THE ROLE OF PLATELET-MONOCYTE BINDING IN MACROPHAGE POLARIZATION AND SUSCEPTIBILITY TO HIV INFECTION

by

Elizabeth L. Engle

A thesis submitted to Johns Hopkins University in conformity with the requirements for the degree of Master of Science.

BIOTECHNOLOGY

Baltimore, MD

April, 2016
Abstract

Despite 30 years of research, we still do not fully understand what drives the development of disease in human immunodeficiency virus (HIV) infected individuals. An estimated 36.9 million people are living with HIV today. Platelets (PLTs) associate directly with the majority of CD16+ monocytes within the blood during acute HIV infection. CD16+ monocytes (Mos) become infected with HIV and are believed to initiate organ-based disease early in infection more than other Mo subtypes, based on studies in animal models. Once in an organ, Mos become macrophages that can harbor latent HIV in sanctuary tissues. Depending on environmental influences on the Mos, monocyte-derived macrophages (MDMs) can be polarized into subtypes with different susceptibilities to HIV infection. The effect of PLT interactions with Mos on the polarization of the progeny MDMs and the MDM’s susceptibility to HIV infection is unknown. To examine this we isolated whole blood from healthy human donors, isolated Mos, and cultured them for 7 days with or without exposure to PLTs. These MDMs were then either phenotyped or infected with HIV. When we phenotyped these MDMs we found a decrease in the percentage of M1 MDMs concurrent with an increase in expression of the HIV co-receptor CXCR4, along with a decrease in the activation markers HLA-DR, CD80, and CD163. When we infected these MDMs with HIV, we found an increase in the rate of viral production when the MDMs were exposed to PLTs, with the lowest ratio of PLTs to Mos showing the greatest effect early during infection. We believe that PLTs are affecting the polarization of the MDMs leading to an increase in the expression of receptors necessary for HIV entry, while also decreasing the ability of MDMs to initiate an immune response.
Acknowledgements

Foremost, I must thank my mentor, Kelly Metcalf Pate. Not only did she give me the opportunity to perform this research project through her lab, but she also adopted me into her lab when Chris went on sabbatical and reduced her work hours. Kelly has allowed me the opportunity to develop as an independent researcher by giving me the guidance and mentorship necessary to develop and shape this complex project. She is always a beacon of positivity and I feel like we complement each other well. Through working with Kelly, I have greatly expanded my scientific knowledge and breadth of experimental techniques.

We are platelet people in the Pate lab, which is why Lucio Gama was so instrumental in the design of this project through his expertise in monocyte and macrophage experimentation. Along those lines, I would not have been able to complete this project without the help of Sarah Price, who showed me how to successfully culture and differentiate monocytes, kept me up to date on any protocol changes that would further our experiments, and answered all of my questions along the way. I also need to extend my gratitude to our resident flow cytometry guru Erin Shirk. Erin was instrumental in the establishment of the monocyte and macrophage panels and gating strategies, training me on the Fortessa, and editing this paper. Celina Abreu made an exceptional virus and infection protocol along with Zhaohoa Liao, who gave me his guidance with the p24 ELISA and has been a good friend over the last several years. I also need to thank Dionna Williams for her immense macrophage knowledge and guidance along the way. Also, Kevin Najarro for this help in teaching me all the platelet related protocols over the last two years.
I must extend a special thank you to our phlebotomists Suzanne Queen, Claudia Avalos, and Dillion Muth, as without them I wouldn’t have any starting materials to work with. Additionally, Woodland Pomeroy coordinated all the donor scheduling.

Of course I wouldn’t have had this opportunity if it weren’t for the Retrovirus Lab as a whole. I have to thank Chris Zink for mentoring me along the way and giving me an opportunity to grow and develop after college. I also need to acknowledge Joe Mankowski and Janice Clements for the opportunities they have provided me with in lab and keeping me employed all these years along with the rest of the retrovirus faculty Ken Witwer, David Graham, and Sarah Beck have shared with me their professionalism, guidance, and friendship over the years.

The Retrovirus Lab wouldn’t function as a whole unit without a great technical staff. Our lab managers Brandon Bullock and Suzanne Queen keep the lab running and were also available whenever I needed help on this project. Of course I couldn’t have done this without the fellow technicians past and present who offered not only their help with various projects over the years, but also gave their friendship and commiseration: Erin Shirk, Sarah Price, Rachel Weinberg, Kevin Najarro, Ellen Forsyth, Bonita Powell, Greg Brinsley, Julia Szeto, Claire Lyons, and Jamie Dorsey.

Lastly, I have to thank my friends, Kerri, Steve, and Joey for being so understanding while I finished this rather intensive project. I especially need to thank my parents for all their support and time, driving me from place to place, helping me move on multiple occasions, and being a wealth of knowledge and compassion over the years. I also need to thank my wonderful partner for all her support throughout this project and pushing me to undertake this journey.
Table of Contents

Abstract ................................................................................................................................. ii
Acknowledgements ................................................................................................................ iv
List of Tables ........................................................................................................................ vii
List of Figures ......................................................................................................................... viii
Introduction ............................................................................................................................ 1
Materials and Methods ............................................................................................................ 5
Results .................................................................................................................................... 10
Discussion ............................................................................................................................... 14
Conclusion ............................................................................................................................... 19
Bibliography ............................................................................................................................ 20
Tables ...................................................................................................................................... 25
Figures ..................................................................................................................................... 27
Curriculum Vitae ..................................................................................................................... 36
List of Tables

Table 1 .................................................................................................................................... 25
Table 2 .................................................................................................................................... 26
List of Figures

Figure 1 ................................................................................................................................... 27
Figure 2 ................................................................................................................................... 29
Figure 3 ................................................................................................................................... 31
Figure 4 ................................................................................................................................... 33
Figure 5 ................................................................................................................................... 35
Introduction

HIV infects immune cells and eventually leads to the development of acquired immune deficiency syndrome (AIDS) if left untreated. As of 2014, the United Nations estimates that 36.9 million people are living with HIV, with 25.8 million of infected individuals living in sub-Saharan Africa (UNAIDS 2015). Of the 36.9 million individuals infected with HIV, only 15.8 million are receiving antiretroviral therapy (UNAIDS 2015). More than 1.2 million infected individuals are living in the United States, and an estimated 50,000 new HIV infections occur in the United States annually (CDC 2014).

