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Functional and Phenotypic Analysis of B- and T-Helper Cells in Rhesus Macaques and African Green Monkeys

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FUNCTIONAL AND PHENOTYPIC ANALYSIS OF B- AND T-HELPER CELLS IN RHESUS MACAQUES AND AFRICAN GREEN MONKEYS

by

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ABSTRACT

FUNCTIONAL AND PHENOTYPIC ANALYSIS OF B- AND T-HELPER CELLS IN RHESUS MACAQUES AND AFRICAN GREEN MONKEYS
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Human immunodeficiency virus (HIV) is one of the most challenging infectious agents at the current time. To date, many vaccine trials have been conducted. However, there has not been a fully successful trial. This is due, in part, to the gap in knowledge of the protective immune response against HIV. African green monkeys (AGMs) serve as an interesting model to study immune protection in primate immunodeficiency virus infections due to their resistance to AIDS. Experimental SIV infection in rhesus macaques (RMs) resembles HIV infection in humans with chronic immune activation and progression to AIDS well characterized in both. Understanding the immune systems of AGMs and RMs and how they differ is likely to advance our knowledge of immune protection and immunopathogenesis in SIV/HIV disease. B cells are responsible for the humoral arm of acquired immunity, which results in the production of specific antibodies. In lentiviral infections, neutralizing antibodies block viral entry and are, therefore, essential for protective immunity. Other antibody-mediated functions, such as antibody-dependent cellular cytotoxicity, have gained increased interest after the recent demonstration of enhanced antibody-dependent cell-mediated cytotoxicity (ADCC) in vaccinees in a vaccine trial commonly known as the Thai trial. T helper cells provide co-stimulatory signals that are essential for B cell activation and antibody production. Blocking CD40/CD40L interactions impairs antiviral humoral response, which emphasizes the importance of B and T helper interaction for protective

humoral response. The current study aims to identify key B-cell and T-cell-related differences that separate African green monkeys and rhesus macaques in term of constitutive function (i.e. in the absence of infection) and *in vitro*, SIV-induced dysfunction. In this study, multiparametric flow cytometry approaches were used to identify any differences in the distribution of B and T cell subsets. B cells were stimulated with anti-IgM/G and T cells with phorbol myristate acetate and ionomycin (PMA/I) to assess the expression of their activation markers. These are unspecific stimuli that give an idea about the potential responses of the cells following specific stimulus. Also, B cells were evaluated for the expression and phosphorylation of intracellular signaling proteins. In addition, T cells were evaluated for the production of IFNɣ, TNFα, IL-2, expression of CD107a, and their ability to have more than one function. The results showed that AGMs have lower frequencies and absolute numbers of B and CD4+ T cells than RMs. Moreover, B cells of AGMs showed higher expression levels of CD40 than RMs, a molecule that plays an essential role in antibody production and Ig class switching. Furthermore, the analyses of B cell signaling proteins showed that AGMs express higher levels of these proteins and they get phosphorylated in higher levels than RMs. This may indicate a more robust signaling activity of AGMs' B cells as compared to RMs, which may play a role in an early antibody response that controls the infection. T cells data analyses revealed that CD4 T cells of AGMs produce less IFNɣ, TNFα, and IL-2 than RM, which may indicate another immediate response, anti-inflammatory, rather than pro-inflammatory response. Also, this may explain the lack of chronic immune activation in AGM. DN T cells of AGM showed higher expression levels of CD107a than the DN T cells of RM. This may indicate the cytotoxic function of these cells in addition to their helper functions. DN T cells are resistant to SIV infection, and their multifunctionality give insights into new cellular therapeutic approaches.

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LIST OF ABBREVIATIONS

HIV, Human immunodeficiency virus

AIDS, Acquired immunodeficiency syndrome

SIV, Simian immunodeficiency virus

AGM, African green monkey

RM, Rhesus macaques

SM, Sooty mangabeys

CD, Cluster of differentiation

DP. Double positive

SP, Single positive

EC, effector cells

EM, effector memory

CM, Central memory

BCR, B cell receptor

ART, Antiretroviral therapy

ITAMS, Immune-receptor tyrosine-based activation motifs

Syk, Src-tyrosine kinase

BLNK, B cell linker protein

PLCγ2, Phosphorylates phospholipase C gamma 2

DCs, Dendritic cells

GC, Germinal center

ADCC, Antibody-dependent cellular cytotoxicity

SAC, *Staphylococcus aureus* Cowan

PWM, Pokeweed mitogen

EBV, Epstein–Barr virus

PMA, Phorbol myristate acetate

I, Ionomycin

TNF-α, Tumor necrosis factor-alpha

IL, Interleukin

Ag, antigen

Ig, immunoglobulin

IFN-γ, Interferon gamma

LAMP-1, Lysosomal-associated membrane protein-1

PTM, Pigtailed macaques

PBMCs, peripheral blood mononuclear cells

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CHAPTER I: INTRODUCTION

B Cells

Activation and Dynamics

B cells are one of the main components of acquired immunity. The main function of B cells is secretion of antibodies that protect against pathogens. B cells recognize an antigen through the B cell receptor (BCR). The interaction of the antigen and the BCR triggers phosphorylation of immune-receptor tyrosine-based activation motifs (ITAMS), which are located on the cytoplasmic tail of CD79A and CD79B of BCR. These activate Src-tyrosine kinase (Syk) that phosphorylate B cell linker protein (BLNK) (Rowley et al. 1995; Ishiai et al. 1999). BLNK in turn phosphorylates phospholipase C gamma 2 (PLCγ2) (Ishiai et al. 1999). This signaling cascade leads to activation of the B cell.

Naïve mature B-lymphocytes leave the bone marrow and get activated either in peripheral blood or in secondary lymphoid organs. B cell in the secondary lymphoid organs encounter antigens presented by Dendritic cells (DCs), proliferate and either differentiate into short lived plasma cells with low affinity antibodies or enter germinal center (GC) reaction. In the GC, B cells proliferate to produce high affinity antibody-secreting plasma cells and memory cells. Memory cells are responsible for antibody secretion during secondary immune responses, in which they rapidly expand and differentiate into plasma cells in response to recall antigen encounter (reviewed in (LeBien and Tedder 2008)).

The distribution of B cell subsets in the peripheral blood of healthy donors is composed of 56-60% naïve B cells, 28 to 40% memory cells (Klein, Rajewsky, and Kuppers 1998; Spaks, Spaka, and Rivkina 2011), 3% plasma cells (Spaks, Spaka, and Rivkina 2011), and 2-3% immature B

cells (Sims et al. 2005). Defects in B cell function as antibody-producing cells and alterations in the distribution of B cell subsets have been found in some diseases and infections.

The Humoral Response

B cells are responsible for the humoral arm of acquired immunity, which results in the production of specific antibodies. In lentiviral infections, neutralizing antibodies block viral entry and are, therefore, essential for protective immunity (Frankel et al. 1998). During HIV infection, these antibodies are produced against the initial viral strain. However, the HIV virus evolves and escapes rapidly by inducing mutations in the targeted epitopes (Burton, Stanfield, and Wilson 2005). HIV evolution makes this antibody response ineffective in controlling HIV infection. However, neutralizing antibodies that target conserved regions of the virus antigens can neutralize a broader number of strains (Burton, Stanfield, and Wilson 2005). Induction of this type of antibodies is the goal of many HIV vaccine designers. Simek, M.D et al. have reported cases in which such broadly neutralizing antibodies were naturally generated in elite controllers, a rare population of infected persons who are able to control HIV infection without a need for retroviral therapy (Simek et al. 2009). Such broadly neutralizing monoclonal antibodies in this group of HIV infected individuals could help in identifying an effective epitope for HIV vaccine design. In addition, several studies showed that passive administration of neutralizing antibodies could prevent HIV and simian immunodeficiency virus (SIV) infection in non-human primate models (Shibata et al. 1999; Prince et al. 1991; Putkonen et al. 1991).

Other antibody-mediated functions, such as antibody-dependent cellular cytotoxicity (ADCC), have gained increased interest after the recent demonstration of enhanced ADCC in vaccinees in a vaccine trial commonly known as the Thai trial (Bonsignori et al. 2012) . RV144 (Thai-trial) is the only HIV vaccine trial that showed partial protection with 31.2% efficacy (Rerks-Ngarm et

al. 2009). ADCC is mediated by non-neutralizing antibodies, in which the IgG Fab portion binds to the viral antigen presented on the surface of infected cells, leaving the Fc portion of the antibody free to bind to effector cells. This process brings effector cells in close proximity to infected target cells, whereby effector cells lyse or induce apoptosis of target cells.

Elite controllers have showed higher ADCC titers than viremic individuals (Lambotte et al. 2013; Baum et al. 1996). The studies mentioned above indicate the importance and the role of B cells and humoral response in controlling HIV infection. However, most HIV patients are unable to produce broadly neutralizing antibodies, or protective non-neutralizing antibodies. Failure of producing such antibodies by HIV patients is due to HIV induced B-cell pathogenesis. Understanding the mechanisms that could protect against HIV-induced B-cell dysfunction can lead to developing new HIV-treatment approaches.

B-Cells Abnormalities in HIV-Infected Subjects

Alteration in the Distribution of B Cell Subsets

HIV patients demonstrate B cell dysfunctions and alterations in B cell subsets. HIV-infected people showed higher count of immature transitional B cells, exhausted B cells, activated mature B cells, and plasmablasts in the peripheral blood compared to uninfected people (Susan Moir and Fauci 2009). It was found that HIV infected patients demonstrated a reduction in the circulating memory B cells (CD19+, CD27+) (Titanji et al. 2006). Also, there was a correlation between memory B cells and CD4+ T-cell counts, which may indicate that memory B cells can be used as a marker of the progression of HIV infection (Titanji et al. 2006). Rhesus macaques (RMs) also showed loss of memory B cells during SIV infection (Klatt et al. 2011; Kling, Shipley, and Norris 2011). In addition, it was found that HIV-infected patients had higher total frequency of plasmablast compared with uninfected donors (Doria-Rose et al. 2009). Moreover, Susan Moir et

al, described a new subpopulation of B cells detected in the circulating blood of HIV patients, which has been referred to as exhausted cells (Susan Moir et al. 2008). These cells are characterized by CD21 ^{low}, high expression of CD20, and low-to-intermediate expression of CD27 (Susan Moir et al. 2008). In addition, exhausted cells demonstrated high expression levels of inhibitory receptors, and alteration of migration molecules, which give similar pattern to virus-exhausted T cells (Susan Moir et al. 2008). Also, these exhausted cells showed a decrease in proliferation, immunoglobulin diversities, and fewer cell divisions occurred compared with CD27+ B cells (Susan Moir et al. 2008). Moreover, these cells showed a decrease in the response to stimuli, but they showed enriched HIV-specific responses (Susan Moir et al. 2008).

Defects in B cell responses and proliferation

HIV impairs the functions of B cells. Memory B cells of patients with primary and chronic HIV infection showed a defect in responding to measles, and *Streptococcus pneumoniae*, while memory B cells of long-term non progressors showed similar frequency, and antigen –specific antibody responses as those of healthy blood donors (Titanji et al. 2006). Moreover, the antiretroviral therapy did not reverse memory B cell defects in primary and chronic HIV-infected patients (Titanji et al. 2006).

Malaspina et al, tested the ability of B cells of HIV-infected and uninfected subjects to proliferate in response to CpG-B. They found that the B cells of HIV-infected subjects who did not receive antiretroviral therapy had lower proliferative capacity and diminished cytokine secretion compared to uninfected or infected subjects under treatment (Malaspina et al. 2008). In addition, B cells from HIV-infected patients were unable to differentiate *in vitro* following stimulation with *Staphylococcus aureus* Cowan (SAC) and anti-CD40 monoclonal antibodies

(Conge et al. 1998). B cells of SIV infected rhesus macaques also have showed impairment in response to neo and recall antigens (Klatt et al. 2011)

In spite of the hyperactivation of B cells by HIV, B cells showed poor response to other stimuli. Moir et al. compared B cell's ability to proliferate during viremia and after effective antiretroviral therapy (S Moir et al. 2001). They found that B cells showed poor response to various stimuli during viremia, especially $CD21^{low}$ fraction compared to $CD21^{high}$ (S Moir et al. 2001). Also, Miedema et al. investigated the white blood cells of HIV infected homosexual men (Miedema et al. 1988). These men had been infected for two years, and had normal CD4 T cell counts. They found that the B cells of these patients in the presence of allogeneic T-helper cells are not able to produce Ig in response to pokeweed mitogen (PWM) (Miedema et al. 1988). The authors concluded that the HIV infection caused significant defect in the immune cell function very early, before the decline in the CD4+ T cell count (Miedema et al. 1988).

Immunoglobulin Secretion and Cytokines

HIV-infected individuals demonstrate hypergammaglobulinemia, and increases in B cell activation and proliferation, which may lead to B-cell malignancies. When normal B-lymphocytes were incubated with retrovirus, the cells showed high rates of proliferation and differentiation, which was independent of accessory cells. The magnitude of this proliferation was similar to the proliferation rate in response to *Staphylococcus aureus* cowan strain I or Epstein-Barr virus (EBV) (Schnittman et al. 1986). Hypergammaglobulinemia was found in SIV-infected rhesus macaques as well (Kling, Shipley, and Norris 2011; Amos et al. 2013). Rhesus macaques have shown 2-fold increase in their peripheral immunoglobulin within one year of SIV infection (Amos et al. 2013).

The studies mentioned above indicate the significant B-cell dysfunction in HIV-patients. Some studies had reported possible mechanisms behind the B-cell pathogenesis. Macchia et al. found that expressing TNF-alpha by HIV-infected T-cells causes polyclonal B-cell activation and immunoglobulin production, which may contribute to hypergammaglobulinemia and development of B-cell malignancies during HIV-infection (Macchia et al. 1993). In addition, Muller et al. reported a higher level of IL-10 and expression of CD40 ligand by T lymphocytes, which directly correlated with serum IgG. This suggests that IL-10 and interaction of T cells with B cells via CD40 are a possible mechanism of B cells polyclonal activation (Müller et al. 1998). They also reported that injection of Ig intravenously caused an increase in IL-10 levels, which served as a positive feedback mechanism to increase the production of antibodies. This indicates a vicious cycle of IL-10 secretion and antibody production (Müller et al. 1998).

Since the humoral immune response is an important defense mechanism against HIV, better understanding of B cells pathogenesis will help in advancing HIV treatments and vaccine trials. Identifying mechanisms by which B cells can avoid HIV-induced dysfunction can lead to new treatment that complement the current ART.

T helper Cells

Naïve T cells get activated by antigens in secondary lymphoid tissues and differentiate to effector cells and memory cells. Memory T cells can be subdivided into effector or central memory cells. Naïve cells have low immediate functions, and require high costimulatory signals (Croft, Bradley, and Swain 1994). In comparison, T effector memory have immediate effector functions, require low costimulatory signals (Croft, Bradley, and Swain 1994), and they showed faster kinetics of cytokines production and proliferation in response to a lower dose of Ag compared to naïve T cells (Rogers, Dubey, and Swain 2000). Effector T cells are comparable to

T memory cells in their kinetics of cytokines secretion and proliferation (Bachmann et al. 1999). T central memory cells have no immediate functions but they proliferate and differentiate to effector memory upon antigen stimulation in the secondary lymphoid organs (Sallusto, Geginat, and Lanzavecchia 2004).

T helper cells play a major role in the production of protective humoral response. They activate B cells by ligation to CD40 and B7 on the surface of B cells; these ligations provide costimulatory signals that activate B cells. T cells also secrete cytokines that participate in B cell activation, proliferation, and antibody production. Depletion of CD4+ T cells had a sever effect in the ability of B cells to produce antiviral antibodies (Ahmed, Butler, and Bhatti 1988).

In addition, T cells have multiple effector functions such as proliferation and induction of other cells proliferation, secretion of chemoattractants that organize immune responses, and elimination of infected cells by cytolytic activity or secretion of cytokines (Seder, Darrah, and Roederer 2008). The evaluation of T cell cytokine secretion usually assessed by IFN-γ, TNF-α, and IL-2 producing T cells. In addition, other functions such as cytolytic activity can be evaluated by detection of a degranulation marker, CD107a, which also known as lysosomal-associated membrane protein-1 (LAMP-1) (Alter, Malenfant, and Altfeld 2004). T cells that have two or more functions referred to as multifunctional CD4+ T cells. These cells reflect the quality of T cell response (Seder, Darrah, and Roederer 2008). Kannanganat et al evaluated HIV-specific CD4 T cells for the production of IFN-γ, TNF-α, and IL-2 in two HIV-1-infected groups. They found that HIV controller had higher percentages of CD4 T cells producing two or three cytokines compared to non-controllers. These multifunctional T cells showed an inverse correlation with viral load (Kannanganat et al. 2007).

Non-Human Primate Models

Non-human primates are valuable models in biomedical research. This is due the biological and physiological similarities between humans and non-human primates, which make these animals important models of immunological studies and infectious diseases. Importantly, they get infected with some pathogens that infect humans and show similar pathogenesis. For example, Cynomolgus macaques serve as an important model of *Mycobacterium tuberculosis* infection. This is due to the similar symptoms that these animals develop during *Mycobacterium tuberculosis* infection to humans. As a result, these animals are used to study pathogenesis, disease progression, immunological response and vaccine development to *Mycobacterium tuberculosis* (Flynn et al. 2003; J. A. M. Langermans et al. 2005; J. A. Langermans et al. 2001; Capuano et al. 2003). HIV infection in humans is very complicated, which made finding a suitable model to study the pathogenesis of this infection a difficult task. However, non-human primates seem the best model that we have at the current time to study HIV infection. This is due to the studies that showed that chimpanzees (*Pan troglodytes*) are reservoirs of (HIV-1), and sooty mangabeys (*Cercocebus atys*) of (HIV-2). One of the most widely used non-human primate model in HIV research is rhesus macaque. RM develop AIDS during the course of SIV_{mac} infection, similar to human immunodeficiency syndrome. Therefore, this model has helped in understanding HIV pathogenesis (Haase 2010; Smit-McBride et al. 1998; Li et al. 2005; Gao et al. 1999; Picker et al. 2004). Moreover, they help in evaluating HIV vaccines before proceeding to clinical trials. In contrast to RM, there is African green monkey (AGM), a natural hosts of SIV (Kraus et al. 1989; Ohta et al. 1988). Interestingly, AGMs are commonly infected with SIV without developing AIDS (Krugner-Higby et al. 1990). However, SIV_{agm}, which is isolated from AGMs, can cause AIDS in other monkeys (V M Hirsch et al. 1989).

Despite their resistance to SIV pathogenesis, AGMs have moderate to high viral load and a decrease in CD4 count compared to uninfected animals (Vanessa M Hirsch). Understanding the protective mechanisms used by these animals to control the infection will help in more effective vaccine designing and treatment. While some researchers believe that these animals develop an immune response to control the infection, others believe that these animals just developed a tolerance to the SIV (Vanessa M Hirsch). Although there is no clear understanding why these monkeys don't develop AIDS, there are some published characteristics of AGMs that may contribute to their nonpathogenic SIV infection. For instance, AGMs are characterized by paucity of CD4+ T cells that may restrict viral replication due to the low count of targeted cells (Pandrea et al. 2007). Researchers hypothesized that downregulation of CD4 upon stimulation of CD4+ T cells of AGMs is a possible cause of their resistance to SIV pathogenesis (Pandrea et al. 2006; Murayama et al. 1997). In contrast, studies reported that the downregulation of CD4 during SIV infection in AGMs is insignificant (Pandrea et al. 2006). Moreover, Murayama et al. found that CD4 T cells of AGMs co-express a low level of CD8∝ antigen. Also, they found that $CD4CD8^{low}$ cells were resistant to SIV infection when they converted to CD4- cells (Murayama et al. 1997). However, these cells keep their helper functions despite the downregulation of CD4 (Perkins et al. 2014). CD4+ T cells of AGMs are also characterized by low expression of CCR5, a coreceptor of SIV/HIV viruses to invade the cells, which again reduce target cells for viral replication (Pandrea et al. 2007). Another characteristic of the non-pathogenic SIV infection is the low level of immune activation (Kaur et al. 2008), which is believed to have a major role in the control of the virus pathogenesis. However, it was reported that AGMs showed an increase in activated CD4+ during the first 10 days of infection, while rhesus macaques did not show significant activation until day 28 upon infection (Kornfeld et al. 2005). Another study showed

that the acute immune activation in SMs was comparable or higher than RMs (Estes et al. 2008). This may indicate an immediate early immune response in AGMs that may lead to the control of the infection.

Reina et al. showed that AGMs lack antibody response against Gag p27 despite the presence of Gag p27-specific T cells (Lozano Reina et al. 2009). However, another study showed that AGMs develop high titer of neutralizing antibodies (Gicheru et al. 1999). Also, Wilks et al. investigated the adaptive immune responses in the milk of AGMs since AGMs rarely pass the infection to their infants. They found that the milk of AGMs contain specific neutralizing antibodies against the autologous virus, which were absent in the milk of RMs. This may indicate the role of humoral response in protection against the virus transmission (Wilks et al. 2011).

In contrast, Schmitz et al. inhibited the adaptive immune response in AGM and pigtailed macaques (PTM) by depleting CD8+ and CD20+ lymphocytes. PTM showed rapid progression to AIDS while AGMs remained healthy. The investigators of this study concluded that the adaptive immune responses are important in protection of PMT but not AGMs (Schmitz et al. 2009). Despite the underestimation of the humoral response role in protection of AGMs in this study, many studies showed that effective humoral response is protective in HIV/SIV non-progressor humans and rhesus macaques. Also, this study does not obscure the fact that the B cells of AGMs are resistant to SIV-induced dysfunction.

B-cells compartments are considered an important part and defense mechanism of the immune system. They also included significantly in SIV/HIV infection pathogenesis. In addition, T helper function is essential for B cell activation and antibodies production. Studying B and T cell subsets, the expression of their surface molecules, and their functions in RMs and AGMs will

help to identify possible intrinsic differences, which may contribute to the control of the virus pathogenesis in AGMs compared to RMs.

The hypothesis of this study is:

T and B cells of *African green monkeys* have characteristics compatible with more robust immune responses compared to the T and B cells of *Rhesus macaques.*

This hypothesis was tested by accomplishing the following specific aims.

1. Compare B and T cell subsets in rhesus macaques and African green monkeys.
2. Identify B and T cell functional differences between rhesus macaques and African green monkeys.
   A. Comparing the expression of activation markers in T and B cells
   B. Comparing total and phosphorylated intracellular signaling proteins in B cells.
   C. Comparing cytokine secretion patterns in T cells.

CHAPTER II: MATERIAL AND METHODS

Samples and PBMC Purification

In this study healthy RMs and AGMs were used. The exact numbers of monkeys are used in each experiment, age range and genders are shown in (Table.1). The RMs are housed in the Wisconsin National Primate Research Center (Madison, WI), and the AGMs are housed in Wake Forest University-Primates Center (Winston-Salem, NC). Animal care and sample collection were performed based on the guidelines of The Institutional Animal Care and Use Committee (IACUC) of these facilities. A 15 mL whole blood sample was collected from each monkey in an EDTA tube. The PBMCs were separated by using Ficoll-Hypaque gradient centrifugation (Dagur and McCoy 2015). Briefly, the plasma was separated by centrifugation and removed. The remaining cells were diluted with phosphate-buffered saline (PBS) and layered on Lymphocyte Separation Medium (LSM) carefully. Peripheral blood mononuclear cells (PBMC) were separated by centrifugation. The plasma layer above PBMC layer was removed, and the lymphocytes layer were transferred to a new tube. The last step is to dilute PBMC with PBS and wash twice.

Cryopreservation

After separation, the PBMCs were pelleted by centrifugation at 400xg for 10 minutes. The supernatants were removed, and the cells were resuspended in freezing medium (10% DMSO, 20% FBS, 70% RPMI 1640) at concentration 2-10 x 10^6 cells/mL. The cell suspensions were divided as 1 mL per cryopreservation tube and frozen in a slow-freeze container at -80˚C overnight. Then, the cells were stored in liquid nitrogen.

Experiment	Sample Size		Gender	Age Range
B cell Subsets and activation Marker	AGM	12	5 Female, 7 Male	6-12 year
	RM	12	9 Female, 3 Male	2.8-4.5 year
The effect of SIV-infection on B cell Activation Markers	AGM	3	F	6-12 year
	RM	3	F	2.9-3.7 year
T cell Subsets and Activation Markers	AGM	12	5 Female, 7 Male	6-12 year
	RM	12	9 Female, 3 Male	2.8-4.5 year
T cell Cytokine Production	AGM	8	4 Female, 4 Male	6-12 year
	RM	9	7 Female, 2 Male	2.9-5.2 year
Intracellular Signaling proteins	AGM	5	2 Female, 3 Male	6-9 year
	RM	5	4 Female, 1 Male	2.9-3.7 year

Table 1. The number of African green monkeys (AGM) and rhesus macaques (RM) that were used in each experiment, genders, and age ranges.

Cell Revival

The cells were thawed in a 37˚C water bath. Then, the cells were washed in a 15 mL tube with 9 mL of warm (37˚C) RPMI 1640 containing 10% FBS, penicillin (100 IU/mL), streptomycin (100 µg/mL), 1 mM sodium pyruvate, and 50 µM β-mercaptoethanol by centrifugation at 500xg, for 10 minutes. The supernatant was removed and the cells were resuspended in 2.5 mL of the same medium and plated in 12-well plates. Then, the cells were incubated at 37ºC in a 5% carbon dioxide atmosphere overnight. On the second day, the cells were processed for stimulation at a concentration of 5 x 10^6 cell/ mL.

Flow Cytometry

Titration of Conjugated Antibodies

Antibody titration is performed in order to determine the proper concentration that gives the best separation of the positive and negative populations. **Figure 1** shows an example of surface antibody titration (anti-CD20-APC.H7) by using RMs' PBMC. Titer 1.25µl showed a good separation of the positive and negative populations. **Figure 2** shows Anti-pERK-PE titration by using AGMs' PBMC, 10 µl was a good titer to distinguish the two populations of cells.

B cell's Classification and Detection of Activation Markers

In this experiment 12 animals each of healthy RM and AGMs were used as shown in (Table.1). After overnight revival, the cells were washed again as described previously and resuspended in the same medium at a concentration of 5 x 10^6 cells /mL. The cells were stimulated with 10µg/ mL of F(ab')2 Anti-Human IgM+IgG Functional Grade Purified (eBioscience), unstimulated cells from each animal were used as controls. The cells were incubated for 48h in a 96-well flat bottom plate at 37ºC in a 5% carbon dioxide atmosphere

After 48 hours of stimulation, the cells were stained for live/dead by re-suspending in 1:1000 dilution of the violet fluorescent reactive dye (Live/Dead Fixable Dead Cell Stain kit, Invitrogen) and incubated for 20 minutes at room temperature in the dark. After incubation, the cells were washed twice with RPMI 1640 by centrifugation at 500xg for 10 minutes. For surface staining, the appropriate titers of the following fluorochrome-conjugated antibodies were added: anti-CD20-APC.H7 (Clone: 2H7), anti-CD21-PE.Cy5 (Clone: B-Iy4), anti-CD40-FITC (Clone: 5C3), these antibodies were purchased from BD Biosciences, anti-CD27-APC (Clone: 0323) and anti-CD86-PE (Clone: IT2.2) were purchased from Biolegend. The cells were incubated for 20 minutes at room temperature in dark, and then washed twice with RPMI 1640 by centrifugation

at 500xg for 10 minutes. After that, the supernatants were removed and the cells were fixed with 0.5% paraformaldehyde for 20 minutes at room temperature in dark. Then, the cells were washed twice with RPMI 1640 by centrifugation at 500xg for 10 minutes. After that, the cells were resuspended in 50 to 100 µl of RPMI 1640. The data was collected by using flow cytometry FACS Aria III, and the data analysis was performed by using FlowJo software. The unstimulated cells from each animal were used for B cell subsets identification. The cells were gated for singlets, the dead cells were excluded and the lymphocytes gate was performed based on SSC and FSC as shown in (Figure 3, A). The total B cells were identified as CD20+ cells, and the subsets were identified as naïve B cells (CD27-, CD21+), double positive (DP) memory B cells (CD27+, CD21+), single positive (SP) memory B cells (CD27+,CD21-), tissue like memory B cells (CD27-,CD21-) (Figure 3, A) (Das et al. 2011). For activation markers the unstimulated and stimulated cells were further gated for CD86 and CD40hi as shown in (Figure3, B).

In vitro SIV infection and detection of B cell's activation markers:

In this experiment 3 animals each of healthy RMs and AGMs were used as shown in Table. 1. After overnight revival, each sample were divide into three portions and 10µg/ mL of F(ab')2 Anti-Human IgM+IgG Functional Grade Purified (eBioscience) were added to each of them. SIVmac251 was added to one portion (titer 26,900 TCID50), 1x of SIVagm9063-2 was added to the second portion, and the third portion kept uninfected as a control. After 48 hours of incubation at 37ºC in a 5% carbon dioxide atmosphere, the same protocol mentioned previously for detection of B cell's activation markers was repeated to detect any effect of the infection on the expression of activation markers.

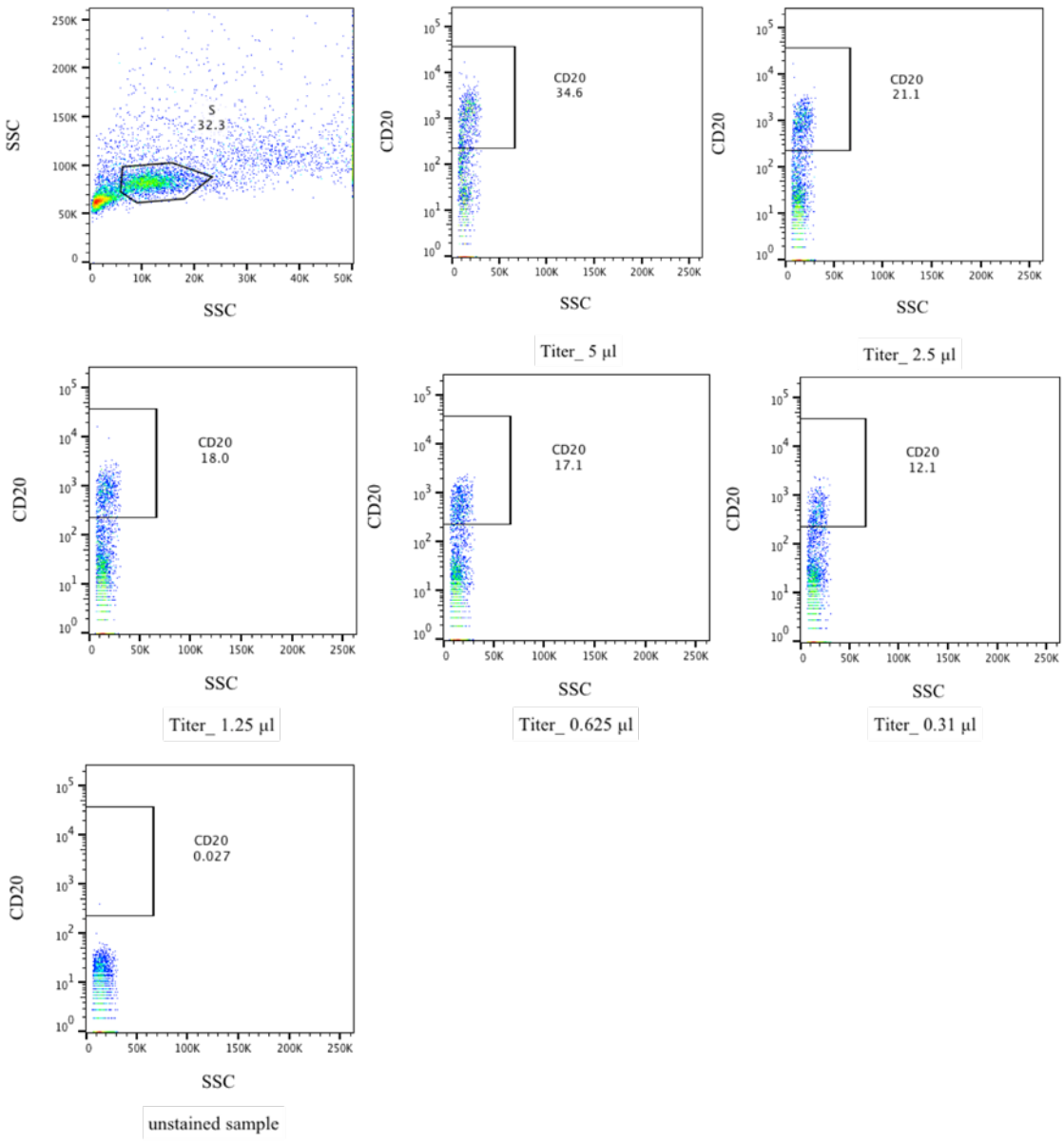


Figure 1 Titration of Anti-CD20-APC.H7 by using RMs' PBMC.

