Platelet-Mediated Modulation of Adaptive Immunity: Unique Delivery of CD154 Signal by Platelet-Derived Membrane Vesicles

Short Title:
PDMV deliver CD154 signals to B cells

Daniel L. Sprague*,†,‡, Bennett D. Elzey†, Scott A. Crist†,‡, Thomas J. Waldschmidt§, Robert J. Jensen*, and Timothy L. Ratliff*,†,‡,¶,

*Department of Urology, University of Iowa
†Medical Scientist Training Program, University of Iowa
‡Department of Microbiology, University of Iowa
§Department of Pathology, University of Iowa
¶Interdisciplinary Immunology Program, University of Iowa
‖Purdue Cancer Center, Purdue University

Keywords: platelets, CD154, B cells, humoral immunity, mouse, innate immunity, knockout

Abbreviations: platelet-derived membrane vesicle (PDMV), platelet-derived microparticle (PMP), activated platelet (AP), unactivated platelet (UAP), platelet (PLT).

1This work was supported by National Institutes of Health Grants AI060924.
2Corresponding Author: Timothy Ratliff, phone: 765-494-9129; fax: 765-494-9193; email: tlratliff@purdue.edu
Abstract

Although mounting evidence indicates that platelets participate in the modulation of both innate and adaptive immunity, the mechanisms by which platelets exert these effects have not been clearly defined. The study reported herein uses a previously documented adoptive transfer model to investigate the ability of platelet-derived membrane vesicles to communicate activation signals to the B cell compartment. The findings demonstrate for the first time that platelet-derived membrane vesicles are sufficient to deliver CD154 to stimulate antigen-specific IgG production and modulate germinal center formation through cooperation with responses elicited by CD4+ T cells. The data are consistent with the hypothesis that platelets modulate inflammation and adaptive immunity at sites distant from the location of activation and that platelet-derived membrane vesicles are sufficient to mediate the effect.
Introduction

Platelets are enucleated cellular fragments produced by megakaryocytes, and are best known for their role in hemostasis. However, accumulating evidence from recent studies indicates an additional role in modulating adaptive immune responses. In this regard, platelets have been shown to modulate dendritic cell activation, enhance T cell responses, induce B cell production of IgG antibodies, and enhance germinal center formation in cooperation with T cells.

In spite of this support for the idea that platelet activation is necessary for modulation of immunity, the underlying mechanisms by which platelets communicate signals have not been clearly defined. Platelets are activated by diverse stimuli, including infection, inflammation, and injury, and these cause the rapid release of numerous bioactive mediators capable of modulating innate immune cells, activating endothelial cells, and influencing systemic immune responses. The exquisite sensitivity of platelets to the environment, their location and number within the circulation, and the variety of chemical modulators released upon their activation make them uniquely suited to play a sentinel role and to provide early signals to immune cells.

For about a half century, researchers have known that platelets modulate inflammatory cell responses. Recently, the list of proposed effector functions for platelets was expanded to include a regulatory role in adaptive immune responses. Most of the studies on which these proposals are based have
focused on CD154 (CD40L).\textsuperscript{15} CD154 is critical to the initiation and propagation of the adaptive immune response and is expressed by a number of cell types, including CD4\textsuperscript{+} T cells, CD8\textsuperscript{+} T cells, \(\gamma\delta\)-T cells, and platelets.\textsuperscript{16} Though a wide variety of cells express CD154 messenger RNA, expression of the protein seems to be tightly regulated, adding further evidence of the importance of this molecule in normal biology. Best known as “signal 2” delivered through ligating to its receptor, CD40, during CD4\textsuperscript{+} T cell-mediated activation of B cells, CD154 is crucial for the development of T cell-dependent humoral immune responses. Humans lacking functional CD154 fail to isotype switch from the IgM antibody isotype, producing a hyper-IgM syndrome. Consistent with this finding, CD154 gene knockout (CD154-/-) mice are incapable of producing IgA, IgE, or IgG in response to T cell-dependent antigens, and are also unable to produce the germinal center response necessary for the differentiation of memory B cells and plasma cells.\textsuperscript{17}

The established paradigm is that the CD154 signal is delivered solely by CD4\textsuperscript{+} T cells to B cells. Recent reports, however, suggest that this paradigm may need to be revised to include a role for platelet-derived CD154. In vitro, platelets are capable of activating B cells to proliferate and produce antibodies\textsuperscript{10}, and in vivo they augment IgG production in CD154-/- mice\textsuperscript{5}. The physiological relevance is supported by several studies. Experiments in which wild type mice were depleted of platelets prior to priming showed a reduction in antigen-specific IgG production, suggesting that platelet-derived CD154 is necessary for an optimized antibody
response. Further experiments showed that under conditions of low precursor T cell numbers, platelets deliver a CD154 signal in cooperation with T cells, enhancing germinal center responses and increasing specific IgG production. These data support the hypothesis that platelets are an important early source of CD154 signals that promote optimal immune response and IgG production.

