AGE-ASSOCIATED B CELL AND T HELPER CELL DYSFUNCTION IN RHESUS MACAQUES

by

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ABSTRACT

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The immune system undergoes drastic changes as we age, known as immunosenescence. An example of this is the dysfunction and subpopulation distribution changes of immune cells. Due to immunosenescence, the elderly have a higher risk of complications and rapid progression of new challenges to the immune system, including human immunodeficiency virus (HIV). In an effort to determine the mechanism of dysfunction of humoral immunity due to age, I compared B cell and T cell responses to non-specific, mitogenic stimuli between aged and young adult rhesus macaque peripheral blood mononuclear cells (PBMCs). Analysis by flow cytometry revealed several differences in B cell population distributions, activation, and signaling that could begin to explain the mechanism of immunosenescence in the elderly. CD4+ T helper cells help activate B cells and CD4-CD8- (double negative) T cells have a role in autoimmune diseases, which are more common in the elderly. Differences in CD4+ and CD4-CD8- T cell subpopulation distributions and CD28 expression with advanced age were also found, along with decreases in multifunctional T cells.

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LIST OF ABBREVIATIONS

ADCC	Antibody-Dependent Cellular Cytotoxicity
AIDS	Acquired Immunodeficiency Syndrome
AM	Activated Memory
BCR	B Cell Receptor
BLNK	B Cell Linker Protein
Btk	Bruton's Tyrosine Kinase
CBC	Complete Blood Count
C	Celsius
CM	Central Memory
DMSO	Dimethyl Sulfoxide
DN	Double Negative
EDTA	Ethylenediaminetetraacetic Acid
EFF	Effector
EM	Effector Memory
ERK	Extracellular Signal-Regulated Kinase
FMO	Fluorescence Minus One
HIV	Human Immunodeficiency Virus
I	Ionomycin
IFN-γ	Interferon γ
Ig	Immunoglobulin

IL	Interleukin
IP3	Inositol-1, 4, 5-Triphosphate
LNTP	Long Term Non-Progressor
MFI	Mean Fluorescence Intensity
NF-κB	Nuclear Factor-kappaB
NIH	US National Institutes of Health
PI3K	Phosphatidylinositol-3 Kinase
PLCγ2	Phospholipase C Gamma 2
PMA	Phorbol 12-Myristate 13-Acetate
RM	Rhesus Macaque
RM (graphs)	Resting Memory
Syk	Spleen Tyrosine Kinase
TCR	T Cell Receptor
Th	Helper T
TL	Tissue-Like
TNF-α	Tumor Necrosis Factor α
WHO	World Health Organization

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INTRODUCTION

Age-Associated Immune Dysfunction

The humoral aspect of adaptive immunity functions to recognize an antigen and mount an appropriate response through antibody production and antigen-specific memory. B cells produce this response after binding of CD40 to its ligand, CD40L or CD154, on CD4+ helper T (Th) cells. This humoral response is also facilitated by pro-inflammatory cytokines produced by Th cells. However, the aging process has profound effects upon the immune system. The collective dysfunctions associated with aging are termed immunosenescence, which causes an increased risk and severity of infectious and autoimmune diseases. Immunosenescence affects the humoral compartment of the immune system in several specific ways, including causing a difference in proportions of B cell subsets (Macallan et al., 2005), impaired responses to vaccination (Goodwin et al., 2006; Hainz et al., 2005), impaired lymphopoiesis (Stephan et al., 1996), and autoimmunity (Rubtsov et al., 2011; Klinman, 1992).

AIDS Risks, Symptoms, and Progression

Acquired immunodeficiency syndrome (AIDS) is a life-long disease that can occur due to infection with the lentivirus human immunodeficiency virus (HIV). Exposure to HIV begins with few symptoms, or commonly with none, in people of any age. Upon infection, it is possible to have flu-like symptoms after a two to four week incubation period (NIH, 2009). This period is known as the primary infection. Afterwards, it may be years before any other symptoms appear; this is known as the latency period where the virus is present but there is no decrease in CD4+ T cells. With antiretroviral therapy, this

period can be extended indefinitely. Without therapy, this period typically lasts around 8 years (Giesecke et al., 1990). After this period, progression to AIDS occurs. The body's ability to mount an immune response is destroyed, and opportunistic infections occur. AIDS is diagnosed by CD4+ T cell count dropping below 200 cells per microliter (US Dept. of Health and Human Services, 2010).

Once HIV is diagnosed, several outcomes have been reported. Long term non-progressors (LTNPs) are individuals who are able to maintain high levels of CD4+ T cells without progressing to AIDS for more than ten years without therapy. A subgroup of LTNPs is elite controllers. Elite controllers are able to maintain limited amounts of virus in addition to high levels of CD4+ T cells in the blood (Okulicz et al., 2009). Alternatively, rapid progressors are diagnosed with AIDS within three years of infection, and one study found that the median age was higher in rapid progressors compared to other groups (Anish et al., 2011). The cause of the differences seen among these groups has not been fully understood.

Older individuals (over 50 years old) are at an increased risk for AIDS because they are diagnosed later in disease and the progression to AIDS is shorter than in younger individuals (Pezzotti et al., 1996; Carré et al., 1994). The reasons for this have not been elucidated.

Humoral Immunity and Vaccine Research

In a phase III trial, the combination of ALVAC and AIDSVAX vaccines against HIV infection with a prime-boost regimen named RV 144 in Thailand resulted in rates of infection dropping almost 30% for the volunteers who received the vaccine compared to

the placebo (Rerks-Ngarm et al., 2009). Because this study was linked to B cell antibody dependent cellular cytotoxicity activity as one of the causes for its success (Bonsignori et al., 2012), research of B cell immunosenescence is necessary to provide a working vaccine in the elderly. More recently, vaccine candidate SAV001 finished a phase 1 trial in humans in September 2013 (Western News communications staff, 2012). This vaccine showed no adverse effects and provoked HIV-1 specific antibody formation. While neutralizing antibodies are found more often in normal progressors and LTNPs than in elite controllers (Doria-Rose et al., 2009), B cells are still necessary because this difference may be attributed to a lack in antigenic stimulation of B cells in elite controllers.

Simian immunodeficiency virus induces a disease homologous to HIV and has a similar structure (Daniel et al., 1985), so using SIV with monkeys is an established model of HIV. In a study by Barouch, et al. (2013), a hybrid of SIV and HIV, termed SHIV, was used as a model for HIV and showed partial success correlated with functional and neutralizing antibody production.

B Cell-Specific Immunosenescence

B cells are typically described as having four main subpopulations. Naïve, activated memory, resting memory, and exhausted (or tissue-like memory) B cells are each studied according to their specific functions, although studying the relative proportions of each can give insight into the mechanism of immunosenescence. For example, circulating memory B cells have been shown to be diminished in old age in humans, although naïve, immature, and total B cell counts have not been found to be

statistically different (Caraux et al., 2010). However, CD27+ (memory) B cells may also have an increased proportion of the B cell count in the elderly (Macallan et al., 2005). In a similar study, CD27+ B cells were found to have a moderately decreased absolute number, but increased percentage of the B cell repertoire in elderly humans (Colonna-Romano et al., 2003). Lastly, a study by Twillert, et al. (2014) found similar results, indicating a decreased number of circulating B cells but a higher memory B cell proportion in humans. However, in a study using Chinese-origin rhesus macaques, advanced age was associated with lower total B cell counts, fewer naïve B cells, and increased resting memory B cells, while activated memory B cell subsets were not different between age groups (Zheng et al., 2014). Furthermore, another study found absolute counts of circulating B cells decreased with age in rhesus macaques (Didier et al., 2012). There appears to be a similar loss in B cells in aged macaques and humans, along with an increased proportion of memory B cells.

The humoral response to antigen is impaired with advanced age. B cell diversity is known to be decreased in the elderly. This was demonstrated through measuring the available antigen-binding regions of the B cell receptors and antibodies (Gibson et al., 2009). Also, serum immunoglobulin G (IgG) and IgA levels are increased in the elderly, while IgD and IgM levels decrease with age; however, the IgG increase was only significant in males and the IgM decrease was only significant in females, which was attributed to a small sample size of males (Listi et al., 2006). In one study by Colonna-Romano et al. (2006), IgD memory B cells were increased in proportion to a naïve cell decrease in the aged humans compared to young adults' B cells. This shift from naïve to memory B cell numbers with age is also seen in macaques (Zheng et al., 2014).

Certain subpopulations of B cells are affected by age as well. It has been shown that CD5+ (B-1, IgM secreting) B cells are decreased in percentage and absolute numbers in the aged (Colonna-Romano et al., 2003). Thus, age and possibly gender can affect the B cell antibody response, which could result in an inefficient reaction to both novel and previously encountered antigens.

Collectively, these results indicate that memory B cells, indicated through class-switching and surface marker expression, are increased in the elderly because the B cells have had more time to differentiate and had more exposures to different antigens. The increase is not likely due to increased activation of naïve B cells, because class-switching is down-regulated with advanced age (Frasca et al., 2005). Another explanation for increased proportions of memory B cell in the elderly is that age-associated B cells within the spleen can secrete tumor necrosis factor alpha (TNF-α), which induces apoptosis of pro-B cells within mice (Ratliff et al., 2013). TNF-α secretion is known to decrease in B cells from aged mice (Frasca et al., 2012), but it has not been studied in aged monkeys or humans. Dysfunctional lymphopoiesis due to TNF-α secretion could explain the lower levels of naïve cells as well as more encounters with antigens leading to differentiation.

Functional defects of B cells, such as non-specific or chronic activation, due to senescence have not been studied in depth. One study found decreased expression of activation marker CD25 in the elderly (Amu et al., 2014). However, the effects of immunosenescent B cell accumulation are noticeable when studying vaccination in the elderly. For example, vaccination against pneumococcal pneumonia with PPV23, a T cell-independent stimulator of the B cell response, is much less effective in the elderly

(Blomberg et al., 2012). The mechanism is not well understood, partially due to inadequate correlates of protection. A similar study found that aged mice produce antibodies against *Streptococcus pneumoniae* that have a lower affinity, avidity, and protective function than young mice (Nicoletti et al., 1993). Similarly, the B cell functional response to the influenza vaccine decreases with age (Blomberg et al., 2012). Interestingly, B cells from aged mice actually suppressed a response to a novel antigen when the responding B cell is of the same heavy chain allotype locus (Klinman, 1981). Proliferation also appears to be diminished in the elderly, which has consequences for protective effects of vaccinations; the study by Whisler, et al. (1991), measured RNA synthesis and DNA replication through non-specific stimulation with immobilized anti-IgM antibodies and found deficiencies in the proliferative response of the B cells in the aged.

As part of the signaling cascade of stimulation of the BCR, several important proteins are phosphorylated. These include Src-tyrosine kinase (Syk) and phospholipase C gamma 2 (PLCγ2) (Ying et al., 2011). Syk is phosphorylated first, which then phosphorylates B cell linker protein (BLNK) (Ying et al., 2011). BLNK then regulates phosphorylation of PLCγ2 by Syk (Fu et al., 1998). Due to this process, extracellular signal-regulated kinase (ERK) becomes phoshorylated and translocates to the nucleus.

Syk is expressed in a multitude of hematopoetic cell types and several non-hematopoetic cell types (Duta et al., 2006). In B cells, phosphorylated Syk continues the BCR signaling cascade by phosphorylating other proteins to generate inositol-1,4,5-triphosphate (IP3), leading to increased intracellular Ca2+ levels from the endoplasmic

reticulum's lumen (Verkhratsky and Petersen, 2002), and mobilizing free calcium ions in the cytoplasm (Takata et al., 1994). Syk is recruited to the B cell receptor (BCR) complex at the plasma membrane upon stimulation of the cell, resembling a cap under fluorescent microscopy (Ma et al., 2001).

PLCγ2 is expressed on platelets (Zheng et al., 2015), hematopoetic cells (Wilde and Watson, 2001), and B cells. In B cells, PLCγ2 becomes phosphorylated by Bruton's tyrosine kinase (Btk) by attaching to the B cell linker protein (BLNK)-Btk complex (Kurosaki and Tsukada, 2000). Activated PLCγ2 causes an increased amount of available Ca2+ ions in the cytoplasm through phospholipase activity on phosphatidyinositol-4,5-bisphosphate to produce IP3 (Berg et al., 2002). After stimulation of B cells, PLCγ2 is recruited to lipid rafts supporting the BCR itself at the cellular membrane within minutes (Aman and Ravichandran, 2000).

The ERK1 (MAPK3) and ERK2 (MAPK1) proteins are mostly ubiquitously expressed. ERK becomes phosphorylated in proportion to the amount of stimulus when used to spur the cell into reversible responses to stimulation; however, when used to go through irreversible processes such as differentiation, ERK becomes phosphorylated in an all-or-nothing manner (Caunt and McArdie, 2012). When the BCR is triggered, ERK phosphorylation peaks very quickly and remains higher than normal for at least half an hour afterwards in a PLCγ2-dependent manner due to PLCγ2's ability to mobilize Ca2+ ions (Jacob et al., 2002). Depending on the stimulus, phosphoERK is translocated into the nucleus in a graded fashion, e.g. as the stimulation increases, ERK levels within the nucleus increases (Caunt and McArdie, 2012). Increased Ca2+ concentration within the cytoplasm halts this translocation, demonstrated by Chuderland et al. (2008) in a study

with rat fibroblasts.

Any dysfunction in this complicated process can lead to an inability of the B cell to activate, or proliferate. However, no studies have been conducted to determine whether there are deficiencies in signaling protein phosphorylation or recruitment to the BCR in the elderly.

B Cell Response to HIV

Since its discovery in the 1980s, human immunodeficiency virus (HIV) has had an immense impact on the world. HIV is the cause of acquired immunodeficiency syndrome (AIDS) that currently has no cure and is lethal without treatment (Barré-Sinoussi et al., 1983; Gallo et al., 1983; Popovic et al., 1984; Sarngadharan et al., 1984). Collectively, approximately 1.8 million people died of AIDS-related causes globally in 2010, with 2.7 million people newly infected with HIV (WHO, 2011). In North America alone, there were twenty thousand deaths due to AIDS and forty eight thousand new cases of HIV infection. In Africa, where the disease is highly prevalent and there is a growing need for medical care, there were 1.2 million deaths and 1.6 million new cases in 2010 (WHO, 2011). Despite much research within the field of HIV, the disease progression is quicker (Phillips et al., 1991) in the elderly regardless of route of infection (Pezzotti et al., 1996), and the mechanisms involved have not yet been found.

B cells produce antibodies that neutralize toxins and some viruses, increase phagocytosis of antibody-covered pathogens, and help in antibody-dependent cell-mediated cytotoxicity (ADCC). ADCC is the process in which antibodies attach to

antigens on the surface of a cell, effector immune cells recognize the Fc portion of the antibody, and the effector cells lyse the target cell. HIV causes B cell dysfunction (Lane et al., 1983), but while the mechanisms are not known, the phenotypes of B cells associated with progression to AIDS have not been studied. Furthermore, HIV surface protein gp120 has B cell superantigen properties (Berberian et al., 1993; Berberian et al., 1994) that can have a prolonged effect on the ability of the host to respond to immunological threats.

B cell dysfunctions during HIV infection vary among reports, but there are certain similarities. Early after HIV-specific antibodies are detectable, B cells become nonresponsive and fail to produce antibodies; after two years, B cells fail to differentiate normally under CD4+ T cell helper activity (Terpstra et al., 1989). After AIDS is diagnosed, B cells which spontaneously produce antibodies are found in the blood, leading to hypergammaglobulinemia (Lane et al., 1983); this may be due to the superantigen properties of gp120 mentioned above (Berberian et al., 1993; Berberian et al., 1994). B cells with diminished capacities of proliferation and differentiation are both found during HIV infection as well (Lane et al., 1983). Even with therapy started early after infection, memory B cell loss still occurs; however, B cell apoptosis and SIV-specific antibodies were abrogated when a therapy regimen was given in an SIV study by Peruchon, et al. (2009). Furthermore, populations of B cells in the periphery are altered in response to HIV infection. For example, there is an increased frequency of plasmablasts and tissue-like B cells circulating in the blood of HIV-infected individuals compared to uninfected individuals (Moir et al., 2010). Activated memory B cells and transitional B cells are increased as well, corresponding to a decrease in resting

memory and naïve B cells (Moir et al., 2010), which might contribute to low-level chronic inflammation. In another study by Amu et al. (2014), the authors found decreased expression of CD25, an activation marker on B cells, in the uninfected aged and HIV infected, aged groups, indicating that there is a defective response to stimulation. They also determined that CD69 expression, another activation marker, is increased on the B cells of younger individuals, and therefore is not able to be a reliable marker for relative proportions of activated B cells compared by ages. However, this study also reveals that CD25 is probably not correlated to B cell hyperactivation seen in the aged and HIV-infected individuals, but perhaps the diminished proliferation in the aged and HIV-infected people. The defect in proliferation, as indicated by decreased expression of CD25, has been seen in the context of HIV in the results of another study by Moir et al. (2003), but it has not been confirmed in the elderly population. Moir et al. (2003) also found the lack of proliferation was observed despite normal levels of CD154 expression on CD4+ T cells; CD40-CD154 interactions stimulate B cells to produce CD25. However, dysfunctional activation and differentiation are of utmost importance for combating HIV infection, but the CD40-CD154 pathway has not been studied at length between age groups and HIV infection. All of these dysfunctions seen contribute to the inability to terminate infection before progression to AIDS.