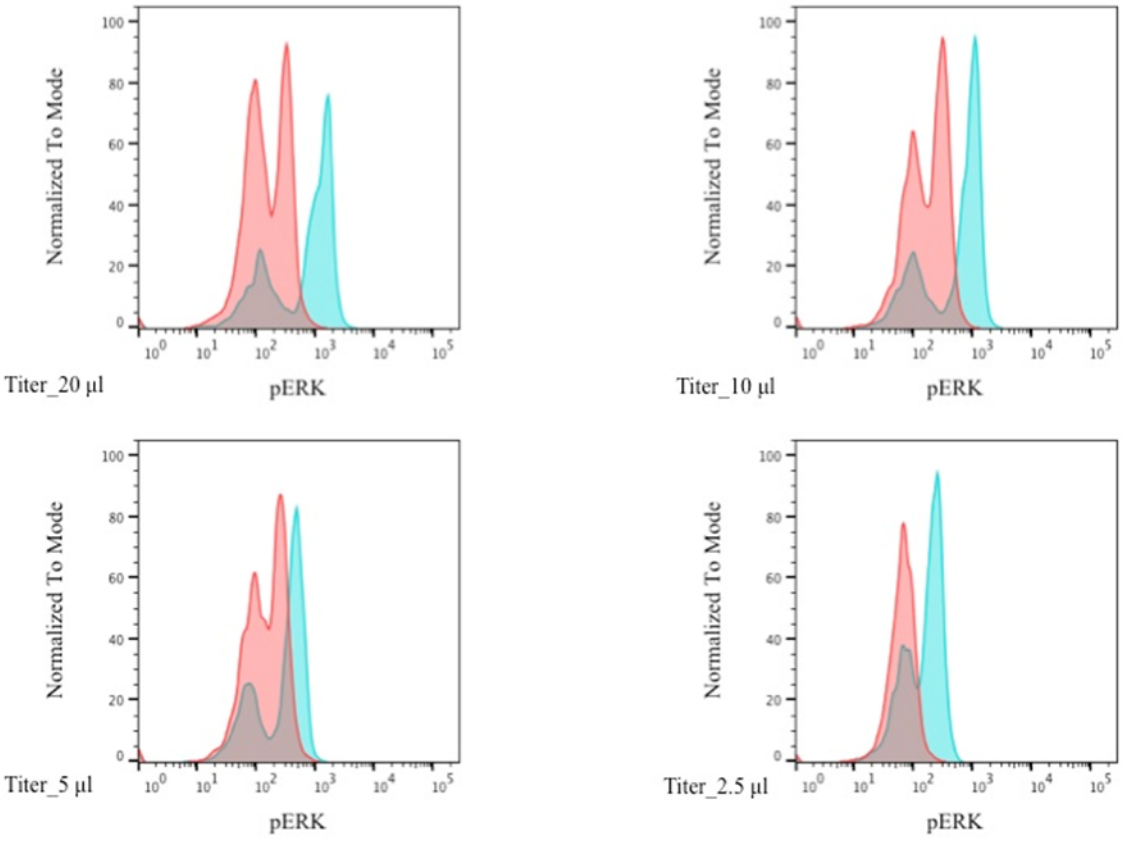


Figure 2. Anti-pERK-PE titration by using AGMs' PBMC.
Red= unstimulated cells, blue= stimulated cells

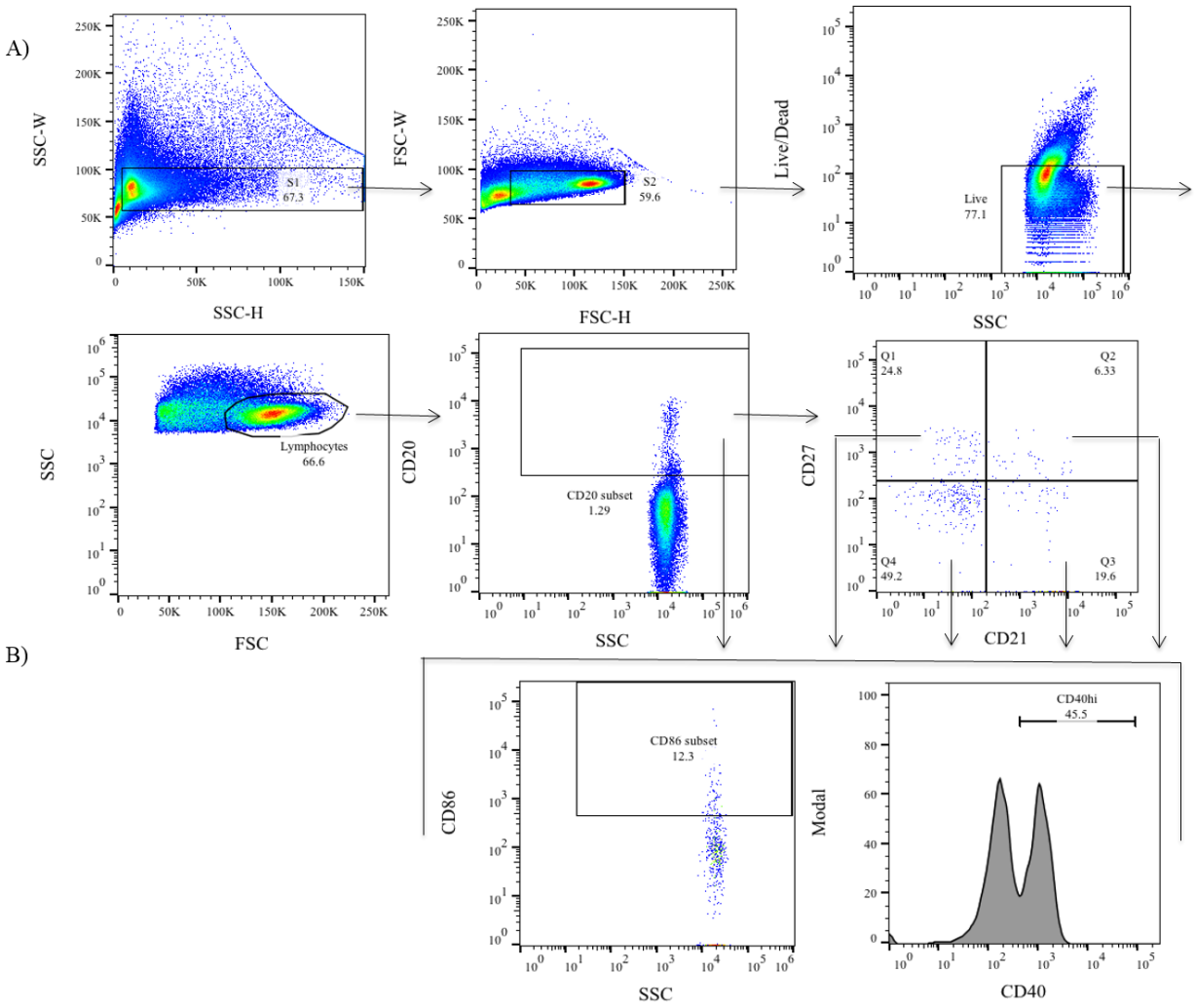


Figure 3. Gating strategy for phenotyping of B cell subsets in the peripheral blood of RM and AGMs, and characterization of surface activation markers.
(A) Unstimulated cells were gated for singlets first, and the dead cells were eliminated. Then, the lymphocytes were gated based on FSC and SSC, and the CD20+ B cells were gated based on SSC and CD20. The total CD20+ B cells were classified based on CD21 and CD27 gaiting as following: naïve B cells (CD27-, CD21+), double positive memory B cells (CD27+, CD21+), single positive memory B cells (CD27+,CD21-), tissuelike memory B cells (CD27-,CD21-). (B) Stimulated and unstimulated total CD20+ B cells and each subset of B cells were further gated for the activation markers CD86+ and CD40hi cells. CD86+%, CD86MFI, total CD40 MFI, and CD40hi MFI were calculated.

T cell's Classification and Detection of Activation Markers

In this experiment 12 animals each of healthy RMs and AGMs were used, as shown in (Table. 1). After overnight revival, the cells were stimulated with 50 ng/mL of phorbol myristate acetate and 500 ng/mL of Ionomycin (PMA/I), DMSO were add to unstimulated cells from each animal as mock stimulus. The cells were incubated for 6 hours in a 96-well flat bottom plate at 37ºC in a 5% carbon dioxide atmosphere. By the end of the stimulation period, the cells were stained with the same procedure mentioned in B cell's classification except that the antibodies were used for surface staining as follows: anti-CD3-PerCP-Cy 5.5 (Clone: SP34-2), anti-CD4-PE-CF594 (Clone: L200), anti-CD8-PE-Cy7 (Clone: RPA-T8), anti-CD25-APC-H7 (Clone: M-A251), anti-CD28-FITC (Clone: CD28.2), anti-CD45RA-PE-Cy5 (clone: 5H9), anti-CD95-PE (clone: DX2), and anti-CD154-APC (clone: TRAP1). All the antibodies were purchased from BD Biosciences. The data was collected by using flow cytometry FACS Aria III, and the data analysis was performed by using FlowJo software. The unstimulated cells from each animal were used for T cell subsets identification. The cells were gated for singlets first, and the dead cells were excluded. Then, the lymphocytes were gated based on FSC and SSC, and T cells were gated based on SSC and CD3. The total CD3+ T cells were further gated based on CD4 and CD8. T helper cells were identified as CD4+, CD8-, and double negative (CD4-, CD8-) cells **(Figure 4. A)**. T helper cell subsets were identified as naïve ($CD28^{lo}$, $CD95^{-}$), central memory CM ($CD28^{hi}$, $CD95^{+}$), and effector memory (EM) ($CD28^{-}$, $CD95^{+}$) **(Figure 4. B)** (Nishimura et al. 2004). For activation markers, total T helper cells and subsets were gated for CD25+ and CD154+. Then, means fluorescence intensity of CD25 and CD154 were calculated **(Figure 5, A)**. EM cells were further gated for the expression of CD45RA **(Figure 5, B)**.

Detection of T cell's Cytokines Production

In this experiment 9 of healthy RMs and 8 AGMs were used, as shown in (Table. 1). After overnight revival, the cells were stimulated with 50 ng/mL of phorbol myristate acetate and 500 ng/mL of Ionomycin (PMA/I), DMSO were add to unstimulated cells from each animal as mock stimulus. Anti-CD107a-APC.H7 (clone: H4A3, BD Biosciences) was added for both stimulated and unstimulated cells. The cells were incubated for 6 hours in a 96-well flat bottom plate at 37ºC in a 5% carbon dioxide atmosphere. BD GolgiPlug protein inhibitor (containing Brefeldin A) was added on the last 4 hours of stimulation. Brefeldin A (BFA) blocks protein transportation, which facilitates cytokines detection by flow cytometry.

The cells were stained for live/dead as mentioned previously. For surface staining, the appropriate titers of the following fluorochrome-conjugated antibodies were added: anti-CD3-PerCP-Cy 5.5 (Clone: SP34-2), anti-CD4-PE-CF594 (Clone: L200), anti-CD8-PE-Cy7 (Clone: RPA-T8), anti-CD45RA-PE-Cy5 (clone: 5H9), anti-CD197 (CCR7)-PE (clone: 3D12). All the antibodies were purchased from BD Biosciences. The cells were incubated for 20 minutes at room temperature in dark, and then washed twice with RPMI 1640 by centrifugation at 500xg for 10 minutes. Cells were fixed and permeabilized by resuspending in 100 µl of Cytofix/Cytoperm fixation/permeabilization solution (BD Biosciences) for 20 minutes at room temperature. By the end of staining, the cells were washed twice by BD perm/wash buffer (BD Biosciences) by centrifugation at 500xg for 10 minutes. Then, the cells were stained with anti-TNF-alpha-BV605 (clone: MAB11), anti-IL-2-FITC (Clone: MQ1-17H12), and anti-IFN-ɤ-APC (Clone: 4S.B3) for 20 minutes at room temperature in dark. All antibodies were purchased from BD Biosciences. Finally, the cells were washed twice by BD wash buffer (BD Biosciences) by centrifugation at

500 xg for 10 minutes before proceeding to flow cytometry analysis. **Figure 6** shows the gating strategy for cytokines analysis.

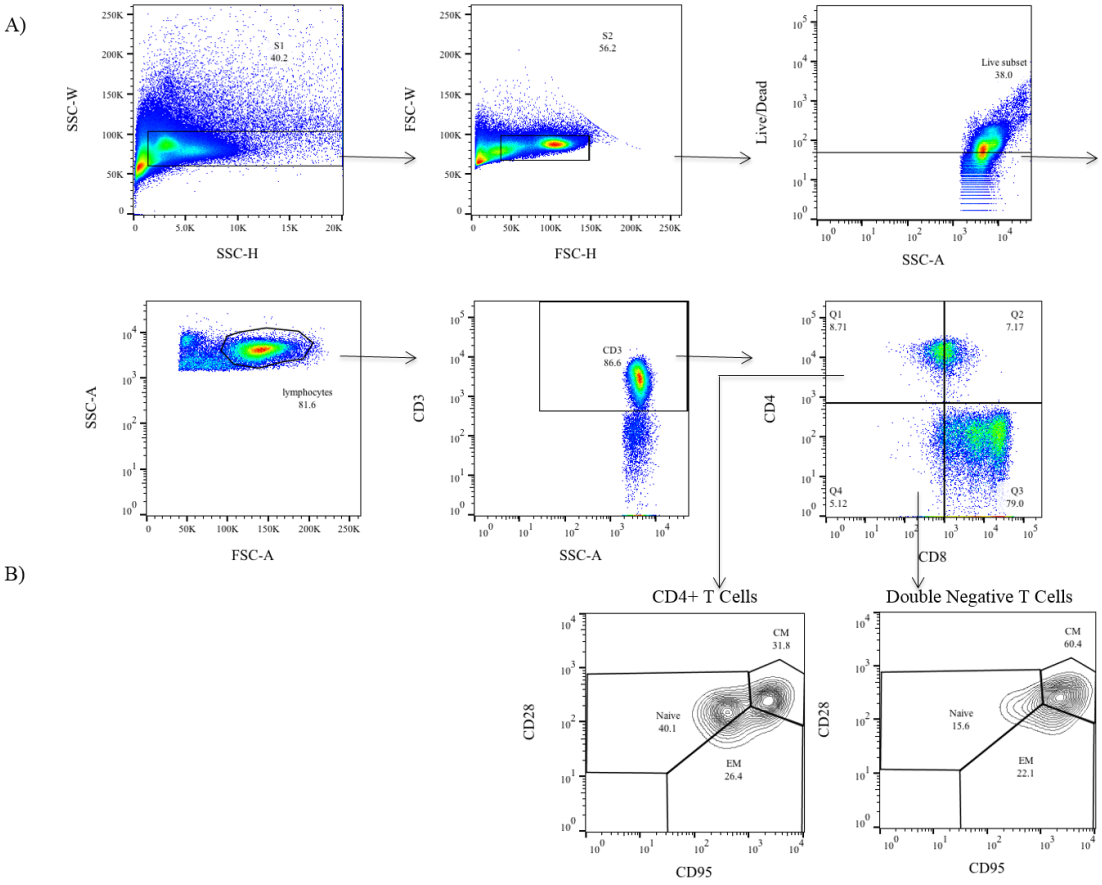


Figure 4. Gating strategy to identify T cells and subsets.
(A) The cells were gated for singlets, the dead cells were excluded, and lymphocytes gating was performed based on FSC and SSC. Total T cells were identified as CD3+. Then, T helper cells were identified based on CD4 and CD8 as CD4+ T cells (CD4+, CD8-) and double negative (DN) T cells (CD4-, CD8-). (B) CD4+ and DN T cells were further classified into subsets based on CD28 and CD95 as naïve (CD28lo, CD95-), central memory CM (CD28hi, CD95+), and effector memory (EM) (CD28-, CD95+)

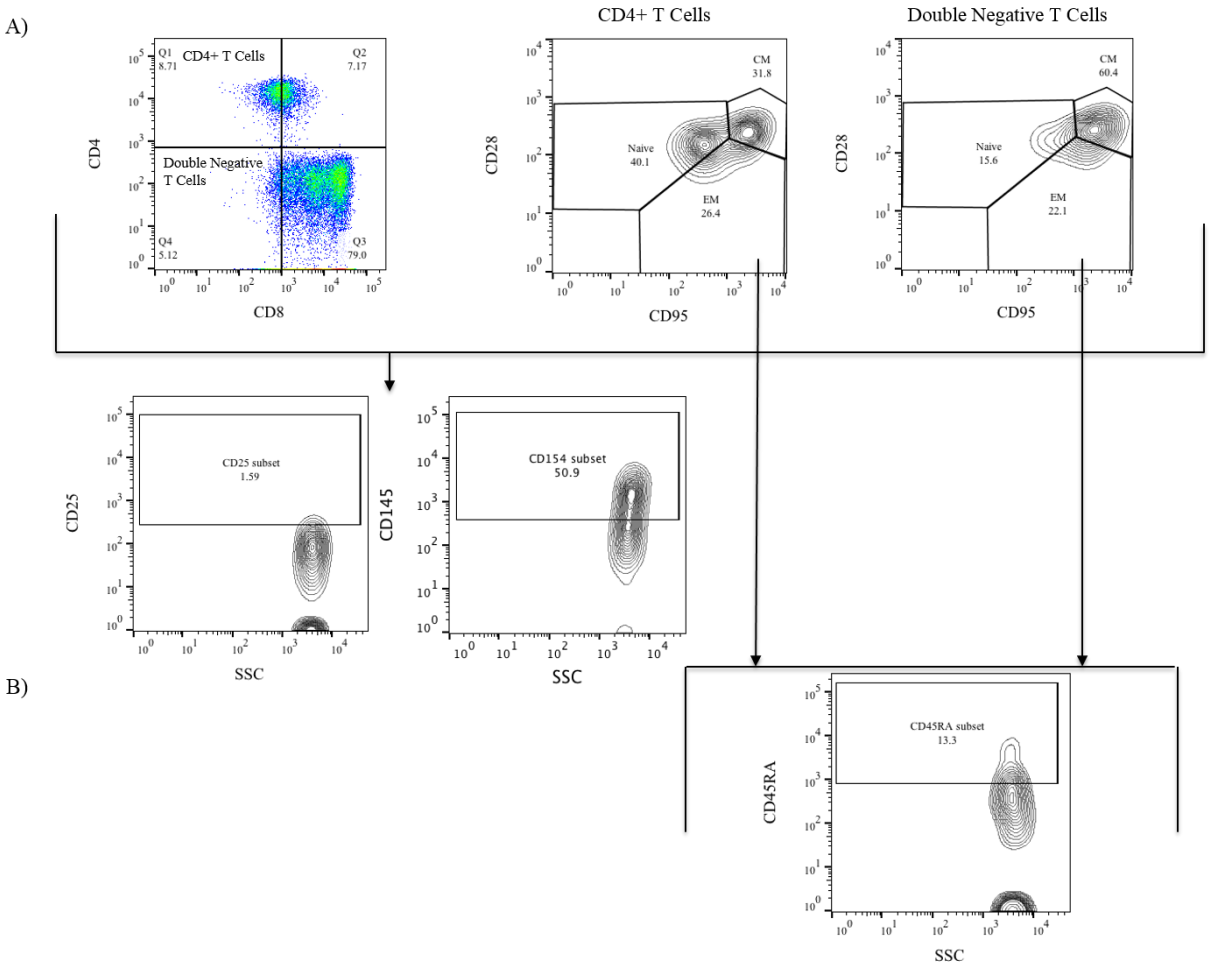


Figure 5. Gating strategy to identify T cell activation markers.
(A) Total CD4+, DN and subsets were gated for the expression of activation markers CD25 and CD145. (B) The effector memory cells were gated for the expression of CD45RA.

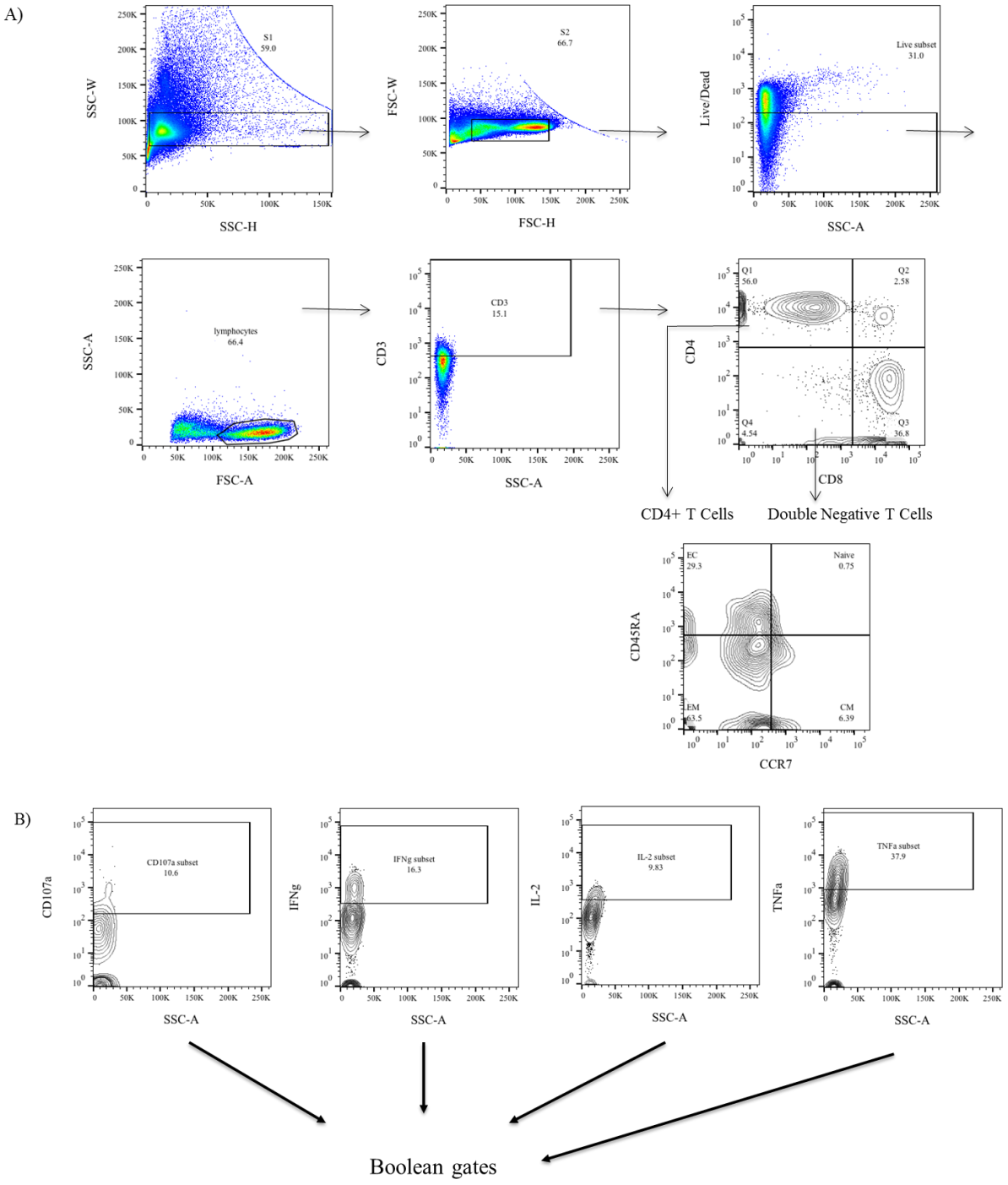


Figure 6. Gating strategy for cytokine analysis.

(A) The cells were gated for singlets, the dead cells were excluded, and lymphocytes gating was performed based on FSC and SSC. Total T cells were identified as CD3+. Then, T helper cells were identified based on CD4 and CD8 as CD4+ T cells (CD4+, CD8-) and double negative (DN) T cells (CD4-, CD8-). CD4+ and DN T cells were further classified into subsets based on

CCR7 and CD45RA as naïve (CCR7+, CD45RA+), central memory CM (CCR7+, CD45RA-), effector memory (EM) (CCR7-, CD45RA-), and effector cells (EC) (CCR7-, CD45RA+). **(B)** Total CD4, DN, and each subset were gated for IFNɣ, TNFα, IL-2, CD107a. Then, Boolean gates were performed

Detection of Phosphorylation of Intracellular Proteins

After overnight revival, cells were serum-starved by washing, and incubation in 5 ml RPMI 1640 containing penicillin (100 IU/ mL), and streptomycin (100 µg/mL), without FBS at 37°C in a 5% carbon dioxide atmosphere for 3 hours. After starvation, the cells were stained with the violet fluorescent reactive dye (Live/Dead Fixable Dead Cell Stain kit, Invitrogen) as mentioned previously. Then, the cells were stained with anti-CD20-APC.H7 (Clone: 2H7, BD Biosciences) and anti-CD27-APC (Clone: 0323, Biolegend) for 20 minutes at room temperature in dark. The cells were washed twice with RPMI 1640 by centrifugation at 500 xg for 10 minutes. Cells stimulation and fixation were taken place in 37°C water bath. The cells were pre warmed in 37°C water path for 10 minutes. After that, 10µg/ mL of F(ab')2 Anti-Human IgM+IgG was added to stimulate the cells, RPMI 1640 was added to the un-stimulated cells as a mock stimulus. The incubation continued to 10 minutes. Then, the cells were fixed with an equal amount of pre-warmed BD Phosflow fix buffer I for 10 minutes. After that, the samples were transferred to ice and washed twice with RPMI 1640. The supernatant were aspirated and the cells were permed with 50 ul of BD Phosflow perm buffer III for 30 minutes in ice. Then, the cells were washed twice with RPMI 1640 and stained with either anti-Syk (pY348)-Alexa Fluor488 (Clone: I120-722) and anti-ERK1/2 (pT202/pY204)-PE (Clone: 20A) from BD Biosciences, or anti-PLC-g2 (pY759)-PE (Clone: REA341, Miltenyi Biotec) for 30 minutes at room temperature. After staining, the cells were washed twice, and analyzed by flow cytometry. **Figure 7** shows the gating strategy that used to analyze phosphorylation of intracellular proteins by using FlowJo.

Detection of Total Intracellular Signaling Proteins

The cells were starved and stained as mentioned above in the *detection of phosphorylation of intracellular proteins*. However, to detect the total intracellular signaling proteins, the cells do not need to be stimulated. The antibodies were used to detect the intracellular proteins are anti-Syk-FITC (Clone: 4D10) and anti-ERK2-PE (Clone: G263-7), or anti-PLC-g2-PE (Clone: K86-1161). All the antibodies were purchased from BD Biosciences. **Figure 7** shows the gating strategy that used to analyze total intracellular proteins by using FlowJo.

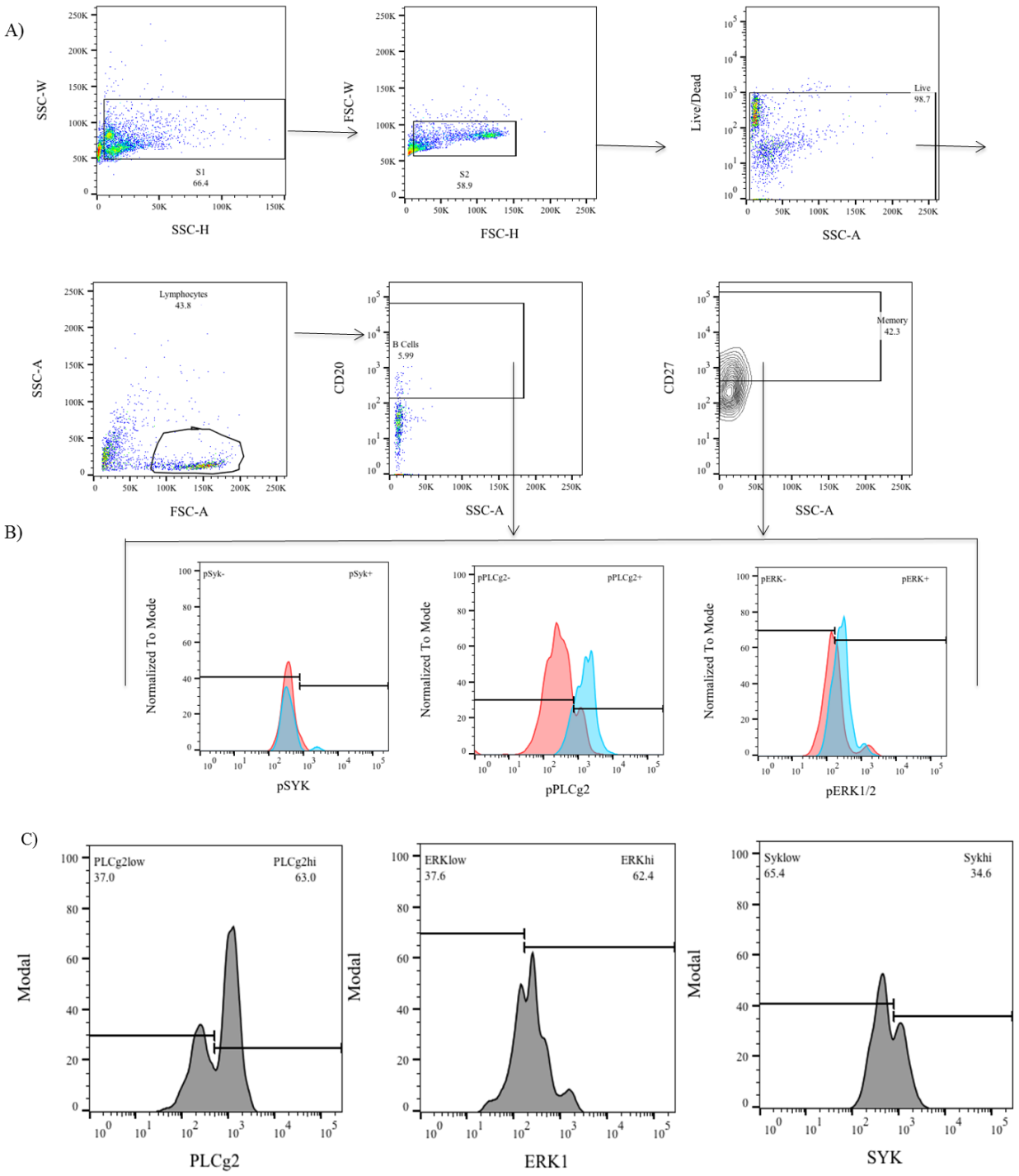


Figure 7. Gating strategy to analyze total and phosphorylated intracellular proteins. (A) Cells were gated for singlets first, and the dead cells were eliminated. Then, the lymphocytes were gated based on FSC and SSC, and the CD20+ B cells were gated based on SSC and CD20. Memory B cells were identified as CD27+ cells. (B) Total B cells and memory B cells were gated for pSYK, pPLC-g2, and pERK1/2. Gates of unstimulated (red) and stimulated cells (blue)

were emerged to identify cells that phosphorylated the intracellular proteins as shown by shifting of the cells as compared to unstimulated cells. (C) Unstimulated total and memory B cells were gated for total SYK, ERK, and PLC-g2 to identify cells hi and low with these proteins.