The mechanism whereby platelet-derived CD154 is delivered to splenic B cells has not been investigated. Upon their activation at a site of injury or inflammation, platelets translocate adhesion molecules to the surface to aid in self-aggregation, and bind to other cells and matrices to prevent the loss of blood and to form a thrombus around the site of injury. The aggregation and binding properties of activated platelets make it difficult for fully activated platelets to travel through the circulatory system, through the lungs and to the spleen to act upon B cells, although circulating platelets expressing activation markers have been reported. An alternative to intact platelets is that factors released after activation mediate platelet-induced responses. Platelet activation results in release of a myriad of soluble factors including the release two types of membrane vesicles: microparticles and exosomes. Upon activation platelets vesiculate, forming cell membrane-derived microparticles expressing and/or containing membrane and cytoplasmic molecules released upon activation. These platelet-derived microparticles (PMP) are about 0.1 to 1.0 μm in diameter in humans and express P-selectin (CD62P) and glycoprotein IIb-IIIa (GP IIb-IIIa). PMP are elevated in peripheral blood as a result of chronic
platelet activation in various disease states. Moreover, PMP adhere to a variety of cells, can activate endothelial cells, leukocytes and other platelets, and deliver signals through chemokines such as RANTES. Another vesicle released by platelets, exosomes, range in size from 0.04 to 0.1 μm and arise from the internal membrane vesicles of multivesicular bodies (MVBs) and granules in platelets. Unlike PMP, exosomes do not share a similar surface phenotype of activated platelets. However, both PMP and exosomes are known to carry and deliver cellular signals, suggesting a potential role in platelet-derived signaling to the adaptive immune compartment that often is far removed from the site of platelet activation. Studies reported herein use a previously documented adoptive transfer model to investigate the ability of platelet-derived membrane vesicles to communicate activation signals to the B cell compartment to augment antigen-specific IgG production and germinal center formation.

Methods and Materials

Mice and Materials. C57Bl/6 (B6) mice were purchased from the National Cancer Institute (Frederick, MD). Breeding pairs of CD154 gene knockout mice (CD154−/−) (H-2b background) and B6.RAG1 knockout mice (RAG 1−/−) (H-2b background) were purchased from Jackson Laboratories (Bar Harbor, ME). Thrombin, apyrase, and PGE1 were purchased from Sigma (St. Louis, MO). Agonistic anti-CD40 antibody 1C10 and the CD154 blocking antibody MR-1 were purified from serum-free
medium (HB101) using conditions that minimize introduction of endotoxin. Anti-
mouse IgG, IgG1, IgG2b, IgG2c, IgG3 and IgM antibodies purchased from Jackson
Immunoresearch Laboratories, Inc. (West Grove, PA). Anti-mouse TNF-α (clone
TN3-19.12) was purchased from eBiosciences, Inc. (San Diego, CA). All adenovirus
vectors were produced by the Gene Transfer Vector Core at the University of Iowa.

Platelet and Platelet-Derived Membrane Vesicle Preparation. Murine platelets were
isolated essentially as described. In brief, mice were anesthetized and bled by
severing the abdominal aorta. Blood was collected into syringes containing 1.0 ml
ACD (12.5 g/L Na Citrate, 10.0 g/L D-glucose, and 6.85 g/L citric acid), added to 6
ml PIPES (150 mM NaCl and 20 mM PIPES [pH 6.5]), and spun at 100 x g for 15
min. The platelet-rich supernatant was collected and 1 U/ml apyrase and 1 μM PGE₁
(final concentrations) were added and spun at 1000 × g for 10 min. The platelet pellet
was resuspended in Tyrodes buffer (134 mM NaCl, 2.9 mM KCL 0.34 mM Na₂PO₄,
12 mM NaHCO₃, 20 mM HEPES, 1 mM MgCl₂, 5 mM glucose, and 0.5 mg/ml BSA
[pH to 6.5]), and counted using a Coulter Particle Counter (Coulter Corp., Miami,
FL). To activate platelets, 0.5U thrombin/ml was added to platelet suspension. All
platelet manipulations were performed at room temperature. Platelets were allowed
to activate for 20 min. at 37°C. The activated platelet suspension was spun at 13,000
x g for 5 min. to pellet whole platelets and platelet aggregates. PDMV were pelleted
by modifying previously described protocols. Briefly, the activated platelet
supernatant (AP Sup) was collected and fractionated into platelet-derived membrane vesicle (PDMV) pellet and PDMV-poor supernatant by centrifugation at 20,000 x g for 2 hours at 4°C. Total protein analysis was performed using BCA™ Protein Assay Kit (Pierce, Rockford, IL) with a stated working range from 0.02 to 2 mg/mL.

**Adenovirus Specific IgG ELISA.** Serum was collected from mice 7–14 days after immunization with adenovirus. 96-well plates were coated overnight at 4°C with 10⁹ Ad5-βgal particles per well in 50 μL 0.1 M NaHCO₃ (pH 9.2). Wells were then washed and blocked 2–4 hours at room temperature with 3% BSA in 0.01% TWEEN 20 and 0.02% NaN₃. Blocking solution was decanted and 100 μL diluted plasma incubated per well for 2–4 hr at room temperature. After six washes, 100 μL peroxidase-labeled secondary antibody was incubated for 1–2 hr at room temperature per well. Wells were washed seven times, after which 100 μL fresh substrate (OPD in 0.04 M Na₂HPO₄ and 0.02 M citric acid [pH 5.0]) was added. Samples were incubated at room temperature in the dark for 30 min. The reaction was then stopped by adding 25 μL 4.5 M H₂SO₄ per well. Sample absorbances were measured at 490 nm. Total IgG was quantified using a standard mouse adenovirus monoclonal IgG₁ from Fitzgerald Industries International, Inc. (Concord, MA).

