.iii) and RELM-α Biv) are shown. Finally, CD41+ were gated into CD41 High and CD41 int populations and the expression of RELM-alpha compared C). Data is representative of 2 experiments, n = 5 mice. Paired t-test.

Chapter 3.2:

Establishing a model of Nb infection in platelet depleted mice and evaluating the role of platelets in protection to Nb-associated pulmonary pathology

3.2.1: Platelet depletion prior to infection with Nb results in markedly worsened pulmonary pathology

To further study the role that platelets are playing in the acute innate immune response to Nb infection we established a model of antibody mediated platelet depletion. This model entailed rendering mice severely thrombocytopenic (less than 10 % of normal platelet levels **Appendix, figure 5.3**) prior to infecting them with Nb.

Studies using antibody mediated depletion of platelets have contributed significant findings to our understanding of the pathophysiology of pulmonary infection with bacterial and viral pathogens and the function of platelets [50]. We hypothesized that depletion of platelets would perturb immunologic and physiologic processes supporting protection to pulmonary Nb infection as well.

In line with our hypothesis, we found that mice that were rendered thrombocytopenic prior to infection with Nb (Nb + anti-CD41 mice) succumbed quickly to infection and had marked pulmonary pathology. Indeed, none of the Nb + anti-CD41 mice survived longer than 24 hours post infection **(figure 3.2.1, A)** and Nb + anti-CD41 mice displayed significantly increased levels of protein and DNA in their BAL fluid relative to non-depleted (Nb only) mice **(figure 3.2.1, B)**. Interestingly, the increased pathology in Nb + anti – CD41 mice correlated with reduced worm burdens in their lungs **(figure 3.2.1, C)**, suggesting that they also had differences in anti-parasitic responses.

Haematoxylin & Eosin staining further revealed significant differences between Nb only and Nb + anti-CD41 mice. Thrombocytopenic Nb + anti-CD41 mice showed extensive haemorrhaging in their lung **(figure 3.2.1, D – upper panels)** which was absent from Nb only mice. Additionally, haemorrhaging in Nb + anti-CD41 localized to regions where Nb larvae were present and was associated with severe alveolar inflammation **(figure 3.2.1, E – lower right panels)**.

Taken together, these data clearly suggest a notable role for platelets in protecting against Nb-associated pathology in the lung during acute infection.
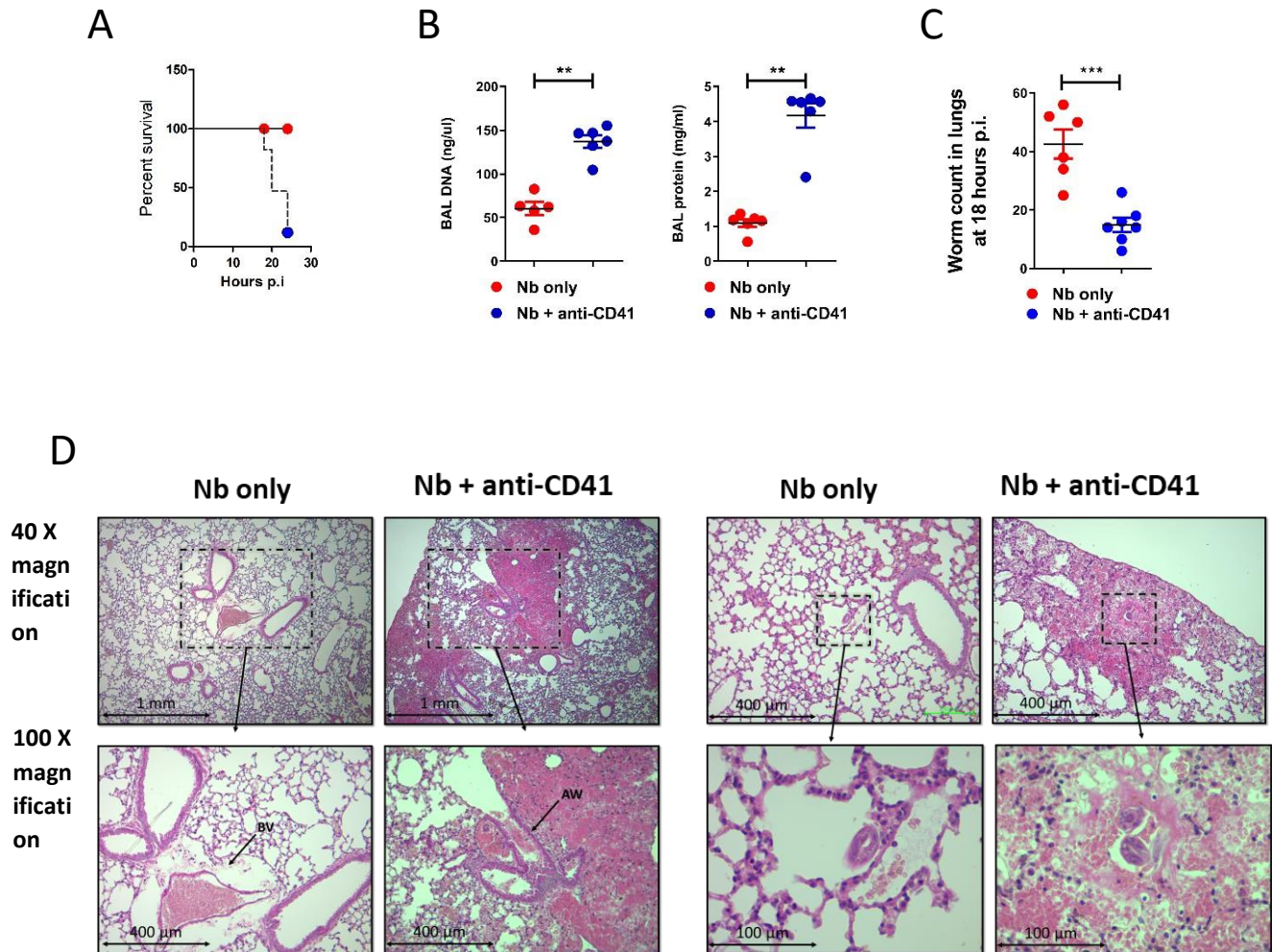
Figure 3.2.1: Platelet depletion prior to infection with Nb results in markedly worsened pulmonary pathology. Mice were rendered thrombocytopenic prior to being infected with Nb. The survival curves of Nb infected and Nb infected + anti-CD41 treated mice are shown -**A**). Pulmonary pathology was quantified using readouts relating to infiltration of protein and DNA **B**) into BAL fluid. Worm counts in the lungs of treated mice were additionally determined **C**). H & E staining on lung sections from Nb only and Nb + anti-CD41 mice was also performed to gain oversight of gross pathology **D**). Arrows point to Nb larvae.BV = blood vessel; AW = airway. Data are representative of three experiments, n = 5 mice. ** represents a p-value < 0.01 (Mann-Whitney u-test).

3.2.2: Platelet depletion prior to Nb infection is associated with changes in circulating immune populations and decreased levels of platelet-leukocyte aggregates.

Having established that platelets are crucial for control of helminth – associated pathology in the lung, we next investigated whether platelet depleted mice also had notable differences in their acute response to Nb infection. To assess this, we performed flow cytometry on circulatory cells in the blood of Nb only and Nb + anti-CD41 mice. Specifically, we focused on establishing potential differences in the myeloid compartment, given our previous findings showing acute changes of myeloid cell populations following infection with Nb **(figure 3.1.2).**

Flow cytometric analyses revealed that while Nb + anti-CD41 mice had similar levels of circulating monocytes **(figure 3.2.2, Bi)** and eosinophils **(figure 3.2.2, Ci)** in their blood, they had significantly increased levels of neutrophils (**figure 3.2.2, Ai**) relative to Nb only mice.

Furthermore and in line with expectation, we observed decreased levels of circulating platelet-leukocyte complexes in Nb + anti-CD41 mice, especially platelet- neutrophil **(figure 3.2.2 , Aii)** and platelet-monocyte complexes **(figure 3.2.2, Bii)** . While the levels of circulating platelet-eosinophil complexes were moderately lower in Nb + anti-CD41 mice **(figure 3.2.2, Cii)**, this was not statistically significant.
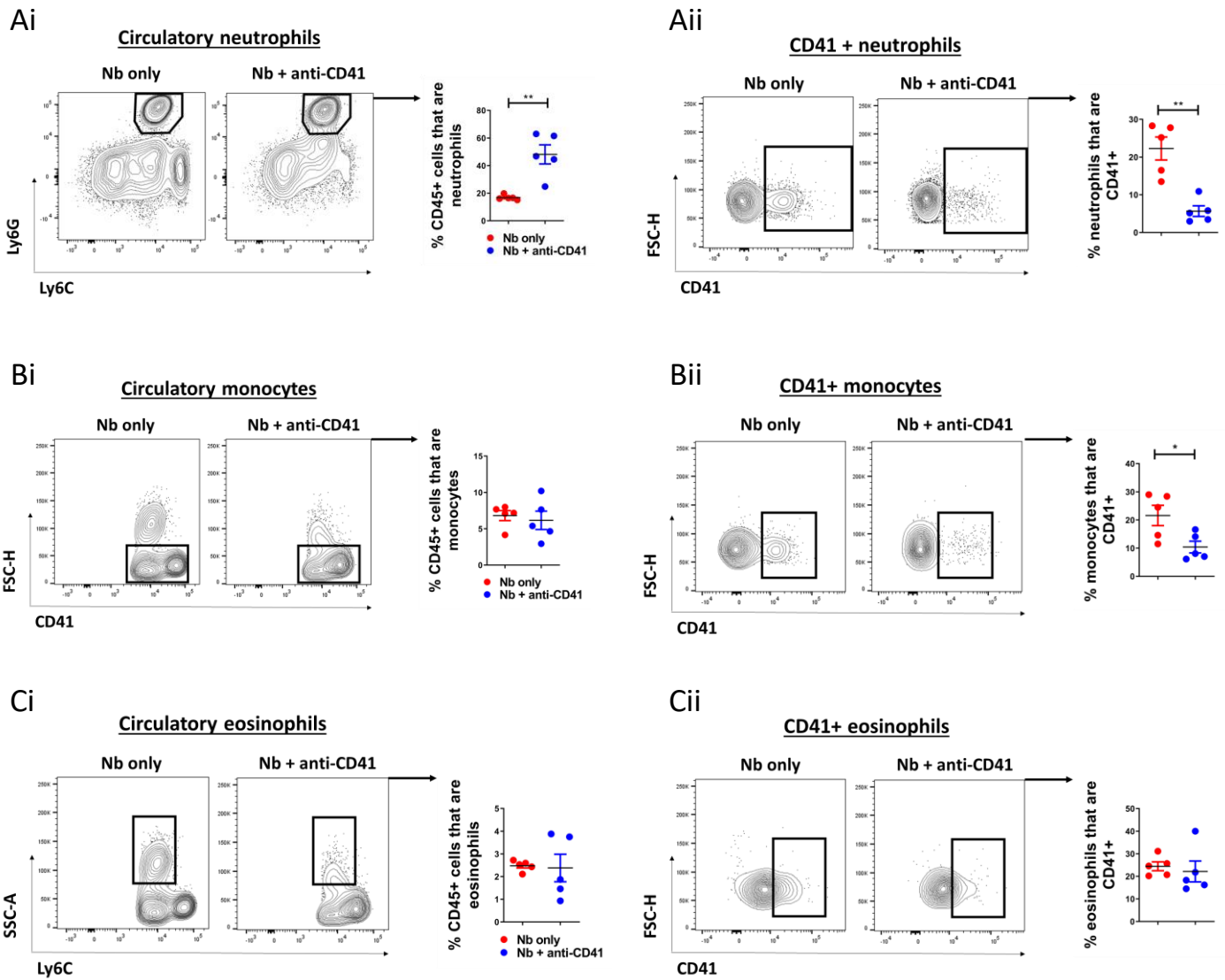
Figure 3.2.2: Platelet depletion prior to Nb infection is associated with changes in circulating immune populations and decreased levels of platelet-leukocyte complexes. Blood was collected from Nb only and Nb + anti-CD41 mice, and circulating myeloid populations were analysed via flow cytometry. Representative flow cytometry plots and quantification of neutrophil **Ai**), monocyte **Bi**) and eosinophil **Ci**) levels are shown. Similarly, the levels of CD41+ neutrophils **Aii**), monocytes **Bii**) and eosinophils **Cii)** are presented. Data is representative of two experiments, n = 5 mice. ** represents a p-value < 0.01 (Mann-Whitney U- test)

3.2.3: Platelet depletion leads to minor changes in the pulmonary myeloid immune landscape

As outlined previously, innate myeloid cells are rapidly recruited to the lung during Nb infection **(figure 3.1.3)** and play crucial roles in mediating acute immune responses [102]. Both earlier and recent studies have indicated that platelets play notable roles in mediating and regulating the extravasation of innate cells out of the circulation and into tissue, including the lung [88]. Platelets mediate leukocyte recruitment by either enhancing their adherence to endothelial sites, or through the production of factors that drive leukocyte chemotaxis [70].

Given these previous studies, we next tested whether platelet depletion prior to Nb infection would also be associated with significant changes in the pulmonary myeloid immune landscape, specifically at 18 hours post infection. Flow cytometric analyses revealed that Nb only and Nb + anti-CD41 mice had only moderate differences in the levels and numbers of lung myeloid cells, with similar levels of neutrophils **(figure 3.2.3, A)**, monocytes (CD45+ , CD11b+ , SiglecF- , Ly6G - , SSC-A^{low} cells) **(figure 3.2.3, B)** and alveolar macrophages   **(figure 3.2.3 , C).** However, we did observe consistently lower levels and numbers of eosinophils in Nb + anti-CD41 mice relative to Nb only mice **(figure 3.2.3, D),** which indicates that platelets may regulate the recruitment of myeloid cell subsets during acute Nb infection in a cell-specific manner.
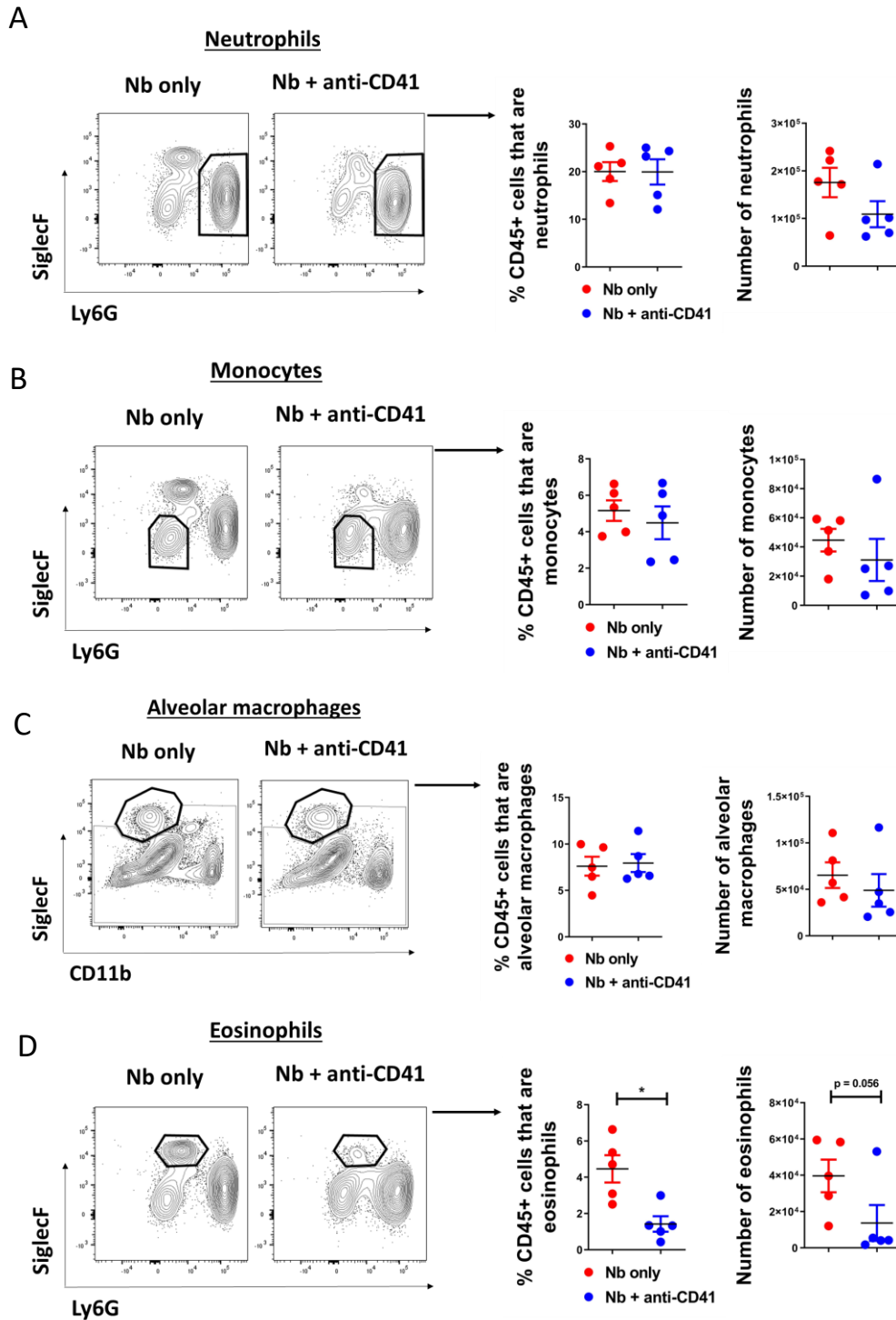
Figure 3.2.3: Platelet depletion leads to minor changes in the pulmonary myeloid immune landscape. Lung cells from Nb only and Nb + anti-CD41 mice were prepared into single cell suspensions and analysed using flow cytometry. The relative proportion (% CD45+ cells) of individual myeloid cells and their total number in prepared suspensions were quantified. Nb only and Nb + anti-CD41 mice were compared in terms of their neutrophils **A**) , monocytes **B**) , alveolar macrophages **C**) and eosinophils **D**) Data are representative of 2 experiments , n = 5 mice. (Mann-Whitney U- test)

