The Role of Inflammatory Calprotectin-Expressing Monocytes/Macrophages in Simian Immunodeficiency Viral Infection

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ABSTRACT:

HIV infection elicits dysregulation of the immune system and is associated with a number of comorbidities. A common link among HIV-associated comorbidities is monocyte and macrophage activation, accumulation, and high turnover. Anti-retroviral therapy (ART), while useful in preventing HIV infection from progressing to immune dysfunction characterized by AIDS, does not eliminate infection. Even with ART, individuals retain a low level of virus that is able to reseed infection and a higher rate of comorbidities than uninfected people. Prior research has revealed an inflammatory monocyte/macrophage cell population that is uniquely in tissues with infection in an HIV model that uses simian immunodeficiency viral (SIV) infection of rhesus macaques. This cell type is characterized by expression of the inflammatory marker calprotectin.

Through measurements of soluble calprotectin present in the plasma of SIV-infected rhesus macaques, I found that calprotectin levels remained low within the first two weeks of infection, sharply increased around three weeks post-infection, typically increased to a maximum during late stage chronic disease, and positively correlated with plasma viral load. Initial calprotectin levels suggests a trend that high pre-infection levels are associated with *not* progressing to AIDS or SIV encephalitis. Through immunostaining monocytes and flow cytometry, I found that calprotectin expression on classical, intermediate, and non-classical monocytes initially decreases with SIV infection, rebounds for most of infection, and sharply

decreases again with late-stage chronic disease. Flow cytometry further showed that the calprotectin-expressing monocyte expresses CD163, CD169, and CCR2, but lacks expression for CX3CR1 and CCR5. Analysis of RNAseq data illustrates trends that suggest an increase in gene expression of genes involved in antiviral/antibacterial and chemotactic functions during conditions when calprotectin gene expression is also increasing. In summary, the data presented in this thesis suggest that the calprotectin-expressing monocyte/macrophage may come from an intermediate monocyte and play a role in inflammation through calprotectin secretion, activation, and increased chemotactic function.

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CHAPTER 1: INTRODUCTION

1.1 HIV

HIV infection is characterized by three phases: an acute phase with sharply decreasing blood CD4+ T cell levels and a sharp rise and subsequent drop in plasma viral load, an asymptomatic phase with gradually increasing viral load and gradually decreasing blood CD4+ T cells, and, finally, acquired immune deficiency syndrome (AIDS), which is defined by a blood CD4+ T cell count dropping below 200 cells per cubic milliliter and is often accompanied by an increase in plasma viral load (1) (see Figure 1 below). Cells expressing CD4 and either the coreceptor CCR5 or CXCR4 are susceptible to HIV infection, making activated CD4+ T lymphocytes the primary target of HIV infection. However, blood monocytes and tissue macrophages are also capable of sustaining HIV infection (1), with potential for productive viral infection only after monocyte differentiation into macrophages (2). Antiretroviral therapy (ART) results in increases in CD4+ T cells and a reduction of virus to below detection in most assays (1). However, despite viral suppression with ART, chronic HIV-associated comorbidities persist (3), suggesting persistent immune activation that drives inflammation and pathogenesis.

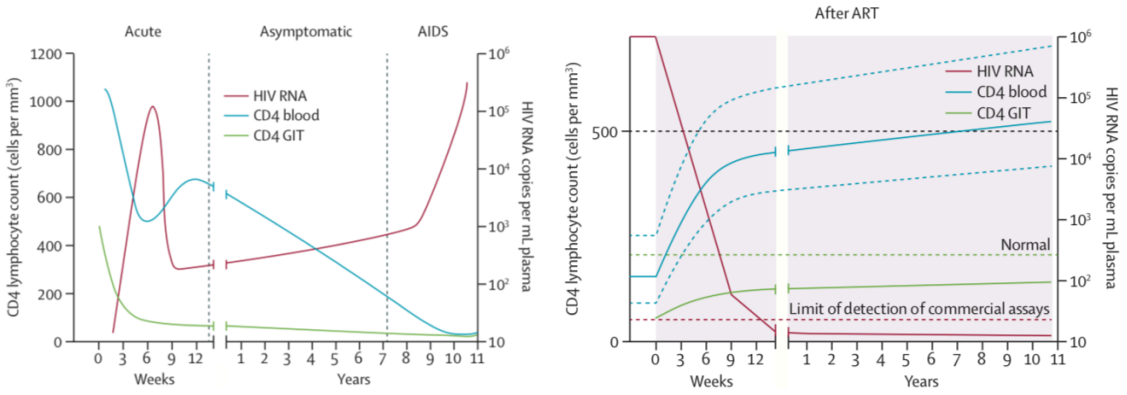

Figure 1. Viral load and CD4 Cell Count in HIV Infection, Untreated and ART Treatment. *Panel A shows the three phases of HIV infection without treatment: acute, asymptomatic, and AIDS. Acute infection is characterized by decreases in CD4+ T cells in the blood and gastrointestinal tract and an increasing viral load with peak viral load around six weeks after infection. Asymptomatic infection is characterized byfurther decreases in CD4+ T cells in the blood and gastrointestinal tract, and a gradual increase in viral load. Immune dysregulation that accompanies AIDS is associated with a more rapid viral load increase as CD4+ T cells in the blood and gastrointestinal tract strongly decreases. Panel B shows the effect of ART on HIV infection. ART adherence results in a decrease in viral load to below the limit of detection of commercial assays, but not 0, evidenced by viral rebound with ART cessation. ART adherence also results in increases in CD4+ T cells in both the blood and gastrointestinal tract. Taken from (1).*

1.2 Monocyte Role in HIV/SIV

Blood monocytes originate in splenic and bone marrow reservoirs, enter blood circulation, and serve an immune function through the release of cytokines and phagocytosis (4). Three major monocyte subpopulations exist, and are defined by their expression of CD14 – a co-receptor for lipopolysaccharide – and CD16 – a Fc Receptor. Monocyte subpopulations, CD14+CD16-, CD14+CD16+, and CD14-CD16+, are referred to as classical, intermediate, and non-classical monocytes, respectively. The relationship among these monocytes is unknown, and cells may be fated towards a particular subpopulation, or may mature from classical to intermediate to non-classical monocytes upon entry into the blood. These three monocyte subpopulations differ in their kinetics of exit from the bone marrow into the blood (5), and in protein and gene expression profiles.

Classical monocytes are unique in that they enter peripheral tissues under non-inflammatory conditions (6) and have higher phagocytic ability and release toxic superoxides

(7). Monocyte chemotactic protein-1 receptor CCR2, the receptor for monocyte chemotactic protein with involvement in monocyte translocation from bone marrow to blood, is most highly expressed on the classical monocyte subpopulation, suggesting differences in migration and response to signals by different monocyte subpopulations (8). Gene expression unique to classical or classical *and* intermediate monocytes is involved in positive regulation of cell proliferation, regulation of protein kinase cascades, endocytosis, and wound healing functions (9).

Intermediate monocytes are transcriptionally intermediate between classical and non-classical monocytes, but also unique in several aspects (9, 10). Intermediate monocytes have the highest level of SIV replication of all monocytes (11). Functions of intermediate monocytes include secreting reactive oxygen species, serving as antigen presenting cells, and contributing to inflammation (12). Gene expression unique to the intermediate *and* non-classical or non-classical monocytes includes genes involved in negative regulation of cell proliferation, cell activation, and regulation of T-cell activation (9), as well as genes involved in cytoskeletal and inflammatory cytokine gene networks (10). Intermediate and non-classical monocytes are more susceptible to HIV infection than classical monocytes, suggesting their similarity to tissue macrophages (13).

Further, gene expression in non-classical monocytes is most similar to expression by tissue-differentiated macrophages, illustrating this monocyte subpopulation as the most mature (9). Non-classical monocytes serve a role as immune patrollers in the blood, mediated by their expression of high levels of adhesion receptors and integrin adhesion molecules (14). Compared to other monocytes, non-classical monocytes express significantly higher levels of CCR5, the receptor for macrophage inflammatory protein 1α, and lack the expression of CCR2 (8).

1.3 Monocyte Expansion in HIV/SIV

Monocyte subpopulations further differ in their rate of turnover from bone marrow to the blood. 5-bromo-2'-deoxyuridine (BrdU) labeling of pre-monocytes in bone marrow shows that BrdU-labeled classical monocytes are the first to peak in blood, followed by intermediate, and, lastly, non-classical monocytes, illustrating turnover and kinetic differences among the subpopulations that is compatible with monocyte subsets representing stages of cell maturation (15). The rate of monocyte turnover is significantly higher in SIV-infected macaques than uninfected controls, and strongly correlates with animal survival, making monocyte turnover a potentially useful predictor of disease progression (16). Monocyte turnover also correlates with the severity of CNS terminal disease and distinguishes rapid and slow AIDS progressors (5). Thus, further characterization and phenotyping of monocyte populations that expand with SIV and HIV infection will help in understanding the functions of monocyte subpopulations and what their roles are in disease progression.

1.4 Macrophage Accumulation in HIV/SIV

Macrophages are a heterogeneous myeloid population found in tissues, and most macrophages are a mature, tissue-differentiated cell type of blood monocytes (17). Mediated by pro-inflammatory cytokines, blood monocytes bind to selectins and intercellular adhesion molecules expressed by endothelial cells to mediate transmigration into inflamed tissue (18). High levels of monocyte traffic across the endothelial barrier into peripheral tissues and the accumulation of macrophages in the tissue results in an expansion of cellular traffic across weakened barriers, including across the blood-brain barrier into the central nervous system (CNS) (19). High levels of macrophage accumulation in the central nervous system (CNS), especially of inflammatory macrophages, is a defining feature of encephalitis (17, 20). Within

the CNS of HIV+ persons, macrophages are the main cell type sustaining HIV infection, which makes this cell type a potential viral reservoir (21). Because of the presence of virally infected macrophages in the brain that can persist even with ART and their recent immigration into tissues, it is critical to understand both tissue macrophages and their immature monocyte precursor. HIV-infected macrophages can be sources of latent HIV infection, in which HIV DNA is integrated into the host DNA (1). Viral sequencing indicates HIV compartmentalization in the brain of HIV-infected people and that the CNS contains residual virus throughout infection (22). Chemotactic signaling increases monocyte- and macrophage-mediated inflammation following HIV replication within the brain, increasing the monocyte entry into tissue and macrophage accumulation (23).

The role of macrophage accumulation and contribution to disease is thus integrally related to monocyte expansion and phenotype. A unique inflammatory macrophage subpopulation, which is identified by the expression of the calprotectin protein, is absent in tissue without inflammation, lesions, or infection, and makes up over 80% of the infiltrating cells during SIV infection (24). These inflammatory calprotectin-expressing macrophages, and monocytes, have a strong correlation with infection and disease, and thus are expected to play an important role in the inflammatory immune response.

1.5 Calprotectin Structure

Calprotectin, alternatively known by the names of the L1 antigen (25) and the cystic fibrosis antigen (26) is a 36.5 kDa protein heterodimer. This calprotectin complex is formed by disulfide-linked MRP-8 and MRP-14 proteins (for which the encoding genes are referred to as S100A8 and S100A9, respectively) (27) (see X-ray crystal structure and binding sites of the complex below, Figure 2). Further complex formation has illustrated the possibility of

homodimerization, trimerization, and tetramerization of these two subunits. Calprotectin contains an EF hand motif on each of its subunits, which facilitate the calcium-binding function of the molecule (25), and the dimerization of the two subunits to form the calprotectin molecule is a calcium-dependent process (28). In humans, the encoding subunits of calprotectin map to chromosome 1q21-25 (29). Calprotectin also has zinc-binding function, which is found on the MRP-14 subunit and does not compete with this subunit's calcium-binding function (30). The MRP-14 subunit also has specific and high-affinity binding to heparan sulfate proteoglycans (31) and carboxylated glycans (32). Because of this affinity and calprotectin expression on the surface of neutrophils during their activation, calprotectin may also play a role in cell extravasation into tissues (32).