Since people are able to live longer with HIV due to improved therapy, there is a growing prevalence of people over 50 years of age living with HIV (Mahy et al., 2014). Progression to AIDS is more rapid in the elderly (Phillips et al., 1991), and the age at seroconversion increases the risk of death due to HIV as well (Babiker et al., 2001). This indicates a need for further research on the mechanism of age-induced

immunosenescence pertaining to HIV.

T Cell-Specific Immunosenescence

While T cell-independent activation of B cells is possible, it does not confer a true memory response (Hosokawa, 1979). T cell-dependent activation of B cells provides the impetus for B cell differentiation into long-lasting memory B cells. Immunosenescence interrupts this dialogue through defective T cell receptor (TCR) signaling and activation (Fülöp et al., 1999), lesser Th proliferative capacity (Haynes et al., 2005), and fewer cytokines produced (Naylor et al., 2005). The dysfunction in TCR signaling is due in part to deficient recruitment and phosphorylation of signaling cascade proteins downstream of the TCR, including ZAP-70 and Lck proteins (Fülöp et al., 1999).

The diversity of the TCR is impaired drastically beyond 65 years of age in humans; this reduction is consistent among naïve and memory T cells (Naylor et al., 2005). Along with this loss of diversity, there is a depletion of the naïve T cell subset found in advanced age due to high turnover rates; this is consistent in humans and rhesus macaques (Čičin-Šain et al., 2007). Other subsets negatively affected by advanced age are increased central memory and decreased effector memory T cell proportions (Kang et al., 2004). This was correlated with a lack of long-term protection against influenza after inoculation (Kang et al., 2004). Furthermore, in order to maintain the functionality of the T cell response despite this depletion, memory T cells in the elderly are generally older than their counterparts in the young. This cellular ageing is at least partially responsible for immunosenescent dysfunction in proliferation and IL-2 production (Haynes et al., 2005).

CD28 is a costimulatory molecule expressed on T cells. Stimulation through interaction with CD80, and to a lesser extent CD86, causes down-regulation of CD28, but its expression is typically restored quickly (Eck et al., 1997). Immunosenescence is also associated with down-regulation and eventual loss of CD28 on T cells (Effros et al., 1994). During ageing, there is an increase in CD4+CD28- T cells that exhibit defective responses to antigens and have been implicated in age-related development of rheumatoid arthritis (Schmidt et al., 1996). CD4+CD28- T cells in the aged have also been found to have decreased CD154 expression, indicating defective B cell stimulatory capacities, and to react to self antigens, indicating a predisposition for autoimmune disorders in the elderly (Weyand et al., 1998). This population is apparent in aged human and non-human primate species *in vivo* and in human cells after *in vitro* ageing (Effros et al., 1994). However, this cell type is typically absent in mice (Weng et al., 2009; Ortiz-Suarez and Miller, 2002), indicating that murine models of immunosenescence are limited in their capacity to correlate to aged humans. CD154 is a costimulatory molecule used in activating B cells through interaction with CD40 on the B cell surface. In advanced age, there is a decrease in CD154 expression on CD4+ T cells, which is correlated to defective B cell activation (Haynes and Eaton, 2005).

Double negative (DN) T cells, expressing neither CD4 nor CD8, can have either regulatory or inflammatory functions. Their regulatory function aids in preventing certain types of autoimmunity such as allograft rejection or autoimmune diabetes (Ford et al., 2007), which may be helpful in the elderly where low-level chronic immune activation is more common. However, these cells have also been implicated as at least partial causes in other autoimmune diseases such as systemic lupus erythematosis (Crispín et

al., 2008) and autoimmune lymphoproliferative syndrome, where they have a functional profile similar to that of CD8+ T cells (Bristeau-Leprince et al., 2008).

T Cell Response to HIV

Because HIV can integrate into the DNA of an infected T cell, eliminating infection is a complicated issue for finding a cure because it becomes hidden to antiviral drugs. The main reservoir of latent HIV infection is the resting memory CD4+ T cell subset, while active replication of the virus is mainly found in activated CD4+ T cells. In blood, DN T cells are the main source of active viral replication in humans (Kaiser et al., 2007). DN T cells have been found to harbor latent HIV and aid in persistent infection in humans (DeMaster et al., 2015).

DN T cells in sooty mangabeys have a large diversity of cytokines secreted in response to a stimulus, with a profile similar to CD4+ T cells (Rout et al., 2014). Rhesus macaques possess DN T cells with a less diverse cytokine repertoire and a profile more similar to CD8+ T cells. This difference between species is seen even after SIV infection, with a further decrease in rhesus macaque DN T cell functionality. These results implicate DN T cells as a possible cause for the lack of development to SAIDS in sooty mangabeys.

While CD28- T cells have been characterized in the elderly, this population has not been studied in the context of HIV infection. CD28 interacts with CD80/86 on B cells, where CD80/86 is known to be an activation marker. Ligand binding of CD28 on the T cell stimulates expression of CD154 and CD25, important molecules for interacting with B cells as discussed above. During HIV infection, B cells express higher

levels of CD86 correlated with higher viremia (Nicholas et al, 2013); but, at the same time, CD86 expression could not be stimulated *in vitro* to the same extent (Malaspina et al., 2003). Dysfunctional CD28-CD86 interactions may contribute to our inability to cure HIV infections, but this pathway may also be dysfunctional during immunosenescence. Age-independent senescent T cells expressing CD4 and CD57 contribute to higher levels of IFN-γ in response to stimulation during HIV infection (Fernandez et al., 2011). Uninfected control samples showed higher levels of IL-2 instead (Fernandez et al., 2011). This shows a marked difference in the CD4+ T cell activation pathways between HIV infected and uninfected samples. IFN-γ is an antiviral cytokine, which would be appropriate against the cytomegalovirus used as a stimulant in the experiment by Fernandez et al. (2011). IL-2 is a multifunctional cytokine used to activate B cells and recruit lymphocytes to the site of infection.

Hypothesis

The hypothesis of this thesis is: *Aged rhesus macaques will have deficient B cell activation leading to less robust B cell, CD4+ T cell, and DN T cell responses.*

Specific Aims

Specific Aim One: Determine if inherent differences between aged and young adult rhesus macaque B cell responses are due to dysfunctional B cell activation. The working hypothesis of this aim is: Aged rhesus macaques will have a higher proportion of memory B cells, but an overall decrease in B cell numbers, and fewer activated B cells after *in vitro* stimulation compared to young adult macaques. This will be

addressed by finding the absolute circulating B cell counts per microliter of blood and frequencies of B cell subpopulations using flow cytometry and complete blood count (CBC) data. Since gender may play a role in age-related immune function, data will be analyzed for comparisons within both male and female samples. For activation, after *in vitro* stimulation, activated B cell counts, frequencies, and MFI values for activation surface markers will be measured and analyzed. Along with this, potential for activation of signaling proteins within the B cell activation signaling cascade will be measured via phosphorylation.

Specific Aim Two: Determine B cell dysfunction differences between young adult and aged rhesus macaques after *in vitro* SIV exposure. The working hypothesis of this aim is: Aged rhesus macaque B cells will have fewer activated B cells than young adults after *in vitro* exposure to infectious SIV. This will be addressed by finding the counts, frequencies, and MFI values for surface activation markers of B cell subpopulations after *in vitro* exposure to infectious SIV co-incubated with a B cell-specific stimulus. By co-incubating with a stimulus, down-regulation or other SIV-induced dysregulation of B cell activation during viral infection may be found worse with advanced age.

Specific Aim Three: Determine differences in T cell functions between aged and young adult rhesus macaques. The working hypothesis of this aim is: Aged rhesus macaques will have fewer naïve CD4+ T cells and fewer activated CD4+ and DN T cells after *in vitro* stimulation compared to young adult macaques. This will be addressed by measuring CD4+ and DN T cell subpopulation proportions (counts and frequencies),

surface marker expression, activation marker expression, and cytokine secretion profiles. Correlations between T cell molecules with B cell ligands will be assessed to see whether deficiencies in the T cell response could lead to diminished B cell responses or hyperactive B cells. Decreased CD4+ T cell aid could explain B cell immunosenescence and diminished capacities to respond to vaccination in the elderly. Since rhesus macaques' DN T cells exhibit CD8+ T cell profiles as mentioned previously (Rout et al., 2014), it would be expected that they would be less effective at clearing viral infections by secreting fewer cytokines and expressing less degranulation marker CD107a.

METHODS

Animal Samples and Collection

Healthy Indian-origin rhesus macaque (RM) samples were provided by the researchers at the University of Wisconsin-Madison from the Wisconsin National Primate Research Center. A sample of 15mL of blood from each animal was taken with an ethylenediaminetetraacetic acid (EDTA) tube according to their Institutional Animal Care and Use Committee (IACUC) protocols. Each sample was assessed for a CBC before arrival at our lab. Peripheral blood mononuclear cells (PBMCs) were separated in each sample by Ficoll-Hypaque gradient centrifugation upon arrival at our laboratory, where they were subsequently cryopreserved. Ten aged macaque samples (>20 years) and fourteen young adult macaque samples (<10 years) were used unless otherwise noted below in Table 1. Ages were chosen based on incidence rates of geriatric conditions found in previous studies and average lifespan (Uno, 1997).

PBMC Revival and Stimulation

Each PBMC sample was revived 12-18 hours in RPMI 1640 medium (HyClone; cat.SH30027.01) supplemented with 10-15% heat-inactivated fetal bovine serum (Corning; cat.35-010-CV), 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 100 U streptomycin, and 100 μg/mL penicillin in a 37 degree Celsius (C), 5% CO_2 incubator; these samples were revived in 2.5 mL of pre-warmed medium in 12-well plates. Cell counts using sample diluted 1:5 in white blood cell counting medium with 0.04% trypan blue staining were made under light microscopy to determine cell concentrations. Distinguishing characteristics of each sample are presented in Table 1. Prior to

stimulation, samples used for phosphoprotein and total protein analysis were incubated in serum starvation medium (RPMI 1640 with 100 U streptavidin and 100 µg/mL penicillin) for 3 hours at 37 degrees C, 5% CO_2.

Table 1: Rhesus PBMC Characteristics

Name	Sex	Age (years)	Date of Sample Collec.	Exp.	**Name**	Sex	Age (years)	Date of Sample Collec.	Exp.
R7021	M	5.29	1/11/13	4	**Rh1938**	M	21.2	7/15/13	1,3,4
R7036	F	4.5	4/24/12	1,3	**Rh1940**	M	23.5	7/15/13	1,3,4
R8001	F	4.2	4/24/12	1,3	**Rh1946**	F	25.2	7/15/13	1,2,3,4,5
R8016	M	3.8	4/24/12	1,3	**Rh1950**	F	24.1	7/15/13	1,2,3,4,5
R8026	F	3.8	1/9/13	4	**Rh2060**	F	28.0	7/15/13	1,2,3,4,5
R8043	M	3.6	4/24/12	1,3	**Rh2071**	M	26.2	7/15/13	1,3,4,5
R8044	M	3.6	4/24/12	1,3	**Rh2085**	M	24.1	7/15/13	1,3,4,5
R9027	F	3.71	1/9/13	1,2,3	**Rh2112**	M	24.1	7/15/13	1,3,4
R9033	F	2.9	4/24/12	1,2,3,4,5	**Rh2255**	F	29.8	7/15/13	1,3,4
R9044	F	3.63	1/9/13	1,2,3,4,5	**Rhan72**	F	26.1	7/15/13	1,3,4
R9046	F	3.63	1/9/13	1,3,4,5					
R9047	F	2.8	4/24/12	1,3,4,5					
R9050	F	3.6	1/9/13	4,5					
R9068	F	2.8	4/24/15	1,3,4					

1- Sample used for B cell surface activation.
2- Used for B cell surface activation co-incubated with SIV.
3- Used for T cell surface activation.
4- Used for T cell surface markers and cytokines.
5- Used for B cell total and phosphoprotein markers.

After revival, the samples were washed once by adding 3 mL RPMI 1640 medium to the sample in a 15 mL tube and then pelleted by centrifugation at 500 xg for 10 minutes. Samples were resuspended in the same medium at a concentration of 5 million cells/ mL. For B cell analysis, 1 µg anti-IgG/IgM antibody suspension (eBioscience; cat.16-5099-85) per 100 µL sample was added to half of the sample; mock stimulation for a negative control was done by adding 1 µL sterile water per 100 µL to the unstimulated half of each sample. Samples were incubated at 37 degrees C, 5% CO_2 for 48 hours for surface activation marker analysis. This same stimulus was used for B cell phosphoprotein and total protein analysis after incubation for 10 minutes at 37 degrees C in a water bath. While this stimulus was human specific, rhesus

macaques show approximately 93% gene homology with humans (Gibbs et al., 2007), and there is a lack of suitable macaque-specific reagents. Preliminary experiments showed adequate B cell activation by flow cytometry.

For T cell analysis, half of each sample was stimulated with 50 ng/mL phorbal 12-myristate 13-acetate (PMA) and 500 ng/mL ionomycin (I) before being incubated for 6 hours at 37 degrees C, 5% CO_2. GolgiStop™ (BD; cat.51-2301KZ) was added at a concentration of 1:1000 by volume to samples after 2 hours of incubation for cytokine analysis. The other portion of the samples was mock stimulated with 1 µL dimethyl sulfoxide (DMSO) in 100 µL RPMI 1640 as a control.

SIV Infection

Samples were plated at a concentration of 5 million cells/ mL in RPMI supplemented as above for revival medium. $SIV_{mac251\text{-}32H}$ (TCID50 of 269,000/ mL; 1X stock) was added at a concentration of 2.69 TCID50/ 50,000 cells and mixed. Separately, 1 µL of $SIV_{agm\text{-}9063\text{-}2}$ (100X stock) per 100µL of cells was added in order to determine whether a strain of SIV that does not induce SAIDS in macaques would affect activation of B cells. Samples were stimulated at the same time with 1 µg anti-IgG/IgM antibody suspension (eBioscience; cat.16-5099-85) per 100 µL sample. Uninfected, stimulated cells were included as a negative control. The plate was incubated at 37 degrees C, 5% CO_2 for 48 hours.

PBMC Antibody Staining

After stimulation, each sample was stained with fluorescently labeled antibodies

for flow cytometry analysis at a concentration of 20 million cells/ mL. A violet reactive dye kit (L/D; Life Technologies; cat.L34955) was used to distinguish living cells. A portion of a sample was kept as an unstained control. The antibodies used to distinguish B cells are as follows: anti-CD27-APC (Biolegend; cat.302810), anti-CD86-PECy7 (cat.305422), anti-CD20-APCH7 (BD; cat.560853), anti-CD21-PECy5 (cat.557327), anti-CD40-FITC (cat.556624), phosphoSyk-Alexa fluor™ 488 (cat.560081), anti-Syk-FITC (cat.552476), anti-phosphoPLCγ2-PE (cat.558577), anti-PLCγ2-PE (cat.560134), anti-phosphoERK1/2-PE (cat.612566), and anti-ERK2-PE (cat.558530). Anti-CD14 antibodies were used in a dump channel when obtaining preliminary data in order to exclude monocytes and provide a purer B cell population when gating. However, it was not used in further data collection due to expression of this molecule on B cells (Labeta et al., 1991). Lymphocytes were gated based on L/D exclusion and forward and side scatter properties. CD20 is a common marker for B cells in rhesus macaques, and was used in this study. Even though CD19 is a more accurate marker for B cells due to its presence throughout their differentiation, and CD20 is downregulated on plasma cells (Anolik et al., 2003), CD19 is inconsistently found on macaque B cells. Furthermore, plasma cells are typically rare in the periphery (Kuhrt et al., 2011). CD21-CD27- cells are considered exhausted or tissue-like B cells, while CD21+CD27- cells are naïve, CD21-CD27+ are activated memory, and CD21+CD27+ are resting memory B cells (Titanji et al., 2010). CD40 and CD86 were used to identify activated B cells.

