NEW INSIGHTS INTO PLATELET AND LEUKOCYTE BIOLOGY

by

Tammy L. Smith

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STATEMENT OF DISSERTATION APPROVAL

The dissertation of **Tammy L. Smith**

has been approved by the following supervisory committee members:

Andrew S. Weyrich	, Chair	**05/09/13** Date Approved
Curt H. Hagedorn	, Member	**05/22/13** Date Approved
Gerald S. Spangrude	, Member	**05/09/13** Date Approved
Janis Weis	, Member	**05/09/13** Date Approved
Guy A. Zimmerman	, Member	**05/09/13** Date Approved

and by **Peter Jensen** , Chair/Dean of

the Department/College/School of **Pathology**

and by David B. Kieda, Dean of The Graduate School.

ABSTRACT

The formed elements of blood perform essential functions and are necessary for survival. Red blood cells transport oxygen to tissues, platelets maintain hemostasis and leukocytes provide host defense. In addition to these autonomous roles, blood cells are able to communicate with one another to execute complex physiological processes. Platelet interactions with leukocytes and endothelium regulate diverse inflammatory responses. Platelet-monocyte interactions result in robust cytokine production in monocytes. Neutrophils receive signals from platelets which can result in transendothelial migration or neutrophil extracellular trap (NET) formation. Many of the mechanisms by which these processes are regulated have only been recently discovered. Here we report that platelets are able to sequester exogenous ribonucleic acid (eRNA). This uptake is time and dose dependent and is unique to platelets of the cells examined so far. Uptake of eRNA does not require activation of platelets or alter the ability of platelets to respond to thrombin. While the fate of this sequestered eRNA has yet to be determined, our observations represent the first report of this novel capacity of platelets. Our interest in platelet and leukocyte biology also led us to develop a genetic screen to identify genes required for NET formation. The nearly haploid human cell line, KBM-7, was determined to be competent to form NETs, and a retroviral gene-trap mutagenesis strategy was employed to generate a pool of mutants to assay for defects in NET formation. Due to technical limitations, the screen was unable to be executed as designed,

but advances in bioinformatics may allow a modified version of the screen to be performed in the near future. Throughout the body of this work, we have used traditional techniques combined with modern innovations to interrogate the functions of platelets and leukocytes. The knowledge gained in these investigations will improve our understanding of these essential cells and provide new avenues for research and therapeutic interventions in the future.

Dedicated to my partner, Brian

&

My parents

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CHAPTER 1

INTRODUCTION

The Formed Elements of Blood

Blood is an essential vertebrate tissue consisting of plasma and formed elements. The formed elements of blood include red blood cells (RBCs), platelets and leukocytes. While each of these elements executes specialized functions on their own, they also communicate with one another as well as various tissues throughout the body to orchestrate complex physiological processes. Understanding the behavior of these formed elements in isolation and in concert with one another is critical for deciphering their contributions to both health and disease.

The study of the formed elements of blood was impossible before the advent of microscopy. The first recorded observation of a blood cell was of an RBC by Jan Swammerdam in 1658[1]. Leukocytes and platelets were not observed until the mid-1800s, a lag explained by the relative abundance of RBCs to platelets and white blood cells (WBCs). Not long after their discovery, the primary function of each of these components was described. The majority of an RBC is composed of hemoglobin, a molecule whose reversible oxygenation was characterized by Felix Hoppe-Seyler in the second half of the 19th century[2]. The ability of platelets to accumulate at the site of vascular injury was described by Bizzozero in the late 1800s[3]. Leukocytes, a diverse

group of cells within themselves, were not readily distinguished from one another until Paul Ehrlich published a method for staining blood films in 1879[1]. The distinction between granulocytes, lymphocytes and monocytes allowed for understanding the distinct role each of these leukocyte subsets plays in innate and adaptive immunity.

While direct microscopic observation provided a wealth of information to early hematologists, the past few decades have given researchers the biochemical, molecular and genetic tools to expand the limits of our understanding of the biology of these formed elements of blood. Platelets, far from simply creating hemostatic plugs, have been shown to participate in inflammatory processes with other cells throughout the body[4,5]. The study of rare hereditary immunodeficiencies not only led to identification of the mutations responsible for the leukocyte defects, but also to a broader understanding of common cell signaling processes[6,7]. Neutrophils, cells long recognized for their importance in killing invading organisms, have only recently revealed their ability to kill microbes using extracellular DNA traps[8]. Continued interrogation of each of these formed elements in isolation and in concert will enable better understanding of the human organism as a whole and provide novel therapeutics to improve health.

Platelets: Central Mediators of Hemostasis and Inflammation

Platelets, the smallest of the formed elements, were easily overlooked in early microscopic studies of blood. When they were noted, they were thought to represent degraded leukocytes or precursors to erythrocytes[9]. This debate was settled by Giulio Bizzozero's careful microscopic examination of blood both *in vivo* and *in vitro*, whereupon he firmly declared platelets to be the third element of blood, along with RBCs

and WBCs. Bizzozero observed the rapid accumulation of platelets in injured blood vessels, as well as characterized the deposition of platelets followed by fibrin on foreign surfaces, providing one of the earliest descriptions of thrombus formation [3].

Thirty years after these seminal observations on the function of platelets, James Wright demonstrated that platelets arise from fragmented megakaryocytes within the bone marrow[10]. Megakaryocytes are terminally differentiated cells which undergo a process of endomitosis before elaborating their cytoplasm and membranes to produce proplatelets[11]. During this process, proplatelets are packaged with organelles such as mitochondria, alpha and dense granules, ribonucleic acid (RNA), proteins and cytoskeletal components. Megakaryocytes do not indiscriminately partition their cytoplasmic constituents into proplatelets, but selectively transfer a subset of these into the nascent proplatelet[12]. The importance of proper trafficking of these components is underscored by the variety of rare platelet disorders caused by genetic defects in cytoskeletal and sorting proteins[13-15]. Global analysis of the platelet transcriptome and proteome has demonstrated little variation between healthy donors[16,17]. However, platelets from diseased patients show altered RNA and protein signatures, suggesting that platelet constituents are dynamically regulated in response to physiological cues[18-21]. Whether this regulation occurs as platelets are produced by megakaryocytes in the bone marrow or represents alterations acquired as platelets circulate within the periphery remains to be determined.

Extracellular cues affect changes within individual platelets in addition to the globally programmed changes described above (Fig. 1.1). Thrombin treatment leads to

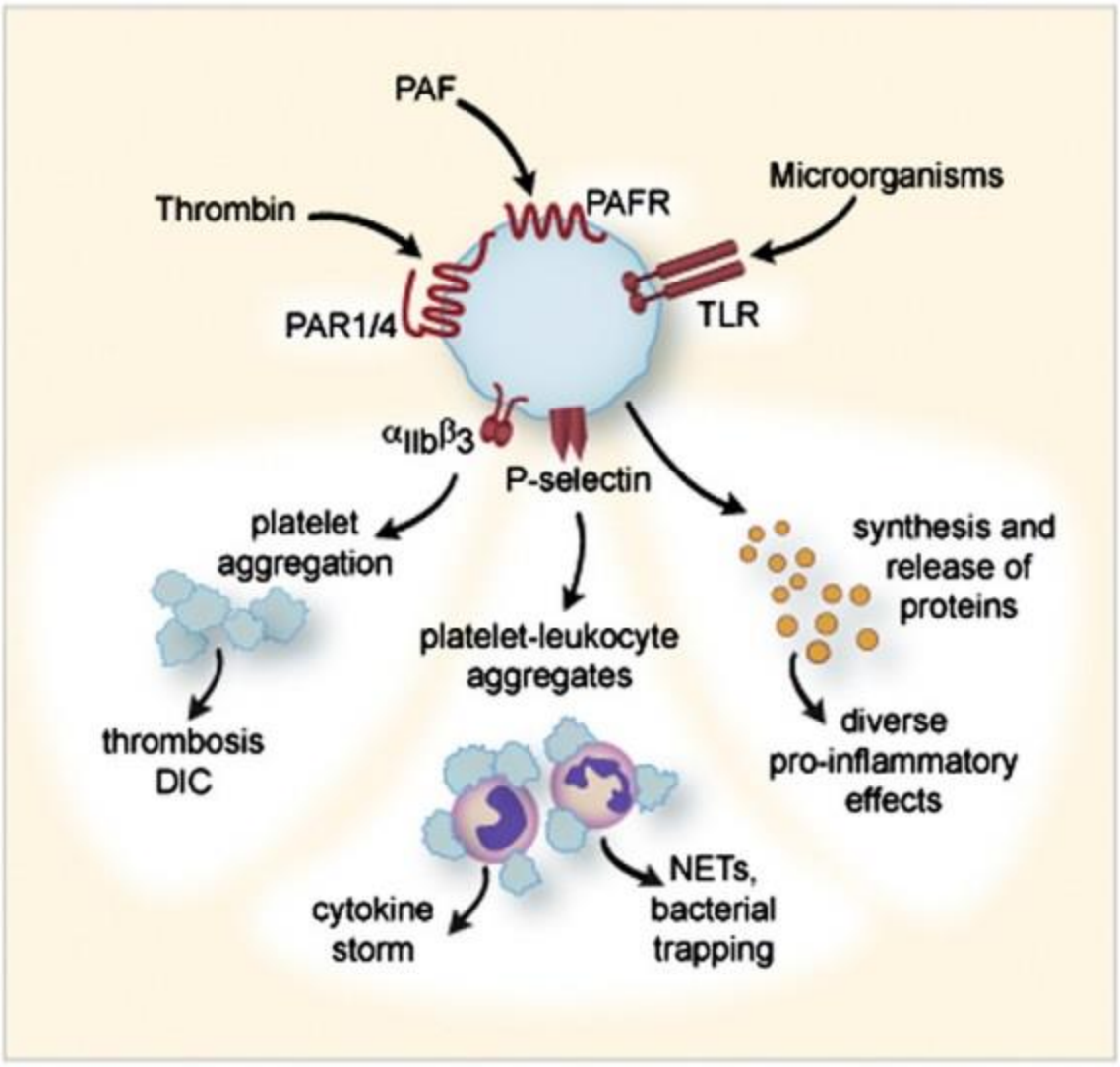

Figure 1.1. Platelets integrate a variety of inputs to mediate pathology in systemic inflammatory responses. Thrombin, PAF, and microorganisms are commonly observed in infectious situations. Each of these factors modulates platelet function, inducing both rapid and prolonged responses that include the formation of homotypic and heterotypic aggregates. Platelet-platelet aggregates and platelet-leukocyte aggregates contribute to DIC, the formation of NETs, and the synthesis and secretion of proinflammatory cytokines that are commonly observed as the host responds to infection. These responses may help fight off infection, but they can also contribute to the pathology of the disease. Abbreviations: DIC, disseminated intravascular coagulation; NETs, neutrophil extracellular traps; PAF, platelet activating factor; PAFR, PAF receptor; PAR, protease activated receptor; TLR, Toll-like receptor.[5] (Reprinted with permission of Elsevier Publishing)

rapid degranulation of platelets and release of vasoactive and prothrombotic factors[22,23], important responses to maintain hemostasis following damage to vessels. These rapid and conspicuous changes overshadowed more long term consequences of platelet activation for years. In the late 1990s, thrombin-activated platelets were shown to synthesize Bcl-3 rapidly, but also to sustain this response for up to 8 hours[24]. Synthesis of Bcl-3 is required for normal retraction of fibrin clots to support recanalization of vessels and reperfusion of tissues after injury[25]. Anucleate platelets also contain precursor mRNAs, which they are capable of splicing and translating in response to activating signals[26-28]. In the case of IL-1β synthesis by activated platelets, the subsequent release of this inflammatory mediator promotes adherence of neutrophils to the endothelium[28].

Activated platelets promote inflammation through interactions with other cells in addition to the cell autonomous mechanisms described above. Platelet binding to monocytes promotes nuclear translocation of NF-κB and proinflammatory cytokine production, such as MCP-1 and IL-8, by monocytes[29]. The induction of COX-2 expression in monocytes by platelets is regulated by an additional posttranscriptional checkpoint requiring COX-2 mRNA stabilization downstream of IL-1β signaling, allowing for tightly regulated synthesis of this protein[30]. Platelet signals to neutrophils can promote extracellular trap formation in a TLR4-dependent manner, an important innate immune response which will be described in further detail later[31]. Cytokines secreted by platelets signal to endothelium to promote leukocyte adhesion, amplifying existing inflammation in a site-specific manner[32]. Taken together, it is clear that platelets serve not only as important mediators of hemostasis, but also as central regulators of inflammatory responses. Deciphering the mechanisms by which these anucleate

cytoplasts communicate with such diverse cells may provide new targets for therapeutics in the future.

Leukocytes: Genetic Deficiencies Illuminate Mechanisms of Immunity

Leukocytes are the most heterogeneous of the formed elements of blood. Leukocytes found in peripheral blood consist of neutrophils, monocytes and lymphocytes. While each of these cells has their own unique characteristics, they are all involved in immune defense. As such, the ability of leukocytes to traffic to sites of inflammation to mediate effector functions is fundamental. Rare genetic defects known as leukocyte adhesion deficiencies (LAD) have provided important insights into the steps required for leukocytes to adhere to activated endothelium, known as the adhesion cascade[6]. In addition, these maladies have illuminated common themes used for intercellular and intracellular communication.

The initial step of the adhesion cascade requires leukocytes to loosely adhere to the endothelium in a process known as rolling, which is mediated by selectins[33]. Inflamed endothelial cells express E-selectin and P-selectin, which interact with fucosylated P-selectin glycoprotein ligand-1 (PSGL-1) on leukocytes to induce rolling[34,35]. A genetic mutation resulting in defective GDP-fucose transport into Golgi vesicles leads to global disruption of fucosylated glycoconjugates and the immunodeficiency known as LAD-II[36,37]. Hallmarks of LAD-II include recurrent infections, severe mental retardation, short stature and facial stigmata[38]. While the immune deficiency is explained by the lack of selectin ligands on leukocytes, the metabolic pathways contributing to the remainder of the phenotype remain unclear and continue to be the subject of study[39].

The importance of integrin signaling for firm adhesion of leukocytes to activated endothelium is underscored by the fact that mutations in the gene *ITGB2*, coding for the β_2 integrin subunit, lead to LAD-1. These mutations result in absent, reduced or nonfunctional expression of β_2 integrin on the surface of leukocytes. While still rare, LAD-1 is the most common of the leukocyte adhesion deficiencies, with over 80 mutations described in the *ITGB2* gene resulting in moderate to severe phenotypes[40]. Clinically, delayed separation of the umbilical cord, severe bacterial infections and the absence of pus formation at sites of infection are hallmarks of LAD-I[39,41]. The absence of viral infections in these patients illustrates the fact that lymphocytes are able to use β_1 integrin-dependent adhesion for transendothelial migration, while neutrophils and monocytes rely on β_2 integrin[42].

The genetic defect responsible for LAD-III is the most recent to be characterized, and represents an important breakthrough in understanding integrin-mediated inside-out signaling [43]. Patients with LAD-III have normal surface expression of integrin heterodimers, yet suffer from recurrent bacterial and fungal infections as well as a severe bleeding tendency due to a platelet defect[44-46]. This compound phenotype affecting the function of β1, β2 and β3 integrins without compromising their expression suggested a defect in a common integrin tail binding protein[39]. The search for this protein ultimately led to the identification of Kindlin-3, which is localized to integrin adhesion sites and expressed primarily in hematopoietic cells[47]. Deletion of Kindlin-3 in mice phenocopies defects seen in LAD-III patients[48]. Genotyping of the index patients rapidly confirmed that mutations in the *FERMT3* gene, which codes for Kindlin-3, were responsible for LAD-III in all of the reported families[7,49-52]. Kindlin-3 binds to the cytoplasmic tail of β

integrins, facilitating the conformational change of αβ heterodimers required for inside-out integrin signaling in platelets, neutrophils and lymphocytes[53]. Whether Kindlin-3 serves as a cofactor, scaffold protein or regulates integrin affinity or avidity is the subject of ongoing research[54]. While the molecular details are not yet clear, LAD-III provides an example of how careful observation of complex clinical phenotypes and animal models can provide insights into fundamental cell biology.

Neutrophils: Leukocytes Specialized for Rapid Defense

Neutrophils are the most abundant leukocytes found in peripheral blood and are widely accepted to provide first-line defense against pathogens. Inflammatory cytokines activate neutrophils, resulting in interaction with selectins and integrins on the endothelium and allowing them to escape the circulation and traffic to sites of infection[55]. Genetic defects affecting this process can result in severe immunodeficiencies (described above). Migration of neutrophils to sites of injury after traversing the endothelial barrier depends on chemoattractant gradients[55].

Upon encountering pathogens, neutrophils use several strategies to protect the host. In the early 20th century, Elie Metchnikoff reported phagocytic killing of pathogens[56]. This receptor-mediated engulfment of pathogens results in the formation of a phagosome within the neutrophil[57]. Granules stored within neutrophils contain hydrolytic enzymes and antimicrobial proteins, capable of fusing with the phagosome to effect cytotoxic killing[58]. These granules may also be released from neutrophils to kill extracellular pathogens. In addition to the preformed antimicrobial agents present within granules, neutrophils produce highly cytotoxic reactive oxygen species (ROS) upon

activation[59,60]. Genetic defects in any of the subunits of the NADPH oxidase enzyme required to generate this "oxidative burst" result in the immune deficiency known as chronic granulomatous disease (CGD)[61-63]. Patients with CGD suffer from recurrent infections due to an inability to generate an oxidative burst to kill phagocytosed organisms. In addition, CGD patients are unable to generate neutrophil extracellular traps (NETs), a recently described tool by which neutrophils ensnare and kill extracellular organisms[8].

NETs form when stimulated neutrophils undergo an active form of cell death, known as NETosis, which is distinct from apoptosis and necrosis[64]. During NETosis, the nuclear membrane disintegrates and chromatin decondenses, mixes with cytoplasmic granules, and is released from the cell[8,64]. This process has been shown to trap and kill bacteria, fungi and protozoan parasites[65]. Neutrophils isolated from patients with CGD are unable to form NETs in response to stimuli unless treated with supplemental ROS, demonstrating that the enzyme activity of NADPH oxidase is required for this process[64]. Gene therapy to replace the defective subunit of NADPH oxidase in a patient with CGD restored NET formation and resulted in clearance of a fulminant *Aspergillus* infection[66]. Importantly, *Aspergillus* hyphae cannot be effectively phagocytosed by neutrophils, suggesting that NET-mediated killing is required to control these infections.

As with all weapons, misuse can be destructive. Failure of neutrophils to properly clear after recruitment can lead to chronic inflammation. Deregulated neutrophil clearance has been implicated in the pathogenesis of autoimmune disorders such as rheumatoid arthritis, autoimmune vasculitis and systemic lupus erythematosus (SLE)[67-69]. The lungs of patients with chronic obstructive pulmonary disease show chronic

neutrophil infiltrates with concomitant tissue damage and dysfunction[70]. NETs, only discovered a decade ago, have been implicated in acute lung injury and organ damage in sepsis[31,71,72]. While neutrophils are capable of providing rapid defense against pathogens, tight control of the weapons in their arsenal is necessary to prevent damage to the host. Understanding the balance between protection and destruction is an important consideration when targeting these powerful cells clinically.

Modern Tools and Approaches for Cell Biology

The tools available to study the formed elements of blood have come a long way since the first look at red blood cells under a simple microscope. Confocal microscopy and fluorescently labeled antibodies allow for specific staining of subcellular structures and high resolution imaging. The revolution of molecular biology enables rapid identification of specific mutations in genes as well as quantitation of the entire transcriptome of a given sample. Thorough clinical characterization of patients with rare heritable diseases combined with molecular techniques provides insight into normal cellular functions. Due to the complexity of multicellular organisms, many genes required for hematopoiesis are required for earlier development, and defects in these genes are not compatible with life. One technique which may allow interrogation of such genes is gene-trap mutagenesis in cell lines.

Gene-trap mutagenesis introduces an engineered vector into the genome of target cells by electroporation or retroviral infection[73]. This vector is designed with a splice acceptor site upstream of a promoterless reporter (such as GFP)[74]. Upon successful integration into a gene, transcriptional activation from the endogenous promoter results in

expression of a fusion transcript consisting of the upstream coding sequence and the reporter gene. Successful mutagenesis can be tracked via expression of the reporter, which also serves as a template for PCR-based cloning of the insertion site within the genome. High throughput sequencing technology allows for identification of millions of mutant alleles within a pool of mutagenized cells, and subsequent identification of candidate genes required for the observed phenotype[75]. Gene-trap mutagenesis only results in disruption of a single allele and diploid mammalian genomes still express one functional copy of the targeted gene after mutagenesis. With the exception of disrupted gene products which have a dominant negative effect on the function of the endogenous gene product, such screens are of limited utility in diploid cells. Interestingly, a mostly haploid human cell line is available and has been used successfully for gene trap mutagenesis screens[76,77]. The availability of technologies such as gene-trap mutagenesis in human cells and high-throughput sequencing may hold the keys to identifying the function of countless new genes important for health and disease.

We have taken advantage of these modern tools and approaches to interrogate the functions of platelets and leukocytes and to better understand these formed elements. Using confocal microscopy and molecular techniques, we characterize for the first time the ability of platelets to sequester exogenous RNA. Gene-trap mutagenesis provided the inspiration behind a genetic screen to identify factors required for NET formation. Through sequencing and western blotting, as well as cell culture techniques, we confirmed that a mutation in *FERMT3* was responsible for LAD-III in the family we had previously identified[7,46]. This body of work serves to answer some fundamental questions

about the biology of blood cells while laying a foundation for new questions to be asked and progress to be made toward improving health for future generations.

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CHAPTER 2

PLATELETS SEQUESTER EXOGENOUS RNA

Abstract

Platelets have long been appreciated for their role in maintaining hemostasis and contributing to thrombosis. Increasingly, new roles for platelets are emerging. The ability of platelets to respond to stimuli and translate messages accordingly has been demonstrated. In addition, it is evident that platelets circulating in an inflammatory milieu such as sepsis have altered mRNA and protein expression patterns when compared to basal conditions. Interactions with other vascular and immune cells also provide a platform by which platelets convey information and affect function within the body. Here we describe a novel function of platelets: the sequestration of exogenous RNA. Using human platelets, monocytes, THP-1 cells and umbilical vein endothelial cells we have shown that this uptake is unique to platelets and represents a previously unrecognized function of this anucleate cytoplast. The kinetics of RNA uptake show a reproducible peak at 2h, and are concentration dependent. RNA is taken up and sequestered within the granulomere of platelets and is not translated under conditions we have tested. Basic platelet functions, including the ability to adhere to matrix, express surface P-selectin, or undergo conformational changes in $\alpha_{IIb}\beta_3$ integrin are unaffected by interaction with exogenous RNA in the presence or absence of thrombin. These data serve to characterize

this novel function of platelets and provide the groundwork for future studies to determine the role of platelet RNA uptake in health and disease.

Introduction

Platelets were first observed at sites of vascular injury by Bizzozero in the late 1800s[1]. Over the next century, the critical role that platelets play in maintaining hemostasis and in maladies such as stroke and myocardial infarction has been elucidated and oral antiplatelet drugs have become a $15 billion a year industry[2]. More recently, platelets have been recognized as effectors of immune function[3], and future research promises to bring new properties of platelets to light.

