Hu5F9 anti-CD47 antibody promotes macrophage phagocytosis of SIV- infected cells in various infection stages

By

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ABSTRACT

HIV-1 infection leads to the acquired immunodeficiency syndrome (AIDS). Antibody-dependent cellular phagocytosis (ADCP) by macrophages was found to contribute to HIV-1 clearance. CD47 is a cell-surface marker regulating macrophage phagocytic activity in both cancer and HIV models, providing "don't eat me" signals preventing phagocytosis. Therefore, it seems reasonable that CD47 blockade (i.e. Hu5F9 anti-CD47 antibody) will enhance macrophage phagocytosis of HIV-1 infected cells. This study investigated whether anti-CD47 antibody treatment of SIV-infected target cells led to their increased phagocytosis by monocyte-derived macrophage (MDM) as compared to SIV-negative cells, suggesting that SIV-infection causes up-regulation of CD47 for immune evasion. We applied Leica confocal microscopy, flow cytometry, as well as EVOS microscopy and imaging system to detect and evaluate the fraction of macrophages containing ingested cells ("phagocytic ratio") under different treatment conditions. We found that SIVmac251-infected T cells treated with Hu5F9 IgG1 anti-CD47 antibody are efficiently cleared by MDMs via ADCP *in vitro*. Also, Hu5F9 and related antibodies (e.g., of different subclasses) were useful in supporting phagocytosis of both the latently infected and reactivated J-Lat cells. In addition, Hu5F9 IgG1 could effectively synergize with 10-1074 broadly neutralizing antibody (bNAb) to encourage engulfment of SHIV-AD8-EO infected CD4+ T cells. Similar to previous cancer studies, another cell surface marker called Calreticulin (CRT) was found to interact with CD47 in the setting of HIV infection to provide balance between pro-phagocytosis and anti-phagocytosis. CRT-related mechanisms may be a topic of future investigation, and especially how such mechanisms might be exploited when combined with anti-CD47 treatment in HIV-1 infection. In conclusion, Hu5F9 IgG1 anti-CD47

antibody promotes monocyte-derived macrophage phagocytosis of HIV/SIV infected cells at several infection stages.

Key Words: HIV-1, monocyte-derived macrophages, CD47, phagocytosis, anti-CD47 antibody, Calreticulin

1. INTRODUCTION

1.1 Human immunodeficiency virus (HIV)

Human immunodeficiency viruses (HIV)-1 and -2 infect humans and lead to the acquired immunodeficiency syndrome (AIDS). HIV-1 infection causes immunodeficiency, opportunistic infections, disturbed sleep patterns, lack of energy and pain [1]. The progress of HIV-1 infection can generally be divided into four major stages, including (a) initial productive infection, (b) latency, (c) deep latency, and (d) reactivation, e.g., following treatment interruption [2]. HIV establishes permanent infection due to its ability to integrate into the host genome and this generates obstacles for spontaneous cure. When HIV enters a target cell, the viral RNA genome is reverse transcribed into double-stranded DNA (dsDNA), which is translocated to the nucleus and may become latent, in which state it can avoid detection by the host immune system [3, 4].

Highly Active Antiretroviral Therapy (HAART) was sufficiently developed by the end of 20th century to dramatically decrease HIV-1 viral load and reduce patients' mortality and morbidity [5, 6]. Anti-viral drugs can be classified into mechanistic categories like non-nucleoside reverse transcriptase inhibitors (NNRTIs), nucleoside reverse transcriptase inhibitors (NRTIs), integrase inhibitors (IIs) and protease inhibitors (PIs) [7]. Although HARRT was proven effective to

counter HIV infection as well as to reduce mortality, adherence to a therapeutic regimen is required for consistent viral suppression [8, 9]. Recently, antibody-based strategies have been developed by scientists to counter HIV infection, inspired by the monoclonal antibodies used in other disease models, like cancer, autoimmune diseases and some infectious diseases [10]. Ibalizumab (anti-CD4 humanized immunoglobulin G4 monoclonal antibody) and Leronlimab (anti-CCR5 humanized immunoglobulin G4 monoclonal antibody) have been investigated to provide another HIV therapy approach [10].

Although much attention has been paid to drugs that act directly on the virus, it is also necessary to focus on the target cells of HIV-1 infection. In humans, $CD4^+$ T lymphocytes and macrophages are considered to be the major targets of HIV-1 [11, 12]. The most apparent result of HIV-1 infection is $CD4^+$ T lymphocyte depletion, the core mechanism of HIV-1 pathogenesis. Due to their shorter life span, however, macrophages have been considered less sensitive to HIV-mediated cytopathic effects and to depletion [11]. It is now known that some tissue-resident macrophages, however, are long lived. Given the heterogeneity in macrophage populations, it now seems possible that these cells could serve as the HIV reservoir for long-term persistence.

1.2 Monocyte-derived macrophages (MDMs) and their potential role in HIV clearance

Macrophages are non-dividing, terminally differentiated immune cells that contribute to both cell-mediated and innate immunity [13]. Monocyte-derived macrophages (MDMs), embryo-derived macrophages (EDMs) and self-renewing macrophages are considered three major types of macrophages. MDMs are short-lived cells recruited into tissues during stress or inflammation and can be generated from monocyte precursors found in human or macaque blood [14, 15, 16].

Due to their important contribution to functioning of the immune system, it is likely that MDMs contribute to countering HIV infection as effector cells capable of clearing virus-infected cells such as $CD4^+$ T cells.

An important function of MDMs is phagocytosis, leading to removal of cellular debris, and dying/dead cells. When macrophages engulf viable cells, the process can be called "programmed cell removal" (PrCR) [17, 18]. Cell targets are engulfed into macrophages and passed into phagosomes, which then develop into phagolysosomes by fusion with lysosomes [12]. Cell targets are finally removed within these phagolysosomes through enzymes and toxic peroxides [12]. MDMs may participate in HIV clearance via PrCR of infected cells.

1.3 CD47 and its relationship with phagocytes

The CD47 pathway has been shown to inhibit MDM phagocytosis. CD47 is a transmembrane protein expressed on many types of cells that regulates phagocytosis. It is the ligand for signal regulatory protein alpha (SIRPα) on both dendritic cells and macrophages. Binding between SIRPα and CD47 provides an anti-phagocytic "don't eat me" signal. The signal seems to be transduced by phosphorylation of immunereceptor tyrosine-based inhibitory motifs (ITIMs) within the cytoplasmic tail of SIRPα, which initiates a signaling cascade that inhibits phagocytosis [19]. CD47 was first identified in ovarian tumor cells [20], and it has been proven that CD47 up-regulation impacts tumor clearance [21, 22, 23]. For multiple types of cancers, those with higher CD47 expression are more resistant to phagocytosis [24]. Expression of CD47 in such cancers and in atherosclerotic plaques is driven at least partly by the TNFα-NF-κB1 signaling pathway [25]. Cancer studies also revealed that the CD47-SIRPα interaction results in

failure of phagocytosis as a result of the protective signal described above [26, 27]. This mechanism of protection from phagocytosis is not restricted to cancer cells, with other cell types, including platelets, red blood cells, and hematopoietic cells showing both CD47 expression and reduced vulnerability to phagocytosis [28, 29, 30, 31]. It is possible that effective phagocytosis of both cancer cells and the normal cell types listed above can be achieved by using CD47 blockade.

1.4 CD47 blockade and resulting antibody-dependent cellular phagocytosis (ADCP) may be useful in the setting of HIV infection

Since CD47 blockade promotes macrophage phagocytosis in the setting of cancer, it is possible that such blockade can be used to encourage phagocytosis and removal of HIV-infected cells. ADCP of HIV-infected cells has previously been demonstrated, via interactions between antibody Fc domains and Fc receptors on the phagocyte surface [32]. One study showed that CD47 upregulation was observed after HIV infection *in vitro* [33].

Many studies of HIV-1 clearance have implicated ADCP as an important mechanism. For HIV-1 infected people, inheritance of a low-affinity allele of FcγRIIa (R/R131), which is deficient in mediating ADCP, predicted HIV-1 disease progression [34]. In addition, HIV-1 vaccine studies showed that Fc-mediated ADCP contributes to protection [35, 36, 37]. Fc receptor genetics, type of phagocytes and antibody characteristics (isotype, subclass, specificity) may also regulate HIV-1 disease progression [32, 38, 39, 40, 41]. Similar findings were reported in SIV infection of *rhesus macaques* [32, 42].

1.5 Hu5F9 anti-CD47 antibody may be useful for promoting MDM phagocytosis during HIV infection

Considering the findings above, we hypothesized that HIV-infected $CD4^+$ T cells treated with anti-CD47 blocking antibody would be efficiently cleared by macrophages via ADCP. Hu5F9 is a humanized anti-CD47 antibody, which carries the complementarity-determining regions from the mouse anti-human CD47 antibody, 5F9 [43]. Hu5F9 (another name Magrolimab) is known as a first-in-class macrophage immune checkpoint inhibitor that targets CD47 on the cancer cell surface and interferes with the CD47-SIRPα interaction [43]. Hu5F9-G4 (Hu5F9 with the IgG4 subclass) augments cancer-cell phagocytosis by macrophages [43, 44]. Hu5F9-G4 was proven effective and safe in patients with advanced cancers via targeting CD47 for cancer clearance [43]. Other types of anti-CD47 antibodies (MIAP301, MIAP410, CC-90002, SRF231, ALX148) have also been proved effective in different infection models [45]. Therefore, it is possible that Hu5F9 is also effective in clearing HIV/SIV-infected $CD4^+$ T cells.

The major aim of this study was to test if MDMs can phagocytose SIV-infected $CD4^+$ T-cell targets, and whether such phagocytosis could be specifically augmented by the 5F9 specificity. The effect of MDM phagocytosis was evaluated by "phagocytosis ratio", which means the percentage of MDMs that have phagocytosed SIV-infected $CD4^+$ T cells under different treatment conditions.

1.6 HIV latency and use of the J-Lat cell line model of HIV latency

Highly active antiretroviral therapy (HARRT) fails to eradicate viral reservoirs that include the latent (transcriptionally silent) HIV provirus [46, 47]. Latently infected T cells harbor

replication-competent HIV and survive for a long time *in vivo* [48]. There are a variety of conceptual approaches to eliminating latently infected cells. One strategy called "shock and kill" refers to stimulation of HIV gene expression followed by immunologic purging of reservoir cells [49]. When latent HIV reservoirs emerge from latency, they are subsequently recognized and cleared by the host immune system [49]. To track the biological characteristics of latently infected cells, cell lines have been used in this setting. Folks *et al* first found the HIV-1 infected cell line called HTLV-III/LAV showing low or absent HIV-1 gene expression, indicating HIV-1 latency [50]. Later investigations of molecular mechanisms showed that transcription from the HIV-1 LTR was stimulated by NF-κB [51, 52]. Epigenetic changes in chromatin that inhibit HIV-1 gene expression; DNA methylation; and transcriptional interference, e.g., interference with RNA Pol II initiation & elongation or with RNA splicing & export, have also been suggested as deeper molecular mechanisms of HIV-1 latency [48].

Several cell lines have been used to examine HIV latency, as these cell lines support easier tracking of HIV latency genetically. Although cell lines may differ in many respects from latently infected cells *in vivo* (e.g., in virus integration sites, mutations, or cycling), they are still useful investigative tools [53]. A number of *in-vitro* cell culture models of HIV latency have been developed, including the Greene model, Lewin model, Patient cells/QVOA model, Pianelles model, Siliciano model, Spina model and Verdin model [53].

The *in-vitro* Jurkat cell line (J-Lat) model of HIV latency is particularly tractable and convenient. J-Lat cells were created by first infecting Jurkat cells with GFP reporter virus (HIV-R7/E$^-$/GFP), and then FACS sorting for GFP-negative cells. The sorting of GFP-negative cells aimed at eliminating productively HIV infected cells. The next step was to stimulate these GFP-negative

cells with TNF-alpha cytokine, followed by another sorting for GFP+ cells, in order to identify the cell clones capable of inducible gene expression from the integrated provirus. Finally, the sorted GFP+ cells were cloned and expanded, resulting in the final J-Lat cell line [54]. When J-Lat cells are activated by TNF-alpha or some other NF-kappa B activators, they produce GFP but not new infectious particles, as HIV-R7/E^-/GFP contains a frameshift mutation in the env gene [55]. The J-Lat cell line is useful for separation of re-activated cells from un-activated cells by different color staining. Also, J-Lat cell lines can be seen as target cells for MDMs, since J-Lat cells have already been infected by HIV. The phagocytosis ratio can be tested assessed for either re-activated J-Lat or un-activated J-Lat cells phagocytosed by MDMs, based upon fluorescent staining. Additionally, inspired by the idea of using antibody-based strategies to control HIV infection [10], it is possible to combine 5F9 anti-CD47 antibody treatment with the J-Lat model.

