Published in final edited form as:

J Autoimmun. 2013 May ; 42: 71–79. doi:10.1016/j.jaut.2012.11.005.

Gammaherpesvirus latency induces antibody-associated thrombocytopenia in mice

Michael L. Freeman^{a,1}, Claire E. Burkum^a, Kathleen G. Lanzer^a, Alan D. Roberts^a, Mykola Pinkevych^b, Asako Itakura^c, Lawrence W. Kummer^a, Frank M. Szaba^a, Miles P. Davenport^b, Owen J.T. McCarty^c, David L. Woodland^{a,2}, Stephen T. Smiley^a, and Marcia A. Blackman^{a,*}

^aTrudeau Institute, 154 Algonquin Avenue, Saranac Lake, NY 12983, USA

^bComplex Systems in Biology Group, Centre for Vascular Research, University of New South Wales, Sydney, NSW 2052, Australia

^cDepartment of Cell and Developmental Biology, School of Medicine, Oregon Health & Science University, Portland, OR 97239, USA

Abstract

Human herpesviruses establish lifelong latency. Viral recrudescence can lead to the development of cancers, immunoproliferative disorders, transplantation complications, and thrombocytopenia. Although platelet-specific autoantibodies have been reported in patients infected with the Epstein-Barr virus (EBV), the mechanisms by which thrombocytopenia is induced remain unclear, as do the relative contributions of lytic viral replication and latent viral gene expression. The human gammaherpesviruses are tightly restricted in their ability to infect other mammals, so they are difficult to study in live animal models. Here we show that infection of mice with murine gammaherpesvirus-68 (γ HV68), a rodent-specific pathogen closely related to EBV, induces the production of platelet-binding antibodies and causes thrombocytopenia. Infection of antibody-deficient mice does not lead to thrombocytopenia, indicating the platelet decrease is mediated by antibody. Additionally, infection with a latency-null recombinant γ HV68 does not induce thrombocytopenia, suggesting factors associated with viral latency drive the infection-induced antibody-mediated thrombocytopenia. These studies describe an important animal model of gammaherpesvirus-induced autoimmune thrombocytopenia and demonstrate that this pathology is mediated by antibody and dependent on viral latency. This model will allow studies of the underlying mechanisms of disease progression and the testing of therapeutic strategies for the alleviation of virus-induced thrombocytopenia.

1. Introduction

A majority of the world's population is infected by gammaherpesviruses such as Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV). After a primary lytic infection, these viruses persist permanently in a quiescent state called latency and are never completely cleared by the immune system. The latent stage of infection is

© 2012 Elsevier Ltd. All rights reserved.

*Address correspondence to: Marcia A. Blackman, Ph.D., Trudeau Institute, 154 Algonquin Avenue, Saranac Lake, NY 12983, Office: (518) 891-3080 x316, Fax: (518) 891-5126, mblackman@trudeauistitute.org.

¹Present address: Department of Immunology, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH 44195, USA

²Present address: Keystone Symposia on Molecular and Cellular Biology, Silverthorne, CO 80498, USA

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

characterized by periodic reactivations of the virus into a lytic form. The consequences of viral infection and reactivation include immunoproliferative disorders such as mononucleosis, the development of cancer, and transplantation complications [1, 2]. Gammaherpesvirus infections have also been associated with the development of autoimmunity; EBV infections are associated with development of multiple sclerosis, systemic lupus erythematosus, and rheumatoid arthritis [3]. An additional pathological consequence of gammaherpesvirus infections is thrombocytopenia, a decrease in the number of platelets in the blood, which can occur transiently or chronically [4]. In patients with chronic active EBV infection, severe thrombocytopenia has been associated with mortality [5].

Clinically significant thrombocytopenia accompanies a variety of diseases [6, 7]. Thrombocytopenia can be mediated by several mechanisms, including impaired platelet production; hemorrhagic loss of blood cells; trapping of platelets in the spleen; increased blood clotting, thereby removing platelets from the circulation; or abnormal destruction of platelets by activated macrophages, adaptive immune cells, anti-platelet antibodies, or direct infection by viruses [6, 8]. How gammaherpesvirus infections induce thrombocytopenia is not known. Autoantibodies specific for platelet glycoproteins have been found in some EBV-infected patients, but it remains unclear whether EBV-induced thrombocytopenia is a direct result of antibodies [9, 10]. Moreover, the relative roles of acute viral replication, latent viral gene expression and/or viral reactivation have yet to be evaluated.

EBV and KSHV are highly species-specific and can only be studied in humans or in humanized animal models that do not recapitulate many aspects of natural human infection. Therefore, to investigate the etiology of gammaherpesvirus-induced thrombocytopenia in a tractable animal model, we infected mice with murine gammaherpesvirus-68 (γ HV68), a rodent-specific pathogen closely related to EBV and KSHV [11–13]. Intranasal γ HV68 infection results in acute viral replication in the lungs, followed by establishment and amplification of viral latency in dendritic cells, macrophages, and B cells in the spleen and elsewhere [14, 15]. This amplification of latent virus is characterized by an infectious mononucleosis-like syndrome, including marked splenomegaly, lymphocytosis in the blood, and expansion of CD8 T cells expressing a $V\beta 4+$ T cell receptor [16–18]. Murine γ HV68 infections have been used to investigate the association of gammaherpesvirus infections with autoimmune disorders – γ HV68 has been shown to exacerbate experimental autoimmune encephalitis (EAE), a mouse model of multiple sclerosis [19, 20]; worsen inflammatory bowel disease in IL-10^{-/-} mice [21]; and may lead to the production of autoantibodies [22, 23]. Alternatively, γ HV68 infection is not always detrimental to the host, as it may protect lupus-prone mice from disease development and even confers transient protection against bacterial infection [23–25]. Here, we investigated the association between γ HV68 infection and thrombocytopenia. In particular, we delineated the relative contributions of lytic viral replication, virus latency, and immune components such as antibody to thrombocytopenia development.

2. Materials and Methods

2.1 Mice and viral infections

Female 6- to 12-wk old C57BL/6 or AID^{-/-} μ s^{-/-} mice [26, 27] were obtained from the Trudeau Institute animal facility and kept under specific-pathogen free conditions. AID^{-/-} μ s^{-/-} mice were kept on sulfamethoxazole-trimethoprim food (Test Diet). Mice were anesthetized with 2,2,2-tribromoethanol and infected intranasally (i.n.) with 400 plaque forming units (PFU) murine gammaherpesvirus-68 (γ HV68, strain WUMS) or AC-RTA [28](a generous gift from R. Sun and T-T. Wu, UCLA) in 30 μ l HBSS. All animal

experiments were approved by the Institutional Animal Care and Use Committee of the Trudeau Institute.

2.2 Infective center assay

Triplicate 10-fold serial dilutions of splenocytes were plated onto monolayers of NIH-3T3 mouse fibroblast cells, incubated overnight at 37°C, and overlaid with carboxymethyl cellulose. Plaques were counted 6 d later after methanol fixation and Giemsa staining. Samples were also assayed following one cycle of freeze/thaw to determine the contribution of lytic virus to the overall viral titers. The number of latently infected cells was calculated as the difference between the total number of infected cells and the number of lytically infected cells.