Platelets are the progeny of megakaryocytes, which reside primarily in the bone marrow. Upon the initiation of proplatelet formation long cytoplasmic extensions protrude from the body of the progenitor into venous sinusoids[4]. Organelles including mitochondria, alpha and dense granules, proteins and RNA traffic from the body of the megakaryocyte to these cytoplasmic extensions, which pinch off to become proplatelets[5,6]. Data suggests that the final stages of platelet maturation occur, in part, in the circulation[7,8]. The end result is the release of individual, discoid platelets that are packed with organelles and cytoskeletal elements. While platelets are devoid of a nucleus, they are capable of a wide range of complex cellular processes, including processing of pre-mRNA to form a mature transcript[9-11], translation[12-14], and anuclear cell division that resembles proplatelet formation in megakaryocytes[15]. The execution of these events serves to diversify the functional repertoire of platelets in response to environmental cues.

Homotypic aggregates of platelets form as fibrinogen binds activated integrin $\alpha_{IIb}\beta_3$ receptors on the surface of platelets[16]. This is an important step in hemostasis, as patients with genetic defects in $\alpha_{IIb}\beta_3$ (Glanzmann's thrombasthenia) have profound bleeding disorders[17]. Platelets also use regulated surface expression of receptors to interact with other cells, including monocytes and neutrophils[18-20]. These heterotypic interactions have been shown by our lab and others to induce functional changes within these cells. For example, platelet interaction with monocytes through a P-selectin/PSGL-1-dependent mechanism induces nuclear translocation of NF-κB and proinflammatory cytokine production, as well as upregulation of integrins which promote adhesion to activated endothelium[21,22]. Activated platelets induce transcription of COX-2 mRNA in monocytes rapidly, but this message is only translated after receiving a secondary IL-1β signal from platelets which stabilizes the transcript[23]. Platelets also adhere to activated endothelium to promote transendothelial migration of neutrophils through interactions with P-selectin, PSGL-1 and Mac-1[24,25]. Taken together, these data and others suggest that platelets are not exclusively cell autonomous, but orchestrate complex interactions with other cells to maintain hemostasis and coordinate inflammatory responses.

Clinically, the role of platelets as biomarkers of disease is increasingly appreciated in addition to their functional properties. Heterotypic aggregates of platelets and leukocytes have been shown to be a more sensitive marker of platelet activation than P-selectin expression[26], and can be used to stratify patients with acute myocardial infarction[27,28]. Surprisingly, platelets have also been reported to contain mutant RNA from tumors. Specifically, a study by Nilsson *et al*. demonstrated that platelets from glioma patients contain tumor-derived mutant RNA and that this RNA is transferred *in vitro* via

membrane microvesicles[29]. As platelets are more readily available for diagnostic testing than glioma biopsies, they may play an important role in personalized medicine and predicting tumor response to therapy.

Tumor-associated RNA has also been reported within the serum of patients with melanoma[30], breast cancer[31] and nasopharyngeal carcinoma[32]. This is surprising given the abundant ribonuclease activity present within human plasma[33], but may represent a protected fraction of RNA bound by proteolipids or other macromolecules[34]. In addition to serving as a marker of disease, extracellular RNA can directly mediate the permeability of the vascular endothelium in an VEGF-dependent manner, and pretreatment with RNase reduces vessel occlusion and edema in animal models of stroke[35,36]. Proteases important for the contact phase of blood coagulation (Factors XII and XI) bind extracellular RNA and have increased activity (˜40-fold) in the presence of RNA[37]. This ability of extracellular RNA to potentiate coagulation responses may play a role in hemostasis in response to massive tissue damage, where lysed cells release their contents into the extracellular space.

Here we report that platelets are able to sequester exogenous RNA (eRNA). Using platelets isolated from human donors, we demonstrate that eRNA uptake is time—and concentration—dependent, and unique to platelets of the cells examined so far. Uptake of eRNA does not require activation of platelets, and occurs independent of transcript size or secondary structure. Microscopy studies allowed us to visualize eRNA within the granulomere of spread platelets after uptake. Platelets exposed to eRNA are able to adhere to fibrinogen, express P-selectin on their surface and bind PAC-1 upon stimulation with thrombin, suggesting that eRNA uptake by platelets does not trigger

traditional platelet activation. The fate of this sequestered eRNA has yet to be determined, as we have not detected translation under conditions tested thus far. Given the recently described role of eRNA in potentiating coagulation[37], it is intriguing to speculate that platelets may scavenge eRNA from the circulation to prevent spurious thrombus formation. Work to test this hypothesis is ongoing. For now, these data serve to characterize this novel function of platelets and, in doing so, provide the groundwork for future studies to determine the physiological role of this process.

Materials and Methods

Cell isolation and culture

All studies were approved by the University of Utah Institutional Review Board committee (IRB #392). Leukocyte-depleted platelets were isolated as previously described[10]. Platelets were resuspended at 100,000/μL in serum-free M199 medium, placed in round-bottom polypropylene tubes and cultured in a 37°C humidified incubator with 5% CO_2. Monocytes were isolated from a Ficoll monolayer using $CD14^+$ beads (Miltenyi Biotec, Auburn, CA) and resuspended at 1000/μL in serum-free M199 medium, and cultured as described for platelets. THP-1 cells were obtained from and propagated as indicated by the distributor (ATCC, Manassas, VA, batch ID 38999776). For experiments, THP-1 cells were centrifuged, washed once in Hank's Balanced Salt Solution (HBSS), resuspended in M199 and cultured under the same conditions as the primary monocytes. Human endothelial cells (HUVECs) were isolated from umbilical veins and cultured as previously described[38]. One hour prior to adding eRNA to HUVECs, they were washed twice with HBSS and cultured in serum-free M199 medium.

In select studies, thrombin (0.1U/mL) was added to cells for the time indicated. RNAse I (1μL/mL) was added when indicated and incubated for 15′ at 37°C.

Constructs

pCS2^{+}5S-Spinach. The pAV-5S-Spinach construct was a gift from the Jaffrey lab at Cornell[39]. It was subcloned into the pCS2^{+} vector (Addgene, Cambridge, MA) downstream of the SP6 promoter using BamHI to cut the vector and BamHI/NotI to remove the insert from its original vector.

pCS2^{+}tRFP. The tRFP construct was synthesized by PCR amplification of tRFP from pRFP-C-RS (Origene, Rockville, MD) with primers containing the restriction sites for ligating into pCS2^{+}: forward primer with BglII restriction enzyme (RE) site (5′ -GATCAGAGATCTCACCATGAGCGAGCTGATCAAGG-3′) and reverse primer with BamHI RE site (5′ -GATCAGGGATCCCTCTTCATCTGTGCCCCAG-3′). The PCR product was digested with the enzymes indicated and ligated into the pCS2^{+} vector downstream of the SP6 promoter.

IRES-NFLAG-tRFP. pT7CFE1-NFtag vector (Thermo Scientific, Rockford, IL) was cut with BamHI. PCR amplification of tRFP from pRFP-C-RS (Origene, Rockville, MD) was done with primers containing the restriction sites for ligating into pT7CFE1-NFtag: forward primer with BglII RE site (note: 1bp smaller than the primer noted for pCS2^{+}tRFP construct to maintain reading frame from FLAG tag to tRFP insert) (5′-GATCAGAGATCTCACATGAGCGAGCTGATCAAGG-3′) and reverse primer with BamHI RE site (5′-GATCAGGGATCCCTCTTCATCTGTGCCCCAG-3′). The PCR

product was digested with the enzymes indicated and ligated into the pT7CFE1-NFtag vector downstream of the T7 promoter.

tRFP-Spinach. The pCS2^{+}tRFP construct was cut with XhoI and XbaI and the pAV-5S-Spinach construct with SalI and XbaI. The fragment from the pAV-5S-Spinach construct was then ligated into the pCS2^{+}tRFP vector to create a construct with Spinach downstream of the tRFP ORF.

Luciferase. The luciferase construct was purchased—#18964 (Addgene, Cambridge, MA)[40].

pCS2^{+}GMCSF. GM-CSF (NM_000758) was PCR amplified from primary human monocyte cDNA using primers with a BamHI site on the forward primer (5′-TGTGTTGGATCCAGGATGTGGCTGCAGAGCCTGC-3′) and a XhoI site on the reverse primer (5′-TGTGTGCTCGAGTCACTCCTGGACTGGCTCCCAG-3′). The PCR product was digested with the enzymes indicated and ligated into pCS2^{+} cut with the same enzymes.

All constructs were sequenced to confirm identity. In addition, constructs were transfected into HeLa cells using Lipofectamine 2000 (Life Technologies, Grand Island, NY) to confirm that the open reading frame coded for a functional protein. For RFP, cells were inspected visually 24h after transfection using an EVOS microscope (AMG, Bothell, WA) to confirm the presence of RFP. For remaining constructs, cells were harvested and used as positive controls in subsequent assays. For example, HeLa cell lysates from cells transfected with the luciferase construct were used as positive controls in luciferase assays.

Protein expression

For western blot analysis, cell lysates were prepared by centrifuging cells and resuspending directly in Laemmli buffer, then sonicating for 5′ to shear DNA and reduce the viscosity of the sample. Sample from approximately $2.5x10^7$ platelets was loaded in each lane of a 12% SDS-polyacrylamide gel and electrophoresed to resolve proteins[41]. Proteins were transferred onto a PVDF membrane and probed with the following antibodies: anti-RFP cat # MA5-15257 (Thermo Scientific, Rockford, IL) at a dilution of 1:1000, anti-actin cat #691002 (MP Biomedicals, Santa Ana, CA) at a dilution of 1:50,000, anti-FLAG cat# F3165 (Sigma-Aldrich, St Louis, MO) at a dilution of 1:5000. Secondary antibody was HRP-conjugated goat antimouse cat# G-21040 (Life Technologies, Grand Island, NY). Luminescence was generated for detection using ECL western blotting detection reagents (GE Healthcare, Waukesha, WI). Membranes were probed independently with antibody, stripped with Restore Western blot stripping buffer (Thermo Scientific, Rockford, IL), and reprobed with another antibody as needed.

For flow cytometry, 10μL of the platelet suspension (at 100,000/μL) was left untreated or stimulated for 15′ with thrombin (0.1U/mL), then stained with CD41a-PE and either CD62P-FITC or PAC-1-FITC (BD Biosciences, San Jose, CA). Isotype controls used were IgM-FITC (eBioscience, San Diego, CA) and IgG1-FITC (BD Biosciences, San Jose, CA). After 10′ incubation with antibodies, cells were fixed with FACS Lysing Solution (BD Biosciences, San Jose, CA) and analyzed using a FACS CantoII flow cytometer (BD Biosciences, San Jose, CA) and FlowJo analysis software (Tree Star, Ashland, OR).

For luciferase detection, the Luciferase Assay System (Promega, Madison, WI) was used according to manufacturer instructions. Briefly, $1x10^8$ platelets were pelleted and resuspended in 20µL of Passive Lysis Buffer, and incubated for 15′ at room temperature. Opaque white flat-bottom 96-well plates were prepared with 50µL LARII/well, and 10µL of sample was added. The plate was read on a Synergy HT luminometer (Bio-Tek, Winooski, VT). HeLa cell lysate from cells transfected with the luciferase construct used for *in vitro* transcription (IVT) were run on the same plate as a positive control.

For the GM-CSF ELISA, a sandwich ELISA kit was purchased (R&D Systems, Minneapolis, MN). Platelets ($1x10^8$) were incubated with 1µg/mL RNA for GM-CSF for 2.5h, 5h and 18h. Where indicated, 0.05U/mL thrombin was added to the platelet suspension 30′ prior to the time when cells and supernatants were harvested. Cells were centrifuged at 12,000xg for 3′ and supernatants were transferred to a new tube. Pellets were resuspended in 200µL RIPA and incubated for 30′ on ice prior to centrifugation to remove insoluble material. The solubilized fraction from the cell pellet was transferred to a new tube and stored at -80°C. The ELISA plate was prepared as directed by the manufacturer, and dilutions of 1:4 and 1:40 of the cell pellet were assayed, as well as 1:2 and 1:20 of the supernatant.

Microscopy

Fluorescence microscopy was performed on a confocal microscope using a 60x/1.42 NA oil objective as previously described[15]. FV1000 image acquisition software Version 5.0 (Olympus, Center Valley, PA) was used for recording images. The small

molecule 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI) (Lucerna Technologies, New York, NY) was added to cells immediately prior to imaging, where indicated.

In vitro transcription (IVT)

RNA was IVT using MegaScript SP6 (pCS2^{+}5S-Spinach, pCS2^{+}tRFP, tRFP-Spinach, pCS2^{+}GMCSF) or MegaScript T7 (IRES-NFLAG-tRFP) kits (Life Technologies, Grand Island, NY) according to manufacturer instructions. 5′-ARCA capped and polyA tailed IVT RNAs were generated using mMessage mMachine ULTRA kits (Life Technologies, Grand Island, NY) as directed by the manufacturer. Alexa-488-labeled RNA was synthesized using FISH Tag RNA Green kit (Life Technologies, Grand Island, NY) as directed by the manufacturer. All transcripts were confirmed by formaldehyde-agarose gel electrophoresis[41] and quantitated using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Rockford, IL).

RNA analysis

Cell pellets were lysed in Trizol, cell suspensions in Trizol LS (Life Technologies, Grand Island, NY) and RNA was isolated as recommended by the manufacturer. Glycogen was added during the isopropanol step to facilitate precipitation. RNA pellets were resuspended in 10µL RNAse-free water and the entire sample was used for cDNA synthesis. Random hexamers and SuperScript II (Life Technologies, Grand Island, NY) were used to synthesize cDNA. Relative transcript levels were determined in a SYBR-Green based real-time PCR assay on a Bio-rad iCycler iQ using

perfeCTa SYBR Green supermix (Quanta Biosciences, Gaithersburg, MD). All RT-PCR shown was performed with primers specific for the luciferase transcript (5′-GAGGTGGACATCACTTACGCTGAG-3′ and 5′-CCCATACTGTTGAGCAATTCACGTTC-3′). Data were normalized to the housekeeping gene β_2 microglobulin using specific primers (5′-AGATGAGTATGCCTGCCGTGT-3′ and 5′-AGCTACCTGTGGAGCAACCTG-3′)

Statistical analysis

Error bars represent the mean +/- the standard error of the mean.

Results

Platelets take up exogenous RNA

Our interest in platelet biology led us to test whether platelets take up an IVT eRNA delivered in their culture medium. Initial studies were performed using an RNA aptamer mimic of green fluorescent protein tagged 5S-ribosomal RNA subunit (5S-Spinach) IVT using our pCS2^{+}5S-Spinach construct[39]. We reasoned that this transcript would not be subject to translation and would allow us to visualize any RNA uptake by the platelets. Confocal microscopy of unstimulated platelets incubated with this eRNA and DFHBI showed evidence of eRNA within the platelets (Fig. 2.1a). Cells incubated with DFHBI alone, a small molecule that requires the RNA aptamer to stabilize it to emit fluorescence, showed a very small amount of background fluorescence, but were clearly distinguished from the cells where the RNA aptamer was present (Fig. 2.1b). This important control ruled out the possibility of DFHBI being stabilized by an endogenous

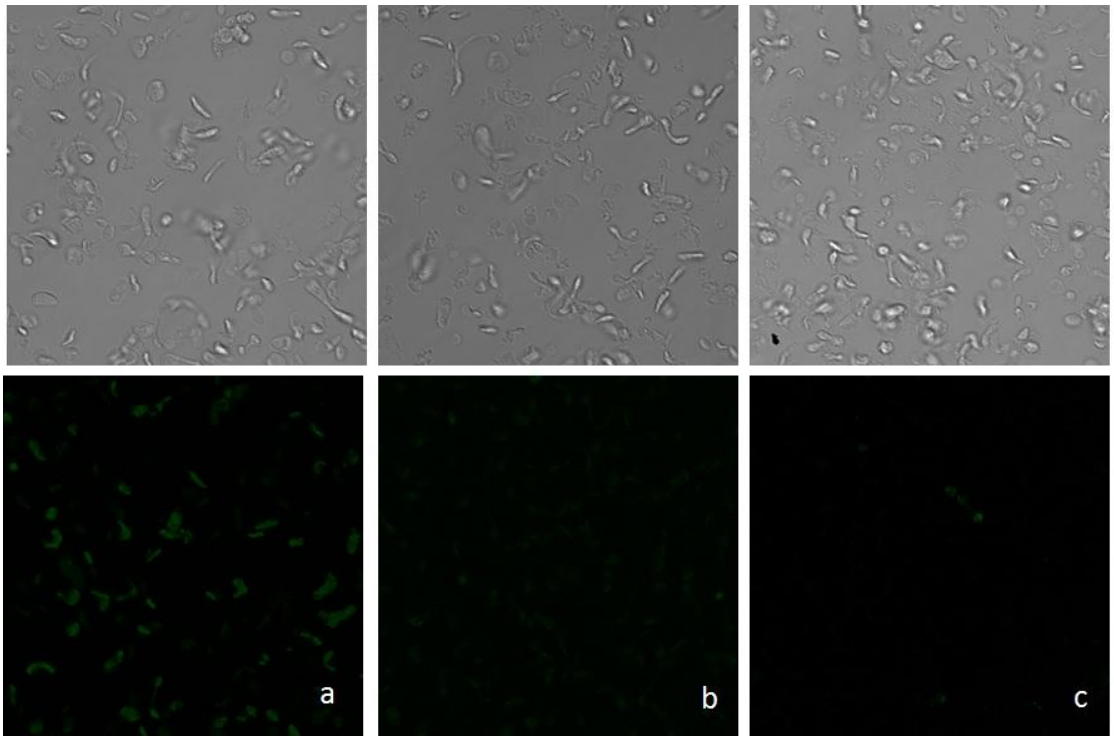


Figure 2.1: Exogenous RNA tagged with a fluorescent aptamer is taken up by platelets. Platelets were incubated in the presence (a, c) or absence (b) of 1µg/mL 5S-Spinach RNA overnight and placed on immobilized fibrinogen for live imaging. DFHBI was added immediately prior to imaging (a, b). Corresponding transmission images are shown in the top row.

RNA within the platelets resulting in fluorescence in the absence of eRNA uptake. Platelets incubated with eRNA but without DFHBI also failed to show fluorescence, although debris present outside of the focal plane resulted in spurious autofluorescence (Fig 2.1c).

As each RNA molecule is only tagged with one fluorophore-binding aptamer, we sought a more robust way to label the RNA for visualization. In addition, we wanted to determine whether eRNA uptake was restricted by size. We generated mRNA for luciferase by IVT, a transcript which is twice as long as the 5S-Spinach transcript, and labeled it with Alexa-488. We determined that each transcript was labeled with 6-7 fluorophores. After incubation with this Alexa-488-eRNA in suspension, platelets were allowed to adhere to fibrinogen-coated coverslips in the presence of thrombin prior to fixation and visualization by confocal microscopy. In these spread platelets, the eRNA was clearly visualized as bright punctae within the granulomere of the platelet (Fig. 2.2a). No green fluorescence was seen within spread platelets incubated with an eRNA which was not labeled with Alexa-488 (Fig. 2.2b). Platelet granules are heterogeneous, and subpopulations of these granules can be identified using granule-specific markers[42]. Future studies will seek to identify whether these eRNAs are located within a specific granule compartment to better understand what role they may be playing there.

eRNA uptake by platelets is time- and concentration-dependent

Uptake of eRNA by platelets has not been previously reported, so we next sought to characterize this uptake. Initial work focused on using the 5S-Spinach IVT RNA for passive uptake, followed by RT-PCR. Despite extensive testing with 7 different primer

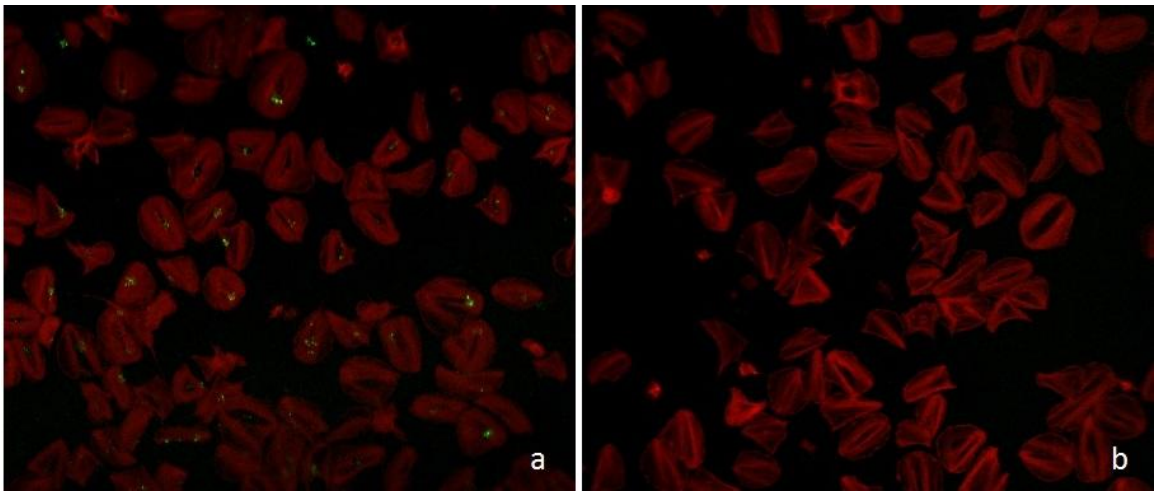


Figure 2.2: Exogenous RNA labeled with Alexa-488 is visible within the granulomere of spread platelets. Platelets were incubated for 2h with 100ng/mL eRNA labeled (a) with and (b) without Alexa-488 after IVT. Samples were stimulated with 0.1U/mL thrombin and plated onto fibrinogen-coated coverslips and allowed to spread for 45' at 37°C prior to fixation and imaging. Green represents labeled RNA. The red stain represents actin.

pairs, however, nonspecific amplification was a consistent problem. Therefore, we used the IVT RNA for luciferase, as we knew it was able to be taken up by platelets (Fig. 2.2). Primers for luciferase were optimized and used to determine the kinetics of eRNA uptake in platelets. This luciferase eRNA rapidly associated with isolated platelets, with a maximum amount detected at 15′ and decreased association over time (Fig. 2.3, bars). Interestingly, RNA was still detectable in these samples after overnight incubation. To determine whether the eRNA we were detecting was associated with the membrane or internalized within the platelets, we added RNase I prior to harvest of the samples. RNase I is a ribonuclease that will robustly cleave any single-stranded RNA not protected from degradation by a membrane barrier[43]. RNase treatment of our samples at 15′ showed that little of the eRNA associated with the platelet membrane had been internalized at this early timepoint, as it is not protected from degradation by RNase I (Fig. 2.3, line). Internalization of eRNA reached a peak at 2h and gradually declined over an 18h period. Of note, we found that the amount of RNase-protected eRNA after overnight incubation is identical to the amount of total RNA associated with the platelets. This suggests that eRNA not internalized by platelets is completely degraded after an overnight incubation.