A major obstacle to curing HIV is the presence of reservoirs of latent virus in tissue sanctuaries within the body that include the brain, lungs, and lymphoid organs; these reservoirs are established during acute infection (Chun et al. 1998). HIV can infect CD4+ T-cells, monocytes (Mos), and macrophages (Mθs) (Stevenson 2003). HIV viral entry into host cells is dependent on both binding of the viral protein glycoprotein 120 to CD4 on the immune cell, and concurrent binding of a co-receptor, either C-C chemokine receptor type 5 (CCR5) or C-X-C chemokine receptor type 4 (CXCR4). There are three HIV-1 tropism variants that can utilize co-receptors for entry: X4, which uses CXCR4, R5, which used CCR5, and X4R5, which uses either co-receptor (Alkhatib 2009).

Monocyte have been classified into three distinct categories: CD14\textsuperscript{hi} CD16- (classical), CD14\textsuperscript{hi} CD16+ (intermediate), and CD14\textsuperscript{low} CD16+ (non-classical) (Ziegler-Heitbrock et al. 2010). CD14 is the LPS receptor for Mos and is responsible for Mo activation in response to bacteria, while CD16, the FC\greek{y}III receptor, is responsible for activation secondary to binding the Fc fragment of antibodies (Williams et al. 2014). CD14\textsuperscript{hi} CD16- Mos represent 90-95% of all circulating Mos in healthy individuals.
(Passlick et al. 1989). CD14 hi CD16+ Mos are generally believed to be the pre-Mθ, pro-inflammatory Mo population (Strauss-Ayali et al. 2007). Upon HIV infection, CD16+ Mos can expand to up to 40% of the circulating Mo population (Thieblemont 1995). CD16+ Mos show a higher rate of viral production than their CD16- counterparts (Ellery et al. 2007). Based on animal model studies, CD16+ Mos are believed to carry HIV to tissues, including the brain, leading to the seeding of viral reservoirs during acute infection (Clay et al. 2007).

Monocytes can differentiate into Mθs upon entry into organs in vivo. In culture, Mos can be polarized into monocyte-derived macrophages (MDMs) and the characteristics of these MDMs are dependent on the growth factors these MDMs are exposed to during maturation. For example, in vitro exposure to granulocyte macrophage colony stimulating factor (GM-CSF), interferon-γ, lipopolysaccharide, or tumor necrosis factor polarizes MDMs towards an M1, pro-inflammatory phenotype, while macrophage colony stimulating factor (M-CSF) or CSF1, interleukin-4, interleukin-10, and transforming growth factor-β (TGF-β) polarizes towards an M2, anti-inflammatory phenotype (Jaguin et al. 2013). M-CSF binding to its receptor, colony stimulating factor 1 receptor (CSF1R), causes receptor dimerization, activation of extracellular signal-regulated kinase, phosphatidylinositol-3 kinase, and phospholipase C leading to Sp1 nuclear translocation (Martinez and Gordon 2014), and eventually to the downregulation of the human leukocyte antigen (HLA) genes –DP, –DR, and –DQ, identified through transcriptome analysis (Martinez et al. 2006). Upon binding to its receptor, TGF-β1 signals through Smad proteins, ultimately driving the MDM towards an M2 phenotype, and can have many differing effects on Mθ activity and function (Akhurst and Hata
2012). MDMs grown in M-CSF and exposed to LPS generate M1 MDMs and show an upregulation of CD80, as compared to M2 MDMs generated by exposure to IL-4 (Jacquin et al. 2013). However, this initial fate is not absolutely fixed, and M1 and M2 MDMs show plasticity and as such can change phenotype and cell surface expression when exposed to different stimuli (Davis et al. 2013).

The MDM phenotype directly affects the ability of the MDM to become infected. M1 macrophages have been shown to be more resistant to CCR5 mediated HIV-1 infection as compared to M2a macrophages (Cassol et al. 2009). This may be due in part to macrophage mannose receptor (MMR) expression, as MMR functions to recognize and bind to mannose on the pathogen’s surface for phagocytic engulfment, and has also been shown to help stabilize HIV-1 on the macrophage cell surface to aid in viral entry (Nguyen and Hildreth 2003).

Even though the first reported case of HIV in the United States was over thirty years ago, more work is needed to understand how sanctuaries of infected cells are established in HIV-infected individuals. Platelets (PLTs) are small, 1-3µm, anucleate peripheral blood cells with a well-known role as drivers of hemostasis (Semple et al. 2011). In recent years, the emerging field of PLT immunology has demonstrated that PLTs play key roles in both the innate and adaptive immune systems (Semple et al. 2011). PLTs act in the innate immune response through both cytokine signaling and direct cell-cell contact (Semple et al. 2011). PLTs contain rich stores of chemokines and cytokines in α-granules, including TGF-β1, that are released into the microenvironment upon activation (Assoian and Sporn 1986). Activated PLTs expressing P-selectin can bind to Mos via P-selectin glycoprotein ligand-1 (PSGL-1) (Fernandes et al. 2003). Upon
binding, PLT-monocyte aggregates (PMAs) are formed, and this induces a change in Mos to a CD16+, pro-inflammatory phenotype (Passacquale et al. 2011). HIV-1 infection has been shown to increase PLT activation as evidenced by increased release of soluble CD40L, a measure of PLT activation and pro-inflammatory status (Damien et al. 2013), and an increase in PMA formation is seen in individuals infected with HIV (Singh 2014). Furthermore, PMA formation during acute infection accounts for the PLT decline observed during this phase in the simian immunodeficiency virus infected macaque model of HIV infection (Pate et al. 2013). Activated platelets have been shown to suppress HIV-1 infection in T cells *in vitro* through the release of CXC chemokine ligand 4 (CXCL4) by PLTs, which presumably blocks HIV entry into cells by binding its cognate receptor CXCR4 (Solomon et al. 2013).