3.2.4: Platelet depletion significantly reduced the number of platelet-leukocyte complexes in the lung

Our previous data indicated that platelets interact with immune populations in the lungs following Nb infection. Based on our analyses showing reduced levels of platelet-leukocyte complexes in the circulation of Nb + anti-CD41 mice, we postulated that anti-CD41 treatment would also significantly decrease the number of platelet-leukocyte complexes detectable in the lung.

In line with our hypothesis, we found that within Nb + anti-CD41 mice a reduced proportion of neutrophils (**figure 3.2.4, A)** monocytes **(figure 3.2.4, B)**, alveolar macrophages (figure 3.2.4, **C**) and eosinophils **(figure 3.2.4, D)** were staining CD41+ positive relative to Nb only mice.

Furthermore, these Nb + anti-CD41 mice also displayed significantly reduced total numbers of CD41+ monocytes and CD41+ eosinophils **(figure 3.2.4, B and D)** and a strong trend for reduced numbers of CD41+ neutrophils and alveolar macrophages **(figure 3.2.4, A and C**).

These data suggest that treatment with anti-CD41 prior to infection with Nb leads to a broad ablation of direct platelet – leukocyte interaction both within the circulation **(figure 3.2.2)** as well as in the lung.
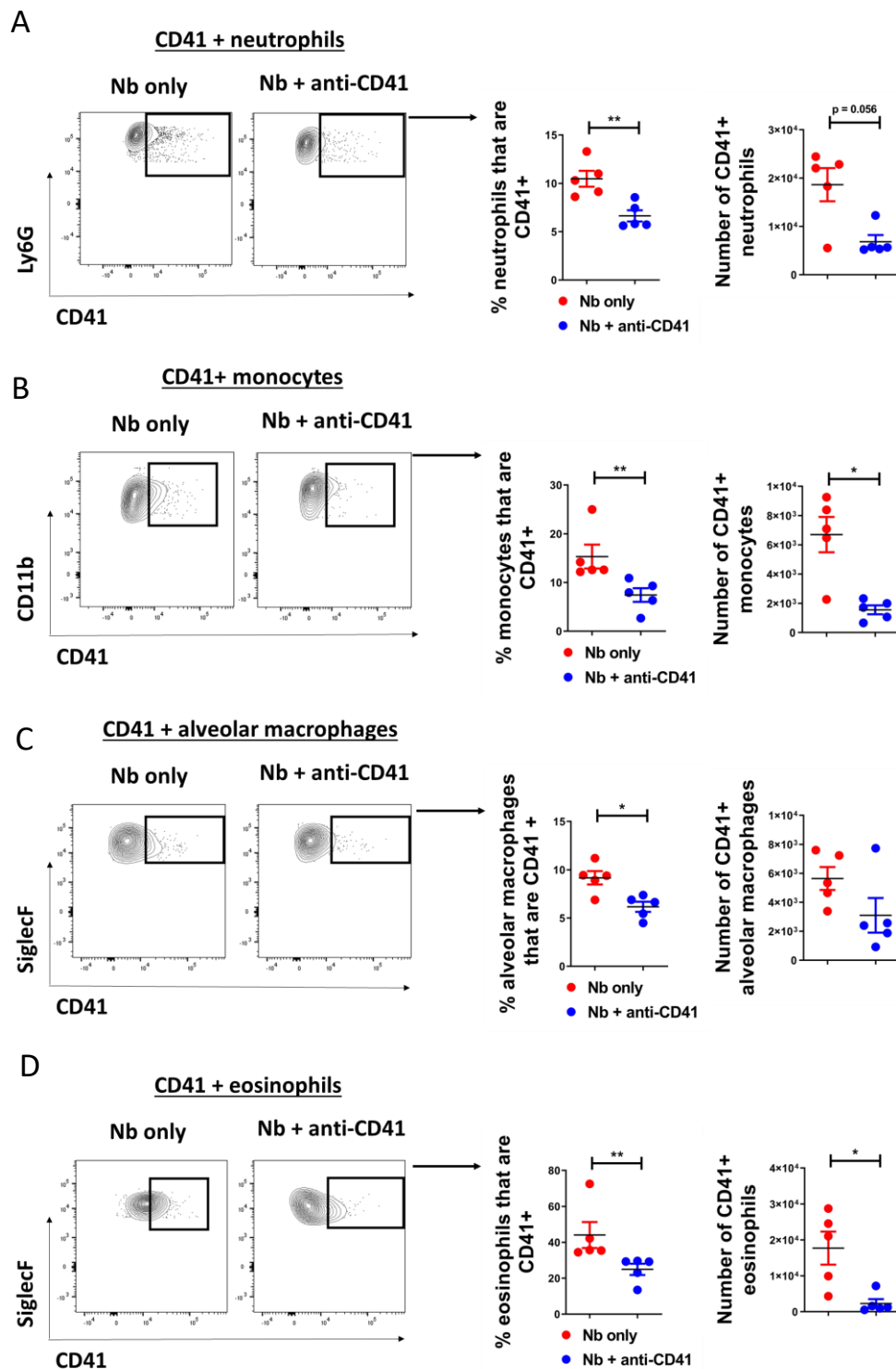
Figure 3.2.4: Platelet depletion significantly reduced the number of platelet-leukocyte aggregates in the lung. Flow cytometric analyses of platelet-leukocyte aggregates in single-cell suspension of lung tissue from Nb only and Nb + anti-CD41 was performed. The relative proportions and number of platelet-neutrophil **A**), platelet – monocyte **B**), platelet- alveolar macrophage **C**) and platelet-eosinophil **D**) aggregates were determined. The data are representative of 2 experiments, n = 5 mice. (Mann-Whitney U- test)

3.2.5: Circulatory neutrophils from platelet depleted mice are similar to CD41 negative neutrophils

To further identify immunologic correlates associated with the severe pathology observed in the lung of platelet depleted (Nb + anti-CD41) mice we next focused our attention on investigating differences in neutrophils. Given our findings indicating increased circulatory neutrophil levels in Nb + anti-CD41 mice (**figure 3.2.1)** we investigated whether neutrophils from platelet depleted mice would display an altered phenotype, and whether they would be similar to CD41 negative neutrophils characterized previously **(figure 3.1.6).**

To test this we employed both supervised and unsupervised flow cytometric analysis on a defined population of neutrophils from both Nb only and Nb anti-CD41 mice and compared them.

Similar to our previous findings showing marginal differences in the expression of Ly6G, Ly6C and CD11b between circulatory CD41 + and CD41 − neutrophils (figure 3.1.6), t-SNE analyses revealed that circulatory neutrophils from Nb only and Nb + anti-CD41 mice exhibited similar expression patterns of Ly6G, Ly6C and CD11b **(figure 3.2.5, A).** In contrast, but also in line with our previous findings, we found that neutrophils from Nb + anti-CD41 mice qualitatively differed in their expression of CD41, IFN-y and granzyme B **(figure 3.2.5, A).** More specifically, t-SNE analysis revealed markedly altered expression of IFN-y in neutrophils from Nb + anti-CD41 mice compared to Nb only mice. Interestingly, IFN-y expression did not localise to single clusters within the landscape, but was dispersed among different clusters, suggesting that IFN-y positive neutrophils may comprise a heterogenous population of cells.

To quantify the differences observed using t-SNE, MFI analyses of the same receptors and effector molecules visualized in the t-SNE landscape were performed. In agreement with our t-SNE analyses, neutrophils from Nb + anti-CD41 mice had similar MFI of Ly6G, Ly6C and CD11b **(figure 3.2.5, Bi – Biii)** and significantly altered expression of CD41 and IFN-y **(figure 3.2.5, Biv and Bv)**. While we did observe a trend for reduced granzyme B expression on neutrophils from Nb + anti-CD41 mice **(figure 3.2.5, Bvi),** this was not statistically significant.

Mirroring these results, using conventional flow cytometric analyses, we also found strong trends for increased proportions of IFN-y + neutrophils and decreased proportions of granzyme B + neutrophils in Nb + anti-CD41 mice (**figure 3.2.5, C**).
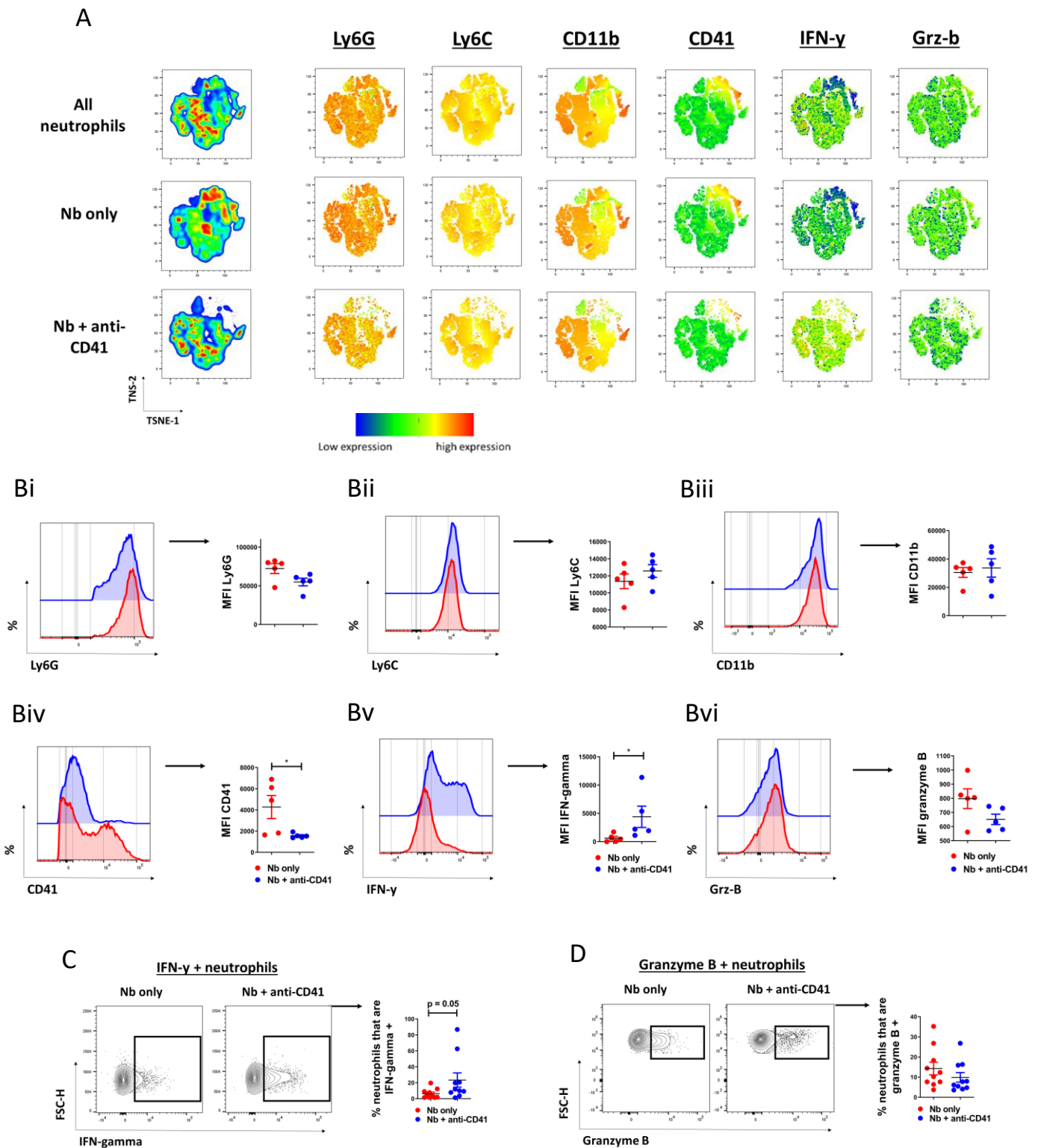
Figure 3.2.5: Circulatory neutrophils from platelet depleted mice are similar to CD41 (-) neutrophils. T-SNE analysis was performed neutrophils from Nb only and Nb + anti-CD41 mice collected at 18 hours post infection. Their visualization on the t-SNE landscape and expression patterns of neutrophil receptors and effector molecules are shown A). The MFI of individual receptors and effector molecules are also presented B.i – Bvi). Additionally, manual supervised analyses of IFN-γ C) and granzyme B D) expression on neutrophils was performed. Data is representative of 2 experiments. For figures C and D , data was pooled. N = 5 mice. (Mann-Whitney u- test)

3.2.6: Lung neutrophils from platelet depleted mice have altered phenotype.

Given our earlier findings showing tight association of platelets with IFN-y and RELM-α expression in neutrophils of Nb infected mice **(figure 3.1.6 and 3.1.7, 3.2.5)**, we next sought to determine whether lung neutrophils from platelet depleted mice would also exhibit altered expression of IFN-y and RELM-α.

To gain insight into the association of platelet depletion with IFN-y expression, lung neutrophils from naïve, CD41 treated, Nb infected (Nb only) and Nb + anti-CD41 treated mice were isolated and, following stimulation , were stained for intracellular IFN-y. Our results indicated that while Nb only mice had similar MFI of IFN-y compared to naïve mice, both CD41 only and Nb + CD41 mice had strong trends for increased MFI of IFN-y **(figure 3.2.6, A)**

In separate experiments, to interrogate the association of platelet depletion with lung neutrophil expression of RELM- α, we used both supervised and unsupervised flow cytometric analysis of neutrophils from Nb only and Nb + anti-CD41 mice.