We investigated whether CD47 blockade would lead to phagocytosis and clearance of HIV-infected cells, especially cells that may have recently reactivated HIV gene expression. We hypothesized that 5F9 anti-CD47 antibody treatment can augment the MDM phagocytosis of both re-activated J-Lat and latently infected cells (un-activated J-Lat) *in vitro*. Stimulation of phagocytosis may vary for different subclasses of 5F9 anti-CD47 antibody, which interact differently with different Fc receptors on the macrophage surface.

1.7 Neutralizing antibodies (NAbs) and broadly neutralizing antibodies (bNAbs)

Neutralizing antibodies (NAbs) prevent a pathogen from infecting host cells. NAbs normally act on the specific sites of pathogens (e.g. viruses) that engage with host-cell surfaces and protect the host cells from infection [56]. NAbs can be used therapeutically in HIV-1 infection. NAbs

intervene HIV transmission by either blocking HIV-1 entry into the host cell receptors, or by antibody-dependent cell-mediated cytotoxicity (ADCC) [56]. Current NAb studies have focused on targeting the trimeric HIV-1 envelope (Env), as Env contains the only known neutralizing epitopes [57]. Although NAbs are generally effective against homologous infections (infection with virus having the same sequence against which the NAb is directed), targeting of different viruses with greater genetic variations is more difficult. HIV-infected people normally develop antibodies that are specific for their own dominant, endogenous virus with limited cross neutralization of other strains [58]. Interestingly, although broad cross neutralization is rare, some very potent broadly neutralizing antibodies (bNAbs) were still observed, which can target HIV-1 with of many genetic subtypes [59]. Halper-Stromberg *et al* found that the bNAbs can be applied to interfere with maintenance and formation of HIV reservoirs [60]. Using bNAbs to potentially neutralize and clear HIV-reservoirs has also been tested, with encouraging results [61].

Evaluation of several generations of anti-HIV bNAbs has proceeded in stages. Initially, several first-generation bNAbs (b12, 2F5, 4E10, 2G12 and KD-247) were used to evaluate how these antibodies could reduce viremia after passive infusion [62-67]. These first-generation antibodies decreased plasma viral loads transiently and delayed viral rebound after analytic treatment interruption (ATI), but unfortunately, virus escape was still observed [64, 68, 69]. Later, a potent bNAb that targets the CD4 binding site of envelope protein was also tested. Two other strong bNAbs called 10-1074 and PGT121 were found to target the V3-glycan of HIV-1 instead of the CD4 binding site [71]. Evidence regarding PGT121 and 10-1074 inhibition of the viral reservoir has been found. PGT121 was shown to block HIV-1 replication in viral reservoir formed by

activated cells [72]. In non-human primates (NHPs), PGT121 was shown to deplete pro-viral DNA [73]. Similar findings were obtained in humanized mice: 10-1074 and 3BNC117 led to below-detectable cell-associated pro-viral load [74]. In addition, HIV-1 can be transmitted by cell-to-cell virological synapses, and most neutralizing antibodies were proved to inhibit cell-to-cell transmission [75]. Although there is no evidence available regarding MDMs participating in the bNAb inhibition, it is possible that any of the known bNAbs (3BNC117, PGT121 and 10-1074) may be useful for either clearing productively HIV/SIV-infected T cells, or removing persistent HIV reservoirs by interacting with MDMs and activating phagocytosis of infected cells.

1.8 SHIV-AD8 and MDM phagocytosis

SIV/HIV chimeric viruses (SHIVs) are useful for investigation of antibody-mediated anti-HIV therapies. SHIVs express the HIV Env glycoprotein (gp120), allowing investigation of bNAbs that interact with the HIV envelope [76]. SHIVs were constructed by inserting a large segment of the HIV genome, especially the Env gene, into SIVmac239 to combine the characteristics of both HIV and SIV [77]. CXCR4-tropic as well as CCR5-tropic SHIVs were both introduced for different co-receptor usage for viral entry [78, 76]. SHIV-AD8 is a CCR5-tropic SHIV [79]. SHIV-AD8 was created by transfecting HeLa cells with the molecular clone, pSHIV-AD8 [80], and subsequently infecting *rhesus macaque* concanavalin A-activated peripheral blood mononuclear cells (PBMC) [81] or PM1 cells [80] with HeLa-derived SHIV-AD8. The R5-tropic SHIV-AD8 system mimics multiple clinical features of human HIV infection [82]. Stable viremia is maintained, leading to the continuous and slow $CD4^+$ T-cell loss as well as eventual opportunistic infections [76].

We used the SHIV-AD8-EO infection model to test broadly neutralizing antibodies in combination with anti-CD47 antibodies. We sought to test (a) whether MDMs can phagocytose SHIV-AD8 infected $CD4^+$ T cells, and (b) whether such phagocytosis could be augmented by different combinations of antibodies, including both Hu5F9 anti-CD47 blocking antibodies and anti-HIV broadly neutralizing antibodies (PGT121, 10-1074 and 3BNC117). These studies will inform selection of a bNAb subtype for pre-clinical and eventually clinical testing of bNAbs in combination with anti-CD47.

1.9 CRT and CD47 may be interacting in HIV/SIV infected cells HIV latently infected cells using J-Lat model

CD47 was first characterized as a pentaspanin cell surface protein that inhibits phagocytosis in tumor cells [83]. Studies have found that such inhibition was achieved by CD47 binding its receptor, SIRPα [83, 84]. SIRPα is expressed commonly on both dendritic cells and macrophages [85]. Specifically, on signaling, myosin-IIA is inhibited by the recruitment and activation of Src homology phosphatase to inhibit macrophage phagocytosis [85, 45]. Anti-CD47 blocking antibodies have been introduced before for both cancer and other infection models [43, 45]. Another protein called calreticulin (CRT) was found to promote recognition and phagocytosis of cancer cells by macrophages when CD47 is blocked [86, 87]. CRT normally regulates adhesion through integrin activation [88], and regulates some cell-surface proteins on folding and assembly [89]. CRT has been seen as a label for "unwanted cells" as well as "pro-phagocytosis signal", and most CD47-expressing normal tissue cells are relatively insensitive to programmed cell removal when they do not express CRT for macrophage recognition [90].

Macrophages are actually responsible for such pro-phagocytosis signal labeling, coordinating with the CD47-SIRPα anti-phagocytosis signal [91]. The balance between pro-phagocytosis signal and anti-phagocytosis signal decides whether the target cells will be preserved or eliminated by host immune system, indicating that CRT signal is counterbalanced by CD47 [90].

Since the importance of CRT-CD47 balance has been proven in cancer studies, it is likely that such balance is similarly at play in the setting of HIV/SIV, HIV latency infection *in vivo* and/or *in vitro*. We were interested in investigating (a) whether CRT could be differentially expressed in developing MDMs or activated/inactivated J-Lat targets; (b) whether an inverse relationship between CRT and CD47 exists before and after co-incubation with MDMs.

2. METHODS

2.1 Animals and virus

*Rhesus macaque*s (*Macaca mulatta*) were obtained from California National Primate Research Center (CNPRC) for our studies. Peripheral blood samples were collected and peripheral blood mononuclear cells (PBMC) were isolated. The studies were approved by the UCD Institutional Animal Care and Use Committee and conducted in compliance with the Health Research Extension Act and the United States Federal Animal Welfare Act. A characterized SIVmac251 viral stock ($2.14*10^4$ /ml PFU), was utilized for assays of productively-infected T cells on phagocytosis ratio. J-Lat 9.2 cells (HIV reagent program, #ARP-9848) were regarded as successful latency infection cells carrying HIV latency infection viral particles. SHIV-AD8-EO (TCID50 $6*10^3$/ml) was used (provided by Mal Martin) for neutralizing antibody assay.

2.2 Cell lines, activators and inhibitor used

Confocal microscopy assessment was done using both RAW264.7 (ATCC, #TIB-71) and K562 (ATCC, #CCL-243) cell lines. Lipopolysaccharide (LPS, Millipore Sigma, #L6511) was used for RAW264.7 cell stimulation to better phagocytose their targets. Cytochalasin D (Millipore Sigma, #C8273) was also used for inhibiting RAW264.7 cell phagocytosis. J-Lat 9.2 cell line (HIV reagent program, #ARP-9848) was used for HIV latency assays. TPA (12-O-Tetradecanoylphorbol-13-acetate, Millipore Sigma, #P8139) and TNF-α cytokine (Miltenyi Biotec, #130094015) were used to activate the J-Lat cells to separate true latency infection cells and reactivated cells.

2.3 Peripheral blood mononuclear cell (PBMC) isolation

PBMC were isolated on the same day after obtaining *Rhesus macaque* peripheral blood samples. Briefly, lymphocyte separation medium (LSM, MPBio #0850494-CF) was initially prepared at the bottom of 50-ml Falcon conical tubes. Then, mixture of both peripheral blood and phosphate-buffered saline (PBS, Gibco #10010023) was gently and slowly overlaid on top of LSM, with a clear distinction between two layers. The tubes were next centrifuged at 2500 rpm, 5/2 for acceleration/deceleration, and at room temperature for 30 minutes. After centrifugation, PBMC from white buffy coat layer lying in the middle were pipetted and removed to new tubes. Pre-cooled PBS was added into the new tubes for the next step centrifugation (1800 rpm, 10 minutes, 4 C°) to wash away the LSM. Finally, the isolated PBMC was counted for further use.

2.4 Magnetic beads purification

The isolated PBMC were used either for T cell culture or monocyte-derived macrophage culture. Miltenyi Biotec CD4 (#130091102), CD8(#130045201) and CD14 (#130091097) beads were used in different experiments following different magnetic separation protocols. Buffer was also prepared by making a solution containing PBS, 0.5% BSA (from BSA Stock solution, Miltenyi Biotec # 130091376) and 2mM EDTA before beads purification. Briefly, the counted PBMC were re-suspended into 80 μl of buffer as well as 20 μl Microbeads per 10 million cells. Then, 15-minute incubation at 4 C° was required to ensure enough antigen attached to the corresponding beads. Finally, cells were processed to magnetic separation with the help of MACS Column (Miltenyi Biotec # 130042401) and MACS Separator (Miltenyi Biotec #130042501).

2.5 Cell culture

All types of cells were cultured in 150i CO_2 incubator (Thermo Scientific #51026282) with 37 C° 5% CO_2 setting. T cells purified from PBMC were cultured in the growth media containing Roswell Park Memorial Institute Medium (RPMI, Gibco #11875101) supplemented with 10% tetracycline-negative fetal bovine serum (FBS), plus IL-2 cytokine (Miltenyi Biotec #130097743). CD14+ purified cells were cultured in KPB-M15-enriched macrophage growth media for monocyte-derived macrophages differentiation. This media includes KPB-M15 cell line growth media supernatant, Dulbecco's Modified Eagle's Medium (DMEM, Gibco #11885084) supplemented with 10% tetracycline-negative fetal bovine serum (FBS), plus IL-1β (Miltenyi Biotec #130093895) and M-CSF (Miltenyi Biotec #130096491) cytokines. CD14+

cells were monitored the growth state every day, with 2 ml KPB-M15-enriched macrophage growth media added every three days to ensure enough nutrition and differentiation support.

2.6 CellTrace staining, co-incubation and cell imaging

For all types of experiments, cells were either stained with CellTrace Violet (ThermoFisher Scientific #C34557) or CellTrace FarRed (ThermoFisher Scientific #C34572) following the staining protocols. Briefly, counted cells were first diluted into single-cell suspension by cold PBS, and then mixed with the staining solution. Cells were incubated for at least 20 minutes at room temperature under dark condition. When staining was done, 5-minute quick quenching using cold growth media removed any free dye remaining in the solution. Finally, the stained cells were counted for further use. Co-incubation of both MDMs and target cells (e.g. SIV-infected T cells, J-Lat cells and SHIV-AD8-EO-infected T cells) was achieved by using either 6-well plate (ThermoFisher Scientific #145380) or 8-well chamber slides (ThermoFisher Scientific #154534PK). Basically, counted MDMs were pre-attached to the plates or slides one day before co-incubation. The CellTrace-stained target cells were counted the following day and were added to each plate or slide, along with treatment conditions (e.g. anti-CD47 antibody treatment or no treatment control) as well as growth media. After co-incubation overnight in 150i CO_2 incubator, cells were fixed by either 4% formaldehyde or 1% paraformaldehyde for 10 minutes, before drying, cooling, and taking images under either EVOS FL Auto 2 Imaging System (Invitrogen, #AMAFD2000) or Leica TCS SP8 Confocal Microscope. For EVOS2, Cy5, DAPI and FITC channels represent red, blue and green fluorescence lights respectively. For Leica SP8 Confocal, emission light wavelengths were adjusted for both Far Red (661 nm) and Violet colors (450 nm).