In contrast to the effect of PLT binding on T cells, we hypothesize that PMA formation results in polarization of progeny macrophages to the M2, anti-inflammatory phenotype due to the PLTs rich stores of TGF-β1, and thus increases the susceptibility of macrophages to infection by HIV. We further hypothesize that PLT binding to Mos will contribute to an increased rate of HIV infection of MDMs in culture. If our hypothesis is correct, such polarization and subsequence increased susceptibility of MDMs to infection by HIV following the formation of PMAs could contribute to an increased rate of infection of the M0s in sanctuary tissues and the establishment of viral reservoirs during acute infection. If this is the case, inhibiting PMA formation early in infection may prevent the establishment of viral reservoirs in sanctuary tissues.
Materials and Methods

Overview

The purpose of this study is to evaluate the effect of PLT binding to Mos on the phenotype and infectability of the resultant MDMs. Monocytes were exposed to different concentrations of activated PLTs in culture or no PLTs as a control, and then induced to differentiate into MDMs. Resultant MDMs were then split into two groups that were either phenotyped to determine whether exposure to different concentrations of PLTs influenced polarization of the MDMs towards M1 or M2 phenotype, or infected with HIV to determine whether exposure to different concentrations of PLTs affected the ability of the MDMs to become infected.

Monocyte Isolation

Whole blood (WB) was obtained from healthy HIV seronegative adult human donors. Citrate-dextrose solution (Sigma-Aldrich) was utilized as the anticoagulant. A sample of WB was removed for flow cytometry analysis. The WB was diluted 1:1 with 2% fetal bovine serum (FBS) (Atlanta Biologicals) in Dulbecco’s phosphate buffered saline (DPBS) without Ca\(^{2+}\) or Mg\(^{2+}\) (Gibco) and added to a Ficoll-Hypaque (GE) gradient in SepMate™-50 tubes (StemCell). This gradient was centrifuged at 1200g for 10min, then resuspended in 2% FBS in DPBS. The cells were washed twice at 300g for 8min. Red blood cell (RBC) contamination of the resulting cells was removed through lysis for 20min at 37°C in RBC Lysis Buffer (39mM NH\(_4\)Cl, (Sigma-Aldrich), 2.5mM KHCO\(_3\) (Sigma-Aldrich), 0.25mM EDTA (Sigma-Aldrich)), and washing by centrifuging at 400g for 10min with 1mM EDTA in HBSS without Ca\(^{2+}\) or Mg\(^{2+}\) (Gibco). The
remaining peripheral blood mononuclear cells (PBMCs) were resuspended in cold 2% FBS in DPBS with 1mM EDTA at 1x10^8 cells/mL. Monocytes were isolated through positive selection for CD14 by the EasySep™ Human CD14 Positive Selection Kit II (StemCell). A small portion of the remaining cell suspension was analyzed using flow cytometry to confirm purity. The remaining cells were resuspended at 8x10^5 cells/mL in macrophage media 20% (MDM20%) (80% DMEM (Gibco), 2mM L-glutamine (Life Technologies), 8mM HEPES (Gibco), 20mM Gentamicin (Life Tech), 20% Human AB Serum (Gemini BioProducts), 1μM C₃H₃NaO₃ (Sigma-Aldrich), and 50ng/mL M-CSF (R&D Systems)).

**Platelet Isolation**

Citrated WB was obtained from the same donor. A subset of WB was removed for CBC and PLT activation flow cytometry. The WB was centrifuged at 1000g for 10min with the brake off. The PLT rich plasma (PRP) was removed, aliquoted, and incubated at 37°C for 10min. 0.5μM Prostaglandin I2 (PGI₂) (Cayman Chemical) was added to the PRP then the PLTs spun at 1000g for 10 mins with the brake off. The PLTs were resuspended in Tyrode’s albumin buffer (TAB) (11.9mM NaHCO₃ (Sigma-Aldrich), 136mM NaCl (Sigma-Aldrich), 11.9mM KCl (Sigma-Aldrich), 0.43mM NaH₂PO₄ (Sigma-Aldrich), 1mM MgCl₂ (Sigma-Aldrich), 2mM CaCl₂ (Sigma-Aldrich), 5mM HEPES (Sigma-Aldrich), 38μM Bovine Serum Albumin (Sigma-Aldrich), 1.38mM D+glucose (Sigma-Aldrich), pH 7.35, osmolality 295mOsm/L) with 100U heparin (Sagent Pharmaceuticals) and 0.5μM PGI₂ to prevent PLT activation. The PLTs were incubated for 10min at 37°C, 0.5μM PGI₂ added, then centrifuged at 1000g for 8min with
the brake off. The PLTs were resuspended in TAB with 0.5μM PGI\textsubscript{2}. The PLTs were incubated at 37°C for 10min, 0.5μM PGI\textsubscript{2} added, and centrifuged at 1000g for 8min brake off. The resultant PLTs were resuspended in MDM20% at 4x10\textsuperscript{8} cells/mL. Two small subsets of PLTs were removed, one was stained to measure baseline PLT activation status by flow cytometry, the other stimulated with the thromboxane A2 receptor agonist U46619 (Sigma-Aldrich for infection, Caymen Chemicals for phenotyping), incubated for 15min at 37°C, then stained to measure PLT responsiveness by flow cytometry.

**Cell Culture**

Monocytes were added to culture plates and centrifuged at 300g for 10min to encourage adherence of the Mos to the bottom of the plate. Then PLTs were added to the wells as indicated, and 20μM of U46619 (Sigma-Aldrich) added to activate PLTs, as shown below in Table 1; all conditions were run in triplicate, except where noted. The different ratios of platelets to monocytes were chosen because they represent a variety of physiologic conditions: 250 PLTs to every Mo is representative of typical PLT:Mo ratio in the circulating blood of a healthy individual, while 125:1 is reminiscent of the ratio seen in HIV-infected individuals and 62.5:1 of that seen in a clinically thrombocytopenic person; 500:1 may be observed locally in response to an inflammatory insult. Co-cultures were incubated for 24hrs at 37°C, washed with TAB to remove unbound PLTs, resuspended in MDM20%, and incubated for 2 days at 37°C. A half change of the media was performed with MDM20% on day 3 post plating, and then the cultures were incubated for 4 days at 37°C. At 7 days post plating the cells were washed with DPBS to
remove any non-adherent (dead or non-MDM) cells and either infected with HIV-1 or harvested for phenotyping.