To determine whether the uptake of eRNA was concentration-dependent, we repeated the experiments with varied concentrations of eRNA. When 10-fold less eRNA was added to the platelets, we see similar kinetics for the eRNA protection, with a peak at 2h and a decline overnight (Fig. 2.4a). However, the amount of eRNA that was protected at this peak is 4-fold less than we saw with the initial experiments (0.12 vs 0.45), suggesting that uptake is concentration-dependent and not saturated at lower concentrations. When 10-fold more eRNA is added to the platelets (Fig. 2.4b), the

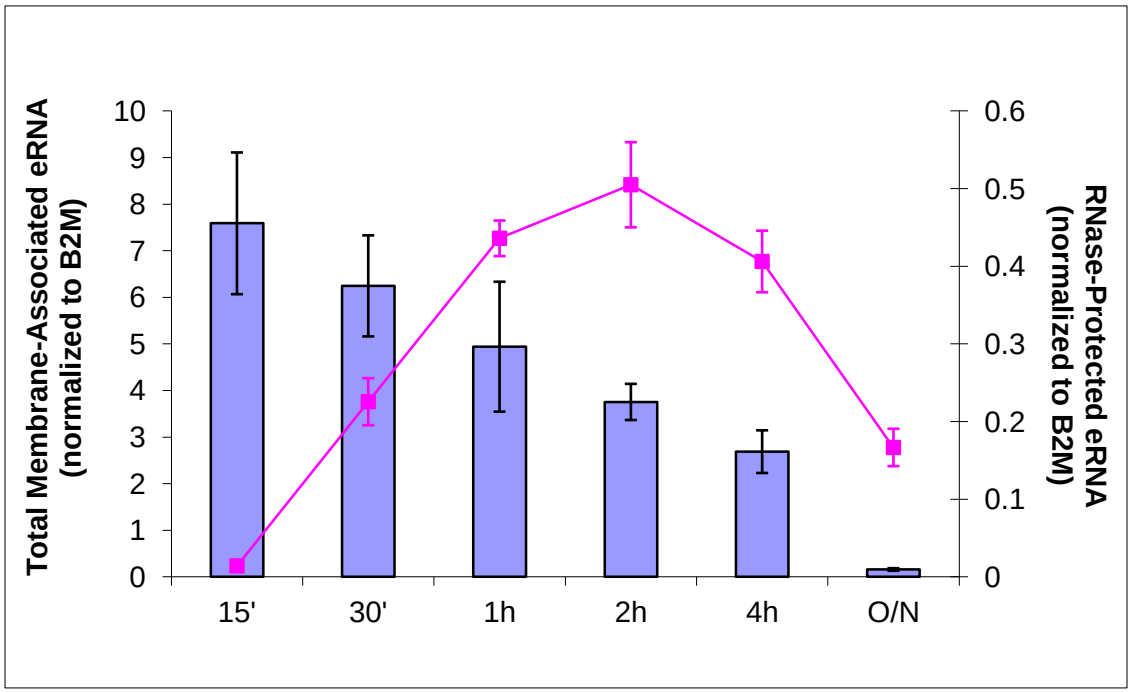
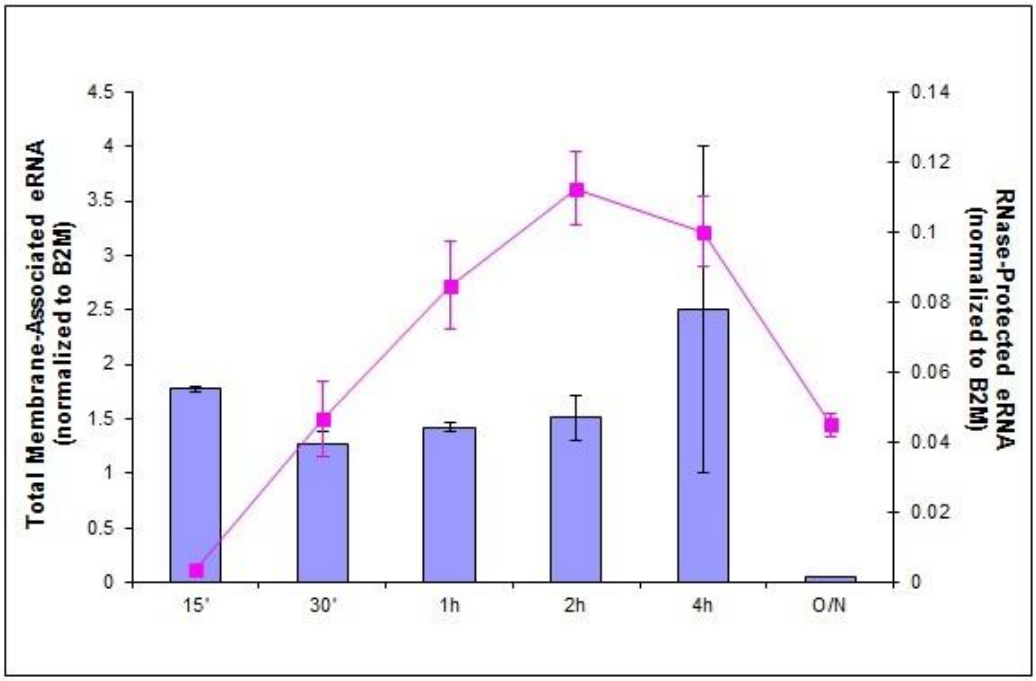


Figure 2.3: Time course of RNA uptake by platelets. Platelets were incubated with 100ng/mL eRNA for the times indicated (O/N- overnight). To harvest total membrane-associated eRNA, cells were centrifuged, washed and resuspended in Trizol prior to total RNA isolation and RT-PCR with primers specific for the eRNA cDNA (bars). To harvest platelet-associated RNase-protected eRNA, samples were incubated with 100U/mL RNaseI for 15' at 37°C, then centrifuged and analyzed as described above (line).

a



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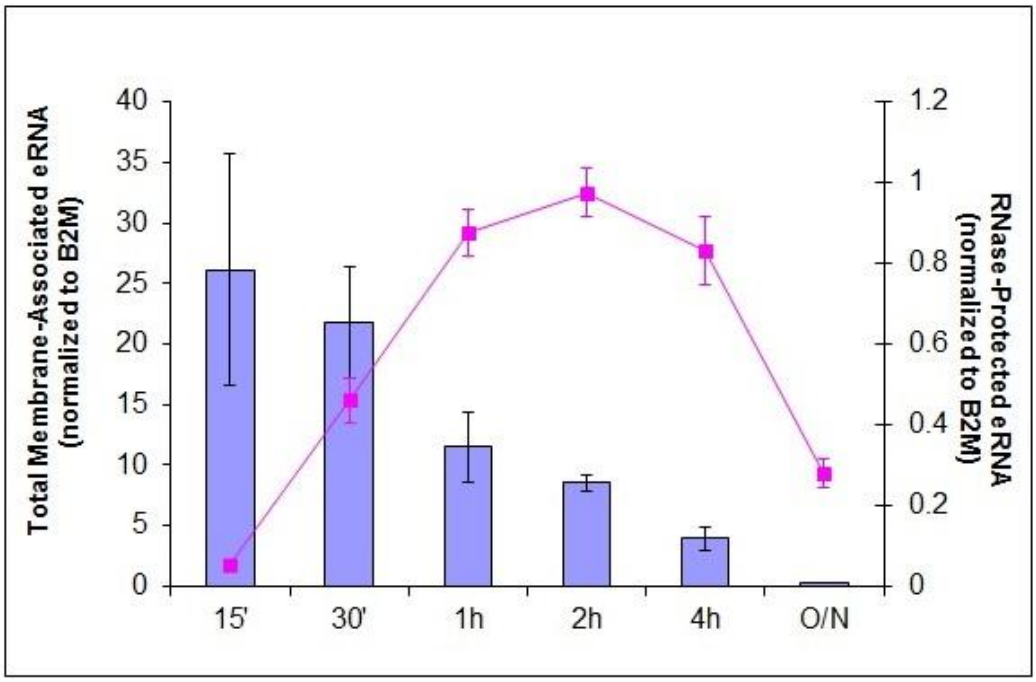


Figure 2.4: Concentration response of RNA uptake by platelets. Platelets were incubated with (a) 10ng/mL or (b) 1000ng/mL eRNA for the times indicated. Total membrane-associated eRNA and RNase-protected eRNA were assayed as described in Fig. 2.3.

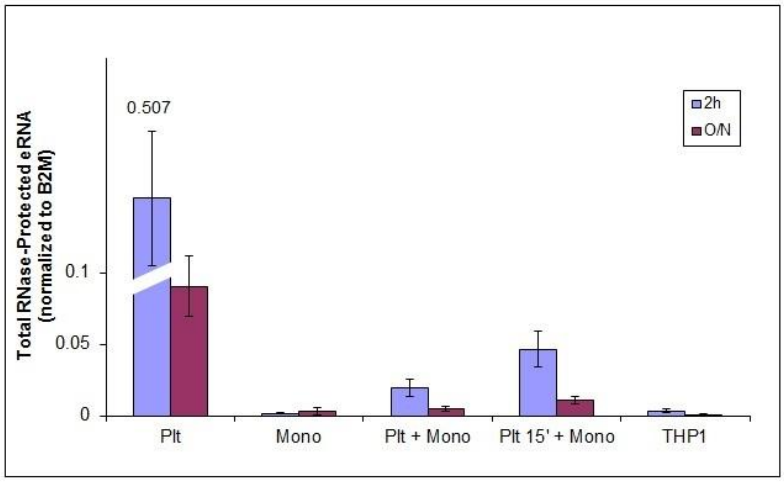
kinetics remain the same, but the protected fraction at 2h is only about 2-fold higher than with the intermediate dose (0.95 vs 0.45). Taken together, these data suggest that uptake of eRNA is a regulated process that is saturable.

eRNA uptake is unique to platelets

Given the robust uptake of eRNA by platelets, we wanted to determine whether this was a property unique to platelets. Primary human monocytes and THP-1 cells, an immortalized monocytic cell line, were each incubated with eRNA for 2h or overnight and the amount of RNase-protected eRNA was determined. Surprisingly, almost no eRNA was protected by either monocytes or THP-1 cells under the conditions tested (Fig. 2.5a). When eRNA was added to a coculture of platelets and monocytes, there was a small amount of protected eRNA detected, but not nearly as much as when platelets and eRNA were incubated without monocytes. Since we saw rapid association of eRNA with platelet membranes in our previous experiment (Fig. 2.3, bars), we also tested whether platelets incubated with eRNA for 15′ prior to addition of monocytes led to significant protection of eRNA after 2h or overnight coculture. As seen in the figure, preincubation led to a slight increase in the amount of protected RNA as compared to when platelets and monocytes were immediately coincubated, but was not able to restore eRNA protection to the level of platelets alone.

The ability of platelets to internalize eRNA, but not monocytes or THP-1 cells, led us to interrogate the fate of eRNA in monocytes and THP-1 cells more rigorously. Specifically, we determined if eRNA simply fails to be internalized by these cells or if it is actively degraded. Here, we isolated total RNA from the cell suspensions incubated

a



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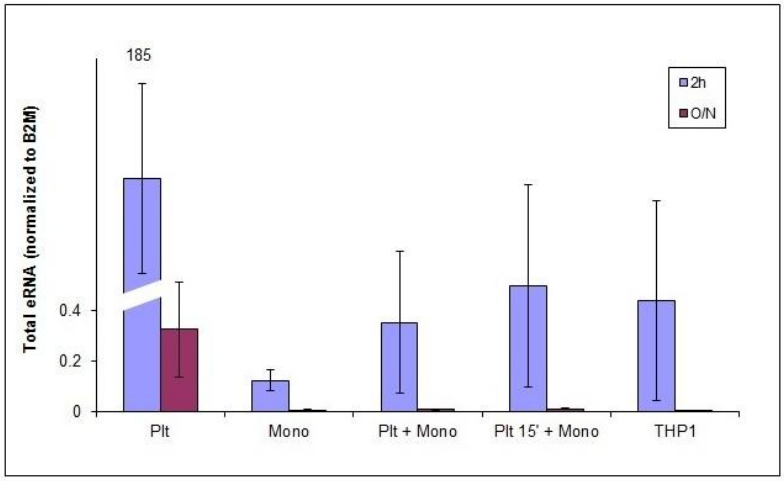

c

	Ct
Baseline	12.6
2h	12.45
8h	12.75
18h	12.7

Figure 2.5: Exogenous RNA uptake is unique to platelets. Platelets, monocytes and THP-1 cells were incubated with 100ng/mL eRNA for the indicated times. For a) 100U/mL RNaseI was added 15' prior to harvest and cells were incubated at 37°C. Trizol LS was added to samples (3:1 v/v), total RNase-protected RNA was isolated and RT-PCR for eRNA was performed. In b) Trizol LS was added to samples (3:1 v/v) without RNase treatment, total RNA was isolated and RT-PCR for eRNA was performed. Values were normalized to β_2 microglobulin (B2M), which was expressed at similar levels in all cells under the conditions of this assay. Panel c) indicates the cycle threshold value for eRNA incubated under identical culture conditions to cells, demonstrating the stability of the IVT RNA during the course of our assay.

with eRNA without RNase-I treatment. The values generated from this experiment are indicative of total eRNA in the sample (extracellular, membrane-associated and intracellular). At 2h, total eRNA in the platelet only sample was 400-fold higher than in any of the other conditions tested (Fig. 2.5b). The dramatic reduction in the total eRNA in the monocyte and THP-1 cultures suggests that these cells actively and rapidly degrade eRNA. Upon overnight incubation with eRNA, the amount of eRNA in the monocyte and THP-1 samples was nearly undetectable, while there was still a significant amount of eRNA in the platelet only cultures. The decrease in total eRNA between 2h and 18h in the platelet cultures suggests platelets degrade eRNA, albeit less efficiently and more slowly than monocytes or THP-1 cells. Importantly, incubating IVT RNA in media overnight under the same conditions as cell culture resulted in no change in cycle threshold by RT-PCR (Fig. 2.5c), indicating that the decreases in signal in this assay are due to an active degradation process and not the inherent lability of RNA. Additional experiments demonstrated that media in which monocytes have been cultured for 2h and then removed is sufficient to induce RNA degradation in this assay. Platelet-conditioned media leads to eRNA degradation to a much lesser extent (data not shown). This result suggests that monocytes and THP-1 cells constitutively secrete a factor capable of degrading eRNA in the media rather than degrading eRNA through an internalization-dependent process.

Exposure to eRNA does not alter platelet activation

Activated platelets express P-selectin on their surface[44], as well as the activated form of the integrin $\alpha_{IIb}\beta_3$, which is measured using the activated integrin-specific

antibody PAC-1[45]. To determine whether exposure to eRNA leads to platelet activation, we performed flow cytometry. After 2h of incubation in the presence of two different concentrations of eRNA, we saw no increase in the expression of P-selectin on the surface (Fig. 2.6c,e) compared to untreated platelets (Fig. 2.6a). Similar results were obtained when using PAC-1 binding to determine integrin $\alpha_{IIb}\beta_3$ activation in these samples (Fig. 2.7). These results indicate that eRNA does not cause platelet activation.

To determine whether exposure to eRNA impacts the ability of platelets to respond to traditional agonists, we preincubated platelets for 2h with eRNA and then stimulated them with thrombin and measured P-selectin surface expression and PAC-1 binding. Platelets exposed to either low (Fig. 2.6d) or high (Fig. 2.6f) concentrations of eRNA expressed similar levels of P-selectin on their surface after thrombin stimulation as thrombin-stimulated platelets not exposed to eRNA (Fig. 2.6b). Using PAC-1 binding to determine integrin $\alpha_{IIb}\beta_3$ activation in these samples yielded similar results (Fig. 2.7). Taken together, these data indicate that eRNA alone does not cause platelet activation and platelets exposed to eRNA are able to activate normally in response to thrombin.

<u>eRNA is not translated</u>

To determine whether eRNA taken up by platelets is translated, several constructs for IVT of red fluorescent protein (RFP) were designed. The standard transcript for tRFP was synthesized without and with a 5′ cap, a modification which is known to improve translation[46]. An additional construct with the Spinach aptamer downstream of tRFP was created with the intention of tracking RNA uptake and translation in live cells[39]. Finally, tRFP was cloned into a construct containing an IRES-FLAG tag, which has been shown

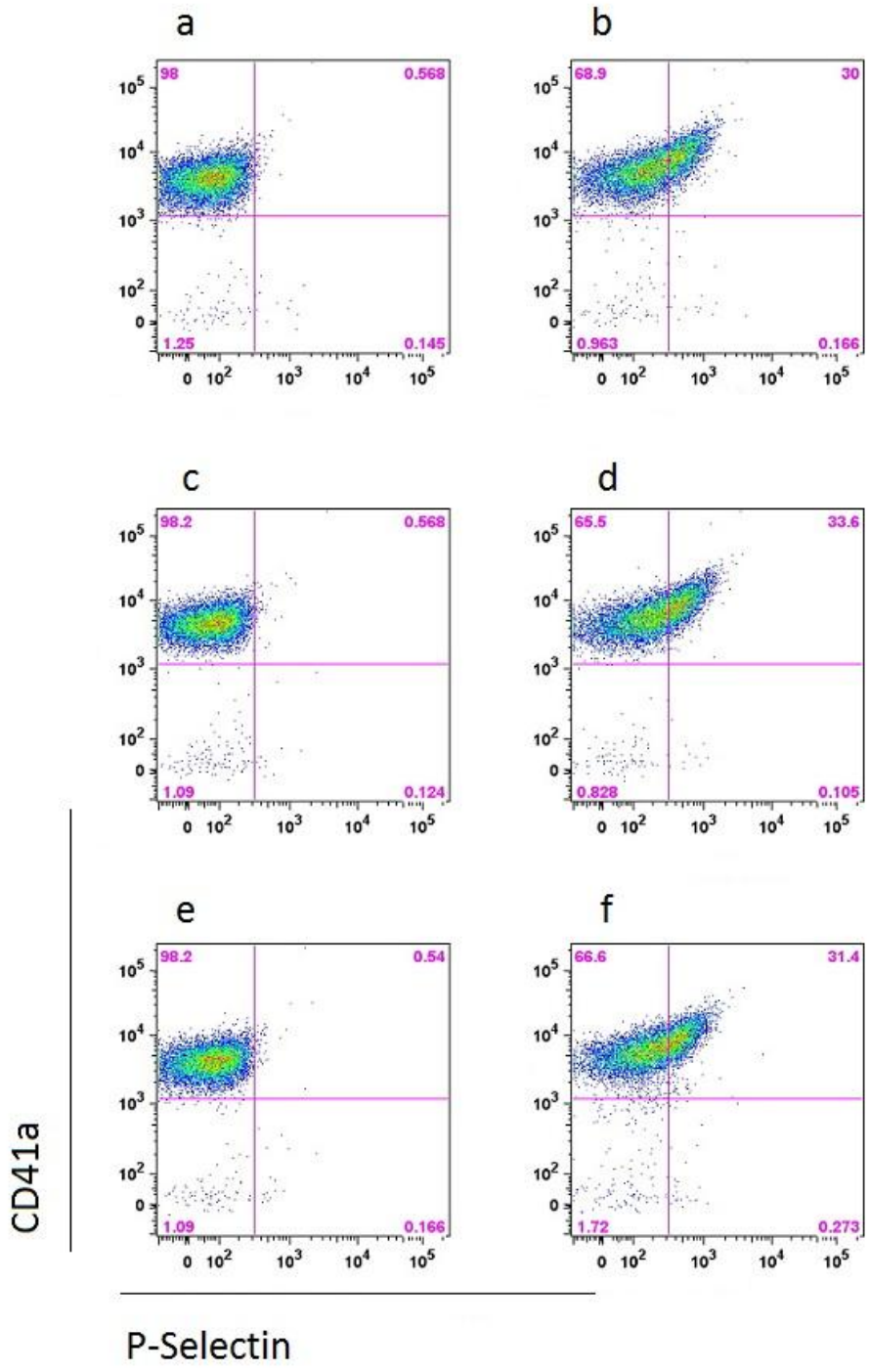

Figure 2.6: Platelets exposed to eRNA translocate P-selectin to their membrane in response to thrombin normally. Platelets were incubated in the absence (a,b) or presence of 100 ng/mL (c,d) or 1000ng/mL (e,f) eRNA for 2h. After incubation, platelets were stimulated with 0.1U/mL thrombin (b,d,f) for 15′ prior to staining with antibodies for P-selectin and CD41a and analyzed by flow cytometry. Figure is representative of three experiments.

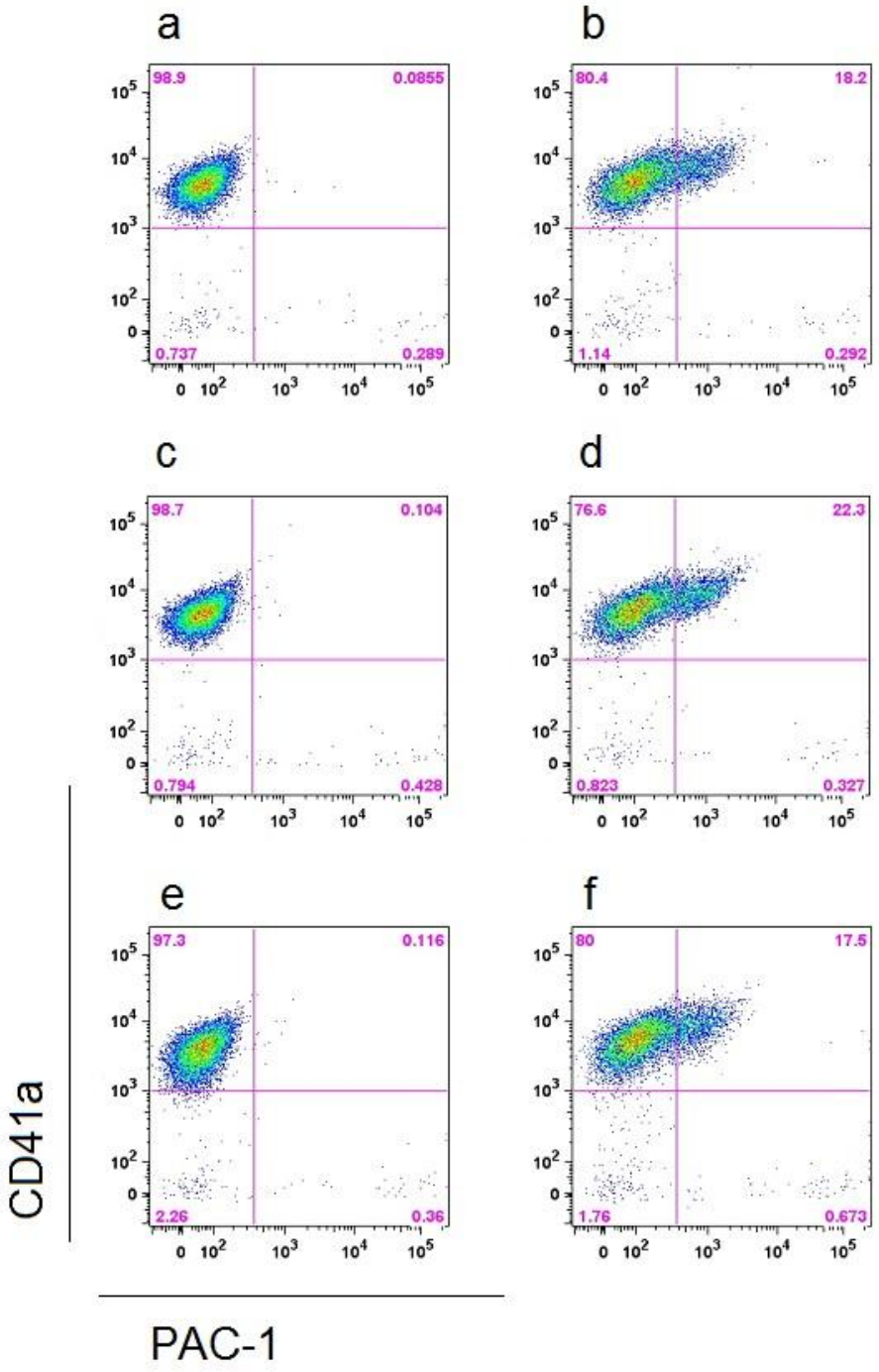

Figure 2.7: Platelets exposed to eRNA bind PAC-1 in response to thrombin normally. Platelets were incubated in the absence (a,b) or presence of 100 ng/mL (c,d) or 1000ng/mL (e,f) eRNA for 2h. After incubation, platelets were stimulated with 0.1U/mL thrombin (b,d,f) for 15′ prior to staining with antibodies for the active integrin $\alpha_{IIb}\beta_3$ (PAC-1) and CD41a and analyzed by flow cytometry. Figure is representative of three experiments.

to promote cap-independent translation[47]. Western blot analysis of platelet lysates incubated with equal amounts of each of these IVT eRNAs showed a multitude of bands cross-reacting with the anti-RFP antibody, but a band induced over time at approximately the correct size for tRFP (Fig. 2.8). Using this same antibody for immunocytochemistry (ICC), we detected a signal in platelets incubated with the eRNA overnight (Fig. 2.9b) that was not present in baseline platelets (Fig. 2.9a). To confirm these results, the experiment was repeated using untreated platelets and platelets incubated with an IVT RNA coding for 5S-Spinach, which should not cross-react with the RFP antibody. Western blot analysis of these lysates showed a band induced at the size expected for RFP under all of the conditions tested (Fig. 2.10). This suggests that the immunoreactive band detected by the anti-RFP antibody is not RFP. An additional western blot was prepared with lysates incubated with the FLAG-tagged RFP IVT RNA and probed with an anti-FLAG antibody. No signal was seen on this blot at any timepoint, further confirming that the eRNA taken up by the platelets was not being translated under these conditions (data not shown). Finally, the immunocytochemistry was repeated using platelets incubated for 18h without eRNA. This experiment showed an equivalent amount of localized signal when stained with the anti-RFP antibody between platelets treated with or without eRNA, providing further evidence that our antibody cross-reacts with a nonspecific band which is induced in cultured platelets (data not shown).