2.7 Flow Cytometry

Flow Cytometry was done for Calreticulin (CRT)-related assays. Briefly, staining antibody mixture including anti-CRT (LSBio #LS-C498182200), anti-CD47 (Abcam #275581), Live/Dead Aqua (ThermoFisher Scientific #L34955) and anti-CD3 (ThermoFisher Scientific #MHCD0328) was made before 15-minute 4C° staining incubation. After staining, cells were fixed by 1% paraformaldehyde for flow cytometry running. BD FACS-Symphony A3 Flow Cytometer was used for primary data collection, and FlowJo v10 was used for detailed gating population analysis.

2.8 Statistical analysis

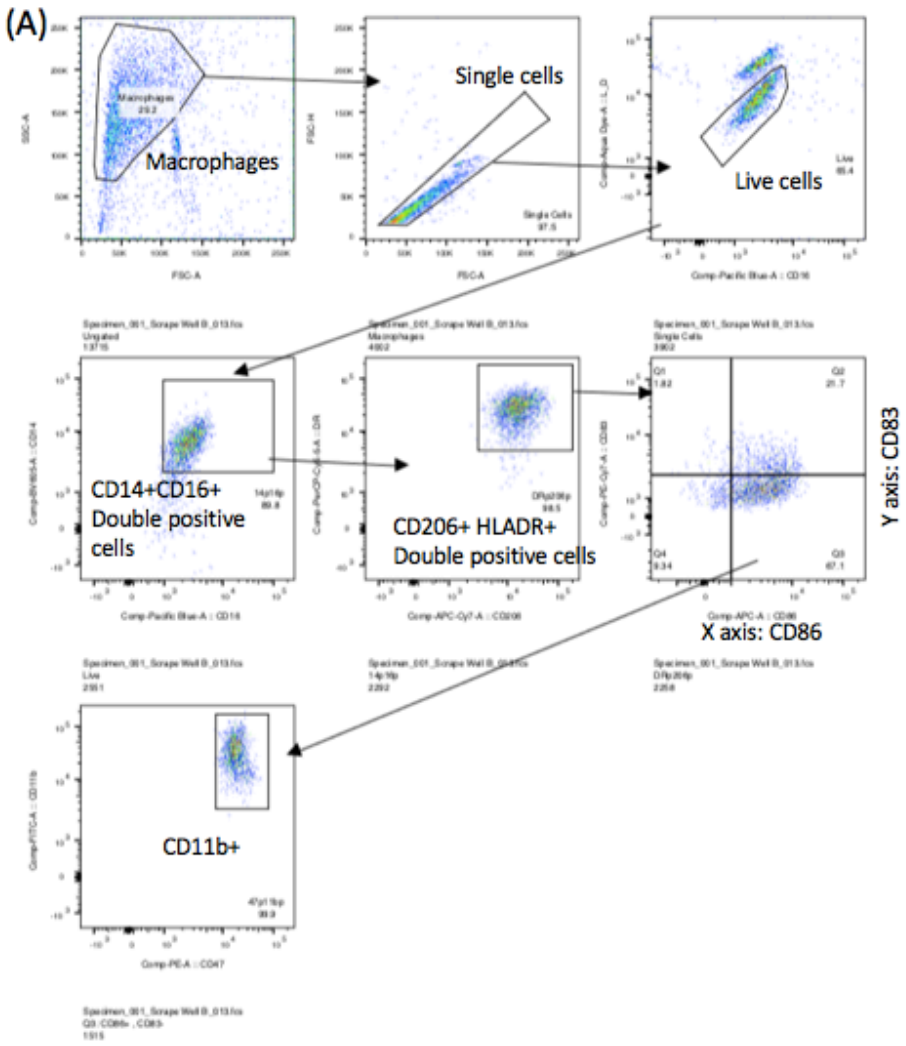
RStudio was used for productively SIVmac251 infection data analysis. Briefly, a mixed model containing both fixed effects and random effects was used, by utilizing lme4 and lmerTest packages. This analysis helped on involving interaction among multiple variables into analysis. Phagocytosis ratio of different latency infection conditions were analyzed on student t-test using RStudio as well. FlowJo v10 assisted on flow cytometry gating and analysis on percentages.

3. RESULTS

3.1 Isolation, cell culture and immunophenotypic characterization MDMs

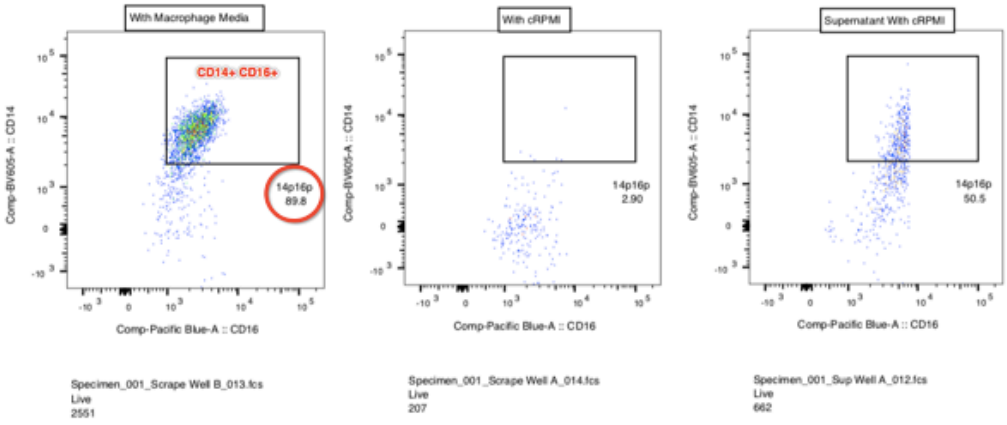
Immunophenotypic characterization was performed after generating CD14+ cells from *rhesus macaque* peripheral blood mononuclear cells (PBMC) using CD14+ beads as well as cell culture *ex vivo*. Such test ensured that the cells generated were functionally effective and could be used for the later study. These cells were cultured in KPB-M15 enriched media and were tested for the expression levels of several MDM phenotype markers using a combination of antibodies, including HLADR, CD206, CD86, CD83, CD11b, CD14 and CD16. Figure 1-A shows the gating strategy of generated cells from blood by flow cytometry and analyzing the data using FlowJo software. Most cells displayed characteristics typical of the MDM phenotype (HLADR+ CD206+ CD86+ CD83- CD11b+ CD14+ CD16+) and were considered functionally effective.

We sought to determine how the phenotypic expression of MDM following the culture using the KPB-M15 enriched media. Please note that M-CSF and IL-1β were added for both two types of media. KPB-M15 enriched media was once proved useful on helping monocytes differentiating into macrophages [95]. So we compared the phenotype differences between with KPB-M15 enriched media and without KPB-M15 enriched media. Figure 1-B shows that nearly 90% of live CD14+ CD16+ double positive cells were successfully generated when KPB-M15 enriched media were included. However, if KPB-M15 enriched media was not included for cell culture, cells become less adherent, and only around 50% of live CD14+ CD16+ double positive cells were detected in supernatant. Such result indicates that KPB-M15 enriched media is necessary for MDM differentiation.



(B) + KPB M15 enriched Media

+ only complete RPMI without KPB enriched



Specimen_001_Scrape Well B_013.fcs
Live
2551

Specimen_001_Scrape Well A_014.fcs
Live
207

Specimen_001_Sup Well A_012.fcs
Live
662

Almost all attached well
After 6-day culture.

Attached cells

Unattached cells in
Supernatant.

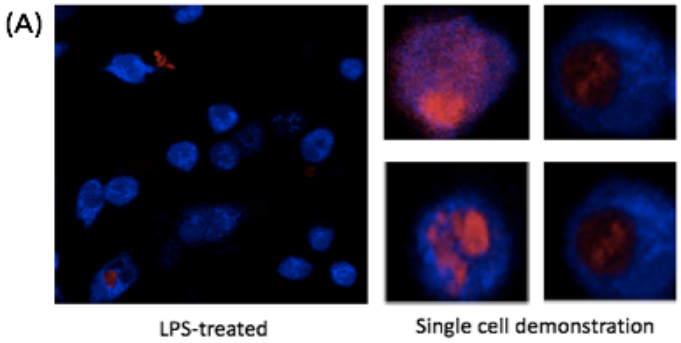
Not all cells attached well.

Figure 1. Phenotypic test for differentiation of monocyte-derived macrophages (MDMs) from *rhesus macaques*. (A) Gating strategy for cultured cells. Most live cells were $HLADR^{+}CD206^{+}$ $CD86^{+}CD83^{-}CD11b^{+}CD14^{+}CD16^{+}$ and represented MDM phenotype. (B) Comparison between cells generated with or without KPB-M15-enriched medium, with percentage of $CD14^{+}CD16^{+}$ double-positive cells.

3.2 Confocal microscopy is a reliable method for quantitative detection of phagocytosis.

We devised a method for measuring phagocytosis by staining both macrophages and targets with different fluorescent dyes, then directly observed engulfed cells under Leica confocal microscopy. We initially used K562 cells as targets, staining them with CellTrace Far Red. Murine immortalized macrophage-like cells (RAW264.7 cells) were stained with CellTrace Violet; we expected that these cells would be capable of engulfing K562 targets. RAW264.7 cells were left untreated (control) or treated with Cytochalasin D, Lipopolysaccharide (LPS), or LPS and Cytochalasin D for one day before co-incubation. Finally, K562 and RAW264.7 cells were stained and co-incubated overnight under RAW264.7 cell growth media to allow enough time for phagocytosis. Successful phagocytosis was defined as CellTrace Far Red cells inside CellTrace Violet cells under confocal microscopy. Figure 2-A demonstrates successful engulfment by RAW264.7 cells of K562 targets after stimulation with 10 ng/ml LPS for 1 day before co incubation. Frequent target-cell internalization and clear phagocytosis were observed with the help of LPS stimulation. However, when RAW264.7 cells were pre-treated with the phagocytosis inhibitor, Cytochalasin D (10 μM), almost no phagocytosis was observed, indicating strong inhibition of a process requiring reorganization of the actin cytoskeleton. For

RAW264.7 cells treated with both Cytochalasin D and LPS, interestingly, we observed close apposition of RAW264.7 cells to targets, but without successful phagocytosis (Fig. 2B). This finding suggested that LPS had successfully stimulated RAW264.7 cells to become more phagocytic, while cytochalasin D prevented successful engulfment. RAW264.7 not treated with LPS were only rarely phagocytic.



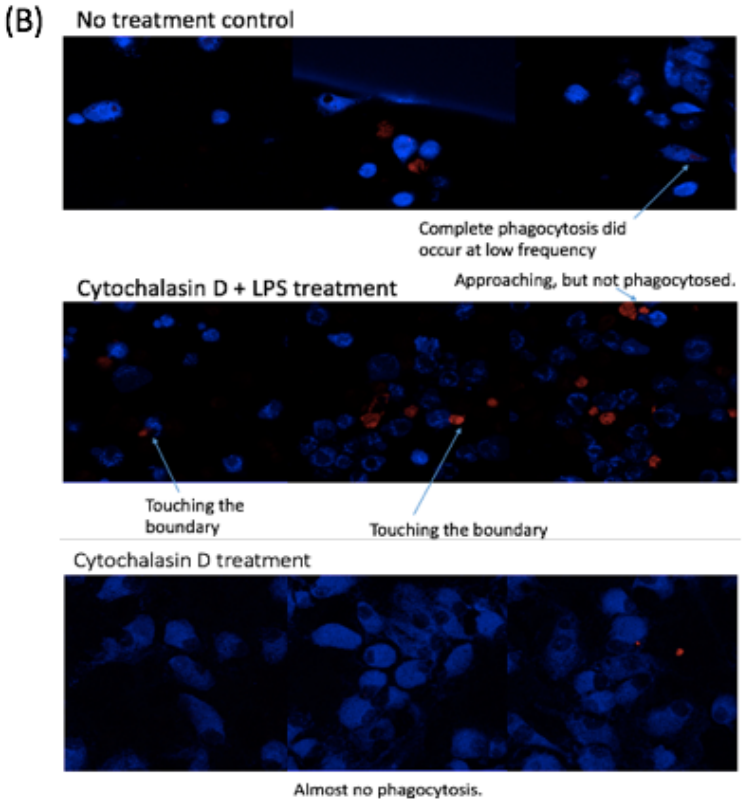


Figure 2. Leica confocal microscopy reveals phagocytosis. Objective: 40X. Red: CellTrace Far Red-stained K562 targets. Blue: CellTrace Violet-stained RAW264.7 cells. (A) Phagocytic activity of LPS-treated (10 ng/ml) RAW264.7 cells with whole demonstration (20X objective) and single cell demonstration (40X objective). Frequent target cell internalization and clear phagocytosis were observed. (B) Phagocytosis activity for untreated condition, 10μM cytochalasin D + 10 ng/ml LPS-treated, and 10μM cytochalasin D-treated conditions. Less phagocytosis activities were observed.