**Phenotyping**

DPBS was removed from the cultures and TrypLE (Gibco) added to remove adherent MDMs from the plate. The culture plates were agitated, and incubated for 10min at 37°C, agitated again and returned to 37°C for 10min. The culture plates were agitated again and incubated for 10min at 4°C, agitated again, then 2% FBS in DPBS was added to each well to neutralize the TrypLE. The harvested MDMs were then pelleted, resuspended in DPBS with 2% FBS, and stained with antibodies for flow-cytometry analysis.

**Flow cytometry**

Samples were stained with antibodies for 20min at room temperature then fixed and lysed for 10min with BD FACS Lysing Solution (BD Bioscience), centrifuged at 500g for 5min, resuspended in DPBS, centrifuged at 500g for 5min, and resuspended in DPBS with 2% FBS. The samples were analyzed on an LSRFortessa (BD Bioscience) with 100,000 events collected per sample. For the MDM panels, fluorescence minus one controls of MDMs were cultured, removed from culture, and stained with antibodies in the same way as above, except each tube was missing one antibody at a time. WB obtained for PLT activation and PLTs were stained for 15min, 2% NBF added, incubated for 30min, and analyzed on a FACSCalibur (BD Bioscience). All data were analyzed using FlowJo software (FlowJo Enterprise).
Infection

DPBS was removed from culture and the X4R5-tropic strain HIV-1 89.6 (AIDS Reagent) was added at a multiplicity of infection (MOI) of 0.1 to the appropriate conditions. The cultures were spinoculated for 2hrs at 1200g, brake off. The virus media was removed from cultures and MDM10% (90% DMEM, 2mM L-glutamine, 8mM HEPES, 20mM Gentamicin, 10% Human AB Serum, 1μM C₃H₅NaO₃, 50ng/mL MCSF) added. Half of the supernatant was collected at days 3, 5, and 7 post-infection for measurement of virus production (PI). At day 10 PI the entire supernatant was collected.

Virus production

Viral production levels were determined in culture supernatants using an HIV-1 p24 ELISA (PerkinElmer) read on an iMark Microplate Reader (BioRad). Supernatants from uninfected culture conditions and blank wells were run as negative controls.

Statistical Analysis

All statistical analyses were performed in Prism 6 (GraphPad Software Inc.) using only non-parametric tests due to the limited number of donors tested. A p-value below 0.05 was used to determine statistical significance.
Results

Plating Purity of Monocytes Used for Phenotyping and Infection Experiments

WB was obtained from a HIV seronegative healthy human donor, and PBMCs isolated through the use of a Ficoll-Hyplaque gradient and SepMate-50 tubes. Monocytes were positively selected for CD14, and plated. To ensure that we were in fact plating Mos, a small sample of WB and plated cells were analyzed using the Mo flow cytometry panel in Table 2. We used a simple gating scheme based on the positive staining in the WB to analyze the plated cells. The gating scheme is depicted in Figure 1. Here, after gating on singlets, we gated on the mononuclear cell (MNC) population (Fig. 1A). From the MNC population, separate gates were drawn around the CD3+ T cells (Fig. 1B), the CD20+ B cells (Fig. 1C), and the CD159A+ NK cells (Fig. 1D). From the MNC population a gate was drawn around the monocytes as shown in Fig. 1E. From the monocytes population we examined the subpopulations by looking at CD14 and CD16 staining (Fig. 1F). In Fig. 1F, the classical Mos (Q1), intermediate Mos (Q2), and nonclassical Mos (Q3) are represented. In each of these subtypes we examined the percentage of cells that stained positively for the PLT marker CD42A (Fig. 1G). As shown in Fig. 1H and 1J, over 90% of plated cells were Mos with the majority of these consisting of classical Mos. Only for Donors 1 and 3 (D1 & D3) were the majority of these plated Mos bound to PLTs, as shown through the high percentage of Mos that stained positively for CD42A.
Activation of Platelets Used for Phenotyping and Infection Experiments

Citrated WB collected from each donor was centrifuged to isolate PLT-rich plasma. This plasma was washed to isolate PLTs using buffers designed to prevent PLT activation and coagulation. A subset of the washed PLTs was stained using the PLT flow cytometry panel in Table 2, while another subset was stimulated with U46619 and stained using the same panel. The gating scheme for PLT flow cytometry analysis is shown Figure 2. Here, all CD42A positive cells were designated as PLTs, then we examined the percentage of PLTs expressing the activation markers P-Selectin, CD40L, HLA-ABC, or HLA-DR. The percentage of PLTs expressing each marker is shown in Fig. 2G-2N, with donors used for phenotyping experiments represented in Fig. 2G-2J, while those donors used for infection are shown in Fig. 2K-2N. The PLTs clearly showed response to stimuli by increasing expression of both P-Selectin (Fig. 2G and 2K) and CD40L (Fig. 2H and 2L), though this response was greater in those donors used for infection experiments. There was no discernable change in the immune activation markers HLA-ABC (Fig. 2I and 2M) or HLA-DR (Fig. 2J and 2N), which was not unexpected following a pro-hemostatic stimulus such as U46619.
The Effect of Platelets on the Polarization of Monocyte-Derived Macrophages

Monocytes were induced to differentiate into MDMs over 7 days in culture, then harvested and stained with antibodies for flow cytometry analysis. MDMs were characterized for polarization into either M1 or M2a MDMs. The gating strategy is represented in Figure 3, where after gating on singlets, the live population (Fig. 3A) was gated on as determined by FMO stained control MDMs. From this live population, the macrophage population (Fig. 3B) was gated. This macrophage population was gated into either M1 HLA-DR+/CD80+ cells (Fig. 3C), or M2a MMR+/CD163+ cells (Fig. 3D). The mean fluorescence intensity (MFI) for each condition was averaged and normalized to the No PLTs condition for each individual donor. Except for the 500PLTs to every Mo condition, there was a non-statistically significant decline in M1 MDMs for all but donor 15 (Fig. 3E). We did not observe an effect on M2a polarization from PLT exposure (Fig. 3F).