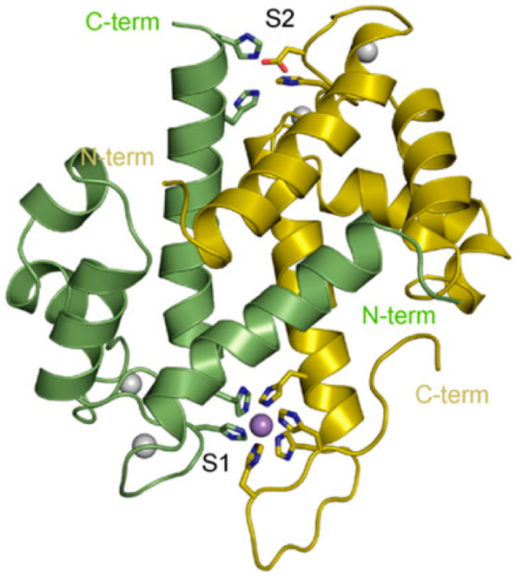

Figure 2. Calprotectin Structure. *X-ray crystal structure illustrates the MRP-8 (green) and MRP-14 (yellow) complex formation to form the calprotectin complex. S2 is the zinc-specific binding site and S1 is a zinc/manganese binding site. Taken from (33).*

1.6 Calprotectin Expression in Neutrophils

Calprotectin is strongly expressed by neutrophils and myeloid cells, and has limited expression in endothelial and epidermal cells (34). Protein expression of calprotectin is highest

in neutrophils, where it is found primarily in the cytoplasm and comprises about 45% of the cytosolic proteins of this cell type (35). About 96% of all granulocytes from healthy individuals are positive for calprotectin, and this expression is ~5% for eosinophils and <2% for basophils (36). The substantial amount of calprotectin in neutrophil and monocyte cytoplasm has indicated the potential role of these cells as 'calcium sinks' (37), suggesting a role in protecting cells from harmful effects of long exposure to high calcium levels and intracellular inhibition of casein I and II, leading to a termination of cellular metabolic activity (38). Through monocyte chemoattractant protein-1 (MCP-1), calcium has been shown to play a role in signaling in the inflammatory process during atherogenesis (39). Another suggested intracellular activity of calprotectin is inhibition of casein kinase I and II, which is itself implicated in regular cellular transcription and translation. Inhibition of these kinases would terminate cellular metabolic activity. During calcium-dependent activation of neutrophils, calprotectin becomes phosphorylated and moves to the cell membrane (40). In neutrophils, the MRP-14 subunit of calprotectin is specifically involved in promoting integrin-mediated cell adhesion (41). Further, neutrophil exposure to MRP-14 results in an elevated and *sustained* increase in neutrophil adhesion to fibrinogen (42). *In vitro* studies further show that the MRP-14 subunit of calprotectin increases neutrophil transmigration both in the presence and absence of chemoattractants (42).

1.7 Calprotectin Expression in Tissue Macrophages

Tissue macrophages expressing calprotectin are generally found near blood vessels, supporting the notion that this macrophage population is recently immigrated into tissue and has a blood monocyte origin (34). Calprotectin is not expressed by normal tissue macrophages, but, under inflammatory conditions or with infection, a population of calprotectin-expressing

macrophages is present in the brain and CNS lesions (24). Macrophage expression of the MRP-14 subunit alone is especially associated with acute inflammation, while macrophage expression of MRP-8 and MRP-14 together is more associated with chronic inflammation (26, 43). The number of macrophages expressing calprotectin is increased in liver tissues of those with hepatitis C infection, relative to uninfected controls (44). In inflammatory neuropathies, including Guillain-Barré patients, the number of calprotectin-positive macrophages increases significantly (43).

Macrophages in tissue lesions expressing calprotectin also represent an important and changing cell population. Cardiovascular tissue immunostaining has shown calprotectin cell expression is most tightly associated with CD68+ macrophage-abundant lesions (45). In human infection with T-cell lymphotropic virus type I, macrophages express calprotectin in active lesions with a short duration of illness, while macrophages lacked calprotectin expression in inactive chronic lesions (46). Calprotectin expression may also occur under non-inflammatory disease states, including ischemic brain lesion formation, where a resident and non-inflammatory macrophage population known as microglia only express MRP-8 and MRP-14 within the first three days in lesions after infarction (47). The lack of MRP expression after 3 days on these microglia suggests MRP expression is an early step in microglial activation and that their expression is very temporally distinct, with an integral role in lesion formation or the early stages of lesion expansion (47). It is suggested that the loss of MRP-8/14 expression in microglia may be a result of cell transformation towards an ameboid cell type with phagocytic functions (47). The role of calprotectin-expressing macrophages in lesions underscores the role of this cell type in chronic disease inflammation and as a contributing cell type to active lesions. Calprotectin expression is also associated with tissue destruction, and expression on muscle tissue

macrophages correlates with muscle fiber destruction (48). Together, the expression of calprotectin by endothelial cells, neutrophils, and monocytes/macrophages suggests its important and widespread role in innate immune protection (49).

1.8 Calprotectin Expression in Monocytes

The calprotectin protein is present in the cytoplasm of virtually all monocytes (36), but is substantially less abundant there than in neutrophils, and comprises about 1% of monocyte cytosolic protein (35). Measures of calprotectin expression between disease states and health is substantially less studied in circulating monocytes than in macrophages. However, increased numbers of blood monocytes expressing calprotectin are associated with mild acute pancreatitis (50).

In those with muscle fiber necrosis, about 90% of monocytes expressing the resident macrophage marker CD68+ were also positive for calprotectin expression (48). However, *in vitro* stimulation with various inflammatory stimuli – LPS, TPA, and IFN-γ – do not increase the number of monocytes expressing calprotectin, but, rather, increase the level of calprotectin expression on monocytes already expressing calprotectin, suggesting that calprotectin-expressing monocytes become activated, rather than a calprotectin-negative monocyte expressing calprotectin as a part of its activation (34). Monocyte expression of either MRP-14 alone or the entire calprotectin complex is associated with cells having increased surface expression of the adhesive molecule CD11b (51). In culture, addition of a pro-inflammatory cytokine that activates monocytes shows that monocytes reach peak calprotectin expression 2-3 days following stimulation (34). However, in the absence of stimulation, monocyte culture conditions do not appreciably alter calprotectin expression by freshly isolated monocytes within three days (19). *In vitro* monocyte stimulation with *Mycobacterium* results in fivefold higher levels of

supernatant calprotectin (52). Furthermore, monocyte incubation with MRP-14 results in cytokine release of the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α and chemokines growth-related oncogene α, IL-8, MCP-1, and MIP-1α, (42). In contrast, neutrophil incubation with MRP-14 was unable to induce these same cytokine and chemokine releases (42), supporting calprotectin-expressing monocytes as particularly important in inflammation.

1.9 Antibody Recognition of Calprotectin

Multiple antibodies against calprotectin have been developed, with a range of specificities, including against MRP-8, MRP-14, or both subunits. The monoclonal antibody MAC387, in use by our lab, was first developed in mouse and demonstrates strong reactivity against infiltrating histiocytes (27). The MAC387 antibody has specificity for the MRP-14 protein, and also recognizes the MRP-8/MRP-14 calprotectin heterocomplex (53, 54). The antibody 27E10 has also been shown to recognize the calprotectin complex, but neither subunit alone (55). Another antibody against calprotectin, mAb 5.5, also reacts only with the MRP-8/MRP-14 complex, but neither independent subunit, suggesting a conformational change with heterodimer formation leading to the formation or exposure of the recognized antigen (53). In addition to calcium-dependent heterodimer formation, antibody recognition of calprotectin is calcium-dependent (53), and neither MRP-8 nor MRP-14 contain a signal or membrane-anchor sequence (26), which suggests the calcium-dependency for calprotectin transmigration to the cell surface.

1.10 Calprotectin Secretion

Without inflammatory stimuli, calprotectin protein is predominantly in the cytoplasm. Following cellular activation, calprotectin is secreted. Adherence of monocytes to extracellular matrix proteins fibronectin and collagen *in vitro* results in a rapid translocation of calprotectin to

the cell membrane, suggesting that monocyte adhesion and entry into tissues is associated with calprotectin surface expression (56). The activation of protein kinase C and an intact tubulin network are required for the process of calprotectin secretion from activated monocytes (57). Secretion of calprotectin by monocytes can be induced by interaction with stimulated endothelial cells, TNF activation, or the presence of extracellular matrix proteins, but interaction with resting endothelial cells is not sufficient to cause calprotectin secretion (58), resulting in the additional recruitment of other inflammatory myeloid cells.

1.11 Calprotectin Function

Upon secretion, calprotectin has a pro-inflammatory role (59), and is present at high levels in the extracellular space with inflammation, including numerous chronic diseases (60). The MRP-8 subunit of calprotectin is a ligand for TLR4 and, through interaction with the TLR4-MD2 complex, amplifies pro-inflammatory phagocyte activation (61). *In vitro* work shows that a calprotectin-expressing monocyte preferentially transmigrates across a blood-brain barrier model (62). Coinciding with surface expression of calprotectin, interleukin-6, tumor necrosis factor α, and superoxide anion release contribute to the pro-inflammatory response (56). Calprotectin also has an inflammatory role in connective tissues, as demonstrated by calprotectin inhibiting fibroblast growth and inducing fibroblast apoptosis *in vitro* (63). Calprotectin secreted from activated macrophages also prevents myoblast proliferation and differentiation, and correlates with myofiber degeneration (48). Calprotectin inhibits the growth of monocytes and macrophages, and even leads to lysis of cells from a tumor line *in vitro*, demonstrating an anti-proliferative role as a negative growth regulator (64). A contributing factor to the anti-proliferative function of calprotectin is explained by its inhibition of intracellular casein kinase II, which is involved in cell growth (37). Together, these functions induced by calprotectin *in*

vitro contribute to cell accumulation observed *in vivo* in response to calprotectin, and may also help explain the pro-inflammatory role of calprotectin-expressing monocytes/macrophages as it relates to inflammation (60). Further, calprotectin is shown to have antimicrobial activity, and is shown *in vitro* to inhibit the growth of fungi, bacteria, and human cell lines (37). Antimicrobial activity by calprotectin is at least partially explained by its competition for zinc ions, which are required for microbial growth (65). This expression contributes to individual cells that express calprotectin being more resistant to bacterial binding and entry (66).

1.12 Soluble Calprotectin As a Marker of Inflammation & Disease

Calprotectin release by neutrophils and monocytes and its accumulation in blood has been associated with a variety of diseases, underscoring the importance of calprotectin secretion with inflammation. Serum and plasma are both the remaining portion of blood after blood cells have been removed; serum further has clotting factors removed. Calprotectin levels in serum and plasma significantly correlate with a substantial number of active infections and inflammatory diseases. In HIV+ people, serum calprotectin is elevated in those with AIDS relative to those in earlier stages of infection, and thus calprotectin serves as a biomarker for acute infection in immunocompromised individuals (67). Plasma levels of calprotectin in those with tuberculosis infection are elevated relative to uninfected controls, and correlate with the severity of tuberculosis disease progression (52). Calprotectin levels are also elevated in the serum of individuals with multiple sclerosis, and, within these patients, are elevated in those experiencing acute relapse relative to those experiencing stable disease remission (68). In serum, MRP-14 concentrations are significantly elevated in those with prostate cancer (69). Plasma calprotectin levels are also elevated in obese individuals, relative to non-obese controls (70) and in those with type 2 diabetes mellitus (71). Active juvenile rheumatoid arthritis results in a 5-

fold increase in serum calprotectin levels relative to those with juvenile rheumatoid arthritis in remission, which is similar in level to those without the disease (58). Serum calprotectin is also elevated in those with Chron's disease, and, within these patients, higher disease activity was associated with higher calprotectin levels in serum (72). Serum calprotectin levels are significantly elevated in patients with systemic lupus erythematosus relative to healthy individuals, and significantly correlate to an index of disease activity (73). Calprotectin levels can also be prognostic of survival, with high levels in plasma correlating with a poor survival rate in those with alcohol-induced cirrhosis (74). With significantly higher calprotectin levels in inflammatory disease states, and also with more severe disease states or progression, the measurement of calprotectin has an extremely important role as a relatively non-invasive diagnostic tool.

1.13 Solubilized Calprotectin In HIV

Previous studies have looked at some correlates with blood calprotectin in the context of HIV. In HIV+ persons, serum calprotectin levels do not correlate with viral load (75). However, secreted calprotectin levels do correlate with other parameters closely tied to immune cell dysfunction. Serum calprotectin levels are inversely correlated with CD4+ T cell count in individuals with CD4+ T cell counts over 50 x 10^6 per liter, with increasing levels of calprotectin correlating with lower CD4+ T cell counts and thus with immunodeficiency (75). Serum calprotectin is significantly elevated in both AIDS and non-AIDS asymptomatic HIV-infected patients relative to HIV-negative individuals (67, 75). Further, serum calprotectin levels are significantly lower during acute HIV infection than in patients with AIDS, and are suggested as arising as part of the neutrophilic antimicrobial defense, resulting in immune activation during the immune dysregulation of AIDS (67). Serum calprotectin is elevated in HIV patients with

moderate to severe clinical conditions associated with HIV, as compared to uninfected individuals (49). In the most severe CDC classification of HIV based on clinical conditions, significantly higher MRP-14 serum levels were found in HIV+ people relative to uninfected people, but no differences were found in the MRP-8 subunit between these groups (49). This association of MRP-14 indicates an association with both progressive immunodeficiency and clinical events, including opportunistic infections (49). In contrast, serum levels of the MRP-8 subunit of calprotectin alone were significantly lower in those with HIV infection relative to uninfected people (67). A comparison of HIV-positive persons with and without oral candidiasis found significantly elevated saliva calprotectin levels in those with oral candidiasis disease, suggesting a defensive role of calprotectin in HIV complications (76). In those with HIV infection, serum calprotectin also significantly correlates with neopterin, a pro-inflammatory molecule produced by stimulated monocytes/macrophages (49).