The antibodies to distinguish T cells are as follows: anti-CD3-PerCPCy5.5 (BD; cat.552852), anti-CD4-PECF594 (cat.562402), anti-CD8-PECy7 (cat.557746), anti-

CD25-APCH7 (cat. 561402), anti-CD28-FITC (cat. 556621), anti-CD45RA-PECy5 (cat.552888), anti-CD95-PE (cat.556641), anti-CD154-APC (cat.560955), anti-CD107a-APCH7 (cat.561343), anti-IL-2-FITC (cat.559361), anti-IFN-γ-APC (cat.551385), anti-TNF-α-BV605 (cat.563915), and anti-CCR7- PE (cat.552176). CD3 is a general marker for T cells, and CD4 is used to identify helper T cells. DN T cells are double-negative for CD4 and CD8. Effector T cells are CD45RA-CCR7-, while memory effector T cells are CD95+CD45RA+/-CD28-CCR7-. Central memory T cells are CD95+CD45RA-CD28+CCR7+. Naïve T cells are CD95-CD45RA+CD28loCCR7+. Staining occurred for 20 minutes at ambient room temperature in the dark followed by washing at 500 xg for 10 minutes in a centrifuge and resuspension in approximately 50 µL medium. Positive and negative compensation controls were utilized for each experiment (Invitrogen; cat.A10346, A10344).

PBMC Fixation & Permeabilization

After staining with surface antibodies, the samples used for cytokine expression were fixed and permeabilized with a kit (BD; cat.554714) before staining with the intracellular antibodies.

Samples used for protein analysis were fixed in equal volumes Fix Buffer I (BD; cat.557870) pre-warmed to 37 degrees C, for 10 minutes. Then the samples were washed at 1500xg for 15 minutes. The samples were next re-suspended in 50 µL Perm Buffer III (BD; cat.558050) at -4 degrees C on ice, following which the samples were washed at 1600xg for 10 minutes and stained with intracellular antibodies.

The samples stained only with surface antibodies and the unstained control cells

were fixed with 0.5% paraformaldehyde for 20 minutes at room temperature in the dark. These samples were washed again at 500xg for 10 minutes and re-suspended in medium before flow analysis.

Flow Cytometry

Samples were run on a FACS Aria III flow cytometer until 500,000 events were collected or until there was no more sample. Gating was accomplished using FlowJo v.X.0.7. Representative gating strategies are provided in the appendices, including lymphocyte (appendix A), B cell and B cell subpopulation (appendix B), B cell signaling (appendix D), T cell surface (appendix I), and T cell cytokine (appendix J) gating. Fluorescence-minus-one (FMO) controls for the B cell surface marker and activation marker panel was used, and the results are depicted in Appendix C; results for the protein panels are shown in Appendices E-H. FMO control results for the T cell surface marker and activation marker panels are shown in Appendix K, and the cytokine panel in Appendix L. Isotype controls were not available.

Statistical Analysis

Statistical significance was determined using Microsoft Excel©. Student's t-tests were used with p-values less than 0.05 considered significant; Welch's corrections and the Holm-Sidak method were utilized where appropriate when multiple t-tests were computed at once. In graphs, error bars represent standard deviation ($p<0.05$*, $p<0.005$**, $p<0.0005$***). Spearman's correlation tests were used with $p<0.05$ considered significant using GraphPad Prism.

RESULTS

B Cell Analysis

Surface Markers and Subpopulations

B cells exhibit several functions which are related to the expression of surface markers CD21 and CD27. By analyzing the subpopulations of B cells using their CD21 and CD27-expression profile, I will be able to determine what effects age have on their respective frequencies and counts. Eight aged and eleven young adult rhesus macaque PBMC samples were analyzed for expression of the surface B cell markers, as listed above in Table 1. The CBC data given to our lab included the numbers of total circulating lymphocytes per microliter of blood for each of the nineteen samples used in this experiment. To begin, I averaged the numbers for each age group, and a t-test determined the decrease in the total lymphocyte population in blood with advanced age was significant ($p=0.003$; data not shown).

Next, in an effort to account for possibilities of gender influence on the immune system, statistical analysis was computed separately for each gender by age group. While the same general trends are evident from comparing age groups when considering both genders together, there are variances of statistically significant differences. For example, the absolute circulating lymphocyte count was only significantly higher in the young adult male group compared to the old male group ($p=0.002$; data not shown) and not for females. Importantly, t-tests comparing the ages revealed that the female macaques' ages were not different from the males' ages for both age groups (young adult male vs. female: $p=0.72$; aged male vs. female: 0.06; data not shown). Thus, since the ages of the young males and young females were not

different and the ages of the old females and old males were not different, the variances can presumably be attributed to gender-specific immunity.

By multiplying the absolute lymphocyte count to the corresponding B cell frequency of each sample from the flow cytometry data, and then averaging the results for each age group, I was able to calculate the absolute count (or number) of circulating B cells per microliter of blood. The results for each age group are shown in Fig. 1(a); this figure depicts a higher count of B cells in the young adult population ($p=0.006$), as expected based on previous results. Thus, total B cell counts are now known to be diminished within the aged macaque samples as expected.

Subpopulations of B cells were also analyzed to determine whether the loss was due to reduction of the naïve or memory subsets. The subpopulations of B cells were identified on CD21 and CD27 expression as described above in Methods (naïve: CD21+D27-; activated memory (AM): CD21-CD27+; resting memory (RM): CD21+CD27+; tissue-like (TL): CD21-CD27-). Overall, young adult macaques had higher numbers of naïve ($p=0.002$) and resting memory ($p=0.02$), B cells (Fig. 1(b)).

Again, samples were separated between the two genders and analyzed. The absolute B cell subpopulation counts in the male RM samples had more significant differences between age groups (naïve: $p=0.0005$; resting memory: $p=0.0001$; tissue-like: $p=0.003$; Fig. 1(c, d, f)), which mimic the overall results. Comparatively, the female age groups showed only one significant difference with a relatively high p-value (naïve: $p=0.049$; Fig. 1(c)), although the same tendency is evident. There was no significant difference of activated memory B cell absolute counts between age groups in either gender or overall.

Subsequently, frequencies were determined by flow cytometry for each of the nineteen samples and averaged for each age group. As noted in the previous study by Zheng et al. (2014) and Fig. 2(a), the frequency of B cells is significantly smaller in aged macaques compared to young adult macaque samples ($p=0.0001$). Not surprisingly, there was also a higher frequency of activated memory B cells within the aged macaque samples compared to the younger age group ($p=0.003$), as demonstrated in previously published studies (Colonna-Romano et al., 2003). There was no significant difference in the other subpopulations, but there was a trend for a higher average in the young adult samples.

Once the subpopulations of B cell frequencies were separated by gender, it became apparent that the same trends were evident for both genders. In the naïve and resting memory subpopulations in the females, the frequency appeared to decline with age, while there was an increase in activated memory B cell subpopulation; only the activated memory difference was significant ($p=0.007$). The male samples exhibited a similar trend, with significance in the resting memory subpopulation ($p=0.02$).

Together, it may seem that aged males have a drastically higher B cell count than young adult males when compared to the female data, but the frequencies of B cell subpopulations remain even between the genders. The same tendencies are evident in both genders as compared to the pooled data. These results lead me to conclude that while analyzing the genders separately may lead to interesting differences, the overall conclusions remain the same.

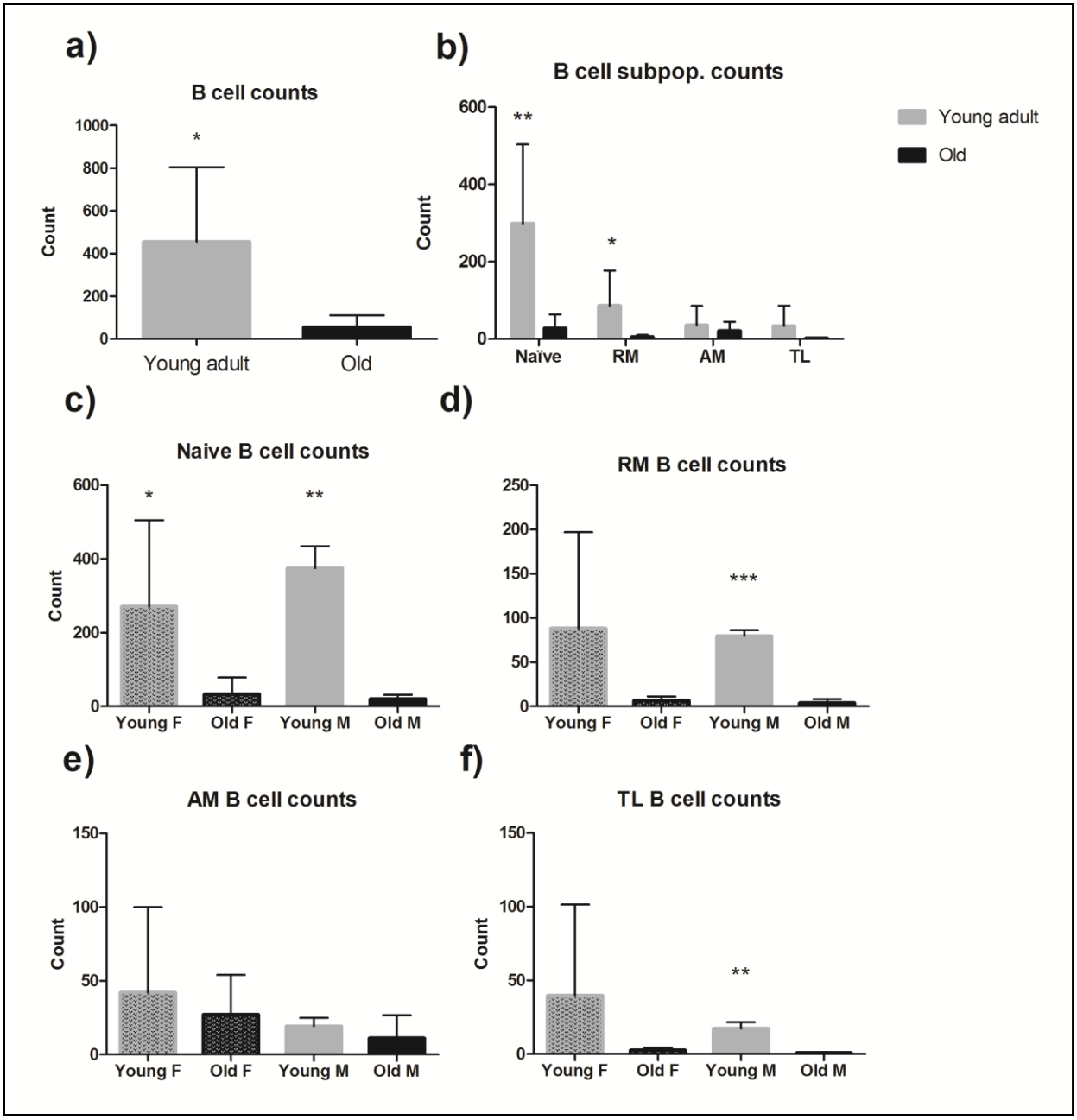

Figure 1: Average B cell absolute circulating counts per microliter of blood compared between 11 young adult (<10 years) and 8 aged (>20 years) rhesus macaques as found by multiplying population frequency from flow cytometry to absolute lymphocyte count from CBC data. (a) B cell counts by age (b) Subpopulation counts by age (c) Naïve B cell counts between 8 young adult and 5 aged female macaques, and between 3 young adult and 3 aged male macaque PBMC samples. (d) RM= resting memory B cell counts compared by age and gender. (e) AM= activated memory B cell counts compared by age and gender. (f) TL= tissue-like B cell counts compared by age and gender. F= Female; M= Male. Error bars represent standard deviation. P-value <0.05*, <0.005**

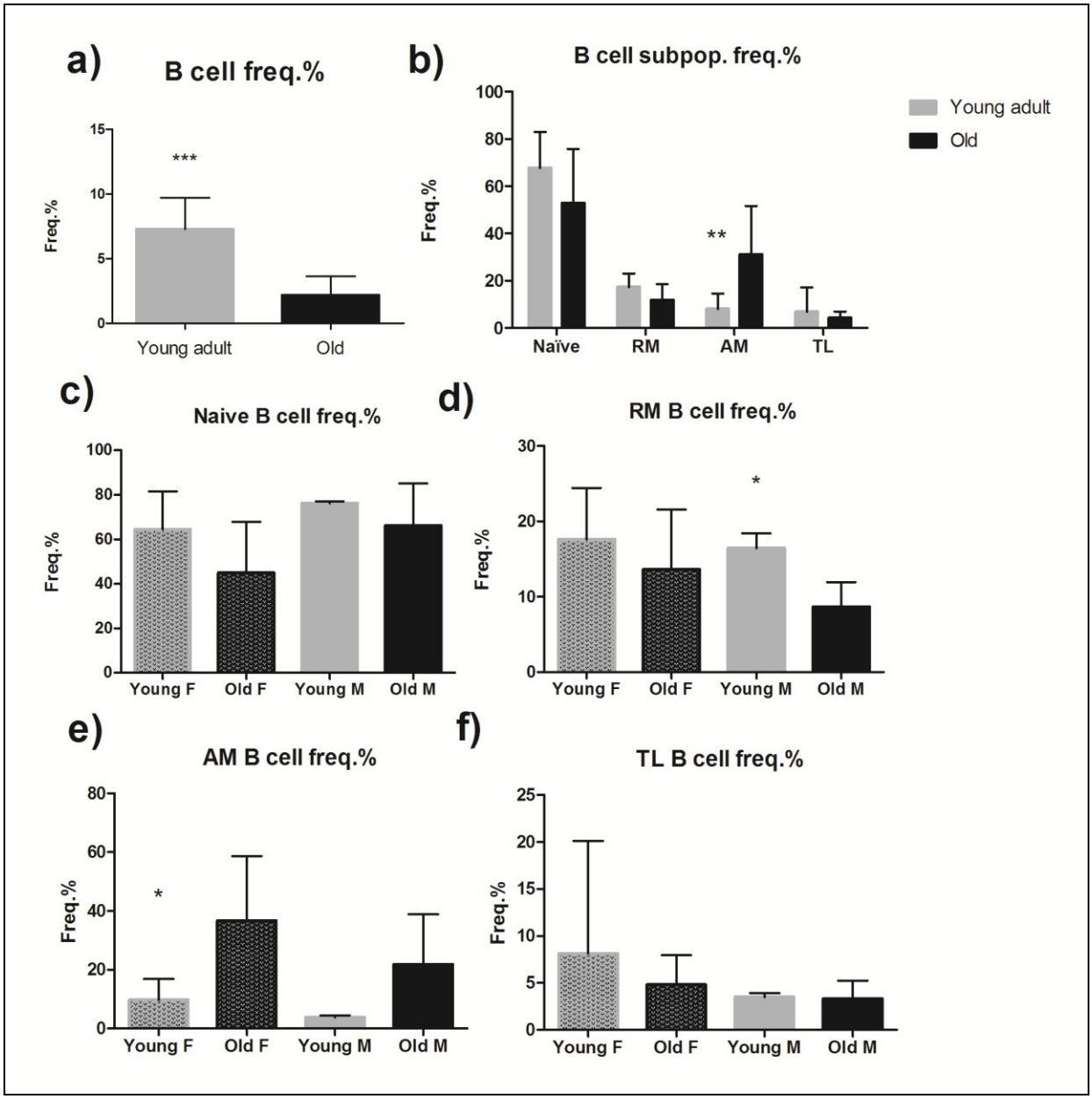

Figure 2: Average B cell frequencies compared between 11 young adult (<10 years) and 8 aged (>20 years) rhesus macaque PBMC samples measured by flow cytometry. (a) B cell frequency by age (b) Subpopulation frequency by age (c) Naïve B cell frequency between 8 young adult and 5 aged female macaques, and between 3 young adult and 3 aged male macaque PBMC samples. (d) RM= resting memory B cell frequency compared by age and gender. (e) AM= activated memory B cell frequency compared by age and gender. (f) TL= tissue-like B cell frequency compared by age and gender. Error bars represent standard deviation. P-value <0.05*, <0.005**

Activation Surface Markers

CD40 and CD86 are known to be expressed during B cell activation. CD40 is constitutively expressed due to its functionality associated with the BCR, and increased expression can be measured through the MFI of anti-CD40 staining antibodies or $CD40^{hi}$ gating. CD86 is expressed upon BCR ligand-coupling, making it another measure of activation. Decreased expression with advanced age could mean deficient B cell activation mechanisms, leading to immunosenescence. It was determined that absolute counts of $CD40^{hi}$ naïve ($p=0.01$) and resting memory ($p=0.02$) B cell subpopulations were higher within the young adult macaque samples without stimulation compared to the aged (shown in Fig. 3(a)). $CD40^{hi}$ naïve B cell counts remained higher in the young adult samples with stimulation ($p=0.04$). So, there are overall decreases in B cells that become activated in the aged.