Data Collection and Statistics

All data were collected by using flow cytometry FACS AriaIII. Flow cytometry data were analyzed by using FlowJo software (Ashland, OR). The data were analyzed using Student's t-test, $p<0.05$ was considered significant. For the *in vitro* SIV infection experiment, repeated measure ANOVA and Tukey test were performed using GraphPad Prism 6.0, $p<0.05$ was considered significant.

CHAPTER III: RESULTS

African green monkeys have fewer B cells compared to rhesus macaques.

African green monkeys have lower counts of total lymphocytes compared to rhesus macaques (data not shown). It is also reported that African green monkeys have few CD4+ cells which might contribute to their resistance to immune deficiency syndrome despite their infection with SIV (Pandrea et al. 2007) . Prior to studying the functional parameters of B cells, we wanted to see if there are any differences in the frequencies and absolute numbers of B cells between African green monkeys and rhesus macaques. The data showed that African green monkeys have significantly lower frequency (Figure.8 A) and absolute number (Figure.8 B) of CD20+ B cells compared to rhesus macaques ($p < 0.05$). These data are consistent with Amos et al in which they showed that AGMs had fewer total B cells than RMs (Amos et al. 2013).

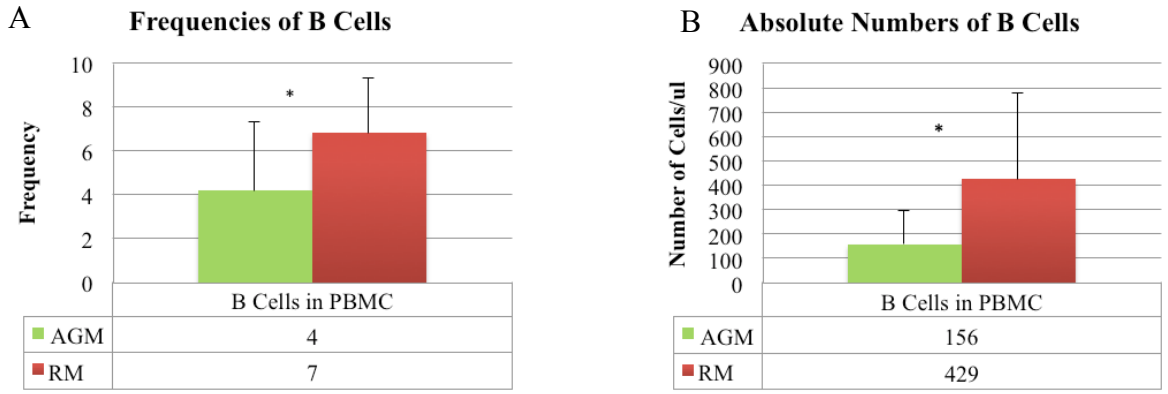


Figure 8. Frequencies and absolute numbers of B cells in the peripheral blood of African green monkeys and rhesus macaques.
African green monkeys have fewer CD20+ B cells in terms of frequencies (A) and absolute numbers (B). Absolute numbers of CD20+ B cells were calculated based on flow cytometry and complete blood counts. The data shown are the means of 12 African green monkeys (AGM) and 12 rhesus macaques (RM). p value <0.05*, or <0.01**, or <0.001***, or <0.0001****.

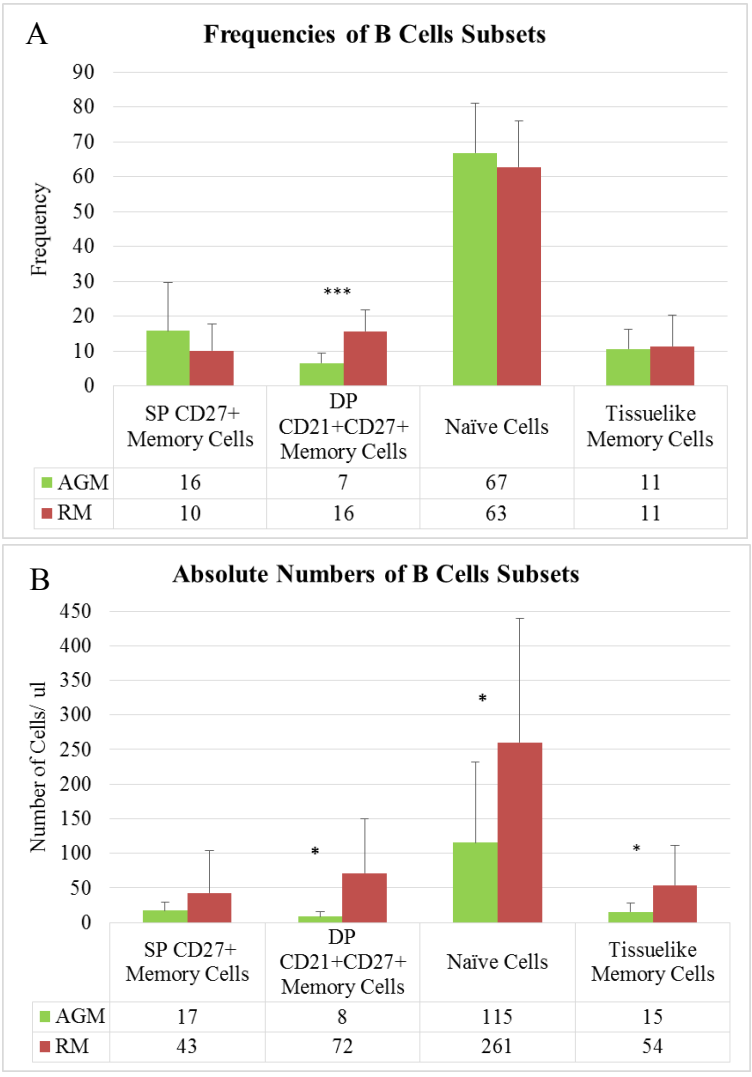


Figure 9. Frequencies and absolute numbers of B cell subsets in African green monkeys and rhesus macaques.

The CD20+ B cells were classified into subsets based on the expression of CD21 and CD27, single positive (SP) memory cells (CD21-, CD27+), double positive (DP) memory cells (CD21+, CD27+), naïve cells (CD21+, CD27-), tissue-like memory cells (CD21-, CD27-). B cell subsets differ between AGM and RM in terms of frequencies (A) and absolute numbers (B)Absolute numbers of B cell subsets were calculated based on the flow cytometry and complete blood counts as count per microliter of whole blood. The data shown are the means of 12 African green monkeys (AGM) and 12 rhesus macaques (RM). p value <0.05*, or <0.01**, or <0.001***, or <0.0001****.

B cell subsets differ between African green monkeys and rhesus macaques

We further analyzed B cell compartment to look to the subsets. The data showed that African green monkeys had higher frequencies of naïve and SP CD27+ memory cells but the differences did not reach statistical significance. The differences were significant only on DP CD21+CD27+ memory cells ($p<0.0001$) when African green monkeys had less frequency compared to rhesus macaques. The two species showed equal frequencies of tissue like memory, 11% of total CD20+ B cells (Figure. 9 A). However, when we compared the absolute numbers, the data showed that African green monkeys have significantly fewer absolute numbers of Naïve, DP CD21+CD27+ memory, and tissue like memory cells compared to rhesus macaques ($p<0.5$). Also, African green monkeys had less activated memory but the difference was not statistically significant (Figure.9 B).

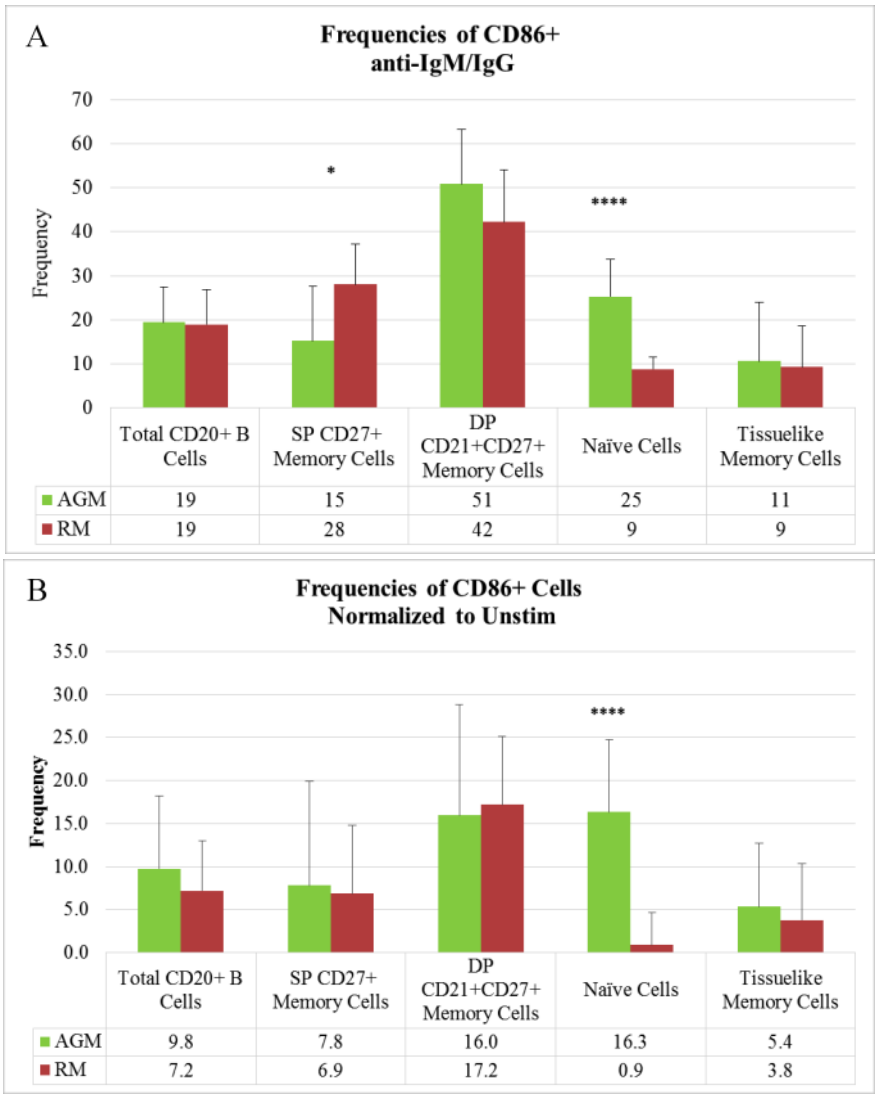


Figure 10. Frequencies of CD86+ cells in total CD20+ B cell and B cell subsets.
PBMC of African green monkeys and rhesus macaques were stimulated with anti-IgM+IgG for 48 hours, ustimulated cells of each monkey were used as control. (A) Frequencies of CD86+ cells based on flow cytometry data. (B) Frequencies of CD86+ cells normalized to unstimulated by subtracting the frequency of CD86+ of unstimulated cells from CD86+ frequency of stimulated cells. The data shown are the means of 12 African green monkeys (AGM) and 12 rhesus macaques (RM). The data was considered significant if the p value <0.05*, or <0.01**, or <0.001***, or <0.0001****.

African green monkeys have higher frequencies of CD86+ naïve, but not single positive memory B cells compared to rhesus macaques.

CD86 is a B cell activation marker which mediates a costimulatory signal through its interaction with CD28, a T cell surface molecule. This signal plays a role in B cell proliferation and antibody production (Jeannin et al. 1997; Sahoo, Rao, and Natarajan 2002). We evaluated the expression of CD86 in African green monkeys and rhesus macaques after stimulating their PBMC with anti-IgM+IgG. The data showed that African green monkeys had higher frequencies of DP memory, naïve and tissue-like B cell expressing CD86. However, only naïve cells showed statistical significance ($p<0.0001$). In contrast, African green monkeys showed significantly less frequencies of SP CD27+ memory expressing CD86 cells compared to rhesus macaques ($p<0.5$). African green monkeys and rhesus macaques showed equal frequencies of total B cells expressing CD86, 19% (Figure.10 A). When the frequency of CD86 expressing cells were normalized to unstim by subtracting the frequency of CD86+ cells in unstimulated cells from stimulated cells, African green monkeys kept their significantly higher frequencies of naïve cells expressing CD86, but they had comparable normalized frequency of other subsets compared to rhesus macaques (Figure.10 B).

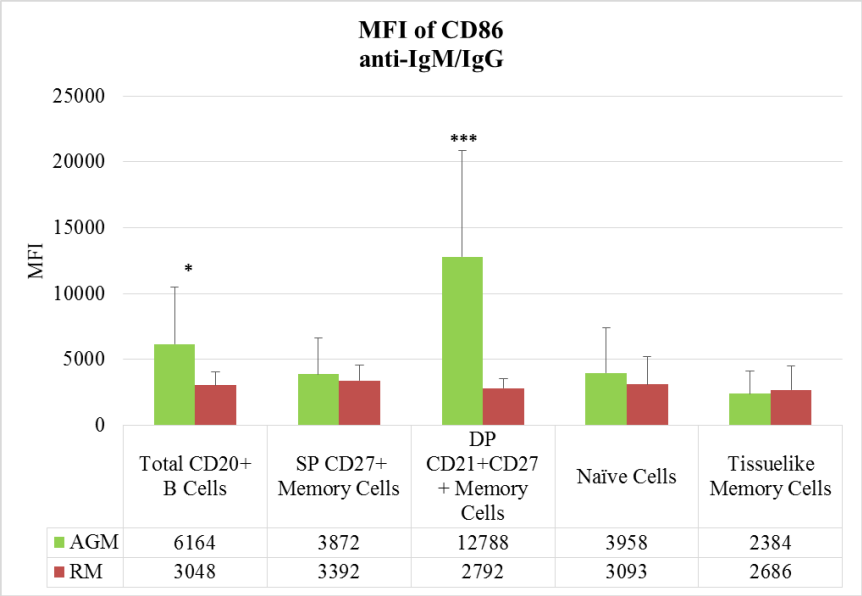


Figure 11. Expression levels of CD86 in CD86+ total B cells and B cell subsets in African green monkeys and rhesus macaques.
PBMC of African green monkeys and rhesus macaques were stimulated with anti-IgM/IgG for 48 hours, ustimulated cells of each monkey were used as control. The expression level of CD86 was evaluated by calculating the mean fluorescence intensity of stimulated cells. The data shown are the means of 12 African green monkeys (AGM) and 12 rhesus macaques (RM). p value <0.05*, or <0.01**, or <0.001***, or <0.0001****.

Double positive memory B cells of African green monkeys express higher levels of CD86 compared to rhesus macaques

The expression of CD86 was further evaluated by calculating the mean fluorescence intensity. The data showed that the DP CD21+CD27+ memory of African green express higher levels of CD86 compared to rhesus macaques ($p<0.001$) (Figure. 11). Higher levels of CD86 expression were also seen in the total CD20+ B cell. There were no statistically significant differences among other subsets.

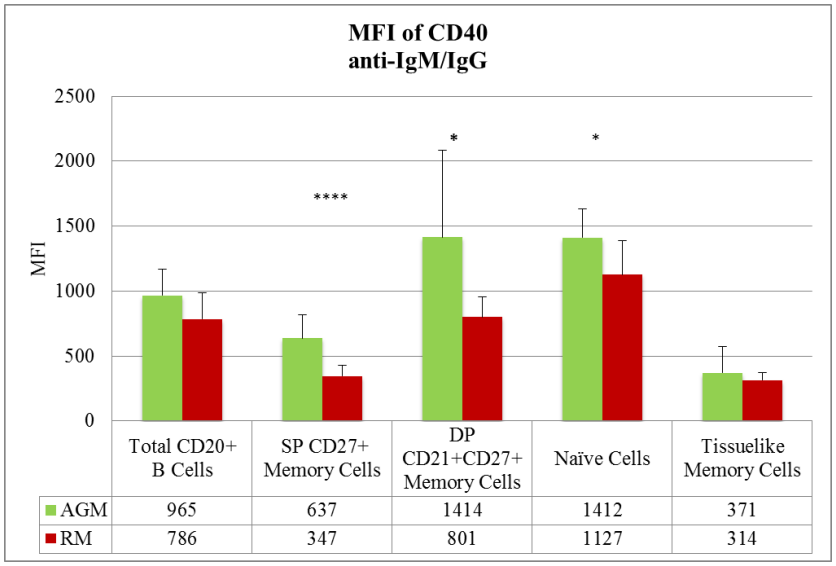


Figure 12. Expression levels of CD40 in total CD20+ B cells and B cell subsets in African green monkeys and rhesus macaques.
PBMC of African green monkeys and rhesus macaques were stimulated with anti-IgM+IgG for 48 hours. Ustimulated cells of each monkey were used as control. The expression levels of CD40 were evaluated by calculating the mean fluorescence intensity of stimulated cells. The data shown are the means of 12 African green monkeys (AGM) and 12 rhesus macaques (RM). The data was considered significant if the p value <0.05*, or <0.01**, or <0.001***, or <0.0001****.

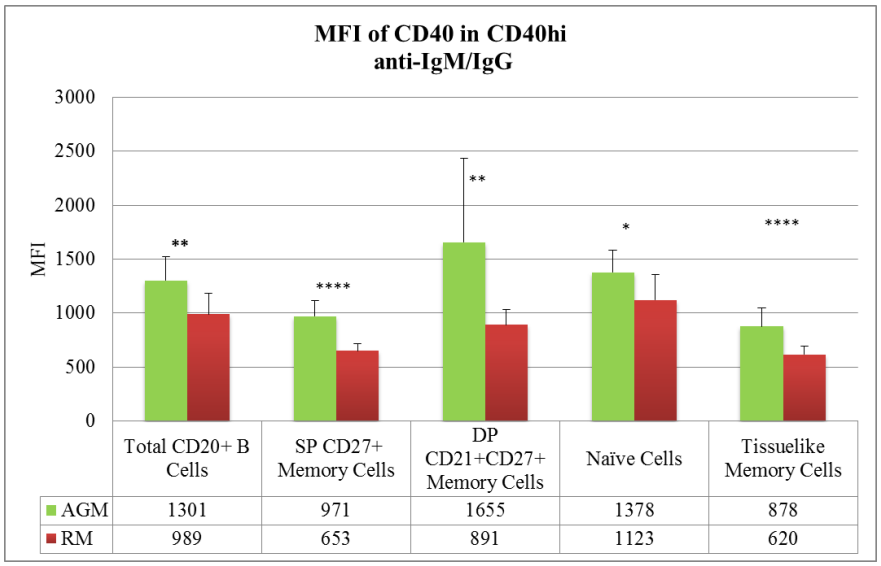


Figure 13. Expression levels of CD40 in CD40hi of total CD20+ B cell and B cell subsets in African green monkeys and rhesus macaques.
PBMC of African green monkeys and rhesus macaques were stimulated with anti-IgM+IgG for 48 hours, ustimulated cells of each monkey were used as control. The expression levels of CD40

were evaluated by calculating the mean fluorescence intensity of stimulated cells. The data shown are the means of 12 African green monkeys (AGM) and 12 rhesus macaques (RM). The data was considered significant if the p value <0.05*, or <0.01**, or <0.001***, or <0.0001****.

B cells of African green monkeys express higher levels of CD40.

CD40 is an activation marker expressed by B cells and other types of cells (Schönbeck, Correspondi and Libby 2001). CD40 binds to CD40L (CD154), this interaction plays a role in immunoglobulin class switching, B cell maturation and proliferation (Zan et al. 1998; Xu et al. 1994; Grewal, Xu, and Flavell 1995). To study the expression of CD40, RMs and AGMs PBMC were stimulated with anti-IgM+IgG. The data showed that naïve, DP CD21+CD27+ memory, and SP CD27+ memory cells of AGMs express higher levels of CD40 compared to RMs when mean fluorescence intensity was used as a measure of expression level ($p<0.05$, $p<0.05$, $p<0.0001$ respectively) (Figure. 12). We further evaluated CD40 expression by calculating the MFI of CD40hi cells. We found that naïve, memory, and tissue like memory cells of African green monkeys express higher levels of CD40 compared to rhesus macaques (Figure.13).

***In vitro* SIV infection has no effect on frequencies and absolute numbers of B cell subsets.**

PBMC of AGMs and RMs were stimulated with anti-IgM+IgG and infected with both SIV9063-2 and SIVmac252 separately; stimulated uninfected cells were used as controls. After 48 hours of stimulation and infection, there were no statistically significant changes in frequencies and absolute numbers of total B cells and subsets in both AGMs and RMs (Figure 14, 15).

SIV infection increases the expression of CD40 in both rhesus macaques and African green monkeys.

We evaluated CD86 expression upon *in vitro* SIV infection to detect any alterations the virus may cause. We found that SIV infection has no effect on the frequencies of cells that express CD86 or on the expression levels of CD86 (Figure. 16, 17). We further evaluated the expression levels of CD40. AGMs' total and naïve B cells showed increases in the expression of CD40 upon infection. RMs' B cell subsets also showed increases in CD40 expression (Figure. 18). Overall, the aim of this experiment was to assess SIV-induced dysfunctions in the expression of activation markers. However, 48h of infection were not enough to see such defects. A longer infection period might be needed to evaluate functional defects.

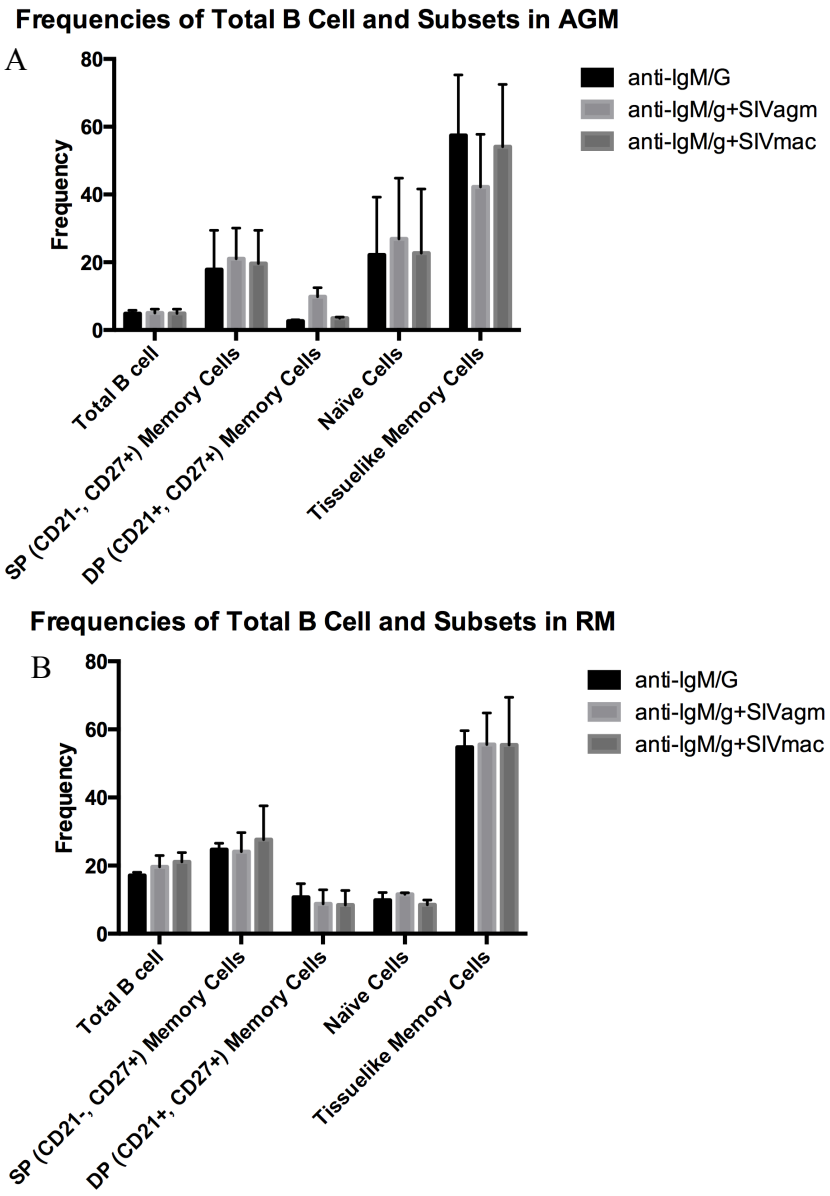


Figure 14. Frequencies of total B cells and subsets following *in vitro* SIV infection.
(A) Frequencies of total B cells and subsets in AGM following three conditions of stimulation, stimulated with anti-IgM/G, stimulated with anti-IgM/G and infected with SIVagm 9063-2, stimulated with anti-IgM/G and infected with SIVmac251. (B) Frequencies of total B cells and subsets in RM following the same conditions of stimulation. The data shown are the means of 3 African green monkeys (AGM) and 3 rhesus macaques (RM). The data was considered significant if the p value <0.05*, or <0.01**, or <0.001***, or <0.0001****.

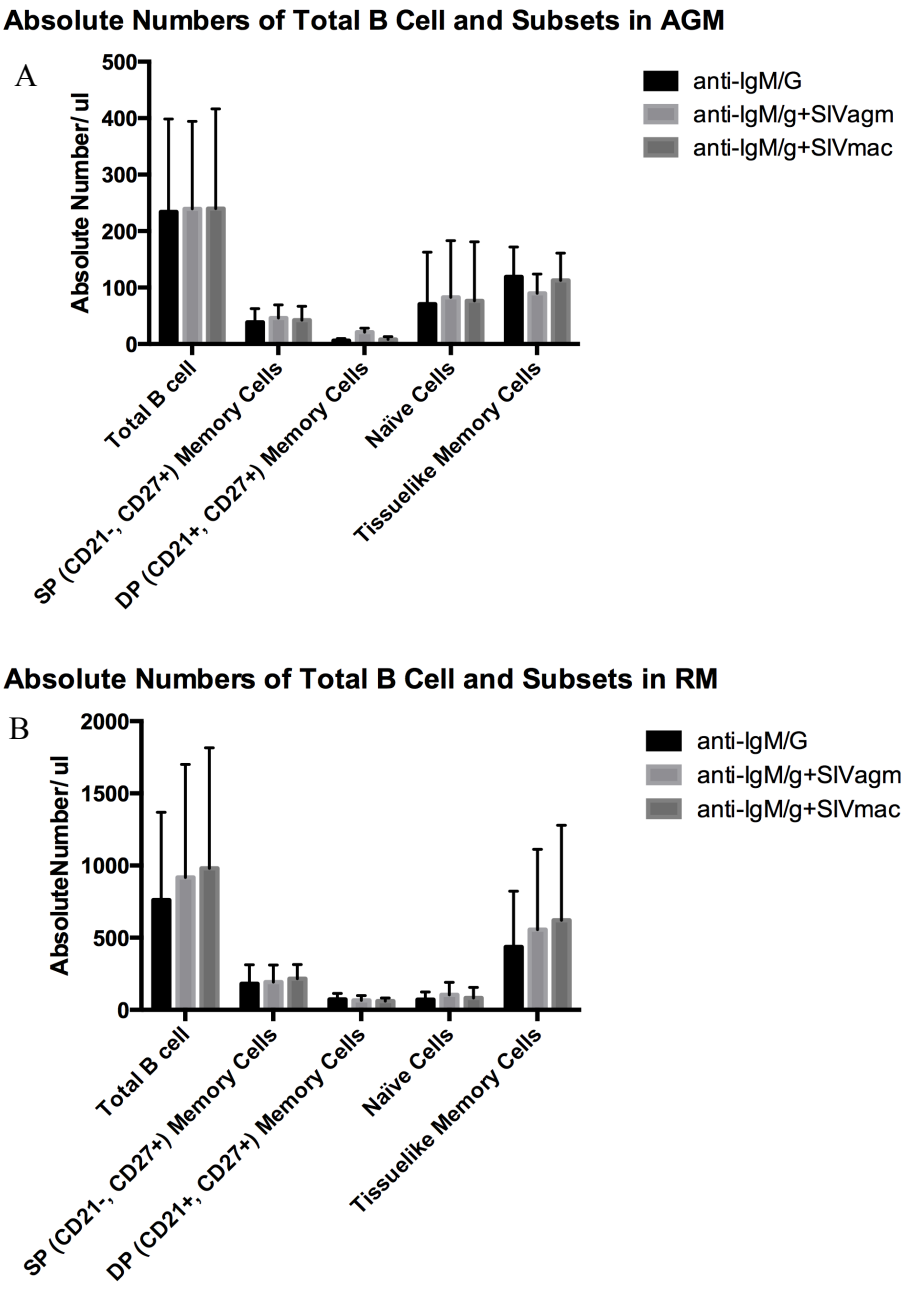


Figure 15. Absolute numbers of total B cells and subsets following *in vitro* SIV infection. (A) Absolute numbers of total B cells and subsets in AGM following three conditions of stimulation, stimulated with anti-IgM/G, stimulated with anti-IgM/G and infected with SIVagm 9063-2, stimulated with anti-IgM/G and infected with SIVmac251. (B) Absolute numbers of total B cells and subsets in RM following the same conditions of stimulation. The data shown are the means of 3 African green monkeys (AGM) and 3 rhesus macaques (RM). The data was considered significant if the p value <0.05*, or <0.01**, or <0.001***, or <0.0001****.