2.3 Quantitative real-time PCR

DNA was isolated from spleens or from platelets pooled from 10 mice, 17 d after mock- or γ HV68 infection. The copy number of the γ HV68 ORF50 gene in 200 ng DNA was determined by quantitative real-time PCR using Taqman Gene Expression master mix (Applied Biosystems) and a standard curve quantitation method on an Applied Biosystems 7500 Real-Time PCR system. Primers, probes, and reaction cycles used were as described previously [29].

2.4 Flow cytometry

Splenocytes and lymphocytes isolated from lung tissue were treated with Fc Block (anti-CD16/CD32) then stained with appropriate antibodies. Allophycocyanin-conjugated MHC class I tetramers specific for γ HV68 epitopes ORF6₄₈₇₋₄₉₅D^b (AGPHNDMEI), ORF8₆₀₄₋₆₁₂K^b (KNYIFEEKL), ORF61₅₂₄₋₅₃₁K^b (TSINFVKI), and ORF75_{c940-947}K^b (KSLTYYYKL) were obtained from the Trudeau Institute Molecular Biology Core Facility. Fluorochrome-conjugated antibodies against CD8, CD19, CD44, and CD69 were purchased from BD Biosciences. Samples were collected on a BD FACSCalibur or FACSCanto II cytometers and analyzed using FlowJo software (TreeStar).

2.5 Hematological measurements

Mouse blood was collected on indicated days, diluted 1:20 in PBS-EDTA, and platelet numbers, white and red blood cell numbers, and hematocrits were determined by Coulter counter (Beckman Coulter). Reticulated platelets in 5 μ l mouse blood were measured by thiazole orange (Retic-Count; BD Biosciences) incorporation for 1 h in the dark at room temperature, then fixed in 1% formaldehyde and analyzed by flow cytometry.

2.6 Warfarin treatment

Mice were treated with 2 mg/l warfarin [3-(α -acetylbenzyl)-4-hydroxycoumarin; Sigma] in the drinking water beginning 10 d p.i. and replenished every 48 h thereafter. We have previously demonstrated that this dose of warfarin significantly anticoagulates mice [30, 31].

2.7 Platelet lifespan determination

Mice were injected intravenously with 5 mM carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) at 17 d post infection. At 3 h, 24 h, 48 h, 72 h, and 94 h after injection, 5 μ L blood was collected by mandibular bleed and mixed with 395 μ L PBS-EDTA containing phycoerythrin-conjugated antibody to CD41 (BD Biosciences) for 30 min in the dark at room temperature. Samples were then fixed with 1% formaldehyde and analyzed by flow cytometry.

In order to characterize the survival of platelets, we used mean platelet lifespan instead of the customary “half-life”, because half-life would be an inaccurate characteristic for non-exponential survival curves. We calculated mean platelet lifespan using the method previously described [32, 33]. Briefly, assuming that a platelet’s life span when released from bone marrow has a lognormal probability distribution, the survival curve would be described by the following survival function,

$$S(t) = \frac{1}{2} (1 - e^{-(m+s^2/2)t}) (1 - \operatorname{erf}(\frac{\ln(t)-m}{\sqrt{2}s})) - \operatorname{erf}(\frac{\ln(t)-(m+s^2)}{\sqrt{2}s})),$$

where t is the time since the beginning of observation, and m, s are the mean and standard deviation of the natural logarithm of lifespans at the production time in bone marrow (parameters of the lognormal distribution). We fitted this survival function to the experimental data using the least squares method implemented in the function *NonlinearModelFit* in Mathematica 8.0 (Wolfram). Mean lifespan at birth was found as $mean = e^{m+s^2/2}$ (the mean of the lognormal distribution).

2.8 Preparation of washed human platelets

Human venous blood was collected from healthy volunteers into sodium citrate (final concentration 0.38% v/v) and acid/citrate/dextrose (10% v/v) to purify the platelets as previously described [34]. Briefly, platelet-rich plasma (PRP) was prepared by centrifugation of whole blood at 200 g for 20 min. The platelets were isolated from PRP from centrifugation at 1000 g for 10 min in the presence of prostacyclin (0.1 $\mu\text{g/ml}$). After centrifugation, purified human platelets were resuspended in modified Tyrode’s buffer (129 mM NaCl, 0.34 mM Na_2HPO_4 , 2.9 mM KCl, 12 mM NaHCO_3 , 20 mM HEPES, 5 mM glucose, 1 mM MgCl_2 ; pH 7.3).

2.9 Anti-platelet antibody detection

Anti-platelet antibodies were detected using modified protocols based on Huang, et al. [35]. Washed human platelets were incubated with 1:20 dilutions of experimental serum samples or human serum type AB (Atlanta Biologicals) for 10 min at 37°C, followed by centrifugation at 1000 g for 10 min in the presence of prostacyclin (1 $\mu\text{g/ml}$). Cell pellets were then resuspended in PBS and centrifuged at 1000 g for 10 min. Cells were then resuspended in modified Tyrode’s buffer and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibody (Southern Biotech) at room temperature (RT) for 20 min. Samples were diluted in modified Tyrode’s buffer and analyzed by flow cytometry. Alternatively, platelets were isolated from sheep whole blood in Na-EDTA (Lampire Biological Laboratories) then incubated with various dilutions of mock-infected or γHV68 -infected serum for 60 min on ice, washed twice with PBS, incubated with FITC-conjugated goat anti-mouse IgG antibody for 40 min on ice, washed twice in PBS, then resuspended in PBS and analyzed by flow cytometry.

2.10 Platelet adhesion assay

Glass coverslips were incubated with a suspension of fibrinogen (50 $\mu\text{g/ml}$) for 1 h at RT. Surfaces were then blocked with denatured bovine serum albumin (5 mg/ml) for 1 h at RT followed by washing with PBS before use in adhesion assays. Washed human platelets were incubated at 37°C for 10 min on fibrinogen-coated coverslips followed by washing with PBS. Subsequently, experimental serum samples or human serum type AB were added to adherent platelets and incubated at 37°C for 10 min. After washing with PBS, adherent platelets were incubated with FITC-conjugated goat anti-mouse IgG antibody in modified

Tyrode's buffer at 37°C for 20 min. Fluorescence and differential interference contrast (DIC) images were recorded using an Axiovert 200M inverted fluorescence microscope (Carl Zeiss) as previously described [34].

2.11 Statistical Analysis

Statistical analyses were performed using Student's *t* test, Mann Whitney test, or one-way ANOVA with Prism 5 software (GraphPad). Differences were considered significant at *P* values less than 0.05.

3. Results

3.1 Murine gammaherpesvirus-68 infection induces thrombocytopenia

To investigate the mechanism(s) by which gammaherpesviruses elicit thrombocytopenia, we intranasally infected C57BL/6 mice with γ HV68. Primary γ HV68 infection resulted in an approximately 40% reduction in platelet number by 13 d, reaching peak thrombocytopenia at 17 d after infection (Fig. 1A). Thrombocytopenia was transient, as by 3 mo after infection, platelet numbers had returned to levels observed in mock-infected mice (Fig. 1B).