Treatment of HIV with the anti-retroviral reverse transcriptase inhibitor Zidovudine results in an expected increase in CD4+ T cells and a decrease in viral load while serum calprotectin levels increase (75). In both asymptomatic HIV-positive patients and HIV-positive patients that have progressed to AIDS, treatment with Zidovudine concomitantly resulted in an initial rise in serum calprotectin levels for the first 3-6 months, followed by calprotectin levels returning to baseline within 12 months after beginning Zidovudine treatment (75). A study found no difference in MRP-8, MRP-14, or calprotectin serum levels between HIV+ people that did or did not receive Zidovudine treatment (49). This study also found that significantly lower calprotectin responses were associated with HIV+ people with an AIDS-defining infection within the first year following Zidovudine treatment, indicating high calprotectin responses are related to a lessened risk of AIDS-defining complications (75). A correlation was found between

the maximum calprotectin response to Zidovudine and the length of survival, suggesting that serum calprotectin levels may be a prognostic marker of the function of anti-retroviral therapy (75). This suggests that calprotectin secretion plays an important role both in acute HIV infection and in the early stages following anti-retroviral treatment.

1.14 Monocyte & Macrophage Calprotectin Expression in HIV & SIV Infection

Microarray analysis of RNA reveals that gene expression for the two subunits of calprotectin – S100A8 and S100A9 – are expressed greater than 2-fold in classical monocytes relative to either intermediate and non-classical monocytes (9). The higher calprotectin RNA expression of classical monocytes is present in uninfected rhesus macaques and SIV-infected rhesus macaques both 26 days after infection and terminally with AIDS (9). However, at the protein level in uninfected macaques, intermediate monocytes have the highest calprotectin expression, followed by classical monocytes, and nearly nonexistent expression by non-classical monocytes (77). This high expression in classical and intermediate monocytes suggests a potential link between this monocyte subset and the inflammatory MAC387+ macrophage. At the protein level, calprotectin expression on both classical and non-classical monocytes tends to decrease from pre-infection levels to AIDS (24), but a characterization of monocyte expression of calprotectin throughout the course of HIV or SIV infection is lacking. However, this does give insight into the specific monocytes that are expressing and potentially secreting calprotectin during the HIV/SIV inflammatory response.

Macrophages staining positive with the anti-calprotectin MAC387 antibody (MAC387+) are a unique inflammatory tissue subpopulation and have distinct surface expression, which includes high CD16 expression, while lacking CD68, CD163, and CCR2 expression (24). These MAC387+ macrophages are absent from the central nervous system (CNS) of SIV-uninfected

macaques, but widespread in CNS tissue by 21 days after SIV infection and their accumulation characterizes early CNS inflammation (78). Expression of calprotectin is limited to this recently infiltrating macrophage population, and is absent on both CD68+HAM56+CD163- resident and CD163+CD68+ perivascular macrophage populations (24). In SIV infection, MAC387+ macrophage accumulation within the CNS is compartmentally distinct, with early accumulation in the meninges and choroid plexus and late accumulation in the perivascular space and encephalitic lesions with the progression to AIDS (78). In the CNS of SIV-infected rhesus macaques, MAC387+ macrophages represent the majority of all recently infiltrated cells, and 30% of these macrophages were infiltrated within 48 hours (24, 78), further supporting the role of this macrophage as inflammatory and blood monocyte-derived. Rhesus macaques infected with SIV also have elevated MAC387+ macrophages during early acute disease within the lymph nodes (79).

In terminal HIV+ patients, MAC387+ macrophages are unique to those with encephalitis (24). The large majority of recently emigrated cells present in lesions of SIV-infected rhesus macaques are macrophages that express calprotectin, present in early acute infection, and are suggestive of a monocytic cell origin (5). In these patients, MRP-8 expression was found in addition to the calprotectin complex, contrasting the lesions of SIV-infected macaques (24). Higher CD163+/MAC387+ macrophages is indicative of AIDS disease progression both in SIV infection (78), supporting MAC387+ macrophages as having a role in the early inflammatory response and lesion formation. Specifically in the case of SIV infection, the MAC387+ macrophage is an inflammatory cell type that is not productively infected, and this cell type contributes more to lesions in mild encephalitis relative to severe encephalitis (24). Immunohistochemistry work shows that in encephalitic lesions, calprotectin-expressing

macrophages are the dominant tissue macrophages during acute infection and inflammation, but there is a shift in lesion macrophage contribution in chronic and severe encephalitic lesions towards CD68+ macrophages and HIV/SIV infected macrophages (24). Phenotyping the MAC387+ monocyte and tracking changes in its expression and levels of soluble calprotectin in blood will likely play a key role in understanding how this crucial cell type contributes to the progression of SIV and HIV disease.

1.15 Open Questions

Prior to this work, an immunophenotype analysis of the calprotectin-expressing blood monocyte throughout the course of HIV or SIV infection was lacking. This research demonstrates the MAC387+ monocyte present in SIV infection as CD163+, CD169+, CCR2+, CCR5-, and CX3CR1-. The expansion of inflammatory, calprotectin-expressing macrophages with SIV infection and elevated blood calprotectin levels in a variety of inflammatory disorders underlines the importance of this cell type in HIV and SIV disease progression. Limited immunophenotyping has been performed on the MAC387+ macrophage, but this analysis is lacking in monocytes, which offer a window into disease progression instead of only the inflammatory conditions after necropsy. While it is established that the classical monocyte subset has the highest calprotectin *gene* expression, it is unknown how calprotectin protein expression corresponds with different monocyte subsets and immune expression. The issue addressed in this thesis and associated work is the contribution of MAC387+ monocytes to SIV disease progression in rhesus macaques. Specifically:

(1) How does the level of secreted calprotectin in blood change over the course of infection?

(2) What is the phenotype of the MAC387+ monocyte and does this change over the course of infection?

(3) What monocyte gene expression changes occur in coordination with calprotectin gene expression increases?

It is hypothesized that levels of secreted calprotectin will peak in blood during acute infection, or approximately the first three weeks following SIV infection. Calprotectin expression on monocytes is expected to accordingly decrease during this time, due to secretion of the molecule, and be highest on the classical monocyte subpopulation. Immunophenotype and gene expression of the classical MAC387+ monocyte are expected to be similar to that of inflammatory MAC387+ macrophage observed in tissue, particularly during the acute phase of SIV infection.

In order to characterize the calprotectin-expressing monocyte, PBMCs isolated from rhesus macaques infected with SIV over the course of infection were immunostained for specific markers of interest. Non-human primates infected with SIV that are CD8+ T cell-depleted to achieve rapid AIDS progression and a higher incidence of encephalitis have routinely been used as model of HIV and AIDS (9, 24, 78, 80). This will both allow a characterization of the MAC387+ monocyte in terms of classical, intermediate, or non-classical monocytes, as well as measuring the changes in immune marker expression over time.

In order to relate monocyte expression of calprotectin to calprotectin levels in blood, ELISAs were performed to measure plasma calprotectin levels in the same animals. The

calprotectin ELISA kit used in this study has previously been used to measure serum calprotectin levels in several primate species, including rhesus macaques infected with bacteria of the genus *Burkholderia* (81, 82), and measuring calprotectin increases with infection. In order to better understand the role of calprotectin levels in blood and expression on monocytes in SIV disease progression, animals were used with varying severities of disease that ranged from no AIDS to SIV encephalitis (SIVE). In order to connect MAC387+ monocyte expression and secreted calprotectin, MAC387+ monocyte expression was compared with plasma calprotectin levels. This thesis describes the phenotype of calprotectin-positive monocytes, the changes of these cells over the course of infection, the expression of these cells on the three recognized monocyte subpopulations, the levels of plasma calprotectin throughout SIV infection and other gene expression increases on monocytes with increasing calprotectin gene expression.

CHAPTER 2: MATERIALS & METHODS

2.1 Animal Cohorts

Peripheral blood mononuclear cells (PBMCs) and plasma samples taken from blood were analyzed from 5 adult rhesus macaques (*Macaca mulatta*). Animals were infected by a 1 mL IV of 1.1 ng/mL SIVmac251. In order to achieve a more rapid progression and higher incidence of AIDS, animals were subcutaneously administered a CD8-depleting antibody at 10 mg/kg bodyweight at 6 days post-infection and intravenously administered the CD8 antibody at 5 mg/kg bodyweight at 8 and 12 days post-infection. One animal, IP79, was found dead in its cage at 63 days after SIV infection, with AIDS and CMV infection. The remaining animals were euthanized between 106 and 126 days after infection by pentoarbital overdose upon presentation of AIDS-defining criteria described in (24), which includes dramatic weight loss and opportunistic infections.

Animal ID	AIDS	SIVE	Other Pathology	DPI at Necropsy
JD29	Yes	Yes	Pneumonia & CMV	126
LB12	Yes	Yes	CMV Infection	115
JE87	Yes	Yes (mild)	None	119
IP79	Yes	No	CMV Infection	63
JL37	No	No	N/A	106

Table 1. *Animal ID, disease pathology of whether an animal had AIDS, SIVE, and/or other relevant disease or infection, and length of survival for the animal cohort used for plasma calprotectin ELISA and PBMC flow cytometry data.*

2.2 Measuring Extracellular Calprotectin

Plasma calprotectin levels were determined with the Calprotectin (serum) ELISA RUO kit from DRG International (cat. # EIA-5111R), according to the manufacturer's protocol. Plasma samples were diluted 1:50 in order to fall within the detection limits of this assay, which

have a minimum of 3.9 ng/mL and a maximum of 250 ng/mL, and absorption was determined at 450 nm against 630 nm.

2.3 Flow Cytometric Analysis of MAC387 on Blood Monocytes

PBMC samples were washed with 20% FBS – 80% PBS washes, and incubated with a surface antibody cocktail, which was made from the antibodies shown below in Table 2 (excluding antibodies against calprotectin – MAC387 – and against BrdU).

Antigen Target	Conjugated Fluorophore	Volume (uL)	Antibody Clone	Vendor
MAC387	RPE	0.2	MAC387 (IgG1 isotype)	Biorad
L/D	QD605	1	N/A	Fisher
CD3	APCCY7	5	SP34-2	BD Pharmigen
CD20	APCCY7	1	2H7	BioLegend
HLA-DR	ECD	2	Immu-357	Beckman Coulter
CD14	PB	2.5	M5E2	BD Pharmigen
CD16	PECY7	0.2	3G8	BD Pharmigen
CD163	PERCPCY5.5	10	GHI/61	BioLegend
CX3CR1	BV650	10	2A9-1	BioLegend
CCR2	A700	5	48607	RnD
CCR5	PEC5	5	2D7/CCR5	BD Pharmigen
CD169	APC	10	7-239	BD Pharmigen
BrdU	FITC	2	N/A	BD Pharmigen

***Table 2**. List of antibodies used for PBMC staining, including vendor, volume used per sample, and specific clone for each antibody.*

Following a cell membrane permeabilization with BD Cytofix/Cytoperm Buffer, cells were incubated with the MAC387 antibody in order to determine intracellular expression of the MAC387 antigen. For the samples from timepoints when animals received BrdU injections, a nuclear permeabilization step was performed with BD Cytoperm Plus Buffer, and cells were incubated with the anti-BrdU antibody. Lastly, cells were fixed with 1% paraformaldehyde. Data was acquired with a FACS Aria (BD Biosciences), and FlowJo software was used to analyze the data. An unstained sample was used for compensation for all markers in order to

minimize the effect of autofluorescence on data. Monocytes were initially selected for based on side and forward scatter, followed by negative selection for L/D stain against free amines in compromised membranes, CD3, and CD20, and positive selection for HLA-DR. Analysis gates were then set that differentiated monocytes into classical, intermediate, and non-classical monocytes on the basis of CD14 and CD16 expression. Monocytes were then immunophenotyped within these subpopulations for expression of MAC387, and the total monocyte subpopulation was immunophenotyped for BrdU (in relevant samples), CD163, CD169, CCR2, CX3CR1, and CCR5. See below for a representative figure of the flow gating scheme.