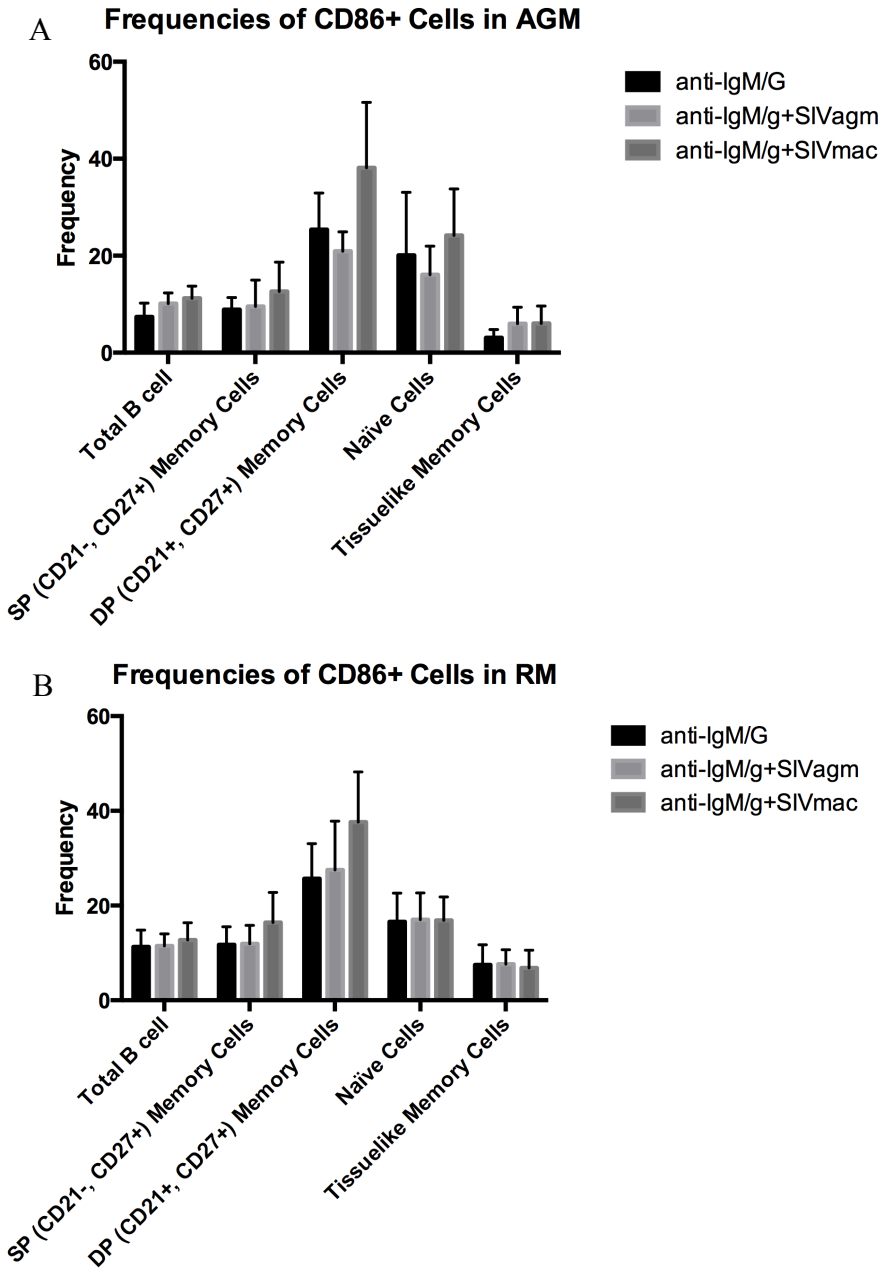


Figure 16. Frequencies of CD86+ cells following in vitro SIV infection.
(A) Frequencies of CD86+ of total B cells and subsets in AGM following three conditions of stimulation, stimulated with anti-IgM/G, stimulated with anti-IgM/G and infected with SIVagm 9063-2, stimulated with anti-IgM/G and infected with SIVmac251. (B) Frequencies of CD86+ of total B cells and subsets in RM following the same conditions of stimulation. The data shown are the means of 3 African green monkeys (AGM) and 3 rhesus macaques (RM). The data was considered significant if the p value <0.05*, or <0.01**, or <0.001***, or <0.0001****.

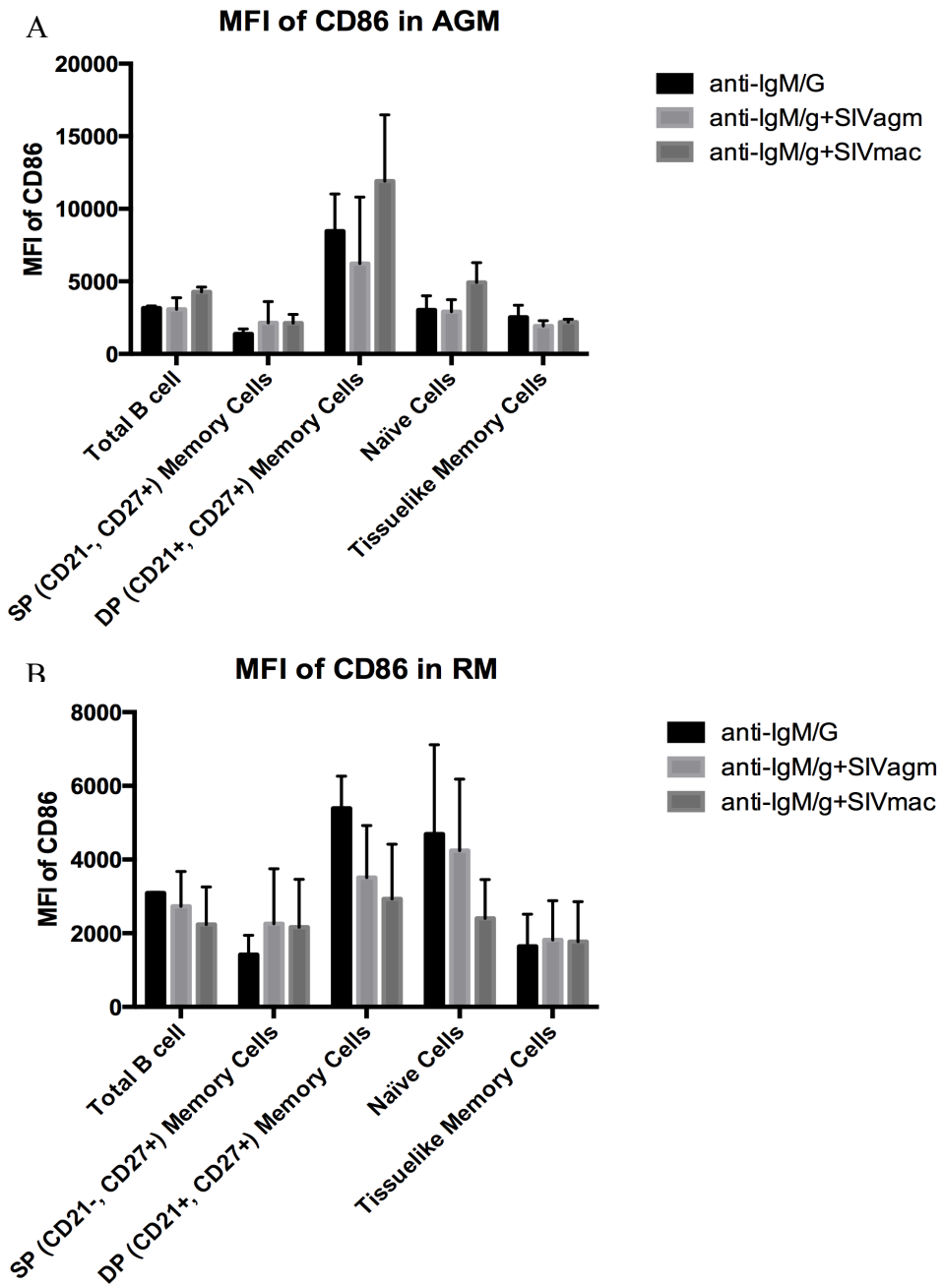
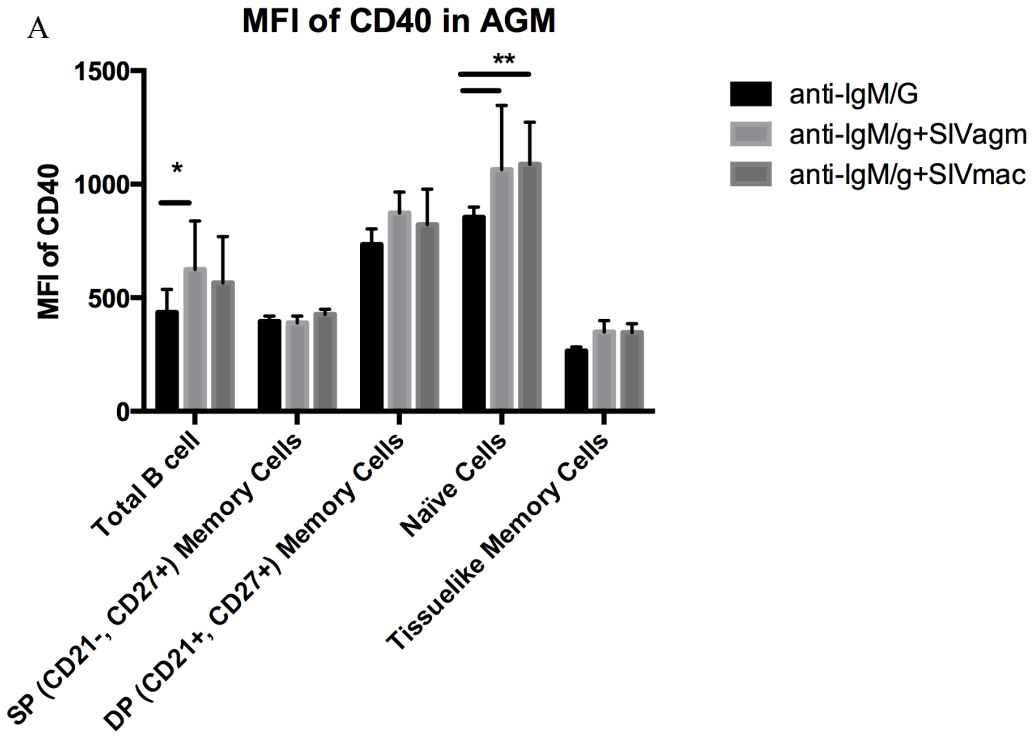


Figure 17. MFI of CD86 following *in vitro* SIV infection.
(A) MFI of CD86 in CD86+ cells of total B cells and subsets in AGM following three conditions of stimulation, stimulated with anti-IgM/G, stimulated with anti-IgM/G and infected with SIVagm 9063-2, stimulated with anti-IgM/G and infected with SIVmac251. (B) MFI of CD86 in CD86+ cells of total B cells and subsets in RM following the same conditions of stimulation. The data shown are the means of 3 African green monkeys (AGM) and 3 rhesus macaques (RM). The data was considered significant if the p value <0.05*, or <0.01**, or <0.001***, or <0.0001****.

A


MFI of CD40 in AGM
MFI of CD40
1500
1000
500
0
*
**
Total B cell
SP (CD21-, CD27+) Memory Cells
DP (CD21+, CD27+) Memory Cells
Naïve Cells
Tissuelike Memory Cells
anti-IgM/G
anti-IgM/g+SIVagm
anti-IgM/g+SIVmac

B

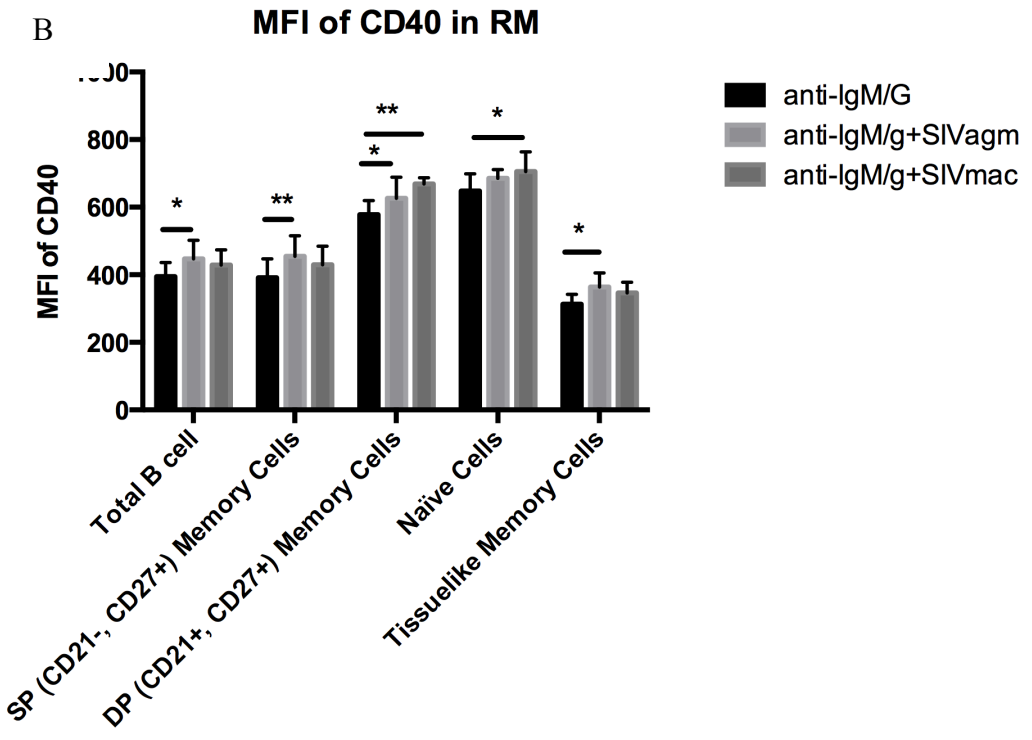

MFI of CD40 in RM
MFI of CD40
800
600
400
200
0
*
**
*
**
*
Total B cell
SP (CD21-, CD27+) Memory Cells
DP (CD21+, CD27+) Memory Cells
Naïve Cells
Tissuelike Memory Cells
anti-IgM/G
anti-IgM/g+SIVagm
anti-IgM/g+SIVmac

Figure 18. MFI of CD40 in total B cells and subsets following *in vitro* SIV infection.
(A) MFI of CD40 in total B cells and subsets in AGM following three conditions of stimulation, stimulated with anti-IgM/G, stimulated with anti-IgM/G and infected with SIVagm 9063-2, stimulated with anti-IgM/G and infected with SIVmac251. (B) MFI of CD40 in total B cells and subsets in RM following the same conditions of stimulation. The data shown are the means of 3 African green monkeys (AGM) and 3 rhesus macaques (RM). The data was considered significant if the p value <0.05*, or <0.01**, or <0.001***, or <0.0001****.

B cells of African green monkeys express higher levels of intracellular signaling proteins and show higher levels of phosphorylation than rhesus macaques.

AGMs and RMs cells were intracellularly stained for total PLC-γ2, ERK1, and SYK proteins. The data showed that AGMs had higher percentages of PLC-γ2hi, and ERK1hi cells than RMs, p<.0.01 (Figures 19A, 20A). Also, these cells showed higher expression levels of PLC-γ2 and ERK1 as compared to RMs (Figures 19C, 20C). In contrast, there were no statistically significant differences in the percentages of SYKhi cells or the expression level of SYK between AGMs and RMs (Figure 21A, C). In addition, the cells were stimulated with anti-IgM+IgG for 10 minutes; unstimulated cells were used as controls. The cells were intracellularly stained for phosphorylated proteins pPLC-γ2, pERK1/2, and pSYK. There were no statistically significant differences between AGMs and RMs in the percentages of total B cells or memory B cells that phosphorylated these proteins upon stimulation. However, total B cells of AGMs showed higher expression level of pPLC-γ2 than RMs, p <0.01 (Figure 22B). Also, memory B cells of AGMs showed higher expression of pSYK than RMs, p< 0.05 (Figure 22F). There were no statistically significant differences in the expression levels of ERK1/2 between AGMs and RMs (Figure 22D).

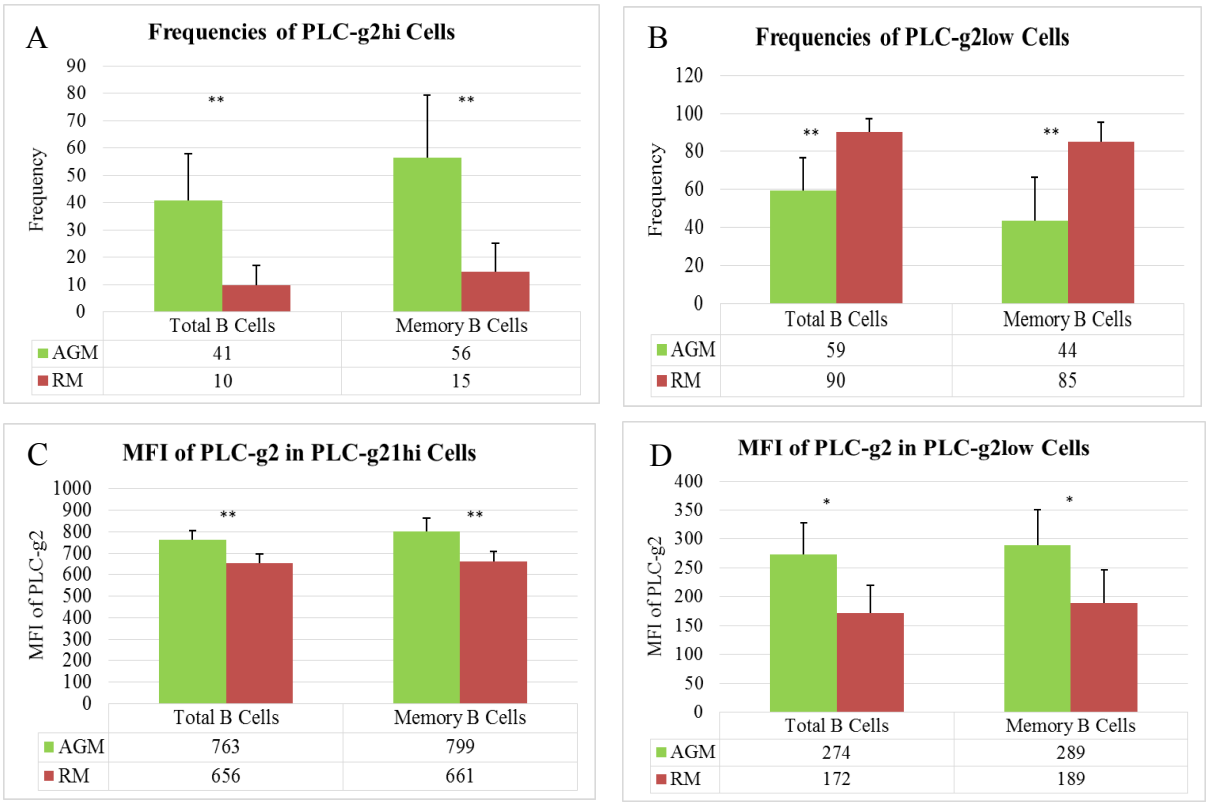


Figure 19. Cells of AGMs and RMs were intracellularly stained with anti-PLC-g2. (A, B) Frequencies of PLC-g2hi and PLC-g2low cells based on flow cytometric gating. (C, D) The Mean fluorescence Intensity (MFI) was calculated for PLC-g2 in of PLC-g2hi and PLC-g2low Cells. The data shown are the means of 5 African green monkeys (AGM) and 5 rhesus macaques (RM). The data was considered significant if the p value <0.05*, or <0.01**, or <0.001***, or <0.0001****.

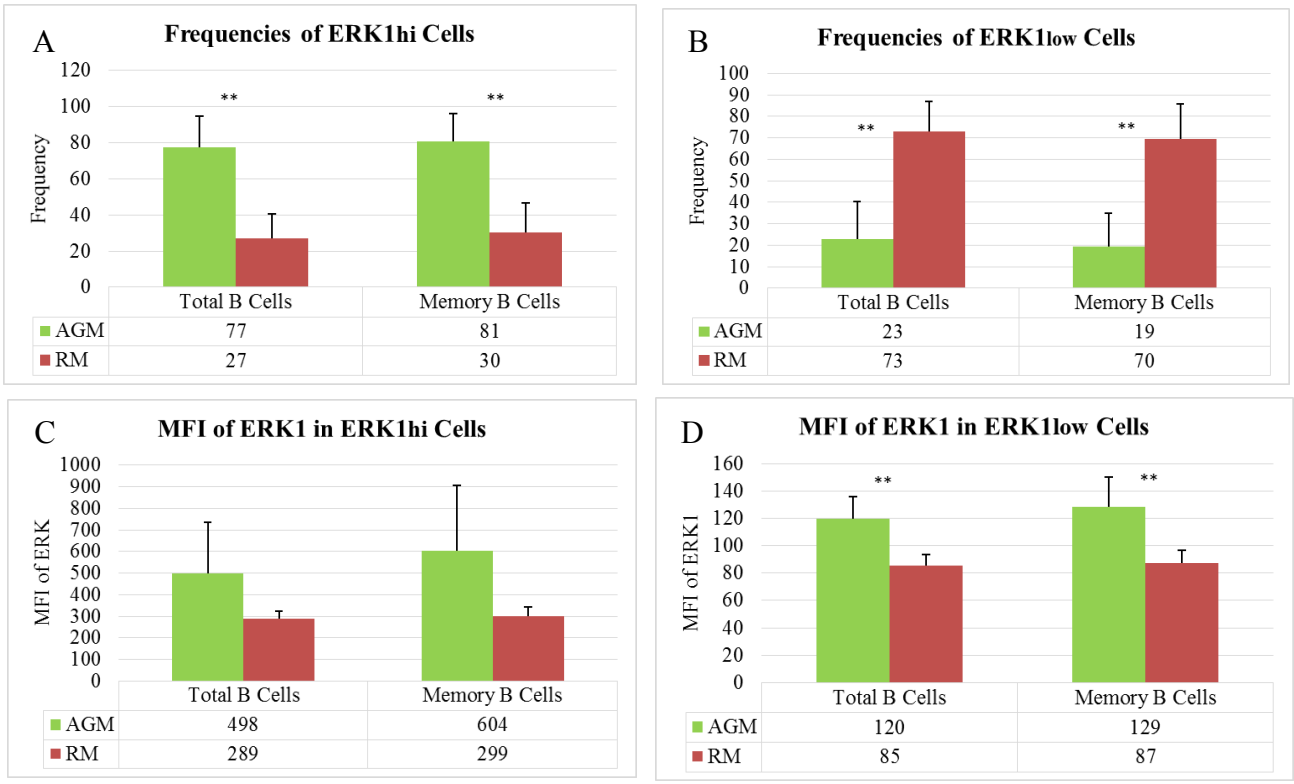


Figure 20. Cells of AGMs and RMs were intracellularly stained with anti-ERK1.
(A, B) Frequencies of ERK1hi and ERK1low cells based on flow cytometric gating. (C, D) The Mean fluorescence Intensity (MFI) was calculated for ERK1 in of ERK1hi and ERK1low Cells. The data shown are the means of 5 African green monkeys (AGM) and 5 rhesus macaques (RM). The data was considered significant if the p value <0.05*, or <0.01**, or <0.001***, or <0.0001****.

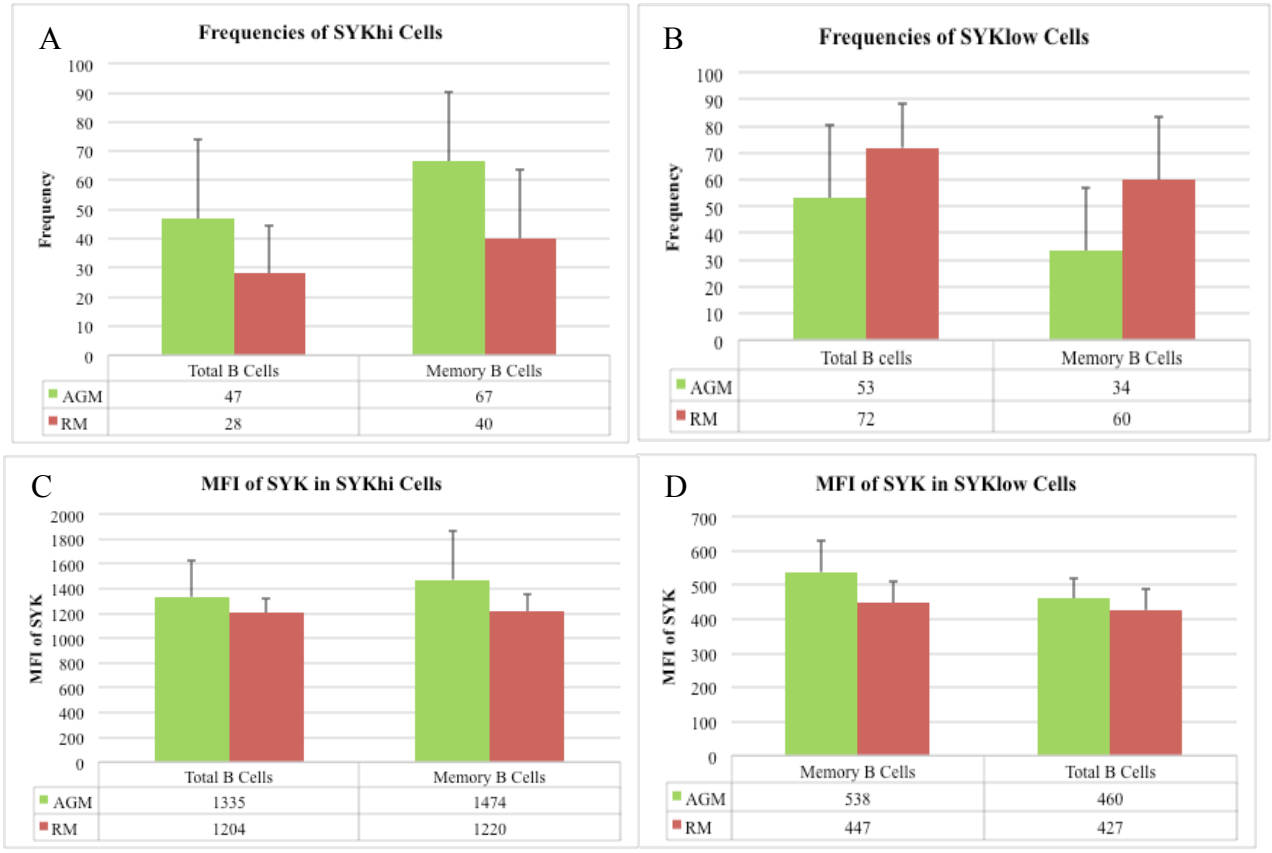


Figure 21. Cells of AGMs and RMs were intracellularly stained with anti-SYK.
(A, B) Frequencies of SYKhi and SYKlow cells based on flow cytometric gating. (C, D) The Mean fluorescence Intensity (MFI) was calculated for SYK in SYKhi and SYKlow Cells. The data shown are the means of 5 African green monkeys (AGM) and 5 rhesus macaques (RM). The data was considered significant if the p value <0.05*, or <0.01**, or <0.001***, or <0.0001****.

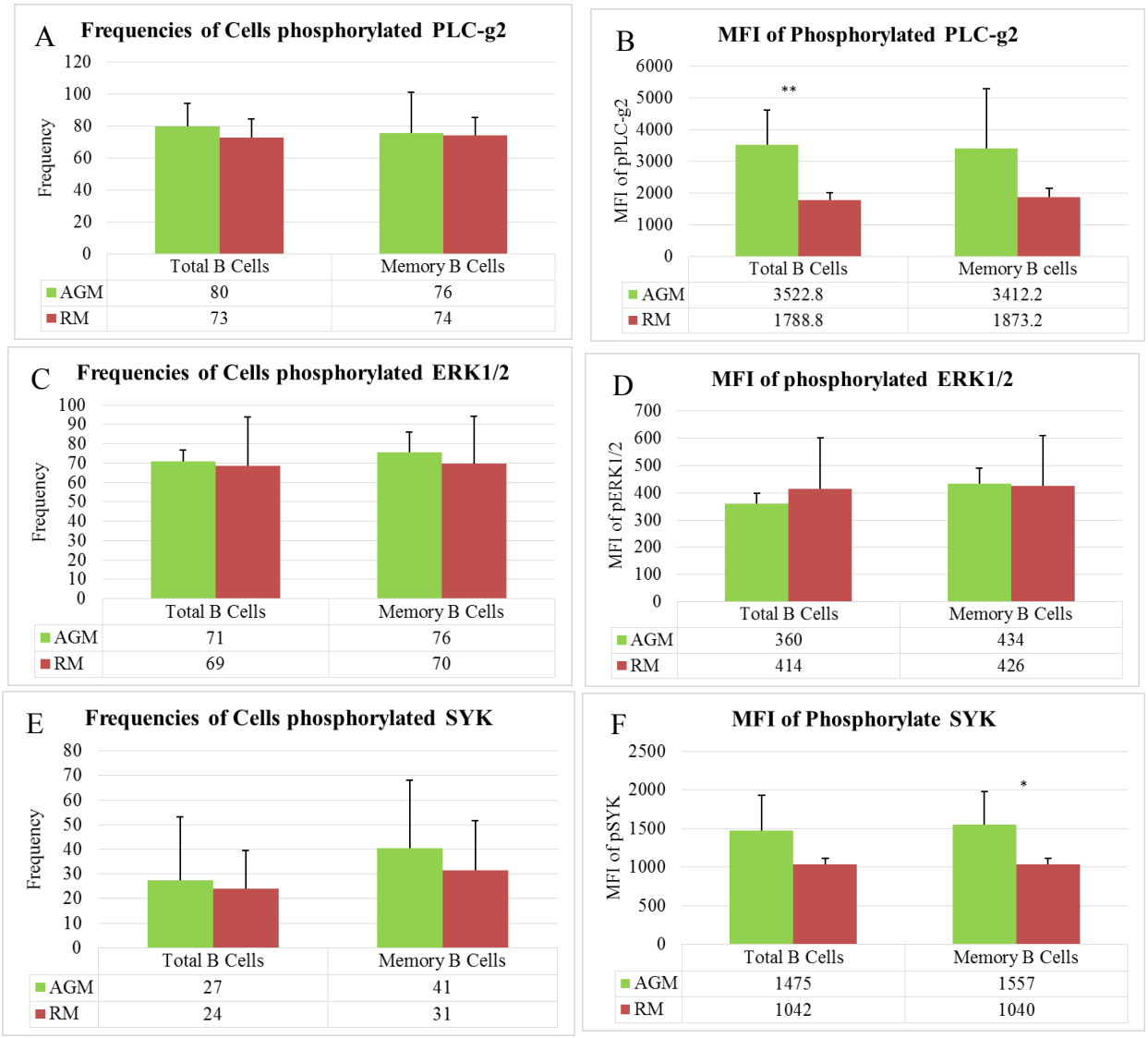


Figure 22. Frequencies and MFI of phosphorylated intracellular proteins.
AGM and RM cells were stimulated with anti-IgM+IgG for 10 minutes; unstimulated cells were used as controls. The cells were intracellularly stained for phosphorylated proteins pPLC-γ2, pERK1/2, and pSYK. (A, C, E) frequencies of cells that showed expression of phosphorylated intracellular proteins. (B, D, F) MFI of phosphorylated intracellular proteins.