Interestingly, t-SNE analysis revealed the presence of significant qualitative differences in the expression patterns of neutrophils from Nb only and Nb + anti-CD41 mice, especially with regard to the expression of the neutrophil markers Ly6G and CD11b , and platelet-associated CD41 **(figure 3.2.6, A)** .However, only minor differences were observed in the expression pattern of RELM-α between groups.

In support of these findings, further analyses demonstrated that Nb + anti-CD41 mice had significantly lower MFI of Ly6G, CD11b and CD41 **(figure 3.2.6, Bi – Biii)**, but similar MFI of RELM-α **(figure 3.2.6 , Biv)** compared to Nb only mice.

In contrast to this however, supervised analyses of the proportion of RELM-α + lung neutrophils revealed that platelet depleted mice had significantly lower proportions of RELM-α + neutrophils in their lungs relative to Nb only mice **(figure 3.2.6, C).**

Collectively these data suggest the existence of a nuanced relationship between platelets and neutrophils in the lung during acute Nb infection and that the absence of platelets alters neutrophil responses in the lung.
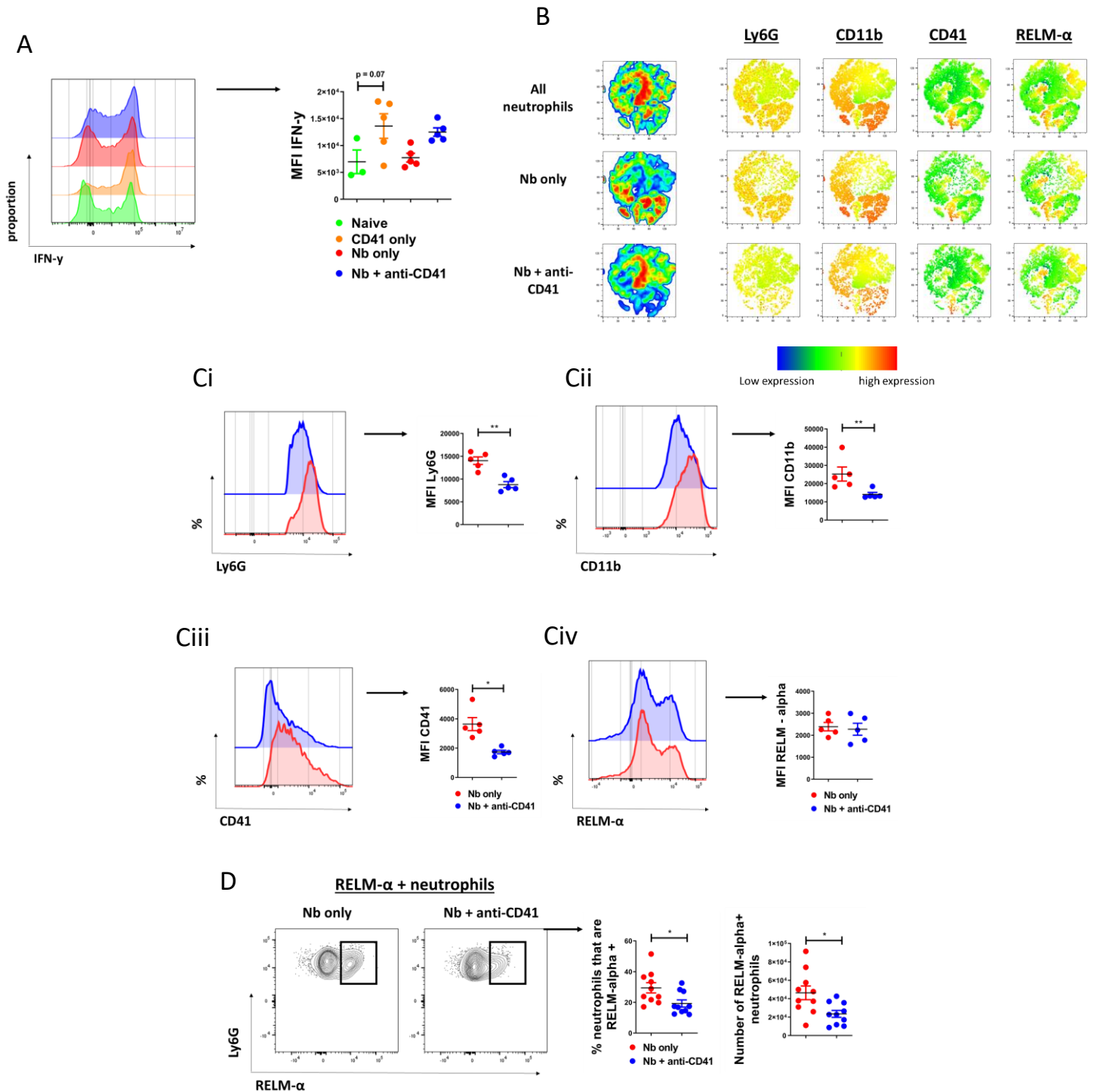
Figure 3.2.6: Lung neutrophils from platelet depleted mice have altered phenotype. IC staining of IFN-y on stimulated neutrophils from lung neutrophils were investigated A). Furthermore t--SNE analysis was performed on lung neutrophils from Nb only and Nb + anti-CD41 mice at a18 hours post infection. Their visualization on the t-SNE landscape and expression patterns of neutrophil receptors and effector molecules are shown B). Additionally, MFI of individual receptors and effector molecules are presented C.i – C.iv) . Finally, supervised gating was performed to determine in the proportion of RELM-alpha + neutrophils in Nb only and Nb + anti-CD41 mice D) Data B -D are representative of 2 experiments. For D) data was pooled. N = 5 mice. (Mann-Whitney u- test)

3.2.7: Treatment with anti-Ly6G leads to significant reductions in circulatory and lung neutrophil populations

Neutrophils are implicated in driving acute immunopathology in pulmonary Nb infection and previous studies have indicated that their depletion leads to reduced Nb -associated pathology at day 2 post infection [160]. Whether their depletion also protects against pathology at an earlier time point (18 hours post infection) and whether neutrophil – mediated immunopathology is modulated by the presence/absence of platelets remains ill-defined, however.

Given the findings of previous studies and our own observation of aberrant neutrophil responses in platelet depleted mice **(figure 3.2.5 and figure 3.2.6),** we postulated that neutrophils were contributing significantly towards enhanced pulmonary pathology in Nb + anti-CD41 mice.

To test this hypothesis, we devised experiments in which we co – depleted mice of their platelets and neutrophils (Nb + anti-CD41 + anti-Ly6G mice) and compared these to mice that had either received no depleting antibody (Nb only), only a platelet depleting antibody (Nb + anti-CD41 mice) or only a neutrophil depleting antibody (Nb + anti-Ly6G mice).

To confirm the efficacy of neutrophil depletion (treatment strategy is outlined in figure 2.3), we performed flow cytometric analyses of blood and lung samples. Our findings indicated that our depletion strategy was effective in significantly reducing circulatory and lung neutrophil numbers in our Nb infection model: Nb + anti-Ly6G mice had significantly reduced levels of circulatory **(figure 3.2.7, A and B)** and lung **(figure 3.2.7, C and D)** neutrophils relative to Nb + anti-CD41 mice, and Nb + anti-Ly6G mice had significantly reduced levels of neutrophils relative to Nb only mice.

Consistent with our previous findings, Nb + anti-CD41 mice also had significantly elevated levels of circulatory neutrophils relative to Nb only mice **(figure 3.2.7, A- B).** Interestingly, while Nb + anti-CD41 + anti-Ly6G mice did exhibit significantly reduced levels of lung

neutrophils compared to Nb only mice **(figure 3.2.7, C -D)**, they had similar levels of circulatory neutrophils **(figure 3.2.7, A- B)**. This suggests that co-administration of a platelet – depleting antibody (anti-CD41), together with a neutrophil-depleting antibody may partially interfere with the efficacy of circulatory neutrophil depletion but is nonetheless a viable method to significantly reduce neutrophil numbers in thrombocytopenic mice.
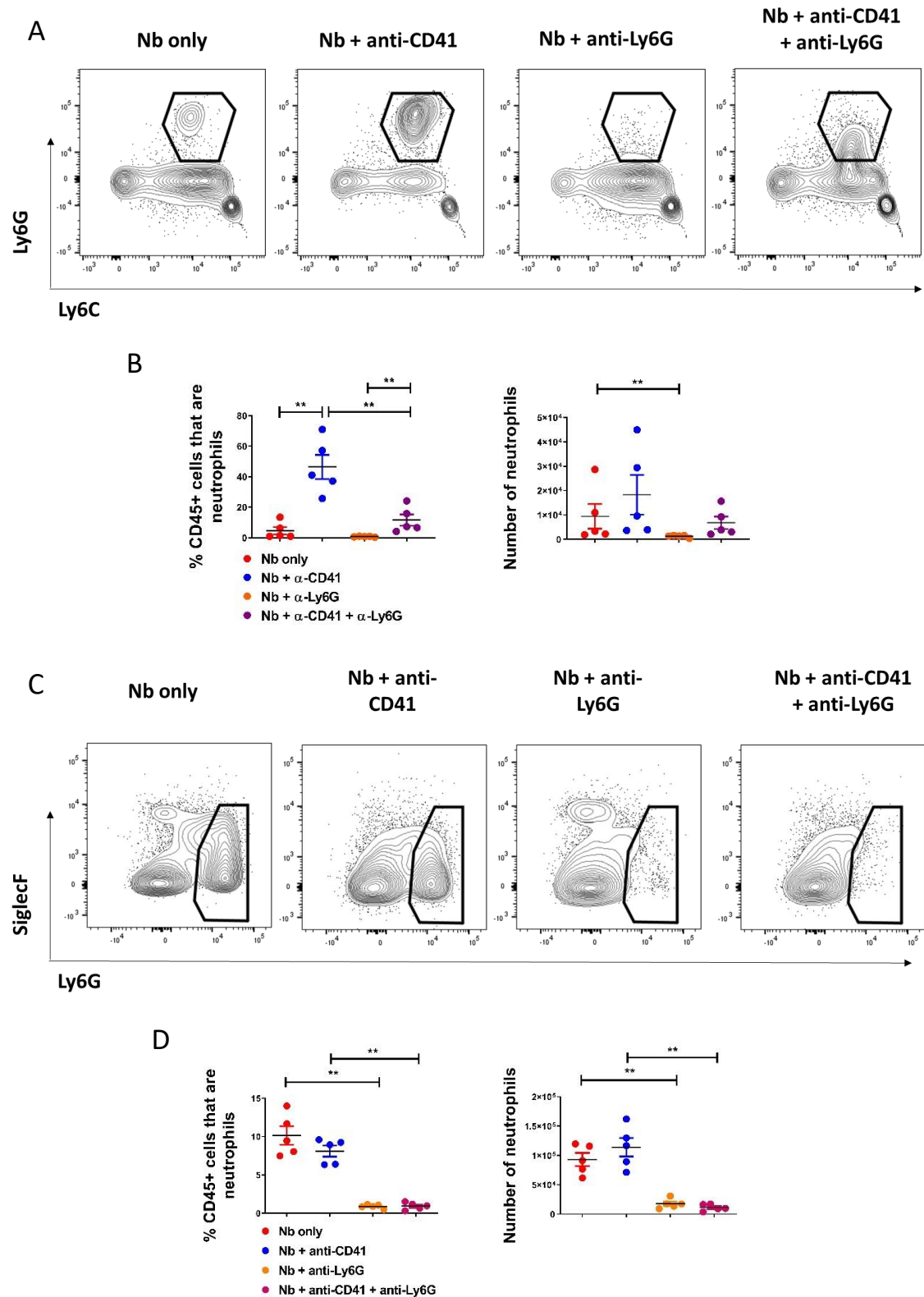
Figure 3.2.7: Treatment with anti-Ly6G leads to significant reductions in circulatory and lung resident neutrophil populations. To test the contribution of neutrophils to helminth-associated pathology in Nb + anti-CD41 mice, neutrophils were depleted using antibodies. Flow cytometric analyses was performed on blood and lung samples of mice to confirm the efficacy of depletion. The groups used for these experiments and representative flow cytometry plots for circulatory neutrophils and lung neutrophils are outlined in **A**) and **C**) respectively. The quantification of the relative levels and numbers of circulatory **B**) and lung neutrophils **D**) are also shown. Data are representative of 2 experiments, n = 5 mice per group. (One way ANOVA).

3.2.8: Co-depletion of platelets with neutrophils significantly decreases acute Nb-associated pathology in the lung

Having established an effective protocol to co-deplete mice of their platelets and neutrophils during acute Nb infection **(figure 3.2.7**), we next explored how co-depletion altered Nb – associated pathology in the lung.

In line with our hypothesis that neutrophils play important roles in modulating acute Nb-associated pathology in platelet depleted mice, we found that co-depleted mice, i.e. Nb + anti-CD41 + anti-Ly6G treated mice , had reduced levels of protein and DNA in their BAL fluid **(figure 3.2.8 , A and B)** relative to Nb + anti-CD41 mice. Indeed, Nb + anti-CD41 + anti-Ly6G mice had comparable levels of protein and DNA in their BAL to mice with normal platelet counts, i.e. Nb only and Nb + anti-Ly6G mice (**figure 3.2.8, A and B).**

Moreover, histological analyses of lung sections from Nb + anti-CD41 and Nb + anti-CD41 + anti-Ly6G mice revealed that co-depleted mice had reduced haemorrhaging and gross pathology **(figure 3.2.8, D)**, although significant haemorrhaging was still observed to occur in these mice.

Furthermore, in line with earlier findings, we observed a strong trend for reduced worm burdens in Nb + anti-CD41 mice relative to Nb only mice **(figure 3.2.8, C).** Interestingly, while not statistically significant, Nb + anti-CD41 + anti-Ly6G mice also displayed reduced worm burdens relative to Nb only mice, which is suggestive of the existence of neutrophil - independent anti-parasitic responses.