We were interested in testing whether platelets could translate a small, biologically relevant protein product from eRNA. Transcriptome and proteomics analysis of platelets have shown that platelets do not express appreciable amounts of GM-CSF[48,49]. In addition, GM-CSF has been demonstrated to reverse the immunosuppression

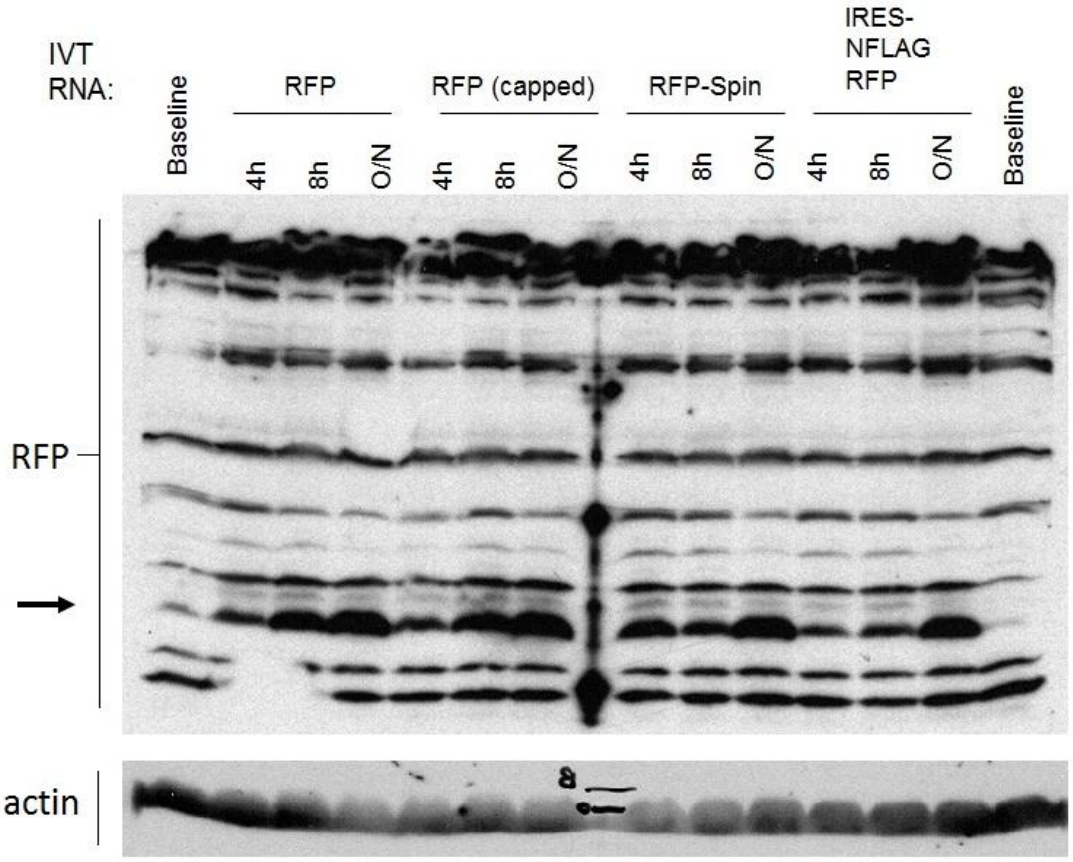

Figure 2.8: Platelets appear to express protein from eRNA by western blot analysis. Platelets were incubated with 1µg/mL eRNA synthesized from the constructs indicated. Samples were lysed and run on SDS-PAGE, transferred to PVDF and probed with antibodies for RFP and actin. The arrow indicates the induced band at approximately 27.5 kDa, the expected size for RFP.

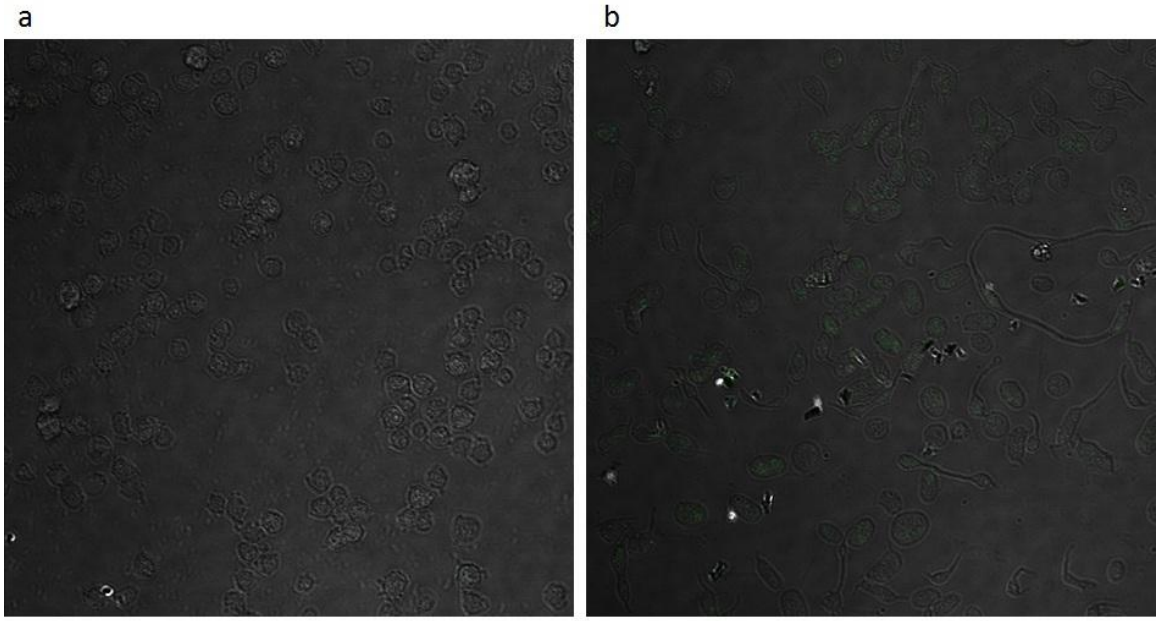

Figure 2.9: Platelets appear to express protein from eRNA by immunocytochemistry. Platelets were incubated with 1µg/mL eRNA coding for tRFP, fixed in suspension and cytospun onto coverslips and stained with anti-RFP. Panel a) platelets at baseline. Panel b) platelets after overnight incubation with eRNA.

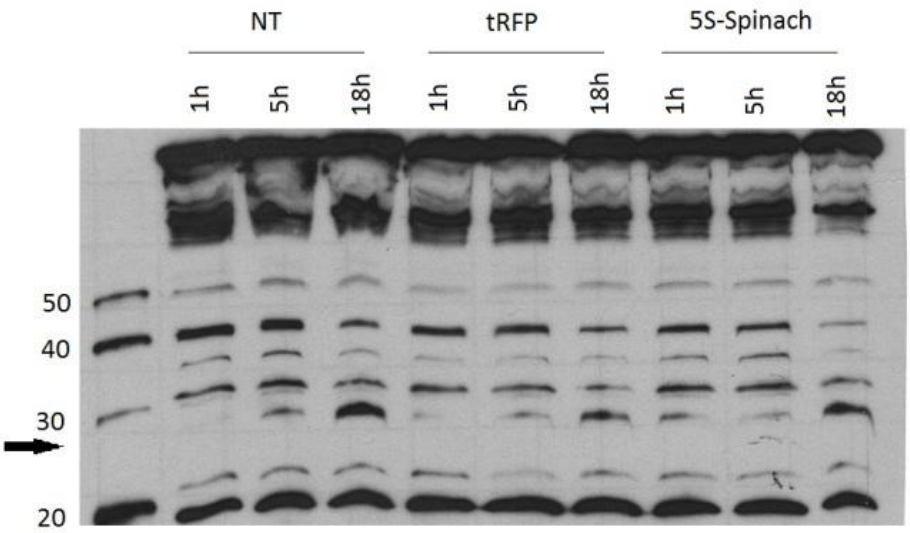

Figure 2.10: Induced band in platelets is not RFP. Platelets were incubated without eRNA, with eRNA which does not code for RFP and with the RFP eRNA. The time-induced band is visible under all conditions, confirming that the RFP antibody is cross-reacting with a nonspecific band induced in platelets under these conditions.

associated with sepsis[50]. We reasoned that this would be an attractive candidate for platelets to synthesize in a clinical setting. A construct was prepared and GM-CSF mRNA was IVT and incubated with platelets in the presence or absence of thrombin. Both supernatants and cell pellets were assayed for GM-CSF protein at 2.5h, 5h and 24h by ELISA. No protein was detected at any of the timepoints under any of the conditions tested (data not shown).

Due to the difficulty with the RFP antibody and the low sensitivity of antibody-based assays, we used a luciferase assay as a more sensitive and specific method for detecting protein expression in platelets from eRNA. The coding sequence for luciferase was IVT with a 5′ cap and a polyA tail and incubated in the presence of platelets. Samples taken at 2h, 4h, 8h and 18h all failed to show any luciferase activity. Despite our evidence that platelets take up eRNA, we have not been able to detect translation of eRNA in platelets under any conditions tested.

Our lab has previously demonstrated the ability of activated platelets to induce synthesis of chemokines by monocytes through a receptor-mediated mechanism[21]. In addition, recent work by another lab suggests that platelet-like particles, a particle created in a cell culture system, are capable of transferring RNA to other cells[51]. We wanted to determine whether platelets take up eRNA to protect it from extracellular ribonucleases and then transfer it to monocytes for subsequent translation. Platelets were incubated with eRNA coding for luciferase for 2h, the timepoint at which we saw maximal eRNA uptake in our earlier experiments (Fig. 2.3). After preincubation with eRNA, monocytes and thrombin were added to the platelets and samples were taken at 2h, 4h, 8h and 18h to assay for luciferase activity. There was no detectable luciferase activity under any of the

conditions tested, suggesting that this eRNA is not transferred to monocytes from platelets for translation.

Discussion

The release of RNA from cultured human cells under basal conditions was first described in 1978[52]. Since then, the presence of eRNA in body fluids including plasma, serum and urine has been detected in both healthy and diseased individuals[53,54]. The majority of this extracellular RNA is likely packaged in membrane-bound microparticles (MP) that are protected from plasma ribonucleases. Consistent with this, filtration or ultracentrifugation significantly decreases the level of detectable RNA in plasma[55]. RNA packaged in MP is enriched for certain RNAs relative to the RNA profiles in the cells from which they originate, and is capable of being translated in target cells[56,57]. Activated platelets are known to be an important source of MP, but multiple other cell types including monocytes, endothelial cells and tumor cells have been shown to release MP as well[58,59]. While MP appear to represent an important means of intercellular communication, the mechanisms by which cargo is packaged into and vesicles are released from cells remain to be characterized and hamper our understanding of their physiological relevance[60].

In addition to uptake of RNA packaged within MP, naked RNA has been shown to be taken up by cells as well. Injection of RNA coding for luciferase into mouse skeletal muscle and intradermally results in expression of luciferase within fibroblasts, dendritic cells and muscle cells which is time and concentration-dependent[61-63]. RNA molecules can serve as transient carriers of information without the risks of genomic

integration and insertional mutagenesis carried by DNA vectors[64]. Vaccination with mRNA in mice has been shown to result in specific immunologic responses against the antigen which the RNA codes for, and the possibility of this technology to generate host antitumor responses is promising[65,66]. Despite the work that has been done to understand exogenous RNA uptake in tissues and in cell culture systems, until the observations presented here nothing was known about platelet responses to eRNA.

Here, we describe for the first time the ability of quiescent platelets to rapidly take up and protect eRNA from RNase in culture. Exposure to eRNA does not lead to surface expression of P-selectin, binding of PAC-1 or alter the ability of platelets to respond to thrombin, suggesting eRNA does not result in direct platelet activation. We observed uptake of eRNA of varying sizes, and uptake was determined to be time and concentration-dependent. In nucleated cells, eRNA uptake has been shown to occur via scavenger receptors, as treatment with ligands for these receptors competes with eRNA for internalization[63]. In addition, double stranded RNA uptake in macrophages has been shown to occur through integrin receptors[67].We have not yet tested whether scavenger receptors or integrins are responsible for eRNA uptake by platelets, but platelets are known to express CD36 and SR-BI as well as integrins, and this possibility will be explored in future studies[68].

Interestingly, other cells we tested not only failed to take up eRNA, but released factors into the culture media that led to rapid RNA degradation. This is in contrast to reports from other groups demonstrating that passive pulsing of mRNA into a variety of cell types leads to detectable translation of the mRNA after 24h[62,63]. As these other reports used 10-fold more eRNA (or more) than we did, it is possible that our assay was below

the limit of detection. It is striking, however, that eRNA uptake by platelets is so robust compared to other cells (Fig. 2.5).

Our lab has previously demonstrated the ability of activated platelets to translate endogenous mRNA in response to extracellular cues[69], leading us to test the hypothesis that eRNAs would be translated within platelets. Using immunocytochemistry, western blotting, ELISA and luciferase assays with the corresponding eRNAs, we were unable to detect translation at any timepoint, even in the presence of thrombin. Our microscopy studies showed localization of eRNA within the granulomere of spread platelets, and more work needs to be done to determine if the eRNA is found within a lysosome, granule or other compartment. It is possible that eRNA taken up by platelets is sequestered within a compartment that is inaccessible to translation machinery. One group has reported localization of eRNA within lysosomes of nucleated cells, with concomitant degradation of the bulk of the eRNA[63]. In addition to localization studies by microscopy, we are undertaking radiolabeled eRNA experiments to determine whether the eRNA within platelets remains intact or represents partially degraded nucleic acids that cannot be translated.

Recently, transfer of platelets from $TLR2^{+/+}$ mice into $TLR2^{-/-}$ mice was demonstrated to lead to detectable levels of TLR2 RNA within the peripheral blood mononuclear cells of recipient mice[51]. This *in vivo* experiment was used to confirm *in vitro* experiments done by the same lab showing RNA transfer from cultured platelet-like particles to THP-1 cells, and subsequent protein expression. While we were not able to detect any luciferase expression in primary human monocytes or THP-1 cells cultured with platelets containing eRNA for luciferase, it is possible that this is due to a technical

limitation of our assay, and further optimization may lead to detectable RNA transfer. We will continue to study the interactions between platelets and leukocytes with the aim of better understanding how the interaction between these cells affects their function.

Translation is not the only means by which eRNA uptake by platelets may exert a physiologic effect. Extracellular RNA has been reported to serve as a scaffold for coagulation factors, with RNase treatment (but not DNase treatment) delaying occlusive thrombus formation in mouse models of vascular injury[37]. As platelets play an active role in thrombus formation, they are poised to interact with eRNA and possibly downregulate the contact phase of coagulation, allowing for previously unrecognized and additional layers of control over hemostasis. To further our understanding of how platelet uptake of eRNA may alter coagulation responses, additional work characterizing the mechanism by which eRNA is taken up by platelets will be needed.

Far from being an inert molecule, extracellular RNA is gaining increased appreciation as not only a marker of disease but also an important player in physiological responses. The work presented here provides the first evidence that platelets take up eRNA and characterizes our incomplete understanding of the outcome of this uptake on platelet function.

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CHAPTER 3

A GENETIC SCREEN TO IDENTIFY NOVEL FACTORS FOR NEUTROPHIL EXTRACELLULAR TRAP FORMATION

Abstract

The ability of neutrophils to release DNA creating extracellular traps (NETs) has been shown to play an important role in innate immune defense against pathogens as diverse as bacteria, fungi and protozoan parasites. Since the first report of this antimicrobial activity nearly a decade ago, much has been learned about this previously unrecognized process. Using primary human neutrophils as well as animal models, a variety of signals including direct interactions with platelets, factors released by platelets and small molecules have been shown to contribute to NET formation in addition to the pathogens mentioned above. Naturally occurring genetic models, such as patients with chronic granulomatous disease, have also established the requirement of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase for this process. Despite rapid progress made in understanding the mechanism by which NETs form, known as NETosis, much remains to be learned. Here, we describe a genetic screen we developed to identify novel factors required for NET formation. Using the nearly haploid human cell line, KBM-7, we determined conditions that were permissive for NET formation in culture. Retrovirus was generated to deliver gene-trap constructs by transduction for

mutagenesis of a pool of KBM-7 cells and a plate-based assay was created to allow us to screen for clones with disruption of genes required for NETosis. Unexpectedly, integration of the gene trap construct failed to result in detectable expression of the marker gene (either GFP by flow cytometry or resistance to puromycin), despite detectable integration by PCR. This limitation made execution of the screen unfeasible as designed, as initial selection for successful mutagenesis is necessary to bring the costs of the screen (in time and reagents) within a manageable level. Despite this setback, future developments in bioinformatics may allow the foundational work described here to be utilized in a pooled screen to identify new genes required for NET formation.

Introduction

Neutrophils are the most abundant white blood cells in humans, accounting for between 50 and 70% of the leukocytes in peripheral blood. Their importance as first-responders in innate immune defense against pathogens is made apparent by an increased susceptibility to bacterial infections in neutropenic patients. The observation that neutrophils are found associated with bacteria in lesions initially led to the false conclusion that neutrophils promoted the growth of bacteria within the host[1]. Using a thorough experimental and evolutionary approach, Metchnikoff clearly demonstrated in the early 20th century that neutrophils engulf pathogens to affect phagocytic killing of invaders[2]. In the subsequent century, the mechanisms by which neutrophils provide innate immune defense were elucidated. Activated neutrophils were demonstrated to either phagocytize microorganisms, exposing them to hydrolytic enzymes and antimicrobial proteins as well as reactive oxygen species within the phagosome, or

release toxic granules into the extracellular space[3]. In either case, the goal is to kill the invading organism while causing minimal damage to the host. In 2004, Brinkmann reported a third and previously unrecognized mechanism by which neutrophils could protect against pathogens: neutrophil extracellular traps (NETs)[4].

NETs consist of decondensed chromatin decorated with proteins from primary, secondary and tertiary neutrophil granules[4]. NET formation can be distinguished from apoptosis by the absence of DNA fragmentation, phosphatidylserine exposure or caspase activation[5]. Rather, cells undergoing NETosis lose their nuclear membrane integrity, whereupon decondensed chromatin mixes with the contents of cytoplasmic granules prior to cell death and release of the NET[5]. This process has been described in humans, mouse and fish as well as several other species[6-10]. In addition to being conserved across multiple organisms, NETs can be induced by a multitude of signals. Bacteria known to induce NETs include *Staphylococcus aureus*, *Haemophilus influenza*, *Mycobacterium tuberculosis* and *Escherichia coli*[11-14]. Fungi such as *Candida albicans* and *Aspergillus nidulans*, in both the yeast and hyphal forms, can be killed by NETs[15,16]. Protozoan parasites including *Leishmania amazonensis*, *Toxoplasma gondii* and *Plasmodium falciparum* have also been reported to induce and be killed by NETs[17-19]. Isolated components of microbial cell walls such as lipopolysaccharide (LPS) and fMLP as well as inflammatory cytokines, activators of protein kinase C and even interactions with platelets and antimicrobial β-defensins released from platelets have all been shown to induce NETosis[4,20-22].

Given the central role of neutrophils in defense against pathogens, the diversity of signals which can lead to NET formation is not surprising. However, it is clear that tight

regulation of such a process is also important to prevent harm to the host. The delicate balance between benefit and harm is illustrated by the association of NETs with both acute and chronic pathologies. Markers of NET formation have been found in the blood of patients with transfusion-related acute lung injury (TRALI) and directly observed in the lungs in mouse models of TRALI[23,24]. Administration of DNase I, either intravenously or by inhalation, improved outcomes in these models. NETs have been shown to damage activated endothelium and contribute to end organ damage in mouse models of sepsis[22]. NETs also provide a scaffold for platelet and red blood cell deposition within the vasculature, contributing to thrombus formation, which can be prevented with the administration of DNase I[25,26]. In the autoimmune disease systemic lupus erythematosus, the serum from a subset of patients with active disease demonstrated a decreased ability to degrade NETs and concomitant complement deposition, suggesting a role for NETs in autoantibody production[27]. Taken together, these studies illustrate how important understanding the molecular mechanisms driving NET formation are for therapeutic purposes.

The initial report describing NETs demonstrated that actin polymerization was not required for NET formation[4]. Subsequent work focused on NADPH oxidase and generation of reactive oxygen species (ROS), a mechanism known to be critical for phagocytic killing by neutrophils. Treatment of neutrophils with the small molecule inhibitor of NADPH oxidase diphenylene iodonium (DPI) blocks NET formation, while treatment with glucose oxidase to generate ROS downstream of NADPH oxidase is sufficient to drive NET formation, even in the presence of DPI[5]. Neutrophils from patients with chronic granulomatous disease (CGD) containing mutations in NADPH

oxidase fail to form NETs in response to agonist, a defect which can be reversed by adding glucose oxidase, providing genetic evidence that ROS are required for NET formation[5]. However, experiments demonstrating that neonatal neutrophils have a developmental defect in NET formation that cannot be rescued upon addition of glucose oxidase indicate that ROS generation is not sufficient to execute NETosis, and additional signaling pathways are likely involved[28]. Mouse knockout models have been used to demonstrate a requirement for neutrophil elastase (NE) and peptidylarginine deiminase 4 (PAD4) in chromatin decondensation; the absence of either of these genes blocks NET formation and increases susceptibility to infection in mouse models of pneumonia and necrotizing fasciitis[29,30]. Treatment of primary human neutrophils with rapamycin demonstrated that mTOR plays a role in NET formation, and shRNA knockdown of HIF-1α in HL-60 cells differentiated into surrogate PMNs confirmed that this was dependent on HIF-1α[31]. Myeloperoxidase (MPO) has also been shown to be required for NET formation, as humans with mutations in the MPO gene leading to an absence of the protein are unable to form NETs[32].

