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Platelet-endothelial associations may promote cytomegalovirus replication in the salivary gland in mice

Alicia M. Braxton[a,b], **Alyssa L. Chalmin**[a], **Kevin M. Najarro**[a], **Jacqueline K. Brockhurst**[a,c], **Karl T. Johnson**[a], **Claire E. Lyons**[a], **Brenna Daly**[a,d], **Catherine G. Cryer**[a,c], **Shefali Vijay**[a], **Griffin Cyphers**[a], **Selena M. Guerrero-Martin**[a], **S. Andrew Aston**[a,e], **Kirstin McGee**[a], **Yu-Pin Su**[f], **Ravit Arav-Boger**[f,g], **Kelly A. Metcalf Pate**[a,*]

[a]Department of Molecular and Comparative Pathobiology, Johns Hopkins University School of Medicine, Baltimore, USA

[b]Department of Oncology, Johns Hopkins University School of Medicine, Baltimore, USA

[c]University of Pennsylvania School of Veterinary Medicine, Philadelphia, USA

[d]Cummings School of Veterinary Medicine, Tufts University, North Grafton, USA

[e]Department of Psychiatry of Behavioral Sciences, Johns Hopkins University School of Medicine, Baltimore, USA

[f]Department of Pediatrics, Johns Hopkins University School of Medicine, Baltimore, USA

[g]Department of Pediatrics, Medical College of Wisconsin, Milwaukee, USA

Abstract

Platelet decline is a feature of many acute viral infections, including cytomegalovirus (CMV) infection in humans and mice. Platelet sequestration in association with other cells, including endothelium and circulating leukocytes, can contribute to this decline and influence the immune response to and pathogenesis of viral infection. We sought to determine if platelet-endothelial associations (PEAs) contribute to platelet decline during acute murine CMV (mCMV) infection, and if these associations affect viral load and production. Male BALB/c mice were infected with mCMV (Smith strain), euthanized at timepoints throughout acute infection and compared to uninfected controls. An increase in PEA formation was confirmed in the salivary gland at all post-inoculation timepoints using immunohistochemistry for CD41+ platelets co-localizing with CD34+ vessels. Platelet depletion did not change amount of viral DNA or timecourse of infection, as measured by qPCR. However, platelet depletion reduced viral titer of mCMV in the salivary glands while undepleted controls demonstrated robust replication in the tissue by plaque assay. Thus, platelet associations with endothelium may enhance the ability of mCMV to replicate within

*Correspondence: kpate5@jhmi.edu; Tel.: +1-410-955-9770.

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the salivary gland. Further work is needed to determine the mechanisms behind this effect and if pharmacologic inhibition of PEAs may reduce CMV production in acutely infected patients.

Keywords

platelet-endothelial association; cytomegalovirus; platelet-leukocyte aggregate; mouse; platelet decline; platelet-monocyte aggregate

Introduction

Platelet decline is a common feature of acute viral infection, including human and mouse cytomegalovirus (CMV).[1–13] Platelets have been shown to respond to viral infection through interactions with leukocytes and endothelial cells.[13–15] This has most extensively been studied in the context of HIV with sequestration of activated platelets in platelet-monocyte aggregates (PMA) driving platelet decline during acute HIV infections.[16] These PMAs subsequently promote monocyte extravasation, potentially facilitating virus entry into tissue.[17,18] Alternatively, platelets can be sequestered in platelet-endothelial associations (PEAs), as seen in dengue virus.[9,13] These PEAs in the brain affect blood brain barrier permeability, directly facilitating virus entry. The mechanisms through which viral infection causes these associations and through which these association affect viral pathogenesis have yet to be fully understood, and it is unclear if these associations are a universal downstream effect of acute viral infection or a specific response to select viruses. We sought to determine if these associations occur concurrent with platelet decline in a murine cytomegalovirus (mCMV) infected mouse model of acute viral infection, and define any downstream effects on viral entry and replication into tissues.

CMV, a beta herpes virus, is highly prevalent, infecting nearly 1 in 3 children by the age of five and over half of adults over the age of 40.[19,20] Following entry of human CMV (hCMV) into mucosal surfaces, acute infection is characterized by evasion of innate immune responses and hematological spread to the salivary gland, lungs, and lymphoid tissues, resulting in cytokine release; importantly, platelet decline is a feature of acute hCMV infection.[11,21] hCMV can infect a broad range of cells, including endothelial cells and myeloid cells, such as monocytes and macrophages.[22–25] Viremia is ultimately controlled by a T cell mediated adaptive immune response, although a prolonged persistent infection is often observed.[21] Infection is usually subclinical in immunocompetent hosts, but clinical disease can become apparent in immunosuppressed or congenitally infected hosts, and cure is prevented by a state of latent infection.[21,26,27]