Frequencies of CD40hi B cells were also assessed to see whether the proportions of activated B cells changed with age. Without stimulation, higher frequencies of $CD40^{hi}$ B cells were found in all subpopulations except for TL (Naïve: $p=0.04$; RM: $p=0.02$; AM: $p=0.08$; Fig 3(b)). Stimulation with anti-IgG/IgM antibodies revealed the same trend, again with a lower $CD40^{hi}$ resting memory B cell frequency found with advanced age (Naïve: $p=0.001$; RM: $p=0.049$; Fig. 3(b)).

CD40 MFI comparisons of unstimulated B cells drew the same conclusions. There was a trend for inherently higher CD40 MFI values within the young adult B cell subpopulations of B cells. The differences were significant for the naïve ($p=0.01$) and resting memory ($p=0.01$) subpopulations, shown in Fig. 3(c). As seen with the $CD40^{hi}$ frequency and count data, there was a general reduction in CD40 MFI with stimulation of anti-IgG/IgM antibodies. This indicates fewer CD40 molecules expressed per cell

after stimulation. Nevertheless, CD40 MFI values were not significantly different with stimulation.

After determining differences in CD40 expression, CD86 expression was analyzed. Absolute counts of CD86+ B cell subpopulations without stimulation are higher in young adults' naïve (p=0.03), resting memory (p=0.03), and activated memory subpopulations (p=0.02; Fig. 3(d)); stimulation revealed the same differences (Naïve: p=0.04; RM: p=0.02; AM: p=0.01). B cells between the macaque age groups revealed differences in CD86+ frequencies with stimulation in both activated memory (p=0.002) and tissue-like B cell subpopulations (p=0.005; Fig. 3(e)). While the frequency was higher in the young adult AM population as expected, the aged macaque CD86+ frequency was higher in the TL subpopulation. The only significant difference in CD86 MFI values was found with stimulation in the resting memory B cell subpopulation (p=0.01; Fig. 3(f)), where it was higher in the aged macaque samples.

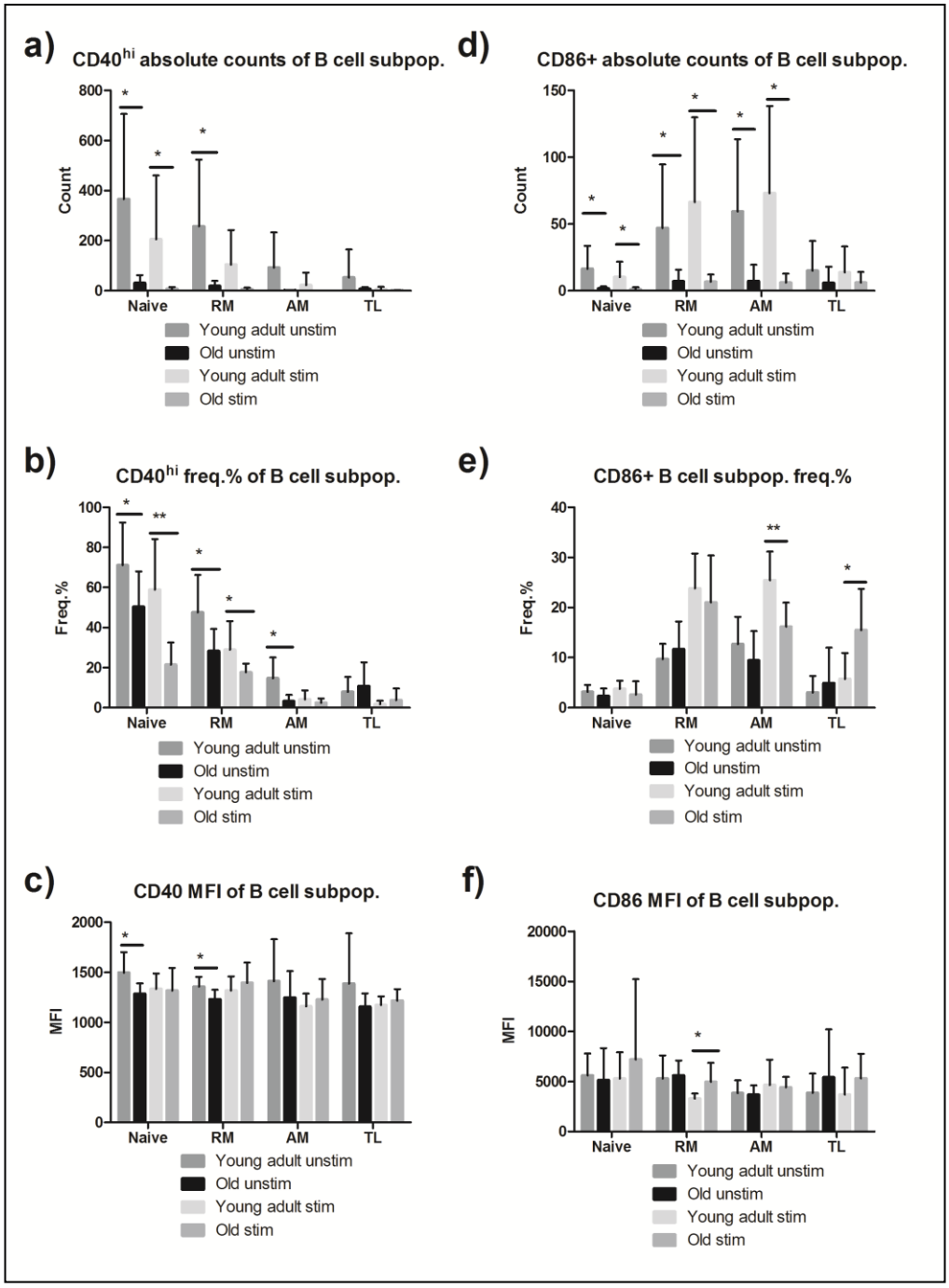

Figure 3: Average B cell activation parameters CD40 and CD86 compared between 11 young adult (<10 years) and 8 aged (>20 years) rhesus macaque PBMC samples. Samples were stimulated with anti-IgG/IgM antibodies for 48hrs. at 37°C, 5% CO_2 with unstim. cells. (a) $CD40^{hi}$ absolute counts in B cell subpopulations by age found by multiplying population frequency from flow cytometry to absolute lymphocyte count from CBC data. (b) $CD40^{hi}$ frequencies in B cell subpopulations compared by age from flow cytometry (c) CD40 MFI values in $CD40^{hi}$ B cell subpopulations compared by age, found by flow cytometry. (d) CD86-positive B cells in each subpopulation compared by age, found by flow cytometry. RM= resting memory; AM= activated memory; TL= tissue-like. Error bars represent standard deviation. P-value <0.05*, <0.005, <0.0005*****

Phosphorylation of BCR Signaling Proteins

In an effort to determine age-associated BCR signaling dysfunction, total Syk, PLCγ2, and ERK2 amounts were analyzed from 5 young adult and 5 aged rhesus macaque PBMC samples. If there were differences beginning at a certain point in the BCR signaling cascade, they might be able to be pinpointed to a certain spot through the measurement of three proteins within the cascade. Activation indicated by phosphorylation was measured for phospho-Syk, phospho-PLCγ2, and phospho-ERK after 10 minute stimulation with anti-IgG/IgM antibodies followed by flow cytometry analysis. Unstimulated PBMCs were used as a negative control.

Total Signaling Protein Levels

First, both inactivated and activated levels of Syk were found. As seen in Fig. 4(a), aged macaque PBMC samples have higher frequencies of both memory ($p=0.02$) and naïve ($p=0.04$) Syk^{hi} B cells. Similar to this, Fig. 4(b) depicts a higher total Syk MFI value in the aged macaque memory B cell subpopulation ($p=0.01$). This indicates a higher potential for activation immediately downstream of the BCR. Fig. 4(c) shows that there is an apparent increase in PLCγ2-positive cells within the aged macaques' PBMC samples that is significant in the memory B cell subpopulation ($p=0.01$), although this was not significant when comparing expression within the cell using MFI values (data not shown). So, while there may be more cells with high levels of PLCγ2 in the aged, the amount of PLCγ2 within the cell is unchanged with age. As seen in Fig. 4(d), the $ERK2^{hi}$ frequencies of memory B cells were higher in the aged macaques ($p=0.007$). While the aged macaque $ERK2^{hi}$ frequencies appeared higher in the naïve B cell

subpopulation as well, this difference was not significant. There was no difference between age groups with ERK2 MFI values (data not shown). Thus, the same can be said to ERK2 that was mentioned for PLCγ2: there may be more cells with high levels of ERK2, but the amount of ERK2 within the cell remains the same.

Activated Signaling Protein Levels

After determining differences in total protein levels, activated protein levels were found by measuring the phosphorylated forms. Baseline levels of phosphorylated Syk+ B cell frequencies are higher in the old macaque naïve B cells and memory B cells ($p=0.01$ and $p=0.02$; Fig. 5(a)). I analyzed the MFI for pSyk for each sample and averaged the amounts in each age group to estimate the relative abundance of pSyk within highly phosphorylated populations of naïve B cells and memory B cells. Fig. 5(b) shows that without stimulation, pSyk MFI is higher in the old macaque memory B cells ($p=0.01$), yet the same for each age group with stimulation. Baseline levels of phosphorylated PLCγ2+ frequencies trended higher in the aged macaque naïve and memory B cell populations, as shown in Fig. 5(c). The difference was significant in the memory B cell population only ($p=0.002$). There were no differences between age groups of pPLCγ2 MFI values (data not shown). Frequencies of phosphorylated ERK1/2+ B cells were higher in the older macaque samples without stimulation in both the naïve ($p=0.006$) and memory ($p=0.00003$) subpopulations (Fig. 5(d)). However, there were no statistically significant differences with stimulation. The results of pERK1/2 MFI values for naïve and memory B cells were not significant either.

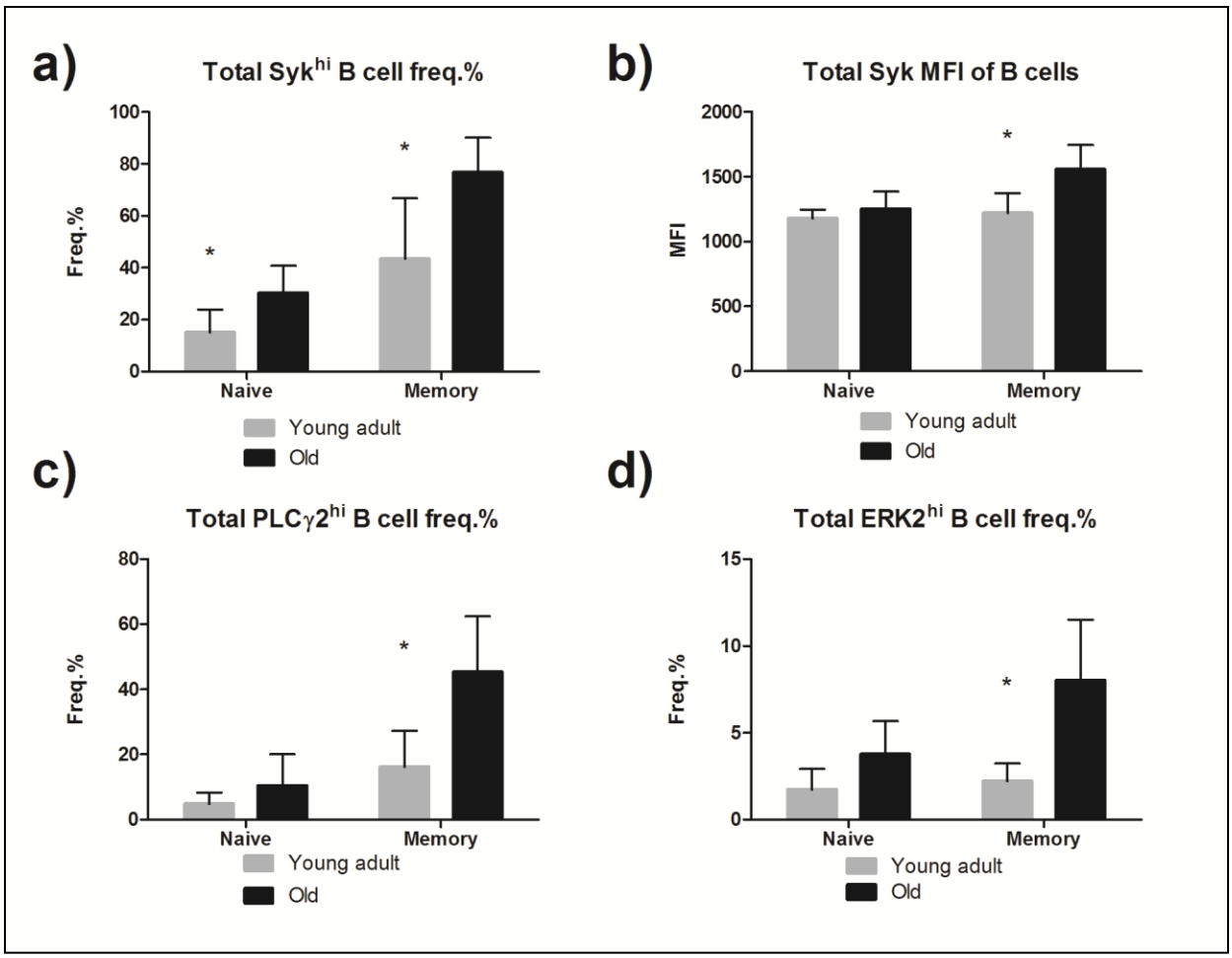

Figure 4: Average B cell total signaling protein analysis compared between 5 young adult (<10 years) and 5 aged (>20 years) rhesus macaque PBMC samples found by flow cytometry. (a) Total Syk^{hi} B cell frequencies in naïve (CD27-) and memory (CD27+) B cells. (b) Total Syk MFI values in Syk^{hi} naïve and memory B cells. (c) Total PLCγ2^{hi} naïve and memory B cell frequencies. (d) Total ERK2^{hi} naïve and memory B cell frequencies. Error bars represent standard deviation. P-value <0.05*