Both small molecule and genetic approaches have helped elucidate key pathways required for NET formation, but these methods have required a candidate approach. To gain new insights into the mechanisms of NETosis, a broader genetic screen is needed. Such an undertaking using animal models is unfeasible due to the time and expense required. A cell culture based assay is not only faster and cheaper than animal models, but has the potential to reach greater genome coverage due to the elimination of confounding effects from genes required for development. However, as human cell lines are generally diploid, any mutagenesis strategy randomly targeting genes would only

affect one of the two alleles, and only identify genes with a dominant effect on NET formation. The human cell line KBM-7, isolated from a patient with chronic myelogenous leukemia (CML) has been reported to be haploid (with the exception of chromosome 8) and has successfully been used to identify genes required for host susceptibility to pathogens using an insertional mutagenesis strategy[33,34]. We speculated that this cell line may be capable of forming NETs *in vitro*, as it is of myeloid origin. Here we describe our work demonstrating that these nearly haploid KBM-7 cells are capable of forming NETs, the design of our genetic screen and the barriers to executing the screen as designed. This foundational work can be built upon with improvements in bioinformatics to identify a host of factors required for NET formation and targets for novel therapeutics in the future.

Materials and Methods

Cell lines and culture

KBM-7 cells were a kind gift from the Brummelkamp lab (Netherlands Cancer Institute) and were cultured in IMDM supplemented with 10% FCS and antibiotics. Cells were grown in suspension and maintained between $0.5x10^6$/mL and $2x10^6$/mL. Phoenix-GP cells were obtained from the National Gene Vector Laboratory Biorepository (NGVB, Indianapolis, IN)[35] and cultured in DMEM supplemented with 10% heat inactivated FBS, 2mM $_L$-glutamine and antibiotics. Cells were maintained below 90% confluency. HL-60 cells were maintained according to distributor's recommendations (ATCC, Manassas, VA). Unless otherwise indicated, cells were maintained in a 37°C humidified tissue culture incubator with 5% CO_2.

Ploidy testing and sorting of haploid cells

Cell ploidy was determined using propidium iodide (PI) staining. KBM-7 cells or HL-60 cells ($1x10^6$) were centrifuged in flow tubes, then resuspended in 500µL cold hypotonic DNA buffer (0.1% sodium citrate, 0.1% Triton X, 20ug/mL RNase A, 50ug/mL propidium iodide), incubated on ice for 20′ and analyzed on a FACSCanto flow cytometer (BD Biosciences, San Jose, CA). Haploid cells were sorted based on size with a FACSAria sorter (BD Biosciences, San Jose, CA) at the University of Utah flow cytometry core facility, and confirmed by PI staining. Where indicated, cells were incubated in the presence of 4µg/mL aphidicolin (Acros Organics, Geel, Belgium) for 16h prior to treatment with hypotonic DNA buffer and analysis.

Plasmids

Gene trap constructs were a kind gift from the Brummelkamp lab (Netherlands Cancer Institute)[34]. As constructs with LTRs frequently recombine during plasmid production within *E. coli*, losing the gene trap region while maintaining resistance to antibiotic selection, all plasmid preparations were confirmed by restriction enzyme digest prior to transfection of packaging cells for virus production. The GT-GFP plasmids contain the coding region for GFP within the LTRs downstream of the splice acceptor site, resulting in GFP expression when integration occurs within an expressed coding gene. The GT-Puro plasmids contain the coding region for the Pac gene within the LTRs downstream of the splice acceptor site, resulting in resistance to puromycin when integration occurs within an expressed coding gene. The VSV-G envelope plasmid for retrovirus was from Addgene (#14888). The CMV-GFP retrovirus positive control

plasmid was a kind gift from Eric Taylor in the Rutter lab (University of Utah). The pAdVAntage plasmid was purchased (Promega, Madision, WI).

Retrovirus production

Phoenix-GP cells were plated the day prior to transfection onto poly-lysine coated tissue culture dishes at a density of 50% in antibiotic-free media. The following day, the media was replaced with fresh antibiotic-free media and cells were transfected at approximately 70-80% confluency. Phoenix-GP cells contain genomic integrations of the packaging genes gag and pol, so transfection only required the envelope pseudotyping plasmid VSV-G, the pAdVAntage plasmid and the gene trap plasmids (either GT-GFP or GT-Puro) or the GFP positive control plasmid. Cells were transfected with plasmids using Lipofectamine 2000 (Life Technologies, Grand Island, NY) according to the manufacturer's instructions and grown at 37°C. Posttransfection (4h), the media was replaced with fresh media containing antibiotics and the cells were incubated overnight. The following morning, the media was replaced again and the cells were cultured at 32°C for 48h. Supernatant containing virus was removed at 48h and 72h posttransfection, filtered through a 0.45 µm filter and stored at 4°C. After the 72h harvest, supernatant containing virus was pooled and ultracentrifuged for 2h at 25,000 rpm, 4°C in a SW 32 Ti rotor (Beckman Coulter, Indianapolis, IN) to concentrate. All but 300µL of the supernatant was removed and the pellet was incubated overnight at 4°C, then resuspended with pipetting and used for transductions.

Transduction

KBM-7 cells were resuspended at $0.5x10^6$/mL the day before infection to ensure that cells would be actively dividing for transduction. Cells were counted on the day of infection, centrifuged and resuspended at a concentration of $1x10^7$/mL; 10µL of cells was used per infection condition. Where indicated, carriers were used to promote infection. Protamine sulfate and polybrene (Sigma-Aldrich, St Louis, MO) were each used at a final concentration of 8µg/mL. Lipofectamine (Life Technologies, Grand Island, NY) was used at a 1:100 dilution. Concentrated virus (100µL) and carrier was added to each tube with cells and centrifuged at 2400xg for 90′ at 4°C (spinfection). After centrifugation, tubes were incubated for 2h at 32°C prior to the addition of 500µL fresh media and overnight incubation at 37°C. The following day the cells were centrifuged, resuspended in fresh media and subjected to a second round of spinfection. Three days after the second round of infection, cells were analyzed for GFP fluorescence by flow cytometry or puromycin resistance. Where indicated, Phoenix GP cells at 60% confluency were transduced with virus as a positive control, as these cells are readily infected by retrovirus.

Microscopy

Cells were washed, resuspended in M199 media and incubated in the presence or absence of the agonists indicated on coverslips for 1h at 37°C. After stimulation, cells were gently washed and stained with a mixture of a cell permeable (SYTO Green) and cell impermeable (SYTOX Orange) DNA dyes (Life Technologies, Grand Island, NY). Fluorescence microscopy was performed on a confocal microscope using both 20x and

60x oil objectives as previously described[28]. FV1000 image acquisition software Version 5.0 (Olympus, Center Valley, PA) was used for recording images.

PCR

Primers for detecting GFP gene trap integration in genomic DNA were 5′-CGGTTCACCAGGGTGTCGCC-3′ and 5′-CGAGCTGGACGGCGACGTAA-3′, which yield a 318bp product. Primers for detecting puromycin gene trap integration in genomic DNA were 5′-ACCGCTCAACTCGGCCATGC-3′ and 5′-CCACATCGAGCGGGTCACCG-3′, which yield a 178bp product. PCR was performed on 100ng of gDNA isolated from transduced cells or 10ng of gene trap plasmid as a positive control using GoTaq polymerase and reagents (Promega, Madison, WI) with 35 cycles and a 72°C annealing temperature.

Puromycin kill curve

Haploid KBM-7 cells ($8x10^4$) were plated into each well of a 24-well tissue culture plate with media in the absence or presence of puromycin at varying concentrations (0.5 to 5µg/mL). Cells were counted every 1-2 days and graphed to determine the optimum puromycin dose and time to kill all nontransduced cells.

Picogreen assay

KBM-7 cells were washed and resuspended at $2x10^6$/mL in HBSS with Ca^{++} and Mg^{++}; 200µL of cells were added to each well of a 96-well round bottom tissue culture plate with either no treatment or stimulation with LPS (100ng/mL), camptothecin (2µM)

or staurosporine (1µM) and incubated at 37°C for 30′ and 2h. Micrococcal nuclease (50U) (New England Biolabs, Ipswich, MA) was added to each well and incubated for 10′ at 37°C. The plate was centrifuged and 100µL supernatant was carefully removed and transferred to an opaque white 96-well plate. Picogreen reagent (Invitrogen, Grand Island, NY) was diluted in TE according to manufacturer instructions and 100µL was added to each well of the assay plate and incubated in the dark for 5′ prior to reading on a Synergy HT luminometer (BioTek Instruments, Winooski, VT). Samples were run in triplicate and the three readings were averaged.

Results

Haploid KBM-7 cells can be isolated by FACS

KBM-7 cells have been selected for their ability to remain haploid in culture for several months, but over time ploidy can increase[33]. The haploid population can be distinguished from the remaining cells based on size. We sorted our initial culture of KBM-7 cells on a FACSAria cell sorter and selected the small cells based on FSC/SSC profiles. Using PI staining we determined that our sorting parameters were sufficient to enrich for haploid cells relative to the mixture of ploidy seen in the unsorted population (Fig. 3.1a). As haploid cells in G2 of the cell cycle are indistinguishable from diploid cells in G1, we treated cells with aphidicolin, a small molecule inhibitor of cell cycle that blocks cells in early S-phase[36], to confirm that our sorted cells were haploid. Aphidicolin treatment of the unsorted cells resulted in a dramatic reduction in the third peak, corresponding to a block preventing the diploid cells in the population from transitioning to G2 (Fig. 3.1b). Treatment of the sorted cells with aphidicolin resulted in a

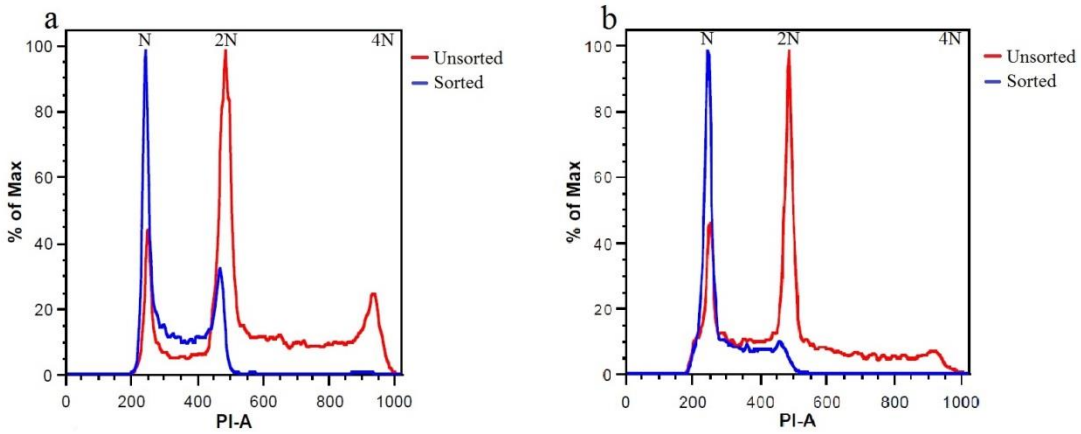


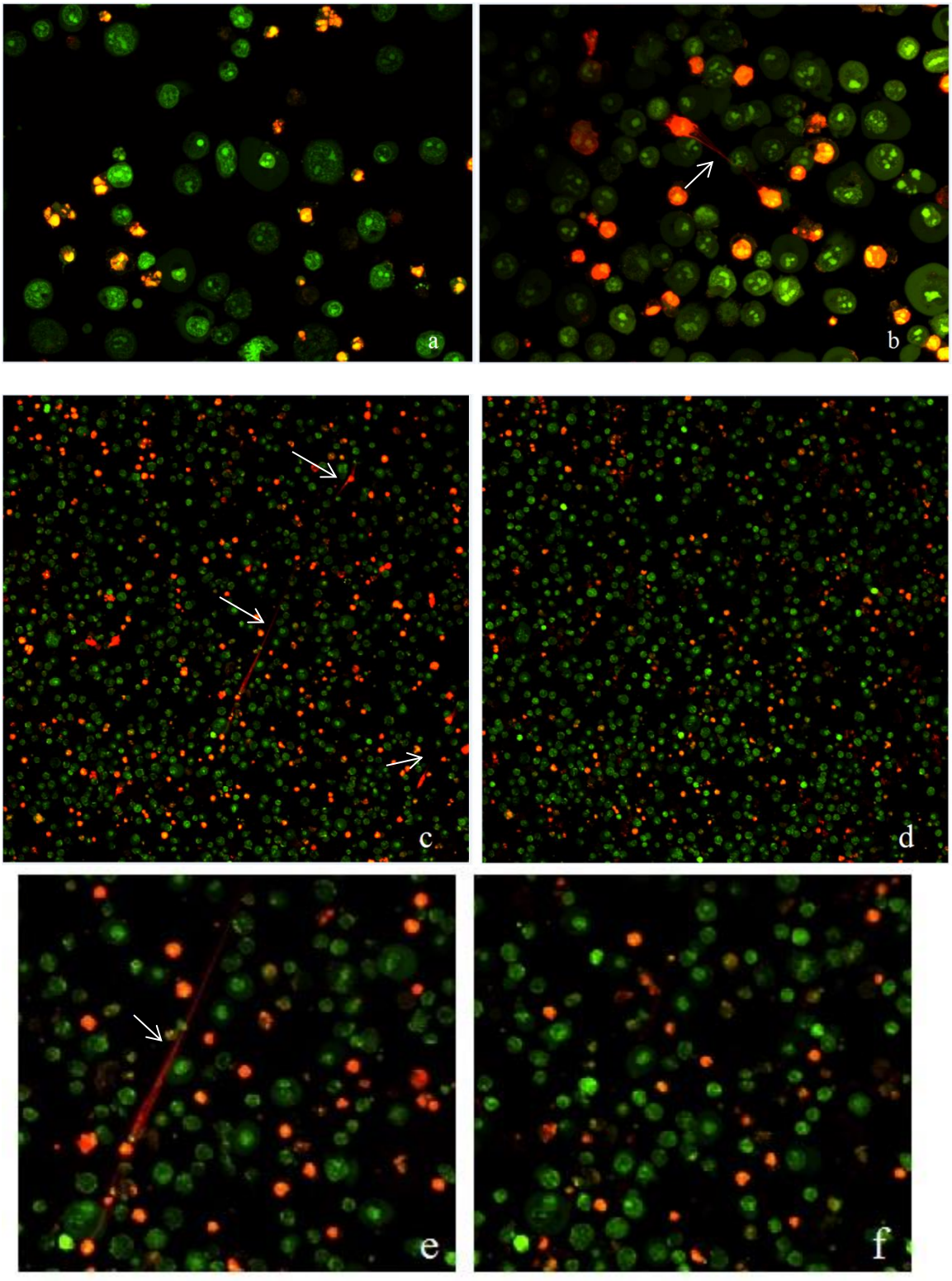
Figure 3.1: FACS sorting of KBM-7 cells enriches for haploid population. Untreated KBM-7 cells (a) or KBM-7 cells pretreated with aphidicolin (b) were stained with propidium iodide before and after sorting based on size, then analyzed by flow cytometry.

loss of the second peak, confirming that this population is indeed haploid (Fig. 3.1b). To ensure that subsequent experiments were performed on an enriched haploid population, these sorted cells were expanded and frozen in liquid nitrogen. Cells maintained in culture were discarded after 2-3 months to avoid potential problems with unstable ploidy.

KBM-7 cells form NETs

Our genetic screen to identify factors required for NET formation relies on the ability of haploid KBM-7 cells to form NETs. While primary neutrophils from several species have been shown to form NETs, as well as the promyelocytic leukemia cell line HL-60 [6-10,37], other immortalized cell lines capable of forming NETs have not been reported in the literature. We tested a panel of agonists shown to induce NETosis in neutrophils on KBM-7 cells and saw that both PMA (Fig. 3.2b) and LPS (Fig. 3.2c) resulted in NET formation, as demonstrated by microscopy. Treatment with DNase resulted in rapid (within 15′) loss of extracellular DNA (Fig. 3.2d). Note that in a population of cells, only a subset form NETs upon stimulation. This is consistent with what has previously been observed in primary neutrophils, with approximately 30% of neutrophils showing extracellular DNA upon stimulation[5]. Whether this represents a heterogeneous population of cells or paracrine feedback preventing excessive activation within the pool of cells has yet to be determined. In addition, we detected expression of the neutrophil markers MPO and NE by western blotting, suggesting that these cells are neutrophil-like (data not shown).

Figure 3.2: KBM-7 cells make NETs. KBM-7 cells were incubated in the absence (a) or presence (b) of 20nM PMA for 1h, washed, and stained with cell permeable (green) and cell impermeable (red) DNA dyes prior to imaging using confocal microscopy (60x magnification). KBM-7 cells treated with 100ng/mL LPS were stained with DNA dyes and imaged (c). The sample shown in panel (c) was treated with 50U of micrococcal nuclease for 15′, and then imaged again (20x magnification)(d). (e,f) Magnified regions of (c,d) to better show NET that disappears after nuclease treatment. Arrows indicate NETs. Images are representative of three independent experiments.


a
b
c
d
e
f

KBM-7 cell NET formation can be quantitated with a plate-based assay

Microscopy-based evaluation of NET formation is useful for qualitative low throughput purposes, but we needed a more quantitative high throughput assay for our genetic screen. One method that has been previously reported is the quantitation of extracellular DNA using Picogreen reagent, which specifically binds dsDNA[5]. This technique uses a fluorescence microplate reader to analyze up to 96 wells simultaneously. Using this method, we saw a 2-fold increase in dsDNA when KBM-7 cells were treated with LPS for 30′, and a similar increase at 2h (Fig. 3.3). Importantly, when cells were treated with camptothecin or staurosporine to induce apoptosis, there was no increase in dsDNA detected over the untreated samples at these time points. This is consistent with the observation that release of DNA from apoptotic cells occurs sometime between 6 and 24h after initiation[38], and reassured us that using the Picogreen plate assay at early timepoints would allow us to effectively distinguish clones which are unable to undergo NETosis from apoptotic cells.

Generation of retrovirus and transduction

Production of high titer retrovirus is essential to efficiently mutagenize the genome of our target cells. We transfected the packaging cell line Phoenix GP, which is a 293T cell line containing a genomic integration of the retroviral gag-pol[35], with a construct for the VSV-G envelope protein and our gene trap constructs. Using the same reagents, we generated a positive control virus, which replaced our gene trap constructs with one containing a GFP marker downstream of a CMV promoter. While our gene trap constructs contain either a GFP marker or puromycin resistance gene, they are

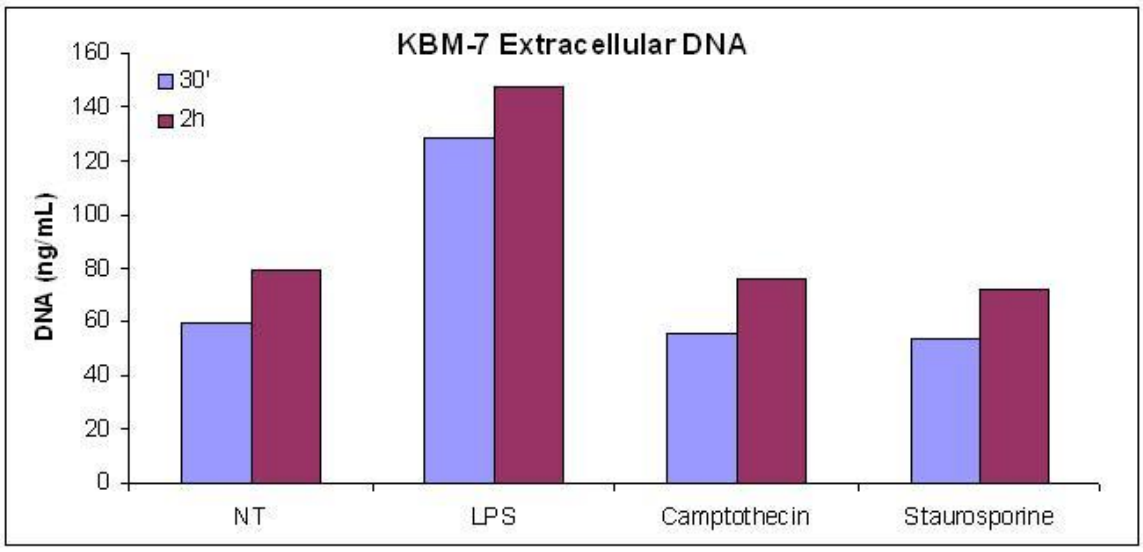


Figure 3.3: Extracellular DNA quantitation by Picogreen plate assay. KBM-7 cells were incubated in the absence of agonist or with LPS (100ng/mL), camptotchecin (2μM) or staurosporine (1μM) for 30′ or 2h. Extracellular DNA was digested with micrococcal nuclease and quantitated using Picogreen reagent. Absolute values varied between experiments, but data are representative of three independent experiments.

promoterless and rely on integration into an actively transcribed gene to be expressed. The CMV-GFP construct produces constitutive expression of GFP and allowed us to optimize general transfection efficiency in the system using a fluorescence microscope during production. Supernatant containing virus was collected at 48 and 72h post-transfection and concentrated via ultracentrifugation as previously described[34].

Concentrated virus was used to infect both Phoenix GP cells, which are permissive to retrovirus infection, and our haploid KBM-7 cells according to detailed protocols from the Brummelkamp lab (personal communication), who have successfully mutagenized these cells previously. Cells transduced with the positive control virus or the GT-GFP virus were assayed 4 days postinfection by flow cytometry. Cells transduced with the GT-Puro virus were assayed 4 days postinfection by treating with 5µg/mL puromycin, and determining cell count relative to nontransduced cells on day 1 and day 4. This concentration of puromycin was used because it was determined to kill 100% of KBM-7 cells lacking the puromycin resistance gene within 4 days (Fig. 3.4). Unfortunately, puromycin treatment of cells transduced with GT-Puro virus showed no survival advantage at day 4 compared to untreated cells (data not shown). This could be due to poor transduction efficiency or integration into poorly expressed regions of the genome, leaving even successfully transduced cells susceptible to puromycin. As GT-GFP virus can be assayed more rapidly and the CMV-GFP virus serves as a good positive control for that assay, we used this virus to optimize our transduction efficiency for insertional mutagenesis.

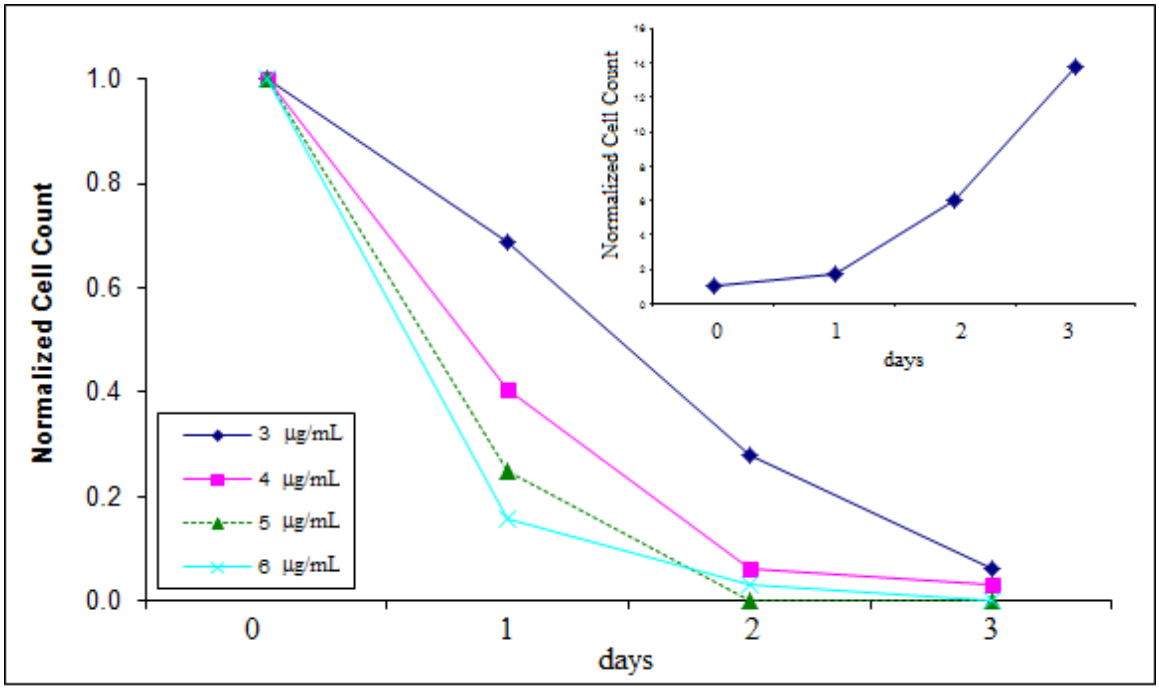


Figure 3.4: Puromycin kill curve of KBM-7 cells. KBM-7 cells were seeded into media containing the indicated concentrations of puromycin and counted daily. Inset: Untreated KBM-7 cells cultured under identical conditions were counted daily to assess normal growth. Data are representative of three independent experiments.