Experimental infection of mice with mCMV is an established model for studies of hCMV infections.[28–33] As seen in acute hCMV infection, mCMV spreads hematologically to multiple organs including the liver, spleen, lungs and salivary glands resulting in viral replication in organs, decreased liver function, release of cytokines, and immunosuppression.[34] mCMV progression can be tracked in murine tissues using real-time PCR as a highly sensitive method for the presence of virus DNA, and plaque assays for the presence of actively replicating virus.[35–43] Latent mCMV infection, defined in the literature as one year post-inoculation when circulating viral DNA cannot be detected in the blood, is outside the

scope of this study.[37,44,45] Platelet decline has previously been documented in mCMV infected mice, making the mCMV-infected mouse model a logical choice in which to further investigate the universality and downstream consequences of platelet sequestration during acute viral infection.

In this manuscript, we seek to determine if sequestration of platelets in platelet-leukocyte or platelet-endothelial associations contributes to platelet decline during acute mCMV infection (days 0 through 21 post inoculation), and to determine if such associations play a role in the pathogenesis of mCMV infection in the mouse. We hypothesize that PMA and PEA formation will contribute to platelet decline during acute mCMV infection, and that the presence of platelets will affect the amount of mCMV that enters into and replicates within key organs such as the spleen, lungs, or salivary gland. We characterize the nature of platelet activation in mCMV, and document the sequestration of platelets in PEAs but not PMAs or other platelet-leukocyte aggregates concurrent with platelet decline and throughout acute infection. We furthermore demonstrate reduced production of mCMV progeny in the salivary glands of mice in the absence of platelets despite normal levels of mCMV DNA. These data imply that platelets sequestered in PEAs during acute infection may influence viral production in the salivary gland, and ultimately may have clinical implications for the development of strategies to prevent active CMV replication in acutely infected patients.

Methods

Animal Work

Male 6 to 8 week old BALB/c mice were purchased from Jackson Laboratory; group size for each time point, organ and condition is available in Supplemental Table 1. BALB/c mice were chosen for this study because they are particularly susceptible to mCMV infection and are commonly used to model the pathogenesis of infection.[29–32,46–49] All mice were group housed in individually ventilated cages within ABSL-2 specific pathogen-free barrier facilities at a fully AAALAC accredited institution. All animal work was approved by the Johns Hopkins University Institutional Animal Care and Use Committee. Mice were inoculated with 3×10^6 PFU of mCMV (Smith strain) via intraperitoneal injection, or mock inoculated with sterile 1x PBS as a control. Viral stocks of mCMV were obtains from American Type Culture Collection (ATCC VR-1399), expanded in SC-1 mouse embryonic fibroblasts (MEFs) (ATCC SC-1 CRL 1404), and harvested from freezer lysed cells through centrifugation at 3000 rpm at 4°C for 10 minutes. Virus was then pelleted from the supernatant at 20,000 g at 4°C for 3 hours and purified using centrifugation of a 15% sucrose / virus standard buffer gradient for 1 hour at 32,000 rpm followed by 0.45 μm filtration. To establish a timecourse of platelet counts following infection, longitudinal platelet counts were determined via staggered every other day mandibular sinus venipuncture in mouse cohorts following inoculation or mock inoculation (N = 5 per cohort, with one cohort starting day 1 post-inoculation and the other on day 2); these data were combined to build a longitudinal timecourse of daily platelet counts for infected and uninfected mice. For the remaining studies, groups were subsequently assigned to platelet depleted and non-platelet depleted groups as indicated. For platelet depletion, animals received 50 μg of mouse platelet-specific anti-GPIb antibody (emfret analytics) every 3 days

via intraperitoneal injection until euthanized (adapted from ref. 49). For non-depleted groups, animals received 50 μg of polyclonal rat IgG (emfret analytics) intraperitoneally as a control every 3 days until euthanized. Pilot studies were completed to optimize and confirm efficacy of this depletion protocol, and efficacy of this depletion protocol was confirmed in study animals using complete blood counts on a machine calibrated for work with mouse blood (IDEXX ProCyte).Platelet depletion was considered successful if platelet count was <500K/μL or plateletcrit < 11%.

Cohorts of mice were euthanized at timepoints throughtout the acute phase of mCMV infection at days 3, 8, or 21 following inoculation, and compared to uninoculated mice (day 0) as a control. Immediately prior to euthanasia, mice were deeply anesthetized with intraperitoneal ketamine (50mg/kg) and xylazine (5mg/kg), and blood was collected via terminal cardiocentesis for complete blood counts and FACs analysis. Blood for complete blood count was placed in EDTA tubes and analyzed the same day. Citrated tubes were used for flow cytometry blood samples; processing occurred the same day and flow cytometry was performed within 24 hours of collection. Immediately following blood collection, mice were perfused with sterile saline, and the salivary gland, lung and spleen were harvested for qPCR and viral plaque assays. Tissue samples were stored at −80°C prior to processing.