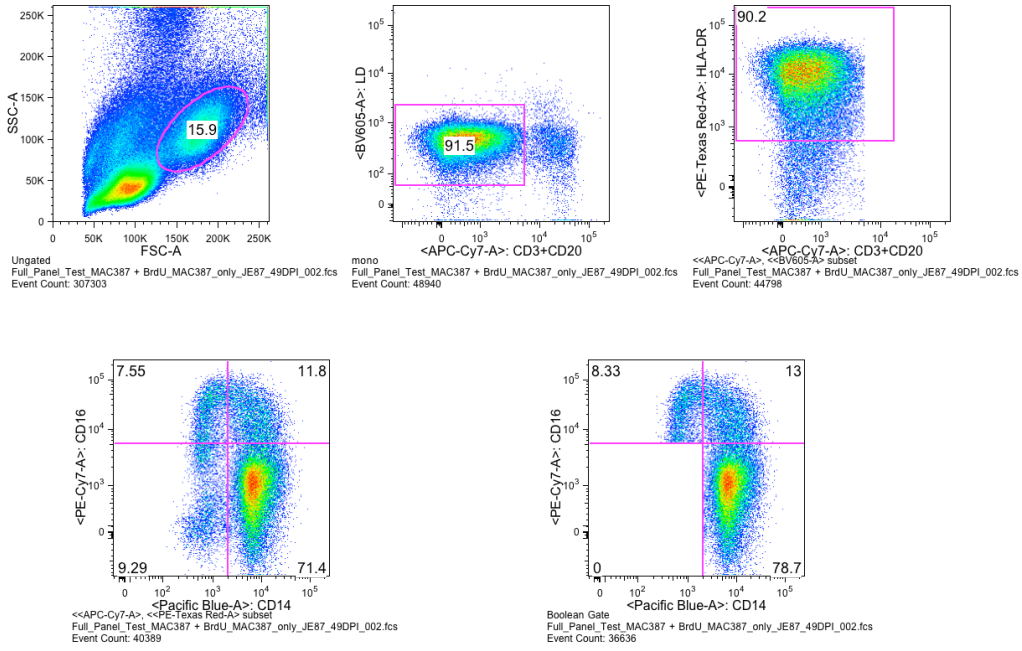

Figure 3. Monocyte Gating Scheme. *Beginning in the top-left, monocytes were selected on a forward scatter/side scatter dot plot, selected for negative expression of a L/D stain and negative for CD3 and CD20, and positively selected for the expression of HLA-DR. Lastly, monocytes were positively selected for based on CD14 and/or CD16 expression. Data on total monocytes used this Boolean gated cell population, and, for data on specific monocyte subsets – classical, intermediate, and non-classical – cell populations were taken from the respective quadrant of this dot plot.*

2.4 Gene Expression Analysis

Transcriptomic data was also utilized which was generated by others from SIV-infected rhesus macaques from 5 treatment groups: no treatment (n=2), placebo (n=2), ART only (n=6), MGBG only (n=4), and MGBG+ART (n=5). Timepoints for samples were 20 DPI, 49 DPI, and necropsy. Since blood draws on day 20 happened prior to the first treatment, these animals are considered untreated. Monocytes were isolated and RNAseq was performed on monocyte samples for each treatment/timepoint, not by single cell sequencing. Monocyte samples had ribosomal RNA depleted and mRNA libraries were prepared for sequencing on the Illumina HiSeq system. Reads were trimmed using Trimmomatic (v 0.36), underwent quality control, and were aligned to the rhesus macaque transcriptome with STAR_2.6.1a_08-27. Quantification of transcript abundance was made with RSEM (v1.2.31). Differential expression analysis between samples was performed with DESeq2 and unsupervised random forest clustering was performed with an *ad hoc* R script. Because of including samples from different studies, direct comparisons were only possible between MGBG only and Placebo; and among ART only, MGBG+ART, and Untreated groups. Differential expression analysis was made between cohorts at the same time points, and between time points for the same cohort. A false discovery rate of p= 0.05 was used. Adapter sequences were trimmed from results with Trimmomatic (v 0.36). Gene networks shown in Figures 11 and 12 were generated by gene comparisons for rhesus macaque at https://string-db.org.

CHAPTER 3: RESULTS

3.1 Plasma Calprotectin Measurement by ELISA

In order to look at the role of calprotectin secretion and blood levels of calprotectin as they associate with SIV disease progression, plasma calprotectin levels were first measured by ELISA. Figure 4A shows plasma levels of calprotectin during early infection, when levels are lower. Figure 4B shows plasma calprotectin levels later in infection, from 20 DPI through necropsy, which is associated with substantially higher levels in blood. The 0 time point here serves as a baseline for each animal, because plasma for this timepoint was taken prior to the injection of SIVmac251. Four out of five of the animals had increases in plasma calprotectin within a week of infection. For all animals, peak plasma calprotectin was either at 20 days post-infection (which is the point of peak viral load), or at necropsy. All animals also had an increase from their second to last data point to chronic late-stage disease at necropsy, most of which were euthanized with AIDS-associated disease. The difference in scale between Figures 4A and 4B here show the higher calprotectin levels that are present from peak infection (around 3 weeks post-infection) until necropsy, compared to early time points.

Rhesus macaques progressing to AIDS (n=4) had an average of an 12.0-fold increase from 0 DPI to 20 DPI, with fold-increase ranging from 3.08-fold to 59.7-fold. Those macaques progressing to AIDS (n=4) had an average of an 18.0-fold increase from 0 DPI to necropsy, with fold-increase ranging from 2.58-fold to 44.9-fold. Standard deviations for plasma calprotectin levels for macaques with AIDS (n=4) was 209.8, 4769.4, and 4630.9 for the respective time points of 0 DPI, 20 DPI, and necropsy. Animals with SIVE (n=3) had an average of a 17.6-fold increase from 0 DPI to 20 DPI, with fold-increase ranging from 5.10-fold to 59.7-fold. Animals with SIVE (n=3) had an average of a 19.0-fold increase from 0 DPI to necropsy, with fold-

increase ranging from 2.58-fold to 44.9-fold. Standard deviations for plasma calprotectin levels for macaques with SIVE (n=3) was 155.7, 5204, and 4924 for the respective time points of 0 DPI, 20 DPI, and necropsy. In contrast, these same time points for macaques without SIVE (n=2), had a fold-increase between 0 DPI and 20 DPI of1.03-fold to 3.08-fold. The range for fold-increase for macaques without SIVE (n=2) between 0 DPI and necropsy was 1.79-fold to 16.5-fold. The standard deviation for plasma calprotectin levels for macaques without SIVE (n=2) was 2441, 1491, and 2940 for the respective time points of 0 DPI, 20 DPI, and necropsy. Statistics are shown in Table 3.

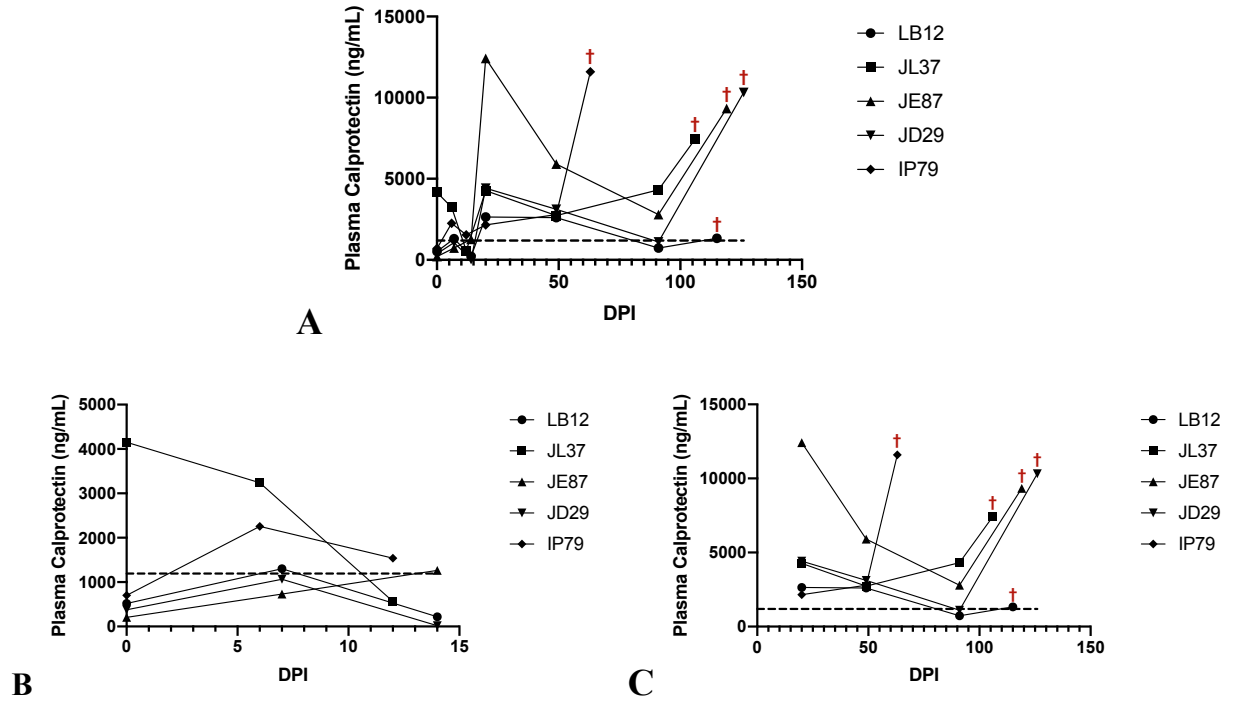

Figure 4. Plasma calprotectin. *Plasma calprotectin levels over the course of SIV infection, broken down by animal. Panel A shows plasma calprotectin for all data points for all animals, from 0 DPI to necropsy. Panels B and C together contain all data present in panel A, but separated for clearer illustration of the early data points within the first 15 days of SIV infection, and therefore with different maximum values on the x-axis. Panel B shows data values from timepoints 0-14 DPI, and panel C shows data values for all later timepoints, beginning at 20 DPI. Red crosses above each animal in panels A and C indicate necropsy timepoint. The dashed horizontal line in all panels represents the average baseline (pre-infection) plasma calprotectin of all five animals of 1192.2 ng/mL.*

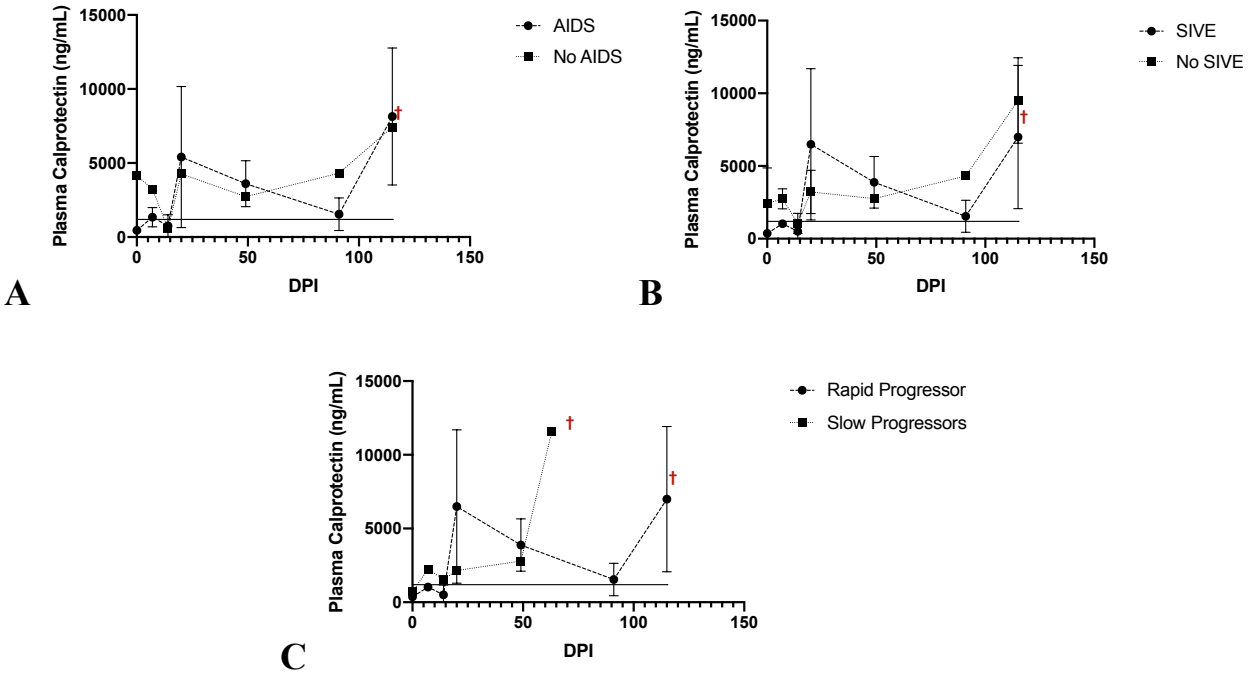

Figure 5. Plasma Calprotectin Comparisons Between Pathologies. *Using the same data as presented in Figure 4, panel (A) compares the average of all animals progressing to AIDS (n=4) to the animal without AIDS (n=1). Panel (B) compares the average of all animals with SIVE (n=3) to the averages of animals without SIVE (n=2). Panel (C) further breaks down those animals with AIDS into IP79, which progressed to AIDS within 63 days, to all other AIDS animals (n=3), which were necropsied with AIDS between 106-126 DPI. For each timepoint, the mean of animals for a particular disease state (i.e. AIDS) is shown, with bars indicating the standard error of the mean. The solid horizontal line in all panels represents the average baseline (pre-infection) plasma calprotectin of all five animals of 1192.2 ng/mL.*