In summary, these data suggest that co-administration of a neutrophil depleting antibody together with a platelet depleting antibody partially protects against enhanced infection - associated pathology observed in Nb + anti-CD41 mice. Additionally, these data point to platelets playing additionally roles in protecting against Nb -associated pathology outside of their potential regulation of neutrophil function.
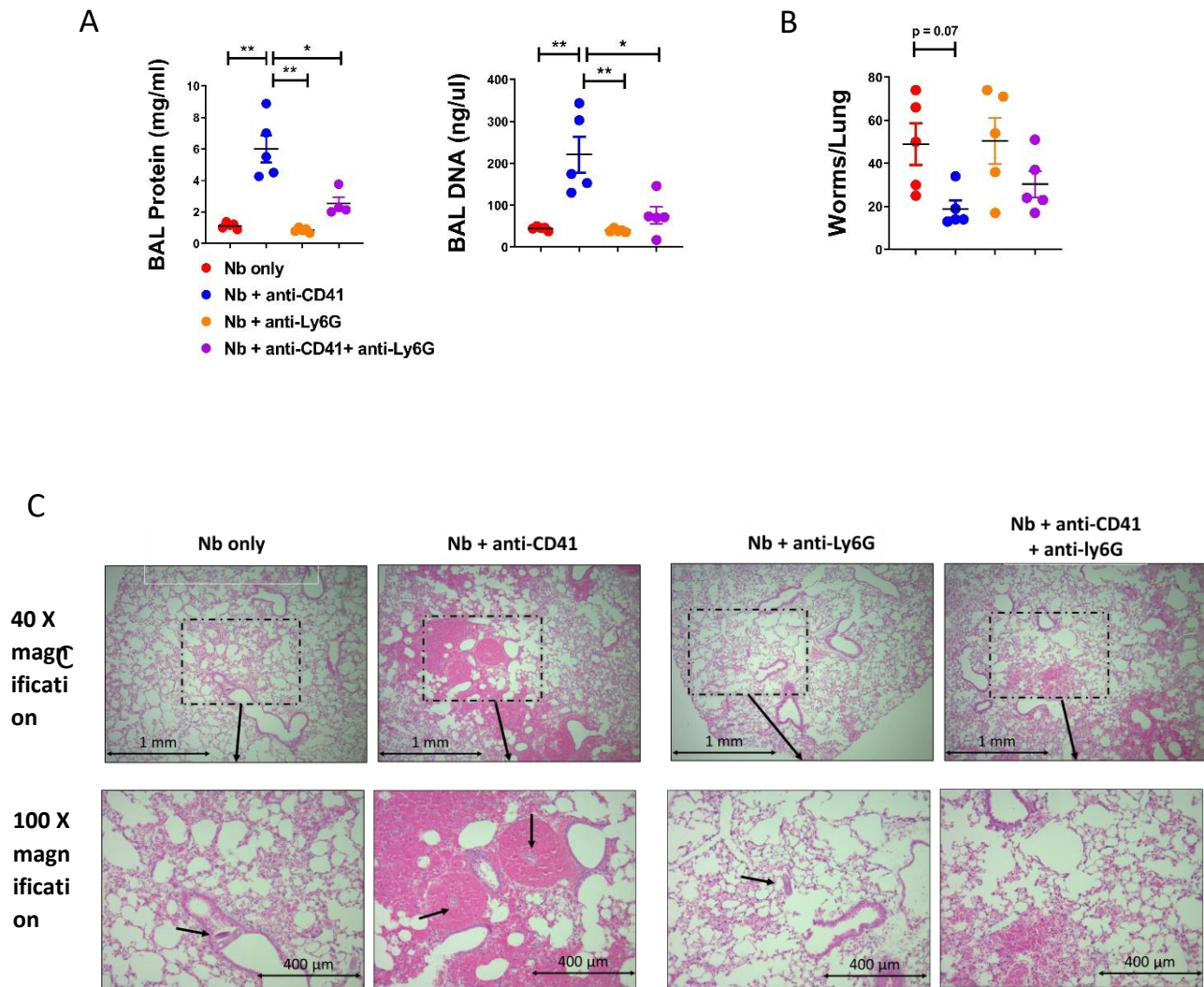
Figure 3.2.8: Co-depletion of platelets with neutrophils significantly decreases acute Nb-associated pathology in the lung. Platelet -depleted mice were additionally treated with a neutrophil depleting antibody to determine the role of infiltrating neutrophils in helminth-associated pathology in the lung. To quantify pathology, the concentration of protein **A**) and DNA **B**) in the BAL fluid was determined. Additionally, worm burdens in the lung were investigated **C**). Lastly, H&E staining of lung sections was performed to give an overview of gross pathology **C**). Arrows point to Nb larvae. Data are representative of 2 experiments, n = 5 mice ** represents a p-value < 0.01 (one- way ANOVA).

Chapter 3.3:

Investigating potential mechanisms underlying platelet regulation of Nb-associated pulmonary pathology.

3.3.1: treatment with anti-CD62P partially disrupts platelet-neutrophil interaction

To gain insight into how platelet regulate Nb-associated pulmonary pathology , we next sought to interfere with platelet-neutrophil interaction .While multiple receptors are implicated in supporting and driving the interaction of platelets with neutrophils, the CD62P(P-selectin)-PSGL-1 axis is the most well studied [69] and research continues to emerge on the importance of this receptor -ligand pair in regulating diverse inflammatory processes. The interaction of platelet with neutrophils via this axis has been shown to modulate diverse neutrophil responses , including the production of ROS , neutrophil degranulation and NET production [161]. Given previous studies implicating direct platelet -neutrophil interaction in modulating immunopathogenic mechanisms in the lung during acute infection [75] and our previous findings showing increased association of platelets with neutrophils in Nb infected mice (**figure 3.1.3**) we postulated that targeting of platelet neutrophil interaction would lead to alter Nb-associated pathology in the lung.

To disrupt platelet-neutrophil interaction we treated mice with anti-CD62P blocking antibodies prior to infection with Nb. The dosing regimen that was used was the same as previously indicated for the anti-CD41 antibody, used in platelet -depletion experiments (see figure 2.3 for details).

To ensure that treatment with the anti-CD62P antibody did not lead to altered platelet responses, we performed flow cytometry on blood from mice that had been treated prior to infection (Nb + anti-CD62P mice) and compared these to non -treated Nb only mice. We found that Nb + anti-CD62P mice exhibited neither altered platelet levels (**figure 3.3.1, A),** nor increased levels of CD62P+ platelets (**figure 3.3.1, B)** compared to Nb only mice.

Furthermore, anti-CD62P treatment was not associated with significant changes to circulatory neutrophil levels (**figure 3.3.1, C).** However, consistent with our hypothesis, we found that Nb + anti-CD62P mice did have a lower proportion of CD41+ neutrophils in their circulation **(figure 3.3.1, D) and** reduced MFI of CD41 on lung neutrophils **(figure 3.3.1, E)** relative to Nb only mice.

In view of earlier data showing altered phenotype in lung neutrophils of platelet depleted mice **(figure 3.2.6)**, we also compared the expression of neutrophil markers between Nb only and Nb + anti-CD62P mice but were unable to find significant differences **(figure 3.3.1, F).**

Taken together, these data suggest that anti-CD62P treatment partially disrupted the association of platelets with neutrophils in the circulation and the lung but was not associated with changes in neutrophil levels or neutrophil phenotype.
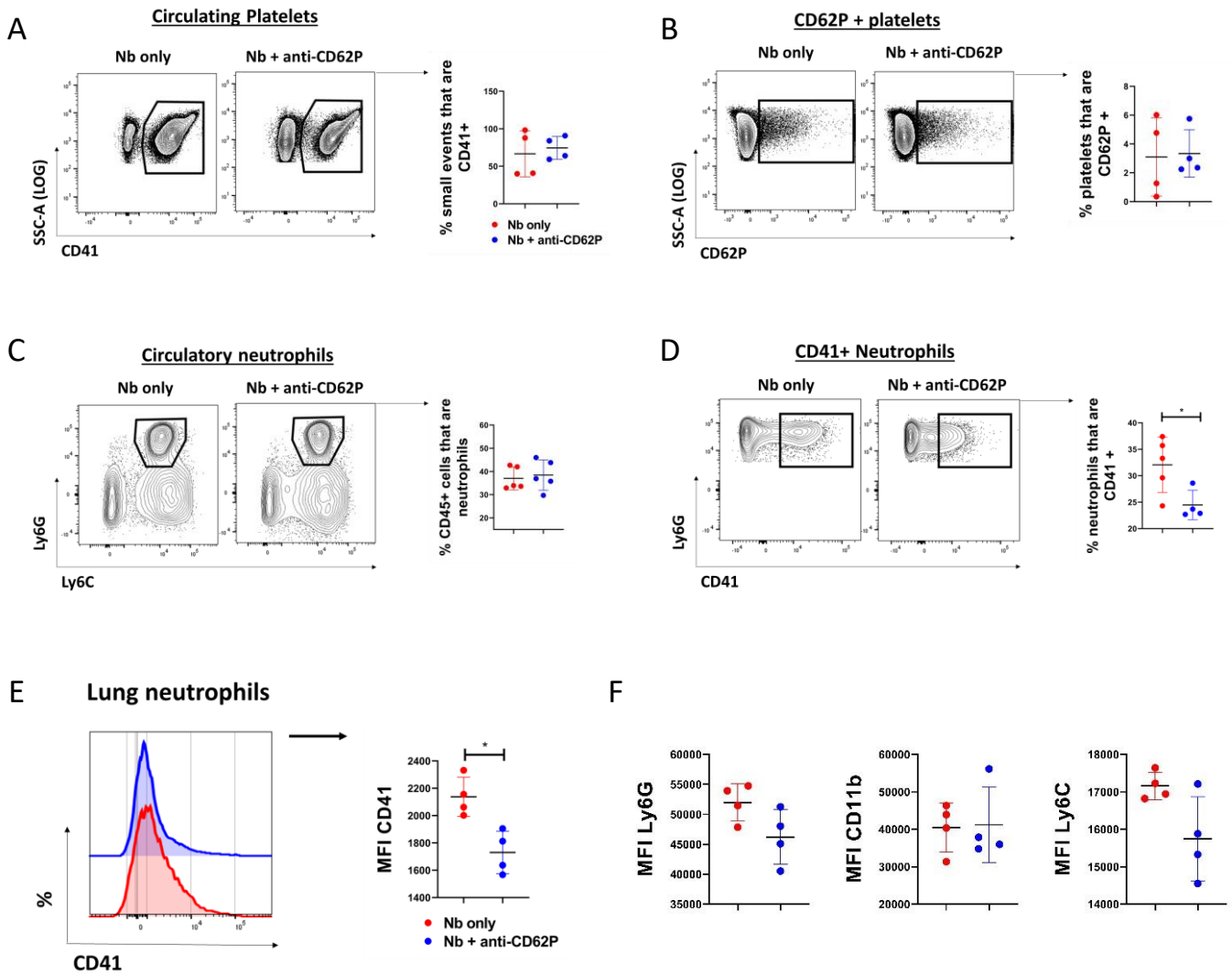
Figure 3.3.1: treatment with anti-CD62P partially disrupts platelet-neutrophil interaction. To investigate potential mechanisms underlying platelet regulation of Nb pathology, mice were treated with anti-CD62P to acutely disrupt platelet–neutrophil interaction. The effect of treatment on platelet levels **A**), platelet activation **B**), circulatory neutrophil levels **C**) and platelet-neutrophil complexes **D**) were determined. Additionally shown, are the effect of CD62P treatment on lung neutrophil CD41 MFI **E**) and lung neutrophil expression of neutrophil markers **F**). Data are representative of 2 experiments. N = 4 mice. (Mann-Whitney U-test).

3.3.2: treatment with anti-CD62P prior to infection does not associate with changes in Nb –associated pathology

After establishing that mice treated with anti-CD62P have reduced platelet-neutrophil interaction (**figure 3.3.1),** we next turned our attention to the effect of anti-CD62P treatment on Nb – associated pathology. Specifically, we wanted to investigate if, similar to Nb + anti-CD41 mice, mice treated with anti-CD62P would exhibit significantly enhanced pulmonary pathology at 18 hours post infection.

As before, to test this, we collected BAL and lung samples from Nb only and Nb + anti-CD62P mice and analysed read outs of pulmonary pathology, including BAL protein and DNA content, BAL haemoglobin concentration and alveolar inflammation. Our results indicate that Nb only and Nb + anti-CD62P mice had similar pulmonary pathology, with comparable levels of BAL protein and DNA **(figure 3.3.2, A)** and BAL haemoglobin **(figure 3.3.2, B).**

Furthermore, investigation into gross pathology using histological staining revealed similar levels of alveolar inflammation in regions surrounding migrating larvae **(figure 3.3.2, D)** and neither Nb only nor Nb + anti-CD62P mice exhibited significant pulmonary haemorrhaging following acute infection. This is in notable contrast to platelet-depleted (Nb + anti-CD41) mice, which exhibited disruption to alveolar -capillary barriers **(figure 3.2.1).**

In line with these observations, Nb only and Nb + anti-CD62P mice also displayed similar worm burdens in their lungs during acute infection **(figure 3.3.2, C)**.

These data suggest that treatment with anti-CD62P, using the dosing regimen outlined previously, does not impact on helminth-associated pathology during acute Nb infection.

Figure 3.3.2: treatment with anti-CD62P prior to infection does not associate with changes in Nb –associated pathology. Mice were treated with anti-CD62P prior to being infected with Nb and lung and BAL fluid were collected at 18 hours p.i. Markers of Nb-associated pulmonary pathology, including BAL protein and DNA **A**) and BAL haemoglobin **B**) concentrations were analysed. Additionally, pulmonary worm burdens were determined **C**). To gain insight into gross pulmonary pathology, H&E staining of lung sections from anti-CD62P treated and non-treated mice were performed **D**). Arrows indicate Nb larvae. Data are representative of 2 experiments. n = 4 mice per group. Data in A -C was pooled. (Mann-Whitney U-test).

3.3.3: treatment with anti-podoplanin modestly alters platelet –neutrophil interaction and associates with altered neutrophil phenotype in the lung

In addition to the CD62P – PSGL-1 axis, several other receptor -ligand pairs have been shown to mediate the interaction of platelets with neutrophils, and modulate immunologic processes. An emerging player in thrombo-inflammatory diseases and infection is the platelet receptor CLEC-2 [17]. This receptor is involved in several aspects of the immunobiology of platelets and also regulated the interaction of platelet with leukocytes [17]. The endogenous ligand of platelet CLEC-2 is podoplanin , which is expressed on a variety of epithelial and immune cells [17]. The CLEC-2 – podoplanin axis is implicated in the regulation of inflammation during infection [94] , and constitutes a mechanism through which platelets may exert protective anti-inflammatory activity [17]. In line with this , previous studies have demonstrated deleterious effects following inhibition of the CLEC-2- podoplanin axis , whether through genetic ablation of the CLEC-2 receptor or through anti-body mediated blockade [94], on pulmonary pathology and inflammation.

We therefore wondered whether platelet- mediated regulation of Nb-associated pulmonary pathology involved the CLEC-2 – podoplanin axis and whether antibody mediated antagonism of podoplanin would result in disruption of platelet-neutrophil interaction and altered neutrophil phenotype.

In order to gain insight into this, we performed flow cytometry on blood and lung samples from mice that had been treated with an anti-podoplanin blocking antibody prior to infection with Nb (Nb + anti-podoplanin mice) , and compared these to non-treated (Nb only) mice (sampling was again at 18 hours post infection).

We found that Nb + anti-podoplanin mice had similar levels of circulatory platelets **(figure 3.3.3, A)** but a trend for increased circulatory neutrophils **(figure 3.3.3, B)** relative to Nb only mice. Moreover, while again statistically insignificant, Nb + anti-podoplanin mice also exhibited a trend for reduced CD41+ circulatory neutrophils **(figure 3.3.3, C).**

Analyses of lung neutrophils however, demonstrated the presence of distinct differences between Nb + anti-podoplanin and Nb only mice: Lung neutrophils from Nb + anti-

podoplanin mice had reduced MFI of CD41, Ly6G , Ly6C and podoplanin **(figure 3.3.3, D – E)**.