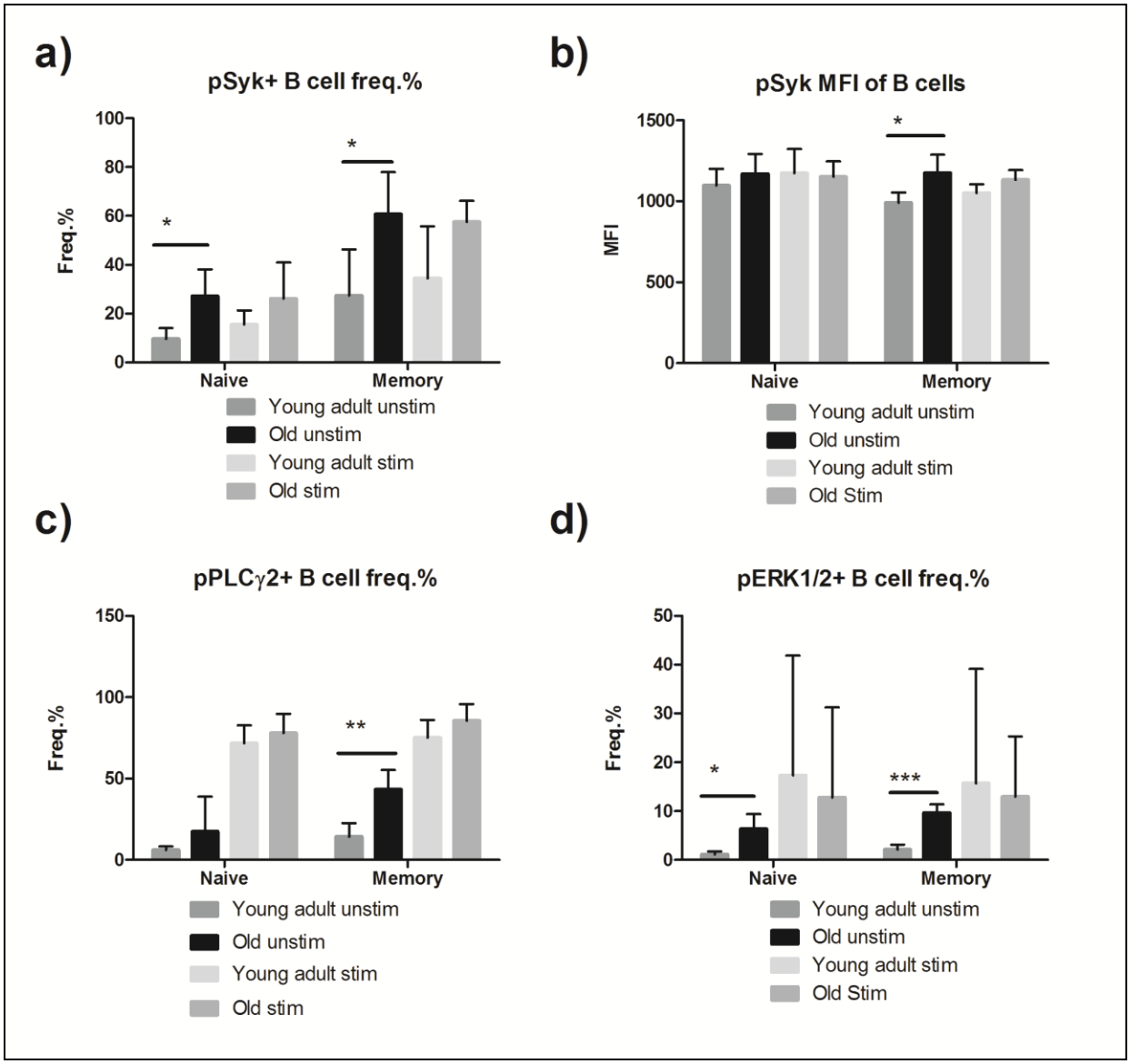

Figure 5: Average B cell signaling phosphoprotein analysis compared between 5 young adult (<10 years) and 5 aged (>20 years) rhesus macaque PBMC samples found by flow cytometry. Half of each sample was stimulated with anti-IgG/IgM antibodies for 10 minutes at 37°C, 5% CO_2 prior to fixation and permeabilization, and the other half was unstimulated as a negative control. (a) Phosphorylated Syk-positive B cell frequencies in naïve (CD27-) and memory (CD27+) B cells. (b) Phosphorylated Syk MFI values in Syk-positive naïve and memory B cells. (c) Phosphorylated PLCγ2-positive naïve and memory B cell frequencies. (d) Phosphorylated ERK1/2-positive naïve and memory B cell frequencies. Error bars represent standard deviation. P-value <0.05*, <0.005**

B Cell Analysis After SIV Co-incubation

SIV-induced immune dysfunction could be caused by alterations in B cell subpopulation distributions or diminished expression of CD40/86 in B cells. If there are alterations in B cells due to SIV infection, and if they are more drastic in the aged macaque samples, then this could be a potential mechanism for the faster progression rate to AIDS seen in the elderly. In order to see whether SIV infection could affect the ability of B cells from aged macaques to be activated, RM PBMC samples from 3 aged and 3 young adults were subjected to one of three conditions: infection with SIVmac251-32H and stimulation with anti-IgG/IgM antibodies, infection with SIVagm and stimulation with anti-IgG/IgM antibodies, or noninfected with anti-IgG/IgM stimulation. SIVmac is a highly virulent strain of virus that causes SAIDS in rhesus macaques, and would therefore be expected to produce drastic changes. SIVagm is a strain that does not cause disease in rhesus macaques, so the changes would be expected to be minimal. After 48 hours of incubation, flow cytometry was used to determine the frequencies of B cell subpopulations and expression of activation markers CD40 and CD86.

No significant differences between the infection conditions were found for B cell subpopulation frequencies infected with SIV, nor were there differences found for $CD40^{hi}$ frequencies, CD40 MFI values, or CD86+ frequencies (data not shown). However, CD86 MFI values of tissue-like B cells were higher in the aged macaques with co-incubation of SIVagm compared to the young adult samples infected with SIVagm ($p=0.001$), aged samples co-incubated with SIVmac ($p=0.04$), and the uninfected, aged samples ($p=0.008$). No differences were found for the other B cell subpopulations.

Intriguingly, this could indicate that the aged macaque B cells express higher levels of CD86 to combat infection that is subverted with the disease-causing SIV strain's virulence factors. Unfortunately, this explanation cannot account for the lack of higher immune response in the young adults' samples with the non-disease causing strain as well. Thus, the most likely explanation is that the small sample size is the cause of the apparently higher CD86 expression, and this effect would be mitigated with more samples.

T Cell Analysis

In order to establish similarities with previously published data, T cell counts, frequencies, and surface marker expression were analyzed to see what impact age had on these parameters within each of the CD4+ and DN T cell subpopulations. CD4+ T cells are known to be potent B cell costimulatory cells, while DN T cells are known to be involved in autoimmunity, which is more common in the elderly.

CD4+ and DN T Cell Subpopulations

Subpopulations of CD4+ T cells include naïve, effector memory (EM), and central memory (CM). By multiplying each frequency with the absolute count for each of the ten aged and eleven young adult samples, I could determine the absolute circulating cell amount for each CD4+ T cell subpopulation (Fig. 6(a)). The naïve ($p=0.009$) compartment was significantly higher in the young adult samples. As shown in Fig. 6(b), the central memory frequency was higher in the old age group samples ($p=0.045$) and

the naïve CD4+ T cell frequency was higher in the young adult samples ($p=0.003$). After calculation of the absolute circulating DN subpopulation cell counts, Fig. 6(c) shows significantly higher DN naive ($p=0.004$) and central memory ($p=0.005$) T cells in the younger age group. The subpopulation frequencies of DN T cells did not show any significant differences, although the data showed a similar trend as that of the CD4+ T cell subpopulation frequencies. This data is summarized in Fig. 6(d).

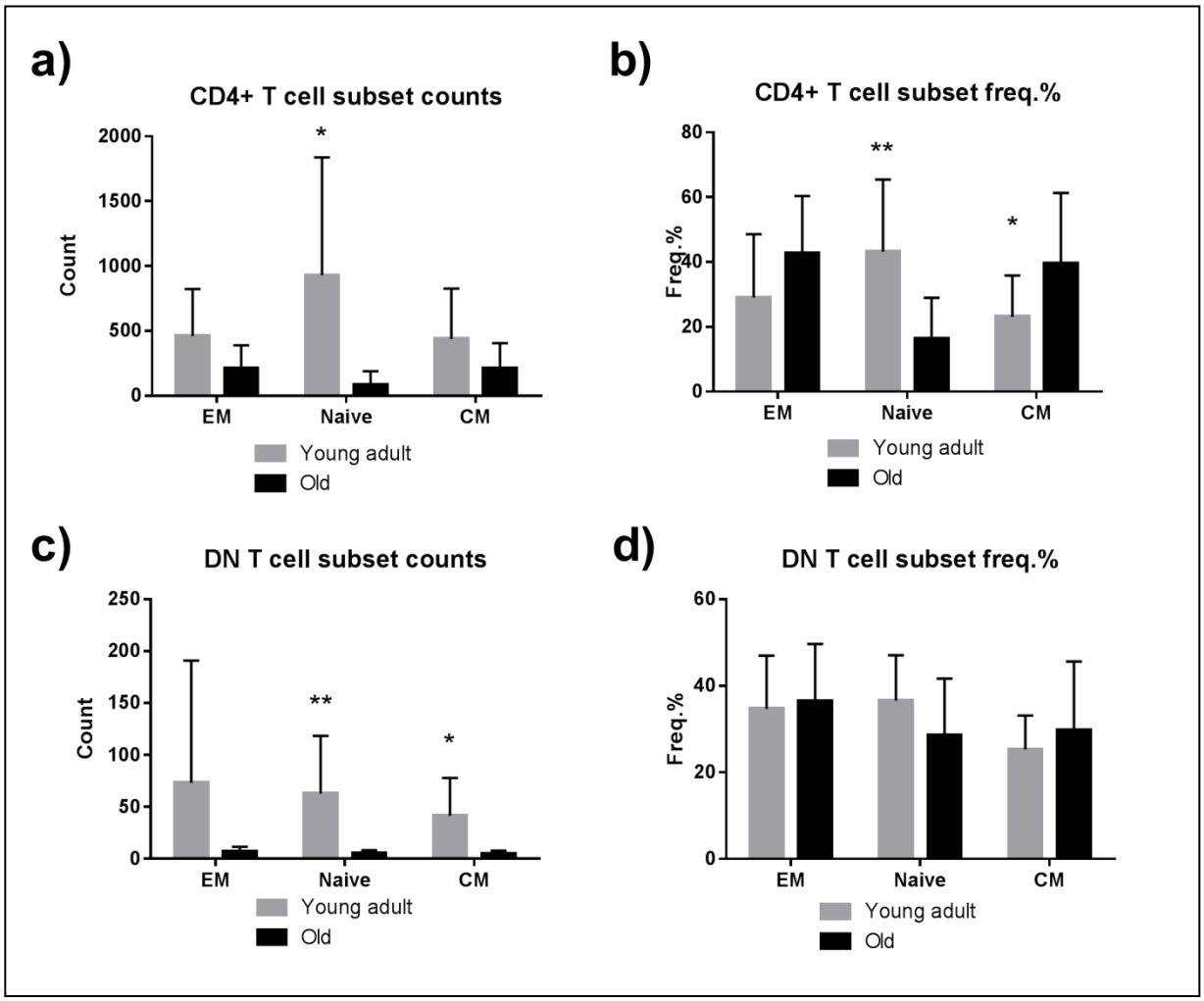

Figure 6: Average T cell counts and frequencies compared between 11 young adult (<10 years) and 10 aged (>20 years) rhesus macaque PBMC samples. (a) Absolute circulating CD4+ T cell lymphocyte counts per microliter of blood found by multiplying subpopulation frequency from flow cytometry to absolute lymphocyte count from CBC data. (b) CD4+ T cell subpopulation frequencies found by flow cytometry. (c) Absolute circulating double negative (DN; CD4-CD8-) T cell counts per microliter of blood. (d) DN T cell subpopulation frequencies. EM= effector memory; CM= central memory. Error bars represent standard deviation. P-value <0.05*, <0.005**

CD4+ T Cell Activation

Surface Markers and Activation Markers

Activation of helper T cells is a preliminary step towards producing co-stimulatory signals for B cell activation. CD25 is an activation marker on lymphocytes and a presumptive marker for regulatory T cells. After PMA & I stimulation for 6 hours, there were no differences between aged and young adult groups in CD25+ CD4+ T cell frequencies or CD25 MFI values (data not shown). Counts of young adult, naïve CD25+ CD4+ T cells were higher than their counterpart in the aged samples ($p=0.04$; Fig. 7(a)). This is likely due to the diminished pool of naïve T cells in the aged macaques' blood available to become activated. Other subpopulations of T cells tended to have higher CD25+ counts in the young adult samples, but this was masked by high variability.

Another T cell activation marker, CD154, is important for B cell activation through ligation with CD40. Despite the evidence of diminished CD4+ T helper cell stimulation of B cells in the aged, there were no differences between age groups in CD154+ frequencies or MFI values (data not shown). Naïve CD4+ T cells had higher absolute CD154-positive counts in the young adults ($p=0.02$; Fig. 7(b)), but no other differences were found. This is most likely due to the loss in naïve T cells circulating in the blood, and could explain the decreased expression of CD154 with age found by Haynes and Eaton (2005), as mentioned in the introduction.

Comparisons of surface molecule expression between aged and young adult rhesus macaques resulted in several differences. CD4+ effector memory ($p=0.006$) and central memory ($p=0.004$) T cells had higher CD3 MFI values in the young adult group (Fig. 7(c)), which is integral for T cell activation. There were no differences in CD4 or

CD28 expression (Fig. 7(d)), which are important for activating antigen presenting cells. CD45RA MFI values were higher in young adult effector memory CD4+ T cells (p=0.03; Fig. 7(e)). Lastly, a marker involved in apoptosis was measured; CD95 expression was higher in the aged macaque samples (p=0.002; Fig. 7(f)).

Stimulation with PMA & I for 6 hours resulted in higher CD3 MFI values in CD4+ naïve T cells in the young adult samples (p=0.0009; Fig. 7(c)). CD28 MFI values in the young adult samples in central memory CD4+ T cells were also higher (p=0.0048; Fig. 7(d)). Fewer molecules of CD28 on the aged macaques' T cells could lead to deficient B cell costimulatory signaling, since CD28 binds to CD86 on B cells. CD45RA MFI values were also higher in the young adult samples in the naïve CD4+ T cell subpopulation (p=0.02; Fig. 7(e)). CD95 expression remained higher in the aged (p=0.003, Fig. 7(f)). There were no differences in CD4 expression (data not shown).

Cytokines and Degranulation Marker

In order to be able to activate B cells and continue an immune response, CD4+ T cells need to have a pro-inflammatory profile of cytokine secretion and degranulation marker expression. I hypothesized that aged macaque CD4+ T cells would produce fewer cytokines and less degranulation marker, leading to immune dysfunction. We measured the production of CD107a, IL-2, TNF-α, and IFN-γ after stimulation with PMA & I for 6 hours. However, there were no differences in the frequencies of single-cytokine producing CD4+ T cells, nor absolute counts (data not shown). IFN-γ MFI values were higher in the young adult samples (p=0.01; Fig. 8(a)). Analysis of multifunctional CD4+ T cells, ones that can produce more than one cytokine, resulted in no differences in

absolute counts, but several multifunctional CD4+ T cell frequencies showed a negative correlation with age (Fig. 8(b)). Interestingly, there was a higher frequency of CD107a+IFN-γ+ T cells in the young adult samples (p=0.03; data not shown), but the average frequencies for both age groups were less than one percent.

Association of T Cell and B Cell Parameters

Association of T cell parameters to B cell parameters could be important for understanding the balance between the two lymphocytes leading to a robust B cell response in young adults. For instance, central memory CD4+ T cell and activated memory B cell frequencies are positively correlated (Fig. 9(a)), while both populations decreased with age. Interactions between these two specific populations of lymphocytes warrant further studies. Ligand interaction analysis revealed several important correlations. CD28 MFI values of CD4+ T cells correlated negatively with its ligand on B cells, CD86 MFI values (Fig. 9(b)). Similarly, CD4+ CD154+ T cell frequency correlated negatively with its ligand on B cells, CD40 MFI (Fig. 9(c)). So, as one molecule may diminish with age, the ligand would be expected to increase, possibly to compensate for the loss. Lastly, CD4+ CD28 MFI values correlated positively with $CD40^{hi}$ frequencies of B cells (Fig. 9(d)).