	AIDS (n=4)		No AIDS (n=1)	
Timepoint	**Mean**	**SD**	**Mean**	**SD**
0 DPI	452	210	4154	0
6/7 DPI	1339	655	3241	0
12/14 DPI	761	754	565	0
20 DPI	5410	4769	4267	0
49 DPI	3608	1548	2730	0
91 DPI	1545	1096	4317	0
Necropsy	8143	4631	7432	0

	SIVE (n=3)		No SIVE (n=2)	
Timepoint	**Mean**	**SD**	**Mean**	**SD**
0 DPI	368	156	2428	2441
6/7 DPI	1033	288	2749	697
12/14 DPI	500	668	1053	690
20 DPI	6493	5204	3213	1491
49 DPI	3879	1776	2762	46
91 DPI	1545	1096	4317	0
Necropsy	6994	4924	9511	2940

	Rapid Progressor (n=1)		Slow Progressors (n=3)	
Timepoint	**Mean**	**SD**	**Mean**	**SD**
0 DPI	702	0	368	156
6/7 DPI	2256	0	1033	288
12/14 DPI	1541	0	500	668
20 DPI	2159	0	6493	5204
49 DPI	2795	0	3879	1776
91 DPI			1545	1096
Necropsy	11590	0	6994	4924

Table 3. Mean and standard deviation for each pathology subset of data presented in Figure 5. Data points at one week (either 6 or 7 DPI) were aggregated for comparison. Data at two weeks (either 12 or 14 DPI) were also aggregated for comparison.

DPI	Calprotectin (ng/mL)					
	AIDS	no AIDS	SIVE	no SIVE	Rapid Progressor	Slow Progressors
	n=4	n=1	n=3	n=2	n=1	n=3
0						
Mean	452	4154	368	2428	702	368
25-75 percentile	210	N/A	156	2441	N/A	156
6/7						
Mean	1339	3241	1033	2749	2256	1033
25-75 percentile	655	N/A	288	697	N/A	288
12/14						
Mean	761	565	500	1053	1541	500
25-75 percentile	754	N/A	668	690	N/A	668
20						
Mean	5410	4267	6493	3213	2159	6493
25-75 percentile	4769	N/A	5204	1491	N/A	5203
49						
Mean	3607	2730	3879	2762	2795	3879
25-75 percentile	1548	N/A	1776	46	N/A	1776
91						
Mean	1545	4317	1545	4317	-	1545
25-75 percentile	1096	N/A	1096	N/A	-	1096
Necropsy						
Mean	8143	7432	6994	9511	11590	6994
25-75 percentile	4631	N/A	4924	2940	N/A	4924

Table 4. *Plasma calprotectin levels throughout SIV infection, broken down by pathology state as shown in Figure 5 to show the 25th/75th percentiles of data. The SIVE group is a subset of the AIDS group, since SIVE is an AIDS-defining characteristic, and thus progression of an animal to SIVE inherently indicates a progression to AIDS as well; three of four animals progressing to AIDS in this study also had SIVE.*

In order to better understand plasma calprotectin levels in the context of SIV disease progression, plasma calprotectin levels were grouped by progression to SIVE, AIDS, or, for animals with AIDS, their rapidity of progression. For the comparison of animals based on AIDS progression, macaques with AIDS (n=4) were compared to the macaque that did not progress to AIDS (only animal JL37). Here, there is not an appreciable difference between plasma calprotectin levels or in trends of increasing or decreasing from two weeks after infection until necropsy. However, as shown in Figure 5A, the initial level of plasma calprotectin is more than four-fold higher for the sole animal which did not progress to AIDS (JL37), relative to the

average of the other four animals progressing to AIDS (JD29, LB12, JE87, and IP79). To look at disease progression another way, comparisons were made between animals progressing to SIVE (n=3) and those without SIVE (n=2); animal IP79 progressed to AIDS, but did not have SIVE. Here, again, there is little difference between the two animal groups with regards to whether or not they developed SIVE, with an initial increase in plasma calprotectin, followed by a more drastic increase by 20 days post-infection, levels decreasing slightly, and increasing again by necropsy. However, by comparing IP79, which progressed to AIDS nearly twice as fast as all other animals with AIDS, to those taking over 100 days after infection to reach AIDS, there is a much earlier drastic increase in plasma calprotectin, as shown in Figure 5C.

In addition to looking at the changes in plasma calprotectin level over the course of SIV infection, calprotectin levels in plasma were also compared to other metrics known to be associated with disease progresson in HIV or SIV infection. As demonstrated by Burdo, *et al.*, (2010), following BrdU injection in SIV-infected macaques, early monocyte expression of BrdU differentiates between slow and rapid AIDS progressors and the percentage of monocytes at necropsy that express BrdU correlates with the severity of SIVE (83). Here, a comparison of the time points for which BrdU staining was available in PBMCs showed a modest positive trend between plasma calprotectin level and the number of BrdU+ monocytes (Figure 6A) ($p=0.2862$). A modest positive trend was also present between the percentage of monocytes that are BrdU+ and plasma calprotectin level (Figure 6B) ($p=0.1057$).

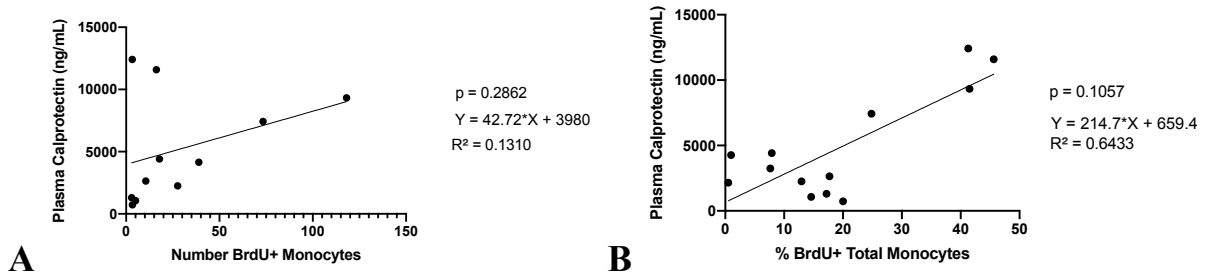

Figure 6. Plasma Calprotectin & Monocyte Turnover. *Data points in panel (A) are corresponding time points for the total number (raw value) of monocytes expressing the thymidine analog BrdU, following a BrdU injection 24 hours to sample collection with the level of plasma calprotectin, as measured by ELISA. Data points in panel (B) are corresponding time points for the percent of total monocytes expressing BrdU with the level of plasma calprotectin. For both panels, p-value and correlation coefficient, r, are given. Neither trend reaches significance at the p=0.05 threshold.*

A comparison of the plasma viral load and plasma calprotectin levels in SIV-infected animals, however, does show a significant positive correlation (p=0.0175) between these two parameters, and higher viral load is positively correlated with higher levels of plasma calprotectin, as shown in Figure 7.

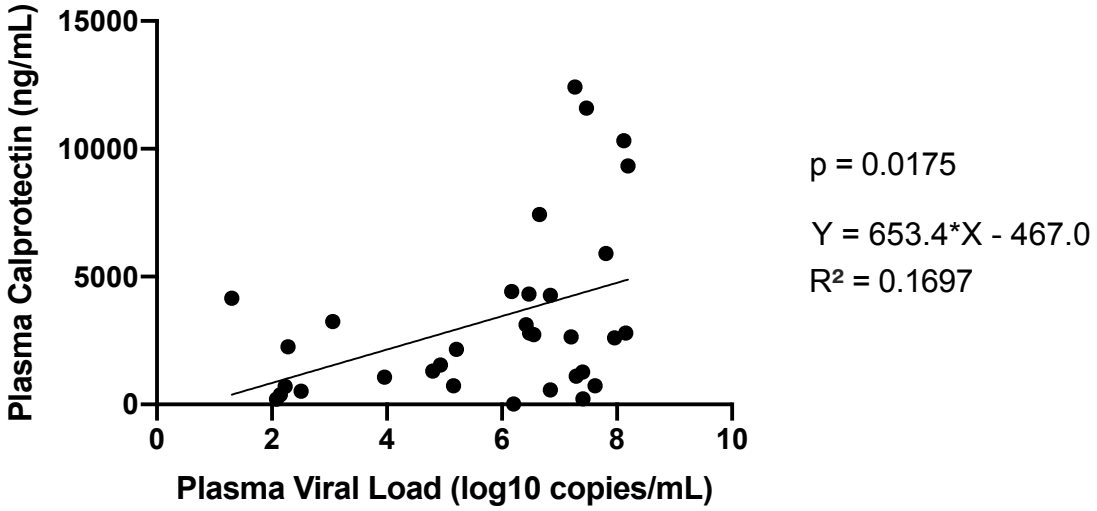

Figure 7. Plasma Calprotectin & Viral Load. *A comparison of plasma viral load values with plasma calprotectin, with each data point representing the same animal and timepoint during infection. There is a signficant (p=0.0175) positive correlation between the viral load and calprotectin in plasma.*

3.2 MAC387 Expression and Immunophenotyping by Flow Cytometric Analysis

In order to address defining MAC387 monocytes and changes in MAC387+ monocytes during the course of infection, PBMCs were isolated from rhesus macaque blood samples,

stained with a panel of fluorescent antibodies for use in flow cytometry to gate for the monocyte population of interest, and immunophenotyped. By looking over the timecourse of infection at the expression of MAC387 on total monocytes, as measured by mean fluorescence intensity (MFI) by flow cytometry, no consistent trends were found that fit the entire cohort (Figure 8). However, the sole animal that did not progress to AIDS (JL37) had a unique several-fold increase in MAC387 protein expression at 20 days post-infection.

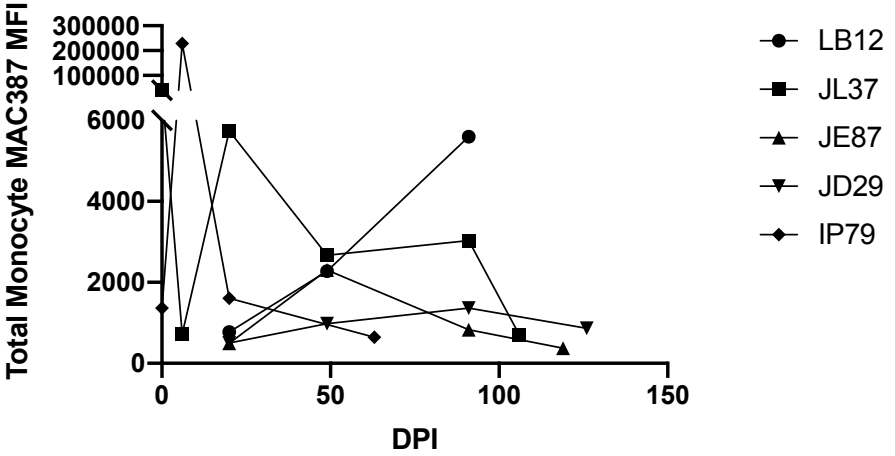

Figure 8. MAC387 Expression in Peripheral Blood Monocytes. *Total monocyte expression of MAC387 over the course of infection is shown, separated by animal. Two massive outliers are included: a CD14+CD16- MFI value of 43166 for animal JL37 and a CD14+CD16- MFI value of 228500 for animal IP79 at 6 DPI. These massive values are the reason for the broken y-axis.*

However, by taking an average of all animals and separating MAC387 MFI data for classical, intermediate, and non-classical monocytes, there is relatively stable expression of MAC387 throughout infection until at least day 91, and then a consistent drop for all animals at necropsy (Figure 9). There is modest change in MAC387 MFI among timepoints 20 DPI, 49 DPI, and 91 DPI. However, the 91 DPI MAC387 MFI values are several-fold greater than their corresponding necropsy values. The 91 DPI MAC387 MFI values are 4.57-, 3.54-, and 3.30-fold greater than necropsy values for classical, intermediate, and non-classical monocytes, respectively.