Flow Cytometry

Staining for platelet activation was performed by incubating whole blood with appropriate antibodies for 15 minutes at room temperature and then fixing them at a 1:20 volume / volume ratio in 2% neutral buffered formalin. For flow cytometry of platelet leukocyte formation, whole blood was incubated at room temperature for 20 minutes with appropriate antibodies, red blood cells lysed and samples fixed through incubation with FACS lysing solution (BD Biosciences), followed by resuspension in 0.5 mL 1x PBS. Samples were analyzed on a Becton Dickinson FACsCalibur and analyzed using FlowJo (Bectin Dickinson). The following monoclonal antibodies were used: CD62P (BD Biosciences monoclonal rat anti-mouse antibody cloneRB40.34 at 0.5 mg/ml), CD145 (BD Biosciences hamster anti-mouse clone MR1 at 0.2 mg/ml), CD41 (BioLegend rat anti-mouse clone MWReg30 at 0.5 mg/ml), H-2Ld/H-2Db (BioLegend mouse anti-mouse H2Ld/H-2Db clone 28–14-8 at 0.2 mg/ml), H-2Kb (eBioscience mouse anti-mouse H-2Kb clone AF6–88.5.5.3 at 0.5 mg/mL), I-A/I-E (BioLegend rat anti-mouse clone M5/114.15.2 at 0.5 mg/mL), CD3 (BD Biosciences rat anti-mouse clone 17A2 at 0.5 mg/mL), CD4 (BioLegend rat anti-mouse clone GK1.5 at 0.5 mg/mL), CD8 (BioLegend rat anti-mouse clone 53–6.7 at 0.5 mg/mL), CD43 (BD Biosciences rat anti-mouse clone S7 at 0.5mg/mL), Ly-6C (BioLegend rat anti-mouse clone HK1.4 at 0.5 mg/mL). Percentage of PMAs and PLAs presented as percentage of a given cell subtype that are bound to CD41+ platelets.

Quantitative PCR

DNA was extracted using the QIAGEN DNeasy Blood and Tissue kit from spleens, salivary glands and lungs harvested from mice of each cohort with the following modifications. Tissues were lysed in Lysing Matrix A tubes (MP Biomedicals) using buffer ATL followed by homogenization for 30 seconds in a FastPrep-24 instrument (MP Biomedicals). After the proteinase K digestion and incubation at 55°C for 90 minutes, RNA was degraded (RNase A

and buffer AL QIAGEN) during a 10 minute incubation at 70°C. DNA was eluted using the QIAGEN spin columns and elution buffer. DNA concentration was determined via a NanoDrop (ThermoFisher) and 100 ng of DNA was used for each PCR reaction.

mCMV glycoprotein B DNA was quantified using the following primers and probe sequences (5' →3'): mCMV <F: AGGGCTTGGAGAGGACCTACA, R: GCCCGTCGGCAGTCTAGTC>; CMV FAM probe <AGATGCTCTATTGATACTCCGCGCG> (adapted from ref. 37) alongside a standard curve with a range of 200 – 2,000,000 copies, and we defined the limit of detection as 500 copies; samples with copy numbers less than 500 were reported as zero copies. Reaction conditions were as follows: 95°C for 2 minutes, followed by 36 cycles of 95°C for 30 seconds and 57°C for 20 seconds, with a 20 second extension at 72°C. Viral DNA was reported out in log scale as copies and is presented normalized to micrograms of DNA.

Plaque Assays

SC-1 mouse embryonic fibroblasts (MEFs) were obtained from ATCC (SC-1 CRL 1404) and passaged fewer than 9 times prior to use in plaque assays. Plaque assays were completed as previously described by Brune, Hengel and Koszinowski (2001). Briefly, 24 well plates were seeded 18–24 hours prior to titration with tissue homogenates, resulting in 50–70% confluency at the time of addition of tissue homogenates.

Spleen, lung and salivary gland tissues were weighed and homogenized in DMEM using a dunce homogenizer. Debris was pelleted with a 5 minute centrifugation at 8,000 rpm at 4°C. Tissue homogenates were titrated to a final concentration of 1% for spleen and lung, and 0.25% for salivary gland using DMEM. MEF subcultures were incubated with titrated homogenates at 37°C for 90 minutes, rotating every 15 minutes. The supernatant was removed and replaced with 1mL of 4% FBS + DMEM containing 2.5 g of carboxymethyl cellulose sodium salt per 500 mL and incubated at 37°C for 4 days (spleen), 6 days (lung) or 7 days (salivary gland) to allow for plaque formation. Positive controls included wells incubated with purified mCMV stock and known positive samples. Negative control wells included media only and known negative samples. Each tissue homogenate at each timepoint were run in duplicate and the number of plaque forming units (PFUs) were averaged for each sample.