The Effect of Platelets on Monocyte-Derived Macrophage Phenotype

We also examined the expression of individual markers of activation on MDMs in each condition. To accomplish this we gated on singlets, then the live population (Fig. 3A), followed by the macrophage population (Fig. 4A), then we examined CXCR4 (Fig. 4B), CCR5 (Fig. 4C), HLA-DR (Fig. 4D), CD80 (Fig. 4E), and CD163 (Fig. 4F) through the use of FMO controls. When we examined the markers necessary for viral entry and binding we found a significant increase in expression of the co-receptor for HIV-entry, CXCR4 (Fig. 4G, p = 0.016), at the clinically thrombocytopenic condition 62.5PLTs to every Mo with a non-statistically significant increase in all other conditions exposed to
PLTs. We did not find any changes in CCR5 expression (Fig. 4H). When examining HLA-DR, we found a non-statistically significant decrease (p = 0.063) at the 62.5PLTs to every Mo condition, along with all but donor 15 showing either a similar decline or no change at the other conditions (Fig. 4I). We saw the same results when examining CD80 with the 62.5PLTs to every Mo condition showing a non-statistically significant decrease (p = 0.063), along with a similar decline at all other conditions except for donor 15 (Fig. 4J). We also found the same trend for CD163 but there was not a significant decline, and donor 15 was elevated at all conditions (Fig. 4K).

Monocyte-Derived Macrophage Infection

Monocytes were incubated for 7 days to allow them to differentiate into MDMs. After 7 days the MDMs were infected with HIV-1 89.6 at a MOI of 0.1. For Donors 3 (Fig. 5B, p = 0.003), 5 (Fig. 5C, p = 0.017), 7 (Fig. 5E, p < 0.0001) we found a statistically significant increase in the rate of viral production of MDMs when exposed to PLTs. Donor 9 (Fig. 5H, p = 0.019) showed a significant decrease in rate of viral production of MDMs in all conditions exposed to PLTs. Donors 1 (Fig. 5A, p = 0.756), 6 (Fig. 5D, p = 0.109), and 8 (Fig. 5F, 0.038) demonstrated an increase in MDM viral production in some of the conditions exposed to PLTs, but not all, and these conditions were not significant. If Donor 9 is removed from analysis, there is a significant increase in MDM viral production at day 5 post-infection when MDMs are exposed to 62.5PLTs per every Mo (Fig. 5H, p = 0.031).
Discussion

To our knowledge, this is the first set of experiments to examine the effect of PLTs binding to Mos on the polarization of MDMs and how that relates to the rates of HIV production by these MDMs following infection. We showed that incubation with PLTs is leading to a polarization away from a M1 MDM phenotype, along with a significant increase in MDM surface CXCR4 expression, a HIV co-receptor necessary for entry. We also found a decrease in markers important in MDM activation and function including HLA-DR, CD80, and CD163. When these MDMs were infected we found an increase in viral production in the conditions exposed to PLTs in some, but not all of the donors. These results imply that PLTs are changing the phenotype of MDMs and thus increasing the susceptibility of MDMs to become infected while also decreasing the MDMs ability to respond to viral entry and become properly activated. This would position these MDMs to be ideal hosts for latent virus.

To perform these experiments we needed to establish a reliable method for Mo isolation without contamination from host PLTs to ensure the changes we found were due to the PLTs we added back into culture. We were able to establish a reliable method for Mo isolation with all but two of the donors having low amounts of platelet-monocyte aggregates upon initial plating ranging from (1-35%). We also needed to verify that we were reliably able to activate the PLTs we obtained from each donor. To accomplish this we used the Thromboxane A2 receptor agonist U46619 to specifically activate the PLTs we isolated and confirmed PLT activation through flow cytometry analysis. All of the donors demonstrated an increase in their expression of P-Selectin and CD40L, which are both important markers of PLT activation. The donors used for infection experiments
mounted a better response to U46619 than those used for phenotyping experiments. We attribute this difference to the fact that we had to switch suppliers for U46619 between performing the infection and phenotyping experiments. In the future we plan to repeat the phenotyping experiments with the more reliable U46619 obtained from Sigma once they start producing this reagent again. Though platelets would doubtlessly activate over time while in culture with Mos, this step will allow us to ensure the results we are seeing are from uniformly activated PLTs.

To examine the phenotype of MDMs following incubation with PLTs, we removed MDMs from culture through TrypLE digestion and agitation to maintain surface antigen expression. We analyzed these MDMs through antibody staining and flow cytometry. We found a trend towards a decrease in the percentage of M1 MDMs at all PLT added conditions for all donors except donor 15 who demonstrated increased percentage of M1 MDMs at the three higher PLT added conditions. Though we did not find any discernable changes in M2a MDMs at any of the conditions, these PLT-stimulated MDMs may be differentiating towards another M2 subtype (i.e. M2b or M2c) or a novel phenotype that has yet to be characterized. Supporting polarization towards a MDM phenotype conductive to HIV infection, we found a significant upregulation of CXCR4 expression following incubation with 62.5PLTs to every Mo, with a trend towards an increase at the other PLT added conditions. CXCR4 is a necessary co-receptor for R4-tropic HIV binding and entry on the MDM surface. There was no discernable change in CCR5 expression. When we examined HLA-DR, CD80, and CD163 we detected a non-statistically significant downregulation of these markers following incubation with 62.5PLTs to every Mo. At the higher PLT concentrations there continued
to be a trend towards a downregulation of these markers in all donors except donor 15. Interestingly, donor 15 had some of the most activated PLTs as indicated by pre-U46619 stimulation P-Selectin levels, but did not show an appreciable increase in activation following U46619, similar to the other donors. The MDM surface antigens HLA-DR, CD80, and CD163 are important for antigen presentation, B cell activation, and phagocytosis, respectively; all roles that are important for the anti-viral response.