3.3 Phagocytosis of SIVmac251-infected T cells was increased by Hu5F9 Hz_IgG1 anti-CD47 antibody treatment.

Based on the successful preliminary results obtained using both RAW264.7 and K562 cell lines, confocal microscopy was next applied to test MDM phagocytosis of SIV-infected T cells *in vitro*. The experimental plan was to initially demonstrate successful phagocytosis, then test if the phagocytosis process could be promoted by different types of anti-CD47 blocking antibodies. Figure 3-A shows a schematic diagram of the experiment. Specifically, PBMC were isolated from *rhesus macaques*. CD14 and CD8 beads were used on the same day after PBMC isolation for either MDM ($CD14^+$) or T-cell culture ($CD14^-CD8^-$). The latter cell population was treated with 1 μg/ml phytohaemagglutinin (PHA) on the PBMC isolation date and two days later. After the 2-day PHA stimulation, the T cells were infected with SIVmac251. $CD14^+$ cells were instead cultured for 6 days in KPB-M15-enriched growth medium that additionally contained the cytokines M-CSF and IL-1β. Both MDMs and SIVmac251-infected T cells were stained and co-cultured overnight to allow time for phagocytosis. Finally, the samples were imaged under Leica

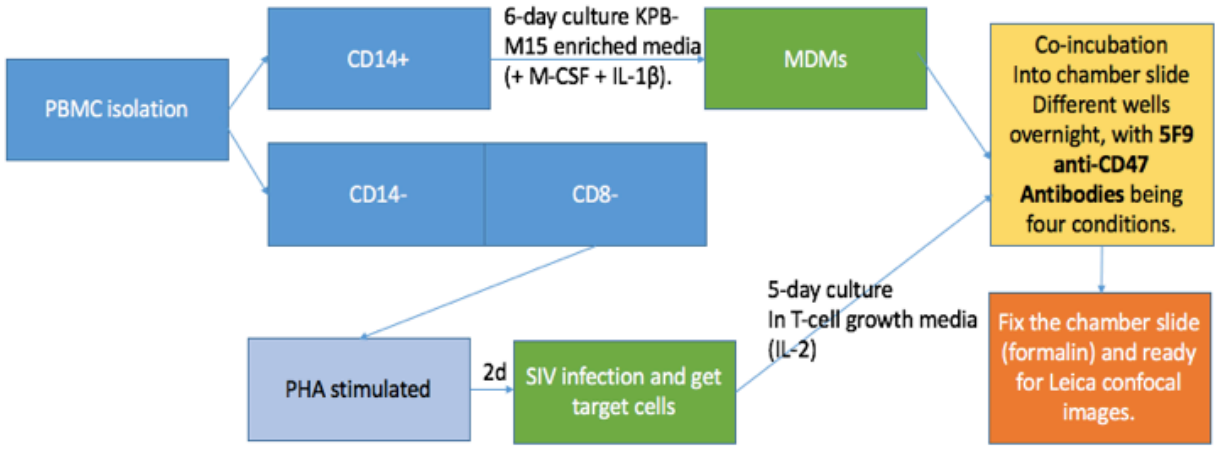
confocal microscopy. The "phagocytosis ratio" was defined as the percentage of MDMs containing T cells and taken to be an indicator of the extent of phagocytosis in each well. Uninfected T cells were also co-incubated with MDMs to see to what extent SIV infection was controlling phagocytosis.

Figure 3-B shows representative Leica confocal microscopy images for phagocytosis of SIV-infected cells in the absence or presence of anti-CD47 antibodies. Four related antibodies derived from 5F9 were tested: humanized IgG1 subclass, humanized IgG4, rhesusized IgG1, or rhesusized IgG4. SIVmac251-infected T cells were stained with CellTrace Far Red, while MDMs were stained with CellTrace Violet. Phagocytosis of SIVmac251-infected T cells was dramatically enhanced by Hu5F9 IgG1 anti-CD47 antibody treatment, compared with all other conditions. Frequent internalization of SIV-infected targets was observed for MDMs of both round and linear morphologies. When SIV-infected T cells were treated with other types of antibodies, MDM phagocytosis was detectable but distinctly lower than observed using the humanized IgG1 antibody. Uninfected T cells appeared not to be actively phagocytosed by MDMs in this assay.

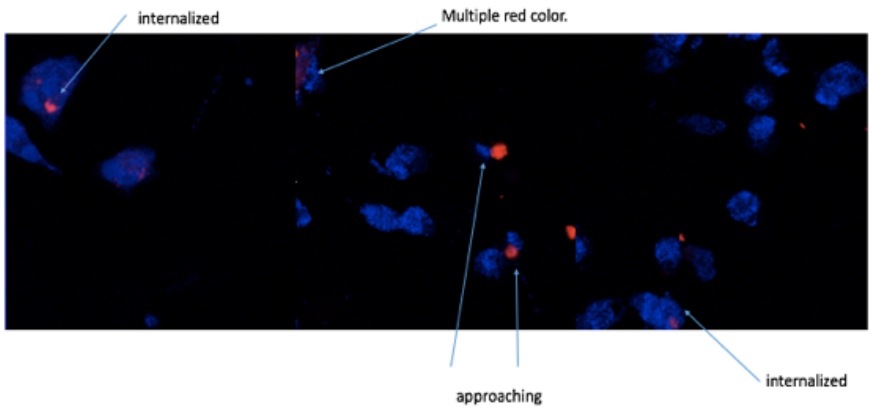
We also employed irrelevant antibodies, DSPR1 and DSPR4, as isotype controls to determine if anti-CD47 specificity was required to encourage phagocytosis. We were interested in whether these isotype controls would achieve similar or different phagocytosis ratios compared to Hu5F9 IgG1. We also tested the impact on phagocytosis of a LALA mutation in the Fc region of the antibody, for both SIV-infected and uninfected T cells.

We calculated the phagocytosis ratio under each different condition after multiple experimental replicates. Figure 3-C and Figure 3-D show the results of phagocytosis assays for the humanized IgG1 anti-CD47 antibody, with or without the LALA mutations that should abolish engagement between the anti-CD47 antibody and Fc receptors, thereby eliminating a potential source of unanticipated side effects *in vivo*. Humanized IgG1 anti-CD47 antibody significantly ($P < 0.05$) augmented MDM phagocytosis of SIV-infected T cells compared to other conditions. For all types of anti-CD47 antibodies, the phagocytosis ratio seemed to be higher when using SIV-infected targets, suggesting that anti-CD47 antibodies were useful in SIV-infected T cell clearance. In these experiments, humanized anti-CD47 clones seemed to work better than rhesusized antibodies. Figure 3-E reveals that the anti-CD47 antibody LALA mutation clearly reduced the phagocytosis of SIV-infected T cells, as compared to the parental antibody, suggesting that candidates with LALA mutations may be much less effective *in vivo*. Figure 3-e shows the comparative effect of multiple anti-CD47 antibody versions.

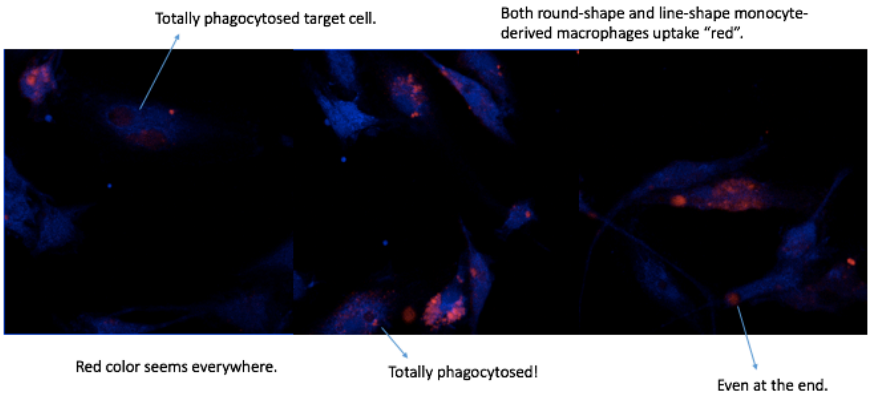
(A)



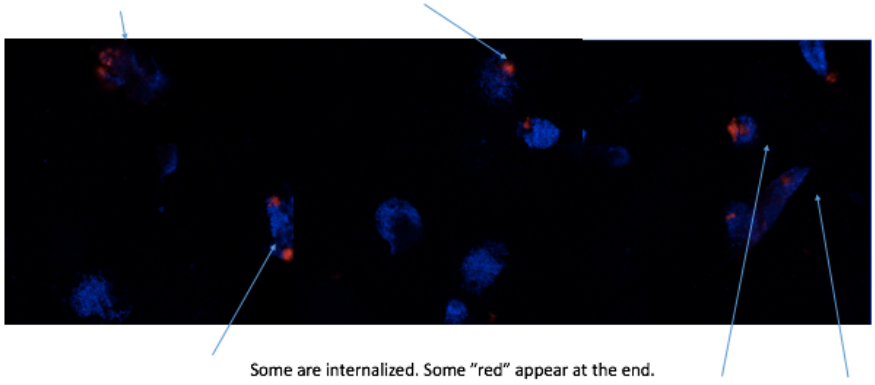
(B) No treatment control



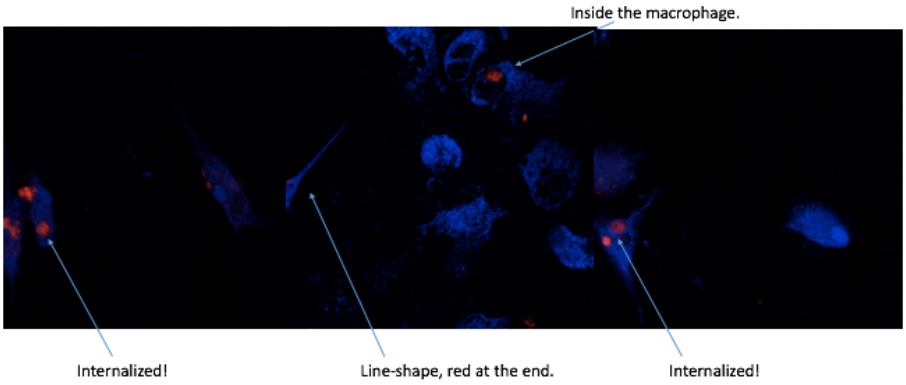
Human IgG1 anti-CD47 antibody treatment



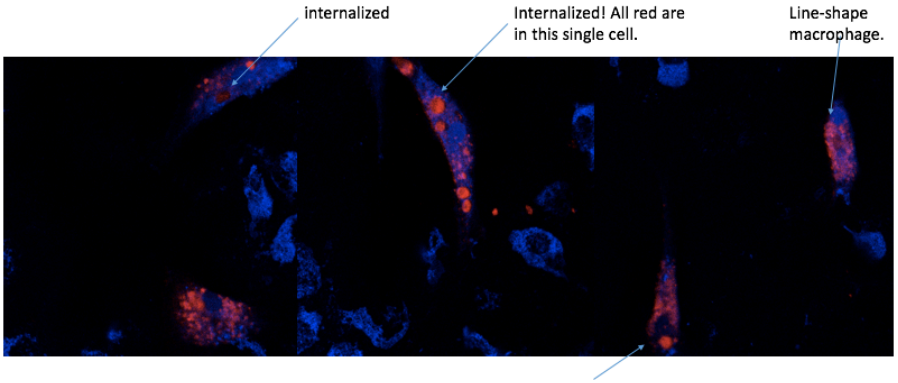
Rhesus IgG1 anti-CD47 antibody treatment



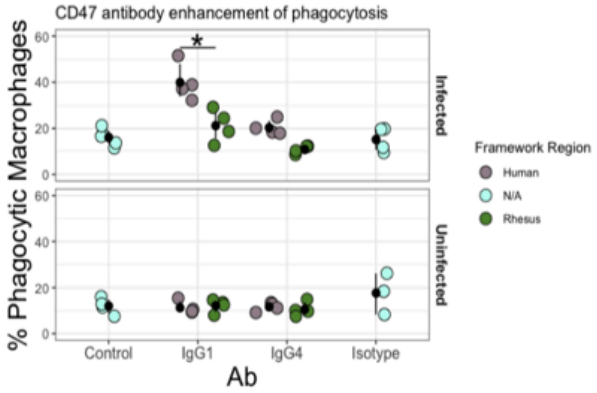
Human IgG4 anti-CD47 antibody treatment



Rhesus IgG4 anti-CD47 antibody treatment

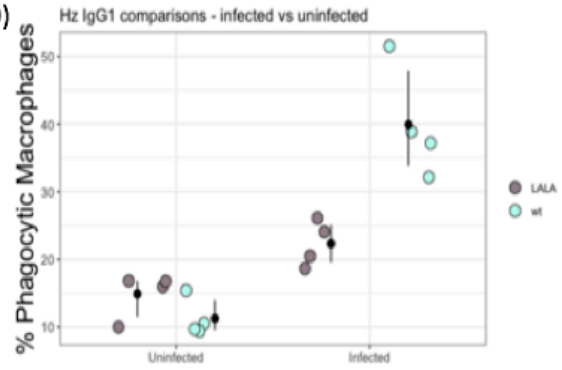


(C)



Y axis: Phagocytosis ratio for macrophages
X axis: anti-CD47 antibody types.