Collectively these data suggest that anti-podoplanin treatment impacts lung neutrophils, while modestly affecting circulatory platelet and neutrophil responses.
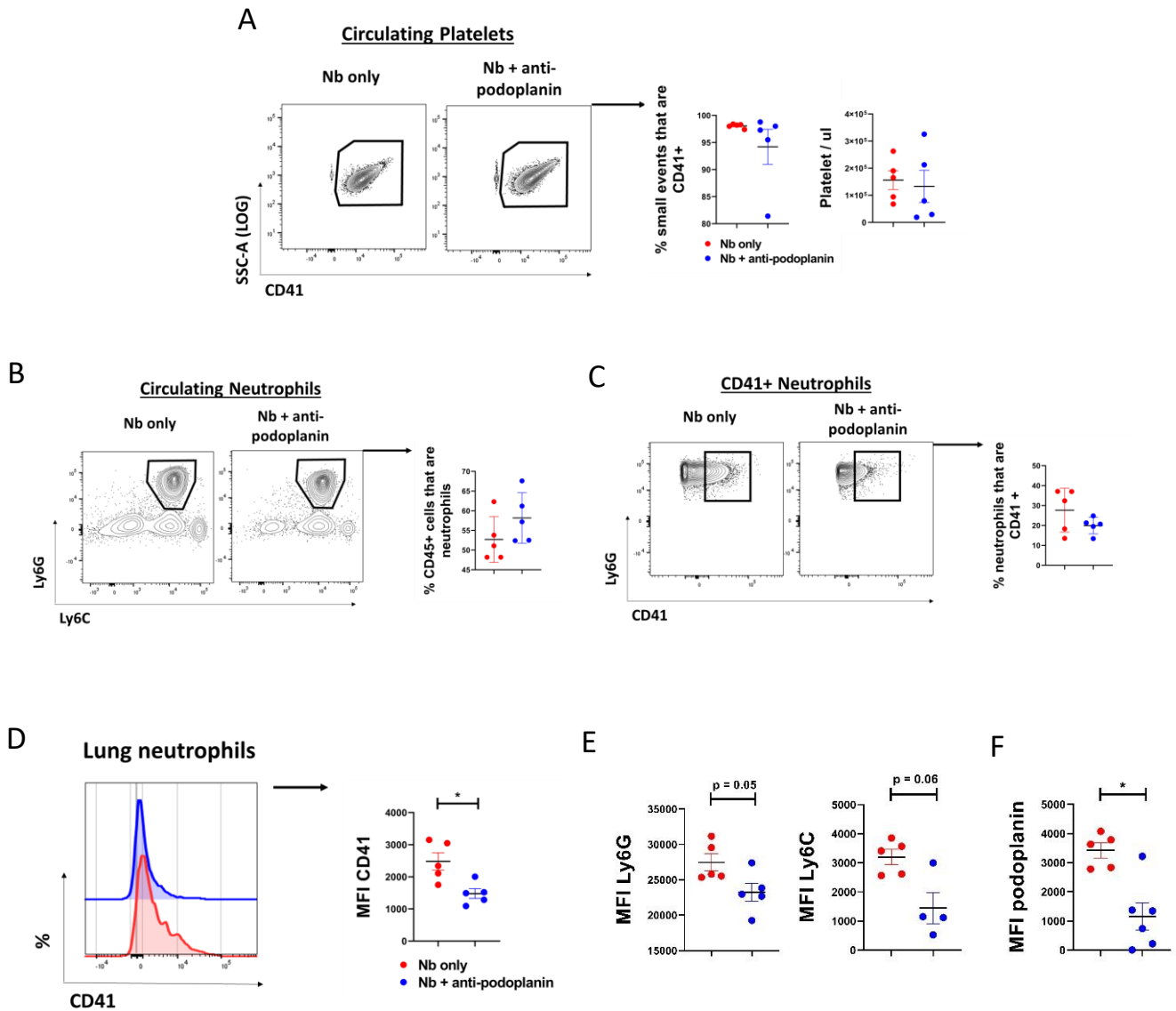
Figure 3.3.3: treatment with anti-podoplanin modestly alters platelet –neutrophil interaction and associates with altered neutrophil phenotype in the lung. Mice were treated with anti-podoplanin antibodies (Nb + anti-podoplanin) prior to infection with Nb. The effect of treatment on platelet levels **A**), circulatory neutrophil levels **B**) and the proportion of CD41+ neutrophils **C**) was determined by flow cytometry and levels compared to non-treated (Nb only) mice. Additionally are shown differences lung neutrophil CD41 MFI **D**) neutrophil marker MFI **E**) and podoplanin expression **F**). Data are representative of 2 experiments. N = 5 mice. (Mann-Whitney U-test).

3.3.4: treatment with anti-podoplanin does not associate with increased Nb – associated pathology

Following on from our analyses on the effect of anti-podoplanin treatment on circulatory platelet and lung responses, we next characterized the impact of treatment on Nb – associated pathology in the lung at 18 hours post infection.

As in previous experiments, analyses of pulmonary pathology involved determining the concentration of BAL fluid molecular infiltrates and alveolar inflammation in Nb only and antibody treated (Nb + anti-podoplanin) mice.

Similar to our findings on the effect of anti-CD62P treatment on acute pulmonary pathology (**figure 3.3.2)**, we found that mice that had been treated with anti-podoplanin prior to infection with Nb had comparable levels of pulmonary pathology compared to non-treated mice. This included comparable levels of BAL protein and DNA (**figure 3.3.4, A)** and a minor trend for increased BAL haemoglobin concentration **(figure 3.3.4, C).**

Moreover, histological analyses revealed Nb + anti-podoplanin mice also had comparable levels of alveolar inflammation and pulmonary haemorrhaging in regions adjacent to migrating Nb larvae (**figure 3.3.4, D**).

Lastly, the number of larvae in the lungs of Nb + anti-podoplanin and Nb only mice were not significantly different (**figure 3.3.4, B).**

These data therefore suggest that treatment with an anti-podoplanin antibodies prior to infection with Nb does not significantly impact on Nb-associated pathology at 18 hours post infection.
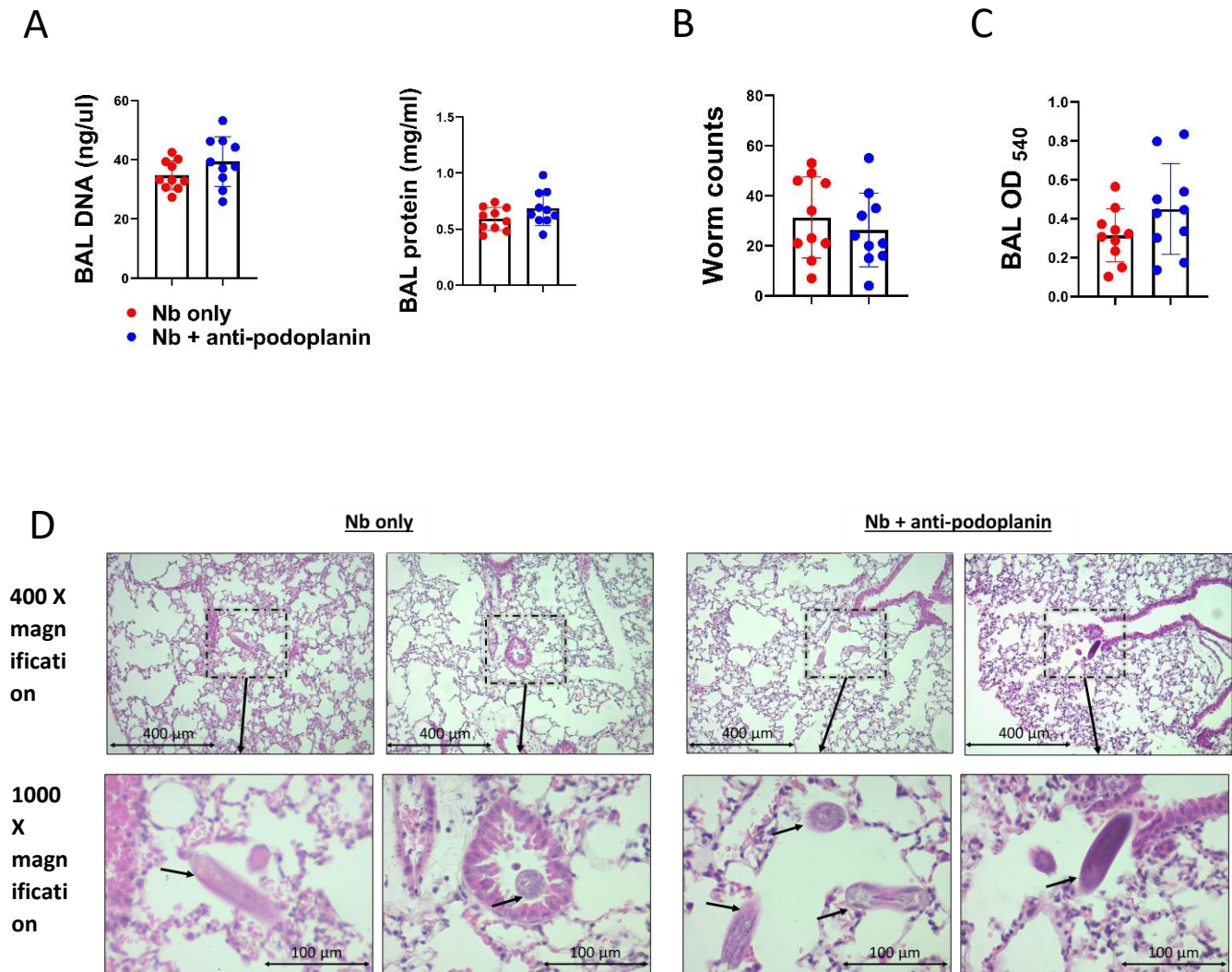
Figure 3.3.4: treatment with anti-podoplanin does not associate with increased Nb – associated pathology. Mice were treated with anti-podoplanin antibodies prior to infection with Nb. Pulmonary pathology was quantified using readouts relating to infiltration of protein and DNA **A**) and haemoglobin **C**) into BAL fluid of untreated (Nb only) and treated (Nb + anti-podoplanin) mice. Worm counts in the lungs of treated mice were additionally determined **B**). H & E staining on lung sections was also performed to gain oversight of gross pathology **D**). Arrows point to Nb larvae. Data are representative of 2 experiments. Data for figures **A – C** was pooled. (Mann-Whitney U-test).

3.3.5: dual treatment with anti-CD62P and anti-podoplanin is associated with altered circulatory neutrophil and platelet responses during acute Nb infection

While our previous experiments suggested that targeting of individual platelet receptors , including CD62P **(figure 3.3.1)** and CLEC-2/podoplanin **(figure 3.3.3)** , did not significantly impact on circulatory platelets and neutrophils phenotype (but possibly lung neutrophils) , in both cases there were trends (though slight) for increased Nb-associated pulmonary pathology **(figures , 3.3.2 and 3.3.4**) While the rationale for these experiments is consistent with previous studies that have demonstrated significant effects on platelet responses through the antagonism of individual receptors [162] , it is also likely that multiple receptors co-ordinate to regulate platelet- mediated functions during infection [48]

We therefore hypothesized that antagonism of multiple platelet receptors at once would enhance disruption of platelet regulated responses relative to single receptor targeting and would potentially allow us to resolve whether platelet signalling via these receptors impacts on pathomechanisms involved in acute Nb infection.

To test this hypothesis, we dual treated mice with an anti-podoplanin (anti-podo) and an anti-CD62P antibody using the dosing regimen outlined previously and compared these to non-treated, Nb only mice. Similar to previous experiments we collected blood at 18 hours post infection and analysed circulatory platelet and neutrophil responses.

We found that Nb + anti-podo + anti-CD62P mice had altered circulatory platelet and neutrophil responses at 18 hours post infection, including altered platelet expression of CD41 (**figure 3.3.5, B**) and altered neutrophil levels (**figure 3.3.5, C).** Furthermore, while Nb + anti-podo + anti-CD62P had proportionally fewer platelets, their absolute platelet counts relative to Nb only mice were similar (**figure 3.3.5, A**).

Interestingly, we did not detect differences in terms of the MFI of CD41 on either circulatory neutrophils or lung neutrophils (**figure 3.3.5, D and F)**, suggesting that, in contrast to previous experiments (**figures 3.3.1 and 3.3.3**), antibody treatment was not leading to altered detection of CD41 on neutrophils. Moreover, circulatory neutrophils from Nb + anti-podo + anti-CD62P and Nb only mice displayed similar MFI of the neutrophil markers Ly6G, Ly6C and CD11b (**figure 3.3.5, E).** We observed a modest trend for reduced MFI of Ly6G on

lung neutrophils from Nb + anti-podo + anti-CD62P mice, although Ly6C expression was unchanged following treatment **(figure 3.3.5, G).**

Together, these data indicate that dual treatment with anti-podoplanin and anti-CD62P significantly modulates circulatory platelet phenotype and is associated with elevated circulatory neutrophil numbers, but does not impact on the detection of CD41 on neutrophils, or circulatory neutrophil phenotype.
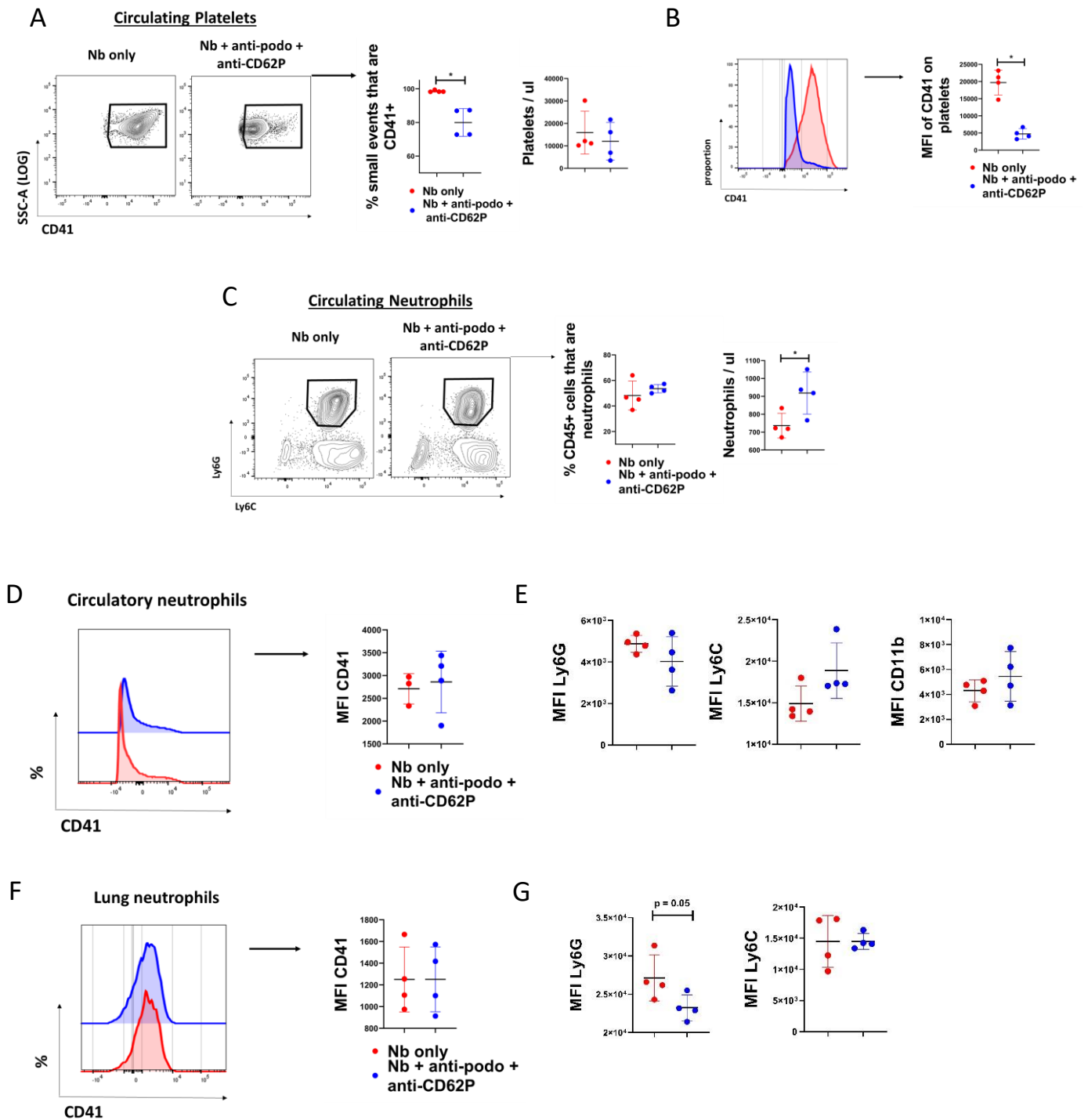
Figure 3.3.5: dual treatment with anti-CD62P and anti-podoplanin is associated with altered circulatory neutrophil and platelet responses during acute Nb infection. Mice were treated with anti-podoplanin and anti-CD62P antibodies (Nb + anti-podo + anti-CD62P) prior to infection with Nb. The effect of treatment on platelet levels **A**) platelet CD41 MFI **B**), circulatory neutrophil levels **C**) and MFI of CD41 **D**) and neutrophil markers **E**) on circulatory neutrophils were determined by flow cytometry and levels compared to non-treated (Nb only) mice. Additionally, are shown, lung neutrophil CD41 MFI **F**) and neutrophil marker expression **G**). N = 5 mice. For **D**, an outlier was removed. (Mann-Whitney U-test**).**

3.3.6: dual treatment with anti-podoplanin and anti-CD62P is associated with a trend for increased Nb –associated pulmonary pathology during acute infection

Finally, to conclude our analyses on the effect of dual antibody treatment with anti-podoplanin and anti-CD62P on the response of mice to Nb infection, we measured the extent of pathology in the lungs of Nb + anti-podo + anti-CD62P mice and compared them to mice that had not been treated with blocking antibodies (Nb only mice).