**Transmission Electron Microscopy.** Platelet and PDMV pellets were washed and fixed with 2% paraformaldehyde/1% glutaraldehyde solution in PBS for 1 hour at
4°C. Rinse 3 times with PBS. Add 1% OsO₄ with 1.5% potassium ferrocyanide in PBS for 2 hours. Rinse 3 times with PBS and once with distilled water. Dehydrate with ethanol through successive steps then embed within epoxy medium. Place in 70°C oven for 8 hours and section using microtome. Examine using a Hitachi H-7000 Transmission Electron Microscope (University of Iowa, Central Microscopy Research Facilities). Images were acquired on Kodak 4489 negative sheet film for image recording.

In vitro Stimulations of B Cells. Primary splenic B cells were isolated from C57BL/6 mice between the ages of 8-10 weeks of age. Mice were anesthetized and sacrificed. Spleens were removed and ground between frosted microscope slides in 1x balanced salt solution (BSS). Cells were washed and resuspended in 2mL of 1x BSS. Percoll stock was made adding 1mL 10x balanced salt solution and 50μL of 7.5% sodium bicarbonate solution to 9mL percoll. Dilutions of 50, 60, 70, and 75% of the percoll stock were made with 1x BSS. The cell suspension was underlayed with the 50, 60, 70, 75, and the percoll stock, in that order, in a 15-mL tube. The tube was centrifuged at 2900rpm in a Jouan CR412 centrifuge for 15 minutes at 4°C. Cells collected from 60-70% interface and 70-75% interface. Cells were washed and resuspended in MACS buffer according to manufacturer’s protocol for isolation of untouched B cells using CD43 microbeads (Miltenyi Biotec, Auburn, CA). Cells
were stained for CD19 and CD45R and analyzed by flow cytometry to assess purity of B cells.

**Quantification of CD154 Activity.** MS-1 cells, an immortalized pancreatic endothelial cell line were obtained from ATCC and routinely cultured in DMEM with 5% FBS. After 6 hour exposure to platelets or sCD154, total cellular RNA was isolated using the RNAeasy kit (Qiagen, Valencia, CA). First strand synthesis was performed using Superscript III reverse transcriptase (Invitrogen) in a reaction using 2 μg of total RNA primed with random hexamers following manufacturer’s instructions. Two microliter of each reverse transcription reaction was subjected to real time quantitative PCR using proprietary TaqMan primer and probe sets for mouse MCP-1, and 18s rRNA (Applied Biosystems, Foster City, CA). For each sample, three PCRs were performed. The resulting relative increase in reporter fluorescent dye emission was monitored by the TaqMan system (GeneAmp 5700 sequence detection system and software; PerkinElmer). The level of MCP-1 mRNA, relative to 18s rRNA, was calculated using the formula: relative mRNA expression = $2^{-(Ct \text{ of } \text{MCP-1} - Ct \text{ of } 18s \text{ rRNA})}$, where Ct is the threshold cycle value.

**Germinal Center Formation Assessment.** Germinal center formation was assessed as previously described. Platelets were depleted using 10 μg of antibody p0p3/4 (Emfret Analytics, Germany) 24hrs before i.v. adoptive transfer of 4x10^6 negatively
selected, naïve B6 CD4+ T cells along with i.v. adoptive transfer of either B6 platelets, B6 PDMV or CD154-/- PDMV. Four hours later the mice received via i.v. injection $10^8$ pfu of adenovirus. Twelve days post-injection, spleens were harvested and processed for histological examination of frozen sections. Germinal Centers (GCs) were visualized and reported as GCs/PALS. A PALS unit is the combined follicular zone/T cell zone surrounding a single central arteriole in thin sections. RESULTS

Activated platelets release functional CD154 into the supernatant

To investigate the role of released CD154 in the induction of IgG production, experiments were undertaken to compare the adenovirus-specific IgG response stimulated in CD154-/- mice by wild-type, intact activated platelets (AP) from B6 mice versus that stimulated by the activated platelet supernatant alone (AP Sup). AP and AP Sup were produced from platelets purified from whole blood. There is between 0.03-0.05% contamination by CD3+ cells (data not shown). This number is equivalent to 150-200 possible T cells in the $5 \times 10^8$ transferred platelets. Serum was collected and tested by ELISA to assess relative adenovirus-specific IgG titers elicited by these and control treatments (Fig 1). When mice were treated with the supernatant from wild-type activated platelets, adenovirus-specific IgG production increased. Supernatant-derived augmentation was enhanced by a second injection, with antibody production approaching the levels observed following injection of
activated platelets. In contrast, supernatant from platelets harvested from CD154-/mice failed to confer this stimulatory effect. These data suggest that activated platelets release factors into the supernatant that are sufficient by themselves to mediate CD154 signaling to B cells for production of IgG.