Very few MDMs were CD42A+ following 7 days in culture, and thus very few demonstrated sustained PLT binding. This is not surprising as the PLT-Mo relationship is suspected to be transient (Singh et al. 2014). Moving forward we would like to examine the MDMs that remained PLT bound and compared them to unbound MDMs, allowing us to see if there are any differences in these MDMs where PLTs have fused. In addition to examining these data we would like to repeat these experiments using PLTs with better levels of activation, to ensure the changes we are seeing are due to uniformly activated PLTs. Given the observed downregulation of CD163 on the MDMs following incubation with PLTs, we would also like to perform FRODO experiments on these MDMs to examine if they are still able to perform phagocytosis properly.

We compared the rates of HIV production from MDMs exposed to PLTs and those not exposed to PLTs. We added PLTs at four different ratios to monocytes that ranged from concentrations similar to those seen in a patient with clinical thrombocytopenia to levels possibly seen in a local tissue inflammatory response. In 3 of 7 donors we found a significant increase in the rates of HIV production from MDMs exposed to PLTs, with 2 of the remaining donors showing increased viral production at several of the PLT exposed conditions but not all. In donor 9 we found the opposite effect
where PLT exposure significantly decreased the rate of HIV production. Interestingly, of all the donors used for infection experiments, donor 9’s PLTs demonstrated lower levels of activation in both P-Selectin and CD40L to U46619, so perhaps this less than robust response to activation accounts for the paradoxical response we observed in this one donor. Our next step will be to repeat donor 9 to see if we observe the same results for this donor again. The rationale for bringing back this donor is that if a person is becoming sick or recovering from being sick, their PLTs will respond to stimuli differently compared to a healthy individual. This has been previously documented in the case of influenza infection as well as HIV infection (Passacquale 2011, Sui et al. 2007).

It is interesting that the most striking changes were observed in MDMs following incubation of 62.5PLTs with every precursor Mo, a condition designed to mimic clinical thrombocytopenia. It should be noted that these numbers are based on an average ratio of circulating cells as captured in a snapshot of single blood draws and complete blood counts. Furthermore, we completed our assay in static culture, which probably enhanced the likelihood of PLT-Mo interaction beyond that which would be afforded by the same ratio within the circulation, where PLT-Mo interactions are likely more fleeting. The true microenvironmental ratio of PLTs to Mos under flow conditions within the body are unknown, so it is quite possible that this ratio is physiologic on the local vascular level.

Moving forward, we will need to increase the number of donors we have for both the phenotyping and infection experiments as there was variability in MDM response by donor and many of the trends we observed were not statistically significant; ideally we will start by using the donors from the phenotyping experiments in infection experiments, and vice versa. This may allow us more insight into the differences we found among
donors. We would also like to include conditions in which we use unactivated PLTs to examine if there are any differences in rates of viral production from unactivated PLTs, given that the donor we saw paradoxical response to PLTs in during the infection experiments had poorly activated PLTs.

To further elucidate the mechanisms underlying the changes that we observed, we would like to examine MDMs that we saved back from donors 6-9 for the presence of integrated viral pro-DNA and viral mRNA transcripts using qPCR to determine whether there are latently infected cells in addition to the productively infected cells in culture. We would also like to use qRT-PCR of the MDM transcriptome to examine if the changes we are seeing in surface marker expression due to PLTs are occurring at the transcriptional level.

If the *in vitro* data presented here is indicative of the role of PLT-Mo binding *in vivo*, with PLT binding changing the phenotype of the resultant MDM to predispose the MDM to infection, this implies that anti-PLT therapy may be beneficial in HIV-infected individuals. If PLTs binding to Mos are causing them to more easily harbor virus and become a latent reservoir, then it would be beneficial to add a drug to the current therapy early during infection to block this interaction. While they do have pharmaceutical compounds that specifically target PLT interactions, aspirin has been shown to decrease PLT aggregation in HIV infected individuals, possibly through a decline in CD40L and P-Selectin (O’Brien et al. 2013), and data from our lab has demonstrated that low dose aspirin therapy is effective in reducing the percentage of circulating PMAs in the SIV-infected rhesus macaque (*Macaca mulatta*) (data not shown). As low-dose aspirin is already a commonly prescribed drug with minor side effects, this would be a rather
simple addition to current therapies for HIV infected individuals. However, it is important to remember that the PLTs can also bind to other PLTs, lymphocytes and endothelial cells, and that the larger role of PLTs in the pathogenesis of HIV infection remains poorly understood. Further study examining the effect of PLT interactions with other cells is necessary to elucidate whether aspirin treatment would have inadvertent adverse effects on the HIV-infected individual.

Conclusions

Our data supports our initial hypothesis; PLTs may indeed cause an increased rate of viral production of MDMs. We believe this is due to a polarization away from the M1 phenotype towards an as yet undefined M2 subtype. This change was further characterized by an upregulation of the HIV co-receptor for entry CXCR4 and a downregulation of MDM activation markers HLA-DR, CD80, and CD163. We believe that PLTs are increasing the ability of MDMs to become infected while also downregulating their ability to combat HIV infection. This may imply that PLT binding predisposes MDMs to latent infection by HIV. Though additional work is needed to identify whether viral latency truly occurs in a subset of these PLT-stimulated MDMs, therapies that prevent the binding of PLTs to Mos may be considered for adjunct therapies for HIV in the future.
Bibliography


### Tables

<table>
<thead>
<tr>
<th>Conditions Plated for Phenotyping</th>
<th>Conditions Plated for Infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>No PLTs added</td>
<td>No PLTs added</td>
</tr>
<tr>
<td>PLTs added at 62.5:1</td>
<td>No PLTs added + HIV-1</td>
</tr>
<tr>
<td>PLTs added at 125:1</td>
<td>PLTs added at 62.5:1 + HIV-1</td>
</tr>
<tr>
<td>PLTs added at 250:1</td>
<td>PLTs added at 125:1 + HIV-1</td>
</tr>
<tr>
<td>PLTs added at 500:1</td>
<td>PLTs added at 250:1 + HIV-1</td>
</tr>
<tr>
<td></td>
<td>PLTs added at 500:1 + HIV-1</td>
</tr>
<tr>
<td></td>
<td>PLTs added at 500:1</td>
</tr>
</tbody>
</table>