To quantify PFUs per microgram of tissue, media was removed and 0.1% crystal violet in 30% ethanol was added for 30 minutes at room temperature. Two independent, blinded counters quantified the number of PFUs per well using an inverted light microscope. Results were averaged and a third person was consulted for counting if greater than 10% variance occurred between the two counts. Viral titers were reported out in log scale of PFUs and normalized to tissue weight.

Immunohistochemistry

To visualize platelet-endothelial associations in the salivary gland, dual-antibody immunohistochemistry was performed on 5 μm thick formalin-fixed paraffin-embedded salivary gland tissue sections on the Leica Bond RX Automated IHC Research Stainer. Briefly, epitope retrieval was accomplished by pretreatment with citrate based buffer (ER1,

pH = 6.0, Leica) for 10 minutes at high temperature. Endogenous peroxidases were quenched with a peroxide block for 5 minutes prior to staining platelets with anti-CD41 antibody (Invitrogen polyclonal rabbit anti-human, product number PA5–79527, diluted to 1 μg/mL in Leica Antibody Diluent) for 15 minutes followed by Bond Polymer Refine Detection kit (DAB chromagen) for 10 minutes. Vessels were then labeled by staining with anti-CD34 antibody (Invitrogen monoclonal rat anti-mouse, product number #14–0341-82, diluted to 10 μg/mL in Leica Antibody Diluent) for 60 minutes followed by Bond Polymer Refine Red Detection kit (red chromagen) for 15 minutes.

To quantify PEAs, two independent, blinded counters used a Nikon Eclipse E600 microscope fitted with a Q Imaging Camera and motorized stage aided by Stereo Investigator software (MBF Bioscience) to quantify the areas of tissue represented on the slide and used non-biased stereology to count vessels with and without associated platelets. Results were averaged and a third blinded counter analyzed the slide if the ratio of vessels with and without associated platelets did not agree. All counts were normalized to tissue area.

Statistical Analysis

All figure preparation and statistical analysis was performed using Prism 4.0 software (GraphPad Software, San Diego, CA). Comparisons of platelet count change from baseline was done using a Two way ANOVA with a Bonferroni post-test for multiple comparisons. All other comparisons of flow cytometry, immunohistochemistry, quantitative PCR and plaque assay data were done using a Kruskal-Wallis analysis with a Dunn's post-test for multiple comparisons. For all analyses, $p < 0.05$ was considered significant.

Results

Platelet decline in acute mCMV infection is associated with an increase in MHC I high platelets and a drecrease in p-selectin and CD40L positive platelets

Platelet decline is characteristic of the acute stage of many viral infections.[1–8,13] To define the kinetics of platelet decline following mCMV infection, platelet counts (K/μL) were quantified longitudinally and examined as change from baseline. A nadir in platelet count was observed at day 3 post-infection, followed by a rebound thrombocytosis occurring at day 8 (Figure 1A), consistent with previous reports of platelet decline in mCMV infected mouse models.[10] Such an observed rebound thrombocytosis is common in viral infection, and has been attributed to inflammation following infection with the acute phase production of thrombopoeitin, coupled to a normal regenerative response to transient platelet decline.[8,50] Timeline for all subsequent experiments were based on these findings, with days 3 (nadir platelet count), 8 (rebound thrombocytosis) and 21 (platelet number stabilization) post-inoculation chosen for further analysis of platelet activation and sequestration during acute infection and compared to day 0 (uninfected controls). A distinct pattern of platelet activation was demonstrated, characterized by a peak in MHC Class I high circulating platelets, corresponding with the nadir of platelets at day 3 post-inoculation and a decrease in p-selectin + and CD40 Ligand + (CD40L) circulating platelets (Figure 1B – D).

PEAs contribute to platelet decline during acute mCMV infection

Platelet associations with other cells sequester platelets from the circulating pool and can contribute to decreased platelet numbers on complete blood count. Platelet-monocyte aggregates (PMAs), the association of activated platelets with monocytes, have been shown to significantly contribute to platelet decline during acute lentiviral infection in the simian immunodeficiency virus (SIV) infected macaque model of HIV.[51] Platelets have similarly been found in association with neutrophils in sepsis, and lymphocytes in the context of antigen presentation.[13,14,52,53] We examined if the decline in circulating platelets during acute mCMV infection can similarly be attributed to the formation of aggregates with leukocytes, including monocytes, granulocytes and lymphocytes. We observed a relative decrease in PMA formation starting at day 8 post-infection in total PMAs, with a decrease in associations with each monocyte subset all contributing to this observation (Fig 2A–D). Platelet-granulocyte aggregate (PGA) formation followed a similar pattern (Figure 2E). However, platelet-lymphocyte aggregate formation increased slightly at day 3 followed by a sustained but less marked decline at day 8, with aggregates containing CD4+ but not CD8+ T cells contributing to this trend (Figure 2F–H).