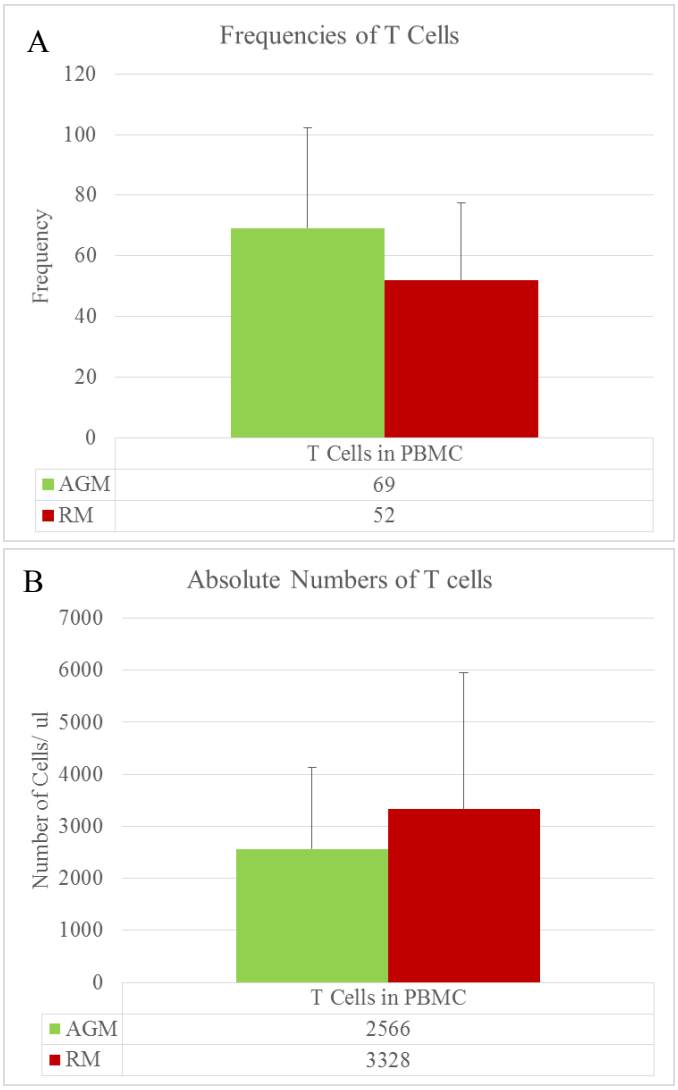


Figure 23. Frequencies and absolute numbers of T cells in the peripheral blood of African green monkeys and rhesus macaques.
African green monkeys and rhesus macaques had comparable total T cells in terms of frequencies (A) and absolute numbers (B). Absolute numbers of T cells were calculated based on flow cytometry and complete blood counts. The data shown are the means of 12 African green monkeys (AGM) and 12 rhesus macaques (RM). p value <0.05*, or <0.01**, or <0.001***, or <0.0001****.

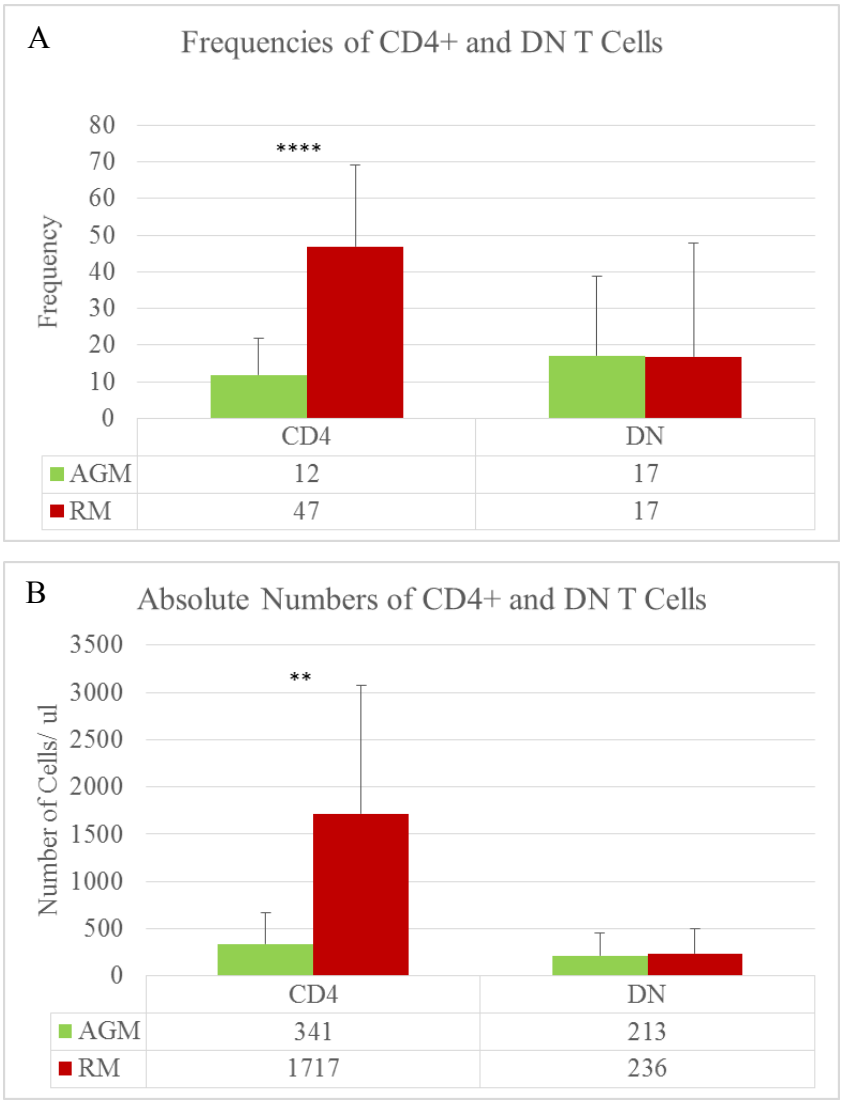


Figure 24. Frequencies and absolute numbers of CD4+ and DN T cells in the peripheral blood of African green monkeys and rhesus macaques.
African green monkeys had significantly fewer frequencies of CD4+ as compared to rhesus macaques, but frequencies of DN (double negative) were comparable in the two species (A). Similar results were found in term of absolute numbers (B). Absolute numbers of CD4+ and DN cells were calculated based on flow cytometry and complete blood counts. The data shown are the means of 12 African green monkeys (AGM) and 12 rhesus macaques (RM). p value <0.05*, or <0.01**, or <0.001***, or <0.0001****.

African green monkeys have fewer CD4 T cells than rhesus macaques

The data showed that AGMs and RMs had comparable percentages and absolute numbers of T cells (Figure 23 A, B). However, AGMs had significantly fewer CD4+ T cells than RMs in terms of frequencies and absolute numbers, $p<0.0001$, $p<0.01$ respectively (Figure 24 A, B). This result is consistent with previous published data (Pandrea et al. 2007). The frequencies and absolute numbers of DN were comparable in AGM and RMs (Figure 24 A, B).

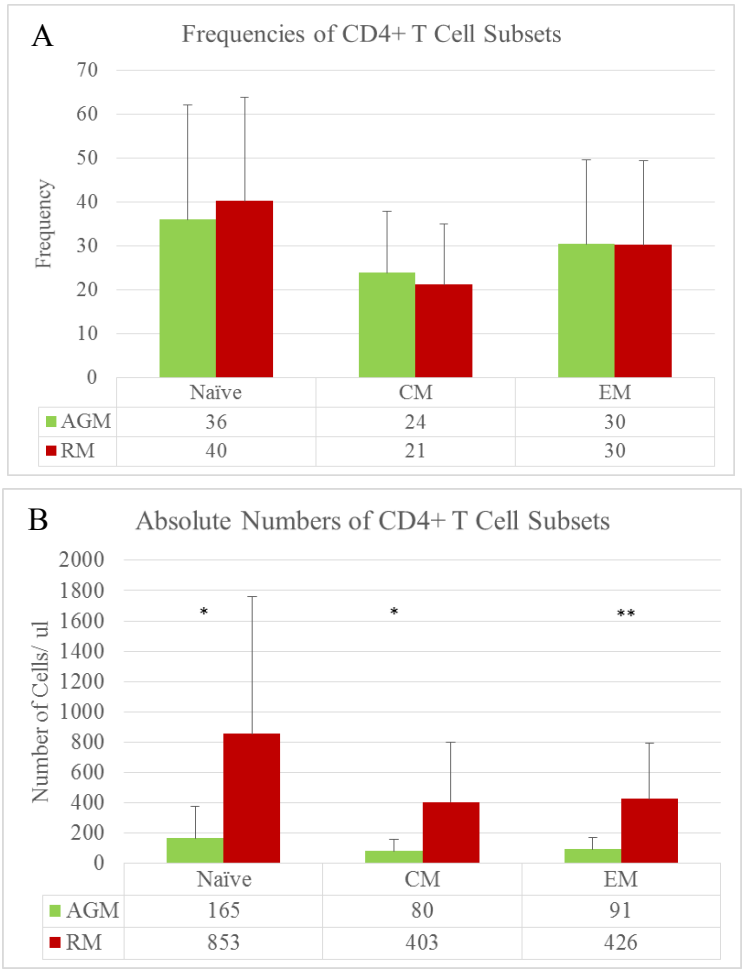


Figure 25. Frequencies and absolute numbers of $CD4^+$ T cell subsets in African green monkeys and rhesus macaques.

The CD4+ T cells were classified into subsets based on the expression of CD28 and CD95, naïve (CD28lo, CD95-), central memory CM (CD28hi, CD95+), and effector memory (EM) (CD28-, CD95+). No significant differences found in CD4+ T cell subsets between AGM and RM in terms of frequencies (A). However, their subsets differ in terms of absolute numbers (B) Absolute numbers of CD4+ T cell subsets were calculated based on the flow cytometry and complete blood counts as count per microliter of whole blood. The data shown are the means of 12 African green monkeys (AGM) and 12 rhesus macaques (RM). p value <0.05*, or <0.01**, or <0.001***, or <0.0001****.

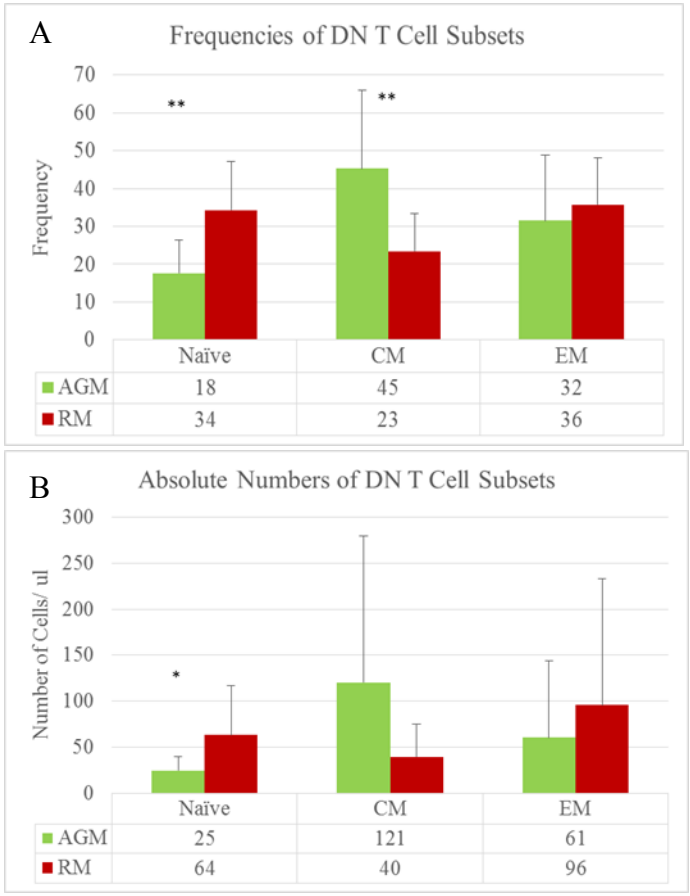


Figure 26. Frequencies and absolute numbers of DN T cell subsets in African green monkeys and rhesus macaques.
The DN T cells were classified into subsets based on the expression of CD28 and CD95, naïve (CD28lo, CD95-), central memory CM (CD28hi, CD95+), and effector memory (EM) (CD28-, CD95+). DN T cell subsets differ between AGM and RM in terms of frequencies (A), and absolute numbers (B). Absolute numbers of DN T cell subsets were calculated based on the flow cytometry and complete blood counts as count per microliter of whole blood. The data shown are the means of 12 African green monkeys (AGM) and 12 rhesus macaques (RM). p value <0.05*, or <0.01**, or <0.001***, or <0.0001****.

T cell subsets differ between African green monkeys and rhesus macaques

CD4+ and DN T cells were further analyzed for subsets, naïve, CM, and EM. The data showed that AGMs and RMs had comparable frequencies of CD4+ T cell subsets. However, there were significant differences in the absolute numbers of naïve, CM, and EM where AGMs had fewer than RMs, $p<0.05$, $p<0.05$, $p<0.01$ respectively (Figure. 25). For DN T cell subsets the data showed that AGMs had significantly fewer DN naïve cells in terms of frequencies and absolute numbers, $p<0.01$, $p<0.05$ respectively (Figure. 26). In contrast, AGMs had higher DN CM in term of frequencies and absolute numbers, but differences were only significant in frequencies, $p<0.01$ (Figure. 26). There were no statistically significant differences in the frequencies and absolute number of DN EM.

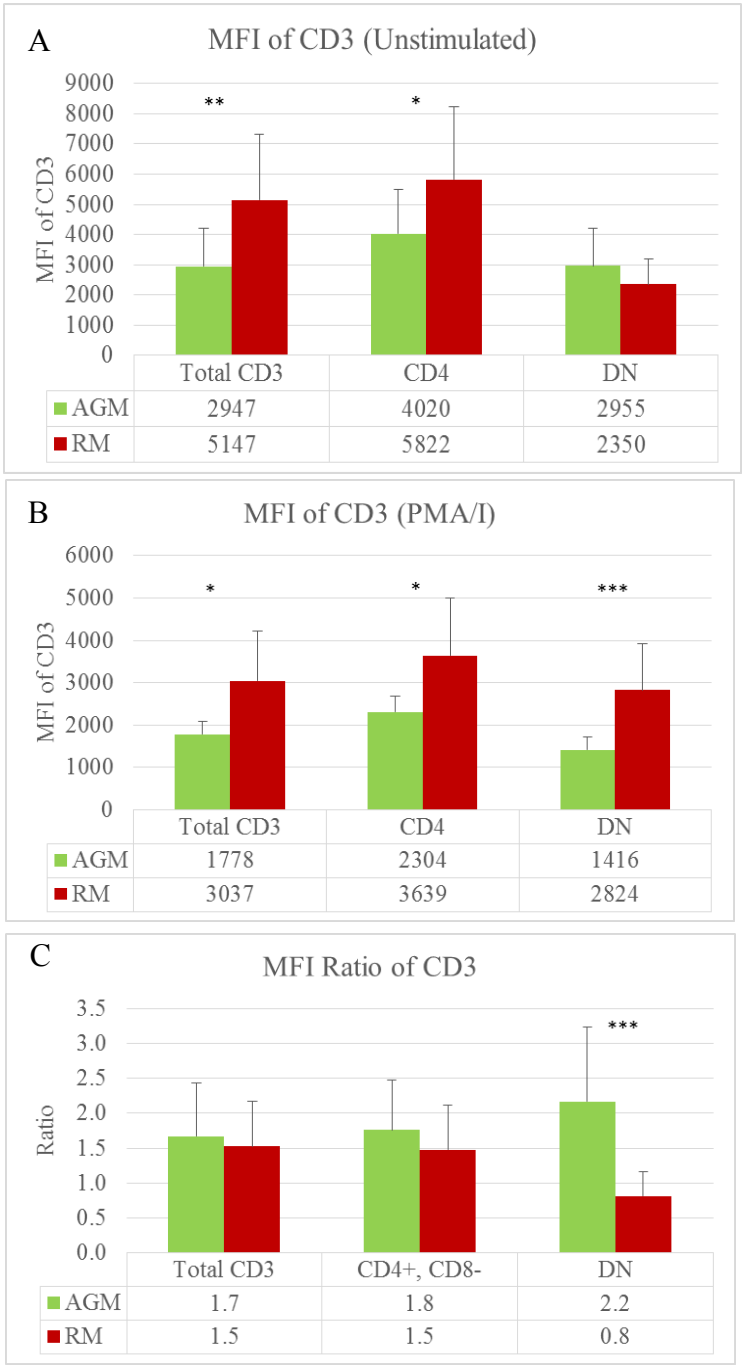
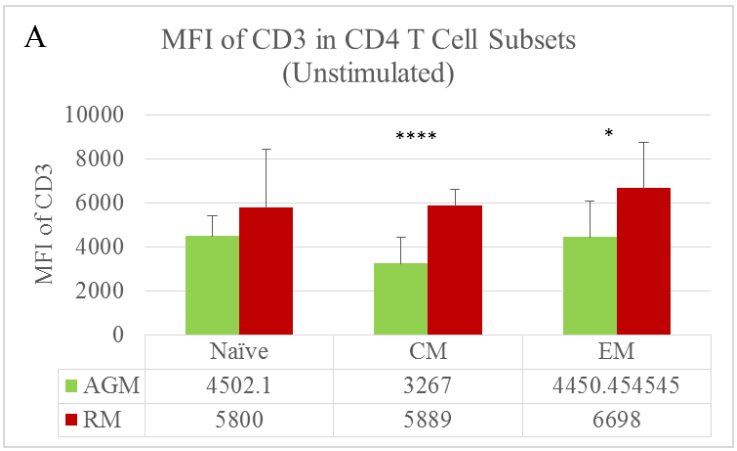
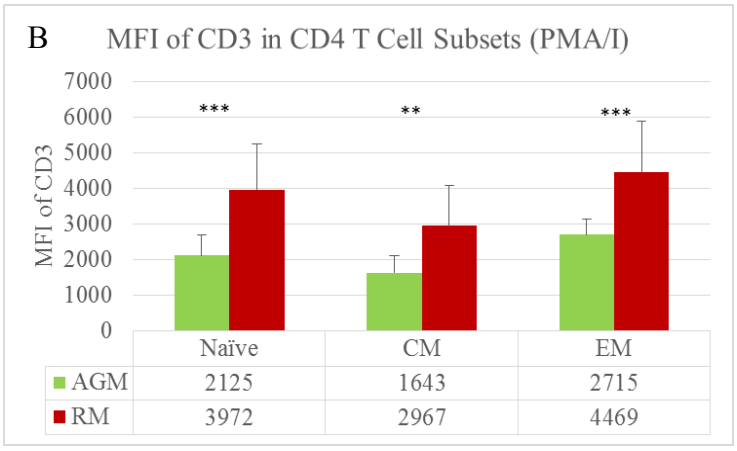


Figure 27 . Expression levels of CD3 on the surface of T cells.
The expression level was evaluated by measuring the mean fluorescence intensity in total, CD4+, and DN T cells of African green monkeys and rhesus macaques. (A) Unstimulated cells. (B) Cells were stimulated with phorbol 12-myristate 13-acetate and Ionomycin (PMA/I) for 6 hours. (C) The MFI ratio was calculated, MFI ratio= MFI of unstimulated cells/MFI of stimulated cells.

The data shown are the means of 12 African green monkeys (AGM) and 12 rhesus macaques (RM). p value <0.05*, or <0.01**, or <0.001***, or <0.0001****.





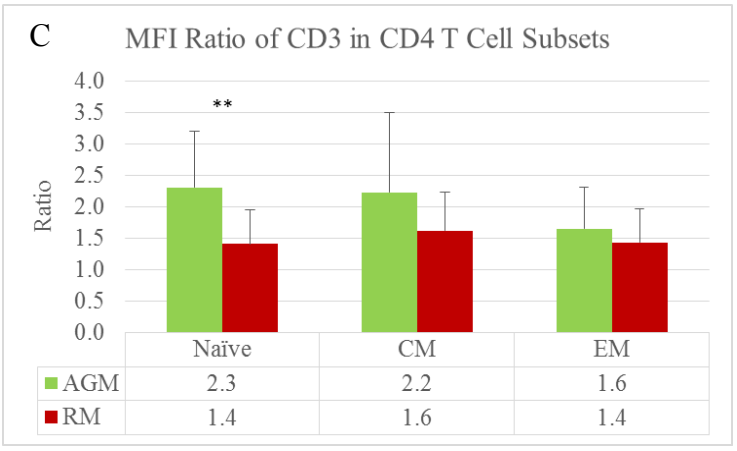


Figure 28. Expression levels of CD3 on the surface of CD4+ T cell subsets.
The expression level was evaluated by measuring the mean fluorescence intensity in CD4+ subsets of African green monkeys and rhesus macaques. (A) Unstimulated cells. (B) Cells were

stimulated with phorbol 12-myristate 13-acetate and Ionomycin (PMA/I) for 6 hours. (C) The MFI ratio was calculated, ratio= MFI of unstimulated cells/MFI of stimulated cells. The data shown are the means of 12 African green monkeys (AGM) and 12 rhesus macaques (RM). p value <0.05*, or <0.01**, or <0.001***, or <0.0001****.

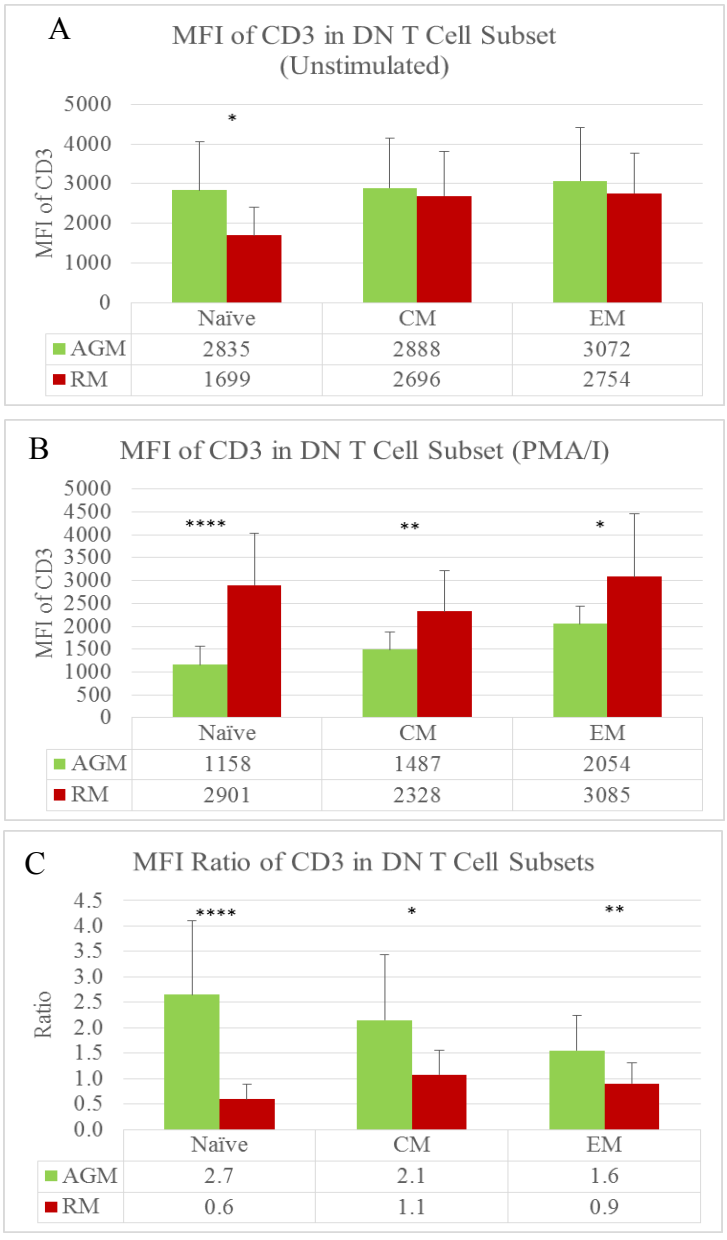


Figure 29. Expression levels of CD3 on the surface of DN T cell subsets.
The expression level was evaluated by measuring the mean fluorescence intensity in DN subsets of African green monkeys and rhesus macaques. (A) Unstimulated cells. (B) Cells were stimulated with phorbol 12-myristate 13-acetate and Ionomycin (PMA/I) for 6 hours. (C) The MFI ratio was calculated, ratio= MFI of unstimulated cells/MFI of stimulated cells. The data

shown are the means of 12 African green monkeys (AGM) and 12 rhesus macaques (RM). p value <0.05*, or <0.01**, or <0.001***, or <0.0001****.

T cells of African green monkeys express lower levels of CD3 than rhesus macaques

The relative expression of CD3 on the surface of T cells was evaluated by the mean fluorescence intensity. In unstimulated cells AGMs expressed lower levels of CD3 on total, and CD4+ T cells than rhesus macaques (Figure.27, A). The differences were considered significant $p<0.01$, $p<0.05$ respectively. The cells were stimulated with PMA/I for 6 hours. Again AGMs showed significantly lower expression of CD3 on total, CD4+, and DN T cells than RMs, $p<0.05$, $p<0.05$, $p<0.001$ respectively (Figure.27, B). The MFI ratio was calculated by dividing MFI of unstimulated cells by that of stimulated cells. The differences in ratios were significant only in DN T cells, which indicate that AGMs downregulated CD3 expression upon stimulation by DN T cells in a greater extent than RMs (Figure.27, C). We further analyzed the expression of CD3 on the subsets of CD4+, and DN T cells (Figure 28 and 29). We found that CD4+ CM, and EM T cells of AGMs express lower levels of CD3 than RMs, $p<0.0001$, $p<0.05$ respectively (Figure 26, A). Upon stimulation with PMA/I, all the subsets CD4+ naïve, CM, and EM of AGMs express lower levels of CD3 than RMs, $p<0.001$, $p<0.01$, $p<0.001$ respectively (Figure 28, B). The differences in ratios were significant only in CD4+ naïve T cells when AGMs had higher ratio than RMs (Figure 28, C). Unexpectedly, the DN naïve T cells of AGMs expressed higher levels of CD3 than RMs, $p<0.05$ (Figure 29, A). Upon stimulation with PMA/I, all the subsets DN naïve, CM, and EM of AGMs showed lower levels of CD3 expression than RMs, $p<0.0001$, $p<0.01$, $p<0.05$ respectively (Figure 29, B). The ratios were significantly higher in AGMs than RMs, $p<0.0001$, $p<0.05$, $p<0.01$ respectively (Figure 29, C). This indicates that DN T cell

subsets of AGMs downregulated CD3 upon stimulation in a greater extent than RMs, which was shown in the total DN as well.

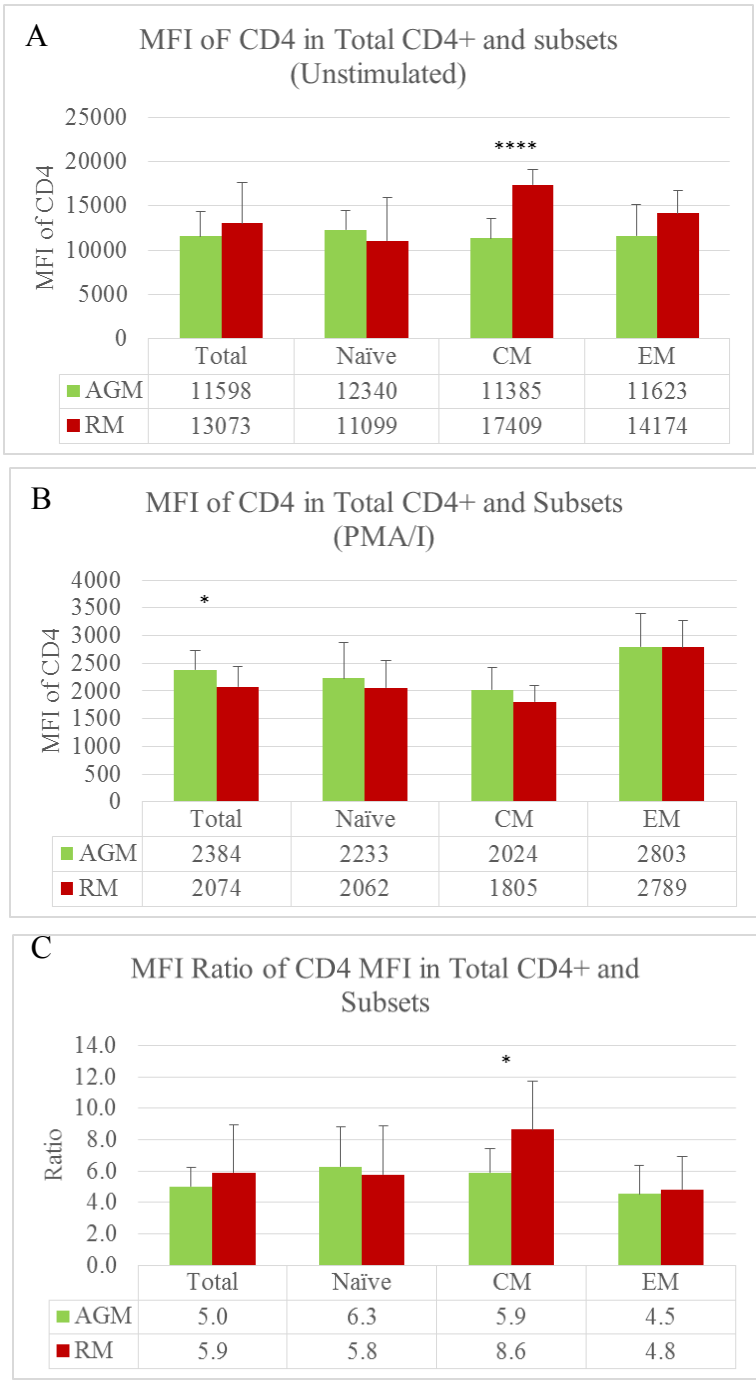


Figure 30. Expression levels of CD4 on the surface of CD4+ T cells.
The expression level was evaluated by measuring the mean fluorescence intensity in total CD4+ and subsets of African green monkeys and rhesus macaques. (A) Unstimulated cells. (B) Cells were stimulated with phorbol 12-myristate 13-acetate and Ionomycin (PMA/I) for 6 hours. (C) The MFI ratio was calculated, ratio= MFI of unstimulated cells/MFI of stimulated cells. The data shown are the means of 12 African green monkeys (s) and 12 rhesus macaques (RM). p value <0.05*, or <0.01**, or <0.001***, or <0.0001****.

Central memory T cells of AGM showed decreases in the expression of CD4 upon PMA/I stimulation but not in rhesus macaques

We compared the expression levels of CD4 by total CD4+ T cells and subsets in African green monkeys and rhesus macaques. In unstimulated cells, we found that central memory T cells of African green monkeys express significantly lower levels of CD4 compared to rhesus macaques $p<0.0001$, while there were no significant differences in the total and other subsets. However, upon stimulation with PMA/I, central memory cells of African green monkeys downregulated the expression of CD4 (Figure. 30). Upon stimulation only total CD4+ T cells showed a significant higher expression of CD4 in African green monkeys compared to rhesus macaques $p<0.05$. When we calculated the ratio of CD4 MFI by subtracting the MFI of stimulated cells from unstimulated cells, only the ratio of central memory cells showed statistically significant when AGMs had lower ratio than RMs $p<0.05$ (Figure. 30C).