Transduction efficiency of KBM-7 cells is low

Using the concentrated GT-GFP and CMV-GFP retrovirus, we tested a multitude of conditions to optimize infection of KBM-7 cells. Carrier agents such as protamine sulfate and polybrene are commonly used during retroviral transduction to promote infection, but we were unable to detect GFP expression by flow cytometry in KBM-7 cells infected with either the GT-GFP or CMV-GFP retroviruses using these reagents (data not shown). It has been reported that infection in media at pH 7.7 can increase transduction efficiency as much as 2-fold in NIH-3T3 cells[39], but we saw no change in GFP expression when our KBM-7 cells were transduced at pH 7.7. In addition to spinfection[34], we also used a transwell system to promote contact between virus and KBM-7 cells[40], but this resulted in undetectable GFP expression in our transduced cells. Despite our efforts, we were unable to detect GFP expression in our KBM-7 cells under any conditions with either the CMV-GFP virus or the GT-GFP virus.

To determine whether the KBM-7 cells were particularly resistant to infection or if there was a problem with our virus production, we tested our CMV-GFP and GT-GFP virus preparations for their ability to infect the Phoenix packaging cell line. Using either protamine sulfate or polybrene to infect Phoenix cells, we were able to achieve approximately 50% transduction efficiency with the CMV-GFP virus (Fig. 3.5a, b). However, the GT-GFP virus showed less than 1% GFP positive cells using the same conditions (Fig. 3.5c, d). These results suggested to us that we were capable of producing competent virus, as the CMV-GFP virus was able to efficiently infect these cells, but that GFP expression from the GT-GFP was undetectable. This may be because viral integration within regions of the genome that are not expressed or poorly expressed may

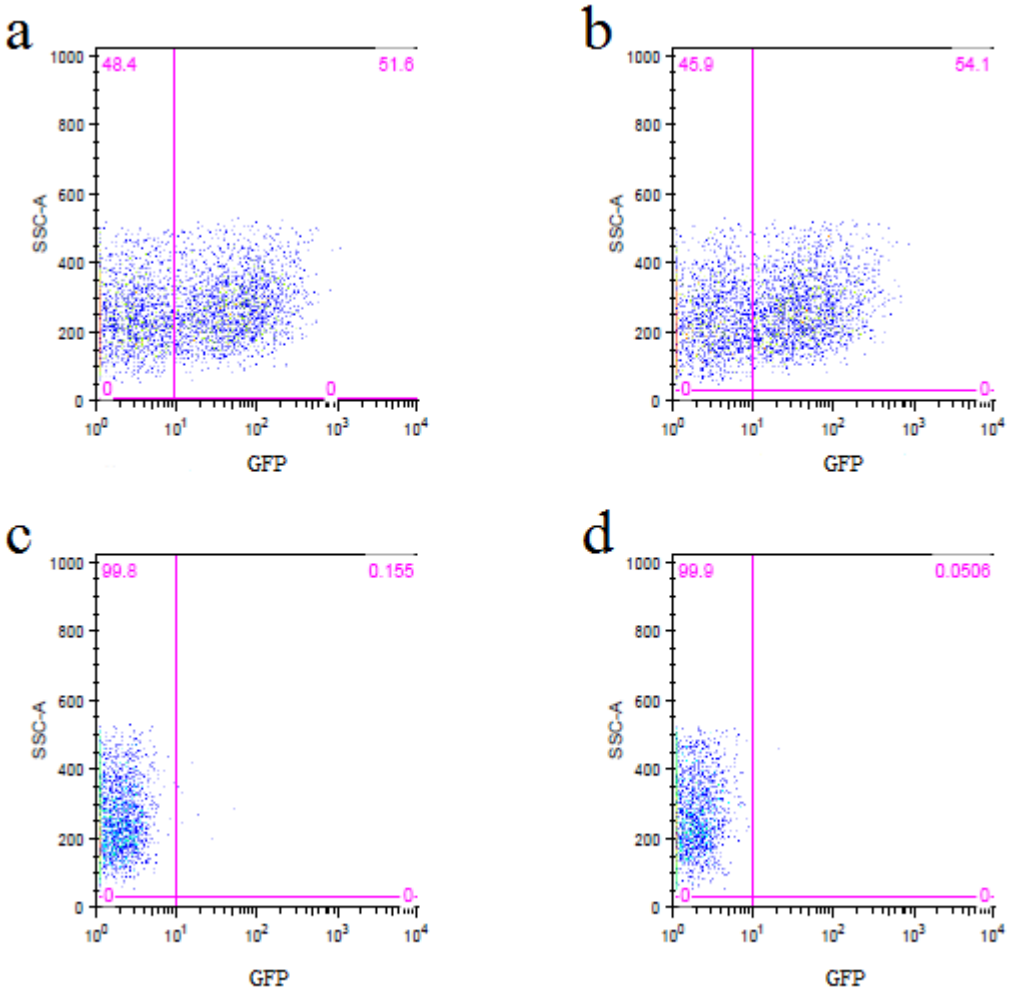


Figure 3.5: Flow cytometry detects GFP expression in transduced Phoenix cells. Phoenix cells were transduced with positive control CMV-GFP virus (a,b) or GT-GFP virus (c,d) in the presence of protamine sulfate (a,c) or polybrene (b,d) and assayed for GFP expression by flow cytometry 3 days later. Data are representative of five independent experiments.

result in GFP expression below the limit of detection. This was a surprise, as the Brummelkamp lab has previously reported that they are able to successfully sort cells mutagenized with this technique using GFP expression[34]. Nevertheless, subsequent genetic screens by this lab have been reported using a pooled lethal selection approach rather than sorting successfully mutagenized cells[41-43], and both successful infection of KBM-7 cells and detection of GFP expression in these cells is technically challenging (personal communication with Dr. Brummelkamp).

To distinguish whether there was a global problem with our gene trap viruses ability to infect KBM-7 cells from difficulty detecting mutagenesis using expression of the reporter gene, we isolated genomic DNA (gDNA) from KBM-7 cells infected with our retroviruses 1 week after infection and used PCR to determine whether the marker gene could be amplified. We were able to detect GFP in gDNA from cells infected with CMV-GFP (Fig. 3.6 lane 2) and GT-GFP (Fig. 3.6 lane 3) virus, but not in uninfected cells (Fig. 3.6 lane 1) or cells infected with GT-Puro virus (Fig. 3.6 lane 4). Interestingly, the signal from the CMV-GFP and GT-GFP viruses is similar. Our experiments with these retroviruses in Phoenix cells showed that the CMV-GFP virus is able to produce a strong GFP signal in the flow cytometry assay, yet in KBM-7 cells this expression is undetectable (Fig. 3.5). While the absolute value for infection is difficult to calculate, these experiments suggest that while the GT-GFP virus is competent to infect KBM-7 cells, it is so inefficient that it is not useful for our genetic screen.

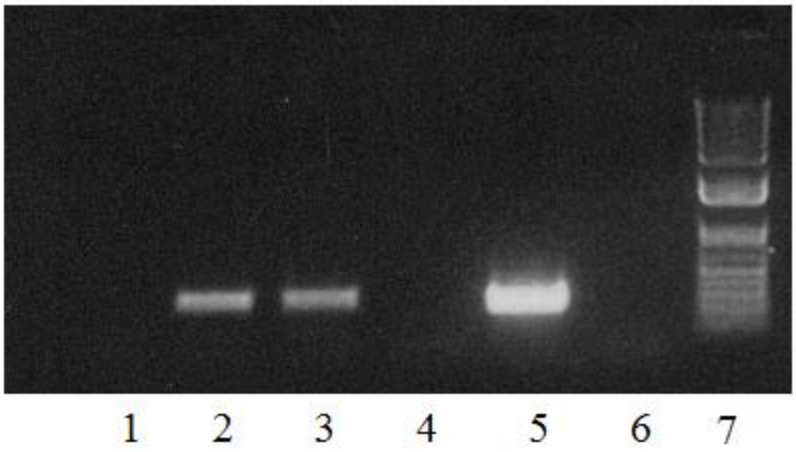


Figure 3.6: GFP is detectable in genomic DNA of transduced KBM-7 cells by PCR. PCR using primers specific to GFP was performed on genomic DNA prepared from KBM-7 cells (lanes 1-4), GT-GFP plasmid (lane 5, positive control) or water (lane 6, no template control), run on an agarose gel and visualized using ethidium bromide. Lane 1: Uninfected cells. Lane 2: CMV-GFP virus infected cells. Lane 3: GT-GFP virus infected cells. Lane 4: GT-Puro virus infected cells.

Discussion

Insertional mutagenesis using retroviral transduction with gene trap plasmids has previously been performed in KBM-7 cells to identify host factors exploited by pathogens[34]. As insertion in a gene required for pathogen intoxication would lead to survival of the mutant cell, treatment of small pools of mutagenized cells with toxin for extended periods of time leads to survival of a single clone, which can be identified by sequencing. Our genetic screen was designed to identify genes required for NET formation. As such, insertions in genes required for NETosis would result in a reduction in extracellular DNA when stimulated with LPS and evaluated in our Picogreen plate assay. However, as not all cells in a population form NETs when stimulated[5] (and Fig. 3.2), treatment with agonist would not be lethal to the population. Therefore, our genetic screen depended on the ability to sort successfully mutagenized cells using either the puromycin resistance marker or the GFP marker and the ability of single KBM-7 cells to survive limited dilution cloning. These clones could then be assayed for NET formation and sequenced to determine genes where mutations disrupt NET formation. We determined that single KBM-7 cells were able to survive and grow as isolated clones (data not shown). In addition, amplification of gDNA from transduced cells showed detectable integration of both of our gene trap viruses. Unfortunately, selection using either puromycin resistance or GFP expression was unsuccessful. This was likely due to poor overall transduction efficiency, as our CMV-GFP virus also failed to demonstrate appreciable GFP expression in these cells.

The Brummelkamp lab, who previously published work using KBM-7 cells in genetic screens, was kind enough to offer reagents and guidance throughout this project.

In personal communications, we were told that transduction of KBM-7 cells is technically challenging, and this is part of the reason that their lab has moved to screening for lethal phenotypes in pooled cells. Indeed, screening pools of cells for resistance to a lethal treatment has been very successful and identified several new genes important for intoxication[41-43]. Unfortunately, the conditions that would allow 100% of NET-competent cells to undergo NETosis have yet to be identified, and as such, our screen cannot be tailored to a pooled approach with current technology. Nevertheless, improvements in bioinformatics may allow our screen to be conducted as designed with a pooled approach in the future. For this to work, a pool of mutagenized cells would be treated with LPS to form NETs. The extracellular DNA would then be treated with DNase to fragment it, insertion sites would be pulled out and amplified using oligos specific to the insertions, amplified and subjected to deep sequencing. With sufficiently powerful bioinformatics tools, the extracellular DNA from the mutagenized cells could be compared to gDNA from the same cells. Insertions only found in gDNA but not in extracellular DNA would indicate genes that are required for NET formation. This kind of approach is beyond the reach of current bioinformatics, but may be feasible in the near future.

Genetic screens offer the promise of an unbiased approach to discover novel biology. While the execution of the screen described here was limited by both our technical ability and current technology, these limitations are likely to be overcome in the near future and this screen can be revisited. Despite these setbacks, the observation that KBM-7 cells are capable of forming NETs is a novel one. As primary neutrophils are not

amenable to genetic manipulation in culture, KBM-7 cells may serve as a useful tool for studying NET formation in culture.

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CHAPTER 4

DISCUSSION

Current Review of Platelet Biology

Platelets play a critical role in maintaining hemostasis, yet antiplatelet therapies are a booming industry[1]. Platelet activation caused by atherosclerotic plaque rupture can rapidly lead to thrombus formation, occlusion of the vessel and hypoxia in downstream tissues. When this occurs in vessels supplying the heart, it results in myocardial infarction, the leading cause of death in the United States[2]. Antiplatelet therapy has reduced morbidity and mortality from cardiovascular disease over the last 50 years[3], but comes at the risk of increased bleeding, which can be fatal[4]. A significant amount of research being conducted on platelets today aims to eliminate the risk of hemorrhage while effectively preventing platelet-dependent thrombus formation to treat cardiovascular disease.

While their contributions to acute thrombus formation are widely acknowledged, a role for platelets in inflammation is emerging. Platelets rapidly release over 300 preformed proteins from their granules upon activation, many of which function in thrombus formation and cell proliferation[5]. In addition, many proinflammatory cytokines are found in platelet releasates. CXCL4 and beta-thromboglobulin (β-TG) were some of the first chemokines identified, partly because the high concentrations of these proteins

released from platelets made them easy to purify[6]. While both of these proteins are known for their procoagulant properties[7,8], they can also act on neutrophils to promote interaction with endothelial cells and the release of secondary granules, and induce oxidative burst within monocytes[9-11]. CCL5 is also released from platelet granules, and has been shown to promote monocyte recruitment to inflamed endothelium in conjunction with CXCL4 as well as monocyte differentiation into foam cells[12-14]. Disruption of this interaction blocks monocyte recruitment to endothelium and attenuates atherosclerosis in mouse models[15].

Platelet granules are a heterogeneous mix of alpha granules, dense granules and lysosomes, each identified by specific subsets of proteins found within them[16]. Time-lapse microscopy has shown that during platelet spreading, alpha granules expressing VAMP-3 move to a central granulomere region, while alpha granules expressing VAMP-7 move to the periphery of the platelet[17]. The differential sorting and release of subsets of granules suggests a regulated process. Given the role of platelets as rapid responders at sites of injury and their ability to regulate the inflammatory responses of other immune cells, it is possible that the differential release of subsets of platelet granules allows for fine regulation of immunologic responses.

In addition to release of secreted molecules, granule fusion with the plasma membrane results in the translocation of receptors, such as P-selectin, to the platelet surface. P-selectin on the surface of platelets binds with PSGL-1 on leukocytes to promote adhesion to endothelium and trafficking to peripheral sites[18-21]. Heterotypic aggregates of activated platelets and leukocytes can also be found circulating in the peripheral blood, where they serve as markers of cardiovascular disease and induce

inflammatory responses[22,23]. Specifically, binding of platelet P-selectin to monocyte PSGL-1 is required for CCL5-dependent expression of inflammatory cytokines such as MCP-1 and IL-8[24], and IL-1β-dependent expression of COX-2[25] in monocytes. Platelet-neutrophil interactions mediated by P-selectin result in neutrophil recruitment to the lung in acute lung injury and blockade of these interactions is capable of halting the development of acute lung injury in mouse models[26]. Platelet TLR4 activation induces platelet-neutrophil aggregation and NET formation capable of trapping bacteria in the vasculature at the expense of endothelial damage[27]. Together, these studies indicate that interactions of platelets with immune effector cells are critical for host defense.

While direct receptor-mediated interactions and release of granule contents allow platelets to affect localized responses, platelets can also release microparticles (MP), which are small membrane vesicles packaged with surface receptors, signaling proteins, mRNA, and miRNA capable of acting on distal target cells[28,29]. A variety of cells are capable of releasing MP, but in healthy individuals >90% of the plasma MP are platelet-derived[30]. As MP display phosphatidylserine (PS) on their surface, they provide a platform for assembly of coagulation factors[31,32] and many hemostatic-based diseases are associated with altered levels of MP[33]. The surface receptors found on MP are able to interact with target cells in a manner similar to that of the host cell, including P-selectin interactions with endothelium resulting in release of cytokines from the endothelium and expression of adhesion molecules[34]. Importantly, MP have been shown to deliver their protein, mRNA and miRNA to target cells and alter the functional responses of the recipient cells[29,35]. The degree to which this form of intercellular communication occurs *in vivo* and its functional relevance have not yet been determined. However, the easy

availability of platelets from phlebotomy makes them attractive candidates for manipulation and delivery of exogenous molecules to target cells.

For small, anucleate cytoplasts, platelets contain an astonishing amount of molecular machinery. Prior to their release from megakaryocytes, proplatelets are packaged with pre-mRNA, mRNA and miRNA as well as the factors necessary for initiating regulated splicing, translation and functional miRNA regulation[36-39]. In response to activation, platelets have been shown to splice pre-mRNA for tissue factor and IL-1β to form mature message, resulting in increased protein expression and procoagulant responses[39,40]. Platelets isolated from septic patients express spliced tissue factor mRNA, while healthy controls do not, suggesting that the regulated splicing of platelet pre-mRNAs has functional consequences in disease states[41]. Platelets are also able to generate functional progeny in a process that resembles proplatelet formation from megakaryocytes, resulting in increased biomass in cultures[42]. This observation serves as a testament to the biosynthetic capacity of platelets and their broader potential to effect functional changes in healthy and diseased states.

Platelets and eRNA

Here, we have presented evidence for the first time that platelets are capable of sequestering exogenous RNA (eRNA). Uptake of eRNA does not require prior activation of platelets nor does it induce surface expression of P-selectin or activation of integrin $\alpha_{IIb}\beta_3$. Furthermore, platelets exposed to eRNA adhere normally and activate appropriately in response to thrombin. Taken together, these data suggest that eRNA uptake does not affect traditional platelet activation responses.

We observed the uptake of eRNA by platelets to be time- and concentration-dependent, with a peak at 2h and a gradual decline overnight. In our initial studies we were surprised to find that eRNA rapidly (within 15′) associates with platelet membranes, but only about 5% of this eRNA is ultimately internalized within the platelet and protected from ribonuclease treatment. When eRNA was incubated with monocytes or THP-1 cells it was rapidly degraded, suggesting that the eRNA sequestration we observed is unique to platelets. Using an *in vitro* transcribed (IVT) 5S-rRNA tagged with an RNA aptamer capable of binding a small molecule and emitting fluorescence[43], we were able to visualize eRNA diffusely within unactivated platelets. Direct fluorophore-conjugation of a longer IVT RNA demonstrated that eRNA localizes to the central granulomere region of spread platelets. We confirmed that platelets are capable of sequestering eRNAs of varying sizes and sequence, suggesting that the process is not dependent on a conserved secondary structure within the transcript. Identifying the mechanism by which platelets take up eRNA is of significant interest to us. In nucleated cells, scavenger receptors have been shown to participate in eRNA uptake[44], and platelets are known to express the scavenger receptors CD36 and SR-BI[45]. Future studies in our lab will address the mechanism by which platelets sequester eRNA.

Despite introduction of a variety of eRNAs coding for proteins which we can readily assay for (RFP, luciferase, GM-CSF), we were unable to detect translation of eRNA in platelets under conditions tested. There are several possible explanations for this result. In nucleated cells, eRNA localizes within lysosomes and only a small fraction of internalized eRNA escapes the lysosome and is translated[44]. Despite the presence of functional translation machinery, platelet synthetic responses are much lower than those

of nucleated cells. If a large fraction of eRNA is sequestered within a lysosome-like compartment, we may be detecting transcript by RT-PCR that is unavailable for translation due to sequestration within a vesicle. Subsequently, if only a small fraction of this message is capable of escaping, the translational response of platelets may not be robust enough to yield detectable protein in our assays. Another possibility is that eRNA is sequestered in platelets and is only translated after platelets receive a secondary signal. Signal-dependent translation has been observed in platelets and represents an important mechanism by which platelets mediate inflammatory and prothrombotic processes[40,46]. We did not detect translation in thrombin-stimulated platelets, but it is possible that alternative signals could lead to translation of eRNA within platelets. Finally, it is possible that the relatively stable pool of eRNA detectable within platelets by RT-PCR is in fact partially degraded and unable to be translated into functional protein. While platelets are known to contain a variety of RNAs, the ability of platelets to degrade RNA has not been previously shown. To examine this possibility, we conducted preliminary experiments using radiolabeled IVT RNA incubated in the presence or absence of platelets or monocytes. RNA incubated with platelets was cut into several fragments of distinct sizes, >100bp each. It is likely that these fragments could represent a stable pool of template capable of generating a signal by RT-PCR, which nevertheless does not represent an intact message capable of being translated. However, a similar pattern was seen in the samples where RNA was incubated with monocytes, a condition where we do not see a signal by RT-PCR (data not shown). This result was confounding, and needs to be repeated with RT-PCR controls in tandem to allow for interpretation. Additionally, work will be done to determine whether the fragments we observe are generated by

sequence-specific cutting or by length and how long they remain within platelets.

The central role that RNA serves in mammalian cells as a means to transmit sequence-specific information for translation can overshadow some of the more basic biochemical features of RNA. Detection of foreign material is an important part of innate immunity, and foreign RNA can be recognized by Toll-like receptors (TLR) -3 and -7 as well as RIG-I and Mda5[47]. While platelets have been reported to express TLR-2, -4 and -9, they are not known to express any of the receptors commonly known to respond to foreign RNA[48,49]. Nevertheless, the ability of platelets to robustly package and secrete MP raises the possibility that eRNA taken up by platelets is sequestered and then released in MP, which can interact with cells capable of inducing an immunologic response. We performed RT-PCR on MP isolated from platelets incubated with eRNA in the presence and absence of thrombin, but did not detect significant levels of eRNA released in MP. Importantly, RT-PCR of the platelet pellet showed that the bulk of the eRNA was sequestered and detectable using this assay demonstrating that the absence of a signal in the MP fraction is unlikely to be due to degradation of the template preventing detection. This suggests that platelets do not release sequestered eRNA in MP. Nevertheless, to rule out the possibility that partially degraded eRNA is released by platelet MP, radiolabeled template can be used to detect partially degraded RNA within a MP fraction.

Extracellular RNA interactions with proteins have been shown to have important functions in hemostasis and thrombus formation. Specifically, RNA is able to bind VEGF and other growth factors with heparin-binding domains, resulting in increased endothelial cell permeability[50,51]. In addition, RNA promotes the activation of contact phase proteins, increasing their activity by 40-fold[52,53]. Administration of RNase (but not DNase) prior to

vascular injury in animal models reduces vessel occlusion and edema[50]. Our observations that platelets rapidly sequester eRNA could have two important physiological consequences in light of these reports. Upon traumatic injury to tissue, cells rupture and their cytoplasmic contents, including RNA, are released. The ability of platelets to rapidly sequester this released RNA may be important to prevent excessive coagulation responses and limit endothelial disruption. In addition, the RNA sequestered within platelets may be released under circumstances we have yet to identify, contributing to coagulation responses to maintain hemostasis. Our observation that the eRNA taken up by platelets is partially degraded would not affect its ability to function as a pro-coagulant.