Alternatively, platelets can associate directly with endothelium, similarly resulting in sequestration of platelets from complete blood count.[13,54,55] Indeed, when we examined the salivary gland for the presence of platelet-endothelial associations (PEAs), we found an increase in PEA formation peaking at day 3 concurrent with the nadir of platelet decline and continuing throughout acute mCMV infection (Figure 3).

Experimental depletion of platelets results in decreased mCMV titers in salivary gland but does not affect viral DNA in tissues

To determine if platelets were necessary for mCMV replication in tissue reservoirs, we compared tissue viral DNA (qPCR) to viral titer (plaques assay) in the key reservoir tissues of spleen, lung and salivary gland in experimentally depleted mice and compared them to mock depleted controls. Temporal patterns of detection of mCMV by qPCR were consistent with those seen in previous studies, with virus DNA appearing first in the spleen at day 3, then in the lung (days 3 and 8) and finally in the salivary gland (day 21) (Figure 4A–C).[37] Though there was no difference in total viral DNA in any organ as measured by qPCR (Figure 4A–C), there was a significant decrease in viral titer in the salivary gland in depleted animals compared to non-depleted animals (Figure 5A–C).

Discussion

We originally undertook this work to determine if platelet activation and platelet decline due to platelet-monocyte aggregates may be a universal truth in acute viral infection, as is observed in acute HIV, influenza and dengue virus infection.[51,56–58] However, we observed platelet sequestration in PEAs rather than PMAs throughout acute infection; no persistent infection timepoints were assessed in the present study. Furthermore, we observed that the nature of platelet activation in acute mCMV infection differs from that previously reported in acute viral infections, with increased MHC I expression of platelets in the absence of increased p-selectin or CD40 Ligand (CD40L) expression.[51,56,58] This is a finding that

implies that the role of platelets in the pathogenesis of acute CMV may be distinct from that in other viral infections. Paired with our data that demonstrates substantially decreased viral production in the salivary gland of mCMV infected mice in the absence of platelets, these data suggest that MHC I high platelets may be required for the salivary gland tissue to foster actively replicating virus during acute infection, and that this effect may be mediated through platelet association with endothelial cells in the salivary gland.

A direct effect of platelets on the pathogenesis of a viral infection would not be unprecedented. Soluble CD40L of platelet origin directly influences the development of CNS disease characterized by increased blood brain barrier permeability with viral entry and inflammation in the brain in a mouse model of HIV infection.[59] Antiplatelet therapy conferred protection against lung inflammation and prolonged survival in a mouse model of influenza A; this effect was independent of the presence of productive virus in the lungs.[60] Given that we observe PEA formation in the salivary glands and a reliance on the presence of platelets for mCMV production in the salivary gland, it is possible that inhibition of the interaction of platelets with endothelial cells could reduce viral production. Interestingly, hCMV production by endothelial cells can directly promote platelet binding to infected endothelium, which, taken with our data, implies that platelet presence may be instigated by initial infection and then provide a feedback mechanism for ongoing viral replication.[11] Further studies are required to determine the mechanisms underlying decreased mCMV production by the salivary gland in the absence of platelets, and the extent that PEAs may influence mCMV production in other organs throughout acute infection. It also remains to be determined if these findings apply to hCMV in addition to mCMV. If these findings do extend to hCMV, they imply that antiplatelet therapy may have an effect on viral production and subsequently the control of clinical signs associated with viremia in immunocompromised or congenitally infected patients.

The nature of platelet binding to endothelium throughout acute mCMV infection has yet to be defined, and may have implications for the mechanism behind the lack of mCMV production in depleted animals. Vessel wall endothelial cells express several surface markers, including podoplanin and p-selectin glycoprotein ligand-1, which allow for interactions with activated platelets apart from hemostasis.[13,14,17,61] These interactions mediate the rolling and adhesion of platelets and leukocytes over activated endothelium, and platelets can facilitate the entry of leukocytes into tissues by directly affecting the permeability of the endothelial barrier through receptor and cytokine signaling and by indirectly acting as a mediator for leukocyte binding and entry into organs.[13,14,17] Additionally, inhibition of vWF – platelet GPIb associations and endothelial ICAM-1 inhibit PEA formation in an in vitro model of hCMV thrombosis.[11] Interactions between platelets and endothelial cells harboring mCMV or hCMV may be mediated through these receptors and others. Thus, platelets have the potential to affect the traffic of infected, permissive or inflammatory cell populations into the organ through direct interactions with endothelium, or to directly influence the ability of the cell to produce virus. Further work is needed to determine if receptors involved in these associations and downstream mechanisms could provide appealing targets for modulation of mCMV and hCMV production.