Our results from these experiments indicate a trend for increased pulmonary pathology in Nb + anti-podo + anti-CD62P mice, including a trend for increased BAL protein and significantly elevated BAL DNA levels **(figure 3.3.6, A)** and a trend for increased BAL haemoglobin **(figure 3.3.6, B).** Furthermore, while qualitative, Nb + anti-podo + anti-CD62P mice also exhibit increased morphological damage to lung tissue (**figure 3.3.6, D**), however, further stereological analyses would be necessary to confirm this.

However, Nb + anti-podo + anti-CD62P mice displayed similar worm counts in their lungs relative to non-treated mice (**figure 3.3.6, C)** .

In conclusion, although further experiments and analyses are required, our analysis of pulmonary pathology in Nb + anti-podo + anti-CD62P mice suggests that dual- treatment with anti-CD62P and anti-podoplanin may modulate pathology in a manner distinct from single treatment.
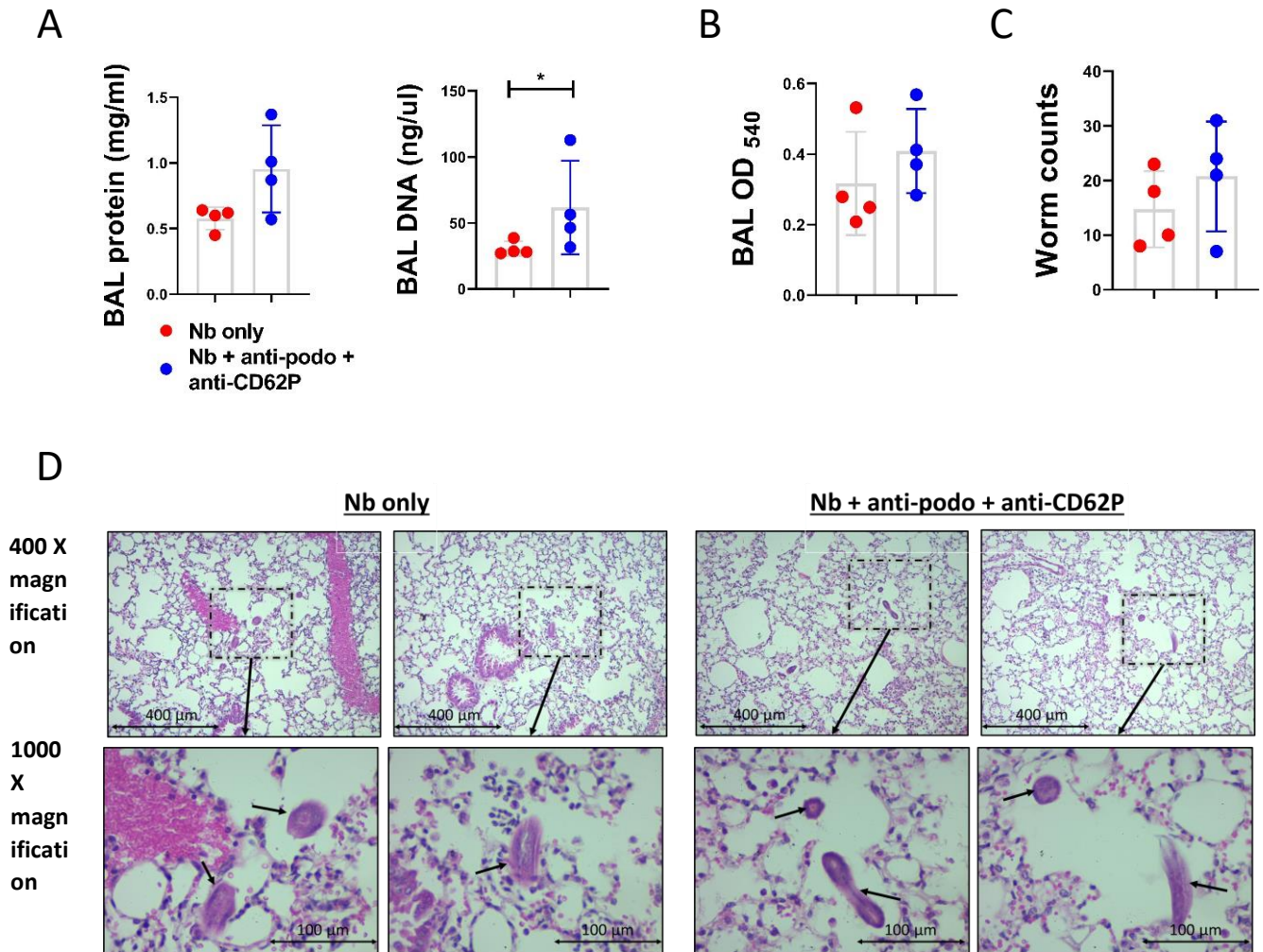
Figure 3.3.6: dual treatment with anti-podoplanin and anti-CD62P is associated with a trend for increased Nb –associated pulmonary pathology during acute infection. Mice were treated with anti-podoplanin and anti-CD62P antibodies prior to infection with Nb. Pulmonary pathology was quantified using readouts relating to infiltration of protein and DNA **A**) and haemoglobin **B**) into BAL fluid of untreated (Nb only) and treated (Nb + anti-podo + anti-CD62P) mice. Worm counts in the lungs of treated mice were additionally determined **C**). H & E staining on lung sections was also performed to gain oversight of gross pathology **D**). Arrows point to Nb larvae. N = 4 mice. (Mann-Whitney U-test).

Chapter 4: Discussion

Helminths parasitise a remarkably high number of individuals globally and are a significant public health concern [109]. The life cycle of many different species of helminths entails migration through pulmonary vasculature and tissue and their migration is often associated with marked pulmonary pathology. This is especially apparent in animal models such as murine *N.brasiliensis* infection, where the pulmonary migration of larvae results in the development of severe and persisting emphysematous lesions [130]. In human helminthiasis, pulmonary migration of helminth parasites is also associated with significant inflammation (Löffler's syndrome) [123] and irreversible lung dysfunction [125]. While significant headway has been made in our understanding of the cells and factors (both host as well as parasite – derived) that impact on the interaction between helminth parasites and their hosts during pulmonary infection [102,105,121], little is known on the function of platelets in this setting, especially with regard to their immunoregulatory capacity. In light of the rapidly growing body of evidence which points to platelets significantly impacting on the pathophysiology and immunity to a variety of other pathogens in the lung [50163], we hypothesized that platelets also have significant function in pulmonary helminth infection. We believe we have provided firm evidence that this is indeed the case.

Chapter 3.1: Platelets are a feature of the acute innate response to N. brasiliensis infection and associate with the induction of protective responses

Given the paucity of data regarding platelets in helminth infection, our first imperative was to establish whether acute infection with the gastro-intestinal nematode *N. brasiliensis* (Nb) was associated with platelet activation and their localisation to infected (pulmonary) tissue. Analyses of circulatory immune populations at 18 hours post infection revealed that Nb infected mice had elevated levels of platelet-leukocyte complexes (CD45 +, CD41 + cells), elevated platelet expression (MFI) of CD41 and CD62P and increased levels of CD62P+ platelets compared to naïve mice **(figure 3.1.1, A-D).** Complimenting these data, immunohistochemical analyses of lung sections also revealed localisation of platelets in extravascular lung compartments of Nb infected mice, which was not observed in naïve mice **(figure 3.1.1, E)**. We found that extravascular platelets were typically found in clusters and tended to co-localize with other cells. However, consistent with previous studies showing platelets recruiting independently into the lungs during pulmonary inflammation [92] , we also observed individual platelets in the lungs of Nb infected mice.

Interestingly, and further supporting the notion that Nb infection led to platelet activation, we were able to detect intravascular thrombi in Nb infected mice **(Appendix, figure 5.2).** However, we did not find evidence that Nb infection was associated with changes in total platelet counts as Nb infected mice had similar platelet levels to non-infected mice throughout the course of infection (**Appendix, figure 5.1**). Given that mild thrombocytopenia has been previously reported to occur in a rodent model of *S. mansoni* infection [164], our data suggest that effect of helminth infection on circulating platelet levels may be parasite -specific.

Having established that platelets become activated and localise to the lung at 18 hours post infection with Nb, we next sought to characterise the acute circulatory and pulmonary innate immune response that develops at this time point. Our rationale behind focusing our analysis on this acute stage of the infection is based on the results of previous studies demonstrating that platelets rapidly respond to infection and critically impact on the initiation of inflammatory processes [50]. Furthermore, as studies have shown that Nb larvae begin reaching the lung in appreciable numbers within 24 hours of inoculation [158], this time point would allow us to establish which factors mediate early host control of parasite migration.

In line with previous studies which have shown that the acute innate response to Nb infection in the skin (at 6 hours post infection) [154] and in the lung (at 2 days post infection) [132] is dominated by a neutrophilic response, we found that Nb infection was specifically associated with an increase in the number of circulating neutrophils, but not monocytes or eosinophils (**figure 3.1.2).** In agreement with this, flow cytometric analyses of lung tissue taken at 18 hours post infection also revealed elevated numbers of neutrophils in Nb infected relative to naïve mice (**figure 3.1.3).** Interestingly we also found evidence of early eosinophilia in the lungs of Nb infected mice. This is noteworthy given that eosinophils are not commonly characterised as important effector cells in acute pulmonary Nb infection and are thought to recruit into the lung only following the expression of type 2 cytokines from ILC2s and other early responders [156]. While our data does contrast with previous findings, it is important to point out that we also observed reduced levels of eosinophils in the lungs of platelet depleted mice following infection with Nb (**figure 3.2.3)**, which suggested eosinophils were indeed being recruited at this stage. However, whether eosinophils also

have important function at this time point remains unknown. This should be a consideration for future investigations.

After characterisation of the acute innate immune response at 18 hours post infection with Nb, we set out to establish if and how platelets are involved in these responses. The interaction of platelets with immune cells is increasingly recognized as an important mechanism underlying platelet immune function [48]. We therefore next tested whether Nb infection led to changes in the association of platelets with immune cells. Corroborating previous reports showing platelet interacting with neutrophils and eosinophils in the context of pulmonary inflammation [66,86] we found that Nb infected mice had increased levels and numbers of CD41+ neutrophils and eosinophils compared to naïve mice at 18 hours post infection **(figure 3.1.4).** Intriguingly, we also observed increased association of platelets with alveolar macrophages at this time point, suggesting that platelets were also readily entering alveoli during infection. However, whether platelets actively traffic to the alveoli during acute Nb infection, or whether their presence is primarily a result of diffuse disruption of the alveolar-capillary barrier caused by tissue damage, requires further investigation.

To further establish how platelets associate with pulmonary immune responses to acute Nb infection we next sought to ascertain whether platelet-association with immune cells also occurs at later time points post infection. Our analyses revealed that at day 2 post infection , which previous studies have indicated is when the number of Nb larvae reaches its peak [158] , there was significant association between platelets and leukocytes, including neutrophils , alveolar macrophages and eosinophils **(figure 3.1.5).** Indeed, we observed that a greater proportion of these immune cells were platelet associated at day 2 compared to 18 hours post infection. Furthermore, platelet association with immune cells remained high at day 3 post infection and was similar to the levels seen on day 2 post infection. These data suggest association of platelets with immune cells in the lung is not restricted to the acute setting (i.e., at 18 hours p.i.), but also occurs during later stages of pulmonary Nb infection. The observation that platelet association with immune cells remained elevated after the majority of larvae have migrated away from the lung, raises the possibility that the association of platelets with these immune cells is not only driven by the direct presence of Nb larvae (and presumably , the tissue damage that they cause) but also occurs in the lung

during the initiation of type 2 responses that are aimed at controlling pathology and inducing wound healing responses. In view of this , it is noteworthy that we were able to detect platelet association with macrophages, as these are known to play important roles in regulation of pulmonary inflammation , and in protection from excessive tissue damage [142]. A recent study by Rossaint et al. [87] demonstrated that platelet association with immune cells, in particular regulatory T cells (Tregs), is an important feature of resolution of neutrophil-dominated pulmonary inflammation during bacterial pneumonia. Whether a similar mechanism is involved in the resolution of pulmonary inflammation after Nb infection , or whether platelets associate with other protective (type 2) responses crucial for protection against pulmonary pathology [121] are both questions deserving of further experimental enquiry.

Based on previous studies which have shown that the interaction of platelets with immune cells is associated with significant transcriptional and functional changes [48,87] we next examined whether platelet-associated (CD41+) and non-associated (CD41-) circulatory neutrophils exhibited altered phenotype. We chose to focus our analysis on neutrophils based on our earlier findings (outlined in **figure 3.1.2**) showing that neutrophils dominate the early circulatory response to Nb infection. To study the association of platelets with neutrophil phenotype, we used both supervised as well as unsupervised (t-SNE) flow cytometric analysis. We found that platelet associated neutrophils were distinct from non-associated neutrophils and exhibited reduced IFN-γ and increased granzyme B expression (**figure 3.1.6**). Moreover, we observed that the of degree platelet association (i.e., neutrophil MFI of CD41) also correlated with these differences, with CD41 [Hi] neutrophils exhibiting reduced expression of IFN-y and increased expression of granzyme B relative to CD41 [Int] neutrophils.

Due to the lack of empirical studies investigating the association of platelets with the expression of these factors in neutrophils, it is difficult to interpret how these findings correlate with previous research. However, IFN-y is known to negatively regulate the expression of CD41 on Basophils [165] and platelets have been shown to associate with interferon responses in other contexts [166]. Our data is also supported by previous findings which show that IFN-y and RELM-α antagonise each other – a relationship that our data also

supports in (**figure 3.1.7**), in which we demonstrate that neutrophil MFI of CD41 strongly associates with the expression of RELM-α.