**Table 1: Cell culture conditions.** Monocytes were plated with or without exposure to stimulated platelets (PLTs). The ratios represent the amount of PLTs added in respect to the number of monocytes plated.
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Fluorophore</th>
<th>Source</th>
<th>Clone</th>
<th>Panel</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>FITC</td>
<td>BD Bioscience</td>
<td>SP34</td>
<td>Mo</td>
</tr>
<tr>
<td>CD42A</td>
<td>PE</td>
<td>BD Bioscience</td>
<td>ALMA.16</td>
<td>Mo/PLT</td>
</tr>
<tr>
<td>CD16</td>
<td>PerCP-Cy5.5</td>
<td>Biolegend</td>
<td>3G8</td>
<td>Mo</td>
</tr>
<tr>
<td>CD20</td>
<td>e450</td>
<td>BD Bioscience</td>
<td>2H7</td>
<td>Mo</td>
</tr>
<tr>
<td>CD14</td>
<td>BV650</td>
<td>BD Bioscience</td>
<td>M5E2</td>
<td>Mo</td>
</tr>
<tr>
<td>CD159A</td>
<td>APC</td>
<td>Beckman Coulter</td>
<td>Z199</td>
<td>Mo</td>
</tr>
<tr>
<td>CD163</td>
<td>FITC</td>
<td>Biolegend</td>
<td>GHI/61</td>
<td>MDM</td>
</tr>
<tr>
<td>MMR (CD206)</td>
<td>BV421</td>
<td>Biolegend</td>
<td>15-2</td>
<td>MDM</td>
</tr>
<tr>
<td>CD80</td>
<td>PE-Cy7</td>
<td>BD Bioscience</td>
<td>L307.4</td>
<td>MDM</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>APC</td>
<td>Biolegend</td>
<td>L243</td>
<td>MDM</td>
</tr>
<tr>
<td>Live/Dead</td>
<td>Near IR</td>
<td>Life Technologies</td>
<td></td>
<td>MDM</td>
</tr>
<tr>
<td>CD42A</td>
<td>FITC</td>
<td>BD Bioscience</td>
<td>ALMA.16</td>
<td>MDM/PLT</td>
</tr>
<tr>
<td>TLR4</td>
<td>PE</td>
<td>Biolegend</td>
<td>HTA125</td>
<td>MDM</td>
</tr>
<tr>
<td>CD4</td>
<td>PerCP-Cy5.5</td>
<td>BD Bioscience</td>
<td>L200</td>
<td>MDM</td>
</tr>
<tr>
<td>CXCR4</td>
<td>APC</td>
<td>Biolegend</td>
<td>12G5</td>
<td>MDM</td>
</tr>
<tr>
<td>CCR5 (CD195)</td>
<td>BV605</td>
<td>BD Bioscience</td>
<td>3A9</td>
<td>MDM</td>
</tr>
<tr>
<td>P-selectin (CD62P)</td>
<td>PE</td>
<td>BD Bioscience</td>
<td>AC1.2</td>
<td>PLT</td>
</tr>
<tr>
<td>CD40L (CD154)</td>
<td>PE-Cy5</td>
<td>Biolegend</td>
<td>24-31</td>
<td>PLT</td>
</tr>
<tr>
<td>HLA-ABC</td>
<td>FITC</td>
<td>BD Bioscience</td>
<td>G46-2.6</td>
<td>PLT</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>PerCP</td>
<td>BD Bioscience</td>
<td>L243</td>
<td>PLT</td>
</tr>
<tr>
<td>IgG</td>
<td>FITC</td>
<td>BD Bioscience</td>
<td>MOPC-21</td>
<td>PLT</td>
</tr>
<tr>
<td>IgG</td>
<td>PE</td>
<td>BD Bioscience</td>
<td>MOPC-21</td>
<td>PLT</td>
</tr>
</tbody>
</table>

**Table 2: Flow cytometry antibodies.** Cells or whole blood were stained using the antibodies above for either the monocyte (Mo) panel for initial plating, or the monocyte-derived macrophage (MDM) panel for phenotyping. Resting and stimulated platelets (PLTs) were stained using the PLT panels.
Figures

H  Purity of Monocytes Cultured for Phenotyping
Figure 1: Monocyte Plating Purity. Whole blood and the plated cells were analyzed for monocyte purity though antibody staining. The gating strategy is shown in which, after gating on singlets, we gated on the mononuclear cell (MNC) population (A). From the MNC population, separate gates were drawn around the CD3+ T cells (B), the CD20+ B cells (C), and the CD159+ NK cells (D). From the MNC population a gate was drawn around the Mos (E). In F, the classical Mos (Q1) are CD14+/CD16-, the intermediate Mos (Q2) are CD14+/CD16+, while the nonclassical Mos (Q3) are CD14-/CD16+. In each of these subtypes we examined the percentage of cells that stained positively for the platelet marker CD42a (G). The percentage of the platelet bound and unbound monocytes of each subtype are shown from either the donors used for phenotyping experiments (H) or those donors used for infection experiments (J).
Platelet Activation and Response to Agonist for Phenotyping Experiments

G

P-Selectin Expression (%)

H

CD40L Expression (%)

I

HLA-ABC Expression (%)

J

HLA-DR Expression (%)
Figure 2: Platelet Activation and Response to Agonist. Washed platelets (PLTs) were analyzed for either baseline activation or response to U46619 using flow cytometry. Panels A-C represent the gating scheme for PLTs stained with the PLT marker CD42A, and the PLT activation markers P-Selectin, and CD40L, while panels D-E represent PLTs stained with CD42A, and the immune activation markers HLA-ABC, and HLA-DR. Panels G-N represent the percentage of PLTs expressing each marker, with panels G-J representing donors used for phenotyping, and panels K-N representing donors used for infection experiments. As expected we found an upregulation in both P-Selectin (K) and CD40L (L) for the donors used for infection experiments, but this effect was not as strong in those donors used for phenotyping experiments (G and H).
Figure 3: Monocyte-Derived Macrophage Polarization. Monocyte-derived macrophages (MDMs) were stained for flow cytometry analysis, with the gating strategy show in panels A-D. (E) M1 MDMs (CD80+/HLA/DR+) and (F) M2a MDMs (CD163+/MMR+) represented as mean fluorescent intensity and normalized to the No PLTs condition for each donor. We found a trend towards a decrease in M1s at all platelet (PLT) conditions except for donor 15 who was elevated at all conditions except 62.5PLTs to every monocyte.
Figure 4: Platelet Effect on the Phenotyping of Monocyte-Derived Macrophages.