The nature of platelet activation in the context of acute mCMV infection may similarly be informative in elucidating the mechanisms behind these findings. In general, platelets have to be activated to associate with other cells, and indeed we observed platelet activation concurrent with the nadir in platelet counts and initial formation of PEAs (Fig. 1A–B). However, the character of this platelet activation differs from that previously reported in other acute viral infections. In the SIV-infected macaque model of acute HIV infection, platelet activation is characterized by p-selectin, CD40L, and MHC Class I high expression, and similarly p-selectin activation was observed following infection with Dengue virus (MHC I expression was not examined).[16,51,58] In the present study, platelets increased expression of MHC Class I concurrent with a trend in decreased expression of p-selectin and CD40L during acute mCMV infection. Platelet expression of MHC Class I allows for antigen presentation to CD8+ cytotoxic T cells, thereby promoting a Th1 inflammatory response, and, interestingly, local inflammation mediated by CD8+ T cells can facilitate viral replication of hCMV.[26,62,63] Further characterization of markers expressed by platelets associating with endothelial cells is needed; although MHC I high platelets increase in circulation, it is not yet known if platelets associating with endothelial cells are MHC I high, or if p-selectin and CD40L expressing platelets are preferentially associating with endothelial cells, sequestering them from circulation. It remains to be determined if recruitment and activation of CD8+ T cells to the salivary gland by MHC I high expressing platelets could represent an additional mechanism through which the presence of platelets could promote viral production.

The sequestration of platelets in PEAs observed in these data are unlikely to be the sole driver of platelet decline during acute mCMV infection. Decreased platelet production due to direct infection and lytic destruction of megakaryocytes has previously been demonstrated in acute mCMV infection.[10] Though PMA and other platelet-leukocyte aggregates were not observed during acute mCMV infection in this model, it is possible that they may occur following inoculation by other routes or they may contribute to platelet decline later in infection as observed in HIV and other viral infections.[9,16] PEA formation in other organs similarly could contribute to platelet decline in acute CMV infection. This study only examined the salivary glands, lungs and spleen; future studies should systematically assess the presence of PEAs in major organs, including the liver, to identify other potential organs of sequestration, thereby contributing to platelet decline. Immune mediated destruction has been observed in hCMV and is likely to similarly contribute to platelet decline later in infection in some individuals, though it is unlikely to affect platelet numbers during acute infection.[64]

Together these data suggest that the interaction of platelets with endothelial cells facilitates virus replication in the salivary gland of mCMV infected mice. Given the myriad differences we see in the nature of platelet activation and association with other cells in acute mCMV in comparison to other viral infections, the mechanisms involved may be unique to CMV. Further work is needed to determine the mechanism by which platelets and endothelial cells interact and contribute to the permissiveness of viral replication in the salivary gland. It remains to be determined if these mechanisms will translate to patients infected with hCMV and if this platelet activation and PEA formation may present novel therapeutic targets to decrease hCMV production in immunocompromised and congenitally infected patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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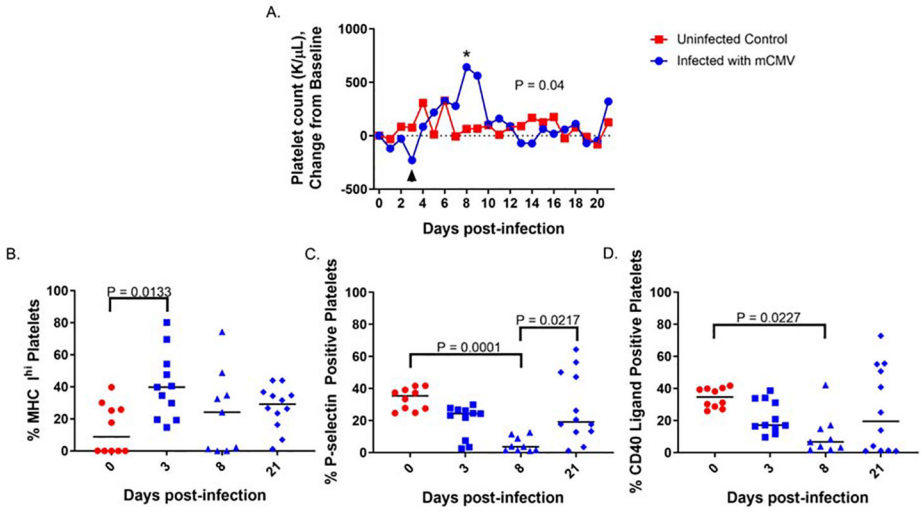

Figure 1.