Characterization of PDMV

To more rigorously ascertain whether platelet components released upon activation are sufficient to signal the adaptive immune compartment, and further to determine which components might be involved, highly purified platelet fractions were isolated. One signaling candidate is the cleaved, soluble CD154 (sCD154), which is released during the degranulation of activated platelets at sites of injury and infection. A second is CD154-containing PDMV released from platelets as microparticles (PMP) and exosomes. PMP, but not platelet-derived exosomes, have been shown to express CD154, although CD154 expression on exosomes from other cell types has been demonstrated. Thus to address whether soluble or PDMV-associated CD154 is responsible for the augmentation of IgG production, PDMV and soluble protein fractions were isolated as described in the Materials and Methods. The literature cites a number of different protocols for the isolation of platelet PDMV. Preliminary studies using varying centrifugation speeds ranging from 10,000 x g to 100,000 x g demonstrated that centrifuging AP Sup at ≥20,000xg for 2 hours was sufficient for the isolation of PDMV of the anticipated
size that contained the CD154 fraction (Supplemental Fig 1; Fig 2). Based on the data reported in Supplemental Fig 1, all PDMV in this report were isolated at 20,000 x g. The purity and efficiency of the PDMV preparation were assessed using electron microscopy (TEM) and protein analyses. TEM visualization of membrane-bound microparticles approximately 100 nanometers in diameter within the purified PDMV preparation demonstrated the isolation of PDMVs (Fig 2A). Due to the lack of an effective means to quantify PDMV prior to adoptive transfer or to track them afterwards, all experiments were standardized based on the number of platelets activated to generate the PDMV sample. Total protein content showed equivalent protein levels in comparable fractions generated from B6 and CD154-/- platelets (Fig 2B). Of note, the lowest protein levels (near the lower limit of detectability) were observed in the PDMV fractions.

Functional quantification of CD154 on PDMV using MS-1 endothelial cell line

Due to the lack of reagents necessary to quantify the amount of mouse CD154 contained within intact or subfractions of activated platelets, we established a biological assay using CD154-induced MCP-1 production by MS-1 endothelial cells. Previous studies showed that both CD154 and TNF induced MCP-1 production thus antibody neutralization was used to segregate activity to the respective stimuli. Recombinant soluble CD154 (Axxora, LLC, San Diego, California) was used as a positive control. Initially, MS-1 cells and PDMV were co-cultured for 6 hours and
assessed for increased expression of the endothelial activation marker MCP-1 mRNA levels using quantitative real time PCR (qRT-PCR). PDMV from B6 mice increased in MCP-1 message about 4-fold which was completely blocked by MR-1, but not anti-TNF-α blocking antibody, indicating that CD154 is the only factor activating MS-1 cells (Fig 2C). Whole activated platelets also induced MCP-1, but this was partially blocked by both CD154 and TNF-α blocking antibodies.

To quantify the relative CD154 activity in the PDMV fraction, we ran the assay with a standard curve and surprisingly, calculated that the CD154 activity per milligram total protein in the PDMV pellet was equivalent to the activity induced by 350 μg of the standard protein (Fig 2D). Whole activated platelets, when blocked with anti-TNF, showed around 100 μg of CD154 activity. This data not only confirms the presence of CD154 activity within the PDMV, but also indicates the CD154 activity becomes concentrated on these structures lending support to the importance of PDMV in the delivery of CD154 signals.

**PDMV delivery of CD154 to B cells**

The isolated fractions were then tested for their ability to activate adenovirus-specific IgG production in our model. Each fraction, including the PDMV pelleted from activated platelet supernatant (PDMV Pellet) and the PDMV-poor supernatant after fractionation, was transferred into CD154-/- mice (Fig 3A) in conjunction with adenovirus immunization. Only the unfractionated supernatant and the PDMV pellet
from B6 mice induced adenovirus-specific IgG production. The fact that neither unfraccionated AP supernatant nor the PDMV fraction from CD154-/- platelets elicited a response, demonstrated that CD154 within the PDMV pellet was sufficient to facilitate the augmentation of adenovirus-specific IgG. This was further verified by the use of the CD154 blocking antibody MR-1 (Fig 3B). Addition of MR-1 to B6 PDMV prior to injection into immunized CD154-/- mice abrogated the antibody augmentation induced by B6 PDMV alone, whereas control Ig had no effect. Taken together, these data confirm the finding that whole activated platelets are not necessary for the delivery of the CD154 signal. Moreover, because total protein analysis of the injected fractions show that PDMV represent a small percentage of the total protein from the initial platelet pellet, these data suggest that CD154 activity strongly fractionates with the PDMV pellet. Finally, the augmentation elicited by the PDMV fraction was comparable to that produced by unfraccionated, activated platelet supernatants, suggesting PDMV may play a role in the delivery of the platelet-derived CD154 signal.

Though the platelets have been purified using the described centrifugation techniques, there is still a minimal amount of contamination by T cells and B cells (0.03 -0.05%; data not shown). Since this level of contamination resulted in only 150-200 T cell in the transferred platelets, it is unlikely that T cell CD154 affected the outcome of the response. However, to determine whether this contamination contributed to the increase in IgG production, we performed the experiments with
platelets from B6.RAG 1<sup>−/−</sup> mice (Fig 3C). The use of RAG<sup>−/−</sup> mice eliminates any possible contribution from T or B cells, which may be contaminating our platelet preparations, as these mice do not produce any lymphocytes. The data show that the increase in IgG is due solely to the platelets in our preparations and the products produced by the platelets during activation and not by any contaminating T or B cells.