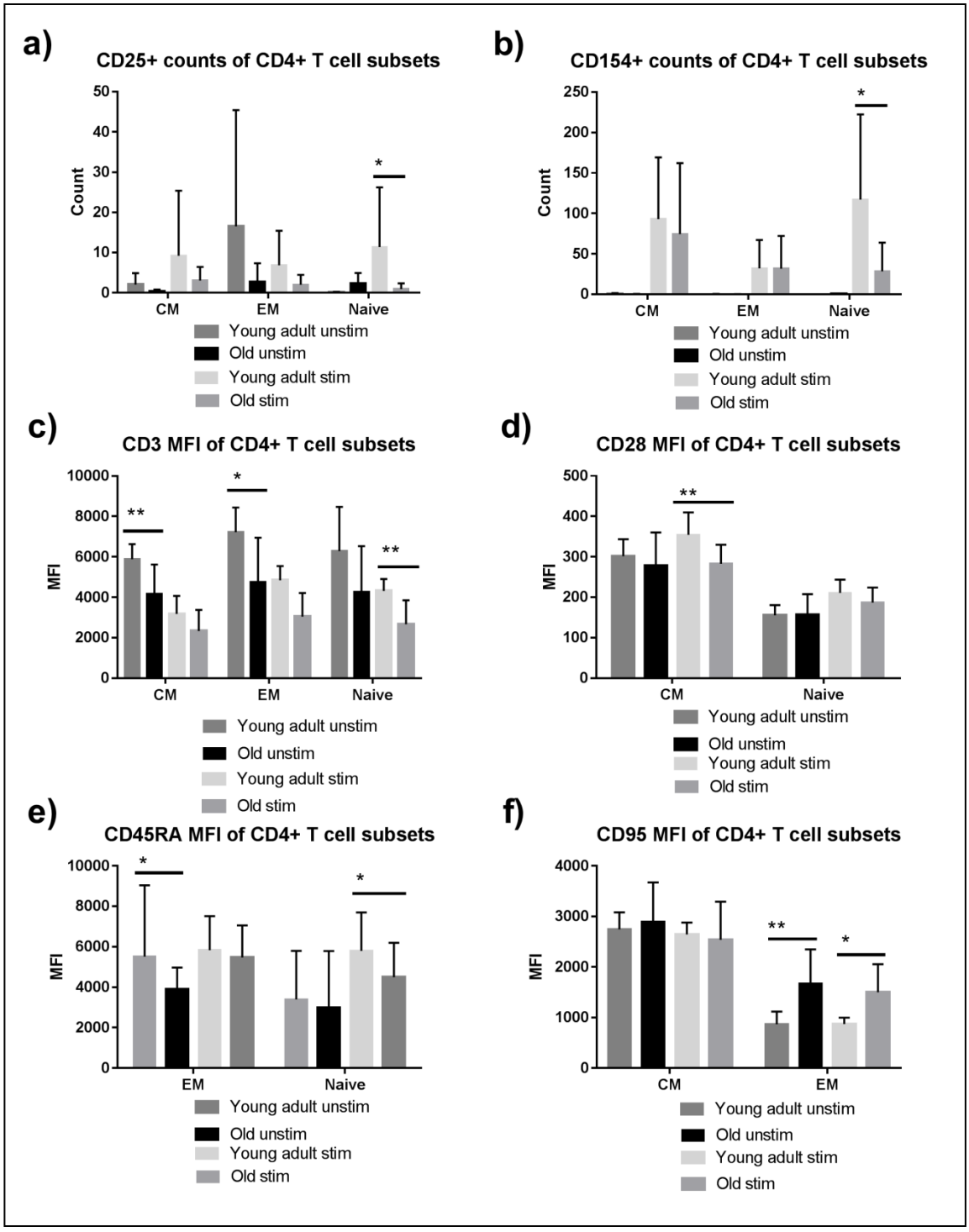

Figure 7: Average CD4+ T cell activation surface marker values compared between 9 young adult (<10 years) and 10 aged (>20 years) rhesus macaque PBMC samples. Samples were stimulated with PMA & I or mock stimulated with DMSO for 6 hours at 37°C, 5% CO_2. (a) Absolute CD25+ T cell subpopulation counts per microliter of blood found by multiplying the subpopulation frequency found by flow cytometry to the absolute lymphocyte count from the CBC data. (b) Absolute CD154+ T cell subpopulation counts per microliter of blood. (c) CD28 MFI values of CD4+ T cell subpop. found by flow cytometry. (d) CD45RA MFI values of CD4+ T cell subpop. EM= effector memory; CM= central memory. Error bars represent standard deviation. P-value <0.05*, <0.005**

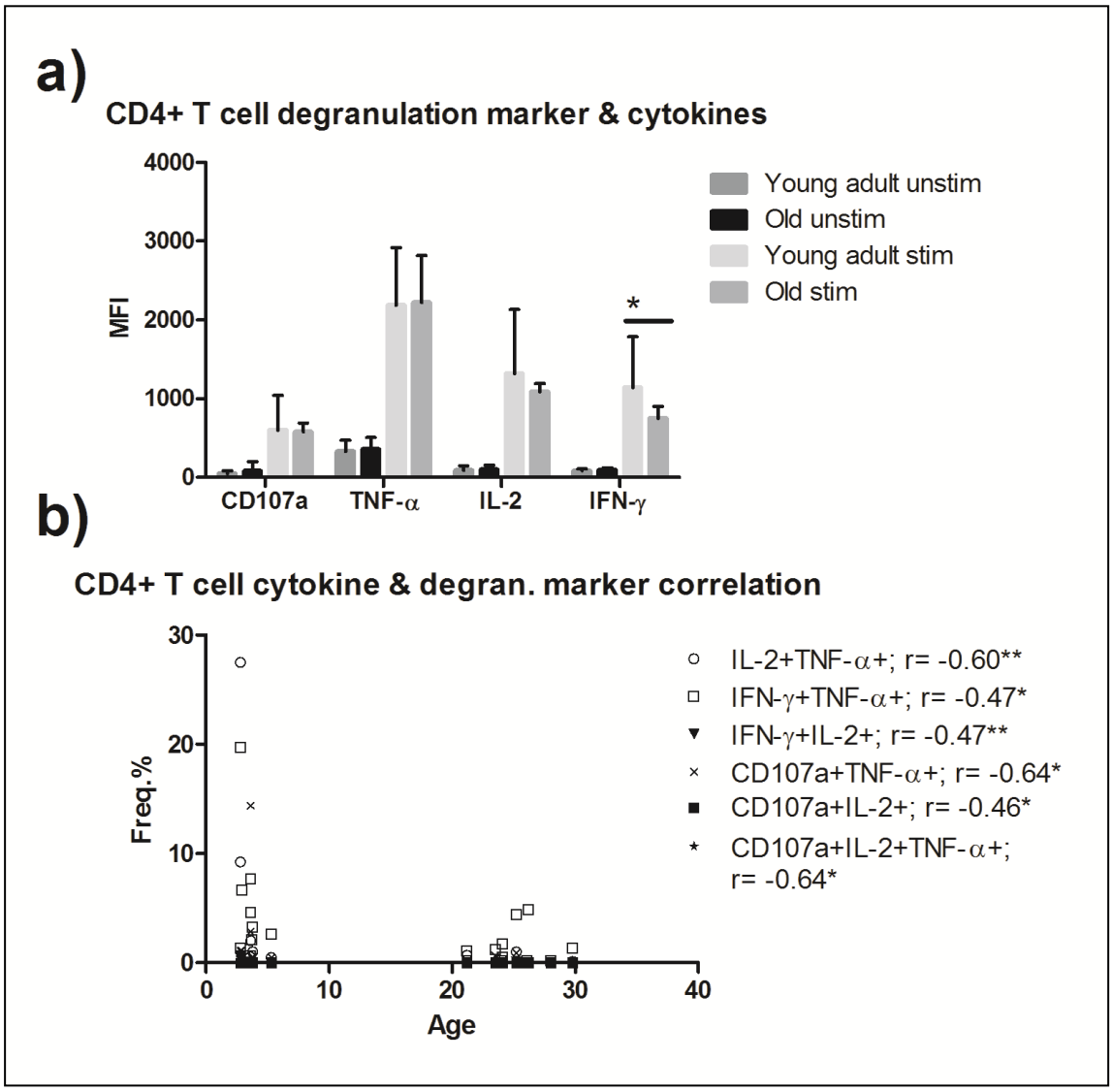

Figure 8: Average CD4+ T cell cytokine and degranulation marker values compared between 9 young adult (<10 years) and 10 aged (>20 years) rhesus macaque PBMC samples found by flow cytometry. Samples were stimulated with PMA & I or mock stimulated with DMSO for 6 hours, with GolgiStop (BD) added after the first two hours of incubation at 37°C, 5% CO_2. (a) MFI values of cytokines TNF-α, IL-2, and IFN-γ and degran. marker CD107a. (b) Correlation of multifunctional CD4+ T cells (which produce >1 cytokine and degran. marker) to age with Spearman's r values shown; n=19 for each r value. Error bars represent standard deviation. P-value <0.05*, <0.005**

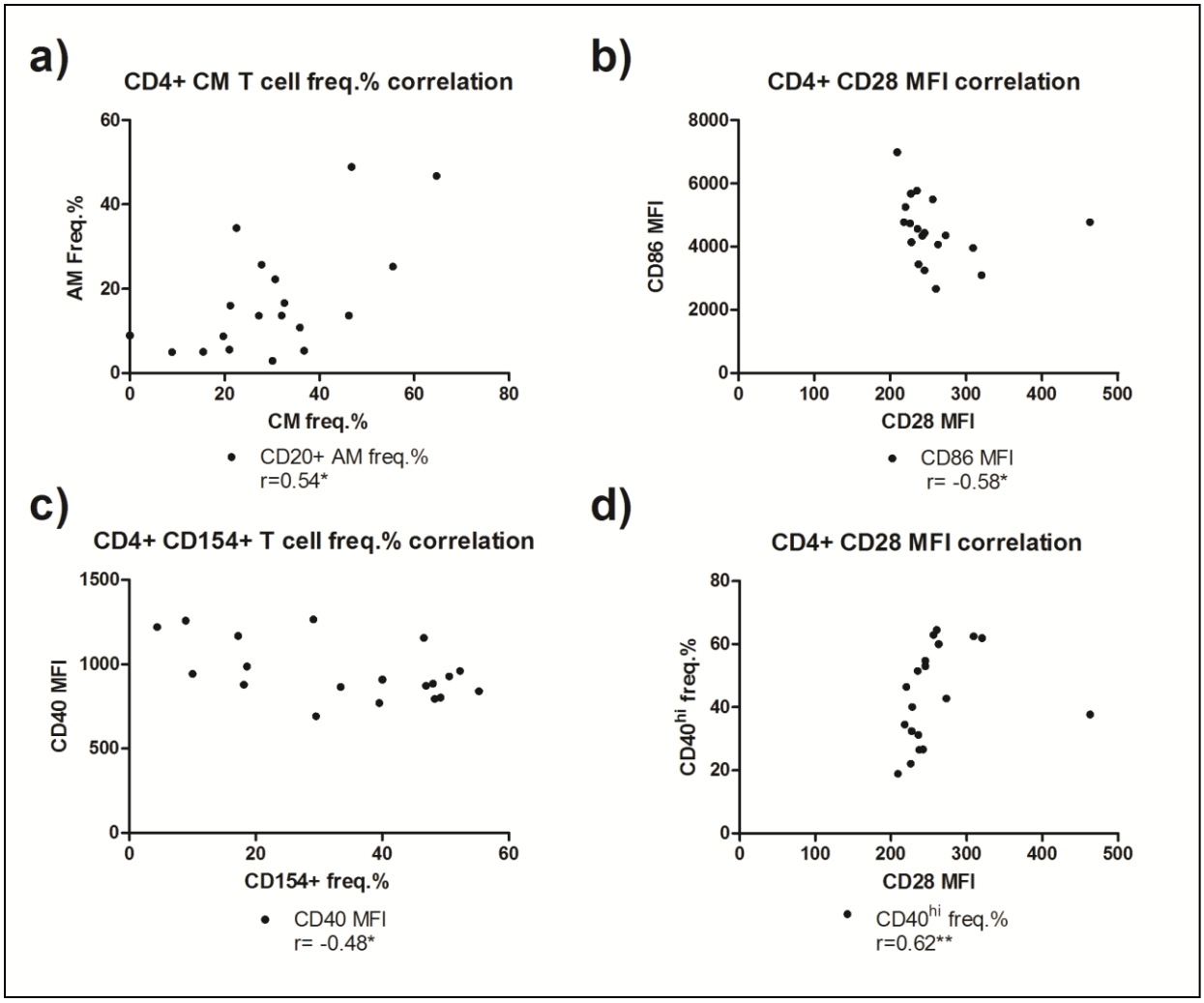

Figure 9: Correlation of CD4+ parameters compared to B cell parameters with Spearman's r values shown. (a) CD4+ CM T cell frequency correlation to B cell AM frequency with a positive association. AM= activated memory B cell; CM= central memory T cell; n=19 (b) CD4+ T cell CD28 expression correlation to stimulated B cell CD86 expression with a negative association; n=19 (c) CD4+ CD154+ (stimulated) T cell frequency correlation to stimulated B cell CD40 expression with a negative association; n=19 (d) Stimulated CD4+ T cell CD28 expression correlated to stimulated B cell $CD40^{hi}$ frequency with a positive association; n=19. Error bars represent standard deviation. P-value <0.05*, <0.005**

DN T Cell Activation

Surface Markers and Activation Markers

Hypoactivity of DN T cells in the aged macaque samples could begin to explain the higher incidences of autoimmunity and B cell dysfunction in the elderly. Stimulation with PMA & I for 6 hours did not result in differences between age groups of CD25+ DN T cell frequencies, absolute counts, or MFI values. CD154+ frequencies and MFI values of DN T cells did not differ between age groups. Central memory ($p=0.008$) and naïve ($p=0.005$) DN T cells had higher CD154+ absolute counts than the aged samples (Fig. 10(a)), parallel to the results seen for CD4+ T cells.

While surface molecule expression on CD4+ T cells appears to be multifaceted in the decline with age, this was not the case with DN T cells. CD3 MFI (Fig. 10(b)), CD28 MFI (Fig. 10(c)), and CD95 values in double negative T cells were not different between age groups. CD45RA, however, had significantly higher MFI values in the younger age group in both effector memory ($p=0.01$) and naïve ($p=0.05$) DN T cell subpopulations (Fig. 10(d)), alluding to possible importance with age.

Surface markers values were compared between age groups after stimulation. CD3 values were higher in the young adult effector memory ($p=0.035$) and naïve ($p=0.001$) subpopulations (Fig. 10(b)). CD28 expression was lower in the aged macaque samples in DN T cell subpopulations (naïve: $p=0.04$; CM: $p=0.001$; Fig. 10(c)). This was also seen in CD45RA MFI values, where both effector memory ($p=0.03$) and naïve ($p=0.008$) subpopulations had higher expression of this marker in the young adults (Fig. 10(d)). Contrarily to data from unstimulated DN T cells, there were no differences in CD95 MFI values.

Cytokines and Degranulation Marker

Lower levels of cytokines and degranulation marker in DN T cells could provide evidence for lack of B cell costimulatory signals in the elderly. Absolute counts of single-cytokine or degranulation marker producing DN T cells were not different between age groups. IFN-γ MFI values in DN T cells were higher in young adult macaques ($p=0.03$; data not shown), as seen with CD4+ T cells. Single-cytokine producing, IL-2-positive DN T cell frequencies correlated negatively with age ($r= -0.49$; $n=19$). Surprisingly, these were the only significant differences found for DN T cells producing cytokines IL-2, IFN-γ, TNF-α and/ or degranulation marker CD107a.

Association of T Cell and B Cell Parameters

There were fewer correlations of DN T cell parameters to B cell parameters as compared to the CD4+ T cell results. CD28 MFI values correlated negatively with their ligand on B cells, CD86 MFI values, and positively with CD40 MFI values (Fig. 11(a)), which was the same trend as CD4+ T cells. CD28 MFI values of DN T cells also correlated positively with $CD40^{hi}$ frequencies of B cells (Fig. 11(b)). CD154 values of DN T cells were not associated with any B cell parameters.