Furthermore , while platelets have , to the best of our knowledge not been previously associated with neutrophil expression of granzyme B, previous studies have indicated that platelets drive apoptotic mechanisms through their own expression of granzyme B [167] and have been shown to markedly alter the expression of granzyme B in other cell types [168]. Moreover , a recent study indicated that granzyme B expression was also upregulated in myeloid cells infiltrating the lung during Nb infection [169], which the authors suggested could play a functional role in anti-parasite immunity. As such, while our results are preliminary and require further exploration, especially with regard to whether platelet interaction drives differences in neutrophil expression of these factors or simply correlates with them, they nonetheless suggest that platelet associated neutrophils are distinct from non-associated neutrophils and therefore that platelets may impact on neutrophil -mediated processes in the context of acute Nb infection.
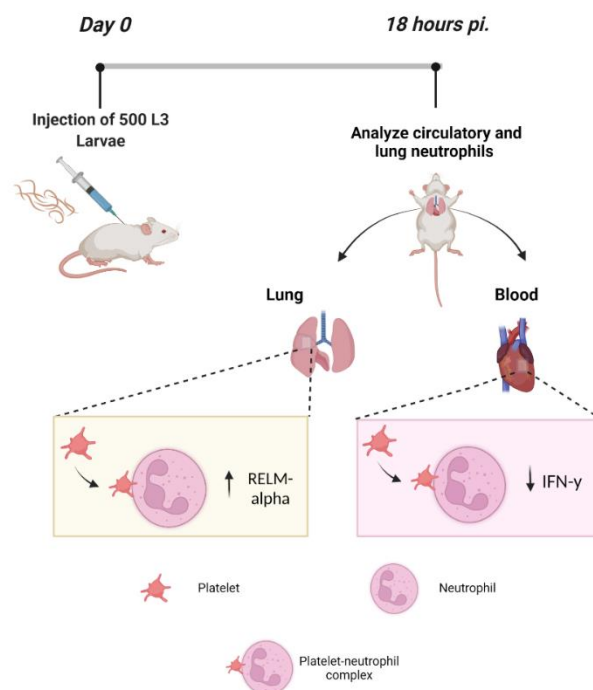
Figure 4.1: Platelet interaction with neutrophils during acute N.brasiliensis infection is associated with changes in their expression of IFN-y and RELM-α.

Chapter 3.2: Platelets are indispensable for the protection against Nb and neutrophil – associated pulmonary pathology

To further clarify the role of platelets in this study we established a model of Nb infection in thrombocytopenic (platelet depleted) mice. Our model entailed depleting mice of their platelets using antibodies prior to infecting them with Nb. Previous studies have demonstrated that treatment with anti-platelet antibodies results in rapid and marked thrombocytopenia , but that continuous treatments are necessary to sustain low platelet counts [170]. In line with these studies, we found that antibody treated mice in our model had severely diminished platelet levels (< 10 % of non-treated mice) but that levels quickly recovered following treatment (**Appendix, figure 5.3**).

After establishing an effective protocol to deplete mice of their platelets we examined how platelet depleted mice compared to non-depleted mice in the context of pulmonary Nb infection. We observed strikingly enhanced pulmonary pathology in thrombocytopenic mice, which was characterised by marked pulmonary haemorrhaging and significant loss to lung integrity **(figure 3.2.1)**. As a result of this, we found that platelet depleted mice rapidly succumbed to infection and that none of the mice survived past 24 hours. These findings are congruous with other recent studies which have shown platelets to be indispensable for maintaining lung integrity during pulmonary infection [78,89,152].

Interestingly, we also consistently observed lower worm burdens in the lungs of platelet depleted mice relative to non – depleted mice. This suggested that the loss of platelets may also have significant ramifications for anti-parasite immunity, as has been indicated in previous studies [149]. However, given previous studies that have demonstrated that the loss of platelets is associated with higher worm burdens in *S. mansoni* infection [149,164], our data suggest that the role of platelets in mediating anti-parasite immunity may be parasite-specific. Future studies should focus on investigating precise regulatory mechanisms supporting anti-parasitic responses that may be modulated by the in vitro or in vivo presence of platelets and the factors that they release.

We reasoned that the differences observed in pulmonary pathology between platelet-depleted and non-depleted mice was possibly a result of changes in innate immune cells

and their phenotype. To this end, we performed detailed flow cytometric analyses on circulatory and lung recruited innate myeloid cells and found that platelet depleted mice exhibited significantly elevated levels of circulatory, but not lung neutrophils and impaired pulmonary recruitment of eosinophils (**figures 3.2.3 and 3.2.4**). In line with expectation, platelet depleted mice also exhibited broad ablation of platelet-leukocyte interaction and had significantly reduced numbers of platelet-leukocyte complexes both in the circulation as well as in the lung. Our observation of circulatory neutrophilia in platelet depleted mice is consistent with other studies using anti-platelet antibodies [162] and aligns with a growing body of evidence that point to an important role for platelets [171] and their pre-cursor cells [172] in shaping haematopoiesis, especially during infection.

Furthermore, our observation that platelets may regulate eosinophil recruitment is in accord with the findings of studies demonstrating an important role for platelets in regulating eosinophil recruitment during allergic airway inflammation [66]. It is important to note that while we did not observe acute differences in neutrophil recruitment to the lung, prior studies do suggest that platelets regulate their recruitment in many other settfuings [161]. As such, more research into the role of platelets in innate cell recruitment during pulmonary Nb infection is warranted, to establish whether our findings are specific to the time point that we chose to analyse (i.e., 18 hours post infection).

Drawing on our previous findings in which we identified differences between platelet associated and non-associated neutrophils, we next explored how circulatory neutrophils from platelet depleted and non-depleted mice compared. We found that, similar to CD41(-) neutrophils, neutrophils from platelet depleted mice exhibited increased expression of IFN-y and decreased expression of granzyme B (**figure 3.2.5** and **3.2.6).** In addition, platelet depleted mice also had significantly elevated numbers of IFN-y expressing circulatory neutrophils relative to non-platelet depleted mice. Given previous studies that have suggested that the suppression of IFN-y is important for the induction of protective host responses during acute Nb infection [160], it is plausible that aberrant expression of IFN-y by neutrophils was contributing towards susceptibility to Nb infection in platelet depleted mice. This was further supported by the observation that platelet depleted mice also had reduced numbers of RELM-α expressing neutrophils in their lungs (**figure 3.2.6**). Interestingly, lung neutrophils from platelet depleted mice also exhibited reduced

expression of the receptors Ly6G and CD11b in comparison to neutrophils from non -
depleted mice. CD11b, which has pleiotropic functions [68,173] ,is known to support the
interaction of neutrophils with platelets [48] and has previously been associated with
protection from pulmonary pathology during Nb infection [174]. The function of Ly6G on the
other hand is less well defined but alterations in Ly6G expression typically align with
differences in neutrophil maturation [175] and aberrant neutrophil responses[176].As such
differences in the expression of these receptors may reflect varied function in neutrophils
[177]. Notably however, differences in CD11b and Ly6G expression between platelet depleted
and non-depleted mice were restricted to lung neutrophils, with few differences being
observed between circulatory neutrophils. While speculative, it is possible that these
differences only arise once neutrophils leave the circulation and become exposed to the
microenvironment of the lung, which is vastly different in platelet depleted versus non-
depleted mice. In support of this hypothesis, we observed marked differences in the
expression (MFI) between circulatory and lung neutrophils (**Appendix, figure 5.4)** indicating
some degree of receptor down-modulation. Taken together, these data suggest that
neutrophils from platelet-depleted mice display aberrant expression of immune-associated
factors that are known to play a role in Nb infection, and we hypothesized that they were
therefore likely contributing towards susceptibility to infection.

To test this hypothesis, we performed experiments in which we co-depleted mice of their
platelets and neutrophils prior to Nb infection. As with platelet – depletion, neutrophil
depletion was mediated by the treatment of mice with monoclonal antibodies. While
(circulatory) neutrophils levels were not entirely diminished in co-depleted mice **(figure
3.2.7)**, we did observe a large reduction in the number of lung neutrophils. Importantly, this
reduction was associated with markedly less pulmonary pathology in co-depleted mice
(figure 3.2.8) , which, as previous studies indicate [132] , further supports the notion that
neutrophils mediate immunopathology during acute Nb infection. Similar to platelet
depleted mice however, co-depleted mice also had reduced worm burdens in their lungs
(figure 3.2.8), which implies that neutrophil-independent mechanisms also contribute
towards anti-parasite host responses during early Nb infection.

In any case, in line with prior investigations [132] , our data suggest excessive inflammation

during Nb infection is associated with increased pulmonary pathology and lower worm burdens.

Collectively, our data therefore argue for an important role for platelets in mediating host-pathogen interaction during pulmonary Nb infection and in supporting responses that not only protect the host, but also benefit invading Nb larvae.
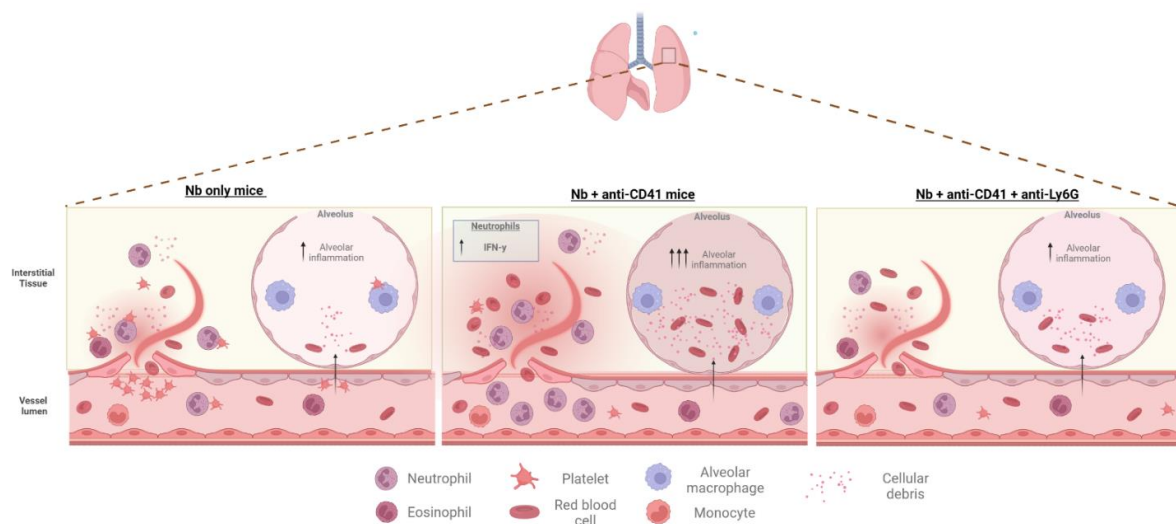
Figure 4.2: The effect of platelet depletion and platelet-neutrophil co-depletion on pulmonary pathology during N. brasiliensis infection.

Chapter 3.3: Blockade of the platelet receptors CD62P and CLEC-2 during Nb infection associate with changes to platelet -neutrophil interaction and neutrophil phenotype

To provide mechanistic insight into how platelets regulated Nb-associated pulmonary pathology we examined the involvement of specific platelet receptors, namely CD62P (P-selectin) and CLEC-2. To study the role of these receptors in Nb infection we used blocking antibodies that either targeted these receptors directly (anti-CD62P) or targeted their major ligand (anti-podoplanin). CD62P is an adhesive protein which plays notable roles in mediating the interaction of platelets with leukocytes and is heavily implicated in the regulation of neutrophil function [178].Platelet CLEC-2 on the other hand has significant function in maintaining vascular integrity during pulmonary inflammation [179] and tightly associates with protective responses in the lung during infection, primarily through its interaction with podoplanin [78,94] .

We hypothesized that blockade of these receptors would be associated with changes in neutrophil association with platelets, neutrophil function and subsequently, worsened pulmonary pathology during Nb infection. We found that while blocking CD62P was associated with reduced numbers of platelet-neutrophil complexes (**figure 3.3.1)** this was not accompanied by differences in neutrophil phenotype and did not correlate with changes to Nb associated pulmonary pathology at 18 hours post infection **(figure 3.3.2)**. We were also unable to identify significant differences in the number of innate myeloid cells in the circulation or lung of anti-CD62P treated mice (data not shown). These data suggest that CD62P blockade is not associated with significantly different immunologic and physiologic outcomes in the context of acute Nb infection. However, based on the view that CD62P is primarily impacting innate responses via supporting platelet-neutrophil interaction, we cannot definitively rule out a function for CD62P in acute Nb infection, as mice treated with anti-CD62P still had an appreciable number of platelet-neutrophil complexes (**figure 3.3.1).** Additionally, given reports of the upregulation of CD62P in other helminth infections [180] , further research into the function of CD62P in Nb infection is warranted**,** especially at other time points in the infectious cycle**.**

The indirect targeting of CLEC-2 through antibody-mediated blockade of podoplanin lead to a modest reduction in platelet-neutrophil interaction and, similar to our experiments with platelet depleted mice, was associated with changes in lung neutrophil phenotype **(figure 3.3.3)**. However, as with CD62P blockade, podoplanin blockade did not lead to lung haemorrhaging or marked differences in pulmonary pathology (**figure 3.3.4).** Our data therefore points to a non-essential role for CLEC-2 in maintaining haemostasis and lung integrity in acute Nb infection and indicates that other receptors , e.g. integrin αIIbβ3 may be important[17].

Additionally, our data also suggest that the CLEC-2 – podoplanin axis impacts on neutrophil phenotype. While , to the best of our knowledge, this has not been previously reported, CLEC-2 deficiency is associated with changes in neutrophil recruitment [78] and with alteration in the expression of factors known to affect neutrophil function [181]. In view of this , and the fact that CLEC-2 signalling has also recently been associated with the functioning of

chitinase-like proteins [182] , it would be of considerable interest to ascertain whether CLEC-2 is involved in pulmonary responses downstream of acute infection.

Finally, to investigate the function of CD62P and CLEC-2 in in our model further, we tested the effect of simultaneous blockade of both CD62P and CLEC-2 on pulmonary pathology and circulatory and lung innate cell responses. In mice receiving both anti-CD62P and anti-podoplanin blocking antibodies, we observed altered platelet responses and modest circulatory neutrophilia, as well as changes in lung neutrophil phenotype **(figure 3.3.5).** Interestingly, simultaneous blockade of CD62P and CLEC-2 was not associated with changes in platelet-neutrophil interaction, which suggests that the blocking antibodies have non-additive effects when administered together. Intriguingly, dual blockade was associated with a strong trend for increased pathology in treated mice **(figure 3.3.6).** While these data, in contrast to our earlier findings, do implicate CD62P and CLEC-2 in platelet regulation of pathology during acute Nb infection, they are limited by our incomplete understanding of how anti-CD62P and anti-podoplanin interact (in their effect) when administered together in our model and by the fact that expression of these receptors is not restricted to platelets [183]. Nonetheless, our data do establish a role for these receptors in regulating platelet-neutrophil responses and indicate that further research into platelet receptors during Nb infection is warranted
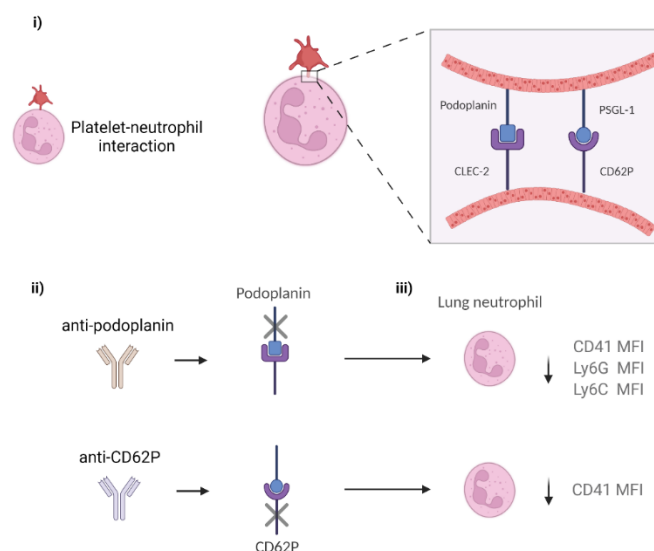
Figure 4.3 : Blockade of platelet receptors leads to changes in the interaction of neutrophils with platelets in the lung and is associated with alterations in neutrophil phenotype .

Conclusion

The data produced throughout this project is some of the first regarding the role of platelets in acute responses to pulmonary helminth infection. While previous studies have implicated platelets in resistance to helminths [184], this study is the first to provide compelling evidence that platelets are essential mediators of immune responses during helminth infection and the first to show that they are inextricably linked to the pathophysiology of pulmonary helminth infection. In doing so, this project notably advances our understanding on the cells and crucial mechanisms underlying host-pathogen interaction in the lung and offers insight into why it is that the migration of these remarkably large parasites does not cause lethal pathology.