The work presented here represents the first report that platelets sequester eRNA. We determined that uptake of eRNA is time- and concentration-dependent and does not depend on or result in platelet activation. The eRNA sequestered within platelets is not translated under conditions we have tested, and appears to be fragmented but not fully degraded. Future work will focus on determining the mechanisms responsible for platelet uptake of eRNA, the fate of the sequestered RNA and the contribution of eRNA to normal platelet functions. In addition, chemical modification of RNA has been shown to make it resistant to degradation, while allowing for translation[54,55]. We will explore this approach to generate functional proteins within platelets exposed to eRNA. This research will contribute to new understanding of platelet biology.

Neutrophil Extracellular Traps: Then, Now and Tomorrow

Upon their discovery, NETs were quickly appreciated for their role in contributing to host innate immune defense. Stimulation of NET formation was shown to promote killing of *Staphylococcus aureus* and *Shigella flexneri*[56]. While compelling, these initial studies were met with some skepticism. Neutrophil-mediated killing of these gram-negative and gram-positive pathogens is easily performed by phagocytosis and oxidative killing. It was difficult to understand why neutrophils would undergo a process that resulted in their demise when less radical means of defense are available. Subsequent studies demonstrated that NETs kill yeast in both the fungal and hyphal forms[57]. Hyphal yeast are too large to be engulfed by a single neutrophil, so it was thought that release of antimicrobial peptides was the primary means of host defense in this case[58-60]. The importance of NETs in defense against fungal pathogens was illustrated by a gene therapy study in a patient with chronic granulomatous disease (CGD) and a fulminant hyphal *Aspergillus nidulans* infection[61]. CGD patients have normal antimicrobial peptides and granule components, but a genetic defect in a subunit of the NADPH oxidase complex prevents oxidative burst as well as NET formation and results in patients with susceptibility to diverse pathogens[62,63]. Restoration of NADPH oxidase activity in this patient resulted in neutrophils that were now capable of forming NETs and clearance of the hyphal infection, a response which could be reversed *in vitro* when DNase was added to the system. Further supporting the role of NETs in normal host defense, it has been shown that neonatal neutrophils are unable to form NETs in response to agonists[64]. Neonates are known to have an incompletely characterized, multifactorial neutrophil dysfunction, which leaves them particularly vulnerable to infections, including life

threatening neonatal sepsis[65]. The defect in neonatal neutrophils preventing NET formation appears to be downstream of NADPH oxidase[64], and further characterization of this pathway may result in treatments which restore NET formation and innate immune function in this vulnerable population.

While the importance of NETs in innate immune defense is now widely accepted, it is also increasingly appreciated that NETs can contribute to disease. In mouse models of sepsis, NET formation has been shown to trap bacteria, but result in damage to the endothelium in liver sinusoids, possibly contributing to the decreased perfusion and end-organ dysfunction seen in sepsis[27]. NETs have also been implicated in pathogen-independent diseases such as transfusion related acute lung injury (TRALI), where markers of NET formation are elevated in the blood of TRALI patients[66,67]. In mouse models of TRALI, administration of DNaseI improves oxygen saturation and prevents neutrophil accumulation within the alveoli, suggesting that NETs contribute directly to disease pathogenesis. Tissue sections from the placenta of patients with preeclampsia show large numbers of NETs in the intervillous space that are not seen in normal placentas, and *in vitro* studies with placental-derived inflammatory factors are able to induce NET formation in culture[68]. NETs have also been implicated in the break in immunologic tolerance seen in the autoimmune disease systemic lupus erythematosus (SLE). SLE is an autoimmune disease characterized by B-cell production of antibodies against nuclear self-antigens (such as DNA and histones), chronic plasmacytoid dendritic cell (pDC) activation, immune complex formation and multiple organ dysfunction[69]. During NET formation, DNA forms complexes with antimicrobial peptides found within neutrophil granules before being released from the cell. Complexes of DNA with one of

these peptides, LL37, have been shown to break pDC tolerance to self DNA resulting in interferon production and the promotion of B-cell autoantibody production through TLR-9[70,71]. Surprisingly, serum from a subset of patients with SLE demonstrated a decreased ability to degrade NETs and activated complement deposition on NETs, which may promote autoantibody production[72]. Taken together, these studies underscore the need for exquisite control over NET formation and how an imbalance can result in life-threatening consequences.

Our interest in developing a genetic screen to identify factors required for NET formation was precipitated by our incomplete understanding of the molecular process driving NET formation. Early studies demonstrated the requirement for ROS generation using small molecules as well as neutrophils from patients with CGD, which are incapable of generating ROS[63]. Additional experiments using mouse models, human variants and drugs established the role of neutrophil elastase, peptidylarginine deiminase 4, mammalian target of rapamycin and myeloperoxidase in NET formation[73-76]. Despite such rapid progress, large gaps remain linking signaling events between pathogens, platelets and inflammatory cytokines and the complex process required to generate NETs.

Using retroviral insertional mutagenesis in the mostly haploid human promyelocytic leukemia cell line, KBM-7, host factors required for pathogenesis and intoxication have been identified[77-79]. We demonstrated that KBM-7 cells are capable of forming NETs, a function which has only been reported in one other immortalized cell line to date[80]. We also successfully developed a plate-based assay to quantitate NET formation in mutagenized clones. Next, we set out to use gene-trap mutagenesis with the goal of generating a large pool of mutagenized KBM-7 cells, with successful insertion

into a coding gene resulting in expression of the reporter GFP. This would allow us to sort individual GFP-positive cells representing unique insertion events into 96-well plates using fluorescence-activated cell sorting and expand these clones in culture until there were sufficient cells to transfer to our plate-based assay and screen for NETs. Insertion into a gene required for NET formation would result in decreased signal in our NET assay. We would then sequence the insertion site to identify novel genes required for NETosis. Unfortunately, we were unable to develop conditions that allowed for successful transduction of KBM-7 cells with gene-trap retrovirus as detected by GFP expression.

Upon close examination of the publications using KBM-7 cells to conduct genetic screens, it is clear that other researchers have focused their efforts on screens that rely on resistance to a lethal phenotype. While NETosis is a terminal process for the individual cell, only a subset of cells within a population generate NETs in response to agonist[63]. The reasons for this observation remain unclear, but we determined that it is not due to heterogeneity within the population, as limited dilution cloning does not produce clones capable of varying degrees of NETosis. Furthermore, prolonged culture in the presence of agonist does not result in a gradual decline in cell counts. As our screen depended on the ability to screen mutagenized cells using expression of the reporter rather than a lethal phenotype, we were unable to execute the screen as designed.

Despite this setback, our interest in identifying novel genes required for NET formation remains. The first paper using KBM-7 cells in a genetic screen reported successful detection of GFP expression in mutagenized cells by flow cytometry. Our failure to detect GFP expression in KBM-7 cells mutagenized with the gene-trap virus as

well as a virus that induces constitutive expression of GFP from a CMV promoter suggests that our transduction efficiency was extremely low. In personal conversations with Dr. Brummelkamp, whose lab originally reported the use of these cells[77], we were told that transduction of KBM-7 cells can be tricky. Using detailed protocols from the Brummelkamp lab we still failed to successfully detect GFP expression in our mutagenized cells, suggesting that additional optimization is necessary.

Modifying our original screen design to allow for a pooled, rather than a subcloned, approach may also prove fruitful. Such a strategy would rely on powerful bioinformatics, but may be possible within the near future. The design of this screen would consist of mutagenesis of a pool of haploid cells, followed by removal of a fraction of these cells and genomic DNA isolation and analysis of insertion sites as a reference population. Subsequently, the pool of mutagenized cells would be treated with agonist to induce NET formation, DNase would be added to digest the extracellular DNA, and insertion sites within the extracellular DNA would be mapped. Insertions in genes within the reference population which are not found within the extracellular DNA would suggest genes that are required for NET formation, and would be further analyzed in additional studies. As the bulk of insertion events are unlikely to affect NET formation, this approach amounts to searching for the proverbial needle in a haystack. Nevertheless, only a decade ago the sequence of the first complete human genome was published in a massive undertaking that took 13 years to complete[81], and now companies advertise complete genome sequencing within a day for a $1,000. As the power to sequence and analyze large data sets improves, a pooled screen to identify factors required for NETosis may be similar to searching for a needle in a haystack with a metal detector.

Kindlins and Integrin Signaling

The identification of mutations in the *FERMT3* gene, coding for Kindlin-3, as the primary defect in all of the reported cases of LAD-III solved a long-standing question in the field of integrin biology[82-86]. Patients with LAD-III suffer from recurrent infections as well as a severe bleeding tendency, implicating a protein that is required for function of β1, β2 and β3 integrins[87]. Nevertheless, as integrins are required for cell-matrix interactions in cells throughout the body, the restriction of the LAD-III phenotype to hematopoietic cells suggested that this protein is expressed in a tissue-restricted fashion. Indeed, of the three members of the Kindlin family (Kindlin-1, -2 and -3) expressed in mice and humans, only Kindlin-3 expression is restricted to hematopoietic cells[88] and deletion of kindlin-3 in mice phenocopies the defects seen in LAD-III patients[89].

Integrins are present as αβ heterodimers on the surface of cells, and require an intracellular signal to undergo a conformational change resulting in an active conformation capable of engaging ligand (inside-out signal)[90]. The intracellular tail domains of β integrins and the proteins they bind, including Talins and Kindlins, are necessary for this process[91]. While Talins bind a membrane proximal motif on β integrin tails, Kindlins have been shown to bind an identical motif on the distal tail[89,92]. Initially, it was unclear whether Kindlin served as a cofactor to recruit Talin to the plasma membrane or if it played a more direct role in activation of the integrin. Studies in Chinese hamster ovary cells using shRNA knockdown of Kindlin showed that loss of Kindlin prevented ligand binding but did not affect Talin recruitment to the membrane, suggesting that Kindlins are important coactivators of integrin signaling[93]. In addition, Kindlins link signals downstream of chemokine receptors to integrin activation to initiate inside-out

signaling[94]. Recent biochemical studies have demonstrated that several distinct regions within Kindlins are necessary for interactions with β integrins, phospholipid head groups within the plasma membrane and PIP3, and all are required for robust inside-out integrin signaling[95-97].

Kindlins have also been implicated in outside-in integrin signaling. Upon ligand binding, integrins transmit signals to the cytoskeleton and act on many intracellular signaling pathways important for development, leukocyte trafficking and hemostasis[98]. The role Kindlins play in inside-out signaling can be separated from outside-in signaling experimentally by bypassing inside-out signals pharmacologically or using constitutively active receptors. Several groups have shown defects in downstream integrin signaling in the absence of Kindlin, suggesting that Kindlins serve to transmit signals downstream of integrin activation to mediate outside-in signals[99-102].

The studies discussed above represent an aggregation of reports looking at Kindlin-1, Kindlin-2 and Kindlin-3 as well as a variety of integrin heterodimers. Nevertheless, both Kindlins and β integrin tails show a high degree of conservation within their respective families, suggesting that the broad concepts regarding function will be generally applicable and specificity will be achieved largely through ligand interactions and tissue-specific expression of additional binding partners[91,103]. The importance of Kindlins in integrin signaling is now widely accepted, and highlighted by the clinical defects seen in LAD-III patients. Our work demonstrating that the LAD-III index family we had previously identified had a mutation in *FERMT3* that resulted in an absence of Kindlin-3 (Fig. 4.1) confirmed that defects in this protein are responsible for every case of LAD-III identified[86,104]. Nevertheless, the Kindlin-3 mutations reported to

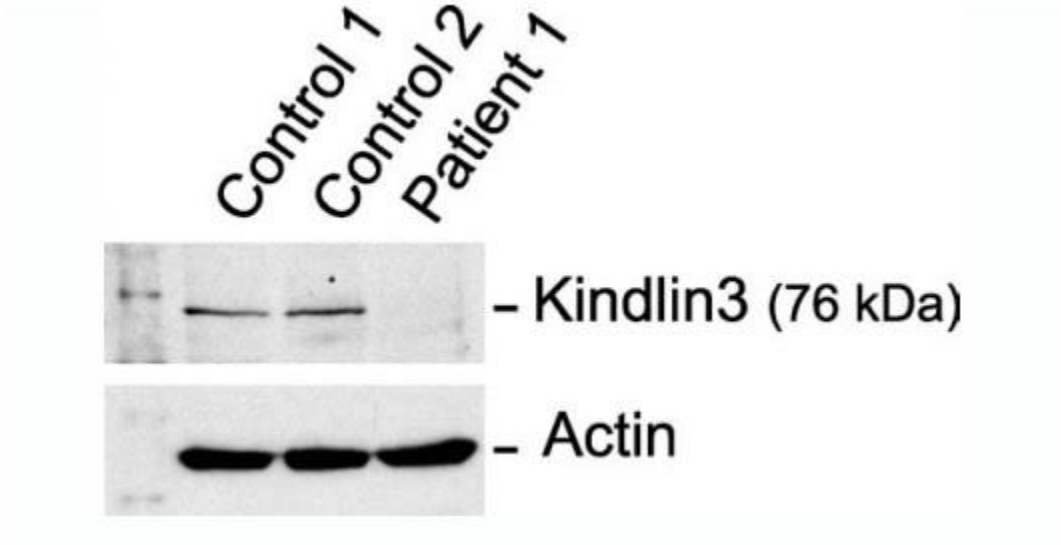

Figure 4.1: Kindlin-3 is absent in EBV-transformed lymphoblasts from Patient 1[86,104]. Lymphoblasts from Patient 1 and two control subjects were lysed, subjected to electrophoresis, and immunoblotted for Kindlin-3 or actin. Reproduced with permission[86].

date are found throughout the gene and in genetically diverse patient backgrounds[105]. While key features of the LAD-III phenotype are shared between patients, there is variation in the severity and bony involvement[106,107]. Additional genetic modifiers may explain these differences and identifying them will further our understanding of integrin signaling.

Perspectives

In science, as in life, there is always more to be learned. The work presented here serves to illuminate previously unrecognized clinically-relevant biology, as well as gaps in our understanding of fundamental hematological processes for future research. Upon first glance, platelets appear to be simple anucleate cytoplasts with the singular function of maintaining hemostasis. However, closer examination reveals a complex capacity to integrate signals from diverse inputs and affect changes both autonomously and in conjunction with other cells within the hemostatic and immune continuum. Neutrophils were thought to have given up their secrets many decades ago, but recently revealed a new mechanism by which they protect the host from infection and contribute to inflammatory pathologies. The complexities of integrin signaling continue to provide insight into genetic diseases and new targets for therapeutics. The privilege and the responsibility of the researcher is to continue to ask questions in the face of answers, and to shine a light in the dark in search of answers to questions that have yet to be asked.

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APPENDIX A

LEUKOCYTE ADHESION DEFICIENCY-I VARIANT SYNDROME (LAD-1v, LAD-III): MOLECULAR CHARACTERIZATION OF THE DEFECT IN AN INDEX FAMILY

Reprinted with permission of the American Journal of Hematology. Harris E, Smith T, Springett G, Weyrich A, Zimmerman G. Leukocyte adhesion deficiency-I variant syndrome (LAD-Iv, LAD-III): molecular characterization of the defect in an index family. *American Journal of Hematology*. 2012; 87(3):311-3.

Leukocyte adhesion deficiency-I variant syndrome (LAD-Iv, LAD-III): Molecular characterization of the defect in an index family

Estelle S. Harris,[1]* Tammy L. Smith,[1,2,3] Gregory M. Springett,[4] Andrew S. Weyrich,[1,2,3] and Guy A. Zimmerman[1]*

Leukocyte adhesion deficiencies are rare clinical syndromes of impaired host defense that provide novel insights into regulation of immune and inflammatory responses [1,2]. Leukocyte adhesion deficiency (LAD)-I variant (LAD-Iv), also called LAD-III, is a unique disorder in which inside-out signaling of β_1, β_2, and β_3 integrins on leukocytes and platelets is disrupted, leading to impaired cellular adhesion, recurrent infections, and bleeding [1–3]. We originally reported the second patient with this disorder to be identified and characterized the adhesive deficiencies and functional phenotype of this subject's leukocytes [4]. Here, we show that the molecular defect in this index subject is a new mutation in *FERMT3* (*KINDLIN-3*) which encodes KINDLIN-3, a cytoskeletal protein that interacts with the cytoplasmic tails of β_1, β_2, and β_3 integrins and is required for inside-out and outside-in signaling of these heterodimers [5,6]. We also report clinical features and previously unrecognized defects in cells from a new patient, a sibling of the original subject that we described who carries the same *FERMT3* mutation. Mutations in *FERMT3* have now been shown to be the basis for LAD-Iv/LAD-III in each of the four original patients or families that established this syndrome [4,7–9], including the family that we describe.

LAD syndromes provide critical insights into molecular regulation of integrin- and selectin-mediated leukocyte adhesion and also reveal other key features of integrin and selectin biology [1,5,6,10]. LAD-I is caused by mutations in the β_2 subunit of the leukocyte integrin heterodimer family ("β_2 integrins"), leading to absent or dramatically reduced levels of β_2 integrins on the surfaces of myeloid leukocytes and lymphocytes; in contrast, LAD-II is a rare disorder of selectin ligand fucosylation [1,11]. LAD-I variant (LAD-Iv), also termed LAD-III (abbreviated LAD-Iv/LAD-III here), is also extremely rare with fewer than two dozen patients reported in the world's literature. LAD-Iv/LAD-III results from molecular defects in inside-out signaling of integrins with preserved surface expression of integrin heterodimers [1–3]. Patients with LAD-I, LAD-II, and LAD-Iv/LAD-III have recurrent, often life-threatening, local and systemic infections secondary to impaired leukocyte adhesion, absent or dramatically reduced accumulation of polymorphonuclear leukocytes (PMNs; neutrophils) at extravascular sites of microbial invasion (deficient pus formation), and other defects in antimicrobial defensive functions [1–3,11]. A striking feature unique to LAD-Iv is bleeding due to impaired platelet adhesive functions. Defective platelet adhesion in LAD-Iv is similar to that in Glanzmann Thrombasthenia and can be severe and life-threatening [1–3].

Original description of LAD-Iv/LAD-III and early characterization of defects in adhesive functions of leukocytes and platelets in this syndrome came from reports of four index patients [4,7–9]. Manifestations in these subjects defined a unique disease phenotype [1,2], with subsequent reports of additional rare patients establishing the natural history of the disorder and its common and variable features [3]. The original index patient that we identified yielded the first evidence for defective inside-out signaling of β_1, as well as β_2 and β_3, integrins [4]. This pattern, together with intact expression and structure of these integrins on leukocytes and platelets, became a defining characteristic of the defective adhesive phenotype in the new syndrome of LAD-Iv/LAD-III [1,3].

The index subject (Patient 1), a boy, is the 3rd child of Hispanic parents who emigrated to the U.S. from Mexico. He presented with recurrent infections, leukocytosis, absent pus formation, mucosal bleeding, impaired platelet aggregation, and absent clot retraction at 3 weeks of age and underwent bone marrow transplantation at 8 months. Analysis of defective inside-out signaling of β_1 and β_2 integrins on his primary PMNs and EBV-transformed lymphoblasts was reported in detail [4]. He has been free of complications of immunodeficiency and bleeding since bone marrow transplantation. Two older sisters were healthy at birth and had no manifestations of immunodeficiency or bleeding problems. Additional information collected after the original report indicated no family history of immunodeficiency but revealed that the grandfathers were cousins.

Subsequently, other investigators reported impaired activation of Rap-1 due to decreased expression of the RAP guanine nucleotide exchange factor, CALDAG-GEF1, as a cause of LAD-Iv/LAD-III [12,13]. Therefore, we examined EBV-transformed lymphoblasts from Patient 1 for this defect. We found normal levels of CALDAG-GEF-1 and Rap-1 in these cells, and no abnormalities when the sequence of *CALDAG-GEF1* was analyzed. Furthermore, overexpression of CALDAG-GEF1 in transformed lymphoblasts from Patient 1 did not rescue defective integrin function (not shown). Expression of other candidate factors that might alter integrin signaling if absent or deficient, including IAP (CD47), UPAR (CD87), CD98, ICP-1, ILK, and RACK-1, was comparable to that in control cells (not shown).

Patient 2, a male sibling born 5 years after Patient 1, was noted to have cutaneous bleeding at sites of minor injury, ecchymoses, and petechiae shortly after uneventful term delivery. He was treated for sepsis at 1 week of age. LAD-Iv/LAD-III was immediately considered because of the clinical presentation and family history; further evaluation revealed leukocytosis and hepatosplenomegaly, consistent with LAD-Iv/LAD-III [3,4]. LAD-I was excluded by flow cytometric analysis, which demonstrated normal expression of β_2 integrins. Defects in inside-out signaling of leukocyte integrins (see below) confirmed the diagnosis of LAD-Iv/LAD-III. Patient 2 was treated for additional infections including oral candidiasis, omphalitis, and two episodes of *Staphylococcal bacteremia* before bone marrow transplantation at 15 weeks of age. Like Patient 1, he remains well after transplantation, with no further evidence of leukocyte dysfunction or thrombasthenia.

In vitro analysis of leukocytes from Patient 2, using approaches similar to those for characterization of cellular defects in Patient 1 [4], demonstrated impaired stimulated adhesion of isolated PMNs to purified fibrinogen and other ligands for β_2 integrins (Fig. 1A and data not shown). In addition, there was a defect in stimulated adhesion of primary PMNs to fibronectin, a ligand for β_1 integrins, whereas an activating anti-β_1 mAb, TS 2/16 [4], induced submaximal adhesion (Fig. 1B). Transformed lymphoblasts from Patient 2 also failed to adhere to immobilized fibronectin when activated with phorbol myristate acetate (PMA) (Fig. 1C). In contrast, lymphoblast adhesion to fibronectin increased four- to eightfold in response to exogenous Mn^{2+}, which induces integrins to shift to the active allosteric conformation and allows them to recognize and bind ligands without a requirement for inside-out signaling [4,8], or when they were incubated with the integrin activating antibody (Fig. 1C). These findings indicate that the basally expressed surface integrins are functional and that the phenotype of impaired adhesion is due to defective inside-out signaling [3,4]. Isolated PMNs from the parents of Patients 1 and 2 adhered as expected when stimulated with fMLP or PMA (not shown).

Although PMNs, lymphocytes, and transformed lymphoblasts from patients with LAD-Iv/LAD-III have been examined intensively, there is a paucity of studies of monocytes. We found that there was a dramatic decrease in adhesion of monocytes from Patient 2 (Fig. 2). In addition, the few residual cells failed to spread and differentiate in culture (Fig. 2) under conditions in which monocytes develop into macrophages [14], suggesting defects in outside-in signaling and inside-out activation of integrins [5], and that deficiencies in monocyte adhesion and differentiation to macrophages may have accounted for some of the features of the clinical phenotype of Patient 2. Nevertheless, neither Patient 2 nor Patient 1 had evidence for osteopetrosis, which has been reported in some patients with LAD-Iv/LAD-III [15–18] and has recently been ascribed to defective integrin signaling in monocyte-derived osteoclasts [19]. We were not able to perform additional studies of monocytes from Patient 2 because of semiemergent bone marrow transplantation.