(D)



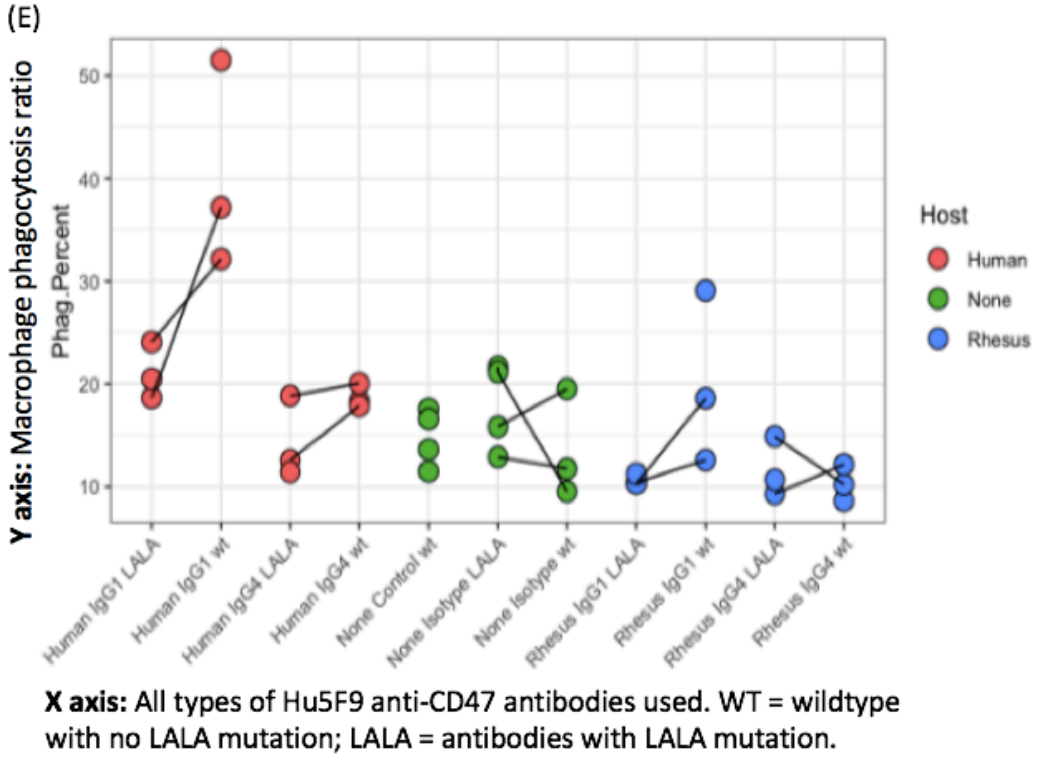


Figure 3. MDM phagocytosis of SIVmac251-infected T cells was increased by Hu5F9 Hz_IgG1 anti-CD47 antibody. (A) Schematic of the experiment. The same color means the steps were done on the same day. (B) Representative images of Leica confocal microscopy. Objective: 40X. Red: SIVmac251-infected T cells. Blue: MDMs. (C) Influence of various anti-CD47 antibodies on phagocytosis of SIV-infected T cells or uninfected T cells. Y axis: Macrophage phagocytosis ratio; X axis: anti-CD47 antibody types. Control = No antibody control. IgG1 = Hu5F9 IgG1 subtype anti-CD47 antibody. IgG4 = Hu5F9 IgG4 subtype anti-CD47 antibody. Isotype = DSPR4 isotype that did not target CD47 antigens. (D) Comparison on phagocytosis ratio between Hu5F9 IgG1 anti-CD47 wildtype antibodies and LALA mutated antibodies. (E) LALA mutation affecting Fc receptor binding generally reduced phagocytosis of SIV-infected T cells.

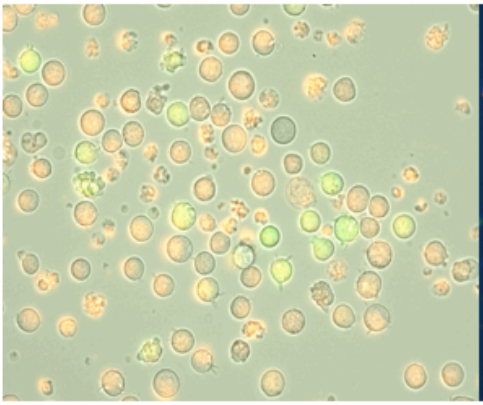
3.4 Hu5F9 IgG1 anti-CD47 antibody promotes phagocytosis of latently infected cells.

Having demonstrated that CD47 blockade encourages phagocytosis of productively infected T cells, we wondered if blockade is useful for latently infected cells as well. J-Lat cells were used as the model for HIV latency, while MDMs were still used as effector cells to phagocytose the J-Lat targets. Activation of J-Lat using "TT treatment" (5 nM TPA plus 5 ng/ml TNF-α) was used to induce cells with reactivated HIV infection, for comparison with the latent population. In this experiment, all J-Lat cells were stained with red fluorescent dye; reactivated cells expressed GFP from the HIV LTR.

To ensure GFP signals of J-Lat were successfully stimulated, one pre-test of J-Lat activation using NF-κB stimulators was necessary. 5nM TPA plus 5 ng/ml TNF-α (collectively "TT") were used for stimulation because these agents were previously proved effective [99]. Figure 4-A shows the successful activation of J-Lat GFP signals, indicating transcription from the HIV LTR. All the cells were stained with CellTrace Far Red, while the successful reactivated J-Lat were green under the FITC channel. The results indicated that TT treatment successfully created a mixure of latently infected and reactivated cells, both visible in the EVOS microscope. Next, co-incubation of J-Lats and MDMs was carried out as before under EVOS in 6-well plates. Figures 4-B, C, D and E are representative of the phagocytosis seen under multiple conditions after co-incubation. MDMs receiving Hu5F9 IgG1 anti-CD47 antibody treatment were most able to phagocytose TT-treated latently infected J-Lats as well as reactivated cells. MDMs in the presence of TT-treated J_Lat cells (but without antibody) were also able to phagocytose cells, but the phagocytosis ratio was much less compared to the TT/5F9 condition. For MDMs mixing

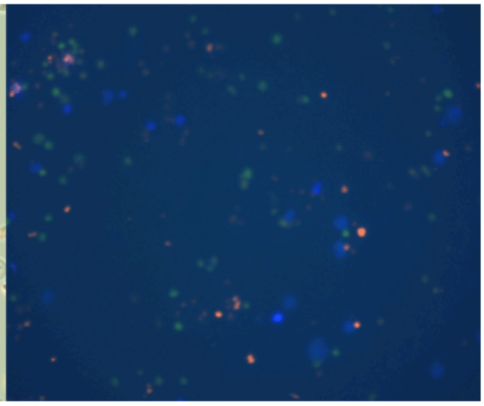
with no TT treatment J-Lat, few or no green signals were detected, as expected. Figure 4-F shows both red and green phagocytosis ratios (i.e., for latently infected or productively infected J-Lats, respectively) after incubation with various antibodies. For J-Lat that remain latently infected after TT treatment, MDMs receiving Hu5F9 IgG1 anti-CD47 antibody treatment manifested a significantly higher ($P < 0.05$) phagocytosis ratio compared to no-antibody controls. Interestingly, with respect to reactivated (green) targets, MDMs receiving Hu5F9 IgG1 anti-CD47 antibody treatment were 5 times more likely to phagocytose targets than those receiving no antibody treatment.

(A)



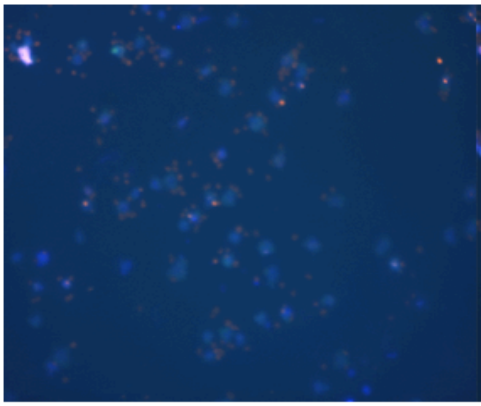
EVOS pre-test results.
Green = successfully stimulated TT-treated J-Lat cells.

(B)



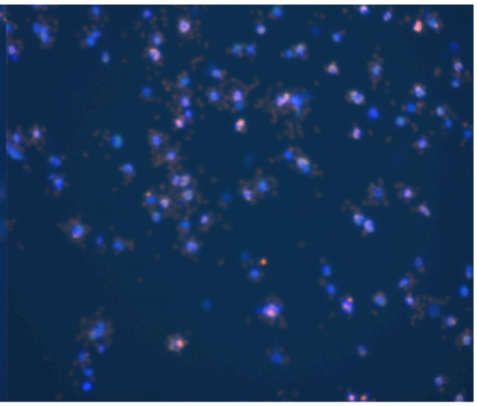
TT_NoCtrl condition
TT_NoCtrl = J-Lat cells receiving 1-day TNF-alpha + TPA treatment but with no anti-CD47 antibody treatment during co-incubation.

(C)



N_5F9 condition.
N_5F9 = J-Lat cells with no TT pre-treatment but with Hu5F9 Hz_IgG1 anti-CD47 antibody treatment during co-incubation.

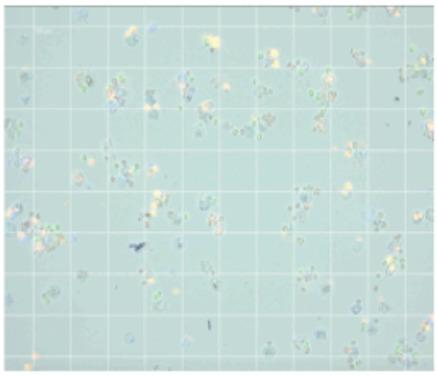
(D)



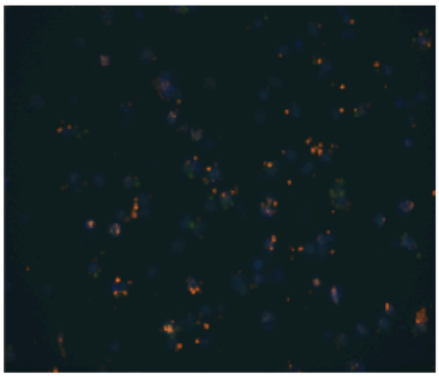
N_NoCtrl condition. N_NoCtrl = J-Lat cells with no TT pre-treatment as well as no Hu5F9 Hz_IgG1 anti-CD47 antibody treatment during co-incubation.

(E)

TT_5F9 condition images



With bright field



With dark field

TT_5F9 = J-Lat cells receiving 1-day pre-treatment plus Hu5F9 Hz_IgG1 anti-CD47 antibody treatment during co-incubation.