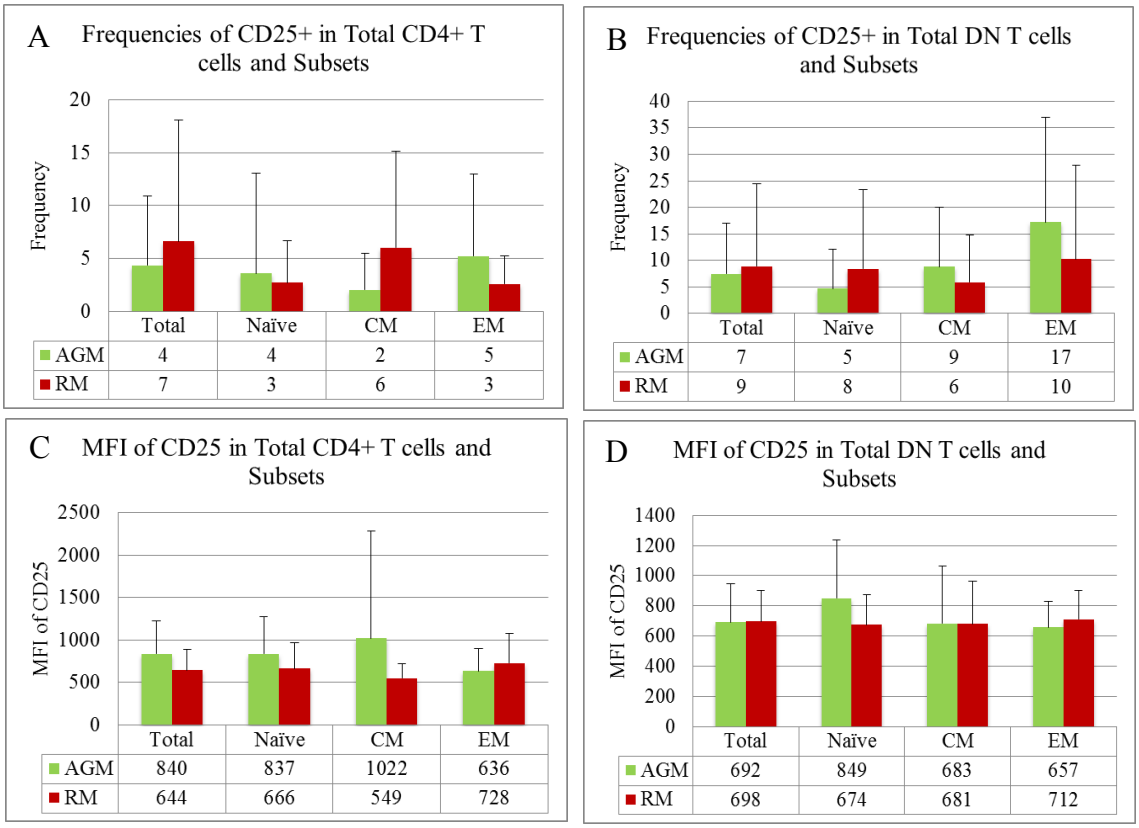


Figure 31. Frequencies of CD25+ and the expression levels in total CD4+, DN and subsets. There were no statistically significance differences between AGM and RM in the frequencies of CD25+ T cells (A, B), or the expression levels as measured by the mean fluorescence intensity (C, D). The data shown are the means of 12 African green monkeys (AGM) and 12 rhesus macaques (RM). p value <0.05*, or <0.01**, or <0.001***, or <0.0001****.

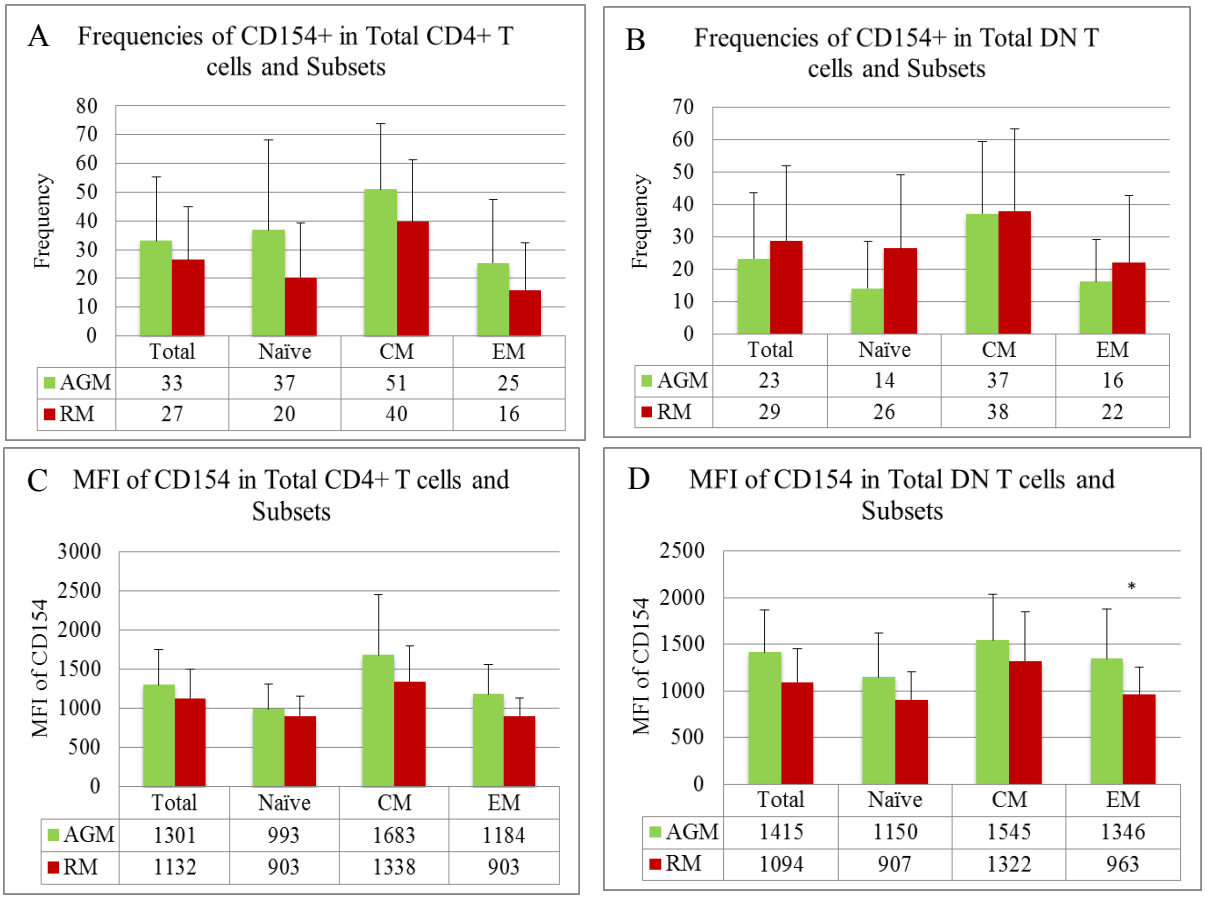


Figure 32. Frequencies of CD154+ and the expression levels in total CD4+, DN and subsets. There were no statistically significance differences between AGM and RM in the frequencies of CD154+ T cells (A, B). There were no statistically significance in the expression levels of CD154 as measured by the mean fluorescence intensity except in DN EM T cells (C, D). The data shown are the means of 12 African green monkeys (AGM) and 12 rhesus macaques (RM). p value <0.05*, or <0.01**, or <0.001***, or <0.0001****.

There are no differences in the frequencies of activated T cells and the expression of activation markers between African green monkeys and rhesus macaques.

The activation markers CD25 and CD154 were evaluated by the frequencies of cells expressing these markers and the expression levels in these cells. These parameters were evaluated in total

CD4+, DN T cells, and subsets. We did not find any statistically significance differences in these parameters between AGMs and RMs except for DN EM T cells, where AGMs had higher expression of CD154 on these cells than RMs (Figures 31, 32).

There are no differences in the expression of CD95 and CD28 between African green monkeys and rhesus macaques.

CD95 is a surface marker that is used to identify effector and central memory T cells. The level of CD95 was evaluated on the surface of these cells in both CD4+ and DN T cells. The data showed no statistically significant differences between AGMs and RMs (figure 33. A, B). However, when the cells were stimulated with PMA/I for 6 hours the DN EM and CM of RMs downregulated the expression of CD95, which mad the differences between the two species significant (figure 33. C, D). When the ratios of CD95 MFI were calculated, there were no statistical significance differences between AGMs and RMs (figure 33. E, F). CD28 is a surface marker that identifies naïve and central memory T cells. The expression levels of CD28 were evaluated in AGMs and RMs. The data showed no statistically significance differences in the expression of CD28 on the surface of naïve and central memory of both CD4+ and T cells (Figure. 34).

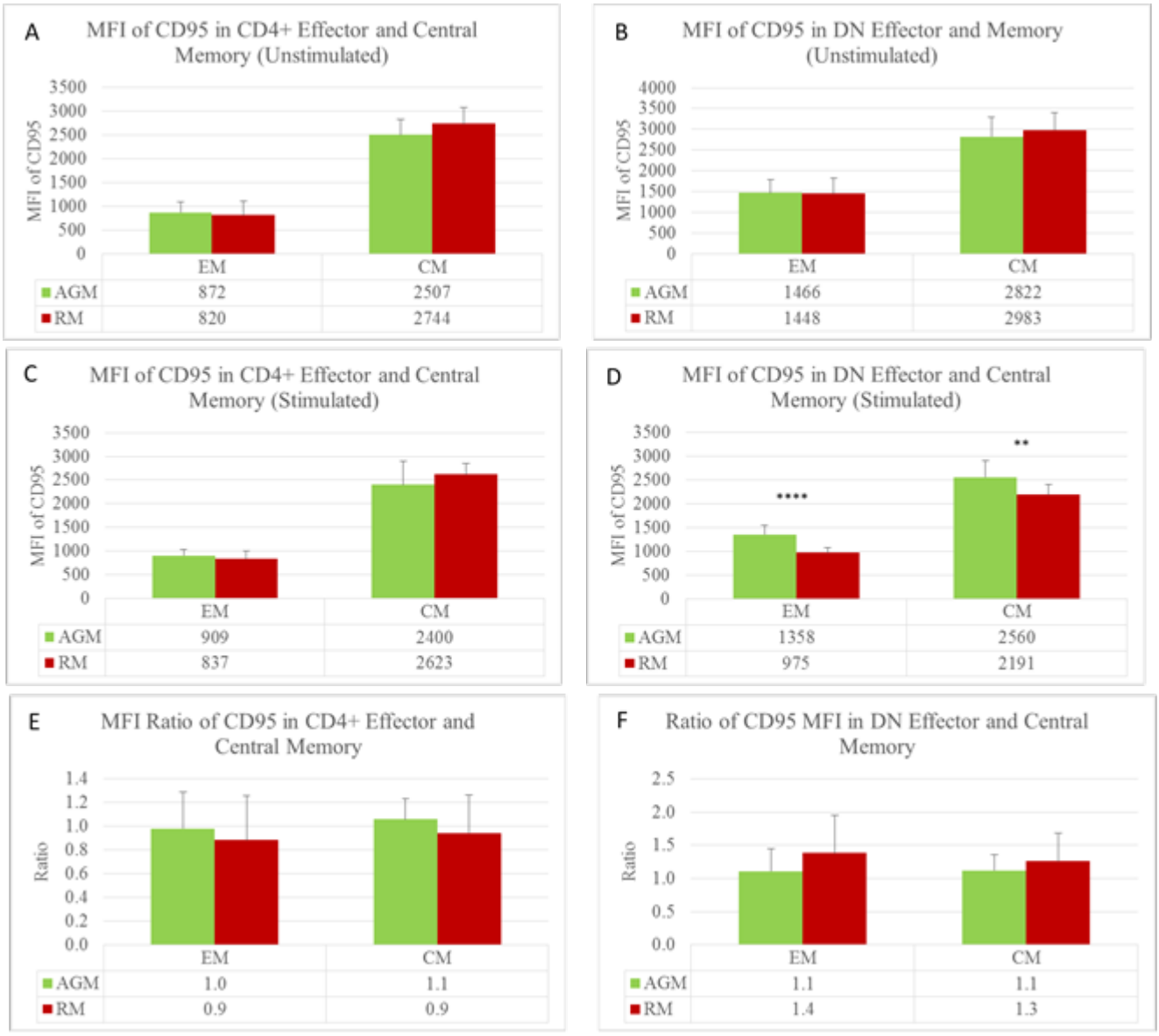


Figure 33. Expression levels of CD95 on the surface of effector and central memory T cells. The expression levels were evaluated by measuring the mean fluorescence intensity in effector and central memory of each DN and CD4+ T cells in both African green monkeys and rhesus macaques. (A) Unstimulated CD4+ EM and CM T cells. (B) Unstimulated DN EM and CM T cells. (C, D) Cells were stimulated with phorbol 12-myristate 13-acetate and Ionomycin (PMA/I) for 6 hours. (E, F) The MFI ratio was calculated, ratio= MFI of unstimulated cells/MFI of stimulated cells. The data shown are the means of 12 African green monkeys (AGM) and 12 rhesus macaques (RM). p value <0.05*, or <0.01**, or <0.001***, or <0.0001****

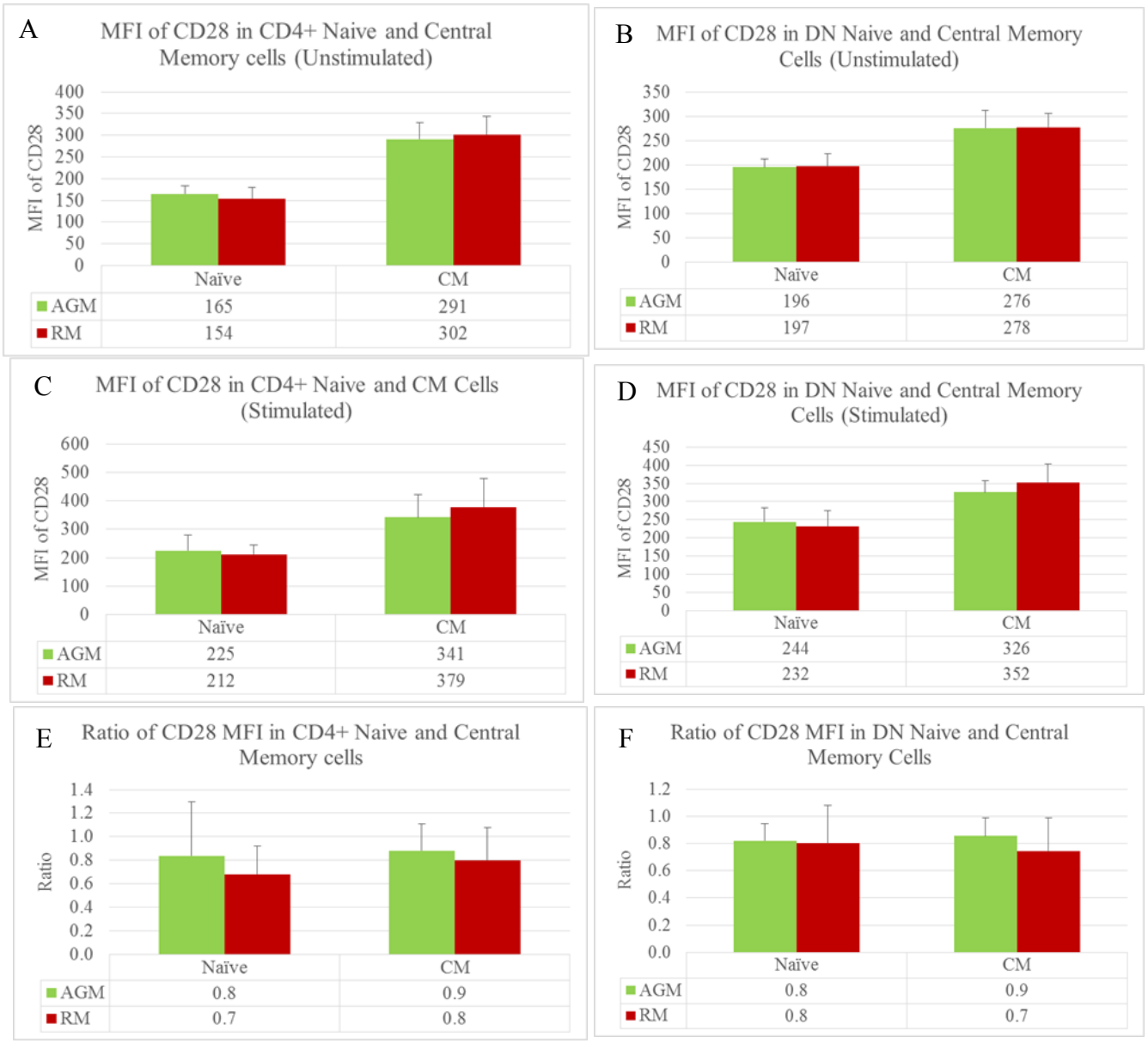


Figure 34. Expression levels of CD28 on the surface of naive and central memory T cells. The expression levels were evaluated by measuring the mean fluorescence intensity in naive and central memory of each DN and CD4+ T cells in both African green monkeys and rhesus macaques. (A) Unstimulated CD4+ Naive and CM T cells. (B) Unstimulated DN Naive and CM T cells. (C, D) Cells were stimulated with phorbol 12-myristate 13-acetate and Ionomycin (PMA/I) for 6 hours. (E, F) The MFI ratio was calculated, ratio= MFI of unstimulated cells/MFI of stimulated cells. The data shown are the means of 12 African green monkeys (s) and 12 rhesus macaques (RM). p value <0.05*, or <0.01**, or <0.001***, or <0.0001****.

T cells of African green monkeys showed less production of cytokines and less multifunctionality than rhesus macaques

AGM showed significantly lower frequencies of CD4 T cells that express CD107a, or produce IFNɣ, $p < 0.05$ (Figure 35A, B). Similar patterns were found in the different subsets of CD4 T cells (Figure. 36). However, AGMs showed higher frequencies of DN T cells expressing CD107a., $p < 0.05$. This pattern was consistent in the different DN T cell subsets as well (Figure 37C). The expression levels of CD107a by DN T cells were higher in AGMs as well, $p < 0.05$ (Figure 35D). The expression levels of TNFα were higher in RMs by both CD4 and DN T cells, $p < 0.05$, $p < 0.01$ respectively (Figure 35B, D). For multifunctional T cells in total CD4, RMs showed higher percentages of cells with CD107a+IFNɣ+IL2+TNfα+, CD107a+IFNɣ+TNFα+, IFNɣ+TNFα+ (Figure 37). CD4 effector cells of RMs showed the same pattern in addition to significantly higher frequencies of IFNɣ+IL2+TNFα+ as compared to AGMs (Figure 38). CD4 EM T cells producing IFNɣ+IL-2+TNFα+, IFNɣ+TNFα+ were significantly higher in RMs than AGMs (Figure. 49). CD4 CM T cells producing IFNɣ+IL-2+TNFα+ were higher in RMs as well (Figure. 40). Overall, CD4 T cells and subsets showed significantly less cytokines producing and multifunctionality in AGMs than RMs. The only higher multifuctionality AGMs showed was in DN T cells producing FNɣ and expressing CD107a (Figure. 42).

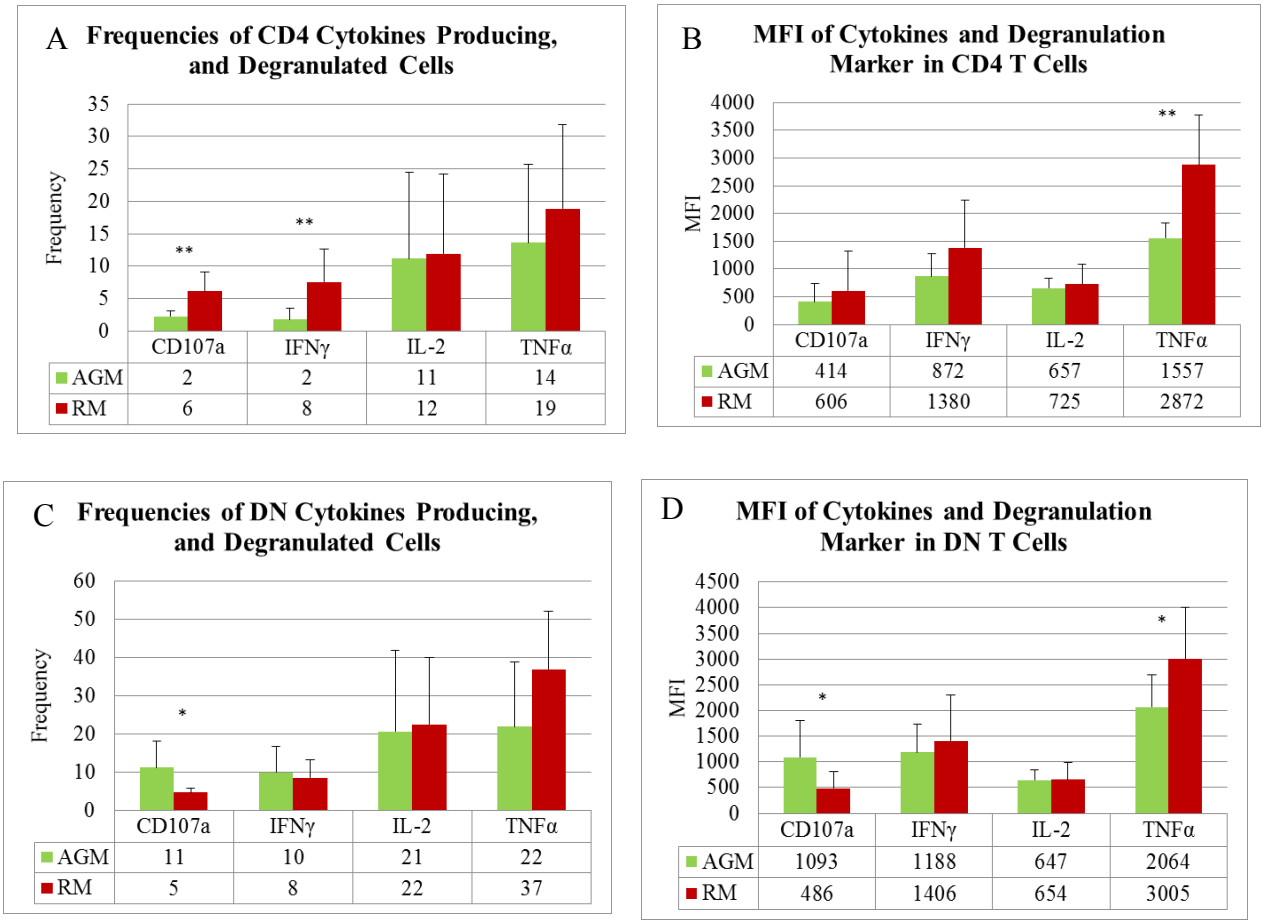


Figure 35. CD4 and DN T cells producing cytokines and expressing CD107a.
(A) Frequencies of CD4 producing IFNɣ, TNFα, IL-2, or expressing CD107a in AGM and RM. (B) MFI of IFNɣ, TNFα, IL-2, and CD107a in CD4 T cells of AGM and RM. (C) Frequencies of DN producing IFNɣ, TNFα, IL-2, or expressing CD107a in AGM and RM. (D) MFI of IFNɣ, TNFα, IL-2, and CD107a in DN T cells of AGM and RM. The data shown are the means of 8 African green monkeys (AGM) and 9 rhesus macaques (RM). The data was considered significant if the p value <0.05*, or <0.01**, or <0.001***, or <0.0001****.

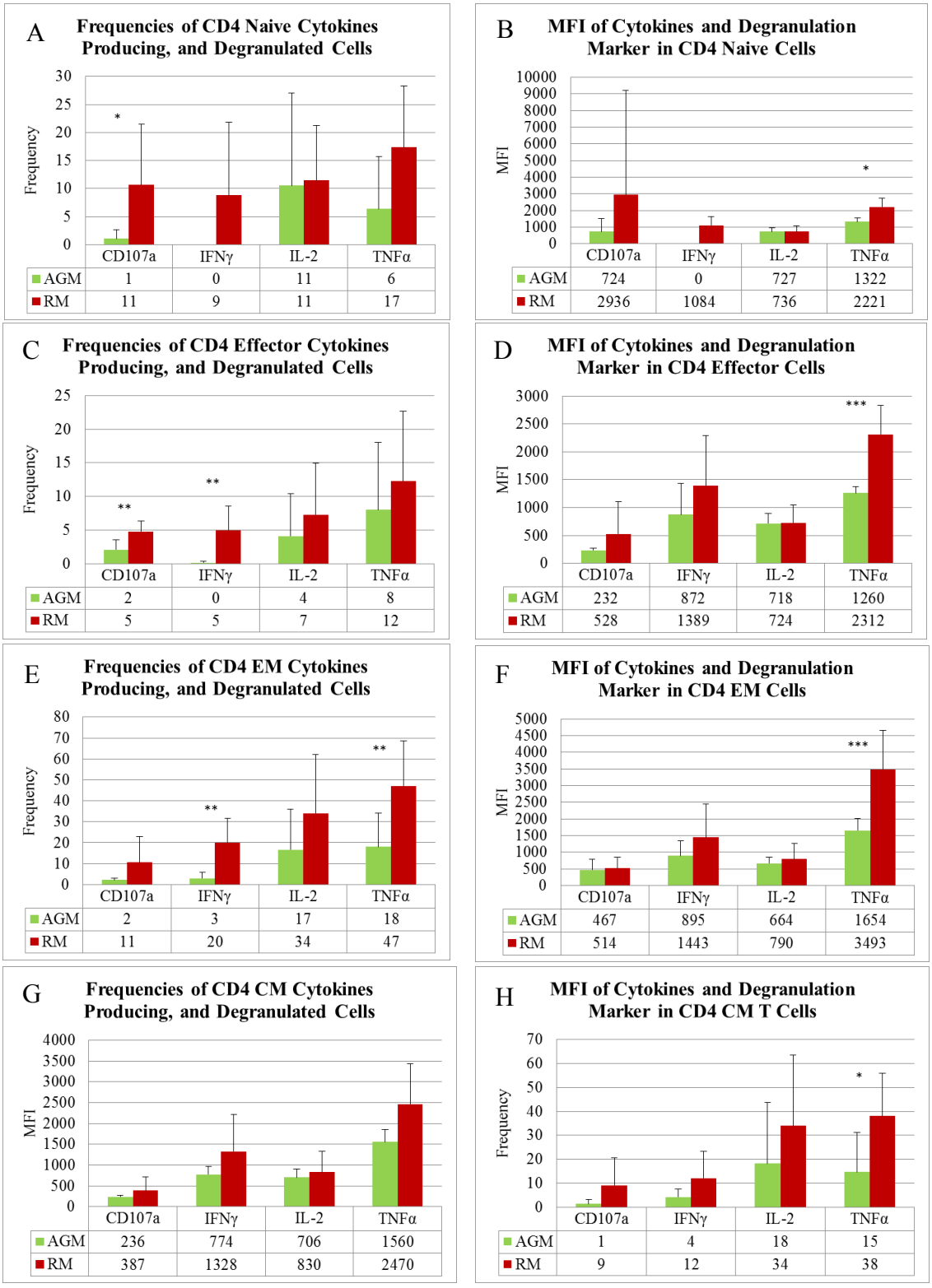


Figure 36. CD4 T cell subsets producing cytokines and expressing CD107a.

(A) Frequencies of CD4 naïve T cells producing IFNɣ, TNFα, IL-2, or expressing CD107a in AGM and RM. (B) MFI of IFNɣ, TNFα, IL-2, and CD107a in CD4 naïve T cells of AGM and RM. (C) Frequencies of CD4 effector cells T cells producing IFNɣ, TNFα, IL-2, or expressing CD107a in AGM and RM. (D) MFI of IFNɣ, TNFα, IL-2, and CD107a in CD4 effector T cells of AGM and RM. (E) Frequencies of CD4 effector memory T cells producing IFNɣ, TNFα, IL-2, or expressing CD107a in AGM and RM. (F) MFI of IFNɣ, TNFα, IL-2, and CD107a in CD4 effector memory T cells of AGM and RM. (G) Frequencies of CD4 central memory T cells producing IFNɣ, TNFα, IL-2, or expressing CD107a in AGM and RM. (H) MFI of IFNɣ, TNFα, IL-2, and CD107a in CD4 central memory T cells of AGM and RM. The data shown are the means of 8 African green monkeys (AGM) and 9 rhesus macaques (RM). The data was considered significant if the p value <0.05*, or <0.01**, or <0.001***, or <0.0001****.

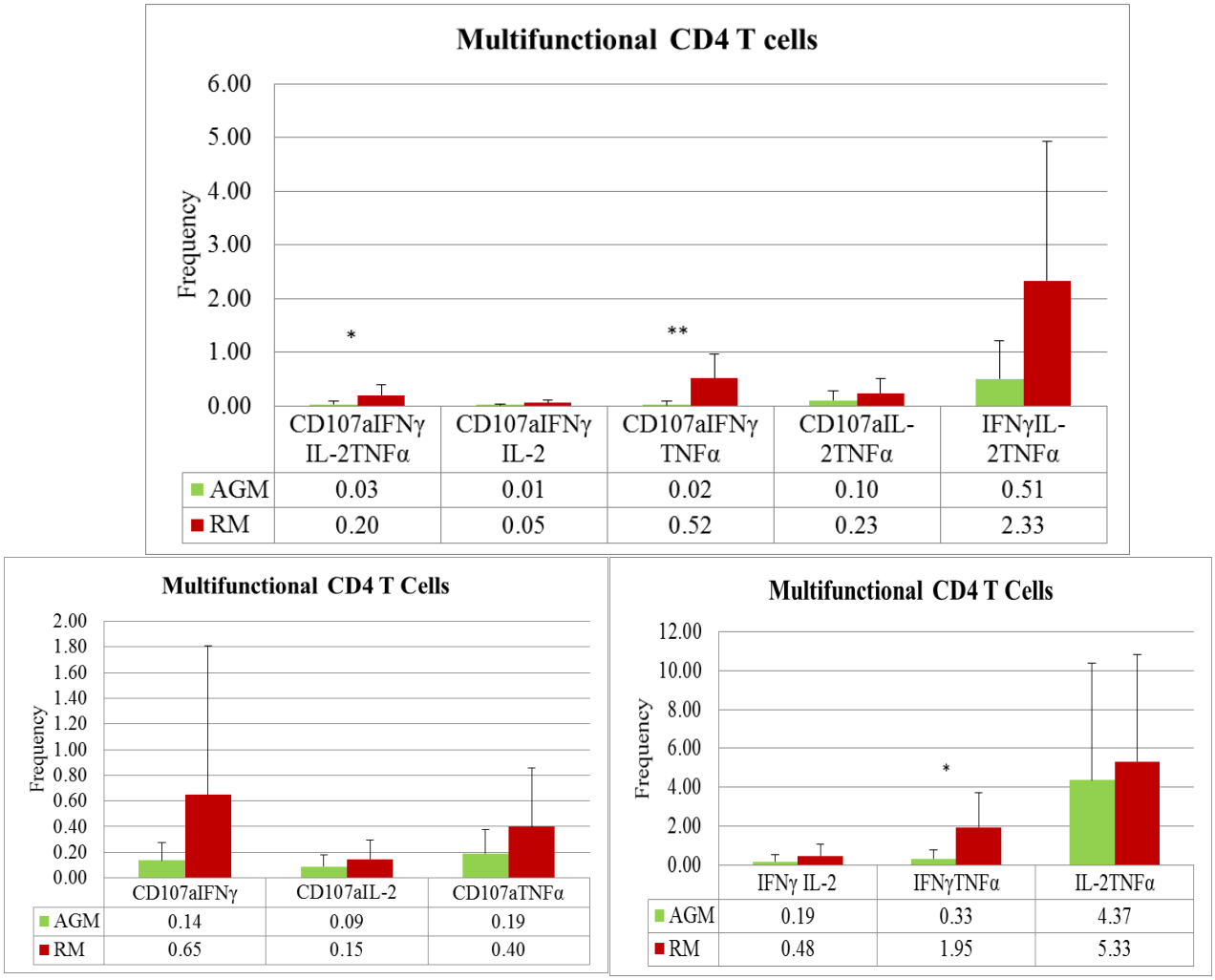


Figure 37. Frequencies of total CD4T cells with different combination of functions include: secretion of IFNɣ, TNFα, IL-2, and expression of CD107a.