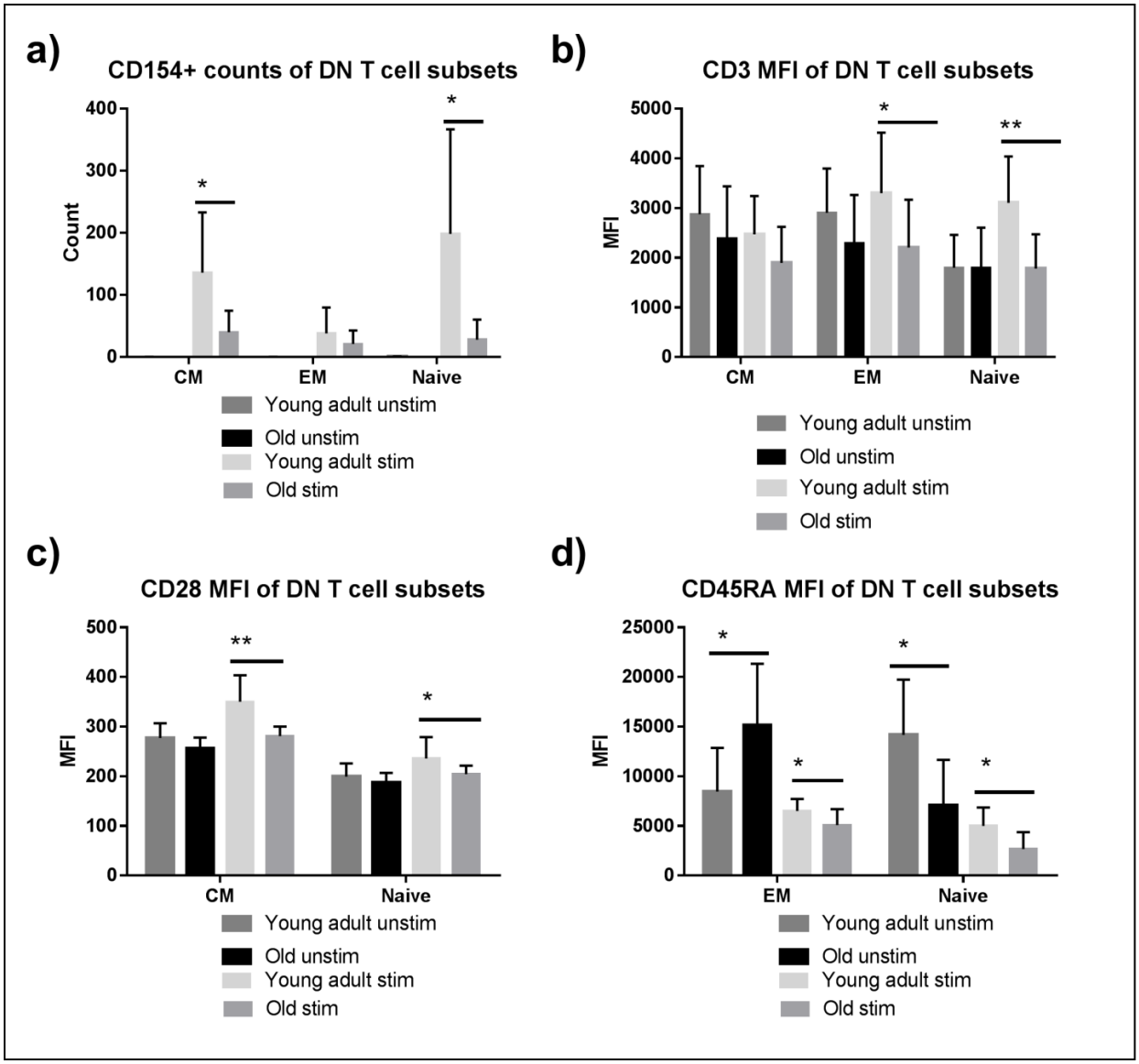

Figure 10: Average measurements of activation surface markers on DN T cells from 10 aged (>20 years) and 9 young adult (<10 years) rhesus macaque PBMC samples. Samples were stimulated with PMA & I or mock stimulated with DMSO for 6 hours at 37°C, 5% CO_2. (a) Absolute CD154+ DN T cell subpopulation counts per microliter of blood found by multiplying the subpopulation frequency found by flow cytometry to the absolute lymphocyte count from the CBC data. (b) CD28 MFI values of DN T cell subpop. found by flow cytometry. (d) CD45RA MFI values of DN T cell subpop. EM= effector memory; CM= central memory. Error bars represent standard deviation. P-value <0.05*, <0.005**

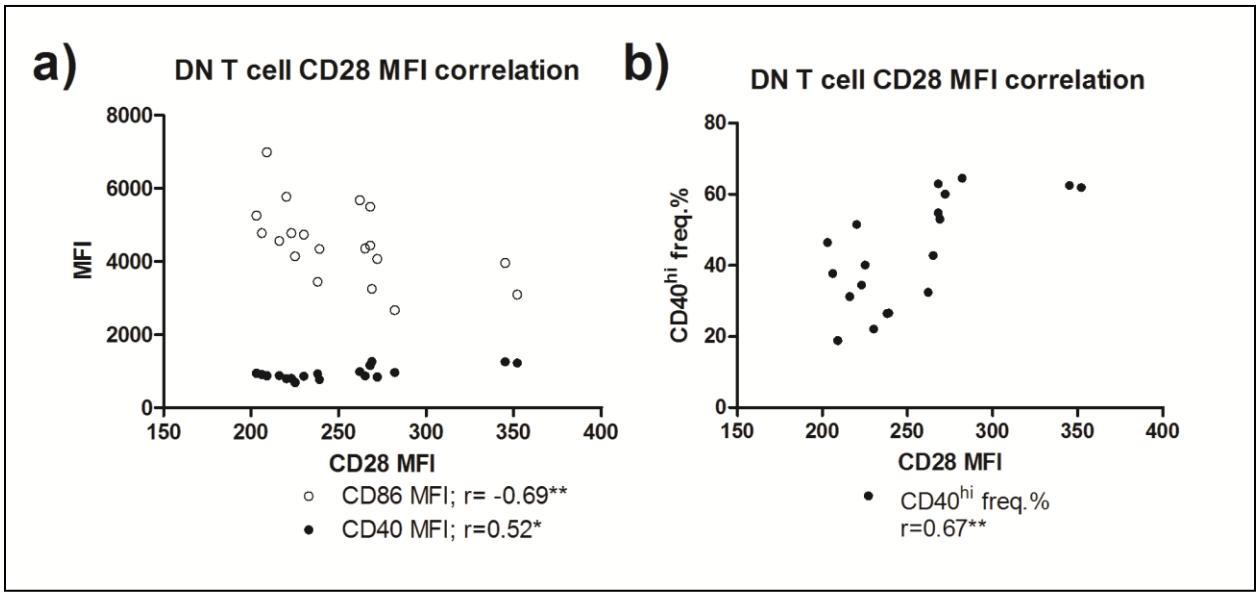

Figure 11: Correlation of DN T cell parameters compared to B cell parameters. (a) DN T cell CD28 MFI correlation with stimulated B cell CD86 expression (n=19) and CD40 expression (n=19) (b) DN T cell CD28 MFI positively correlates with stimulated B cell CD40^{hi} frequency; n=19. DN= Double negative T cell. Error bars represent standard deviation. Spearman's r values shown. P-value <0.05*, <0.005**

DISCUSSION

B Cell Analysis

B Cell Counts and Frequencies

Immunosenescence is a complicated process with an intertwining web of causes within the immune system due to age. In an effort to determine the functional effects of age on humoral immunity, B cell activation aspects were first targeted for study. RM PBMC samples were assessed for lymphocyte and B cell frequency and absolute counts per microliter of blood using flow cytometry. The data indicated a drastic decrease in total circulating lymphocytes with advanced age in RM. This could potentially be caused by a decrease in B cells, T cells, or both, which have all been demonstrated in previous studies (Didier et al., 2012; Čičin-Šain et al., 2007). Analysis of RM PBMC samples revealed that there was a significantly lower frequency of B cells in the aged RM PBMC, which translated to a decrease in absolute circulating B cell count as well.

Loss in humoral immunity function due to these decreases depends on which subpopulation(s) of B cells is affected by age. By measuring B cell subpopulation counts with flow cytometry and CBC data, it is apparent that there is a loss in naïve and resting memory B cells. Decreases in these subpopulations implies that there would be an inherent inability to respond to new antigens, since activated memory B cells are already responding to a threat and tissue-like B cells exhibit an exhausted phenotype. This could explain the deficient responses to vaccinations which are meant to invoke B cell immunity in the elderly. Since B cells in aged macaques have had more time to encounter antigens, this could lead to the trend for lower frequencies of these

subpopulations seen here. These decreases in count are also the same trend seen in the frequency data of naïve and resting memory B cells. Activated memory proportions of B cells were increased in the aged macaque samples, similar to previous studies (Colonna-Romano et al., 2003). The increased proportion of activated memory B cells may contribute to maintaining the humoral aspect of immunity after losses in the other subpopulations.

Perhaps the B cells become less specific, or have increased cross-reactivity to self-antigens, with age. The threshold for activation would be lower, leading to naïve and resting memory B cell activation. This could lead to the chronic, low-level inflammation and increased autoimmunity seen in the elderly. Since B cell diversity decreases with age on a gene-recombination basis (Gibson et al., 2009), these properties of antibodies from the elderly could be compared to the young. Speculation of the threshold for activation of B cells should include mention of the regulatory mechanisms, which would be included in any future studies.

Absolute count analysis also revealed a tendency for lower tissue-like B cell counts with age. These cells have been described as non-proliferative and “innate-like,” and are increased in patients with immunodeficiency (Rakhmanov et al., 2009). In the context of HIV, they exhibit an exhausted phenotype and are also known for being prone to apoptosis (Ho et al., 2006). With more samples, the difference in frequency and counts might become significant. An increased proportion of these cells would indicate a lesser ability to mount a memory response, such as seen with vaccination in the elderly.

Not surprisingly, the changes in B cell frequency with age are seen in both males and females. Males demonstrated more dramatic changes in absolute circulating B cell subpopulation counts. It is generally believed that males age faster than females, but females are more vigorous than males at any age (Blagosklonny 2010). So, absolute counts of immune cells may be the most accurate measure of immunosenescence. While the overall conclusions remain the same when the data is pooled for both genders, this data indicates a potential avenue for future studies into the gender bias of immunosenescence.

B Cell Activation Markers CD40 and CD86

Activation of B cells is the first step towards providing a long-lasting, effective humoral immune response. Expression of CD40 and CD86 is used to measure B cell activation *in vitro.* Using anti-IgG/IgM antibodies to trigger non-specific B cell activation is a viable means to determine differences in activation potential between aged and young adult samples. This experiment revealed higher $CD40^{hi}$ frequencies, absolute counts, and MFI values from the young adult macaques' samples in certain B cell subpopulations. CD40 binds to CD40L, or CD154, on T helper cells to induce activation of the B cell. A decrease in this molecule with age indicates a mechanism for decreased humoral immunity due to resulting deficient T cell costimulatory effects, which is confounded by the decreased frequency and counts of B cells found in the aged. Less CD40 could also explain the decreased diversity found in B cells in the elderly, since CD40 ligation is known to induce class switch recombination in mice; this pathway is also known to be affected by age (Frasca et al., 2007).

However, stimulation caused a decrease in $CD40^{hi}$ frequencies and CD40 MFI values in all B cell subpopulations and both age groups. This could be due to a negative-feedback or down-regulation mechanism that occurred within the 48 hour stimulation time frame. Interestingly, CD40 ligation decreases the half-life of CD40 at the surface of cells (Tucker and Schwiebert, 2008); while the stimulus used does not bind to CD40 directly, the presence of T cells in the PBMC may have caused CD40 ligation and caused the decrease in CD40 expression.

CD86 interacts with the CD28 molecule on T cells, so dysfunctional expression of CD86 could help explain the mechanism of immunosenescence through lack of T cell costimulation. This was demonstrated using CD86+ absolute counts and frequencies. Absolute counts of CD86+ B cells were higher in naïve, resting memory, and activated memory B cells of young adult macaques; these results indicate that since there are fewer B cells circulating in the aged, there is a corresponding decrease in B cells that can become activated. Furthermore, the difference in CD86+ counts in the activated memory population was seen in the frequency data as well, revealing a strong link between CD86 expression and age within this specific subset. Given that there would be a decrease in circulating CD86+ B cells in the aged, IgG1 levels in the blood would be expected to decline as well, since CD86 ligation has a direct relationship with antibody production (Kasprowicz et al., 2000). However, IgG levels appear to increase throughout an individual's lifetime (Bátory et al., 1984), indicating that CD86 ligation is not the major pathway for stimulating IgG production.

Another study analyzing B cell activation found that CD86 expression was missing upon revival in cryopreserved human B cells (Rosa et al., 2005). Nonetheless,

in my experiments, CD86+ frequencies were significantly higher in the stimulated samples compared to unstimulated in the young adult samples, but less in the aged samples (data not shown). Since Rosa et al. (2005) used PBMC samples from humans with an average age above middle age, it is possible their results were affected by age-related defects; the study also assessed activation status directly upon revival, without *in vitro* stimulation, which could explain the differences to my study as well.

Despite the promising results just described, two results did not support my hypothesis. The increase in CD86 MFI within the aged, resting memory B cell subpopulation suggests that while there may be fewer CD86+ B cells in circulation, the expression per cell is increased with age. However, it could also be due to a relatively small sample size, due to the variability in this one sample compared to the rest. Next, there was a higher CD86+ frequency of TL B cells in the aged. Since there would arguably be differences seen in the circulating counts or MFI values if there was a higher proportion of activated TL B cells in the aged, this also may be due to the small sample size.

The fluorescence-minus-one control revealed possible overcompensation of the anti-CD20-APCH7 antibody when analyzing anti-CD40-FITC+ lymphocytes. This is a limitation of the experiment; FITC might be making APCH7 brighter, although the interactions would be expected to be minimal since they are read on different lasers. It is also possible this effect is due to random variation, since only one sample was used for the control. The minus Live/Dead control showed small percentages of CD21/CD27+ populations, but this is most likely due to the infiltration of dead cells that is inevitable with this specific control.

BCR Signaling Proteins

BCR signaling protein activation is imperative for B cell activation. Syk is one of the first proteins to become phosphorylated at the BCR and continue the signaling cascade. In aged RM, total Syk^{hi} frequencies (both phosphorylated and non-phosphorylated forms) are higher compared to the young adults. This is seen in naive B cells and within memory B cells. Total Syk MFI was only found to be higher in the aged macaques' memory B cells. Phosphorylated Syk is at an increased level within the aged macaque B cells as well; this is only evident without stimulation, in both naive and memory B cells. Again, the pSyk MFI value was only higher in the aged macaque sample in the memory B cell subpopulation without stimulation. However, the young macaque B cells increased phosphorylation of Syk after stimulation, while the older samples slightly decreased the average phosphorylation level of Syk. Thus, even though the aged macaque B cells contain higher levels of Syk and baseline activated Syk levels, the overall effect is weaker in comparison to the younger macaque B cells.

Next, Syk activation leads to the phosphorylation of PLCγ2 near the BCR. Total PLCγ2 levels are also significantly higher within the memory B cell population in the aged macaque samples. Surprisingly, this was not the case with PLCγ2 MFI values, which were not different. Baseline frequency of activated PLCγ2 is higher in the aged macaque samples' memory B cells. Overall, the same conclusion can be drawn for PLCγ2 as with Syk: the baseline level may be higher in the aged macaque memory B cells, but it does not equate to a higher level of activation.

Further downstream of Syk, stimulation of the BCR causes phosphorylation and translocation of ERK to the nucleus of the B cell. Total ERK2 levels were higher in the aged macaque memory B cells, similar to Syk and PLCγ2; no difference in ERK2 MFI was found. Phosphorylation of ERK1/2 was comparable to Syk and PLCγ2 as well, such that the differences in the level of phosphorylation were no longer evident after stimulation even though the aged macaque B cells contained higher levels of activated ERK1/2 without stimulation.

Briefly, activated Syk levels maintain a higher baseline level in the aged which does not result in an improved response to stimulation. The higher baseline levels of pSyk may be the cause of the higher levels of pPLCγ2, since it is downstream of Syk, but also does not lead to a more robust response in the aged macaques. Lastly, higher levels of pERK1/2 could be caused by the high levels of upstream activated signaling proteins, but even though this protein has direct effects on antiviral transcription, there was not an enhanced response to stimulation. Therefore, lack of a more robust immune response in the aged could be due to deficiencies in the translation, packaging, secretion, or regulation mechanisms in the aged.

Overall, perhaps the increased baseline levels of phosphoproteins indicate a compensation mechanism within aged B cells. Without the higher baseline levels of proteins, the aged macaque B cells might not be able to reach the same level of activation as the young macaque B cells can. A possible mechanism for the increased signaling proteins in the age is altered transcription regulation in the aged, causing accumulation of proteins as a cell ages. Transcription factor E47, necessary for B cell class-switching, is known to be hindered by advanced age in humans (Frasca et al.,

2008), so it is plausible other transcription factors would be affected as well. In the case of signaling proteins, it would appear that the transcription factors would need to have increased function in advanced age to explain the increased level of proteins.

Alternatively, the accumulation of signaling proteins could be due to deficient proteasome activity. In a study by Grune et al. (2001), proteasome dysfunction resulted in increased levels of oxidized proteins within cells in the aged. If the BCR signaling proteins are negatively affected by oxidative stress, then the accumulation of dysfunctional proteins would not lead to a more robust immune response, such as the results of this paper indicate. Similar to this potential mechanism for increased proteins in aged macaque cells, there is decreased autophagy with age, or the method of breaking down old proteins through lysosome function (Cuervo and Dice, 2000). Thus, the accumulation of signaling proteins could be a measure of dysfunction and not compensation.