A distinct pattern of platelet activation, characterized by an increase in MHC I high platelets and a decrease in p-selectin and CD40L expression, corresponds with nadir platelet counts following infection with mCMV. (A) Longitudinal platelet count (K/μL) following infection, as change from baseline median values (N = 5 per group). Two way ANOVA with Bonferroni post-test. (B) Flow cytometry analysis of percentage of CD41+ platelets expressing high levels of MHC class I (MHC I^{hi}) longitudinally throughout infection. Kruskal-Wallis analysis with Dunn's post-test, bars indicate median. (C) and (D) Flow cytometry analyses of percentage of CD41+ platelets expressing P-selectin and CD40 ligand, respectively, longitudinally throughout infection. Kruskal-Wallis analyses with Dunn's post-test, bars indicate median.

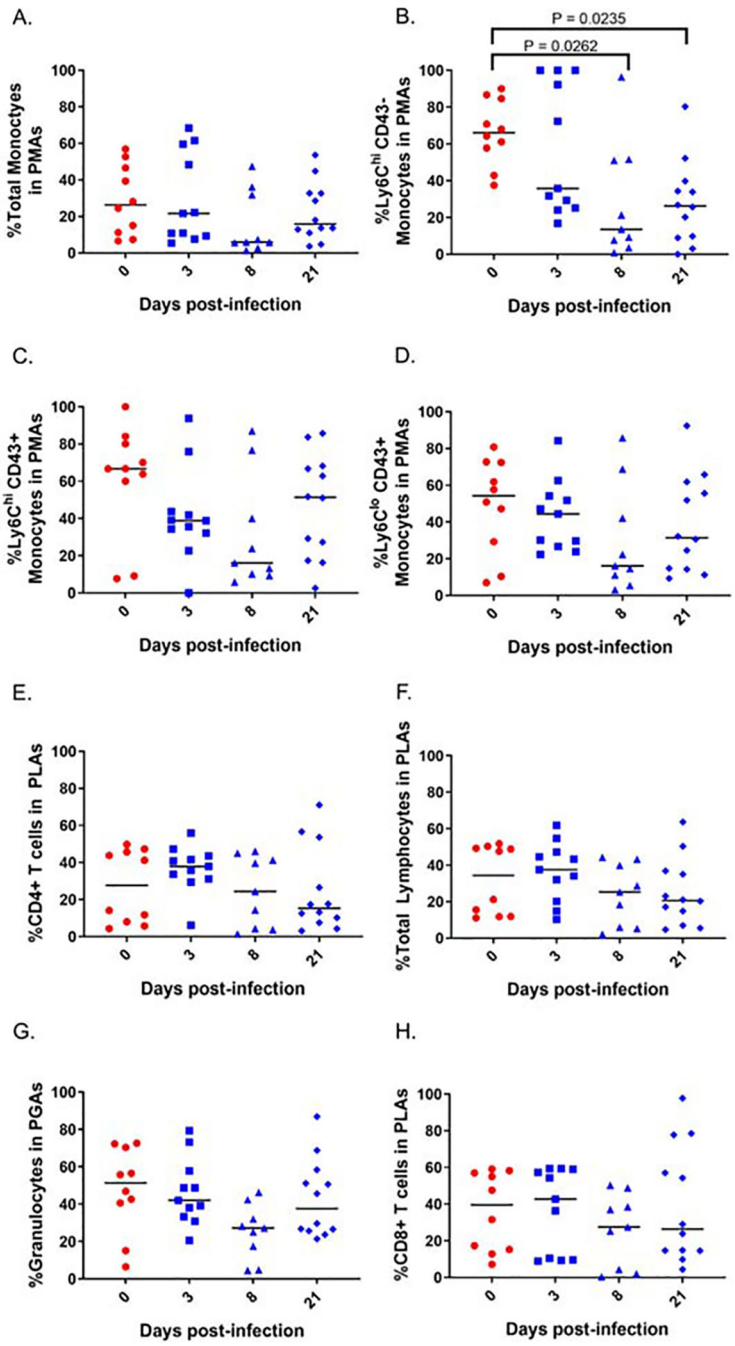

Figure 2.

Platelet sequestration in platelet-leukocyte aggregates does not significantly contribute to platelet decline during acute mCMV infection. (A) Flow cytometry analysis of PMA formation in monocyte subtypes longitudinally throughout infection, and stratified by (B) Ly6C^{hi} and CD43 -, (C) Ly6C^{hi} and CD43 +, and (D) Ly6C^{low} and CD43 + monocyte subset. Platelet-granulocyte (E) and platelet-lymphocyte (F) aggregate formation longitudinally throughout infection, stratified by (G) CD4+ T cell and (H) CD8+ T cell subsets. Percentage of PMAs and PLAs presented as percentage of a given cell subtype that

are bound to CD41+ platelets. Kruskal-Wallis analyses with Dunn’s post-test, bars indicate median values.