Though PDMV induces IgG production <i>in vivo</i>, it is not known whether PDMV are able to interact and stimulate B cells directly. To determine whether PDMV and B cells are capable of interacting directly, the impact of CD154 signaling on B cell proliferation was assessed in an <i>in vitro</i> co-culture using primary splenic B cells isolated from B6 mice. Proliferation was measured by tritiated thymidine incorporation into DNA (Fig 3C). Under conditions with or without anti-IgM acting as “signal 1” in B cell activation, wt PDMV were observed to induce B cell proliferation. While significant proliferation was observed with or without signal 1 activation, the proliferative response driven by PDMV was weak. These data suggest that in vivo signaling by PDMV is more complex that simply engaging B cell CD40. Interestingly, the data repeatedly showed that CD154<sup>−/−</sup> PDMV seemed to inhibit proliferation of B cells.

**Characterization of the PDMV-mediated response**

To verify that the time course of PDMV-induced antibody production observed in our system is similar to what had been reported previously for whole,
activated platelets (5), we performed an adoptive transfer experiment evaluating adenovirus-specific IgG production over time (Fig 4A). PDMV produced a response that peaks at day 7 and decreases rapidly to background, as previous studies investigating platelet induced IgG production have shown, suggesting the mechanism of delivery is the same. Dose-dependence of the PDMV-induced IgG production was determined using PDMV that were prepared from platelets suspended at 4.5x10^8 platelets/mL and serially diluted 2-fold before transfer into CD154-/- mice (Fig 4B). PDMV from the equivalent of 4.5x10^8 platelets represents the undiluted, neat sample. Due to the significant loss of PDMVs during sample preparation (see figure 2B) and the inability to quantify the amount of PDMV in the final samples, it cannot be assumed that PDMV from 2.25x10^8 platelets is the minimum effective dose. Rather, it is likely that only a fraction of the starting sample remains by completion of the preparation. Thus these data demonstrate that the effect of PDMVs is titratable but does not provide a basis for determining the number of PDMV necessary to deliver an activating signal.

To further characterize PDMV-induced IgG stimulation, serum antibody production was analyzed at the isotype level, which based on the isotype secretion pattern might also provide clues to the subset of splenic B cells activated. IgG1, IgG2b, IgG2c, IgG3 and IgM were analyzed by ELISA and the results were consistent with previous reports characterizing IgG production in response to adenovirus. PDMVs strongly induced IgG2b, IgG2c and IgG3 (Figure 4C),^42,43 as
well as leading to a slight increase in IgG1. IgG3 is associated with marginal zone (MZ) B cells, which play a significant role in T-cell independent responses and are among the first B cells to respond to blood-borne pathogens. IgG2b, IgG2c, and IgG1 are produced primarily by follicular B cells.44

**PDMV enhances the GC reaction**

Further studies were performed to determine whether PDMV functions in conjunction with T cells to augment germinal center formation. Previous studies have shown that, in a non-transgenic system in which antigen-specific T cell precursors are limiting, platelet-associated CD154 enhances germinal center (GC) formation and antibody production in cooperation with CD4+ T cells.45 To further validate a role for PDMV in immune modulation, experiments were performed to determine whether PDMV are sufficient to cooperate with CD4+ T cells in stimulating GC formation (Fig 5C). CD154-/- mice injected with B6 PDMV showed a similar percentage of germinal-center positive periarteriolar T-cell-rich lymphoid sheath (PALS) units compared to mice injected with B6 platelets, indicating that PDMV are in fact able to enhance T cell-mediated germinal center formation in a CD154-dependent fashion. Taken together, these data begin to unravel a possible mechanism by which PDMV deliver platelet-derived CD154 to B cells to modulate IgG production and germinal center formation.
Discussion

Previous studies showed that platelet activation results in the modulation of T- and B-cell immunity; however, the mechanism(s) by which the signal is delivered remains unclear.\textsuperscript{5,45} When platelets become activated, they express a variety of surface adhesion molecules, leading to aggregation at the site of activation. Aggregation limits the mobility of platelets, and thus also their capacity to communicate with sites distant from the activation site. We hypothesized that platelet communication with the adaptive immune compartment is mediated at least in part by PDMV. The data presented herein demonstrate that PDMV are sufficient to induce IgG production and enhance germinal center formation \textit{in vivo}, and induce proliferation of B cells and activation of MS-1 cells \textit{in vitro}, in a CD154-dependent manner. Intravenous injection of PDMV under the experimental conditions described here mimics PDMV release during platelet activation, suggesting that PDMV are sufficient to carry a CD154 signal through the circulatory system to distant sites where signaling can occur. Though our platelet preparations contain minimal contaminating T cells and B cells, we showed that RAG\textsuperscript{\textminus\textminus} preparations, which are free of T and B cells, function as well as PDMV isolated from wt mice. Likewise, in data previously published, we showed that in platelet and T cell cooperative studies a minimum of $4 \times 10^6$ T cells alone was necessary to observe an increase in germinal center formation and IgG production.\textsuperscript{11} Taken together these data demonstrate the
sufficiency of platelet-derived CD154 in PDMV preparations to mediate IgG production.