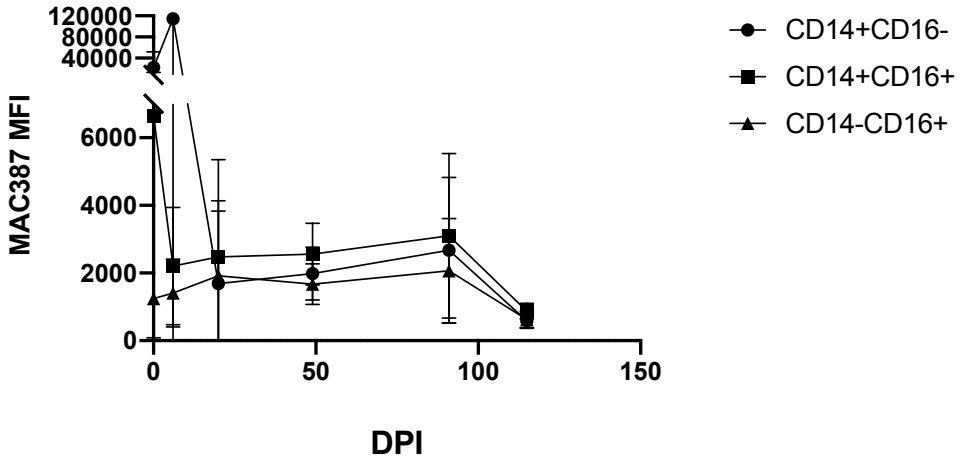

Figure 9. MAC387 Expression in Monocyte Subpopulations. *The CD14+CD16- monocyte is the classical monocyte, the CD14+CD16+ monocyte is the intermediate monocyte, and the CD14-CD16+ monocyte is the non-classical monocyte. For each timepoint, data were averaged for all animals. Two massive outliers are included: a CD14+CD16- MFI value of 43166 for animal JL37 and a CD14+CD16- MFI value of 228500 for animal IP79 at 6 DPI. These massive values are the reason for the broken y-axis. Exclusion of these data points would result in intermediate monocytes having the highest MAC387 MFI value for all timepoints. Inclusion of these two data points results in classical monocytes having the highest MAC387 MFI at 0 and 6 DPI, and the highest MAC387 MFI by intermediate monocytes for all other data points.*

	CD14+CD16-	
Timepoint	**Mean MFI**	**SD**
0 DPI	22271	29550
6/7 DPI	114307	160786
20 DPI	1692	2137
49 DPI	1983	779
91 DPI	2673	2153
Necropsy	585	227

	CD14+CD16+	
Timepoint	**Mean MFI**	**SD**
0 DPI	6648	6565
6/7 DPI	2208	1736
20 DPI	2477	2879
49 DPI	2558	910
91 DPI	3010	2431
Necropsy	876	228

	CD14-CD16+	
Timepoint	**Mean MFI**	**SD**
0 DPI	1247	166
6/7 DPI	1407	1001
20 DPI	1918	2217
49 DPI	1672	599
91 DPI	2069	1547
Necropsy	627	242

Table 5. Mean and standard deviation values for MAC387 MFI for each time point, separated for monocyte subpopulation, as shown in Figure 9. In the absence of the outliers mentioned in the figure legend of Figure 9, the mean for CD14+CD16- 0 DPI would be 1376 and for CD14+CD16- 6/7 DPI would be 614.

BrdU-labeling and expression of the CD163, CD169, CCR2, CX3CR1, and CCR5 immunomarkers were then analyzed over the course of infection. In order to determine the phenotype of the MAC387+ monocytes, and how they change over the course of SIV infection, the total monocyte population (classical, intermediate, and non-classical) were divided based on positive expression of MAC387, or lack thereof. Within the monocyte population staining positive for MAC387, the relative MFI for BrdU and each of the of the immunomarkers

measured was graphed against respective timepoint, as shown in Figure 10. The MAC387+ population consistently included about 70-90% of monocytes, indicating that this is the percentage of monocytes that are MAC387+ and express calprotectin. Because of the limited availability of samples with BrdU injection prior to sample acquisition, BrdU staining was only available at 20 days post-infection and at necropsy. Between these time points, there was a modest increase in BrdU expression by MAC387+ monocytes (Figure 10A). MAC387+ monocyte expression of CD163, CD169, and CCR2 followed similar patterns of expression with an initial increase in expression, followed by a decrease at 91 days post-infection, and increasing to the highest level at necropsy timepoint.

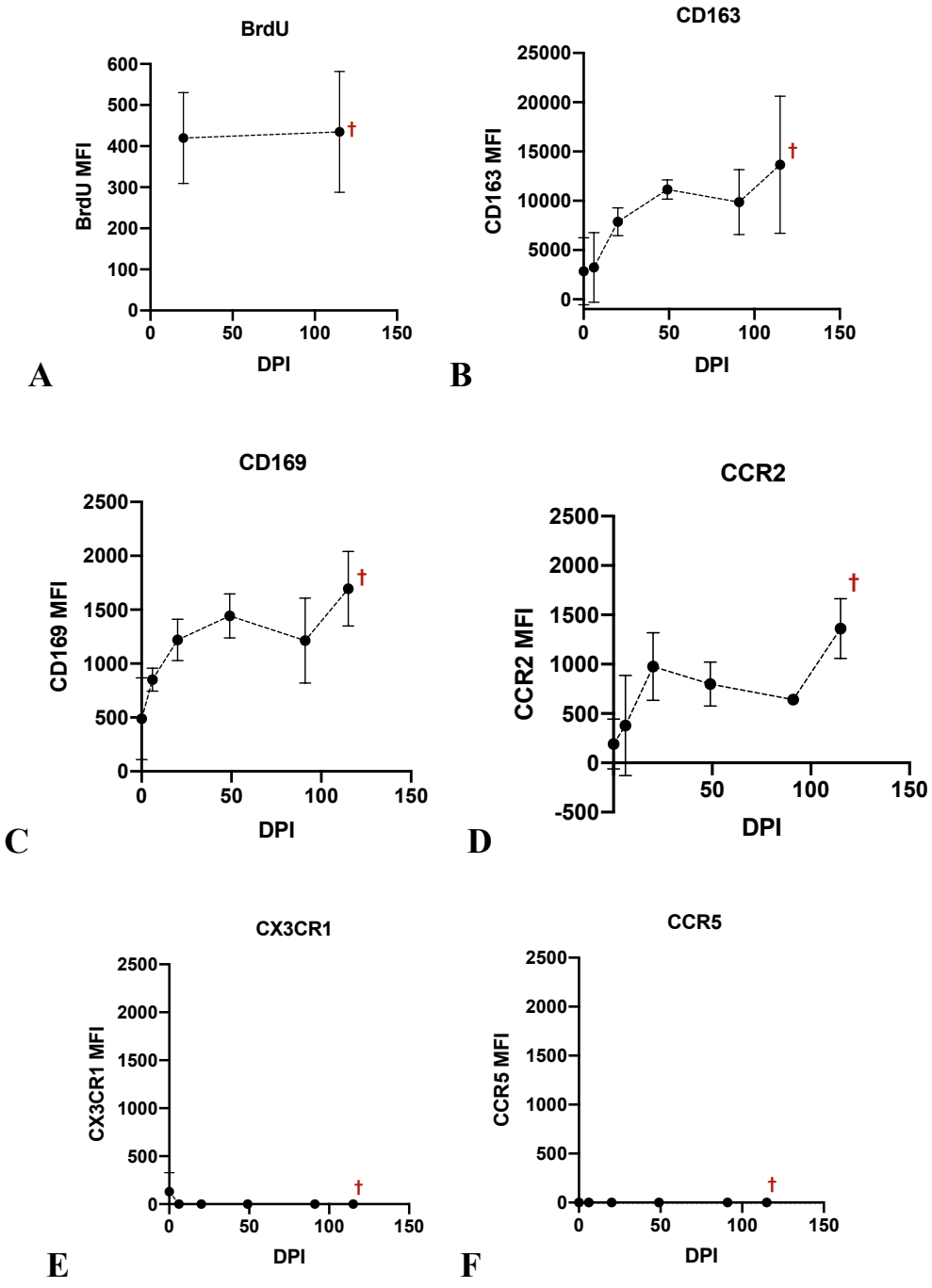

Figure 10. Monocyte Immunophenotyping. *Following the gating selection for total monocytes, this population was further divided into MAC387+ and MAC387- monocyte populations. Using the MAC387+ monocyte subpopulation, immunophenotype marker MFI was looked at longitudinally. For each immunomarker, data for all animals was averaged for each timepoint and standard error bars are shown. Figures are from n=5 animals, but not every timepoint has all five samples available. Following compensation for markers CX3CR1 and CCR5 (Figures 10E and 10F), numerous data points generated a negative MFI value. Such negative values are a physical impossibility, and so are treated as a '0' MFI for this arbitrary unit and negative expression for the monocytes of respective animals at the respective timepoint.*

DPI	Immunophenotype Marker MFI					
	BrdU	CD163	CD169	CCR2	CX3CR1	CCR5
0						
Standard Deviation		3405	380	254	198	1220
Range		4815	538	359	280	1726
Mean		2851	488	192	130	0
6/7						
Standard Deviation		3546	107	510	63.6	812
Range		5015	152	721	56.7	1148
Mean		3231	852	380	0	0
20						
Standard Deviation	110	1406	193	343	78.2	90.3
Range	252	3478	430	803	110	249
Mean	420	7864	1219	975	0	0
49						
Standard Deviation		980	205	223	309	176
Range		2283	489	524	97	420
Mean		11148	1443	799	0	0
91						
Standard Deviation		3309	394	57.3	292	305
Range		6561	951	133	186	725
Mean		9872	1214	642	0	0
Necropsy						
Standard Deviation	147	6957	346	304	249	321
Range	283	15627	361	683	325	701
Mean	435	13652	1695	1361	0	0

Table 6. *Values for standard deviation and range for respective immunophenotype markers for respective timepoints. Based on available samples and BrdU injection schedule, BrdU standard deviation, range, and as illustrated in Figure 10 use n=5 at 20 DPI and n=3 at Necropsy. For all other markers, available data used is from n=2 at 0 DPI; n=2 at 6 DPI; n=5 at 20 DPI; n=3 at 49 DPI; n=3 at 91 DPI; and n=3 at necropsy. For data points that had a 'negative' mean MFI as shown in Figure 10 and were treated as 0 MFI, the range and standard deviation shown in this table are for the values prior to standardization as a value of 0.*

3.3 Gene Expression Analysis Among SIV Infection Treatments and Timepoints

With the aim of determining genes and functions associated with elevated levels of calprotectin gene expression, RNAseq data were analyzed for flow-sorted monocytes from five

treatment groups: MGBG only (n=4), Placebo (n=2), ART only (n=6), MGBG+ART (n=5), and Untreated (n=2). Data were acquired from three infection timepoints for analysis: 20 DPI, 49 DPI, and necropsy. Gene expression was compared among time points within the same treatment group and among treatment groups at the same timepoint. RNAseq comparisons ART-20-49; ART-UNTREATED-D49; ART-UNTREATED-Nec; MGBGART-20-49; and MGBGART-20-Nec show significant differential expression of both S100A8 and S100A9 (the two subunits of calprotectin).

These five gene expression comparisons were analyzed to look at the genes, gene networks, and functions that increased in expression in tandem with gene expression increases of S100A8 and S100A9. Of the identified gene ontology biological processes associated with genes present here, APOBEC3A is highly expressed by monocytes and macrophages with interferon stimulation and IL1B is produced by activated macrophages. APOBEC3A protein product has restriction activity against viruses and foreign DNA and IL1B is a pro-inflammatory cytokine important in neutrophil influx and activation. Figure 11, below, shows the gene networks generated by https://string-db.org. From a random assortment of genes of this size, there is an expectation of three connections. The 47 observed connections shown below more than meet the threshold for significance. The protein-protein interaction (PPI) enrichment p-value of <1.0e-16 measures the significance of biological interaction of protein products for the respective genes, and, in Figures 11 and 12, shows the high functional relatedness of identified genes. This parameter looks at the significance of related function among genes of interest. In the five abovementioned conditions/timepoints where S100A8 and S100A9 increase expression, there is a relatedness among a large number of those genes also increasing in expression. However, the functional related network does not have any established connections to S100A8 or

S100A9. Such upregulated genes and their functions may be important in the MAC387+ monocyte or the monocyte population at large with acute infection. See Table 7 for a more complete list of the gene functions of genes with increased expression with increased calprotectin gene expression. These represent the genes most in direct correlation to S100A8 and S100A9.