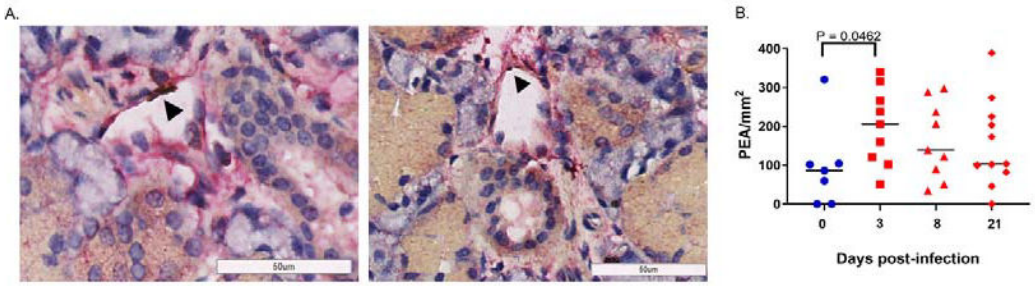

Figure 3.

Sequestration of platelets in PEAs corresponds with platelet decline during acute mCMV infection. (A) Representative immunohistochemistry images (400x) of CD41+ platelets (brown, indicated by black arrowhead) bound to CD34+ vessels (red) in the salivary gland of a mCMV infected mouse. (B) Quantification of PEA formation in the salivary gland longitudinally throughout acute mCMV infection. Kruskal-Wallis analyses with Dunn's post-test, excluding outlier as determined by Grubs outlier test, bars indicate median values.

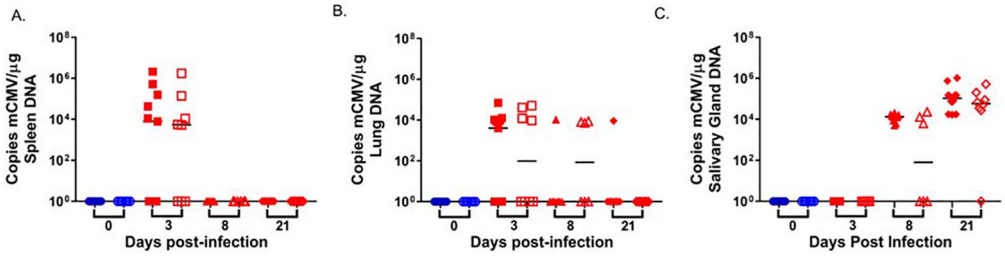

Figure 4.

The effect of platelet depletion on mCMV tissue viral DNA as measured by qPCR. Organs were harvested at the indicated timepoints during acute infection from platelet depleted (hollow symbols) and mock depleted (solid symbols) mice. Tissue viral DNA was determined by qPCR for mCMV glycoprotein B DNA in the (A) spleen, (B) lung and (C) salivary gland. Vertical axis means the logarithm of the number of mCMV DNA copies per µg of DNA. Kruskal-Wallis analyses with Dunn's post-test, bars indicate median values.

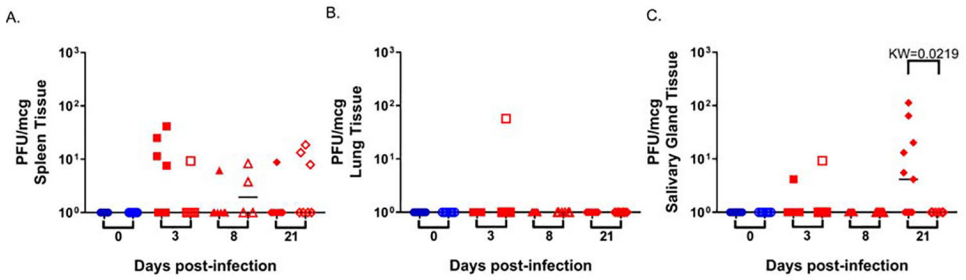

Figure 5.

The effect of platelet depletion on mCMV titer as measured by plaque assay in tissues. Organs were harvested at the indicated timepoints during acute infection from platelet depleted (hollow symbols) and mock depleted (solid symbols) mice. mCMV titer was determined by plaque assay in the (A) spleen, (B) lung and (C) salivary gland. Vertical axis means the logarithm of the number of mCMV PFU per μg of tissue. Kruskal-Wallis analyses with Dunn's post-test, bars indicate median values.