In an attempt to show the presence and quantity of CD154 in the platelets and PDMV preparations, we had purchased and used ELISA kits from two different manufacturers (Bender MedSystems and R&D Systems). However, neither kit was able to quantify mouse CD154 from lysed platelets or PDMV. We were unable to confirm the presence or quantity of CD154 by electron microscopy or western blot, as well, due to the general lack of quality antibodies to detected fixed or denatured mouse CD154. The use of CD154-/- platelets or PDMV and also neutralization of CD154 function in wild type platelet preparations directly links CD154 in PDMV to IgG production. Using a functional assay to quantify CD154 activity compared to a standard, we showed that PDMV contain CD154 and that it becomes concentrated within this fraction after platelet activation.

It is clear that PDMV are available for carrying platelet-derived signals to other cells under physiological conditions. Platelets release two types of membrane vesicles: platelet-derived microparticles (PMP) and exosomes. PMP bud from the plasma membrane of activated platelets and hence are suspected of carrying surface markers from activated platelets as membrane-bound molecules. Early studies showed PMP to be an important source of integrins and selectins for leukocyte attachment to endothelial cells, a process that is important for recruitment to and transmigration at sites of injury. In addition, platelets and PMP are known to
modulate the activity of the cells with which they interact, including monocytes, neutrophils, and endothelial cells. For example, PMP are important for platelet-derived CD154 to stimulate the maturation of monocyte-derived dendritic cells. Since circulating PMP and CD154 increase during inflammation, understanding the role of PMP in the delivery of CD154 signals and the impact of this delivery on damage has broad implications. Unlike PMP, exosomes would not acquire surface markers from the plasma membrane of activated platelets.

Rather, exosomes bud into the granules themselves during development. Platelet-derived exosomes have not been shown to have any function in vivo or in vitro. Since circulating PDMV and CD154 increase during inflammation, understanding the role of PDMV in the delivery of CD154 signals and the impact of this delivery on damage has broad implications. Given the current demonstration of the sufficiency of PDMV to modulate B cell responses, PDMV-associated CD154 signaling likely plays a role in inflammation and other conditions associated with platelet activation.

Signal delivery via membrane vesicles is not a property unique to platelets. Endothelial microparticles have been shown to activate monocytes and to promote adhesion. Membrane vesicles released by dendritic cells and B cells have also been shown to enhance antigen presentation. Mast cells have been shown to produce exosomes, which express a variety of immunoregulatory molecules including CD154. These mast cell-derived exosomes are capable of activating both B
and T cells.\textsuperscript{57} One interesting feature common to microparticles from many cell types including platelets is their ability to transfer membrane-associated molecules to other cell types including B cells.\textsuperscript{58-62} Our study expands the role of membrane vesicles in general, and of PDMV specifically, by showing that platelet-derived PDMV are able to deliver a CD154 signal that is able to modulate the activity of immune cells and the subsequent immune responses.

The mechanisms by which PDMV communicate with the adaptive immune compartment are not known. Our experiments, which take advantage of the availability of CD154/- mice, have shown that platelet or PDMV-induced antibody production is dependent upon platelet-derived CD154. However, the experiments do not address whether the CD154 signal is delivered by PMP or exosomes. Likewise, it is not known whether the CD154 signal is delivered directly to B cells or indirectly through other cell types. A direct interaction would involve platelet-derived PDMV interacting directly with B cells, as has been shown for membrane vesicles derived from other cell types.\textsuperscript{63} Alternatively, PDMV could signal indirectly through phagocytic cells present in the spleen. In this regard, it is interesting to note that CD154 ligation to CD40 activates monocytes and dendritic cells,\textsuperscript{64,65} and that activated macrophages and dendritic cells modulate antibody production by B cells.\textsuperscript{66-69} Similarly, PDMV could indirectly deliver a signal to B cells via PDMV-associated CD154 activated phagocytes, either through another molecule such as BAFF (B-cell activating factor of the TNF family) or by using these phagocytes as a scaffold for
platelet-derived CD154 signaling to B cells. Taken together, these observations support the potential for platelets and PDMV to signal B cells in an indirect manner. Further studies are in progress to more clearly define the signaling mechanism.