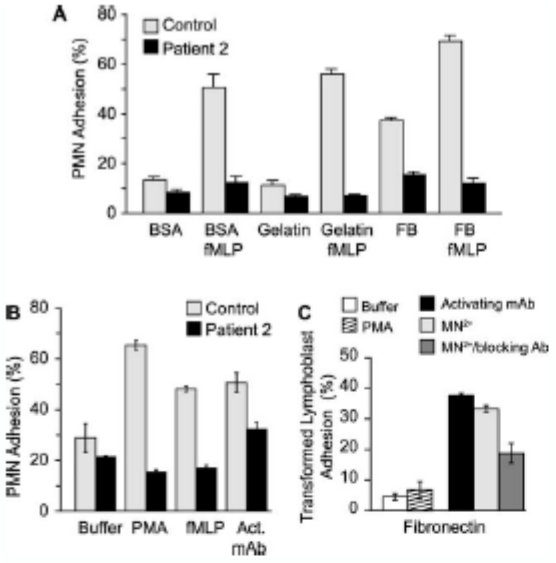

Figure 1. Leukocytes from Patient 2 demonstrated the LAD-Iv/LAD-III phenotype of impaired inside-out signaling of β_1 and β_2 integrins. A: PMNs from Patient 2 did not adhere to β_2 integrin ligands when activated. PMNs from Patient 2 or a healthy age-matched infant were incubated for 10 min at 37°C in wells coated with bovine serum albumin (BSA), gelatin, or fibrinogen (FB) in the absence of an agonist or with *n*-formyl-methionyl-phenylalanine (fMLP) (10^{-7}M). Adhesion was quantitated as described in Methods. This figure illustrates results from a single experiment (performed in triplicate). The same pattern was demonstrated in two other experiments in which PMNs from Patient 2 and control subjects were activated with fMLP or PMA (10^{-7}M) (data not shown). B: Activated PMNs from Patient 2 did not adhere to fibronectin. PMNs from Patient 2 or a control subject were incubated on wells coated with fibronectin in the presence or absence of PMA or fMLP as in panel A. In parallel, PMNs in some wells were treated with the β_1 integrin activating antibody TS 2/16 ("act mAb"; 10 μg/ml). This figure indicates the pattern of adhesion in a single experiment (performed in triplicate) and is representative of two additional experiments examining primary PMNs from Patient 2. C: Adhesion of EBV-transformed lymphoblasts from Patient 2 to immobilized ligands was induced by extracellular Mn^{++} or mAb TS 2/16 ("activating mAb"; see legend to panel B). Lymphoblasts from Patient 2 or a control subject were incubated for 15 min at 37°C in wells coated with fibronectin with buffer alone, or with PMA (10^{-7}M) (performed in triplicate). In parallel wells, lymphoblasts were treated with activating mAb, Mn^{++} (1 μM), or Mn^{++} together with a blocking anti-β_1 mAb (mAb p4C10, 10 μg/ml). A similar pattern was seen in a second experiment. In additional experiments, extracellular Mn^{++} induced adhesion of lymphoblasts from Patient 2 to immobilized ICAM-1, a ligand for β_2 integrins; this was inhibited by a blocking anti-β_2 mAb (mAb 60.3; 10 μg/ml) ($N = 2$, data not shown).

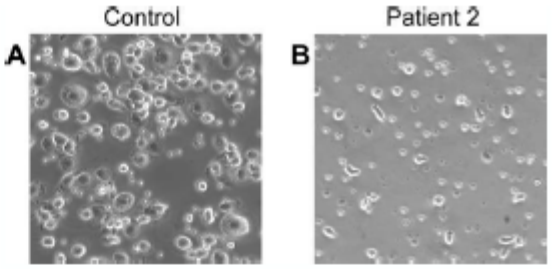

Figure 2. Monocytes from Patient 2 did not adhere or differentiate in culture. Mononuclear cells from a healthy volunteer (*A*) or a Patient 2 (B) were incubated in culture wells for 45 min, nonadherent cells were removed, and the adherent monocytes were washed, covered with fresh medium, and incubated at 37°C in 5% CO_2 for an additional 48 hr. Representative fields were then photographed. These figures illustrate the cellular morphology in one experiment examining monocytes from this subject.

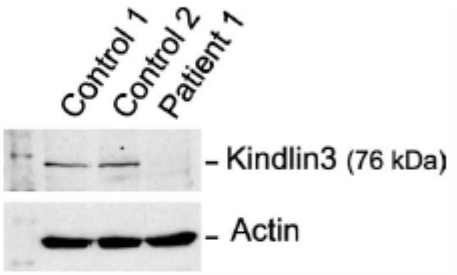

Figure 3. KINDLIN-3 is absent in EBV-transformed lymphoblasts from Patient 1. Lymphoblasts from Patient 1 and two control subjects were lysed, subjected to electrophoresis, and immunoblotted for KINDLIN-3 or actin as described in Methods.

Subsequently, reports by other investigators indicated that kindlin-3 is required for activation of β_3 integrins and $\alpha_{IIb}\beta_3$ on murine leukocytes and platelets [20,21]. Kindlin-3-deficient murine platelets and PMNs have adhesive defects that phenocopy those of cells from subjects with LAD-Iv/LAD-III [20,21]. In addition, mutations in *FERMT3* (*KINDLIN 3*), which encodes human KINDLIN-3, were identified in patients with LAD-Iv/LAD-III [15,22–24]. KINDLIN-3 is largely restricted to hematopoietic cells and activates integrins by directly interacting with the cytoplasmic tails of β subunits, as do other members of the evolutionarily conserved Kindlin family [5,6]. Therefore, we examined *FERMT3* by sequence analysis of RT-PCR products from transformed lymphoblasts from Patient 1 and found a point mutation in codon 476 of exon 12, *CAG→TAG* (Supporting Information Fig. 1). Sequence analysis of RT-PCR products from genomic DNA in samples from Patients 1 and 2 again revealed this mutation, whereas DNA from each parent demonstrated heterozygosity for the *CAG→TAG* variation (Supporting Information Fig. 1). The mutation in Patients 1 and 2, which has not been previously reported in LAD-Iv/LAD-III patients, is predicted to create a premature stop codon in the sequence of *FERMT3* encoding the PERM F2 subdomain of KINDLIN-3 [5]. Consistent with this, KINDLIN-3 was undetectable in lysates of transformed lymphoblasts from Patient 1 by western analysis (Fig. 3).

Mutations in *FERMT3* have now been identified in the four index LAD-Iv/LAD-III patients [2–4,7–9], each with similar clinical features, including subjects of Hispanic [4] (this report), Turkish [7], Maltese [8], and Israeli Palestinian [9] descent. These mutations are in exon 12 [22], the splice acceptor site of exon 14 [24], and exon 2 [25] (A. Etzioni, personal communication) of the *FERMT3* gene, in addition to the new mutation in exon 12 that we report here. Each leads to reduced levels or absence of *KINDLIN3* mRNA and KINDLIN-3 protein in leukocytes and/or platelets [22,24,25] (Fig. 3). Transfection of one subject's EBV-transformed lymphoblasts with murine *kindlin-3* complementary DNA rescued the adhesion defect in these cells [24]. In addition to these four original subjects and, in two instances, siblings of these index patients [25] (this report), mutations in *FERMT3* have been found in all LAD-Iv/LAD-III patients identified to date, including subjects of Turkish [15,22,24], Arabic [23], African-American [17,18], and Gypsy [26] heritage. Thus, the unifying molecular basis for LAD-Iv/LAD-III from currently available genotyping information is mutation of *FERMT3* in one or more [17] regions that disrupts expression of KINDLIN-3, alone or together with other sequence variations [22,26]. Nevertheless, there is also variability in severity, bony involvement, and natural history among LAD-Iv/LAD-III patients [3,22,23], suggesting that there may be genetic or molecular alterations that are not yet apparent [27,28].

Methods

Cells and cellular assays. Isolation of primary PMNs, preparation of EBV-transformed lymphoblasts, adhesion assays, and antibodies and reagents were previously described in detail [4]. These studies were approved by the University of Utah Institutional Review Board. Adhesion of monocytes and their culture to monocyte-derived macrophages were done as previously reported [14].

Sequence analysis of CALDAG-GEF1 and FERMT3. Messenger RNA (mRNA) was isolated as previously described [29]. We analyzed mRNA-derived cDNA from transformed lymphoblasts from Patient 1 (Supporting Information Fig. 1, Panel A) and from control EBV-transformed lymphoblasts [4]. Genomic DNA isolated from Patients 1 and 2 (buccal mucosal cells) and both parents (peripheral blood leukocytes) (Supporting Information Fig. 1, Panel B) was also examined for sequence abnormalities in all exons and intron-exon boundaries for *FERMT3* using primer sequences provided by

Kuijpers et al. [22]. Sequencing was accomplished in the University of Utah School of Medicine sequencing core facility using standard methods. Genomic DNA from transformed lymphoblasts from Patient 1 and control EBV-transformed lymphoblasts was also analyzed for sequence alterations in all exons and intron-exon boundaries of *CALDAG-GEF1* using sequences from GENBANK (not shown).

Western analysis. EBV-transformed lymphoblasts (2×10^7/mL) were lysed in RIPA (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% SDS, 2 mM EDTA, 1% Triton X-100) and mammalian protease inhibitor cocktail (Sigma–Aldrich). Thirty-five micrograms of cleared protein lysate were reduced in Laemmli buffer and separated by 12% Tris-glycine SDS-polyacrylamide gel electrophoresis, and transferred to Hybond ECL Nitrocellulose membranes (GE Healthcare). Blots were probed with anti-KINDLIN3 polyclonal antibody (Sigma–Aldrich) or anti-ACTIN monoclonal antibody (MP biomedicals) and goat anti-mouse IgG conjugated to HRP as a secondary antibody (Invitrogen) and detected using ECL western blot detection reagents (GE Healthcare).

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[1]Department of Medicine, University of Utah School of Medicine, Salt Lake City, Utah; [2]Program in Molecular Medicine, University of Utah School of Medicine, Salt Lake City, Utah; [3]Department of Pathology, University of Utah School of Medicine, Salt Lake City, Utah; [4]Drug Discovery Program, Moffitt Cancer Center, Tampa, Florida

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**Correspondence to: Guy A. Zimmerman, Eccles Institute of Human Genetics, 15 North 2030 East, Rm 4220, Salt Lake City, UT 84112. E-mail: guy.zimmerman@u2m2.utah.edu or Estelle S. Harris, Eccles Institute of Human Genetics, 15 North 2030 East, Rm 4220, Salt Lake City, UT 84112. E-mail: estelle.harris@u2m2.utah.edu*

Additional Supporting Information may be found in the online version of this article.

Conflict of interest: Nothing to report.

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APPENDIX B

PLATELETS AS CENTRAL MEDIATORS OF SYSTEMIC INFLAMMATORY RESPONSES

Reprinted with permission of Elsevier Publishing. Tammy L. Smith and Andrew S. Weyrich. Platelets as Central Mediators of Systemic Inflammatory Responses. *Thrombosis Research*. 2011; 127(5): 391-394.

Review Article

Platelets as Central Mediators of Systemic Inflammatory Responses

Tammy L. Smith [a,c], Andrew S. Weyrich [a,b,c,*]

[a] Program in Molecular Medicine, University of Utah, Salt Lake City, Utah 84112
[b] Department of Internal Medicine, University of Utah, Salt Lake City, Utah 84112
[c] Department of Microbiology and Immunology, University of Utah, Salt Lake City, Utah 84112

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ABSTRACT

Systemic inflammatory responses are associated with high morbidity and mortality and represent a diverse and clinically challenging group of diseases. Platelets are increasingly linked to inflammation, in addition to their well-known roles in hemostasis and thrombosis. There is agreement that traditional functions of platelets, including adherence, aggregation, and secretion of preformed mediators, contribute to systemic inflammatory responses. However, emerging evidence indicates that platelets function in non-traditional ways. In this review, we focus on new functions of platelets that may be involved in the host response to infection.

Contents

Introduction

The role of platelets in the host response to infection is increasingly appreciated. Platelet function is often compromised in diseases as diverse as human immunodeficiency virus (HIV), dengue and sepsis and disease severity and mortality correlate with the degree of thrombocytopenia that is clinically observed [1–3]. The etiology of thrombocytopenia in infectious diseases is complex but is typically due to decreased production of platelets and/or increased sequestration and clearance of activated platelets in the periphery. In regards to production, platelets are formed from megakaryocytes, which typically reside in the bone marrow but have also been observed in the circulation and lungs [4]. Several studies have shown that megakaryopoiesis and/or thrombopoiesis become disrupted during infection, contributing to reduced platelet counts [5]. There is also evidence that platelets continue to develop in the circulation [6]. In this regard, our group recently reported that circulating platelets themselves have the ability to divide and form functional progeny [7]. Whether or not the formation of progeny contributes to circulating platelet counts is unknown. However, when platelets encounter thrombin or *E. coli* they fail to produce progeny raising the possibility that this new function of platelets may contribute to the thrombocytopenia observed in infectious situations [7].

Although the production of platelets is unquestionably affected as the host encounters pathogens, it is more recognized that systemic infections are associated with increased platelet activation in the bloodstream. Activated platelets bind other platelets and leukocytes in the bloodstream setting off a cascade of events that contribute to the development, evolution, and resolution of the systemic inflammatory response (Fig. 1). Abnormal sequestration of platelets in the microcirculation induces thrombocytopenia and often leads to disseminated intravascular coagulation (DIC). In critically ill patients, DIC participates in the development of multiple organ failure and often death. Understanding how platelets become activated and the

* Corresponding author. Eccles Institute of Human Genetics, Bldg 533, Rm 4220, University of Utah, Salt Lake City, Utah 84112, USA. Tel.: +1 801 585 0702.
E-mail address: andy.weyrich@u2m2.utah.edu (A.S. Weyrich).

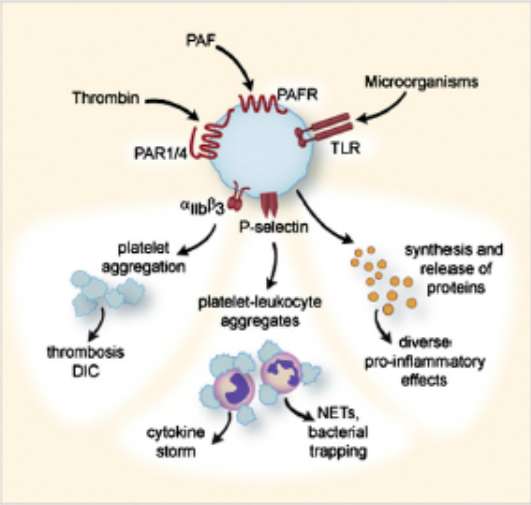

Fig. 1. *Platelets integrate a variety of inputs to mediate pathology in systemic inflammatory responses.* Thrombin, PAF, and microorganisms are commonly observed in infectious situations. Each of these factors modulates platelet function, inducing both rapid and prolonged responses that include the formation of homotypic and heterotypic aggregates. Platelet-platelet aggregates and platelet-leukocyte aggregates contribute to DIC, the formation of NETs, and the synthesis and secretion of proinflammatory cytokines that are commonly observed as the host responds to infection. These responses may help fight off infection, but they can also contribute to the pathology of the disease. Abbreviations: DIC, disseminated intravascular coagulation; NETs, neutrophil extracellular traps; PAF, platelet activating factor; PAFR, PAF receptor; PAR, protease activated receptor; TLR, Toll-like receptor.

downstream consequences of platelet activation will contribute to the treatment of infectious diseases and the development of new therapeutics. In this review, we briefly focus on mechanisms by which platelets become activated during infectious states, paying particular attention to the emerging role of toll-like receptors (TLRs) in this process. We then discuss downstream consequences of platelet activation, focusing on recently identified prolonged actions of platelets that are turned on during the host response to infection.

The Road to Platelet Activation in Infectious Disease

The infectious milieu provides a variety of signals that lead to platelet activation (Fig. 1). It is well known that platelet-activating factor (PAF) and thrombin are generated by host cells as they encounter pathogens [8]. Both PAF and thrombin signal through G-protein coupled receptors inducing rapid changes in platelets including secretome release and translocation of P-selection to the surface of the platelets, where it serves as a tether for leukocytes. PAF and thrombin also activate integrin $\alpha_{IIb}\beta_3$, allowing it to bind fibrinogen and bridge platelets to one another. Homotypic platelet aggregates deposit in the vasculature, leading to thrombosis, DIC and consumption of platelets. Because of their established roles in platelet activation and coagulation, PAF and thrombin have been the targets of clinical trials designed to improve outcomes in patients diagnosed with sepsis [9,10].

While the effects of PAF and thrombin are well characterized, new agonists that induce platelet activation are continually being identified. Among these are TLR agonists, including lipopolysaccharide (LPS). Human and murine platelets express TLR2, -4 and -9 [11,12]. TLR5 has also been identified in human platelets [13]. Unlike classical agonists, however, TLR-dependent signaling typically does not have a direct effect on traditional platelet functions, such as aggregation or secretion [14]. However, studies in knockout mice (TLR4-/-) demonstrate that platelet TLR4 is responsible for mediating LPS-induced thrombocytopenia [15]. Furthermore, thrombocytopenic mice inadequately produce tumor necrosis alpha (TNF-α) in the presence of LPS unless they are simultaneously transfused with platelets [16]. Together, these *in vivo* data indicate that activation of platelet TLR4 is involved in the clearance of circulating platelets and the generation of proinflammatory cytokines in endotoxemia.

Recent studies from Washington and colleagues [17] demonstrate that LPS markedly increases plasma levels of sTLT-1 (soluble Triggering Receptor Expressed on Myeloid Cells-like [TREM] transcript-1) in mice. sTLT-1 is also increased in patients diagnosed with sepsis compared to healthy controls. The exact functions of TLT-1 in platelets, which is stored in α-granules and translocated to the surface in response to thrombin, collagen, and LPS, are still emerging. To date, it is known that activated platelets release sTLT-1, which binds fibrinogen and augments platelet aggregation. Mice that lack TLT-1 have defects in platelet aggregation and more readily succumb to challenges with LPS. TLT-1 deficient mice also display higher plasma levels of TNF-α and D-dimers when compared to their wild-type counterparts, indicating that platelet-derived TLT-1 functions to dampen the inflammatory response to infection.

Recently Identified Consequences of Platelet Activation in Infectious Disease

Activated Platelets Synthesize and Secrete Pro-inflammatory Cytokines

In professional phagocytes, such as neutrophils and macrophages, LPS signaling is linked to *de novo* gene expression. A decade ago, it was heretic to think that platelets were capable of regulated gene expression. However, our thinking has evolved. It is now well accepted that platelets have central roles in inflammation. In addition to rapid release of inflammatory mediators that are stored in granules and immediately released upon activation, platelets also synthesize proteins in response to extracellular cues [18]. Our first clue into the role of protein synthesis by platelets was in 1998 when we demonstrated that thrombin-activated platelets synthesize B-cell lymphoma 3 (Bcl-3) [19]. Synthesis of Bcl-3 begins within 15 minutes of activation and lasts for at least 8 hours, demonstrating that stimulated platelets continue to function over time. As Bcl-3 is synthesized by platelets, it binds to Fyn and regulates cytoskeletal-mediated processes [19]. In follow-up investigations, we demonstrated that targeted deletion of Bcl-3 prevents platelets from retracting fibrin-rich clots [20]. This suggests that protein synthetic events in platelets may be important for recanalization of thrombosed vessels.

In addition to Bcl-3, platelets synthesize other proteins that play critical roles in the host response to infection. One of these is interleukin-1β (IL-1β). In 2005, we discovered that circulating platelets contain precursor mRNA (pre-mRNAs) for IL-1β [21]. In response to activating signals, platelets splice IL-1β pre-mRNA into mature message. Spliced IL-1β mRNA is subsequently used as a translation template to produce new protein. In our original studies, we used thrombin to induce pre-mRNA splicing in platelets. More recently, Shashkin and colleagues demonstrated that LPS is a potent inducer of pre-mRNA splicing in platelets [22]. Using IL-1β and cycloxygenase-2 (COX-2) as index messages, these investigators demonstrated that the magnitude of pre-mRNA splicing and the ensuing translational response is far greater in LPS-stimulated platelets compared to thrombin-stimulated platelets. After it is synthesized, IL-1β is primarily packaged into microparticles that promote the adherence of polymorphonuclear leukocytes (PMNs) to endothelium [23]. Our group and others [24–26] have also demonstrated that human platelets express pre-mRNA for tissue factor (TF), a gene that drives coagulation responses in sepsis [26]. Several agonists active in the septic milieu induce TF pre-mRNA splicing and translation of the mature message into bioactive protein, including

PAF, thrombin, gram negative and positive bacteria, α-toxin, and LPS ([26] and manuscript submitted).

Activated Platelets Mediate Responses in Target Leukocytes

As described above, agonists found in the infectious environment induce traditional and non-traditional activation responses within platelets that contribute to the pathogenesis of sepsis and related disorders. Activated platelets also drive responses in target leukocytes that modulate the host response to infection. As platelets become activated, they express P-selectin on their surface [27]. Membrane-expressed P-selectin engages its receptor, P-selectin glycoprotein-1 (PSGL-1), on PMNs and monocytes [28]. Zarbock and coworkers demonstrated that selectin-mediated platelet-PMN interactions are a critical step in activation and recruitment of leukocytes to the lung in acute lung injury (ALI) [29]. Their work demonstrated that platelet P-selectin mediates PMN sequestration and lung injury, linking platelets directly to inflammatory lung disease [29].

Interactions between platelets and PMNs are critical for cell trafficking, but they also serve as a means for the delivery of molecular signals. A prime example is the formation of neutrophil extracellular traps (NETs) in sepsis. Clark and colleagues recently demonstrated that platelets signal PMNs to extrude DNA-rich NETs, although the signaling factor in platelets that triggers this response is unknown [30]. NETs are extracellular chromatin lattices that are studded with elastase and other antimicrobial factors [31]. The primary function of NETs is to ensnare and kill microbes. Plasma from sepsis patients facilitates platelet-induced NET formation and studies in sepsis mouse models indicate that TLR4-dependent platelet activation is involved in this process [30,32]. In these studies, the authors also observed interactions between platelets and NETs in the pulmonary circulation. These NETs, however, also damaged endothelial cells raising the possibility that platelet-induced NET generation may ignite microvascular injury in sepsis [32]. Recent work also suggests that NETs recruit red blood cells and promote fibrin deposition, providing another link from platelets to thrombus formation [33].

In addition to interacting with PMNs, platelets use P-selectin to bind monocytes. In *in vitro* models, platelets adhere to monocytes for hours [34,35]. Consistent with this observation, platelet-leukocyte aggregates are markedly increased in the bloodstream of patients diagnosed with sepsis [36]. One consequence of prolonged interactions between platelets and monocytes is the expression of genes that encode for inflammatory proteins and enzymes involved in infectious processes. These include the synthesis of proinflammatory cytokines such as monocyte chemotactic protein-1 (MCP-1), IL-1β, and IL-8 [30,34]. Platelet-monocyte aggregates also produce COX-2, matrix metalloproteinase-9 (MMP-9), and TF [34,35,37,38].

Conclusions

Systemic inflammatory responses are complex clinical phenomena that present a major challenge to medicine. Specifically, sepsis presents us with a paradox where innate inflammatory responses evolved to fight infection do significant damage to the host, and DIC leads to platelet consumption and prolonged clotting times in the presence of thrombosis. These complexities provide us with a unique opportunity to study the dynamic role played by the host in the pathogenesis of this disease. We are reminded that platelets are not simple cytoplasts with the sole role of maintaining hemostasis over the course of minutes. Instead, platelets contribute to diverse processes that occur over hours to days and, during this time, change their own phenotype as well as the phenotype of nearby cells. The mechanisms by which diverse inputs differentially regulate platelet outputs are growing. Upcoming discoveries will undoubtedly cement the role of platelets as central mediators of systemic inflammatory responses, shine new insight into their functions and provide opportunities for clinical intervention in the future.

Conflict of interest statement

None.

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