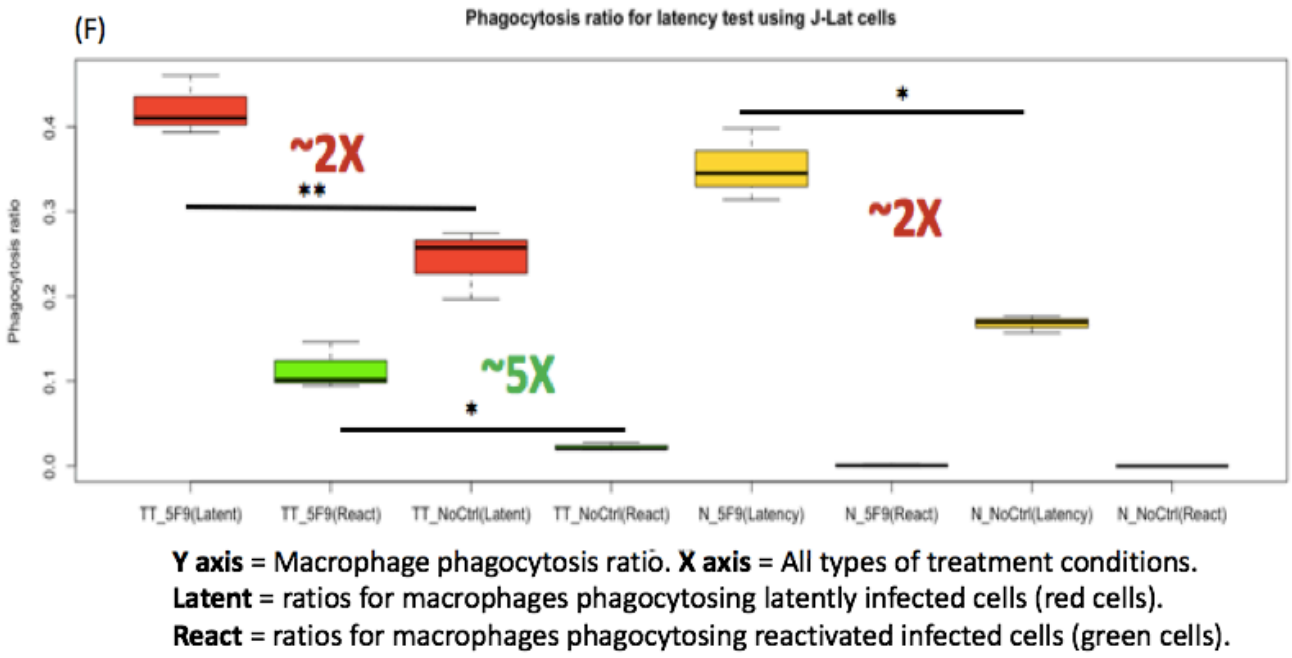


Figure 4. HIV latency test results. (A) Assessment of latency and reactivation by fluorescence microscopy. Red: latently infected J-Lats; green: reactivated J-Lats expressing GFP. (B)-(E) Representative images of phagocytosis occurring under four conditions. *TT_5F9*, cultures received both TT treatment and Hu5F9 IgG1 anti-CD47 antibody treatment. *TT_NoCtrl*, cultures received only TT treatment. *N_5F9*, cultures received only Hu5F9 IgG1 anti-CD47 antibody treatment. *N_NoCtrl*, cultures received neither anti-CD47 treatment nor TT treatment. (F) Box plot demonstration of both red phagocytosis ratio and green phagocytosis ratio for four major conditions. *Latent*, phagocytosis ratio for latently infected J-Lat population (red cells in images). *React*, phagocytosis ratio for reactivated J-Lat population (green cells in images).

3.5 10-1074 coordinated well with Hu5F9 IgG1 anti-CD47 to remove SHIV-AD8-EO infected CD4 + T cells *in vitro*

To explore another potential way for HIV-1 infection, several types of bNAbs (3BNC117, PGT121 and 10-1074) and SHIV-AD8-EO were introduced, because bNAbs were proved useful on neutralizing HIV Env. From the above results, Hu5F9 IgG1 anti-CD47 antibody was helpful on removing both the productively SIV-infected T cells and the latently infected J-Lat targets. We first hypothesized that the bNAbs were useful on promoting MDM phagocytosis towards the SHIV-AD8 infected CD4 + T cells from blood. Based on this hypothesis, it is also possible that the Hu5F9 IgG1 anti-CD47 antibody treatment led to the further augmentation on MDM phagocytosis ratio. We hoped to see which neutralizing antibody type worked the best among all types of conditions.

Figure 5 shows the results of phagocytosis ratio for all conditions. Efficacy of Hu5F9 IgG1 anti-CD47 antibody treatment for stimulating phagocytosis was confirmed. Hu5F9 IgG1 anti-CD47 antibody treated SHIV-AD8-EO infected cells were generally higher on phagocytosis ratio compared to uninfected cells. Generally, 10-1074 was the ideal choice for three types of bNAbs used. 10-1074 treatment slightly increased the phagocytosis ratio in infected cells, and a great synergism effect was shown when 10-1074 was combining the Hu5F9 IgG1 anti-CD47 antibody treatment. For PGT121, a weak synergism effect was also shown. Although 3BNC117 treatment alone led to the most increase on SHIV-AD8-EO infected T cell phagocytosis ratio among all three types of bNAb, 3BNC117 failed to coordinate Hu5F9 anti-CD47 antibody to have synergistic effect.

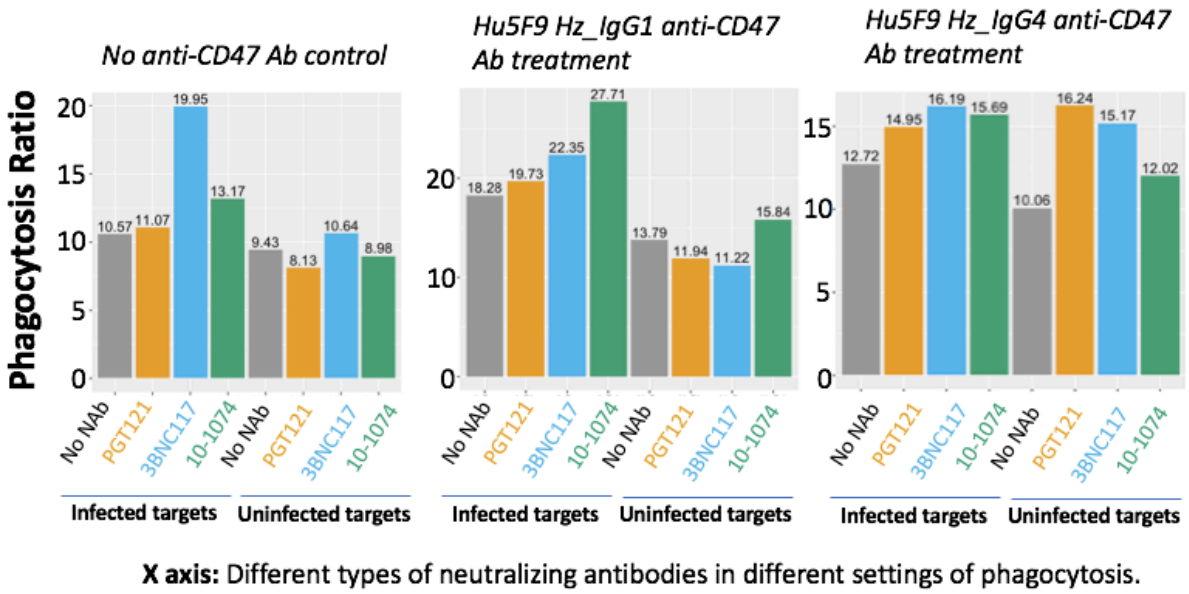


Figure 5. 10-1074 bNAb and Hu5F9 IgG1 anti-CD47 antibody cooperated for maximum clearance of SHIV-AD8-EO-infected $CD4^+$ T cells. X axis shows different types of neutralizing antibodies in different settings. Three panels represent No anti-CD47 antibody control, Hz_IgG1 anti-CD47 antibody treatment and Hz_IgG4 anti-CD47 antibody treatment respectively. Grey bars = No neutralizing antibodies used; orange bars = PGT121 neutralizing antibody type; blue bars = 3BNC117; green bars = 10-1074. For each type of neutralizing antibody, they target both SHIV-AD8-EO infected CD4+ T cells and uninfected J-Lat targets. For example, the second bar shown in the second panel means that MDMs treated by PGT121 + Hz_IgG1 anti-CD47 antibody had 19.73% phagocytosis ratio towards SHIV-infected CD4+ T cells.

3.6 A possible dynamic balance between CRT and CD47 in the J-Lat-MDM model *in vitro*

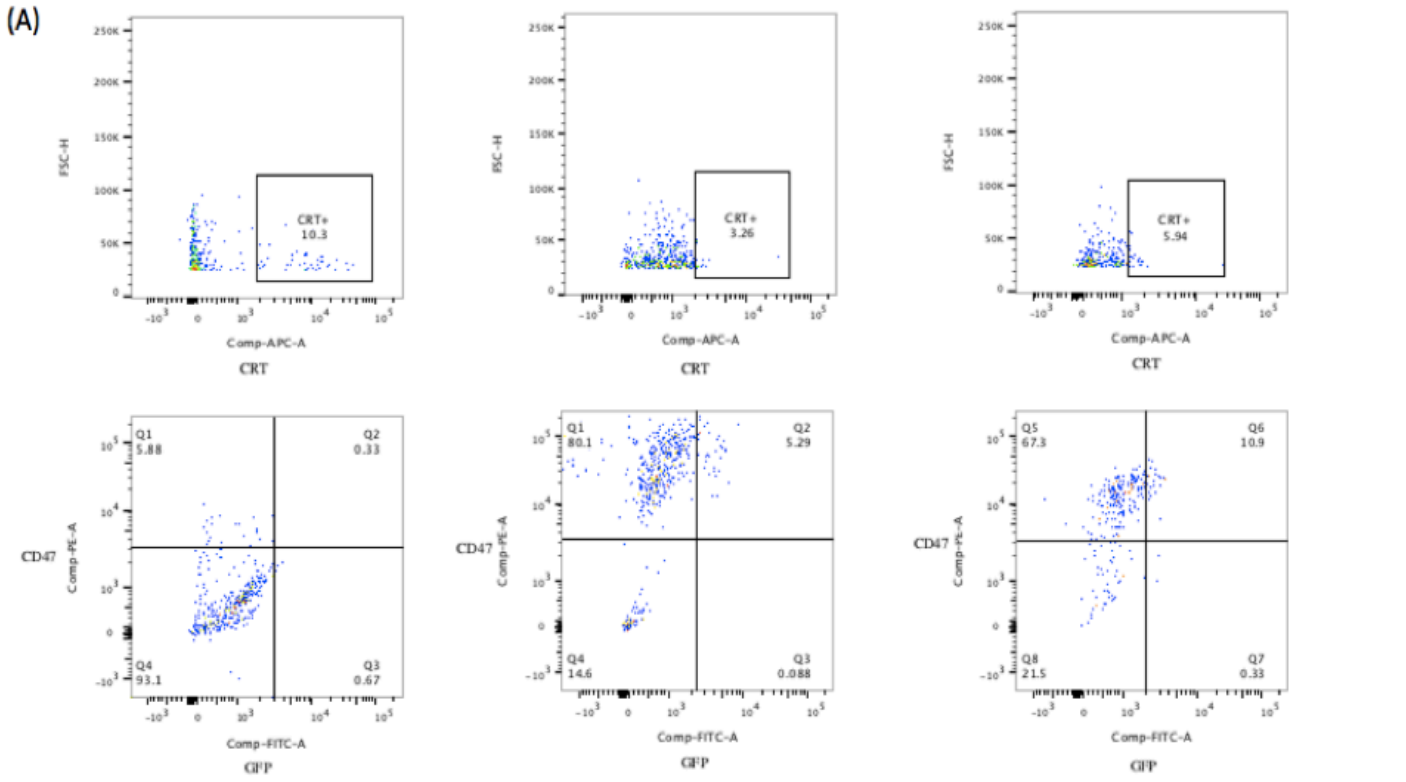
CRT (Calreticulin) serves as a pro-phagocytic signal so that unwanted or dead cells can be cleared by macrophages [90]. CD47 normally provides the anti-phagocytic signal to protect cells and to counter the effect of CRT. The balance between these two signals was proven in cancer studies. To investigate whether a similar balance exists in the setting of latently infected cells *in*

vitro, we had one hypothesis shown in Figure 6F that how anti-CD47 antibodies may interfere the balance in the setting of HIV latently infected cells. We detected the percentage of CRT-, CD47-, or GFP-positive cells among J-Lat cells or MDMs under different conditions. We used FlowJo v10 software to gate on different cell populations for data analysis.