Here, we report that platelets become activated following infection with the nematode *N. brasiliensis* (Nb) and traffic to the lung, where they interact with principal immune cells, such as neutrophils, to modulate their expression of factors that drive acute innate responses. Furthermore, we show that the depletion of platelets prior to Nb infection results in markedly enhanced pulmonary pathology and associates with aberrant neutrophil responses, which can be partially corrected through co-depletion of platelets with neutrophils. Finally, we demonstrate that the platelet receptors CLEC-2 and CD62P regulate the interaction of platelets with neutrophils and potentially neutrophil function during Nb infection but have little impact on protecting against Nb-associated acute pathology.

Future work

While this project has contributed significant knowledge towards our understanding of platelets in the context of helminth infection, much is still to be learned and numerous questions yet remain. In the specific setting of the model used to explore platelet-regulated functions in this project, many lingering questions relate to platelet-regulation of neutrophils and how this impacts immunopathology and anti-parasitic immunity. This could be explored in the future by investigating the association of platelets with specific neutrophil effector responses , such as their production of ROS and NETs, which are

regulated by platelets and the factors that they produce [185] in other settings[75] and are known to contribute towards immunity to Nb [154]. Closely tied to this, it would also be of great interest to understand how the absence of platelets impacts on other known markers of neutrophil heterogeneity [175] and whether platelets preferentially associate or influence neutrophil subpopulations, such as MDSCs [186] or LDNs [177].   Additionally, to further examine the mechanism proposed in this project it would be crucial to investigate the effect of IFN-γ and granzyme B blockade on pathogenesis of in platelet depleted mice, and to correlate differences in the expression of these factors with altered immunity during primary and secondary infection in non-depleted mice.

Further to this, future studies should seek to identify specific responses that associate with the lower worm burdens observed in severely thrombocytopenic mice, and to delineate how absolute platelet count, platelet-derived factors [187] and Nb–associated haemorrhaging [188] impact on worm viability. These features could be elucidated through complimentary murine models of thrombocytopenia [152], or through specific adaptations of the model employed within this project, for instance, by temporally controlling for neutrophilia mediated by treatment with platelet depleting antibodies. It may also prove insightful to alter the infectious dose used in the model to increase the sensitivity of identifying specific immunologic correlates of protection, as has been demonstrated in other studies [189]. Equally, altering the time point at which platelets are depleted will give important insight into time dependent effects – which are known to occur in other settings [190,191].

Moreover, as outlined previously, it would be of significant interest to further investigate how platelets associate with the induction of type 2 immune responses in the lung following acute infection, and how they influence fibrotic and emphysematous pathology. Closely tied to this, it would also be of major interest to explore how platelets interface with mechanisms involved in helminth–mediated immunomodulation, given platelet association with TGF-β production [31] and regulatory T cell (Treg) responses in other contexts [87].

Ultimately, to establish a more holistic understanding of platelets in helminth infection however, future investigations will necessarily have to explore the immunologic functions of platelets at other sites of infection. In the context of Nb infection, this should entail focused investigations into how platelets impact on immune responses in the intestines, as this is where patency, and in many cases, clinical disease develops. In particular, given the vast

variety of growth factors [26] and factors associated with neuronal functions [16] that platelets store in their granules, it would be salient to understand how they impact on epithelial cell responses and smooth muscle contractions. As in the lung, a detailed comprehension of platelets in this setting would also have significant impact on our understanding of the factors supporting recall immunity during helminth infection. Given these theoretical associations and our empirical data, it therefore seems extremely likely that platelet-related research in the future will provide important insight into mechanisms underlying the interaction of helminth parasites with their hosts.

Appendix
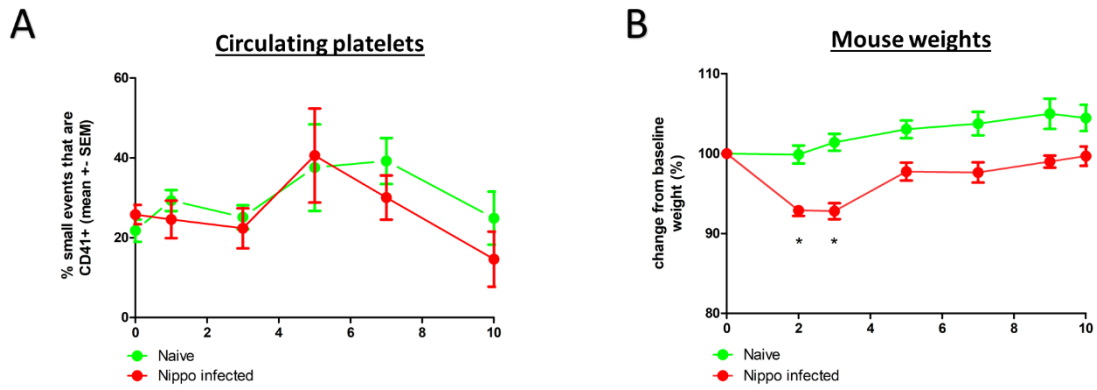
Figure 5.1: Nb infection is not associated with changes in circulatory platelet counts. Mice were infected with Nb, and tail blood was collected at several time points post infection. Flow cytometric analyses of platelet in the circulation of Nb infected mice was performed and levels compared to naïve mice **A**). Mouse weights were also taken at respective time points **B**).
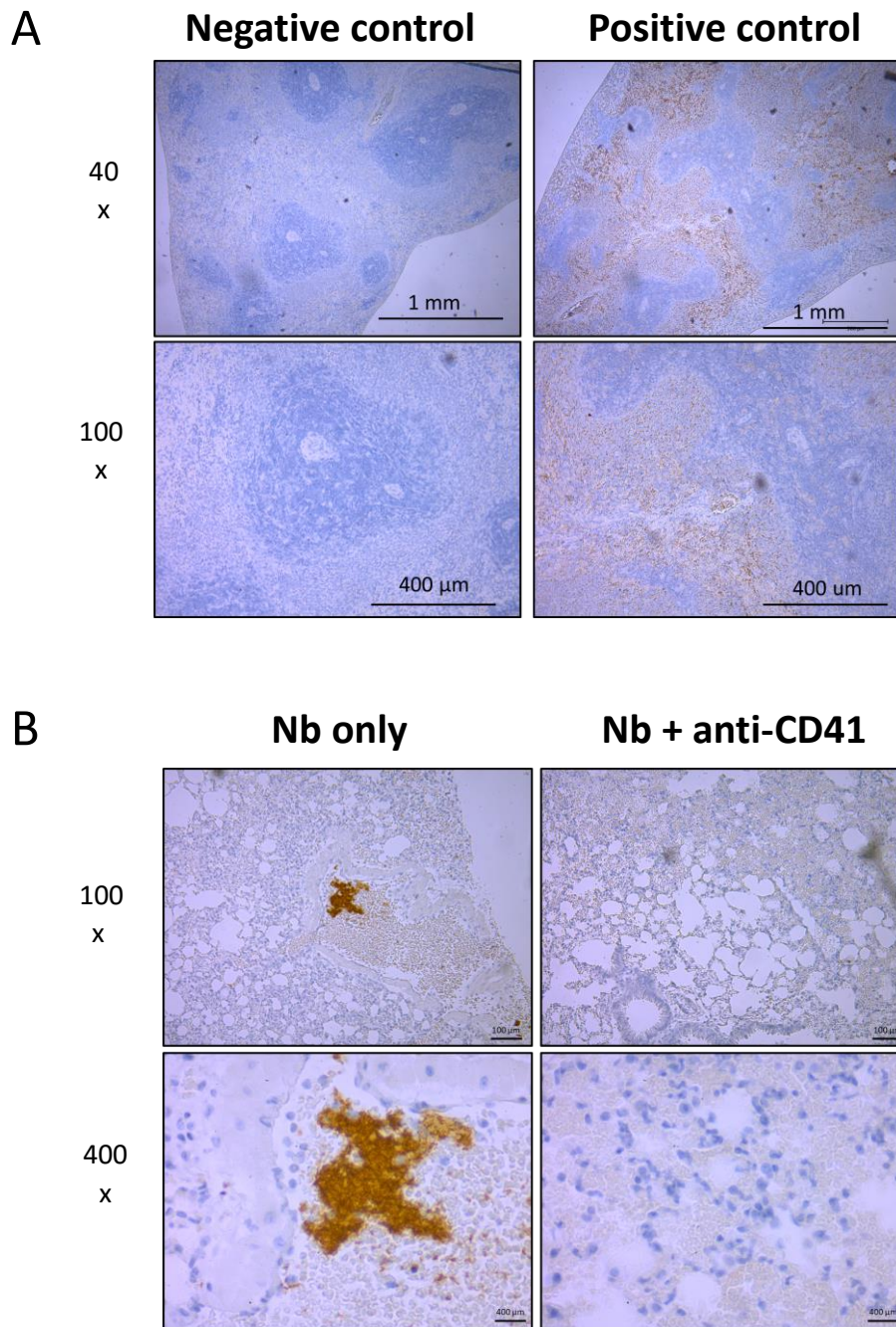
Figure 5.2: Immunohistochemical staining of CD41 in the lungs of Nb infected mice. Lungs were sampled at 18 hours post infection with Nb and stained with polyclonal anti-CD41 antibodies. Spleen tissue was used as a positive control (negative control = no anti-CD41 antibody **A**). Additionally, Lung staining was compared between platelet depleted and non-depleted mice **B).**
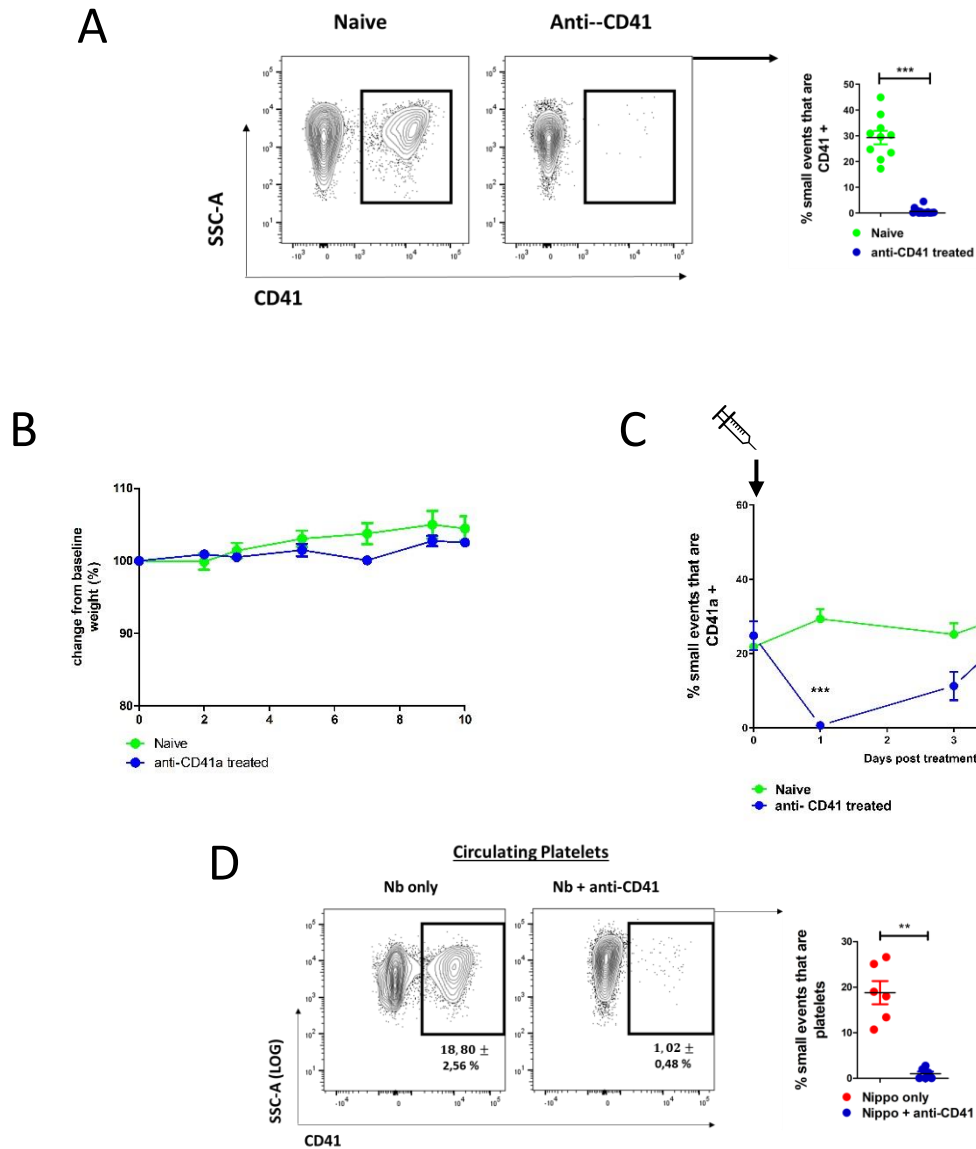
Figure 5.3: treatment with anti-CD41 leads to severe thrombocytopenia. Mice were treated with anti-CD41 antibodies to deplete platelets. To confirm the efficacy of depletion, blood was collected from treated and naïve mice, and the levels of platelets determined by flow cytometry **A**). Treated mice were also monitored for several days to confirm safety of the treatment **B**). Flow cytometric analysis of blood samples taken at day 1, day 3 and day 5 post treatment was performed to determine the duration of platelet depletion **C**). Platelet depletion was also confirmed in the context of Nb (Nippo) infection **D**). Statistical differences were investigated with Mann-Whitney U-test.
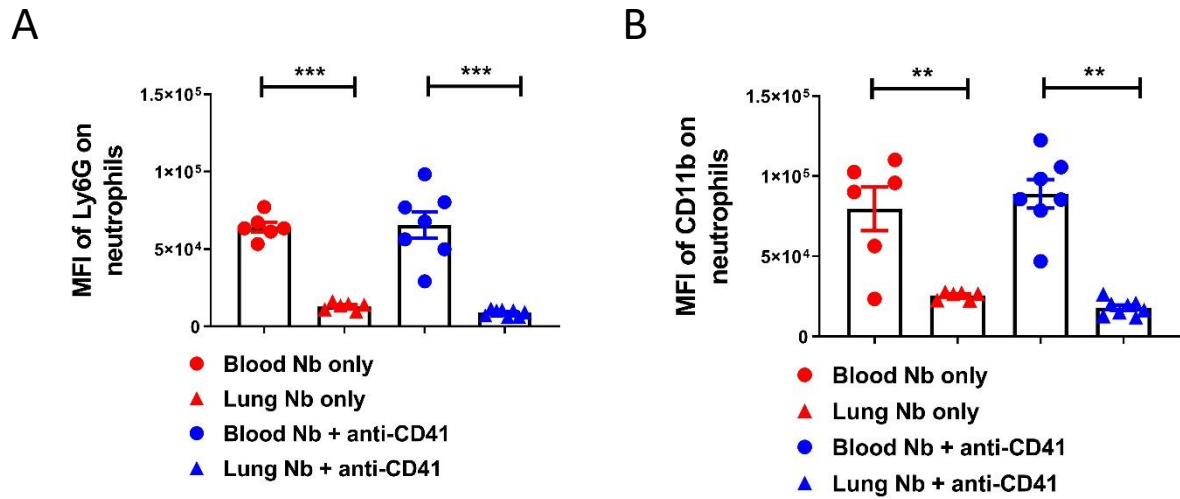
Figure 5.4: Circulatory and lung neutrophils differentially express neutrophil receptors. Flow cytometric analysis of blood and lung neutrophils from Nb only and Nb + anti-CD41 mice was performed and the MFI of Ly6G **A**) and CD11b **B**) was determined. Paired t-tests were used to test for statistical differences.

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