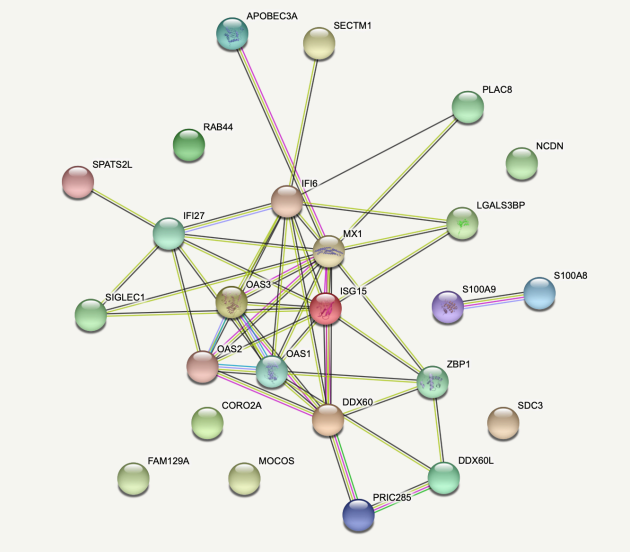

Figure 11. Stringent Network of Genes Upregulated with Calprotectin Upregulation. *Gene networks for genes differentially expressed in* ***all 5 condition*** *comparisons for which both S100A8 and S100A9 were differentially expressed. Among these genes, by random chance, there is an expectation of only 3 connections, but the 47 connections identified represents a PPI enrichment p-value of <1.0e-16. This means that the gene list pulled out is not random. Significance threshold is genes with an absolute fold change of greater than or equal to 1.3 and a permutation FDR of less than or equal to 0.05. Fold-change thresholds are arbitrary, but log2(fold change) values <-1.3 or >1.3 are usually reported in papers as significant (summary of written statement by collaborator Dr. Marco Salemi). Connecting lines indicate the two genes have known interactions from curated databases (teal) or experimental determination (purple); predicted interactions by gene neighborhood (green), by gene fusions (red), or gene co-occurrence (blue); or by textmining (light green), co-expression (black), or protein homology (light blue).*

However, by considering a lower threshold, any genes with increased expression in a minimum of four of the five conditions under which S100A8 and S100A9 had increased expression, Figure 12 was generated. Here, the genes under consideration increased from 30 genes, in Figure 11, to 75 genes, shown below in Figure 12. The expected number of connections here based on a random assortment of genes is 14, but the 162 observed gene

connections illustrates the connected and functionally associated nature of the genes more highly expressed with high calprotectin gene expression. Figure 12 illustrates several things. First, the strong interconnectivity in the gene network in Figure 12 (well above a threshold for significance of interrelated genes) illustrates that monocytes with elevated calprotectin expression also have increases in the expression of other genes, which are functionally related that makes the calprotectin-expressing monocyte a unique and inflammatory cell type with a consistent functional role. Secondly, in Figure 12, CD163 – a strong indicator of cell activation – is the functional link between the calprotectin gene subunits S100A8 and S100A9 and the central hub of genes with elevated expression, indicating the role of CD163 and cell activation in the inflammatory calprotectin-expressing monocyte subpopulation. Thirdly, the gene network in Figure 12 reveals which genes and which functional networks are involved in monocytes with elevated calprotectin expression. Details on these functions are explored in depth in the discussion, with functions and expression of individual genes of interest shown in Table 7.

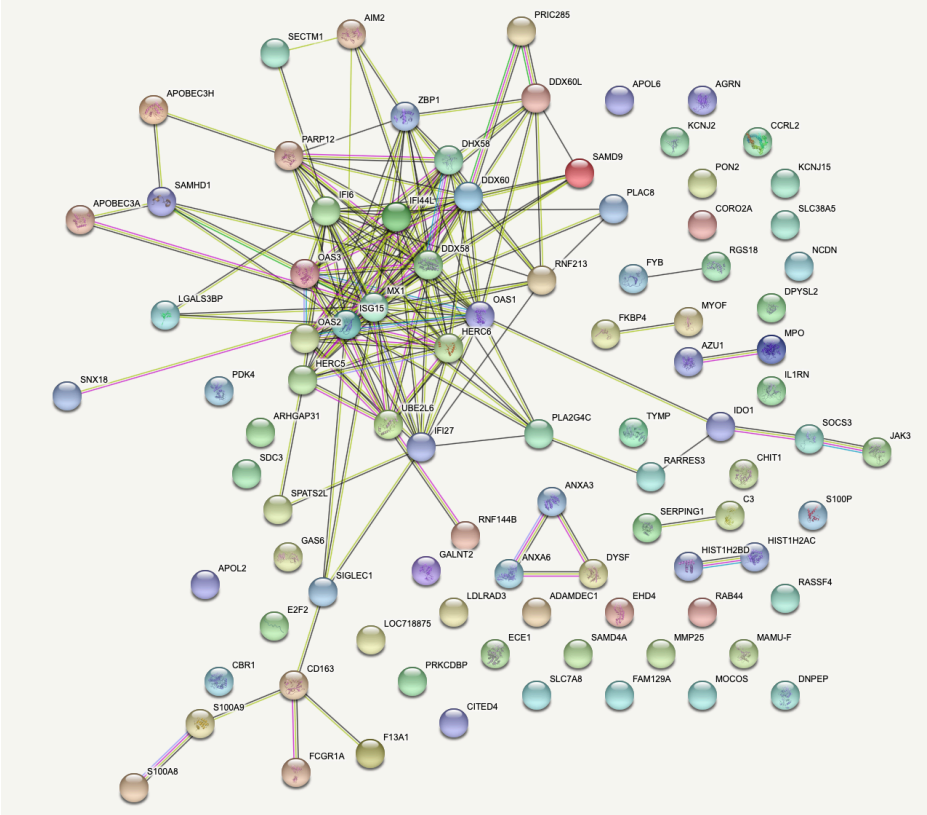

Figure 12. Broad Network of Genes Upregulated with Calprotectin Upregulation. *Gene networks for genes differentially expressed in at least **3** of the 5 condition comparisons for which both S100A8 and S100A9 were differentially expressed. Among these genes, by random chance, one would expect only 14 connections, but the 162 connections identified represents a PPI enrichment p-value of <1.0e-16. Significance threshold is genes with an absolute fold change of greater than or equal to 1.3 and a permutation FDR of less than or equal to 0.05. Fold-change thresholds are arbitrary, but log2(fold change) values <-1.3 or >1.3 are usually reported in papers as significant (summary of written statement by collaborator Dr. Marco Salemi). Connecting lines indicate the two genes have known interactions from curated databases (teal) or experimental determination (purple); predicted interactions by gene neighborhood (green), by gene fusions (red), or gene co-occurrence (blue); or by textmining (light green), co-expression (black), or protein homology (light blue).*

Using the expanded gene list presented in Figure 12, I further examined those genes that are expressed by, or play a role in, monocytes and monocyte-derived macrophages. Below, Table 7 lists these genes, their involvement in monocytes & macrophages in terms of expression, and their functions.

Gene	Myeloid Expression	Function
	Differentially Expressed Genes in 5 Conditions (Figure 11)	
APOBEC3A	highly expressed in monocytes & macrophages upon interferon stimulation	restriction activity against viruses & foreign DNA
CHIT1	occurs only at a late stage of differentiation of monocytes and activated macrophages; secreted by activated macrophages	degrades chitin of microorganisms
IFI27	upregulated in monocyte-derived macrophages by HIV-1 Tat & Vpr proteins	involved in signaling pathway leading to apoptosis; antiviral activity in innate immunity
IL1B	produced by activated macrophages	proinflammatory cytokine; induces neutrophil influx & activation, T-cell activation & cytokine production, and B-cell activation and antibody production
ISG15	expressed by monocytes	chemotactic activity towards neutrophils, antiviral activity
SIGLEC1	macrophage-restricted adhesion molecule	receptor for clathrin-dependent endocytosis; adhesion molecule on macrophages which mediates sialic-acid dependent cell binding
	Differentially Expressed Genes in 4 Conditions (Figure 12)	
ADAMDEC1	upregulated during differentiation of monocytes into macrophages; highly expressed in macrophages	induced by LPS; modulates macrophage polarization
AZU1	Monocyte chemotactic activity	Encodes a preprotein with antibacterial/cytotoxic activity against gram-negative bacteria; may have role in monocyte recruitment
CD163	marker of monocyte/macrophage lineage; exclusive to this lineage; soluble form is a marker of disease	receptor involved in clearance of hemoglobin/haptoglobin complexes; shed with LPS exposure
CPM	expression associated with monocyte to macrophage differentiation	control of hormone & growth factor activity; involved in degradation process of extracellular proteins
FCGR1A	expressed by macrophages & monocytes; downregulated on these cells by HIV-1 Nef protein	Fc Gamma receptor involved in both innate and adaptive immunity
IDO1	expressed by monocytes & macrophages	involved in amino acid catabolism; modulates T cell behavior and is involved in self-tolerance in immunity
MPO	expressed in monocyte liposomes	antimicrobial activity through production of hypohalous acids; involved in phagocytosis
OSM	synthesized by stimulated monocytes	inhibits proliferation, and regulates cytokine production
SERPINB2	present in monocytes/macrophages	metabolism regulation & responds to increases in cytosolic calcium *note that S100A8 & S100A9 are calcium-binding, and their dimerization to form calprotectin is calcium-dependent
TLR8	expressed and undergoes proteolytic processing in both monocytes and monocyte-derived macrophages	role in pathogen recognition (through PAMPs) and activating innate immunity

***Table** 7. Gene name, role with monocytes/macrophages, and function for genes with differential expression between conditions for which both subunits of calprotectin also exhibit differential expression.*

CHAPTER 4: DISCUSSION

Blood calprotectin levels have been extensively studied across a variety of diseases, infections, and disease severities, and calprotectin levels in blood are consistently associated with disease and inflammation. However, monocyte expression and secretion of calprotectin is understudied. Using rhesus macaques infected with SIV, I analyzed longitudinal plasma calprotectin. A previous study in HIV blood samples had shown some of the blood calprotectin level trends, but only had limited timepoints (75).

The work in this thesis presents blood calprotectin levels from 0 DPI until necropsy at late-stage chronic disease in SIV-infected rhesus macaques and includes breakdowns for different disease comparisons. Figure 4 illustrates the large increase in calprotectin levels from pre-infection to necropsy, which, in some cases, increase by greater than ten-fold, suggesting an important role of the calprotectin protein in SIV infection and disease progression. Calprotectin secretion by both neutrophils and monocytes contributes to the level of extracellular calprotectin in the blood, and neutrophils are responsible for the majority of such calprotectin (82). Elucidating the difference in contribution of secreted calprotectin from monocytes versus that from neutrophils would be a difficult endeavor, but here is best addressed by a comparison of secreted levels to calprotectin protection expression observed on monocytes. Following the exclusion of dead cells, CD3+, and CD20+ cells by flow gating, neutrophils can be selected for by CD66b expression (84), and cytometry for a measure of cell numbers. Further work should look into the changes in calprotectin expression on neutrophils and the number of neutrophils expressing calprotectin throughout SIV infection. Monocyte expression of calprotectin would logically decrease with calprotectin secretion, which is a process that occurs during the inflammatory processes associated with SIV infection, and thus is expected to have an inverse

relationship over the course of infection. In fact, the two biggest trends of calprotectin expression in monocytes are a decrease in expression from 0 DPI to 6 DPI, and a decrease in expression in samples immediately prior to necropsy (Figure 9). Figure 4 illustrates the increasing secreted calprotectin levels in blood from 0 DPI to 6 DPI, and the sharp increases in all animals with the necropsy time point, suggesting important monocyte contribution to the calprotectin levels observed by ELISA. Peak viremia occurs around 21 DPI, and the large increases in plasma calprotectin for all animals coinciding with this time point suggests an activation of the MAC387+ monocyte and secretion of calprotectin at this timepoint. For all animals, the maximum calprotectin level in blood was either at 20 DPI or at necropsy, which indicates that animals may take one of two general paths with respect to monocyte secretion of calprotectin: a rapid response to virus, or a persistent secretion that accumulates, and accumulates very rapidly in chronic disease progression and the development of AIDS.