Thrombocytopenia can be due to impaired production of platelets, loss of platelets due to excessive hemorrhagic bleeding, consumption of platelets due to systemic blood clotting, or pathologic destruction of platelets by immune cells or autoantibodies [6]. γ HV68 can infect bone marrow [36], which could lead to a deficiency in platelet synthesis. However, platelet production was not impaired during thrombocytopenia; γ HV68-infected mice contained about 4-fold more reticulated platelets than mock-infected mice as measured by thiazole orange-incorporation by the residual nucleic acids of newly-formed reticulated platelets (Fig. 2A). One caveat of this assay is that we may be detecting viral nucleic acid within the platelets. Human platelets express C3d complement receptor type II (CR2), a receptor for EBV, suggesting platelets might also be infected by γ HV68 [37]. Importantly, murine platelets are not thought to express CR2 [38], making it unlikely γ HV68 directly infects them *in vivo*. Consistent with this observation, we were unable to detect viral genome copies above the limit of detection in platelet preparations from γ HV68-infected mice (Fig. 2B), strongly supporting the conclusion that the nucleic acid we measured is from reticulated, and not virus-infected, platelets. We next measured platelet lifespan by injecting CFSE intravenously and measuring the loss of CFSE-labeled platelets over time. We found a significant reduction in the lifespan of platelets at 17 d post infection (p.i.) as compared to mock-infected mice, consistent with faster clearance of platelets during γ HV68-induced thrombocytopenia (Fig. 2C). The hematocrit was similar in γ HV68-infected and mock-infected mice 17 d p.i., suggesting that uncontrolled bleeding was not the cause of the reduced platelet lifespan (Fig. 2D). Additionally, suppression of coagulation by treatment with the anticoagulant warfarin did not increase platelet numbers in mice infected with γ HV68, suggesting that systemic activation of a coagulation response does not cause the reduced platelet lifespan and thrombocytopenia (Fig. 2E).

3.2 Virus-induced thrombocytopenia is mediated by antibody

Given that virus-induced thrombocytopenia was not due to impaired platelet production, excessive blood loss, or wholesale activation of the coagulation response, we investigated whether γ HV68-infected mice developed platelet-specific antibodies. Using an assay to identify serum platelet-binding activity [35], we observed IgG antibodies that were cross-reactive with human and sheep platelets in the serum of γ HV68-infected mice, but not in serum from mock-infected mice (Fig. 3A). Serum from γ HV68-infected mice also displayed platelet-binding activity on surface adherent human platelets (Fig. 3B). These observations demonstrate the cross-reactivity of anti-platelet antibodies from γ HV68-infected mice and

are consistent with prior reports of polyclonal antibody production after γ HV68 infection, as well as the detection of anti-platelet antibodies in EBV-infected patients [9, 10, 22, 23]. Our laboratory is currently investigating the identity of the protein targets of γ HV68-induced thrombocytopenia-mediating antibodies.

To confirm a necessary role for antibodies in mediating thrombocytopenia *in vivo*, we infected antibody-deficient activation-induced cytidine deaminase (AID)/secretory μ -chain (μ s) double-knockout (AID^{-/-} μ s^{-/-}) mice. First described by Ploegh and colleagues, AID^{-/-} μ s^{-/-} mice cannot secrete IgM and are unable to undergo class switch recombination or somatic hypermutation, but still express immunoglobulin on the B cell surface [26]. These mice make an excellent model for antibody-deficiency during γ HV68 infection, since they are devoid of circulating antibodies yet retain a complete B cell compartment, the major reservoir for latent virus. In the absence of circulating antibody, γ HV68 infection did not cause thrombocytopenia (Fig. 4A). This result suggests that antibody is important for the development of thrombocytopenia, but does not formally rule out several other possibilities, including: 1) that viral infection is abortive in AID^{-/-} μ s^{-/-} mice, 2) that AID^{-/-} μ s^{-/-} mice do not develop splenomegaly, a potential cause of thrombocytopenia, and 3) that AID^{-/-} μ s^{-/-} mice are deficient in antiviral T cells, that may be cross-reactive with platelets.

With regard to viral infection, we have recently shown that γ HV68-infection of AID^{-/-} μ s^{-/-} mice leads to a prolonged lytic infection in the lungs [39]. To determine whether latent virus persists in the spleens of these mice, we compared latent viral loads in the spleen 21 d after infection in C57BL/6 or AID^{-/-} μ s^{-/-} mice by infective center assay, an *ex vivo* measure of reactivatable latent virus (Fig. 4B). Both strains of mice had similar levels of latent virus indicating similar infection efficacy. Thus, reductions in lytic or latent virus were not the reason for the lack of thrombocytopenia in AID^{-/-} μ s^{-/-} mice.

Sequestration of platelets in the spleen during the profound splenomegaly that accompanies the amplification phase of γ HV68 latency is a potential cause of platelet reduction in the blood [40]; however, we were able to rule out splenomegaly as a direct cause of thrombocytopenia in this system because AID^{-/-} μ s^{-/-} mice exhibited significant splenomegaly ($P=0.0057$) in the absence of thrombocytopenia 14 d after infection (Fig. 4C).

We next asked whether AID^{-/-} μ s^{-/-} mice established an antiviral CD8 T cell response following γ HV68 infection. We observed similar CD8 T cell responses for three out of four γ HV68-specific epitopes in the lungs of C57BL/6 and AID^{-/-} μ s^{-/-} mice 17 d after infection (Fig. 4D,E). The other response (to ORF61₅₂₄K^b) was only slightly decreased in AID^{-/-} μ s^{-/-} mice, consistent with the antibody-deficient mice establishing a robust antiviral CD8 T cell response. A possible cell-mediated mechanism for thrombocytopenia development could be the expansion of activated CD8 T cells expressing V β 4 T cell receptors that accompanies the virus-induced infectious mononucleosis-like syndrome, as these cells have been shown to be important in preventing allograft tolerance and in mediating immunopathology of IFN γ R^{-/-} mice [41–43]. However, the expansion of these cells is not observed until 21 d p.i., when platelet numbers are already recovering [16]. It is possible that T cells could potentiate thrombocytopenia via an indirect, antibody-dependent mechanism. Although these data do not completely rule out the possibility of cell-mediated contributions to the development of thrombocytopenia, the evidence strongly supports a major role for antibody in the reduction of platelets following γ HV68 infection. Nevertheless, it should be noted that we were unable to induce thrombocytopenia in naïve mice (or γ HV68-infected AID^{-/-} μ s^{-/-} mice) by injection of serum from γ HV68-infected C57BL/6 mice. This could be due to a number of reasons, including, but not limited to, low level reactivity of anti-platelet antibodies with mouse platelets or a more complex

mechanism of thrombocytopenia induction by γ HV68 infection such as one that involves a contribution by T cells.