In a wild-type mouse, adenovirus is cleared by a Th1-mediated response with an antibody profile made up of IgG2a, in BALB/c mice, or IgG2c, in C57BL/6 mice, and IgG2b. In the PDMV-induced response to adenovirus, IgG2b and IgG2c are the predominant isotypes, with some IgG3 being produced as well. IgG3 is also induced in a Th1 response, suggesting that PDMV can induce the same type of response in CD154-/- mice as found in wild-type mice immunized with adenovirus. The presence of a high level of IgG3 is interesting due to the possible role of MZ B cells in PDMV-induced antibody production. IgG3, and to a lesser extent IgG2a and IgG2c, are strongly associated with marginal zone B cell activation. However, other B cells, including follicular B cells, are also capable of producing these isotypes in significant quantities. MZ B cells, along with B1 cells, are thought to be the major source of “natural antibodies” linking innate and adaptive immune responses. IgG3 antibodies from marginal zone B cells appear early in infection, independent of T cell help. Since this response does not require T cell help, it is limited in duration and lacks memory. This is similar to the characteristics of the PDMV-induced antibody response, suggesting a possible role for platelet-derived CD154 in inducing an early antibody response by MZ B cells in mice, though this has not been fully investigated.
Platelet depletion data show a role for platelets in the early splenic antibody response in wild-type and CD154-/- mice. However, the physiological role platelets and/or PDMV play in the modulation of IgG production during an infection remains to be clearly defined. Our studies clearly demonstrate the sufficiency of PDMV to signal the B cell compartment; however, in the context of a normal animal, the contribution of PDMV in establishing antibody responses to pathogens remains to be clearly established. The current study indicates that PDMV may play an important role by acting as a vector for the CD154 signal by concentrating the CD154 activity and carrying it away from the site of platelet activation and aggregation, and thereby making it possible for it to encounter the cells necessary to produce the antigen specific response.

**Authorship**

T.L. Ratliff contributed vital reagents, designed research, and analyzed data. R.J. Jensen performed research. T.J. Waldschmidt contributed vital reagents, designed research, and analyzed data. S.A. Crist designed research, performed research and analyzed data. B.D. Elzey performed research and analyzed data. D.L. Sprague designed research, performed research, analyzed data, and wrote paper.

The authors declare no competing financial interests.
References


platelets is temporally limited by coexpressed CD40. Blood. 2001;98:1047-1054.


Figure 1. Whole platelets are not necessary for the delivery of CD154 signal.

CD154-/- mice were injected with 5x10^7 activated platelets (AP) or activated platelet supernatant (AP Sup) from 5x10^8 wild-type (B6) or CD154-/- platelets. Positive controls were given 500 μg anti-CD40 antibody (1C10) IP. Mice were immunized on day (-1) with 10^8 pfu of adenovirus. On day 0, all mice received one injection of either AP or AP Sup. A second injection was given to half the mice on day 6 (X1 indicates mice receiving one injection, whereas X2 indicates mice receiving two injections). Serum was collected on day 9 for adenovirus-specific IgG analysis by ELISA. This experiment was performed three times with 5 mice per group. The above graph is data from a representative experiment. (Statistics: Wilcoxon Rank Sum Test, * indicates a two-tailed p-value of 0.0002, ** indicates a two-tailed p-value of 0.0006.)

Figure 2. Characterization of PDMV. PDMV were isolated and visualized by TEM and analyzed for total protein. (A) TEM images of whole platelets (top) and PDMV (bottom). The bar is 100 nanometers. (B) Total protein analysis was performed on 5x10^8 unactivated platelets (UAP) and activated platelets (AP); and activated platelet supernatant (AP Sup), PDMV pellet (PDMV) and PDMV-poor supernatant (PDMV-poor Sup) made from 5x10^8 platelets. *** Total protein values (mg/mL) above each bar. (C) 1x10^5 MS-1 cells were plated in each well of a 24-well plate and allowed to grow to near confluence over 2 days. PDMV from 4.5x10^8
CD154 wt or ko platelets, or 1x10^8 platelets were added to each well. 10μg/ml of anti-CD154 and/or anti-TNF-α antibodies added to designated wells. Experiment was performed in triplicate. (D) Quantitative RT-PCR was performed using purchased primers for MCP-1 and 18s mRNA. Standard curve ranged from 10μg/mL to 4.9ng/mL. (Statistics: Wilcoxon Rank Sum Test, * indicates a two-tailed p-value of 0.0122, ** indicated a two-tailed p-value of 0.0195)

**Figure 3. PDMV delivery of CD154 signal.** (A) CD154-/- mice were immunized on day (-1) then injected on days 0 and 6 with activated platelet supernatant (AP Sup), PDMV pellet, or PDMV-poor supernatant from 5x10^8 wild-type (B6) or CD154-/- platelets, or 500μg 1C10 ip. Serum was collected on day 9 for quantification of total adenovirus-specific IgG using a commercially available mouse anti-adenovirus IgG standard. (B) PDMV derived from 5x10^8 B6 platelets were injected iv into CD154-/- mice 24 hrs after immunization with 10^8 particles Ad-OVA. 10μg/mL CD154 blocking antibody MR-1 was added to AP Sup prior to isolation of PDMV and to PDMV after resuspension. Mice were injected with 100μg MR-1 just prior to receiving PDMV + MR-1. Serum was collected on day 7 for quantification of total adenovirus specific IgG production by ELISA. (C) PDMV derived from 5x10^8 platelets injected 24 hours after immunization. Serum collected on day 7. (D) Primary B cells were isolated from spleens of 8-week-old C57BL/6 mice by percoll gradient enrichment and negative selection over magnetic beads (Miltenyi). 6x10^5 B
cells were plated in each well of a 96-well plate and PDMV from 4.5x10^8 wt or ko platelets added to each designated well, with or without anti-IgM, 5 wells per experimental condition. PDMV and B cells were co-incubated for 48 hours with final 6 hours in presence of 1μCi of ^3H-T. Cells were harvested and thymidine incorporation measured. (Statistics: Wilcoxon Rank Sum Test, * indicates a two-tailed p-value of 0.0122, ** indicated a two-tailed p-value of 0.0195)