Monocyte-derived macrophages (MDMs) were antibody stained for flow cytometry analysis, with the gating strategy determined by fluorescence minus one control stained MDMs. The gating strategy is shown in which after gating on the macrophage population (A) each antibody was gated as shown in B-F. The mean fluorescence intensity for each antibody normalized to the No PLTs condition is shown in G-K. Wilcoxon tests were used to determine p-values. We found a significant upregulation in CXCR4 (G), and a non-statistically significant decline in HLA-DR (I), CD80 (J), and CD163 (K) at the 62.5PLTs to every monocyte condition, along with a trend towards a decline at all other conditions except for donor 15.
Figure 5: Effect of Platelets on Infection of Monocyte-Derived Macrophages.

Monocyte-derived macrophages were infected with a multiplicity of infection of 0.1 of the X4R5 tropic virus HIV-1 89.6. Supernatants were collected at days 3, 5, 7, and 10 post-infection. A p24 ELISA was run to obtain HIV p24 protein concentrations represented in pg/mL. The data for each individual donor (A-G) were analyzed using a Two-Way ANOVA with a Tukey post-hoc correction for multiple comparisons. The data in H was analyzed by a Wilcoxon test. We found a significant increase in the rate of viral production for donors 3 (B), 5 (C), and 7 (E) due to exposure to PLTs. Donors 6 (D) and 8 (F) had higher rates of viral production for some PLT exposed conditions. Donor 9 (G) showed a significant decrease in rates of viral production of PLT exposed conditions. If donor 9 is removed from analysis we see a significant increase in the viral production rate of the 62.5PLTs to every Mo condition compared to No PLTs at day 5 post-infection.
Curriculum Vitae

Elizabeth L. Engle

EDUCATION

Master of Science, Johns Hopkins University Kreiger School of Arts & Sciences, 2016
Graduate Program: Biotechnology with Thesis
Mentor: Kelly Metcalf-Pate, DVM, PhD, DACLAM
Department of Molecular and Comparative Pathobiology
Johns Hopkins University School of Medicine, Baltimore, MD

Bachelor of Science, University of Maryland, Baltimore County, 2009
Degree: Biochemistry and Molecular Biology
Degree: Biology

EMPLOYMENT

Research Specialist, Johns Hopkins University, School of Medicine, Department of
Molecular and Comparative Pathobiology (2009-Present)
Principal Investigator: Kelly Metcalf Pate (2014-Present)
• Examined the interaction between platelets from simian immunodeficiency virus (SIV) infected macaques and a brain endothelial cell line through the use of a transwell assay system to block platelet passage and binding
• Established a protocol for immunohistochemical staining of mouse tissues with mouse antibodies
• Mentored a summer high school student through the Center for Talented Youth (2015)
• Trained in the handling, necropsy, dissection, and tissue processing of NSG mice

Principal Investigator: Joseph Mankowski (2014-Present)
• Demonstrated and established protocols for automated immunohistochemical and in situ hybridization on the Leica Bond
• Established the ultra-low copy in situ hybridization technique RNAscope for two different SIV strains both by hand and on the Leica Bond
• Developed a multitude of both single and double immunohistochemical staining protocols on the Leica Bond
Principal Investigator: Janice Clements (2014-Present)
- Created a triple immunohistochemical staining technique to examine the different macrophage subsets in over 60 macaque spleens during the acute, latent, and terminal stages in an accelerated macaque model of SIV associated central nervous system disease
- Developed a double immunohistochemical staining with in situ hybridization technique to determine which macrophage subset was harboring SIV in the spleens of infected, untreated macaques throughout the course of infection with SIV
- Managed several technicians in the preparation of cART cocktails necessary for treatment of SIV infected macaques
- Created and maintained a database to track the pharmaceuticals necessary for treatment of SIV infected macaques including ordering necessary vehicles for administration

Principal Investigator: M. Christine Zink (2009-Present)
- Used immunohistochemical and image analysis techniques to determine differences in staining of various markers between uninfected macaques, infected, untreated macaques and macaques treated with fluconazole and paroxetine
- Designed a Nanostring codeset for 116 macaque genes to examine changes in spleen of macaques at various timepoints throughout infection with SIV along with treated macaques and uninfected macaques
- Established nitrotyrosine and immunohistochemical techniques to examine differences in staining patterns in deep white matter between macaques infected with SIV and treated with Minocycline compared to untreated, infected macaques and uninfected macaques
- Established a variety of immunohistochemical staining techniques in the gastrointestinal tract of macaques infected with SIV

General Retrovirus Laboratory Responsibilities (2009-Present)
- Trained graduate students, technicians, undergraduates, and high school students in the proper use and maintenance of the Leica Bond Rxm, Nikon TE2000 confocal microscope, Nikon E400 inverted microscope, Nikon Eclipse 90i automated microscope, and the associated software Nikon Elements Advanced and Basic, and EZ-C1
- Coordinated, trained, and assisted in the processing of over forty tissues removed from macaques at necropsy and ensure that each tissue was fixed in the appropriate tissue fixative or frozen, then catalogued and stored properly
- Coordinated and oversaw the labeling of specimen containers required for macaque necropsies
- Prepared required surgical equipment packages, necessary supplies, and the BSL3 facility for macaque necropsies
- Organized, established, and maintained a freezer database for 20-80°C freezers to track tissue samples obtained from macaques at necropsy
- Performed routine maintenance on a variety of laboratory equipment
- Coordinated and placed orders, while communicating with sales representatives, to ensure efficient lab spending
• Organized and condensed a sample library for thousands of stained slides to create an easy access inventory encompassing samples from 1986-present
• Presented data graphically and performed statistical analyses through the use of both GraphPad Prism and Microsoft Excel
• Developed and edited figures using both Adobe Photoshop and Microsoft Powerpoint

PUBLICATIONS


POSTERS