3.3 Viral latency is required for γ HV68-induced thrombocytopenia

Intranasal infection of C57BL/6 mice results in peak viral titers in the lungs around 6 d and clearance of lytic virus from the lungs by 10 d p.i. [44]. Since the onset of thrombocytopenia was not until 13 d after infection, when lytic virus is cleared but latency is undergoing amplification in the spleen, we tested whether a latency-deficient virus was capable of triggering thrombocytopenia. We infected mice with β -actin/chicken-replication and transcription activator (AC-RTA) virus, a latency-null recombinant γ HV68 that undergoes transient lytic infection in the lungs but cannot establish latency in the spleen, and does not induce latency-associated phenomena such as splenomegaly or the expansion of CD8 T cells with a V β 4 T cell receptor component [28, 45]. AC-RTA infection did not lead to a loss of platelets, likely ruling out direct viral infection of platelets as a cause of γ HV68-induced thrombocytopenia (Fig. 5A). Also, the absence of thrombocytopenia was not due to a complete lack of antibody production, as AC-RTA infection has been shown to generate high titers of virus-specific IgG antibody [28, 39]. Interestingly, this finding suggests that *bona fide* virus-specific antibodies following γ HV68 infection are not cross-reactive with platelets. To determine whether viral latency specifically induced thrombocytopenia rather than a generalized leukopenia, we measured whether infection with WT γ HV68 or AC-RTA lead to a loss of other circulating cell types. Acute infection with either virus resulted in a drop in white blood cell numbers, beginning 5 d p.i. and recovering by 24 d p.i. (Fig. 5B). Neither virus induced a loss in red blood cell (RBC) numbers, demonstrating that γ HV68 infection does not induce a pan-cytopenia (Fig. 5C). Taken together, the data suggest that leukopenia appears to be a consequence of acute γ HV68 infection whereas thrombocytopenia is likely reliant on a viral latency-associated component. So what property of latent viral infection leads to the development of autoimmunity? To investigate this, we measured polyclonal B cell activation in the spleen following infection with γ HV68 or AC-RTA (Fig. 5D). Wild-type γ HV68 but not AC-RTA induced a significant upregulation ($P < 0.001$) of the early activation marker CD69 on B cells 12 d after infection. Taken together, these data strongly support a model in which polyclonal B cell activation induced by the amplification of viral latency in the spleen leads to the synthesis of either cross-reactive virus-specific antibodies or anti-platelet autoantibodies during γ HV68 infection, which in turn leads to the development of thrombocytopenia.

When administered prophylactically, AC-RTA has been shown to prevent both acute infection and latency after wild-type γ HV68 challenge [28, 39]; therefore, vaccination with AC-RTA would also be expected to prevent γ HV68-induced thrombocytopenia. Indeed, AC-RTA vaccination prevented the decreases in platelet numbers and white blood cells following γ HV68 challenge (Fig. 5E,F). These data demonstrate the value of the mouse γ HV68 infection model for testing therapies and treatment strategies against gammaherpesvirus-induced thrombocytopenia.

4. Discussion

Human gammaherpesvirus infections can lead to many complications including mononucleosis, transplant rejection, development of malignancies, and autoimmune disease. The association of gammaherpesvirus infections and autoimmunity is still a contentious issue, however. In mice and rats, γ HV68 infection can exacerbate experimental autoimmune encephalitis [19, 20] and γ HV68 infection of mice is associated with autoantibody production [22, 23], but autoantibody expression wanes over time, and γ HV68-infected lupus-prone mice are actually protected from lupus development [23]. Similarly, we found that infection of C57BL/6 mice with γ HV68 resulted in a prolonged, but ultimately

transient, thrombocytopenia, beginning about 13 d and lasting until 3 mo p.i. and the presence of anti-platelet antibodies in the serum, consistent with the production of autoantibodies specific for chromatin, dsDNA, Smith antigen, and type II collagen seen previously [22, 23]. Mice genetically deficient in antibody production and secretion did not develop thrombocytopenia following γ HV68 infection - further evidence of a role for antibody. Although we cannot formally rule out a T cell-mediated component to the development of thrombocytopenia, we observed robust CD8 T cell responses in C57BL/6 and AID^{-/-} μ s^{-/-} mice after γ HV68 infection, suggesting antiviral T cell responses are not sufficient to induce thrombocytopenia. Infection with AC-RTA, a recombinant γ HV68 that is unable to establish latency, did not induce thrombocytopenia, thereby identifying a role for viral latency in the etiology of virus-induced thrombocytopenia. Importantly, AC-RTA infection did not lead to polyclonal activation of B cells, providing a key clue to the source of viral infection-induced thrombocytopenia-mediating antibodies.

Our data fit in a growing body of evidence linking viral infections to acute and chronic thrombocytopenia. Viruses such as HIV, HCV, paramyxoviruses, and herpesviruses can induce thrombocytopenia by direct infection of megakaryocytes or platelets, or indirectly by triggering autoimmune mechanisms that lead to platelet destruction and clearance [6, 8, 46, 47]. Even transient virus-induced thrombocytopenia could present serious complications for patients, such as hyperhemolysis following vaccination during acute EBV-associated thrombocytopenia [48]. The association of pathogens with thrombocytopenia is not always detrimental, however, as there is evidence that viral or bacterial infections may ameliorate chronic thrombocytopenia by promoting platelet production [49–51].

It should be noted that non-infectious disorders can also lead to antibody-mediated thrombocytopenia. Heparin therapy may lead to thrombocytopenia through binding of antibodies to complexes of platelet factor 4 and heparin. These antibodies then bind to Fc receptors on the platelet surface, activating the platelets, leading to increased thrombin production and thrombosis [52]. Thrombocytopenia is also one of many consequences of anti-phospholipid syndrome, when antibodies specific for phospholipids such as β 2-glycoprotein I (also called apolipoprotein H) bind to and activate platelets, leading to thrombosis [53]. Autoimmune hemolytic anemia, a loss of circulating RBCs due to anti-RBC autoantibodies, often manifests with antibody-mediated thrombocytopenia, as both can be driven by polyclonal B cell activation [54]. Notably, following γ HV68 infection, we do not see evidence for autoimmune hemolytic anemia, even in the presence of putative platelet reactive-antibodies and antibodies to collagen type II, dsDNA, and Smith antigen [22, 23].