**Figure 4. Characterization of PDMV induced augmentation of IgG production.**

(A) Time course of PDMV response. CD154-/- mice injected with PDMV pellet from 5x10^8 B6 or CD154-/- platelets. Serum collected on days 3, 5, 7,and 9. Total IgG quantified by ELISA using commercial mouse anti-adenovirus IgG standard. (Statistics: Wilcoxon Rank Sum Test, * indicates a two-tailed p-value of 0.020 and ** indicates a two-tailed p-value of 0.0122 comparing the B6 PDMV time point to the corresponding CD154-/- PDMV time point.) (B) Dose response study. Neat samples contain PDMV from 4.5x10^8 activated platelets. Dilutions made from resuspended PDMV pellets. Total IgG quantified by ELISA using commercial mouse anti-adenovirus IgG standard. (Statistics: Wilcoxon Rank Sum Test, * indicates a two-tailed p-value of 0.0122. **indicates a two-tailed p-value of 0.0119. “ns” = not significant) (C) Analysis of antibody isotypes produced. Samples pooled from neat groups in dose response experiment. ELISAs performed for each antibody isotype. Each experiment performed with 5 mice per group and repeated once.
Figure 5: B6 PDMV enable limiting number of normal B6 CD4+ T cells to induce germinal center reactions in CD154-/- mice depleted of platelets. Platelets were depleted using 10 μg p0p3/4 24hrs before i.v. adoptive transfer of 4x10⁶ negatively selected, naïve B6 CD4⁺ T cells i.v. and either 10⁸ B6 platelets, B6 PDMV from 5x10⁸ platelets, or CD154-/- PDMV from 5x10⁸ platelets. Twelve days post-injection, spleens were harvested and processed for histological examination of frozen sections. Blue = T cell zone (anti-CD4 and CD8 staining); red = B cell follicles (anti-B220); green = peanut agglutinin (PNA; GC B cells stain PNA⁺). GCs are visualized as B220 PNA double-positive (green-yellow) areas of cells. Representative histology from the wild-type control consisting of B6 mice immunized with adenovirus alone (A) and CD154-/- mice immunized with adenovirus and treated with naïve B6 CD4⁺ T cells with CD154-/- PDMV (B) or B6 PDMV (C). GCs were visualized and reported as GCs/PALS (D). A PALS unit consists of the follicular and T cell zones surrounding a central arteriole. An example of a PALS unit is delineated in panel B with a dashed white line. Experiment performed with 5 mice per group and repeated once. (* indicates a two-tailed p-value of 0.0140. ** indicates a two-tailed p-value of 0.0058.)
Figure 1

[Graph showing Total Adenovirus-Specific IgG (OD490) for different genotypes and conditions.]

- Ad
- Ad/B6 AP X1
- Ad/B6 AP sup X1
- Ad/B6 AP sup X2
- Ad/CD154-/- AP sup X1
- Ad/CD154-/- AP sup X2
- Ad/C10
- Naive

Legend:
- B6
- CD154-/-

Significance:
- * p < 0.05
- ** p < 0.01
Figure 2

A. Figure showing MS-1 alone with PDMV ko, MS-1 with wt PDMV, and MS-1 with Platelets.

B. Bar graph showing total protein (mg/mL) across different fractions:
- Untx: 16.0, 14.5
- AP Sup: 3.6, 3.6
- AP: 4.9, 4.8
- PDMV: 0.03, 0.03
- PDMV-poor Sup: 1.9, 1.2

C. Diagram showing fold induction of MCP-1 mRNA with MS-1 treatment (6 hours):
- Untx
- anti-CD154
- anti-TNF
- anti-CD154/TNF
- Isotype

D. Graph showing relative CD154 activity (µg) per mg total protein:
- ko PDMV
- wt PDMV
- wt Platelets + anti-TNF
Figure 3

A. [Legend and data for Figure 3A]

B. [Legend and data for Figure 3B]

C. [Legend and data for Figure 3C]

D. [Legend and data for Figure 3D]
Figure 4

A. [Graph showing Adenovirus-Specific IgG (ng/mL) over different days for B6 PDMV, CD154-/- PDMV, and 1C10.]

B. [Bar chart showing Adenovirus-specific IgG (ng/mL) at different days with dilutions for B6 PDMV and CD154-/- PDMV.]

C. [Bar chart showing Total Adenovirus-Specific IgG (OD490) for antibody isotypes IgM, IgG1, IgG2b, IgG2c, IgG3, B6 PDMV, CD154-/- PDMV, Ad-OVA, 1C10, and Naive at different dilutions.]
Figure 5

A. T cell Zone

B. PNA+ GC B cells

C. B cell zone

D. %GC Positive PALS Units

Wild-type Control CD154-/- PDMV B6 platelets B6 PDMV

* **
Platelet-mediated modulation of adaptive immunity: unique delivery of CD154 signal by platelet-derived membrane vesicles

Daniel L. Sprague, Bennett D. Elzey, Scott A. Crist, Thomas J. Waldschmidt, Robert J. Jensen and Timothy L. Ratliff