Figure 6-A shows the frequencies of CD47, GFP or CRT expression among MDMs, untreated J-Lat cells, or TT-treated J-Lat cells before co-incubation. Live MDMs rarely expressed CD47, but up to about 10% of MDMs were CRT+. About 85% of untreated J-Lat were $CD47^+$, but only 1.6% of them expressed CRT. For TT-treated J-Lat cells, fewer $CD47^+$ cells (77%) but more CRT^+ (5.6%) were detected. Repeated experiments had similar results (n=3). Figure 6-B presents further analysis of expression of these markers. For J-Lat cells, a majority of CRT^+ cells were also $CD47^+$, indicating some form of co-regulations.

We further investigated expression of these markers in co-incubation, in order to better characterize the interaction between MDMs and J-Lat cells. Figure 6-C shows the percentages of CD47, CRT positive cells for both untreated J-Lat and TT-treated unattached J-Lat after co-incubation with MDMs. After co-incubation, for both treated and untreated J-Lat, more CD47+ cells were detected. In addition, TT-treated J-Lat were more often CRT^+ after co-incubation. Increased expression of CD47 suggests intrinsic resistance to MDM phagocytosis for all J-Lat cells after co-incubation. It seems CRT tried to match up with CD47 effect to keep a balance dynamically based on flow results on two time points. However, looking back at the reference [90], the slightly increase on CRT+ cell population could be related with the original high CRT+ levels on MDMs before co-incubation.

We also detected how MDMs would change expression of these markers after co-incubation with J-Lat cells. Relatively lower CRT expression (from about 10% to nearly 1% or 4%) was detected for MDMs after co-incubation overall (Figs. 6D-E). Combining the result that CRT+ had slightly increase in unattached J-Lat cell population after co-culture, the down-regulation of CRT+ for MDMs after co-culture again indicated the possible CRT transfer from MDMs to J-Lat targets. Possibly, MDMs in the culture are "donating" their own CRT to coat the surface of J-Lat cells. MDM population were having higher CD47+ after co-incubation. This could be explained by some J-Lat cells were successfully phagocytosed by MDMs, carrying their own CD47 antigen to MDM populations.

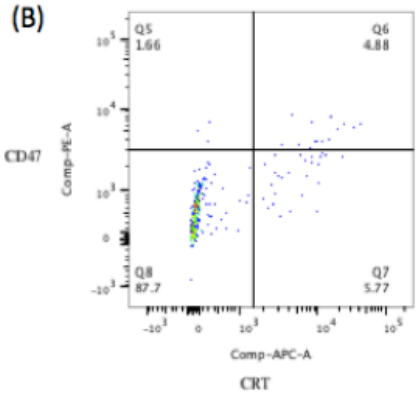


First column: MDM only before co-incubation. Upper: CRT, Lower: CD47 vs GFP.

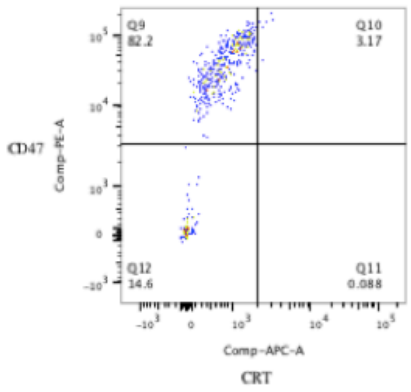
Second column: Untreated J-Lat only before co-incubation. Upper: CRT, Lower: CD47 vs GFP.

Third column: TT-treated J-Lat only before co-incubation. Upper: CRT, Lower: CD47 vs GFP.

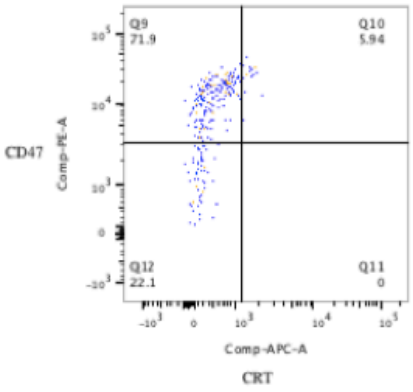
(B)



MDM only before co-incubation.
CD47 vs CRT.

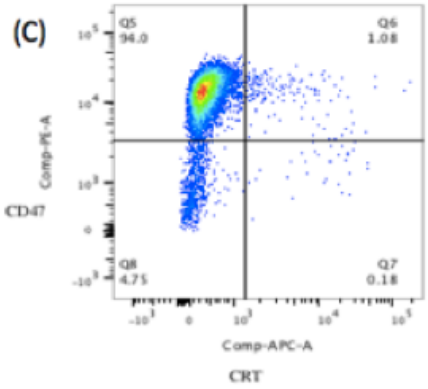


Untreated J-Lat only before co-incubation.
CD47 vs CRT.

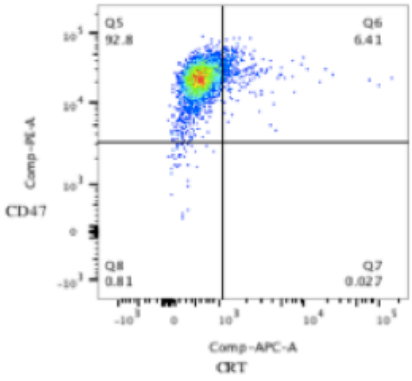


TT-treated J-Lat only before co-incubation.
CD47 vs CRT.

(C)

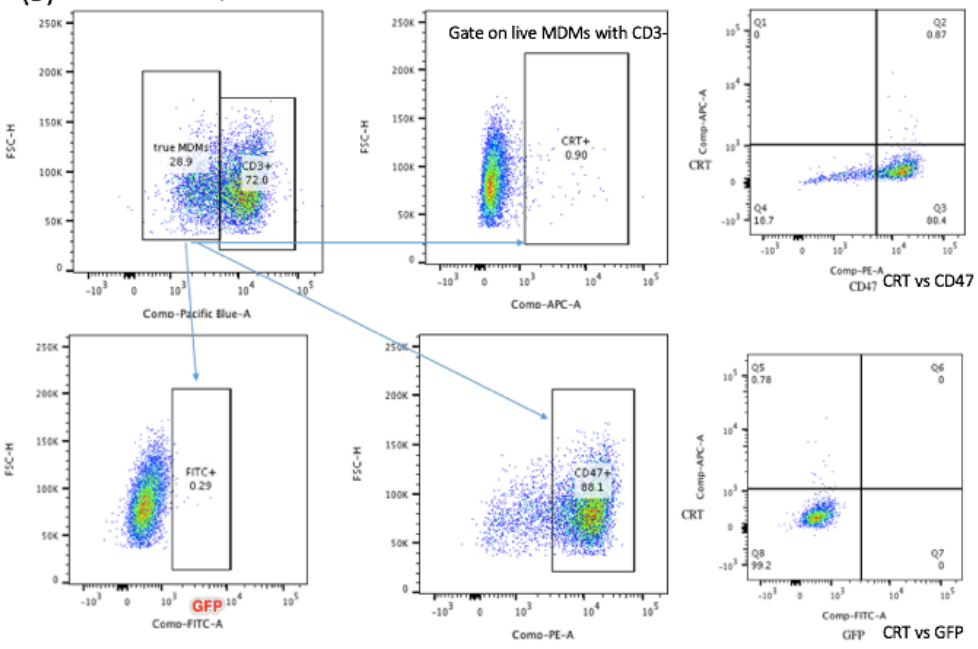


Untreated J-Lat after co-incubation.

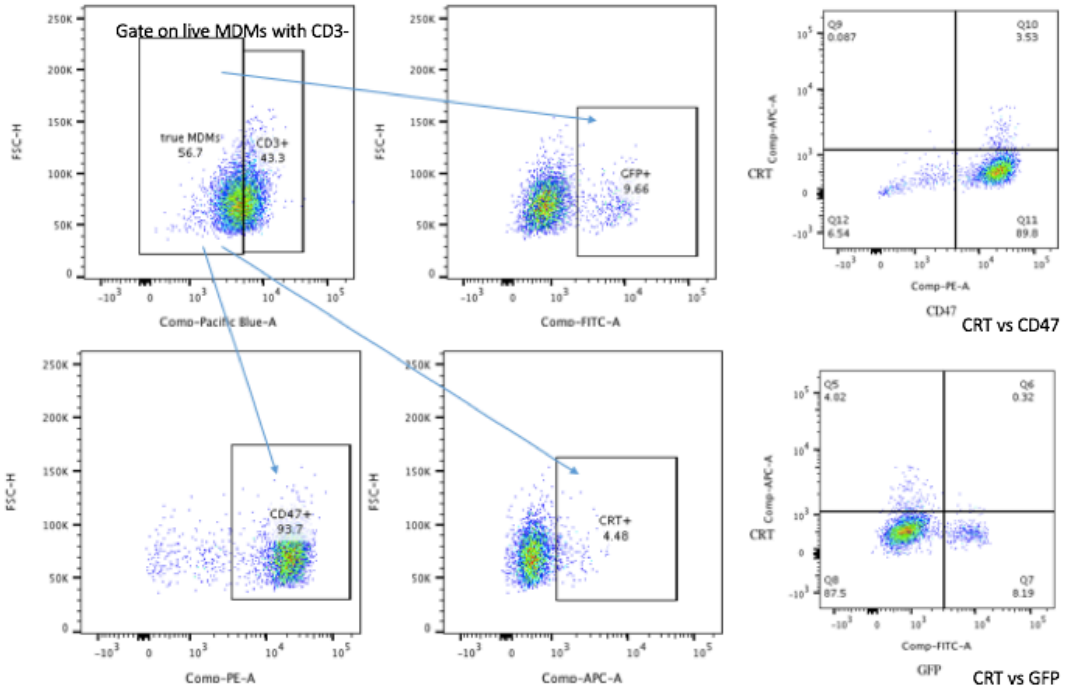


TT-treated J-Lat after co-incubation.

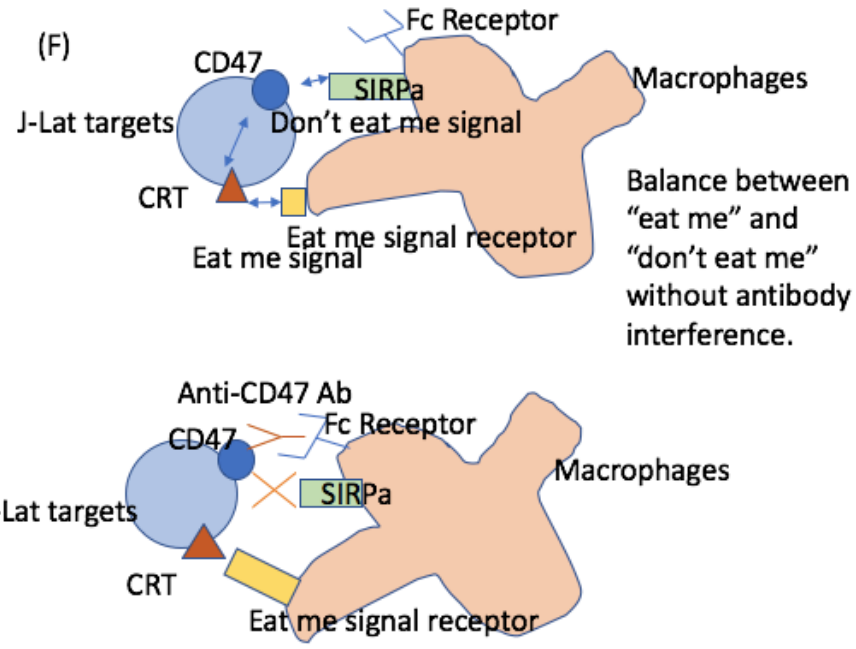
(D) MDM CRT, CD47 and GFP levels after co-incubated with untreated J-Lat cells.



(E) MDM CRT, CD47 and GFP levels after co-incubated with TT-treated J-Lat cells.



(F)



Anti-CD47 Ab destroys the interaction between CD47 and SIRPa to interfere the balance. J-Lat targets are promoted to be phagocytosed.