We do not yet know the specificity of the platelet-reactive antibodies that arise after γ HV68 infection; it is possible that they could be virus-specific antibodies that happen to cross-react with platelets. This phenomenon, known as molecular mimicry, may be important in the association of EBV with autoimmune disorders such as multiple sclerosis or systemic lupus erythematosus [3]. It will be important to determine whether the anti-platelet antibodies are specific for platelet glycoproteins as has been observed in EBV-associated thrombocytopenia [9], as this may provide a clue to which viral protein the antibodies may cross-react. However, because γ HV68 infection results in robust virus-nonspecific antibody production (and AC-RTA infection leads to robust anti-viral antibodies without thrombocytopenia) the anti-platelet autoantibodies may not be virus-specific and cross-reactive; rather, they might only bind platelets, as antibodies specific for other autoantigens have been observed following γ HV68-mediated polyclonal B cell activation [22]. And, as noted above, thrombocytopenia-mediating antibodies could act indirectly through an as-yet-unidentified mechanism leading to platelet clearance. Establishment and amplification of latent γ HV68 infection lead to a transient gamma-interferon (IFN γ)-dependent activation of macrophages that provides protection from lethal bacterial challenge [24, 25], and it has

recently been reported that stimulation with IFN γ can act directly on macrophages to induce anemia and thrombocytopenia [55]. In addition, a humanized mouse model of EBV infection has shown thrombocytopenia associated with hemophagocytosis [56]. Thus, one potential mechanism is the removal of antibody-coated platelets during latent γ HV68 infection by IFN γ -activated macrophages. Importantly, thrombocytopenia was transient, with platelet numbers returning to mock-infected levels by 3 mo p.i. Platelet recovery over time could be due to improved bone marrow hematopoiesis or attenuated platelet clearance – we favor the latter explanation, as we do not observe impaired platelet production during thrombocytopenia, and the timing of recovery is consistent with the waning of the highly inflammatory environment that occurs during γ HV68 latency amplification [23, 25].

Which viral genes are important for inducing polyclonal B cell activation? The obvious candidates from this study would be the major latency locus genes removed from the AC-RTA virus: ORF72 (viral cyclin), M11 (viral bcl-2), and ORF73 (latency-associated nuclear antigen) [28]. Additionally, several other viral genes have been shown to be important for optimal establishment of or reactivation from latency [57–60]. Importantly, the polyclonal B cell activation likely results from a combination of viral protein effects, and as such, identifying which components of γ HV68 latency amplification lead to polyclonal B cell activation (and to thrombocytopenia) may prove difficult.

It is interesting to speculate which participant benefits more from polyclonal B cell activation - the virus or the host? γ HV68 could induce B cell activation to optimize the number of susceptible target cells, as germinal center B cells are the preferential target for infection, memory B cells appear to be the main reservoir of latent virus, and plasma cells have been shown to facilitate viral reactivation [14, 61–63]. Alternatively, the increase in irrelevant antibodies may benefit the virus by diluting out antigen-specific antibodies, thereby delaying the antiviral immune response long enough for latent γ HV68 to propagate. However, polyclonal B cell activation may instead be a host strategy to increase natural antibody production, diversify antibody specificities, or even provide an enhanced co-stimulatory milieu for the optimal generation and regulation of antiviral T cells [64]. Thus, it is important to consider whether thrombocytopenia resultant from the polyclonal B cell activation is beneficial to the virus or is a potentially pathological consequence of the host response.

We can now use this model of virus-induced thrombocytopenia to address preventative and therapeutic strategies that may be directly applicable to the clinic. Prophylactic vaccines against gammaherpesviruses would provide protection not only from the acute infection but also from the establishment and amplification of latency and latency-associated complications, such as infectious mononucleosis and thrombocytopenia. Our findings here with AC-RTA vaccination demonstrate the efficacy of one such prophylactic treatment strategy. Therapeutic strategies can also be tested during γ HV68 infection in this model. One method used to combat thrombocytopenia is splenectomy [65]. Given that the polyclonal B cell activation is prominent in the spleen, and splenic B cells are a major reservoir for latent gammaherpesviruses, splenectomy in this system could help alleviate the thrombocytopenia. Alternatively, since we observed splenomegaly without thrombocytopenia after γ HV68 infection in antibody-deficient AID^{-/-} μ s^{-/-} mice, splenectomy by itself may prove an ineffective strategy. Another clinical therapy is treatment of patients with intravenous immunoglobulin (IVIg), which is proposed to act through Fc receptors on macrophages or dendritic cells [66, 67]. By using IVIg treatment in conjunction with mice genetically deficient in components of Fc receptor complexes during γ HV68-induced thrombocytopenia, it may be possible to systematically identify the mechanism of IVIg-mediated protection. A third strategy that can be tested in this mouse model is treatment with a monoclonal anti-CD20 antibody (rituximab) [65]. Rituximab

treatment leads to the depletion of B cells, which in this model are not only the probable source of thrombocytopenia-mediating antibodies but are also the major reservoir of latent virus.

In conclusion, we describe here an important small animal model of gammaherpesvirus-induced thrombocytopenia mediated by antibody and dependent on viral latency. This model will allow for the further investigation of immunopathology of gammaherpesvirus infections and the testing of preventative and therapeutic treatment strategies, as well as provide insight into the fine line between a highly active antiviral immune response and autoimmunity.

Acknowledgments

The authors wish to thank Tori C. Freitas and Meghan K. Jensen for excellent technical assistance and Jacob E. Kohlmeier and William M. Baldwin III for critically reading the manuscript. This work was supported by NIH grants AI084327 (M.L.F.), HL101972 (O.J.T.M.), AI049823 (D.L.W.), AI071295 (S.T.S.), AI042927, AI082919, and CA148250 (M.A.B.), and funding from the Trudeau Institute and the National Health and Medical Research Council (Australia). A.I. is a Vertex Scholar.

References

1. Rickinson, AB.; Kieff, E. Epstein-Barr Virus. In: Knipe, DM.; Howley, PM., editors. *Fields Virology*. Philadelphia: Lippincott Williams and Wilkins; 2001. p. 2575-627.
2. Doherty PC, Christensen JP, Belz GT, Stevenson PG, Sangster MY. Dissecting the host response to a gamma-herpesvirus. *Philos Trans R Soc Lond B Biol Sci*. 2001; 356:581-93. [PubMed: 11313013]
3. Niller HH, Wolf H, Minarovits J. Regulation and dysregulation of Epstein-Barr virus latency: implications for the development of autoimmune diseases. *Autoimmunity*. 2008; 41:298-328. [PubMed: 18432410]
4. Jenson HB. Acute complications of Epstein-Barr virus infectious mononucleosis. *Curr Opin Pediatr*. 2000; 12:263-8. [PubMed: 10836164]
5. Kimura H, Morishima T, Kanegane H, Ohga S, Hoshino Y, Maeda A, et al. Prognostic factors for chronic active Epstein-Barr virus infection. *J Infect Dis*. 2003; 187:527-33. [PubMed: 12599068]
6. Cines DB, Liebman HA. The immune thrombocytopenia syndrome: a disorder of diverse pathogenesis and clinical presentation. *Hematol Oncol Clin North Am*. 2009; 23:1155-61. [PubMed: 19932425]
7. Stasi R, Willis F, Shannon MS, Gordon-Smith EC. Infectious causes of chronic immune thrombocytopenia. *Hematol Oncol Clin North Am*. 2009; 23:1275-97. [PubMed: 19932434]
8. Flaujac C, Boukour S, Cramer-Borde E. Platelets and viruses: an ambivalent relationship. *Cell Mol Life Sci*. 2010; 67:545-56. [PubMed: 20012669]
9. Tanaka M, Kamijo T, Koike K, Ueno I, Nakazawa Y, Kurokawa Y, et al. Specific autoantibodies to platelet glycoproteins in Epstein-Barr virus-associated immune thrombocytopenia. *Int J Hematol*. 2003; 78:168-70. [PubMed: 12953814]
10. Pipp ML, Means ND, Sixbey JW, Morris KL, Gue CL, Baddour LM. Acute Epstein-Barr virus infection complicated by severe thrombocytopenia. *Clin Infect Dis*. 1997; 25:1237-9. [PubMed: 9402388]
11. Virgin HW, Latreille P, Wamsley P, Hallsworth K, Weck KE, Dal Canto AJ, et al. Complete sequence and genomic analysis of murine gammaherpesvirus 68. *J Virol*. 1997; 71:5894-904. [PubMed: 9223479]
12. Efsthathiou S, Ho YM, Hall S, Styles CJ, Scott SD, Gompels UA. Murine herpesvirus 68 is genetically related to the gammaherpesviruses Epstein-Barr virus and herpesvirus saimiri. *J Gen Virol*. 1990; 71 (Pt 6):1365-72. [PubMed: 2161903]
13. Blackman MA, Flano E. Persistent gamma-herpesvirus infections: what can we learn from an experimental mouse model? *J Exp Med*. 2002; 195:F29-32. [PubMed: 11927639]