The data shown are the means of 8 African green monkeys (AGM) and 9 rhesus macaques (RM). The data was considered significant if the p value <0.05*, or <0.01**, or <0.001***, or <0.0001****.

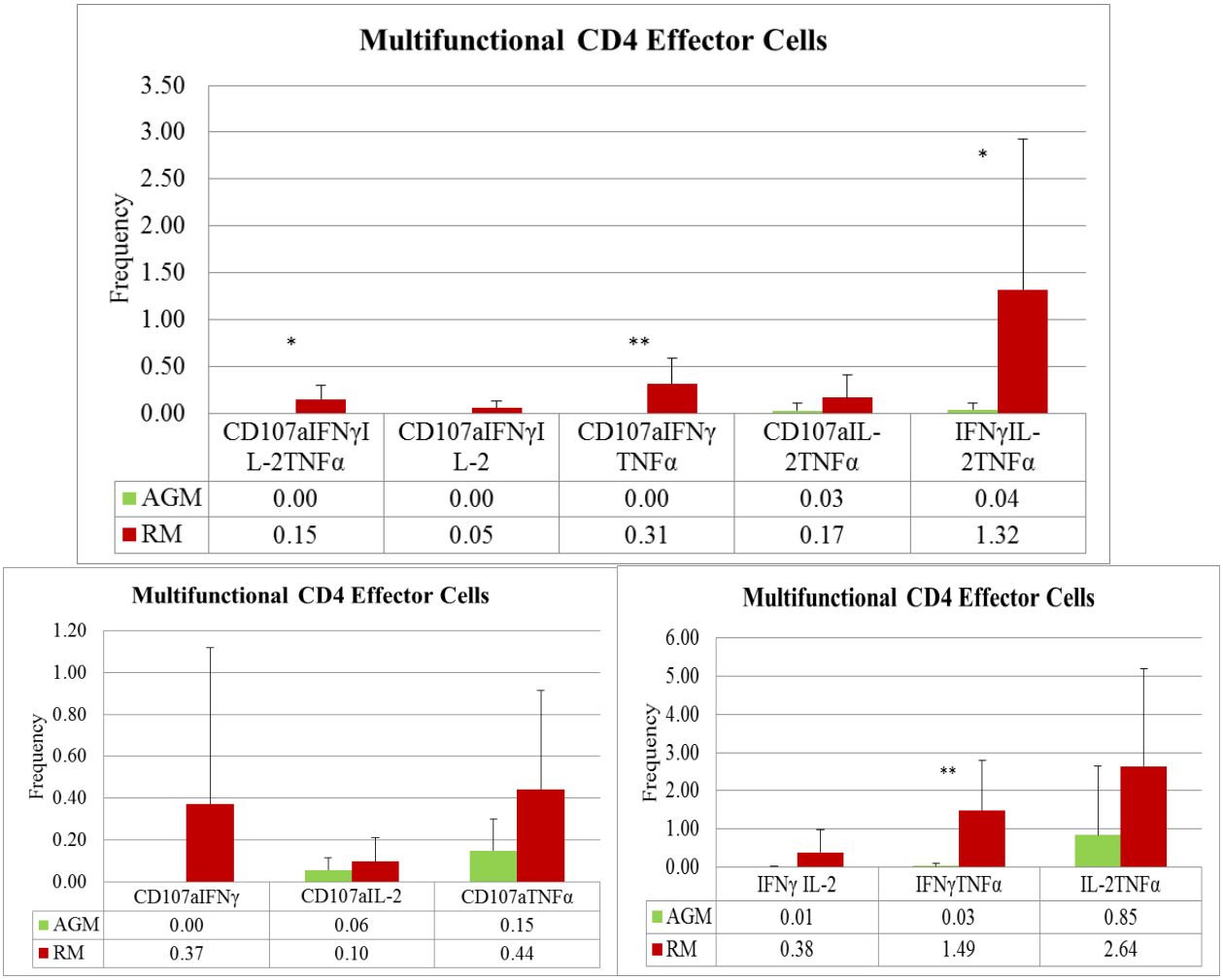


Figure 38. Frequencies of CD4 effector T cells with different combination of functions include: secretion of IFNɣ, TNFα, IL-2, and expression of CD107a.
The data shown are the means of 8 African green monkeys (AGM) and 9 rhesus macaques (RM). The data was considered significant if the p value <0.05*, or <0.01**, or <0.001***, or <0.0001****.

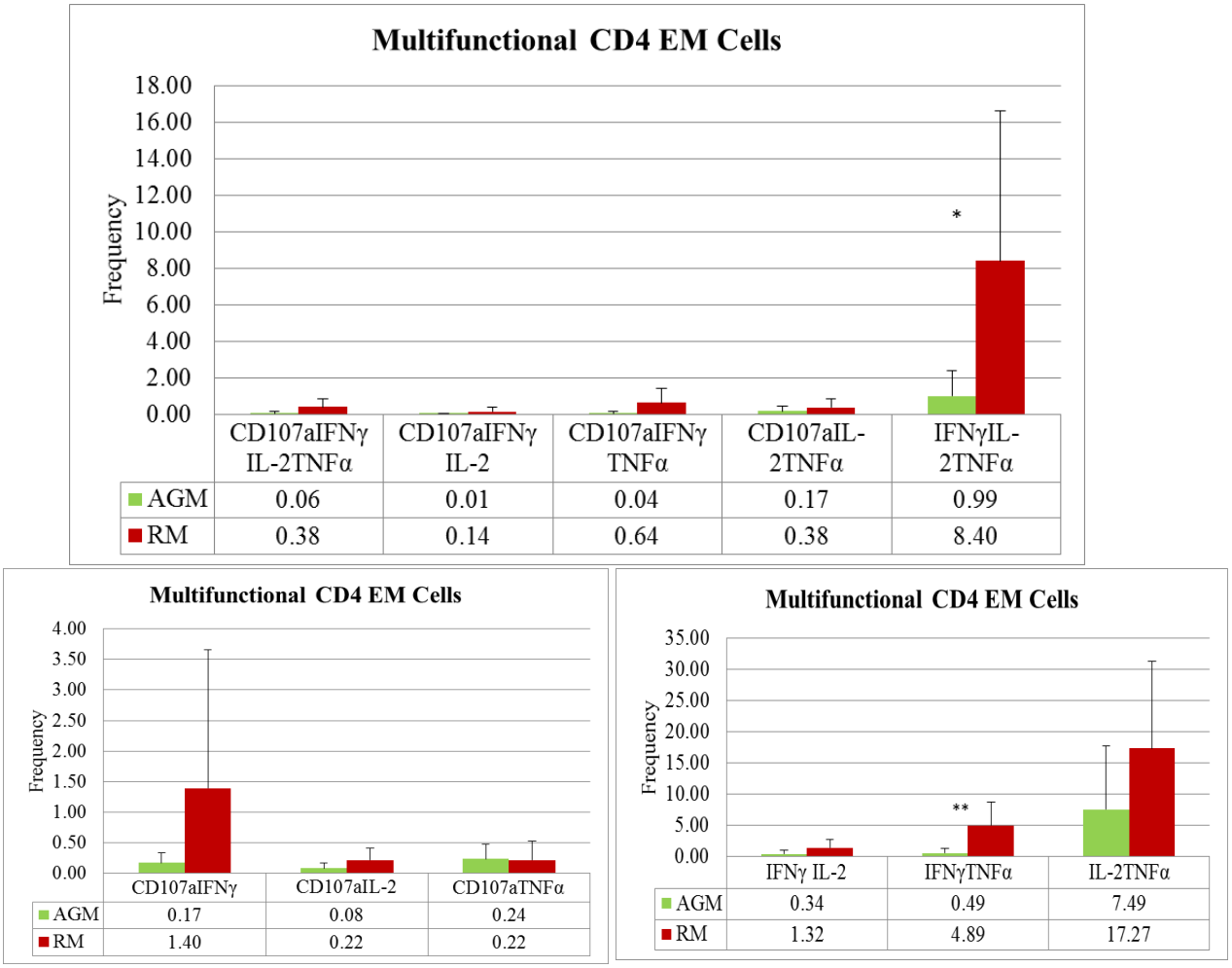
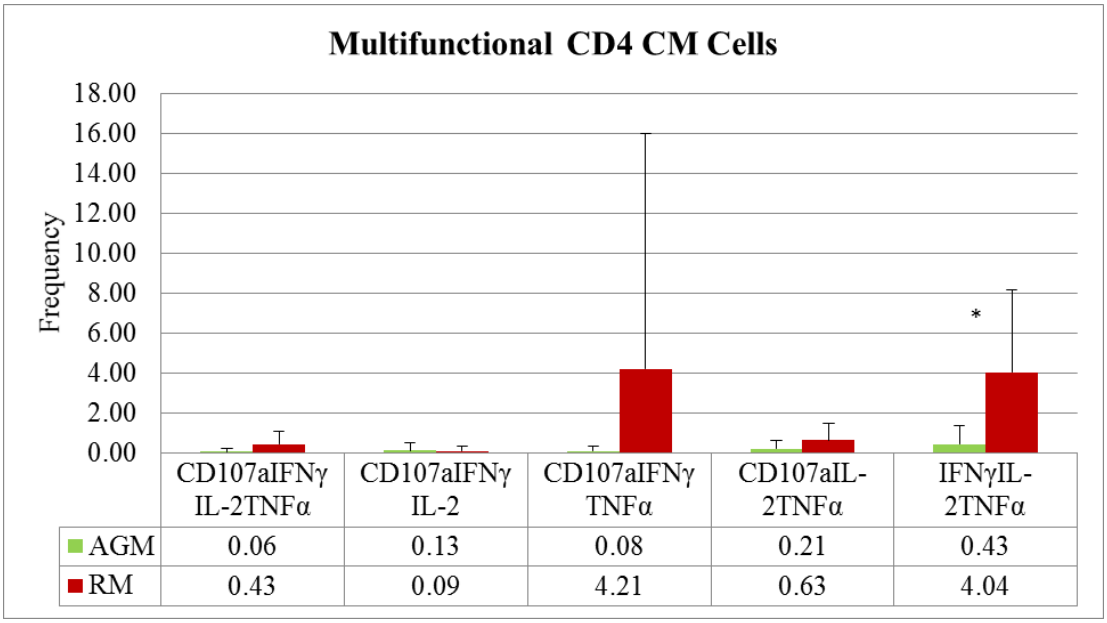
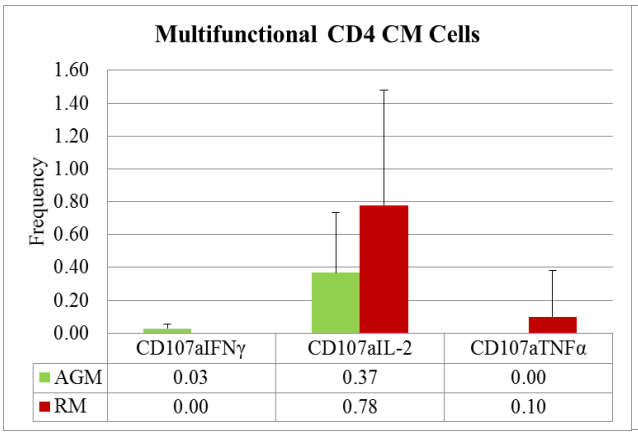


Figure 39. Frequencies of CD4 effector memory T cells with different combination of functions include: secretion of IFNγ, TNFα, IL-2, and expression of CD107a.
The data shown are the means of 8 African green monkeys (AGM) and 9 rhesus macaques (RM). The data was considered significant if the p value <0.05*, or <0.01**, or <0.001***, or <0.0001****.





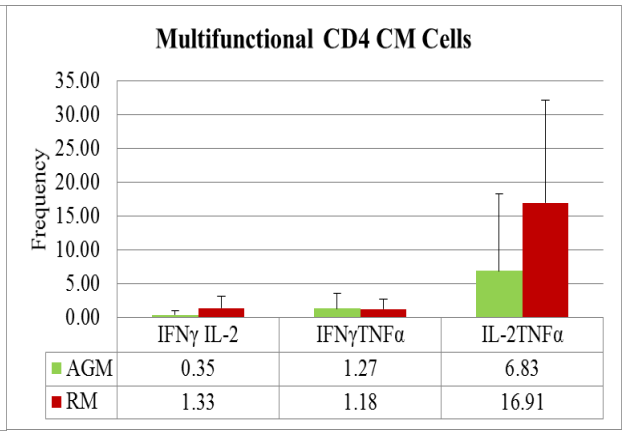
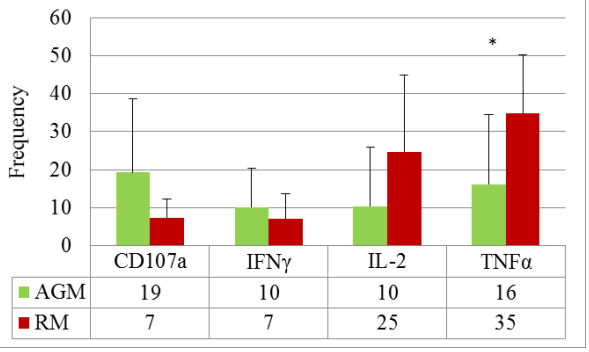


Figure 40. . Frequencies of CD4 central memory T cells with different combination of functions include: secretion of IFNγ, TNFα, IL-2, and expression of CD107a.
The data shown are the means of 8 African green monkeys (AGM) and 9 rhesus macaques (RM). The data was considered significant if the p value <0.05*, or <0.01**, or <0.001***, or <0.0001****.

A

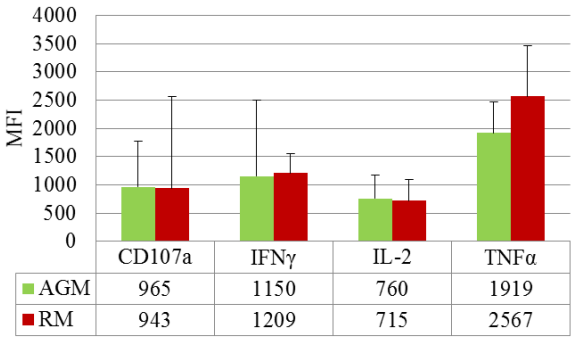
Frequencies of DN Naive Cytokines Producing, and Degranulated Cells



	CD107a	IFNγ	IL-2	TNFα
AGM	19	10	10	16
RM	7	7	25	35

B

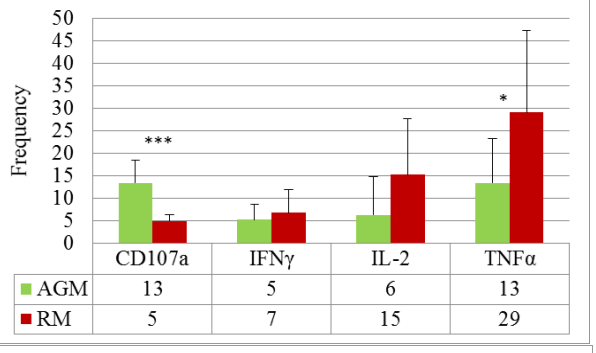
MFI of Cytokines and Degranulation Marker in DN Naive Cells



	CD107a	IFNγ	IL-2	TNFα
AGM	965	1150	760	1919
RM	943	1209	715	2567

C

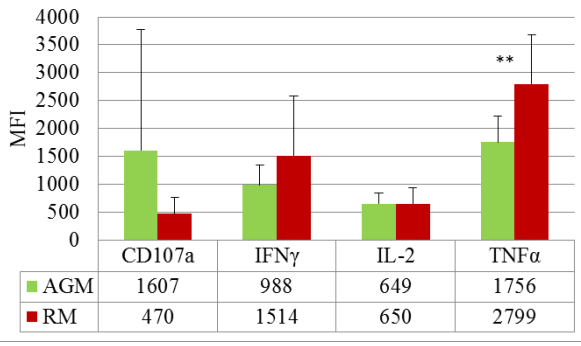
Frequencies of DN Effector Cytokines Producing, and Degranulated Cells



	CD107a	IFNγ	IL-2	TNFα
AGM	13	5	6	13
RM	5	7	15	29

D

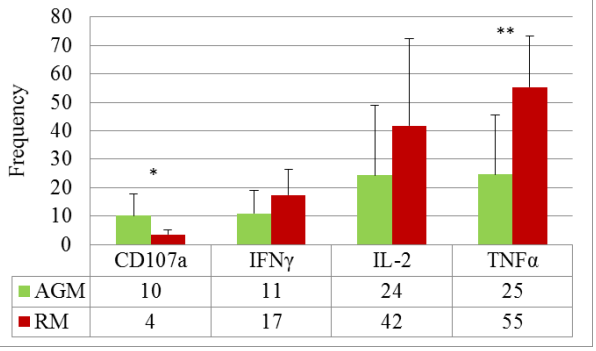
MFI of Cytokines and Degranulation Marker in DN Effector Cells



	CD107a	IFNγ	IL-2	TNFα
AGM	1607	988	649	1756
RM	470	1514	650	2799

E

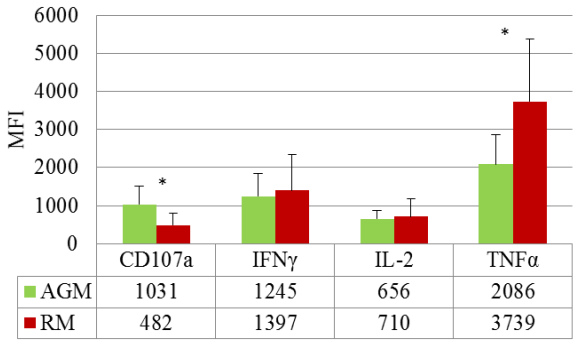
Frequencies of DN EM Cytokines Producing, and Degranulated Cells



	CD107a	IFNγ	IL-2	TNFα
AGM	10	11	24	25
RM	4	17	42	55

F

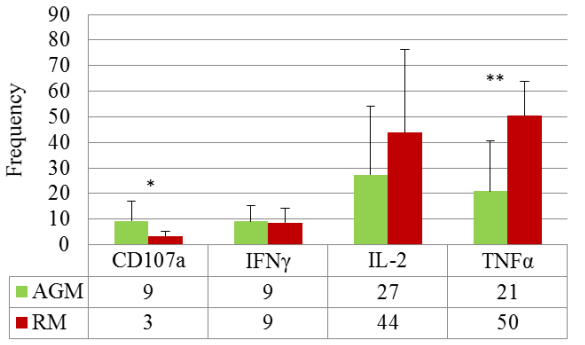
MFI of Cytokines and Degranulation Marker in DN EM Cells



	CD107a	IFNγ	IL-2	TNFα
AGM	1031	1245	656	2086
RM	482	1397	710	3739

G

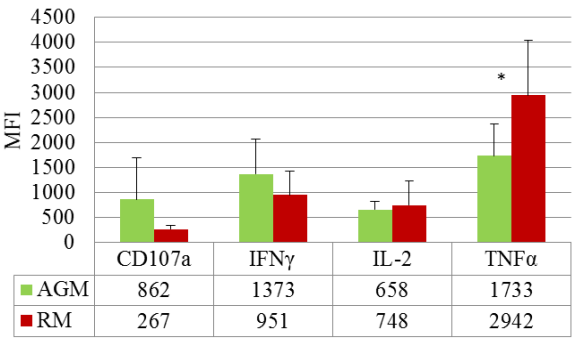
Frequencies of DN CM Cytokines Producing, and Degranulated Cells



	CD107a	IFNγ	IL-2	TNFα
AGM	9	9	27	21
RM	3	9	44	50

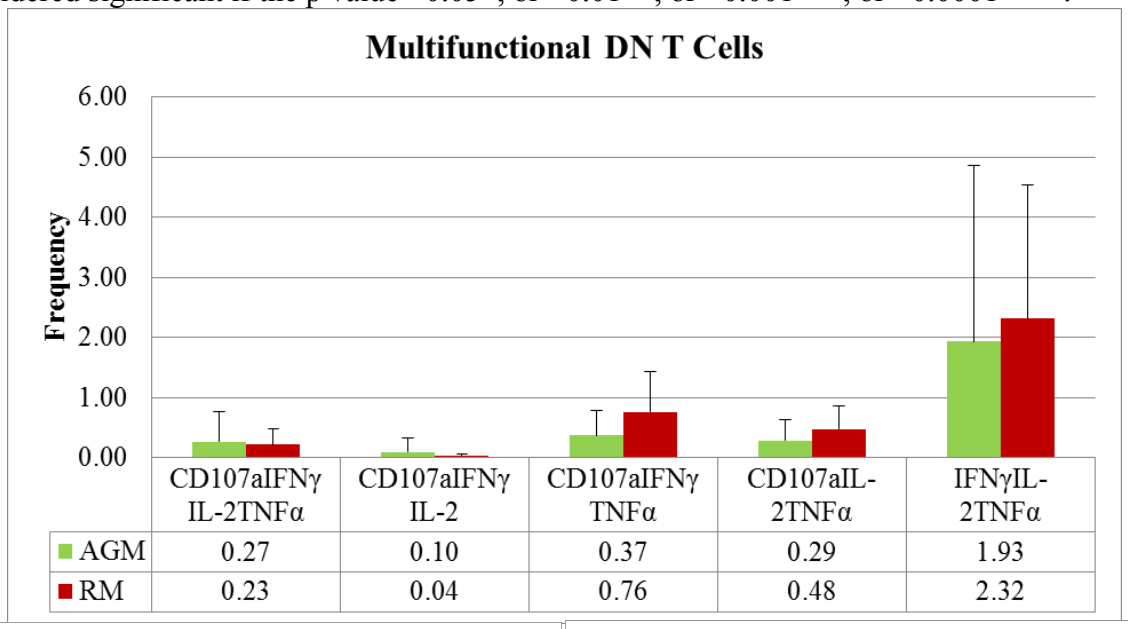
H

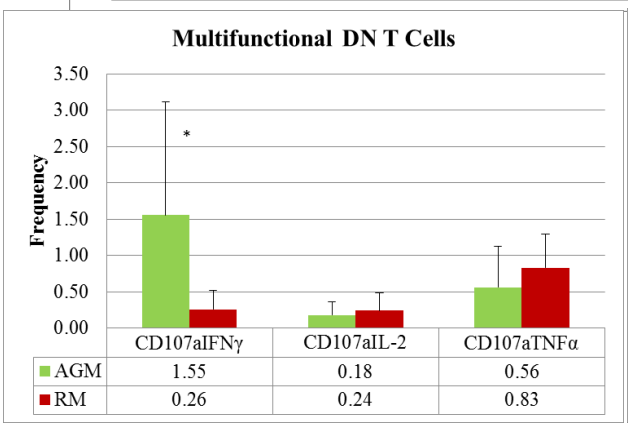
MFI of Cytokines and Degranulation Marker in DN CM Cells



	CD107a	IFNγ	IL-2	TNFα
AGM	862	1373	658	1733
RM	267	951	748	2942

Figure 41. DN T cell subsets producing cytokines and expressing CD107a.
(A) Frequencies of DN naïve T cells producing IFNɣ, TNFα, IL-2, or expressing CD107a in AGM and RM. (B) MFI of IFNɣ, TNFα, IL-2, and CD107a in DN naïve T cells of AGM and RM. (C) Frequencies of CD4 effector cells T cells producing IFNɣ, TNFα, IL-2, or expressing CD107a in AGM and RM. (D) MFI of IFNɣ, TNFα, IL-2, and CD107a in CD4 effector T cells of AGM and RM. (E) Frequencies of DN effector memory T cells producing IFNɣ, TNFα, IL-2, or expressing CD107a in AGM and RM. (F) MFI of IFNɣ, TNFα, IL-2, and CD107a in DN effector memory T cells of AGM and RM. (G) Frequencies of DN central memory T cells producing IFNɣ, TNFα, IL-2, or expressing CD107a in AGM and RM. (H) MFI of IFNɣ, TNFα, IL-2, and CD107a in DN central memory T cells of AGM and RM. The data shown are the means of 8 African green monkeys (AGM) and 9 rhesus macaques (RM). The data was considered significant if the p value <0.05*, or <0.01**, or <0.001***, or <0.0001****.





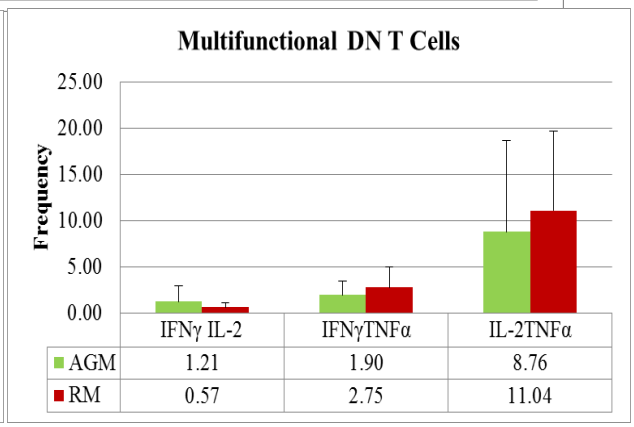


Figure 42. Frequencies of total DN T cells with different combination of functions include: secretion of IFNɣ, TNFα, IL-2, and expression of CD107a.

The data shown are the means of 8 African green monkeys (AGM) and 9 rhesus macaques (RM). The data was considered significant if the p value <0.05*, or <0.01**, or <0.001***, or <0.0001****.

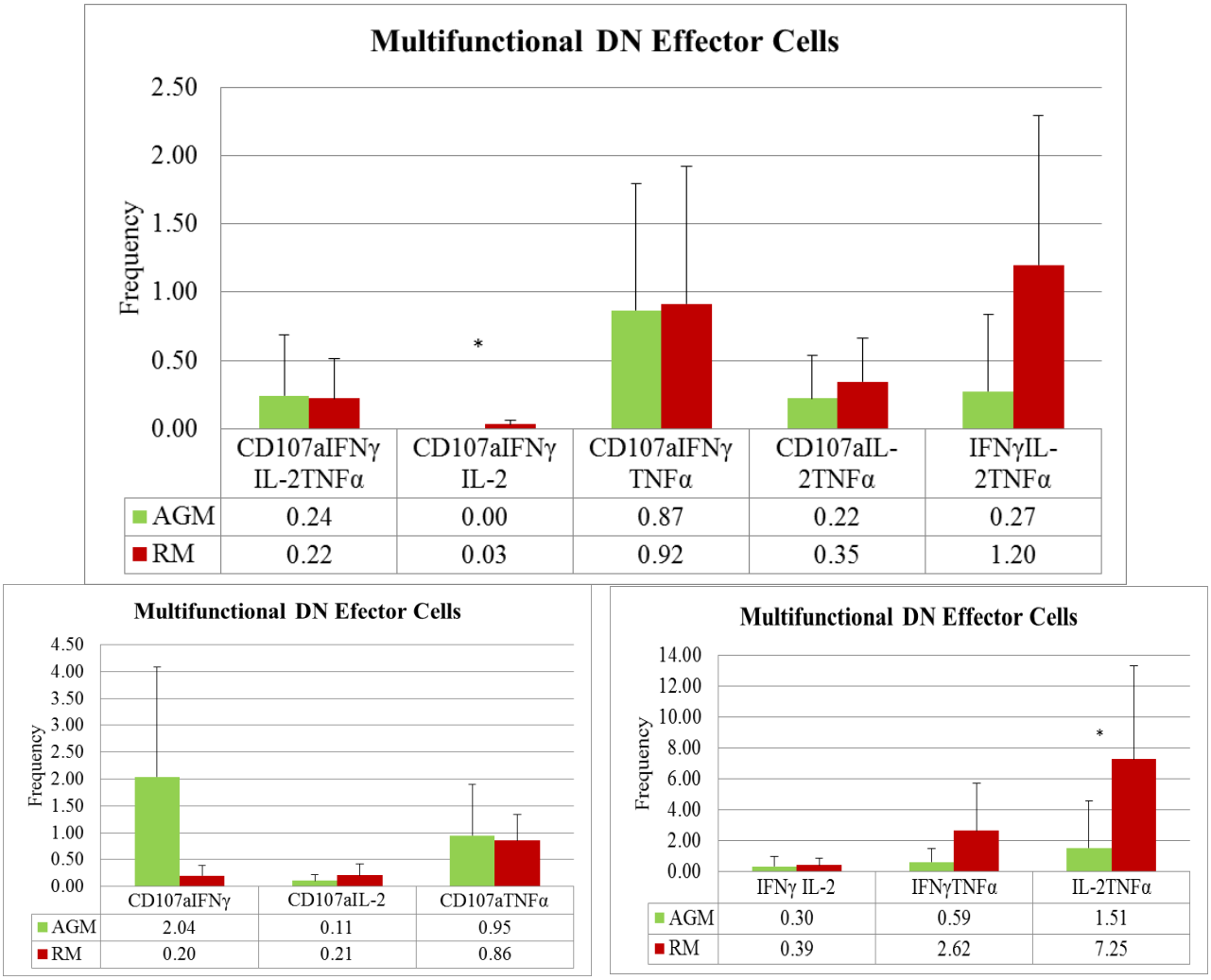


Figure 43. Frequencies of DN effector T cells with different combination of functions include: secretion of IFNɣ, TNFα, IL-2, and expression of CD107a.
The data shown are the means of 8 African green monkeys (AGM) and 9 rhesus macaques (RM). The data was considered significant if the p value <0.05*, or <0.01**, or <0.001***, or <0.0001****.

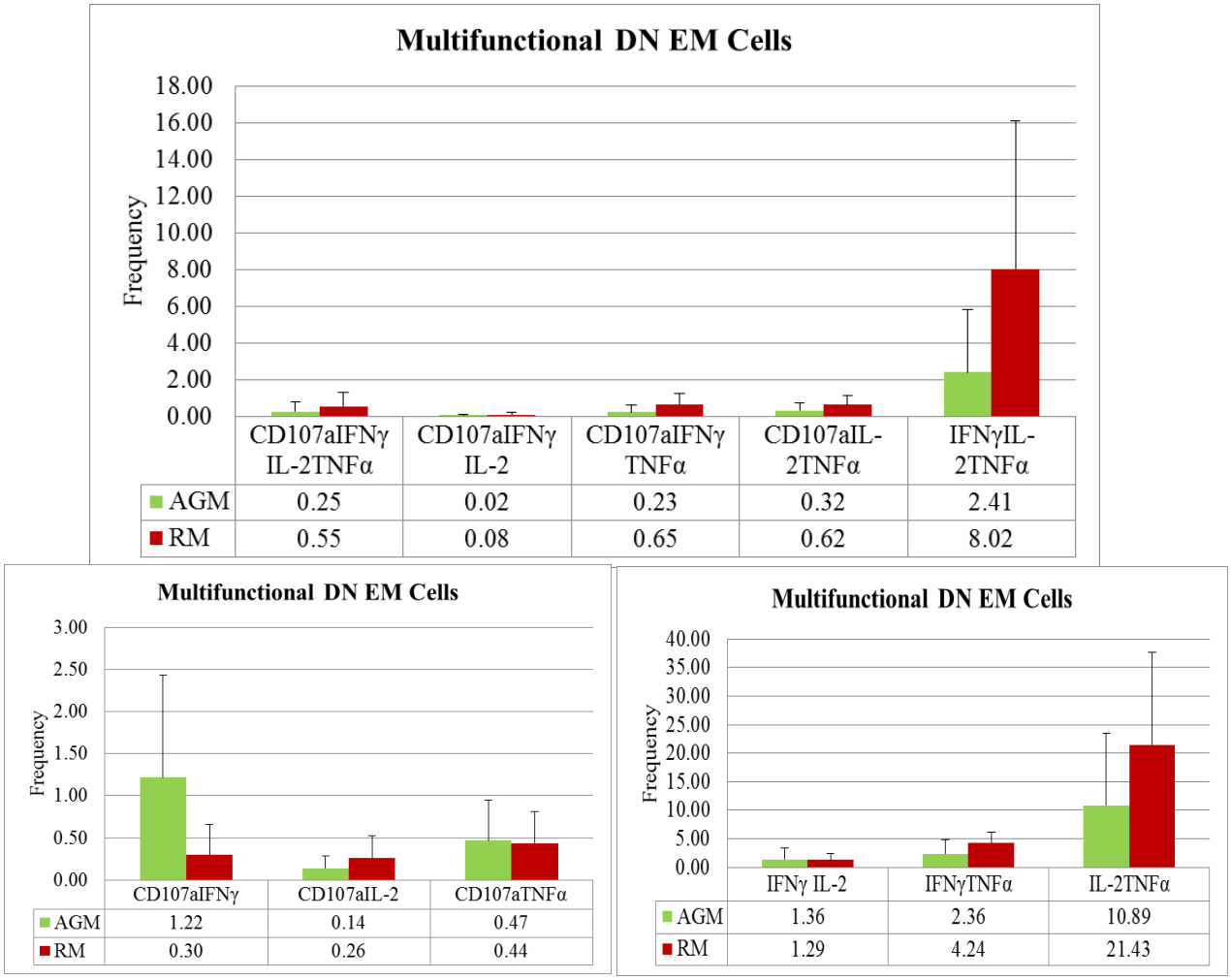


Figure 44. Frequencies of DN effector memory T cells with different combination of functions include: secretion of IFNγ, TNFα, IL-2, and expression of CD107a.
The data shown are the means of 8 African green monkeys (AGM) and 9 rhesus macaques (RM). The data was considered significant if the p value <0.05*, or <0.01**, or <0.001***, or <0.0001****.