Further analysis of plasma calprotectin levels show that, while disease progression to AIDS (Figure 5A) does not have much difference in calprotectin levels over the course of disease, calprotectin levels could play a significant diagnostic role in predicting the rapidity of AIDS progression. A greater than four-fold increase in plasma calprotectin occurred in animal IP79 (Figures 4B and 5C), which was found dead in cage at 63 DPI with AIDS-defining characteristics and had a higher necropsy calprotectin value than the necropsy calprotectin level in any other animal shows the importance of the level of calprotectin secretion. The high calprotectin levels in Figure 5C, which compares plasma calprotectin in the rapid AIDS progressor IP79 to those animals taking over 100 days to progress to AIDS, suggests that the shedding of calprotectin and its accumulation in blood may be a driver or effect of progression towards AIDS and indicate an earlier progression to this disease state. The level of calprotectin

in blood prior to infection may have an important effect on disease progression and opportunistic infections. It is counterintuitive that the single animal which did not progress to AIDS – JL37 – would be the only animal to have a substantial decrease in plasma calprotectin within the first week following infection, while all other animals had increases in plasma calprotectin from their pre-infection baseline. A high plasma calprotectin level in blood during health (pre-infection) may be an indicator of a robust immune system and high expression and secretion of calprotectin by MAC387+ monocytes. Animal JL37 has a baseline blood calprotectin level of *over four times* that of any of the other animals (Figure 4A), all of which have tightly clustered calprotectin baselines. In the plasma calprotectin ELISA data, the necropsy level of calprotectin is *always elevated* relative to the immediately preceding data timepoint. The progression to AIDS, which in this case corresponds to necropsy timepoints, seems to be indicated by a spike in calprotectin secretion that remains elevated with late-stage disease.

The association established between monocyte turnover and the severity of SIVE highlights the importance of monocyte turnover and immigration into tissues during SIV disease progression (83). Here, moderate trends between plasma calprotectin and both the number of BrdU+ monocytes, and the percent of all monocytes expressing BrdU, suggest that the secretion of calprotectin during immune defense is involved with monocyte activation that leads to monocyte/macrophage infiltration into tissues by a calprotectin-expressing monocyte (Figure 6). Better powered studies will be needed to determine if this trend is meaningful, and whether the secretion of calprotectin is involved in the immune defense response associated with monocyte activation. However, the positive and significant correlation between plasma viral load and plasma calprotectin level could indicate that calprotectin is released, at least in part, in response to presence of virus or high levels of infected cells (Figure 7). This may further indicate that in

the case of SIV infection, calprotectin molecules play an antiviral role, which would be supported by their established antimicrobial role (65).

This study is the first that looks at longitudinal expression of calprotectin protein on monocytes, immunophenotypes these cells to define them and their changes over time, and is able to relate this data to plasma levels of calprotectin for corresponding animals and timepoints. The several-fold increase in MAC387 expression by animal JL37 at 20 days post-infection, shown in Figure 8, suggests that an initially strong response in calprotectin expression in monocytes may either be a driver or an indicator of resilience to disease progression. As shown in Figure 9, there is a clear drop in mean expression of MAC387 on all three monocyte subpopulations that distinctly occurs at necropsy. This suggests that something is occurring as an animal is approaching AIDS disease in monocytes where expression stops, or calprotectin is secreted that represents an extreme loss of immune control. The monocyte immunostaining work presented here at the protein level parallels the gene expression work of Nowlin *et al.*, (2018) that shows the highest calprotectin expression in classical monocytes, which is also observed in Figure 9.

Despite the highest gene expression of calprotectin by classical monocytes, this work suggests that at the protein level, intermediate monocytes may be playing an especially significant role with regards to calprotectin secretion. Results shown in Figure 9 illustrate how high the initial calprotectin expression by intermediate monocytes is, which then substantially decreases within a short period following SIV infection. A possible explanation here is that the intermediate monocyte subpopulation is secreting calprotectin. This monocyte subpopulation then continues to have the highest expression of calprotectin with chronic disease (samples taken immediately prior to necropsy), at which point there is a notable decrease in calprotectin

expression by all monocytes. The final decrease in calprotectin expression by monocytes, coupled with the high plasma levels observed, suggests that monocytes may reach an "exhaustion" of calprotectin secretion with the extreme immune disfunction associated with progression to AIDS. Already high blood calprotectin levels and the monocyte's inability to secrete more calprotectin may further exacerbate the immune disfunction of AIDS.

Decreases in CD163, CD169, and CCR2 around 91 DPI – shortly before reaching chronic AIDS disease – supports the MAC387+ monocyte/macrophage as an important, activated, and inflammatory cell type in acute infection, which decreases with late-stage chronic disease. The susceptibility of cells to HIV or SIV infection through the coreceptor CCR5, and the low expression of CCR5 on classical and intermediate monocytes, and the data in Figure 10F showing no CCR5 expression by MAC387+ monocytes supports the notion of the MAC387+ monocyte as the precursor to the MAC387+ macrophage, which does not sustain viral infection. The decrease of CD163, CD169, and CCR2 around 91 days post-infection, prior to rising to the highest expression of any of these markers at necropsy, suggests that MAC387+ monocytes begin to decrease their expression of these markers prior to immune disfunction that results in AIDS (Figure 10). The increases in CD169 expression – an adhesion molecule – and CCR2 – involved in monocyte chemotaxis – by MAC387+ monocytes over the course of SIV infection also suggests a changing role of this cell type: one of adhesion and infiltration into tissue with the inflammatory response. This, coupled with increases in expression of activation marker CD163, suggests this monocyte is likely to enter tissue with inflammation, and is a likely candidate for the MAC387+ tissue macrophage observed with infection and inflammation. This MAC387+ monocyte is also observed to have high turnover both at peak viremia (20 DPI) and at necropsy, suggesting a persistent inflammatory response both in early and late disease.

In addition to immunophenotyping these MAC387+ monocytes with a handful of useful immune-markers, changes in gene expression were examined by RNAseq of bulk populations. Given the caveat that single-cell sequencing was not performed, some potentially interesting connections were observed. Looking across timepoints within a particular treatment, and across different treatments at a particular timepoint, revealed several conditions for which gene expression of S100A8 and S100A9 – which encode the two calprotectin subunits – increases. Of course, these genes were not alone in increased expression in these conditions, and an analysis of which other genes had upregulated expression in tandem added to potentially define the MAC387+ monocyte and its function. An important feature to notice in Figures 11 and 12 is that the genes that change in coordination with calprotectin are highly interconnected. This suggests particular genes with related function may change in coordination with calprotectin gene upregulation and their protein products may suggest functions of MAC387+ monocytes or other monocytes during the acute phase of SIV infection.

Whether some of these genes are changing in response to changes in calprotectin regulation, or calprotectin, along with these other genes, are all changing in response to particular environmental stimuli, such as infection, is not known. With a stringent threshold of which genes change in every instance (treatment *or* timepoint comparison) under which calprotectin significantly changes, the significance of gene interconnection reaches a protein-protein enrichment interaction of p = <1.0e-16. Analysis of the roles of these genes in myeloid cells reveals two general functions important to the immune response to infection: an antiviral/antibacterial role, and pro-inflammatory chemotaxis. In tandem with calprotectin upregulation, APOBEC3A, CHIT1, IFI27, and ISG15 have antiviral or antibacterial roles. Further, the upregulation in IL1B observed as well is likely responsible for amplifying the

response by inducing the activation of several immune cell types and the production of pro-inflammatory cytokines. ISG15 and SIGLEC1 play roles in myeloid cells for chemotaxis and serve as adhesion molecules, respectively.

By expanding the genes that may have important associated functions with the expression of calprotectin in monocytes, a less stringent approach was taken to additionally include any genes changing expression in tandem with calprotectin in at least 80% of conditions where calprotectin had differential regulation. This expansion confirms earlier conclusions that the MAC387+ monocyte has a role in antiviral activity during SIV infection, with AZU1 and MPO having established antimicrobial function. Other genes are present in these cases as well, including genes that serve roles in combating infection. CPM is involved in extracellular protein degradation and TLR8 serves a role in pathogen recognition. One especially important gene in the analysis in Figure 12 is CD163, which connects S100A8 and S100A9 to the central hub of activity, thus linking these proteins to a large portion of the genes that change in expression. CD163 is the scavenger receptor for hemoglobin-haptoglobin and is an important biomarker of disease. Solubilization of CD163 occurs with monocyte/macrophage activation, and CD163 levels in plasma are elevated in neurocognitively impaired HIV patients (85). CD163 is especially important in its relationship with MAC387 in the context of SIV and HIV, where a higher ration of CD163:MAC387 macrophages in lesions is associated with progression to SIV encephalitis, as opposed to animals without encephalitis and animals sacrificed after three weeks of SIV infection (78).

A notable difference between the expression of these two markers on monocytes is that MAC387 expression sharply decreases with necropsy, which is the point with the lowest MAC387 expression across all monocyte subpopulations (Figure 9), while CD163 expression

increases with necropsy, which is the timepoint with highest expression (Figure 10). Analysis of monocytes and plasma protein levels may serve as a noninvasive measure of clinical conditions of a person over the course of HIV infection and the success of an anti-retroviral regimen. This analysis of RNAseq data is limited and does not look specifically into any particular treatment or timepoint. The five sample comparisons with changes in S100A8 and S100A9 are ART-20-49; ART-UNTREATED-D49; ART-UNTREATED-Nec; MGBGART-20-49; and MGBGART-20-Nec. Looking at two of these comparisons – ART-UNTREATED-D49 & ART-UNTREATED-Nec – highlights differences observed between ART and untreated at two different time points. The higher expression of calprotectin with ART treatment at these two different time points suggests the improvements observed with ART (including a decrease in viral load) are associated with an increase in the protective function of calprotectin and cells expressing calprotectin. Future work should look into seeing if this observed increase in calprotectin RNA expression in later-stage SIV infection is also observed at the protein level with ART treatment. The other three sample comparisons with increases in calprotectin gene expression – ART-20-49; MGBGART-20-49; and MGBGART-20-Nec – illustrate the higher calprotectin expression at peak infection relative to both 49 DPI and necropsy. The inflammatory monocyte/macrophage is present at high levels in tissue in acute infection. Higher calprotectin expression at the gene level around 20 DPI relative to both 49 DPI and necropsy supports the notion of an expanding inflammatory population, and that this cell population is responsible for a large portion of tissue entry by monocytes during early inflammation.

This study does have limitations in relating the findings to the role of the MAC387+ monocytes in HIV infection in people. With a sample size of five animal, this work identifies potentially important trends relating to MAC387+, but requires validation with a greater sample

size that will increase the statistical power of results. Additionally, the ELISA measurements of secreted calprotectin in blood may not be exclusively secreted from monocytes, but a substantial proportion may be the result of neutrophil secretion, which, in and of itself, does not change the inflammatory nature of the implications of this data. However, this data must be used with caution when relating measured secreted levels specifically to monocytes and correlation of the two. One must also consider that the MAC387 antibody from Biorad has specificity for the MRP-14 subunit alone, in addition to the MRP-8/MRP-14 calprotectin complex, and thus MAC387 must cautiously be used to indicate the calprotectin complex (53, 54). Further, the monocytes analyzed by RNAseq here were not analyzed by single-cell sequencing, which may provide an inappropriate average of cells and gene expression when there are important subpopulations, which may be lost in this method of cell sequencing for gene expression.

Future experiments could take several forms. A particularly interesting future step would be to relate terminal MAC387+ monocyte expression and plasma calprotectin level with MAC387+ macrophage staining and distribution. Understanding this particular connection would allow analysis of tissue damage – especially lesion formation and accumulation of inflammatory cells – to parameters in blood that would be less invasive. Another particularly useful future strategy would be to use flow gating to select for MAC387+ and MAC387- monocytes to get a more complete picture of the gene expression differences between these cell types, and to see if the gene expression changes observed matches these observations in protein expression. Using the principle of MAC387+ cell isolation, the unique technique of laser capture microdissection for selective isolation of cells based on particular immunostaining could further be used to select for MAC387+ and MAC387- macrophages in tissue, and for MAC387+ cells contributing to lesions, versus those not in lesions, to further characterize the gene expression of

MAC387+ myeloid cells. One of the most relevant future studies for better understanding gene expression of MAC387+ monocytes would be to specifically incorporate MAC387+ and MAC387- monocytes for comparison, both among treatments and among infection timepoints. This work greatly advances our understanding of calprotectin in the context of SIV infection. Future steps to further advance this work could be used to relate individual monocyte subpopulations and MAC387+ monocytes with the population of MAC387+ infiltrating macrophages observed with infection and lesion formation, and to expand the immunophenotyping of the MAC387+ monocyte.

SUPPLEMENTAL INFORMATION

Methods S2.1 Sample Storage

Approximately 2 mL blood samples for respective timepoints were obtained from the animals and ethylenediaminetetraacetic acid was added as an anticoagulant. Blood samples were centrifuged in Ficoll in order to separate PBMCs and plasma. PBMCs were stored in in 10% DMSO – 90% PBS and plasma was stored as is, both at -80C.

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