14. Flano E, Husain SM, Sample JT, Woodland DL, Blackman MA. Latent murine gamma-herpesvirus infection is established in activated B cells, dendritic cells, and macrophages. *J Immunol.* 2000; 165:1074–81. [PubMed: 10878386]
15. Flano E, Jia Q, Moore J, Woodland DL, Sun R, Blackman MA. Early establishment of gamma-herpesvirus latency: implications for immune control. *J Immunol.* 2005; 174:4972–8. [PubMed: 15814726]
16. Tripp RA, Hamilton-Easton AM, Cardin RD, Nguyen P, Behm FG, Woodland DL, et al. Pathogenesis of an infectious mononucleosis-like disease induced by a murine gamma-herpesvirus: role for a viral superantigen? *J Exp Med.* 1997; 185:1641–50. [PubMed: 9151901]
17. Coppola MA, Flano E, Nguyen P, Hardy CL, Cardin RD, Shastri N, et al. Apparent MHC-independent stimulation of CD8+ T cells in vivo during latent murine gammaherpesvirus infection. *J Immunol.* 1999; 163:1481–9. [PubMed: 10415050]
18. Hardy CL, Flano E, Cardin RD, Kim IJ, Nguyen P, King S, et al. Factors controlling levels of CD8+ T-cell lymphocytosis associated with murine gamma-herpesvirus infection. *Viral Immunol.* 2001; 14:391–402. [PubMed: 11792068]
19. Peacock JW, Elsaawa SF, Petty CC, Hickey WF, Bost KL. Exacerbation of experimental autoimmune encephalomyelitis in rodents infected with murine gammaherpesvirus-68. *Eur J Immunol.* 2003; 33:1849–58. [PubMed: 12811845]
20. Casiraghi C, Shanina I, Cho S, Freeman ML, Blackman MA, Horwitz MS. Gammaherpesvirus Latency Accentuates EAE Pathogenesis: Relevance to Epstein-Barr Virus and Multiple Sclerosis. *PLoS Pathog.* 2012; 8:e1002715. [PubMed: 22615572]
21. Nelson DA, Petty CC, Bost KL. Infection with murine gammaherpesvirus 68 exacerbates inflammatory bowel disease in IL-10-deficient mice. *Inflamm Res.* 2009; 58:881–9. [PubMed: 19544045]
22. Sangster MY, Topham DJ, D'Costa S, Cardin RD, Marion TN, Myers LK, et al. Analysis of the virus-specific and nonspecific B cell response to a persistent B-lymphotropic gammaherpesvirus. *J Immunol.* 2000; 164:1820–8. [PubMed: 10657630]
23. Larson JD, Thurman JM, Rubtsov AV, Claypool D, Marrack P, van Dyk LF, et al. Murine gammaherpesvirus 68 infection protects lupus-prone mice from the development of autoimmunity. *Proc Natl Acad Sci U S A.* 2012; 109:E1092–100. [PubMed: 22474381]
24. Barton ES, White DW, Cathelyn JS, Brett-McClellan KA, Engle M, Diamond MS, et al. Herpesvirus latency confers symbiotic protection from bacterial infection. *Nature.* 2007; 447:326–9. [PubMed: 17507983]
25. Yager EJ, Szaba FM, Kummer LW, Lanzer KG, Burkum CE, Smiley ST, et al. gamma-Herpesvirus-induced protection against bacterial infection is transient. *Viral Immunol.* 2009; 22:67–72. [PubMed: 19210230]
26. Kumazaki K, Tirosh B, Maehr R, Boes M, Honjo T, Ploegh HL. AID^{-/-} mice are agammaglobulinemic and fail to maintain B220-CD138+ plasma cells. *J Immunol.* 2007; 178:2192–203. [PubMed: 17277124]
27. Carragher DM, Kaminski DA, Moquin A, Hartson L, Randall TD. A novel role for non-neutralizing antibodies against nucleoprotein in facilitating resistance to influenza virus. *J Immunol.* 2008; 181:4168–76. [PubMed: 18768874]
28. Jia Q, Freeman ML, Yager EJ, McHardy I, Tong L, Martinez-Guzman D, et al. Induction of protective immunity against murine gammaherpesvirus 68 infection in the absence of viral latency. *J Virol.* 2010; 84:2453–65. [PubMed: 20015983]
29. Usherwood EJ, Ward KA, Blackman MA, Stewart JP, Woodland DL. Latent antigen vaccination in a model gammaherpesvirus infection. *J Virol.* 2001; 75:8283–8. [PubMed: 11483773]
30. Johnson LL, Berggren KN, Szaba FM, Chen W, Smiley ST. Fibrin-mediated protection against infection-stimulated immunopathology. *J Exp Med.* 2003; 197:801–6. [PubMed: 12629066]
31. Mullarky IK, Szaba FM, Berggren KN, Parent MA, Kummer LW, Chen W, et al. Infection-stimulated fibrin deposition controls hemorrhage and limits hepatic bacterial growth during listeriosis. *Infect Immun.* 2005; 73:3888–95. [PubMed: 15972474]
32. Dowling MR, Milutinovic D, Hodgkin PD. Modelling cell lifespan and proliferation: is likelihood to die or to divide independent of age? *J R Soc Interface.* 2005; 2:517–26. [PubMed: 16849210]