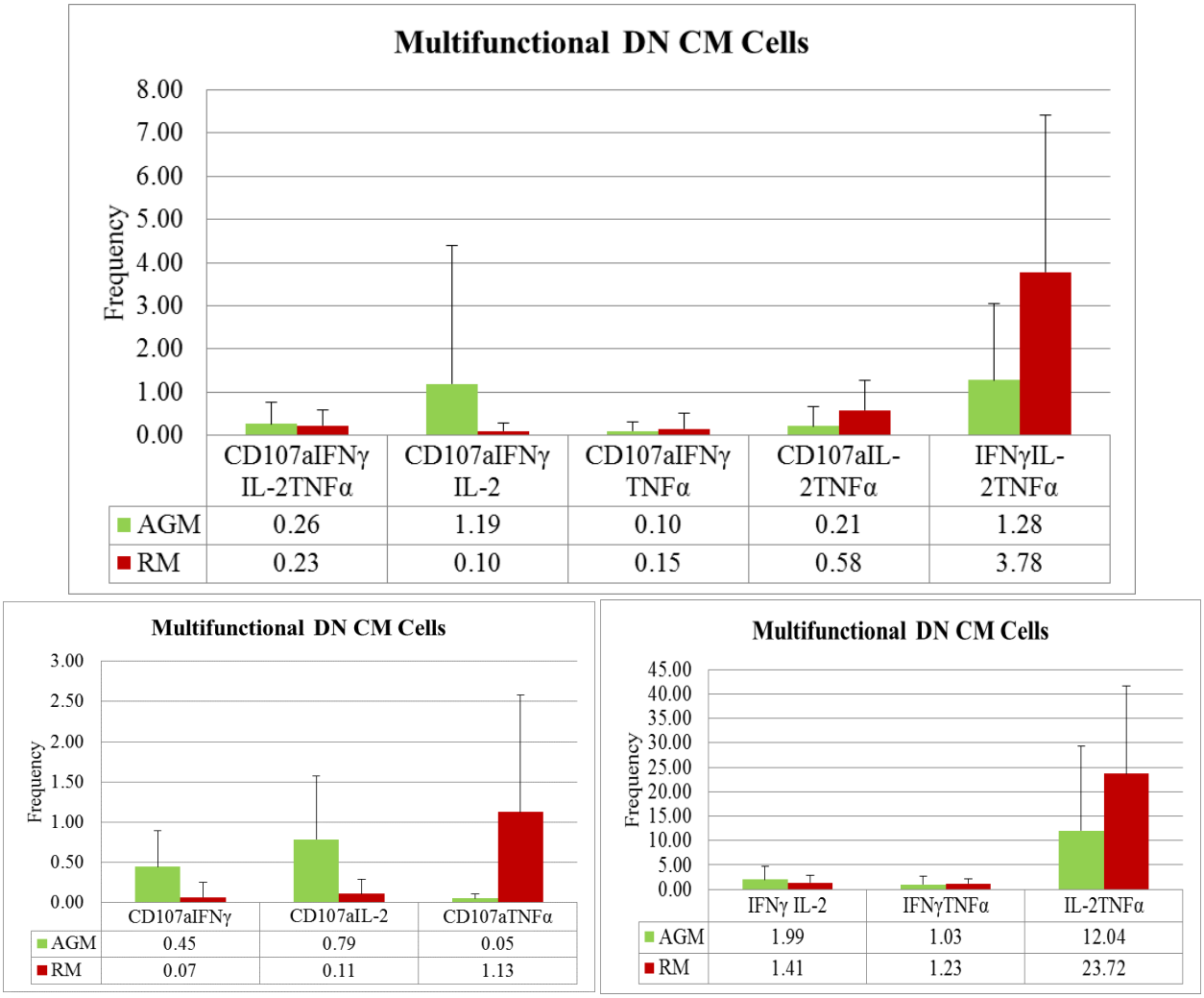


Figure 45. Frequencies of DN effector memory T cells with different combination of functions include: secretion of IFNγ, TNFα, IL-2, and expression of CD107a.
The data shown are the means of 8 African green monkeys (AGM) and 9 rhesus macaques (RM). The data was considered significant if the p value <0.05*, or <0.01**, or <0.001***, or <0.0001****.

CHAPTER IV: DISCUSSION

B cells play an important role in controlling infections by producing antibodies. Activation of B cells to produce protective antibodies requires signals through BCR and co-stimulatory signals provided by T helper cells. The important role of both B and T helper cells have been also emphasized in controlling one of the most serious infections, HIV infection, when the Thai RV144, the only vaccine trial that showed partial efficacy revealed that recipients showed strong CD4+ T cell response and antibody-dependent cellular cytotoxicity (ADCC) (Rerks-Ngarm et al. 2009). In order to understand the protective immune mechanisms against HIV, non-human primates are widely used due to their biological and physiological similarities to humans. Moreover, they get infected with some pathogens that infect humans and show similar pathogenesis. Rhesus macaques (RMs) develop immunodeficiency syndromes similar to humans when they get infected with SIV. In contrast, African green monkeys (AGMs) are resistant to developing AIDS despite their chronic viremia. Studies have shown that AGMs had high titers of neutralizing antibodies (Gicheru et al. 1999), which also may play a role in the rarity of mother-infant virus transmissions via lactation (Wilks et al. 2011) as compared to RMs. This emphasizes the importance of the humoral response and the helper role of CD4+ T cells. In this study, we hypothesized that there are intrinsic differences between AGMs and RMs that are present in the absence of the infection and play a role in the apathogenic nature of SIV infections in AGMs compared to RMs. To explore these differences, we studied different B and T cell parameters in uninfected AGMs and RMs. We explored any variation in the distribution of B and T cell subsets and the expression of activation markers. Furthermore, we investigated T cell cytokine production patterns to identify any functional differences. Additionally, we assessed the activation of B cells by evaluating the phosphorylation of intracellular signaling proteins (plcg2,

ERK1/2, Syk) that function in Ig signaling pathways and play a role in B cell activation and antibody production.

AGMs are characterized by lower counts of lymphocytes compared to RMs. Herein we compared the frequency and the absolute number of T and B cells in these two species. We found that AGMs had fewer total B cells compared to RMs and the differences were statistically significant (Figure. 8). The mean frequency of AGMs B cells is around 50% lower than RMs, while the mean absolute number is around 30 % lower than RMs. Amos et al showed that AGMs had fewer CD20+ B cells than RMs (Amos et al. 2013). However, we did not find statistically significant differences in either the frequency or the absolute number of T cells between AGMs and RMs (Figure. 23). When we further investigated the frequencies and absolute numbers of CD4, and DN T cells, we found that AGMs had statistically significant lower frequencies and absolute numbers of CD4 T cells than RMs (Figure. 24). This is consistent with previous reports that showed that AGMs have fewer CD4 T cells than RMs (Pandrea et al. 2007). However, there were no significant differences in DN T cells (Figure. 24), although studies have shown that AGMs had higher frequencies of DN T cells than RMs (Vinton et al. 2011). Some researchers argue that the low counts of CD4 T cells in AGMs might be a mechanism behind the AGMs resistance to developing simian immunodeficiency syndrome due to the limitation of target cells (Pandrea et al. 2007). However, based on our results AGMs have lower counts of other cells such as B cells. This may indicates a general paucity of immune cells in AGMs.

We further investigated B cell subsets in these models. AGMs showed lower absolute numbers of all subsets (Figure. 9, B). This might be due to the general lower total B cell absolute numbers that AGM had compared to RMs. In terms of frequencies, the only significant difference was in DP memory B cells (Figure. 9, A). These cells are identified as resting memory cells (Susan

Moir et al. 2008; Titanji et al. 2010). However, Das et al showed that these cells are highly activated and had high proliferation and immunoglobulin secretion rates (Das et al. 2011). They also stated that these cells might have a role in SIV infection by maintaining a functional memory B cell subset since they had a high turnover rate (Das et al. 2011). In our study we showed that these cells had a higher percentage of CD86+ (Figure. 10, A), and higher expression levels of CD86 (Figure. 11) and CD40 (Figure. 12) than SP memory cells which are known as activated memory B cells. These data support the study mentioned above by showing that DP memory B cells, which are commonly known as resting memory are highly activated. Naïve B cells of AGMs showed a high percentage of CD86+ cells compared to RMs (Figure. 10, A). This may indicate that AGMs naïve cells have a higher potential to get activated, proliferate and differentiate to memory cells upon antigen stimulation than in RMs. In contrast, the SP memory B cells of AGMs showed significantly lower percentage of CD86+ compared to RMs (Figure. 10, A). However, AGMs showed higher expression of CD40 by naïve, SP, and DP memory B cells than RMs (Figure. 12). CD40/ CD40 ligand interaction is critical for antibody class switching and antiviral humoral responses (Borrow et al. 1996). Also, activation of B cells via CD40 is essential for antigen-specific antibody production (Nonoyama et al. 1993). In addition, blocking CD40/CD40L interaction by anti-CD40L had resulted in a decrease of antibody producing B cells (Huang et al. 2002). The higher expression of CD40 by AGMs may help in a better humoral response as compared to RMs.

In order to study the effects of SIV infection on B cell subsets and the expression of activation markers, we did *in vitro* SIV infection of AGMs and RMs cells. The cells from each species were stimulated with anti-IgM/G and were infected with both SIVmac251 and SIVagm9063-2 separately. We did this to see if the species specific strain has the same effect as a heterologous

strain. We found that SIV infection has no effect in frequencies and absolute numbers of B cell subsets (Figures14, 15). When we evaluated CD86 and CD40 following SIV infection, we found that both viruses increased cell activation in both species. We did not find distinct patterns of alterations of activation markers expression between the two species (Figures 16, 17, 18). Detecting of pathogenic effects of SIV infection on the expression of activation markers might be not detected in this experiment. This might be because of the short infection period, 48hours, and such defects might need longer period of infection. Also, in this experiment we added the stimulus and the virus at the same time. Infecting the cells first and then testing their ability to response to stimulus might be a better approach to detect the effect of the infection on activation markers expression.

For T cell analysis, we further analyzed CD4+ and DN T cells for subsets and the expression of activation markers. We did not find any statistically significant differences in frequencies of CD4 T cell subsets, naïve, central memory, and effector memory (Figure 25A). However, in terms of absolute numbers, AGMs showed significantly lower absolute numbers of the three subsets (Figure 25B). This might be a result of general lower absolute numbers of total CD4 in AGMs.

For DN T cell subsets, AGMs showed significantly fewer DN naïve cells in terms of frequencies and absolute numbers (Figure 25). In contrast, AGMs had significantly more DN central memory cells as compared to RMs (Figure 26). The differences were significant in terms of frequencies but it did not reach statistical significance in terms of absolute numbers. This is consistent with previous reports showed that DN T cells are predominantly memory cells in contrast to CD4 T cells which comprised naïve and memory cells (Vinton et al. 2011; Milush et al. 2011). It is found that DN T cells in SIV natural host such as AGMs and sooty managabeys (SMs) are characterized by helper function and production of Th1, Th2, and Th17 cytokines (Milush et al.

2011; Beaumier et al. 2009). Also, it is found that CD4 T cells of AGMs downregulate CD4 at the memory stage, which turn them to SIV resistant cells (Beaumier et al. 2009). Milush et al found that SM had high frequencies of DN T cells in lymph nodes, bronchoalveolar lavage, and rectum; upon SIV infection DN frequencies increased in these sites (Milush et al. 2011). Also, they found that DN T cells kept central memory phenotype during the infection. Based on that, they hypothesized that these are CD4 and/or CD8 T cells downregulated their markers upon interactions with antigens. In our study, we found that these cells downregulate CD3 as well upon stimulation in a greater extent as compared to RMs (Figure 27). In the Milush et al study, they also found that SIV-infected SM were able to produce protective antibodies upon influenza vaccination despite the low CD4 T cells count during SIV infection. As a result, they hypothesized that DN T cells compensate CD4 T cells loss during SIV infection by providing helper functions (Milush et al. 2011). In this study, we found that DN EM express significantly higher level of CD154 upon stimulation as compared to RMs (Figure 32), which might indicate the helper function of these cells and their role in B cells activation and humoral response. In addition, we found that DN EM and CM express higher level of CD95 upon stimulation as compared to RMs (Figure 33). Further investigation is needed to determine whether this elevated expression indicates that these cells are more susceptible to Fas-FasL mediated apoptosis, or indicates their cytotoxic ability to induce apoptosis to other cells. Also, whether this elevation is a transient acute upon stimulation or persistent needs to be elucidated.

Multifunctional T cells are used to evaluate the quality of T cell responses following vaccination. Also, studies showed that long-term non-progressors had higher frequencies of CD4+IFNɣ+TNFα+IL-2+ or CD4+IL-2+IFNɣ+ cells than progressors who had higher frequencies of cells producing IFNɣ only (Seder, Darrah, and Roederer 2008; Kannanganat et al.

2007). In addition, CD107a is used as a marker of degranulation and the ability of T cells to immediate cytolytic activity by releasing perforin and granzymes (Seder, Darrah, and Roederer 2008). As a result, adding CD107a to the panel we used in our study gave us the ability to assess additional function of T cells. We stimulated PBMC with mass stimulator, PMA/I, which stimulate T cells by passing the TCR receptor. We are assuming that the results we got by using PMA/I will gave us a clue about the potentiality of T cell responses upon encountering specific antigens.

We found that CD4 T cells of AGMs showed lower frequencies of cells producing IFNy and expressing CD107a as compared to RMs (Figure 35). The pattern was similar in the different subset of CD4 T cells: naïve, effector cells, effector memory, and central memory of AGMs (Figure 36). Also the MFI of TNFa in total CD4 and DN were lower in AGMs as compared to RMs (Figure 35); similar patterns were found in different T cell subsets (Figures 36, 41). We further assessed the mutlifunctionality of T cells. We found that AGM had lower frequencies of CD4T cells that produce more than one cytokine as compared to RMs (Figure 37). Although we hypothesized that AGMs will show a stronger immediate response in terms of cytokines secretion. Our results did not support this hypothesis. There is a study that showed AGMs had an immediate anti-inflammatory response when were compared to RMs (Kornfeld et al. 2005). AGM showed higher level of IL-10 and TGF-β (Kornfeld et al. 2005). Our results may indicate that CD4 T cells of AGM have the potentiality of an immediate different function rather than pro-inflammatory response. Further investigation of IL-10, TGF-β, and the frequencies of T-reg will give a clear picture of the nature of AGMs' T cells response. Also, repeating the experiment with specific antigens is needed to define antigen specific responses. In addition, investigating IL-4 secretion will give us information about the T helper role for B cells and antibody

production. Although RMs showed higher frequencies of multifunctional T cells, the challenge is to produce SIV specific multifunctional T cells, which most of progressors fail to do. Also, despite that multifunctional T cells seem to play a role in controlling HIV/SIV infection in humans and RMs non-progressors, the same mechanism is not necessary to be used by AGMs. Interestingly, AGMs control the infection in a different phenomenon. While human and RMs non-progressors characterized by low undetectable viral loads, AGMs have similar viral loads as progressors but they are disease free. Furthermore, the lower levels of pro-inflammatory cytokine secretion by AGMs explain why these monkeys do not develop chronic immune activation following SIV infection. However, AGMs showed higher percentages of DN T cells expressing CD107a than RMs (Figure 35). This pattern was consistent in the different DN subsets as well (Figure 41). Also, CD107a MFI were higher in total DN and EM cells of AGMs than RMs (Figures 35, 41). In addition, DN T cells of AGMs showed higher frequencies of CD107a+IFNɣ+ cells (Figure 42). This may indicates the cytotoxic function of DN T cells in addition to the helper functions. Furthermore, there is a study that showed CD107a+, and CD107a+ FNɣ+ CD4 T cells produced upon experimental SIV vaccine are resistant to depletion following SIV infection (Terahara et al. 2014). Although RMs in our study showed higher frequencies of CD4+CD107a+ and CD4+CD107a+IFNɣ+, the published studies mentioned that there is no correlation between the levels of these cells prior the vaccination and the development of SIV specific CD4 CD107a+, and CD4 CD107a+IFNɣ+ following the vaccination. More studies are needed to compare the ability of both AGMs and RMs to develop SIV specific CD4 CD107a+, and CD4 CD107a+IFNɣ+ cells. On the other hand, since our results showed that AGMs had higher percentages of DN CD107a+, and DN CD107a+IFNɣ+, this may indicate another mechanism by which DN T cells are resistant to SIV infection.

B cell activation and proliferation require signaling cascades that are initiated through antigen-BCR interaction, and additional signals provided by cytokines and T helper cells. The interaction of the antigen and the BCR triggers phosphorylation of the cytoplasmic tails of BCR, ITAMS, which results in phosphorylation of SYK. SYK phosphorylates BLNK, which recruits PLC γ2 that phosphorylate extracellular signal-regulated kinase (ERK). Each one of these signaling proteins plays important roles in B cell development and activation. SYK is essential for B cells clonal expansion, differentiation and maturation (Cheng et al. 1995). PLC γ2 is required for antibody production. Mice deficient in PLC γ2 showed impairments in B cell proliferation and antibody production (Wang et al. 2000). Also, HIV envelope proteins showed an inhibition effect of PLC γ2, which may contribute to B cell pathogenesis during HIV infection (Cefai et al. 1990). The expression of PLCg2 gene was higher in B cells with stronger antibody response (Kasturi et al. 2011). ERK signaling is essential for differentiation of pro-B cells into pre-B cells and subsequent proliferation, development and activation (Gold 2008).

Activation of T cells also requires signaling cascades initiated by antigen-TCR interaction and costimulatory signals provided by antigen presenting cells. Bostik et al compared between the phosphorylation of signaling proteins in CD4 T cells of humans, RMs, and SMs, a natural host of SIV (Bostik et al. 2001). Their results showed that while humans and RMs T cells required both signals (signal through TCR and costimulatory signal), T cells from SM were able to get activated and produce IL-2 by receiving a signal through TCR only, and they were resistant to immune anergy. Also, SM's CD4 T cells showed higher amount of phosphorylated ERK compared to T cells from humans and RMs (Bostik et al. 2001). This study showed differences in the TCR signaling of CD4 T cells between RMs and SMs, which is a possible mechanism of avoiding SIV pathogenesis. Since B cells of AGMs are resistant to SIV induced dysfunction, we

wanted to elucidate any differences in the concentrations of intracellular signaling proteins and their phosphorylation between AGMs and RMs. We found that AGMs express higher levels of signaling key proteins, PLCg2, ERK1, and SYK than RMs (Figures 19, 20, 21). Also, when the cells were stimulated with anti-IgM/G, the cells of AGM showed higher expression of phosphorylated PLCg2 and SYK than RMs (Figure 22). This may indicate the possibility that the B cells of AGMs have lower co-stimulatory signal requirements than RMs, similar to the mechanism mentioned by Bostik regarding SM T cells. Further investigation of the effect of different stimulation conditions on B cells signaling proteins phosphorylation, activation, and function between RMs and AGMs will help to elucidate any differences in BCR signaling between the two species.

In conclusion, this study has identified inherent functional and phenotypic differences of B and T helper cells between the two species, which may contribute to distinct immune responses following SIV infection. AGMs B cells showed characteristics of lower activation thresholds than RMs by expressing higher levels of CD80 and CD40. Furthermore, AGMs B cells showed higher expression levels of total and phosphorylated signaling intracellular proteins, which may indicate lower co-stimulatory signal requirements compared to RMs. This might be a mechanism for an early antibody response to control the infection. In addition, the low levels of pro-inflammatory cytokine secretion may indicate anti-inflammatory responses and explains the lack of chronic immune activation in AGMs. DN T cells in AGMs are characterized by T helper functions. In our study, these cells showed higher expression levels of CD107a as compared to DN T cells of RMs, which may indicate their cytotoxic function. The multifunctionality of these cells in addition to their resistance to SIV may give insights into new cellular therapeutic approaches.

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APPENDIX: Summary of Results

Parameter	Total B Cells	SP CD27+ Memory Cells	DP CD21+CD27+ Memory Cells	Naïve B Cells	Tissue like Memory B Cells
Frequency	AGM<RM (*)	No diff	AGM<RM (***)	No diff	No diff
Absolute Number	AGM<RM (*)	No diff	AGM<RM (*)	AGM<RM (*)	AGM<RM (*)
Frequency of CD86+ cells upon anti-IgM/IgG for 48h	No diff	AGM<RM (*)	No diff	AGM>RM (****)	No diff
Frequency of CD86+ cells upon anti-IgM/IgG for 48h (Normalized to Unstim)	No diff	No diff	No diff	AGM>RM (****)	No diff
MFI of CD86 upon anti-IgM/IgG for 48h	AGM>RM (*)	No diff	AGM>RM (***)	No diff	No diff
MFI of CD40 upon anti-IgM/IgG for 48h	No diff	AGM>RM (****)	AGM>RM (*)	AGM>RM (*)	No diff
MFI of CD40 in CD40hi cells upon anti-IgM/IgG for 48h	AGM>RM (**)	AGM>RM (****)	AGM>RM (**)	AGM>RM (*)	AGM<RM (****)
	Total B cells	**Memory B cells**			
Frequency of PLCg2hi Cells	AGM>RM (**)	AGM>RM (**)			
MFI of PLC-g2 in PLC-g2hi Cells	AGM>RM (**)	AGM>RM (**)			
Frequency of PLCg2low Cells	AGM<RM (**)	AGM<RM (**)			
MFI of PLC-g2 in PLC-g2low Cells	AGM>RM (*)	AGM>RM (*)			
Frequency of ERK1hi Cells	AGM>RM (**)	AGM>RM (**)			
MFI of ERK1 in ERK1hi Cells	No diff	No diff			
Frequency of ERK1low Cells	AGM<RM (**)	AGM<RM (**)			
MFI of ERK1 in ERK1low Cells	AGM>RM (**)	AGM>RM (**)			
Frequency of SYKhi Cells	No diff	No diff			
MFI of SYK in SYKhi Cells	No diff	No diff			
Frequency of SYKlow Cells	No diff	No diff			
MFI of SYK in SYKlow Cells	No diff	No diff			
Frequency of Cells phosphorylated PLCg2 upon anti-IgM/IgG for 10 min	No diff	No diff			

MFI of pPLcg2 upon anti-IgM/IgG for 10 min	AGM>RM (**)	No diff	
frequency of Cells phosphorylated ERK1/2 upon anti-IgM/IgG for 10 min	No diff	No diff	
MFI of pERK1/2 upon anti-IgM/IgG for 10 min	No diff	No diff	
Frequency of Cells phosphorylated SYK upon anti-IgM/IgG for 10 min	No diff	No diff	
MFI of SYK upon anti-IgM/IgG for 10 min	No diff	AGM>RM (*)	

Table 2. Summary of B cell analysis results.
p value <0.05*, or <0.01**, or <0.001***, or <0.0001****. No diff= no statistically significant differences were found.

Parameter	Total CD4 T cells	Naïve CD4 T cells	CM CD4 T cells	EM CD4 T cells	Total DN T cells	Naïve DN T cells	CM DN T cells	EM DN T cells
Frequency	AGM<RM (****)	No diff	No diff	No diff	No diff	AGM< RM (**)	AGM>RM(**)	No diff
Absolute Number	AGM<RM (**)	AGM<RM (*)	AGM<RM (*)	AGM>RM (**)	No diff	AGM< RM (*)	No diff	NO diff
MFI of CD3 (Unstimulated)	AGM<RM(*)	No diff	AGM<RM (****)	AGM<RM (*)	No diff	AGM> RM (*)	No diff	No diff
MFI of CD3 (Stimulated with PMA/I for 6 hours)	AGM<RM (*)	AGM<RM (***)	AGM<RM (**)	AGM<RM (***)	AGM<RM (***)	AGM< RM (****)	AGM<RM (**)	AGM< RM (*)
MFI of CD3 (Ratio)	No diff	AGM>RM(**)	No diff	No diff	AGM>RM (***)	AGM> RM (****)	AGM>RM(*)	AGM> RM (**)
MFI of CD4 (Unstimulated)	No diff	No diff	AGM<RM (****)	No diff	Not tested	Not tested	Not tested	Not tested
MFI of CD4 (Stimulated with PMA/I for 6 hours)	AGM>RM (*)	No diff	No diff	No diff	Not tested	Not tested	Not tested	Not tested
MFI of CD4 (Ratio)	No diff	No diff	AGM<RM (*)	No diff	Not tested	Not tested	Not tested	Not tested
MFI of CD95 (Unstimulated)	Not tested	Not tested	No diff	No diff	Not tested	Not tested	No diff	No diff
MFI of CD95 (Stimulated with PMA/I for 6 hours)	Not tested	Not tested	No diff	No diff	Not tested	Not tested	AGM>RM (**)	AGM> RM (****)
MFI of CD95 (Ratio)	Not tested	Not tested	No diff	No diff	Not tested	Not tested	No diff	No diff
MFI of CD28 (Unstimulated)	Not tested	No diff	No diff	Not tested	Not tested	No diff	No diff	Not tested
MFI of CD28 (Stimulated with PMA/I for 6 hours)	Not tested	No diff	No diff	Not tested	Not tested	No diff	No diff	Not tested
MFI of CD28 (Ratio)	Not tested	No diff	No diff	Not tested	Not tested	No diff	No diff	Not tested
Frequencies of CD25+ Cells following PMA/I stimulation for 6h	No diff	No diff	No diff	No diff	No diff	No diff	No diff	No diff
MFI of CD25 following PMA/I stimulation for 6h	No diff	No diff	No diff	No diff	No diff	No diff	No diff	No diff
Frequencies of CD154+ Cells following	No diff	No diff	No diff	No diff	No diff	No diff	No diff	No diff

PMA/I stimulation for 6h								
MFI of CD154 following PMA/I stimulation for 6h	No diff	No diff	No diff	No diff	No diff	No diff	No diff	AGM> RM (*)

Table 3. Summary of T cell analysis results.
p value <0.05*, or <0.01**, or <0.001***, or <0.0001****. No diff= no statistically significant differences were found. Not tested= this subset of cells were not analyzed for this parameter.

Parameter		Total CD4 T cells	Naïve CD4 T cells	CM CD4 T cells	EM CD4 T cell	EC CD4 T cells	Total DN T cells	Naïve DN T cells	CM DN T cells	EM DN T cells	EC DN T cell
Frequency	***CD107a+***	AGM< RM (**)	AGM< RM (*)	No diff	No diff	AGM< RM (**)	AGM> RM (*)	No diff	AGM> RM (*)	AGM> RM (*)	AGM> RM (***)
	IFNγ+	AGM< RM (**)	No diff	No diff	AGM< RM (**)	AGM< RM (**)	No diff	No diff	No diff	No diff	No diff
	IL-2+	No diff	No diff	No diff	No diff	No diff	No diff	No diff	No diff	No diff	No diff
	TNFα+	No diff	No diff	No diff	AGM< RM (**)	No diff	No diff	AGM< RM (*)	AGM< RM (**)	AGM< RM (**)	AGM< RM (*)
	IL-2+TNFα+	No diff	Not tested	No diff	No diff	No diff	No diff	Not tested	No diff	No diff	AGM< RM (*)
	IFNγ+TNFα+	AGM< RM (*)	Not tested	No diff	AGM< RM (**)	AGM< RM (**)	No diff	Not tested	No diff	No diff	No diff
	IFNγ+IL-2+	No diff	Not tested	No diff	No diff	No diff	No diff	Not tested	No diff	No diff	No diff
	CD107a+TNfα+	No diff	Not tested	No diff	No diff	No diff	No diff	Not tested	No diff	No diff	No diff
	CD107a+IL-2+	No diff	Not tested	No diff	No diff	No diff	No diff	Not tested	No diff	No diff	No diff
	CD107a+IFNγ+	No diff	Not tested	No diff	No diff	No diff	AGM> RM (*)	Not tested	No diff	No diff	No diff
	IFNγ+IL-2+TNFα+	No diff	Not tested	AGM< RM (*)	AGM< RM (*)	AGM< RM (*)	No diff	Not tested	No diff	No diff	No diff
	CD107a+IL-2+TNFα+	No diff	Not tested	No diff	No diff	No diff	No diff	Not tested	No diff	No diff	No diff
	CD107a+IFNγ+TNFα+	AGM< RM (**)	Not tested	No diff	No diff	AGM< RM (**)	No diff	Not tested	No diff	No diff	No diff
	CD107a+IFNγ+IL-2+	No diff	Not tested	No diff	No diff	No diff	No diff	Not tested	No diff	No diff	AGM< RM (*)
	CD107a+IFNγ+IL-2+TNFα+	AGM< RM (*)	Not tested	No diff	No diff	AGM< RM (*)	No diff	Not tested	No diff	No diff	No diff
MFI	***CD107a***	No diff	No diff	No diff	No diff	No diff	AGM> RM (*)	No diff	No diff	AGM> RM (*)	No diff
	IFNγ	No diff	No diff	No diff	No diff	No diff	No diff	No diff	No diff	No diff	No diff
	IL-2	No diff	No diff	No diff	No diff	No diff	No diff	No diff	No diff	No diff	No diff
	TNFα	AGM< RM (**)	AGM< RM (*)	AGM< RM (*)	AGM< RM (***)	AGM< RM (***)	AGM< RM (*)	No diff	AGM< RM (*)	AGM< RM (*)	AGM< RM (**)

Table 4. Summary of T cell cytokine patterns.
p value <0.05*, or <0.01**, or <0.001***, or <0.0001****. No diff= no statistically significant differences were found. Not tested= this subset of cells were not analyzed for this parameter.