Figure 6. CRT, CD47 and GFP levels of MDMs and J-Lats *in vitro*. (A) MDMs, untreated J-Lats, and TT-treated J-Lats **before** co-incubation. MDMs rarely express CD47 while J-Lat were highly expressed. (B) CD47 vs CRT double gating for all the cells **before** co-incubation. CRT+ were almost all CD47+ as well, indicating these two markers are interacting each other. (C) CD47-, CRT- positive cells demonstration for **unattached** untreated and TT-treated J-Lat cells **after** co-incubation with MDMs. (D) CD47-, CRT- or GFP-positive cells for MDMs (with attached J-Lat) **after** co-incubated with untreated J-Lat. (E) CD47-, CRT- or GFP-positive cells for MDMs (with attached J-Lat) **after** co-incubated with TT-treated J-Lat. (F) Schematic showing the hypothesis that how anti-CD47 antibodies are destroying the balance between "eat me" signal and "don't eat me" signal. Upper: CRT and CD47 interact with each other to keep the phagocytosis balance when anti-CD47 antibody is not involved. Lower: The balance is destroyed when anti-CD47 blocks the interaction between CD47 and its receptor SIRPα on macrophages.

4. DISCUSSION

MDMs are required to be cultured in the right growth media to ensure that they are appropriately differentiated and functionally phagocytic. Our result showed that KPB-M15-enriched medium allowed MDMs to become more adherent. Combination of this conditioned medium with IL-1β and M-CSF cytokines promoted differentiation of more $CD14^{+}CD16^{+}$ double positive cells. Previous papers showed that the effect of KPB-M15-enriched medium was due to presence of two factors. Stem cell growth factor (SCGF), originally discovered as a product of the KPB-M15 cell line, promotes granulocyte-macrophage colony activity [92]. IL-6, also produced by KPB-M15 cells, was found useful on up-regulating functional M-CSF receptors on monocytes and macrophages [93] , which further regulates the proliferation, differentiation and CD14+ cell

maturation [94]. Due to these or other unknown factors, KPB-M15-enriched medium provides an important boost to monocytes from *rhesus macaques*, promoting proliferation, differentiation and maturation of $CD14^{+}CD16^{+}$ double positive cells [95]. When generated in the presence of KPB-M15-enriched medium the cells were strongly adherent. As IL-1β triggers the rapid differentiation of monocytes into macrophages, IL-1β was also added to our growth medium [96]. Thus, growth medium supplemented with KPB-M15-enriched medium plus additional cytokines (IL-1β and M-CSF) was ideal for MDM generation.

Antibody-dependent cellular phagocytosis (ADCP) is an essential mechanism for the host immune system to counter all types of virus infection, including HIV-1 [32]. Fc receptor genetics is strongly related to the host ADCP and was proved useful on indicating HIV-1 disease progression or risk of HIV-1 infection [32, 33]. Vaccine studies showed that Fc receptor-mediated ADCP indeed led to protection against HIV-1 [35, 36, 37]. However, type of phagocytes and antibody isotypes were also determining how effective ADCP could be [32].
To explore whether anti-CD47 antibody treatment would lead to increased phagocytosis of SIV-infected T cells by MDMs via antibody-dependent cellular phagocytosis, we compared several types of Hu5F9 anti-CD47 blocking antibodies. We tested effects on phagocytosis of either SIVmac251-infected or uninfected T cells. We first found that confocal microscopy was a reliable and easily interpretable method for assessment of phagocytosis, vs. flow cytometry. The method was tested using K562 targets and the (murine) RAW264.7 macrophage-like cells as effectors. Clear phagocytosis was observed for RAW264.7 cells stimulated by LPS, while almost no phagocytosis occurred when a phagocytosis inhibitor—Cytochalasin D was introduced (Figure. 2). The application of two contrasting fluorescent dyes (CellTrace Violet or Celltrace

Far Red) proved reliable as a method of labeling targets vs. effectors and thus measuring phagocytosis before fixation.

This analytic method was used to test how anti-CD47 antibodies were affecting the phagocytosis ratio (percentage of effectors containing engulfed targets). Hu5F9 IgG1 anti-CD47 antibody significantly ($P< 0.05$) augmented MDM phagocytosis of SIVmac251-infected T cells compared to other conditions. Although the IgG1 subclass of IgG1 gave the most impressive results, all types of anti-CD47 antibodies yielded some discernible increase in the phagocytosis ratio. Furthermore, anti-CD47 treatment had a greater effect on engulfment of SIV-infected cells than on that of uninfected cells, suggesting possible specificity that might facilitate development of anti-CD47 for inducing HIV remission. The higher phagocytosis ratio in this setting may result from the Fcγ receptors on MDM surface, ensuring the ADCP process to carry on. MDMs were originally attached to the bottom of chamber slides; HIV/SIV-infected targets were only phagocytosed as a matter of opportunity/luck *in vitro* if antibodies were not introduced. But when anti-CD47 antibodies were used, Fcγ receptors on MDMs were identified by antibodies, resulting in more MDMs being recruited as well as activated. Application of anti-CD47 antibody might be expected to further promote phagocytosis due to the blockade of the anti-phagocytosis signal that is derived from CD47 signaling. In addition, we found that anti-CD47 antibodies of the IgG1 subclass most efficiently promoted phagocytosis. This result may be explained by higher affinity of the IgG1 subclass for Fc receptors vs. IgG4 [97]. If this hypothesis is correct, then anti-CD47 antibodies constructed from an IgG1 scaffold would be expected to more efficiently recruit macrophages via Fcγ receptor binding, leading to more infected cell removal. Figure 3-E shows that the anti-CD47 antibody LALA mutation clearly reduced phagocytosis

SIV-infected T cells. This result fits with published literature demonstrating that LALA mutation of antibodies hinders contact between Fcγ receptors and effector MDMs, disrupting antibody-dependent cellular phagocytosis [98].

Since CD47 blockade using anti-CD47 antibodies stimulated phagocytosis of productively SIV-infected T cells, we speculated that such blockade might also be effective in the setting of HIV latency. We used the J-Lat cell line, with or without activating agents to force HIV transcription, to create mixed populations of reactivated and latent cells. Several adjustments were required to assess phagocytosis of J-Lat cells by microscopy, due to the bleaching that occurred with formalin fixation. First, formalin was replaced by 1% paraformaldehyde (PFA); second, we used 6-well plates instead of 8-well chamber slides, to gather all the possible green signals; third, the EVOS microscope was used instead of a confocal microscope, because the EVOS instrument is better suited to imaging GFP fluorescence.

Figure 4-A demonstrates successful activation of J-Lat cells using 5nM TPA and 5 ng/ml TNF-α. In the HIV latency assay, MDMs treated with Hu5F9 IgG1 anti-CD47 antibody demonstrated significantly higher ($P < 0.05$) phagocytosis of J-Lats, as compared to no-antibody controls. Thus, Hu5F9 IgG1 anti-CD47 antibody was effective in promoting MDM phagocytosis of latently infected cells *in vitro*. Furthermore, when we examined phagocytosis of reactivated (green/GFP^+) cells, those receiving Hu5F9 IgG1 anti-CD47 antibody treatment were phagocytosed five times more efficiently than untreated cells. This result might be explained by reactivated J-Lat cells having more CD47 expression than latently infected cells. Indeed, this hypothesis was supported by data from the flow cytometric experiments shown in Figures 5-A

and 5-C flow results. We found that almost all reactivated (GFP^+) J-Lat cells were CD47+ as well, both before and after co-incubation with MDMs. In addition, on the absolute values, we found that the reactivated J-Lat (green cells) phagocytosis ratios were lower than latently infected (red cells) conditions. Indeed, J-Lat cells were constructed using HIV-R7/E–/GFP, with GFP engineered near 3' LTR end of pseudovirus genome [99]. Such structure may make it relatively more difficult to detect GFP expression in stimulated J-Lats because GFP can only be expressed following the upstream elements. GFP signal intensity was generally lower than the very bright CellTrace colors.

To investigate another approach for treating HIV-1 infection that might leverage synergistic activity of two agents, several types of bNAbs (3BNC117, PGT121 and 10-1074) were assessed together with anti-CD47 antibody for activity against SHIV-AD8-EO. Hu5F9 IgG1 anti-CD47 antibody treatment clearly promoted the MDM phagocytosis of infected cells, having a better effect than the IgG4 subtype. Neutralizing antibodies also promoted phagocytosis, whether used alone or in combination with anti-CD47. 10-1074 bNAb was the best choice among bNAbs, because it could not only increase SHIV-AD8-EO phagocytosis on its own, but also exhibited slight synergy with Hu5F9 IgG1 anti-CD47 antibody treatment. PGT121 and 3BNC117 showed less or even no synergism with Hu5F9 IgG1 anti-CD47 antibody. This result may be explained by the epitope specificity of different bNAb types. 3BNC117 targets the CD4 binding sites of HIV-1 while PGT121 and 10-1074 target the V3 glycan [70, 71]. Stuart et al showed that CD4-binding sites are located at the more central part of HIV-1 gp120, while V3-glycans are located at more distal part of HIV-1 gp120 [100]. 3BNC117 alone was useful in promoting PrCR of

SHIV-AD8-EO infected T cells, although no synergism with Hu5F9 IgG1 anti-CD47 antibody was found.

To explore whether a CRT-CD47 balance exists in regulating PrCR of SIV productively/latently infected cells *in vitro*, we tested expression of the two markers by either J-Lat cells or MDMs under different conditions using flow cytometry. We first found that TT-treated J-Lat cells had overall lower CD47 expression but higher CRT expression compared to untreated J-Lat, suggesting a mechanism for efficient phagocytosis of reactivated cells. A potential inverse relationship between CD47 and CRT seemed to exist. However, almost all CRT+ cells were $CD47^{+}$ as well (Fig. 6B), indicating that CD47 and CRT were likely to counter each other's activities in the same cell. J-Lat cells expressed clearly more CD47 after co-incubation with MDMs. This finding could be explained by J-Lat cells providing "don't-eat me" signals towards MDMs after co-incubation to establish resistance to phagocytosis. Interestingly, slightly more J-Lat cells expressed CRT after co-incubation, as well; thus, CRT and CD47 may be expressed in dynamic balance. However, the involvement of MDMs should not be ignored, and further evidence as to effect of MDMs on the surface characteristics of J-Lats should also be tested. The fraction of MDMs expression CRT drastically dropped after co-incubation, which led to the hypothesis that MDMs were donating their CRT to J-Lat cell populations. Indeed, Feng et. al found that macrophages were producing and secreting CRT to label neutrophils for programmed cell removal, mentioning a theory called "CRT transfer" from macrophages to their targets [101]. It is possible that the J-Lat cells were providing both pro- and anti-phagocytic signals, while MDMs were responding to their signals with appropriate CRT transfer. Human tumor cells provided stronger pro-phagocytic signals to macrophages, and macrophages were also in

responsible of coordinating the anti-phagocytosis signals from CD47-SIRPα [90]. It seems clear that in some situations a CRT-CD47 balance exists, as a means of regulating MDM activity after co-incubation in the setting of HIV infection. CRT-related mechanisms may be something we may investigate in the future for combination with anti-CD47 treatment.

Macrophages are themselves targets of HIV/SIV infection. But scientists have found that high levels of the deoxynucleotide-triphosphate hydrolase, SAMHD1, were expressed by infected macrophages, which limits the viral DNA synthesis process [102]. SAMHD1 restricts HIV replication by dNTP depletion, thus restraining viral DNA synthesis [103]. More specifically, it was found that macrophages are non-permissive to HIV-1 during G0 due to SAMHD1 activity, but were vulnerable in G1 [102]. Under our experimental conditions, we were using a low dose of virus for all sections of experiments, allowing only a maximum of 12 hours' co-incubation time. Based on this information, it is unlikely that the MDMs we were using were infected to any significant degree by SIVmac251 or SHIV-AD8-EO.

5. Conclusions

A. SIVmac251-infected T cells treated with Hu5F9 IgG1 anti-CD47 antibody are efficiently cleared by MDMs via PrCR *in vitro*.

B. Hu5F9 IgG1 anti-CD47 antibody stimulated phagocytosis of both latently infected and reactivated J-Lat cells.

C. Hu5F9 IgG1 could effectively synergize with 10-1074 broadly neutralizing antibody (bNAb) to encourage phagocytosis of SHIV-AD8-EO-infected $CD4^+$ T cells.

D. Calreticulin (CRT) was found to provide a balance between pro-phagocytic and anti-phagocytic signals towards J-Lat cells.

E. Hu5F9 IgG1 anti-CD47 antibody promotes monocyte-derived macrophage phagocytosis of HIV/SIV infected cells in different infection stages.

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