33. Dowling MR, Josefsson EC, Henley KJ, Hodgkin PD, Kile BT. Platelet senescence is regulated by an internal timer, not damage inflicted by hits. *Blood*. 2010; 116:1776–8. [PubMed: 20530288]
34. Itakura A, Aslan JE, Sinha S, White-Adams TC, Patel IA, Meza-Romero R, et al. Characterization of human platelet binding of recombinant T cell receptor ligand. *J Neuroinflammation*. 2010; 7:75. [PubMed: 21059245]
35. Huang KJ, Li SY, Chen SC, Liu HS, Lin YS, Yeh TM, et al. Manifestation of thrombocytopenia in dengue-2-virus-infected mice. *J Gen Virol*. 2000; 81:2177–82. [PubMed: 10950974]
36. Cardin RD, Brooks JW, Sarawar SR, Doherty PC. Progressive loss of CD8+ T cell-mediated control of a gamma-herpesvirus in the absence of CD4+ T cells. *J Exp Med*. 1996; 184:863–71. [PubMed: 9064346]
37. Nunez D, Charriaut-Marlangue C, Barel M, Benveniste J, Frade R. Activation of human platelets through gp140, the C3d/EBV receptor (CR2). *Eur J Immunol*. 1987; 17:515–20. [PubMed: 2952514]
38. Kinoshita T, Takeda J, Hong K, Kozono H, Sakai H, Inoue K. Monoclonal antibodies to mouse complement receptor type 1 (CR1). Their use in a distribution study showing that mouse erythrocytes and platelets are CR1-negative. *J Immunol*. 1988; 140:3066–72. [PubMed: 2966205]
39. Freeman ML, Burkum CE, Woodland DL, Sun R, Wu TT, Blackman MA. Importance of antibody in virus infection and vaccine-mediated protection by a latency-deficient recombinant murine gamma-herpesvirus-68. *J Immunol*. 2012; 188:1049–56. [PubMed: 22198955]
40. Sandler SG. The spleen and splenectomy in immune (idiopathic) thrombocytopenic purpura. *Semin Hematol*. 2000; 37:10–2. [PubMed: 10676918]
41. Stapler D, Lee ED, Selvaraj SA, Evans AG, Kean LS, Speck SH, et al. Expansion of effector memory TCR Vbeta4+ CD8+ T cells is associated with latent infection-mediated resistance to transplantation tolerance. *J Immunol*. 2008; 180:3190–200. [PubMed: 18292543]
42. Evans AG, Moser JM, Krug LT, Pozharskaya V, Mora AL, Speck SH. A gammaherpesvirus-secreted activator of Vbeta4+ CD8+ T cells regulates chronic infection and immunopathology. *J Exp Med*. 2008; 205:669–84. [PubMed: 18332178]
43. Ebrahimi B, Dutia BM, Brownstein DG, Nash AA. Murine gammaherpesvirus-68 infection causes multi-organ fibrosis and alters leukocyte trafficking in interferon-gamma receptor knockout mice. *Am J Pathol*. 2001; 158:2117–25. [PubMed: 11395389]
44. Stewart JP, Usherwood EJ, Ross A, Dyson H, Nash T. Lung epithelial cells are a major site of murine gammaherpesvirus persistence. *J Exp Med*. 1998; 187:1941–51. [PubMed: 9625754]
45. Freeman ML, Lanzer KG, Cookenham T, Peters B, Sidney J, Wu TT, et al. Two kinetic patterns of epitope-specific CD8 T-cell responses following murine gammaherpesvirus 68 infection. *J Virol*. 2010; 84:2881–92. [PubMed: 20053740]
46. Winiarski J. Mechanisms in childhood idiopathic thrombocytopenic purpura (ITP). *Acta Paediatr Suppl*. 1998; 424:54–6. [PubMed: 9736220]
47. Onan E, Uskudar O, Coskun Y, Akkiz H. Higher hepatitis C virus concentration in platelets than in plasma in a patient with ITP. *Platelets*. 2012; 23:413–4. [PubMed: 22010990]
48. Cserti-Gazdewich CM, Pendergrast JM, Reis M. Postvaccination hyperhemolysis coinciding with remission of Epstein Barr virus (EBV)-associated immune thrombocytopenic purpura (ITP). *Am J Hematol*. 2009; 84:612–3. [PubMed: 19610018]
49. Bordin JO, Smith JW, Hayward CP, Warkentin TE, Wasi P, Kelton JG. Bacterial infection-associated improvement of platelet counts in two patients with chronic and unresponsive idiopathic thrombocytopenic purpura with normal platelet survival studies. *Br J Haematol*. 1995; 90:332–5. [PubMed: 7794752]
50. Costello C. Virus infection-associated improvement of platelet counts in chronic ITP. *Br J Haematol*. 1995; 91:517–8. [PubMed: 8547108]
51. Proctor SJ, Reid MM, Galloway MJ. Virus infection-associated improvement of platelet counts in chronic ITP. *Br J Haematol*. 1995; 91:516–7. [PubMed: 8547107]
52. Warkentin TE. Heparin-induced thrombocytopenia: diagnosis and management. *Circulation*. 2004; 110:e454–8. [PubMed: 15520327]
53. Ruiz-Irastorza G, Crowther M, Branch W, Khamashta MA. Antiphospholipid syndrome. *Lancet*. 2010; 376:1498–509. [PubMed: 20822807]

54. Semple JW, Freedman J. Autoimmune pathogenesis and autoimmune hemolytic anemia. *Semin Hematol.* 2005; 42:122–30. [PubMed: 16041661]
55. Zoller EE, Lykens JE, Terrell CE, Aliberti J, Filipovich AH, Henson PM, et al. Hemophagocytosis causes a consumptive anemia of inflammation. *J Exp Med.* 2011; 208:1203–14. [PubMed: 21624938]
56. Sato K, Misawa N, Nie C, Satou Y, Iwakiri D, Matsuoka M, et al. A novel animal model of Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis in humanized mice. *Blood.* 2011; 117:5663–73. [PubMed: 21467545]
57. Clambey ET, Virgin HW, Speck SH. Disruption of the murine gammaherpesvirus 68 M1 open reading frame leads to enhanced reactivation from latency. *J Virol.* 2000; 74:1973–84. [PubMed: 10644370]
58. Macrae AI, Usherwood EJ, Husain SM, Flano E, Kim IJ, Woodland DL, et al. Murid herpesvirus 4 strain 68 M2 protein is a B-cell-associated antigen important for latency but not lymphocytosis. *J Virol.* 2003; 77:9700–9. [PubMed: 12915582]
59. Herskowitz JH, Jacoby MA, Speck SH. The murine gammaherpesvirus 68 M2 gene is required for efficient reactivation from latently infected B cells. *J Virol.* 2005; 79:2261–73. [PubMed: 15681428]
60. Tarakanova VL, Stanitsa E, Leonardo SM, Bigley TM, Gauld SB. Conserved gammaherpesvirus kinase and histone variant H2AX facilitate gammaherpesvirus latency in vivo. *Virology.* 2010; 405:50–61. [PubMed: 20557919]
61. Sunil-Chandra NP, Efstathiou S, Nash AA. Murine gammaherpesvirus 68 establishes a latent infection in mouse B lymphocytes in vivo. *J Gen Virol.* 1992; 73 (Pt 12):3275–9. [PubMed: 1469366]
62. Flano E, Kim IJ, Woodland DL, Blackman MA. Gamma-herpesvirus latency is preferentially maintained in splenic germinal center and memory B cells. *J Exp Med.* 2002; 196:1363–72. [PubMed: 12438427]
63. Liang X, Collins CM, Mendel JB, Iwakoshi NN, Speck SH. Gammaherpesvirus-driven plasma cell differentiation regulates virus reactivation from latently infected B lymphocytes. *PLoS Pathog.* 2009; 5:e1000677. [PubMed: 19956661]
64. Montes CL, Acosta-Rodriguez EV, Merino MC, Bermejo DA, Gruppi A. Polyclonal B cell activation in infections: infectious agents' devilry or defense mechanism of the host? *J Leukoc Biol.* 2007; 82:1027–32. [PubMed: 17615380]
65. Godeau B, Michel M. Treatment of chronic immune thrombocytopenic purpura in adults. *Ann Hematol.* 2010
66. Samuelsson A, Towers TL, Ravetch JV. Anti-inflammatory activity of IVIG mediated through the inhibitory Fc receptor. *Science.* 2001; 291:484–6. [PubMed: 11161202]
67. Siragam V, Crow AR, Brinc D, Song S, Freedman J, Lazarus AH. Intravenous immunoglobulin ameliorates ITP via activating Fc gamma receptors on dendritic cells. *Nat Med.* 2006; 12:688–92. [PubMed: 16715090]

Highlights

- Murine gammaherpesvirus-68 infection leads to thrombocytopenia
- Platelet-reactive antibodies develop after virus infection
- Antibodies are required for virus-induced thrombocytopenia
- Virus-induced thrombocytopenia requires the establishment of viral latency
- Polyclonal B cell activation is a likely source of anti-platelet antibodies

Fig. 1. γHV68 infection induces thrombocytopenia

(**A and B**) Blood from mock- (open circles) or γHV68 -infected (closed circles) C57BL/6 mice was collected and measured for platelet number ($\pm$ SD) at the indicated times after infection. $n = 7\text{--}15/\text{group}/\text{time point}$, combined from 2–3 experiments; ** $P < 0.01$; *** $P < 0.001$; ns, not significant using Student's t test.

Fig. 2. Virus-induced thrombocytopenia is not due to impaired platelet production, increased hemorrhage, or enhanced clotting
(A) Blood from mock- or γHV68-infected C57BL/6 mice was collected and measured for percent reticulated platelets at 21 d p.i. $n = 2-4$ /group; * $P < 0.05$ using Student's t test. **(B)** The number of viral ORF50 gene copies per 200 ng DNA from spleens or platelets of mice 17 d after γHV68 or mock infection. The dotted line indicates the limit of detection, the average copy number from mock-infected spleens. $n = 2$ /group. **(C)** Blood from mock- or γHV68-infected C57BL/6 mice injected with CFSE at 17 d p.i. was collected 3 h, 24 h, 72 h, and 96 h post injection, and measured for CFSE-labeled platelets. The calculated platelet lifespans are shown. $n = 3-5$ /group; * $P < 0.05$ using Mann Whitney test. **(D)** Blood from mock- or γHV68-infected C57BL/6 mice was collected and measured for hematocrit at 17 d p.i. $n = 8-9$ /group; ns, not significant using Student's t test. **(E)** Blood from warfarin- or vehicle-treated mock or γHV68-infected C57BL/6 mice was collected and measured for platelets 20 d after infection. $n = 5$ /group, all data are representative of at least 2 independent experiments; * $P < 0.05$; ** $P < 0.01$ using Student's t test.

Fig. 3. Anti-platelet antibody in γ HV68-infected mice

(A) Anti-platelet antibodies were detected by staining washed human platelets (left) or sheep platelets (right) that had been incubated with human serum type AB (human serum) or PBS (no serum), with a 1:20 (human platelets) or 1:40 (sheep platelets) dilution of serum from mock-infected mice (mock), or with the same dilution of serum from γ HV68-infected mice 17 d p.i. (γ HV68), with FITC-conjugated goat anti-mouse IgG antibody and analysis by flow cytometry. Histograms are representative of 2 experiments. (B) Immobilized human platelets were incubated with 1:20 final concentration of human serum type AB (human serum), normal mouse serum (mock), or serum from γ HV68-infected mice 17 d p.i. (γ HV68) for 10 min at 37°C. After subsequent washing, platelets were incubated with FITC-conjugated goat anti-mouse IgG antibody in modified Tyrode's buffer at 37°C for 20 min. Cells were image using DIC and fluorescent microscopy. Scale bar: 10 μm.

Fig. 4. γHV68 infection-induced thrombocytopenia is mediated by antibody

(A) Blood from mock- or γHV68 -infected AID^{-/-}μs^{-/-} mice was collected and measured for platelet number ($\pm$ SD) at the indicated times after infection. $n = 4$ –5/group, representative of 3 experiments. (B) Viral latency was measured by infective center assay in the spleen 21 d after γHV68 infection in C57BL/6 or AID^{-/-}μs^{-/-} mice. $n = 3$ /group, representative of 3 experiments; ns, not significant using Student's t test. (C) Spleen weight was measured in mock- or γHV68 -infected AID^{-/-}μs^{-/-} mice 14 d p.i. $n = 3$ –5/group, representative of 2 experiments; ** $P < 0.01$ using Student's t test. (D) Lungs were harvested from γHV68 -infected C57BL/6 or AID^{-/-}μs^{-/-} mice 17 d p.i. and cells were stained with indicated MHC class I tetramers and antibodies to CD8 and CD44. Dot plots are gated on CD8⁺ T cells and are representative of at least 2 experiments. (E) Tetramer-specific CD8⁺ T cell numbers in lung tissue. $n = 5$ /group, representative of at least 2 experiments; * $P < 0.05$; ns, not significant using Student's t test.

Fig. 5. Virus latency is required for thrombocytopenia development

(A–C) Blood from mock-, γHV68 -, or AC-RTA-infected C57BL/6 mice was collected and measured for platelet number ($\pm$ SD) (A), white blood cell number ($\pm$ SD) (B), or red blood cell number ($\pm$ SD) (C), at the indicated times after infection. Black asterisks indicate significance between mock and γHV68 infections. Grey asterisks indicate significance between mock and AC-RTA infections. $n = 8\text{--}15/\text{group}/\text{time point}$, combined from 3 experiments; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ using Student's t test or one-way ANOVA with Dunnett's multiple comparison test. (D) Spleens from mock-, γHV68 -, or AC-RTA-infected C57BL/6 mice were harvested 12 d p.i. and cells were stained with antibodies to CD19 and CD69. The mean fluorescence intensity (MFI) of CD69 on CD19⁺ B cells is shown. $n = 5/\text{group}$, representative of 2 experiments; $***P < 0.001$ using one-way ANOVA. (E and F) Blood from mock- or AC-RTA-vaccinated C57BL/6 mice was collected and measured for platelet number (E) or white blood cell number (F) at the indicated times after γHV68 challenge infection. $n = 9\text{--}10/\text{group}$, combined from 2 experiments; $***P < 0.001$ using Student's t test.