Increased phosphorylation could be caused by higher amounts of proteins available to become phosphorylated, increased kinase functioning, decreased phosphatase activity, or higher amounts of kinases and/ or lower amounts of phosphatases in the aged. For example, increased activity of phosphatidylinositol 3-kinase (PI3K) in CD4+ T cells leads to up-regulation of transcription factor nuclear factor- kappaB (NF-κB) in the aged, and is therefore partially responsible for chronic inflammation in the elderly (Bektas et al., 2014). NF-κB is downstream of the BCR, and may be linked to B cell dysfunction in age due to dysregulation of BCR kinases. Analysis of the transcriptome between aged and young human immune cells revealed enrichment of pro-inflammatory pathways in the aged, although this was not the case

for the BCR complex itself and T cell receptor signaling proteins (Fourati et al., 2016). BCR signaling proteins were not studied in that article, although this type of analysis might be able to confirm the results of my study and whether kinase or phosphatase function are linked to BCR signaling protein phosphorylation. Lastly, leptin is a protein associated with inducing phosphorylation of ERK1/2. It is increased in serum with age; this leads to B cell-mediated chronic inflammation (Gupta et al., 2013), and could possibly be linked to higher BCR signaling protein phosphorylation upstream of ERK1/2.

While these differences in signaling proteins between age groups were statistically significant, it is important to note that without an isotype control for the flow cytometry, the antibodies could have stained more than the protein they were designed against due to adhesion of the constant region of the antibodies. This could indicate a higher level of protein in general in the aged macaque cells, or the effects of signaling protein levels were masked by an overabundance of a cross-reactive protein. While this is unlikely due to the conservative gating of the flow cytometry data and clear separation of stimulated and unstimulated samples, the next step would be to confirm these results. Additionally, phosphatases were not removed from the PBMC cell samples due to incompatibilities with the flow cytometry protocol. However, removal of phosphate groups by active phosphatases would be unlikely due to the short time frame of experimentation; the fixation procedure might also mitigate phosphatase activity, although this has not been tested. The FMO control results showed possible overcompensation of anti-CD20-APCH7 with APC (CD27); this would indicate a shift upwards of CD20+ cells with the addition of anti-CD27 antibody. The difference was likely minimal, since there was still clear separation of CD20-negative and CD20-

positive populations. The addition of CD27 was necessary for B cell subpopulation analyses. Variations in the pSyk-FITC and pERK-PE FMO results could be due to the small sample size since it was done using a young adult macaque sample, but this is a limitation of the study. Similar inconsistencies were seen with the minus (total) Syk and ERK FMO results; the Syk^{hi} and ERK^{hi} populations were shifted lower than the pSyk+ and pERK+ populations, which caused difficulties in gating for both with the same unstained control. Low frequencies could be due to the low sample size. The total PLCg2 panel had similar results, which limits the data from being conclusive.

Conclusion

The B cell counts and frequencies found here mirrored previously published studies relating age to immune cells, and thus supported my hypothesis for specific aim one. Gender did not appear to affect the overall conclusions of age-related immune changes for either count or frequency. Next, activation measured by CD40 declined in advanced age, supporting my hypothesis, but CD86 was not a clear marker for immunosenescence. Levels of BCR signaling proteins were higher in the aged, contrary to my hypothesis that they would be lower. However, the activation of the signaling proteins was not different between age groups with stimulation. While this also did not support my hypothesis, it reveals that the amount of protein may be compensating for a less robust response; this would need to be confirmed by further studies including a western blot, since there are many possible reasons for increased levels of proteins in aged cells. Further confirmatory tests would also be necessary to address the inconsistencies in the FMO controls.

B Cell Analysis After SIV Co-incubation

B Cell Activation Markers after SIV Exposure

A virulent strain of SIV was added to RM PBMC samples along with the control stimulus to detect any virus-specific inhibition of B cell activation related to age. If inhibition was found, the role of B cells in the rapid progression of the elderly to AIDS would be implicated as a cause. Rhesus macaques are known to be elite controllers of SIVagm infections (Pandrea et al., 2011), so I expected no significant differences between infection with this strain and the control, consisting of PBMC with stimulus only added. In opposition to my hypothesis, no differences in B cell subpopulation distributions or CD40 expression with infection were found. Activation marker CD86 MFI was higher in aged macaque, activated memory B cells with the non-virulent strain of SIV compared to the young adult macaque samples; it was also higher compared to the uninfected, aged macaque samples. Without further differences, the function of CD86 expression during SIV infection is tenuous due to the small sample size (n=3) and large variability within age groups.

The structure of the experiment may not be able to accurately encompass the role of B cells during the infection with HIV. The faster progression to AIDS in the elderly may indeed involve B cells, but involve different B cell functions. Potential other functions and avenues for future studies include B cell cytokine production and antibody responses. Furthermore, the timeframe may take longer for the dysfunction of B cells to appear, since the progression to AIDS does not occur within the first few days of HIV infection. There could be a gradual accumulation of B cell dysfunction that hastens the

deterioration of the T cell compartment of the immune system. Finally, the mechanism for B cell dysfunction during SIV infection could involve different activation markers than the ones selected in this study.

Conclusion

Since there were no differences in B cell subpopulation counts or frequencies, these results did not support the hypothesis that virulent SIV exposure would affect the B cell subpopulations worse with advanced age. The lack of differences in CD40 and CD86 expression also did not support the hypothesis that age plays a role in SIV infection. While there was a difference with a non-disease causing strain of SIV, this was unexpected and most likely due to small sample size. Thus, B cell activation alone is most likely unaffected by age when exposed to SIV and not involved in the rapid progression to AIDS in the elderly.

T Cell Analysis

T Cell Counts, Frequencies, and Surface Markers

In order to determine whether any differences in B cell activation due to age could be due in part to deficient T cell activity, several parameters of T cell populations and activation were measured. As expected, there were fewer circulating T cells on average in the aged samples, although the frequency of total T cells was not different. CD4+ T cell frequency analysis showed a significant decrease in naive T cells with age, and a corresponding increase in central memory T cells. The absolute CD4+ T cell counts showed significant decreases in the naive subpopulation as well with advanced

age, and also a decrease in the effector memory subpopulation. Naïve and central memory double negative T cells had lower absolute counts in the aged. The shifts in T cell subpopulations indicate a defect in the potential of T cells to respond to new antigen, since the naïve T cell subset is depleted in both CD4+ and DN T cells; this is most likely due to thymic involution. Since CD4+ T cells interact with B cells, this could affect the potential activation of B cells to new antigens, too.

Baseline expression of surface markers can relate to the functionality of T cells. CD3 is an integral part of the T cell receptor. Effector memory and central memory CD4+ T cells have lowered expression of CD3, which could cause decreased ability to activate. As mentioned in the introduction, CD28 expression on T cells is known to be diminished in the elderly (Effros et al., 1994); this was seen in CD4+ CM T cells and naïve and CM DN T cells with stimulation. Its ligand interaction with B cells' CD86 would therefore be expected to be hindered. CD45RA, a tyrosine phosphatase used to differentiate T cell subpopulations, was also found to be lower in the effector memory and naïve populations of CD4+ T cells. MFI levels of CD45RA were consistently lower in DN T cells in both the effector memory and naïve subpopulations. This is important because CD45RA ligand is expressed on a majority of B cells, and ligation is known to inhibit proliferation of the B cell (Morikawa et al., 1991). So, without this regulation, B cells may be more likely to become hyperactive, leading to low levels of chronic inflammation, or auto-reactive. CD95 is also known as Fas; it is used to differentiate T cell subpopulations and is a receptor well known for inducing apoptosis. CD95 MFI values were actually higher in the aged macaque CD4+ EM T cells, indicating a potential for increased apoptosis.

T Cell Activation Markers CD25 and CD154

Stimulation causes increased expression of CD25 and CD154, a molecule known to interact with B cells. While CD25+ frequencies and MFI were not different between age groups, the absolute count of CD25+ naïve CD4+ T cells was higher in the young adult macaques. Importantly, low CD25 (also known as IL-2Rα) expression was linked to decreased ability to respond to a protozoan parasite *in vitro* (Chizzolini et al., 1991). Expression of this molecule should be researched further to see whether it is involved in the deficient immune responses to vaccination in the elderly.

CD154 expression mimicked CD25 expression. The only significant difference was a higher absolute count of naïve CD25+ CD4+ T cells. This tendency was reflected in the DN T cell subset, wherein absolute counts of CD154+ central memory and naïve T cells were higher in the young adults. Thus, the smaller pool of T cells in the blood results in a smaller amount of T cells available to become activated. With regards to vaccination, decreased CD154 expression on CD4+ T cells was linked to a less robust immune response to influenza vaccination (Seidel et al., 2011). As mentioned, the elderly have a diminished capacity to mount a memory response to the influenza vaccine (Blomberg et al., 2012). CD154-CD40 interaction deficiencies could be responsible.

While the FMO control results generated acceptable results, there was a higher frequency of unstained cells in the minus CD4 control. This is likely due to the small number of cells in the sample, which increased the frequency inordinately.

T Cell Surface Marker Expression with Stimulation

Several surface markers of T cells were found to change significantly with age after stimulation with PMA & I. Despite there being no difference in baseline levelsof CD28 expression, CD4+ central memory T cells from young adult macaques reached a higher level of expression of this molecule with stimulation. Both central memory and naïve double negative T cells from young adult macaques showed higher CD28 expression with stimulation. CD28 ligation is known to develop Th2-type responses in CD4+ T cells, which in turn stimulate strong antibody responses (King et al., 1995). Higher expression of this molecule in the young adults may help explain the deficiency in B cells responses in the aged, i.e. due to lack of costimulatory signals from T cells. Furthermore, CD28 ligation of T cells is a determinant of HIV resistance *in vitro* (Creson et al., 1999); with less CD28 available to aid in Th2 differentiation, there would be a deficient B cell response to HIV in advanced age as well.

Whereas CD45RA MFI was lower in the effector memory CD4+ T cells in the aged macaques, stimulation caused lower MFI within the naïve T cell subset instead. Stimulation did not change CD45RA expression in DN T cells; expression remained lower in the aged macaques' naïve and EM T cell subpopulations. Because this molecule has been consistently found to be decreased with age, the role of CD45RA in T cell immunosenescence and its role in B cell regulation could be investigated more thoroughly in future studies.

T Cell Cytokine Secretion and Degranulation Marker Expression

CD4+ T cells activate B cells through ligand interactions and through pro-inflammatory cytokine secretion, so cytokine production and degranulation marker CD107a were analyzed by flow cytometry. TNF-α is known to induce apoptosis in immune cells. Because of this function, further studies into the level of TNF receptor levels in aged B and T cells should be done to see if a higher sensitivity to this cytokine develops with age. IFN-γ MFI was higher in young adult macaques' CD4+ and DN T cells, which is known for having antiviral action. Antiviral vaccinations could be affected negatively by this decreased expression in the elderly. IL-2+ frequencies of DN T cells decreased with age. Since this cytokine acts as an autocrine, DN T cells may be more difficult to activate in the elderly.

Multifunctional CD4+ T cell frequencies decrease with age, yet DN T cell analysis did not reveal any correlation with multifunctional cells, including triple and double cytokine producing T cells. Decreases in multifunctional CD4+ T cells would negatively affect the ability of the elderly to fight active tuberculosis infections (Caccamo et al., 2010), especially considering their relatively higher rates of infection (Jappar and Low, 2015). The role of multifunctional CD4+ T cells in viral infections or other diseases inordinately represented in the elderly have not been studied.

The FMO results showed a higher CD107a+ frequency in the minus Live/Dead control, most likely due to the additional dead cells autofluorescing in the APCH7 channel which is an inevitable occurrence with this control. Other effects seen were decreased IFN-γ+ and IL-2+ frequencies in the minus CD3 control, minus cytokine controls, and the minus none control. This would mean that there is a masking effect of

some of the antibodies, causing the frequencies to be a more conservative estimate of the true frequency.

T Cell and B Cell Parameter Correlations

Association of T cell parameters to B cell parameters would provide evidence for the compensation of one for the other in the elderly. Notably, B cell CD40 MFI values correlated negatively with its ligand, CD154 frequency, of CD4+ T cells. Therefore, increased amounts of activated T cells would appear to cause a decrease of CD40 on B cells; this is expected, since ligation of CD40 decreases its half-life (Tucker and Schwiebert, 2008). $CD40^{hi}$ B cell frequency was also positively associated with the TCR co-stimulatory molecule, CD28 MFI; this was also the case for DN T cells. CD4+ CD28 MFI values correlated negatively with its ligand, CD86 MFI on B cells, which was seen in DN T cells as well. Previous studies have shown decreased CD28 expression with immunosenescence, so this ligand relationship could be indicative of a compensation of increased CD86 expression on B cells. However, this speculation would need further research to be confirmed.

Importantly, CD4+ central memory T cells were correlated positively with activated memory B cell frequency. Both of these populations had declining frequency with age. It remains to be seen whether the decrease in central memory T cells is a partial cause of the decrease in AM B cells due to insufficient costimulatory signals. Regardless, the interaction between these two subpopulations of lymphocytes warrants further research with respect to immunosenescence.

Conclusion

The biggest cause of deficient T cell costimulation of B cells appears to be due to the overall decrease in available, circulating T cells. While I expected more drastic differences, this still supports my hypothesis that aged macaques' T cells would have a less robust immune response. Expression of CD3, CD28, CD45RA, and CD95 were implicated in T cell immunosenescence to varying degrees as well. The next step for T cell cytokine analysis would be to measure Th2-type cytokine secretion, since these proteins have a direct effect on B cell function; further research into the role of multifunctional T cells in immunosenescence could also be done. Correlation of T and B cell ligand pairs revealed the negative feedback mechanism between CD154 and CD40, and indicated CD28-CD86 as having a similar mechanism.

CONCLUSION

B cells are an integral part of the immune system responsible for humoral memory responses. Dysfunction in B cells due to age has been acknowledged, but the mechanism had been unknown. In an effort to identify the mechanism of B cell immunosenescence, several B cell activation parameters were measured by flow cytometry in aged and young adult rhesus macaques.

Differences in B cell subpopulation distributions were found, which supported previous studies. Activation marker CD40 expression declined with age, indicating a possible mechanism of immunosenescence involving deficient T cell costimulation. CD86 expression is involved in age-related dysfunction in RM due to the general decrease caused by lack of B cells in general. Interestingly, there was also a decline in activated memory B cell frequencies with age. BCR signaling proteins were increased in the aged macaques, but this did not equate to a more effective immune response.

Even though B cell immunosenescence was implicated as a possible mechanism for the faster progression rate to AIDS in the elderly, no differences in B cell activation were found. Further studies may be needed to rule out their involvement completely, including the use of a longer-term study.

Because of the intimate link between helper T cells and B cell activation, pro-inflammatory activity of CD4+ and DN T cell were analyzed by flow cytometry. Differences in T cell subpopulation numbers and surface marker expression due to age were found and correlated with B cell activation markers. Multifunctional T cells decrease with age, indicating their importance in immunosenescence.

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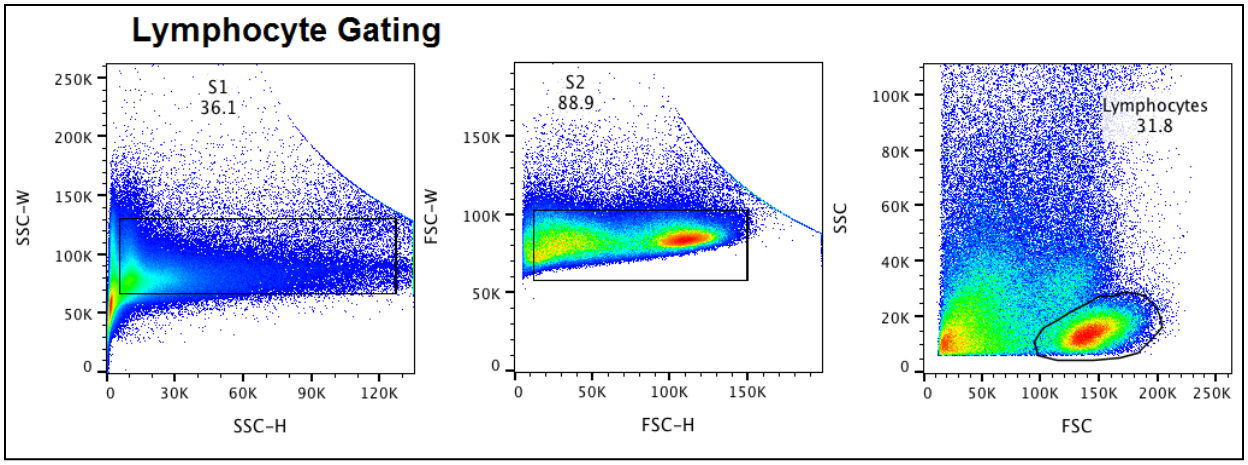
APPENDIX A

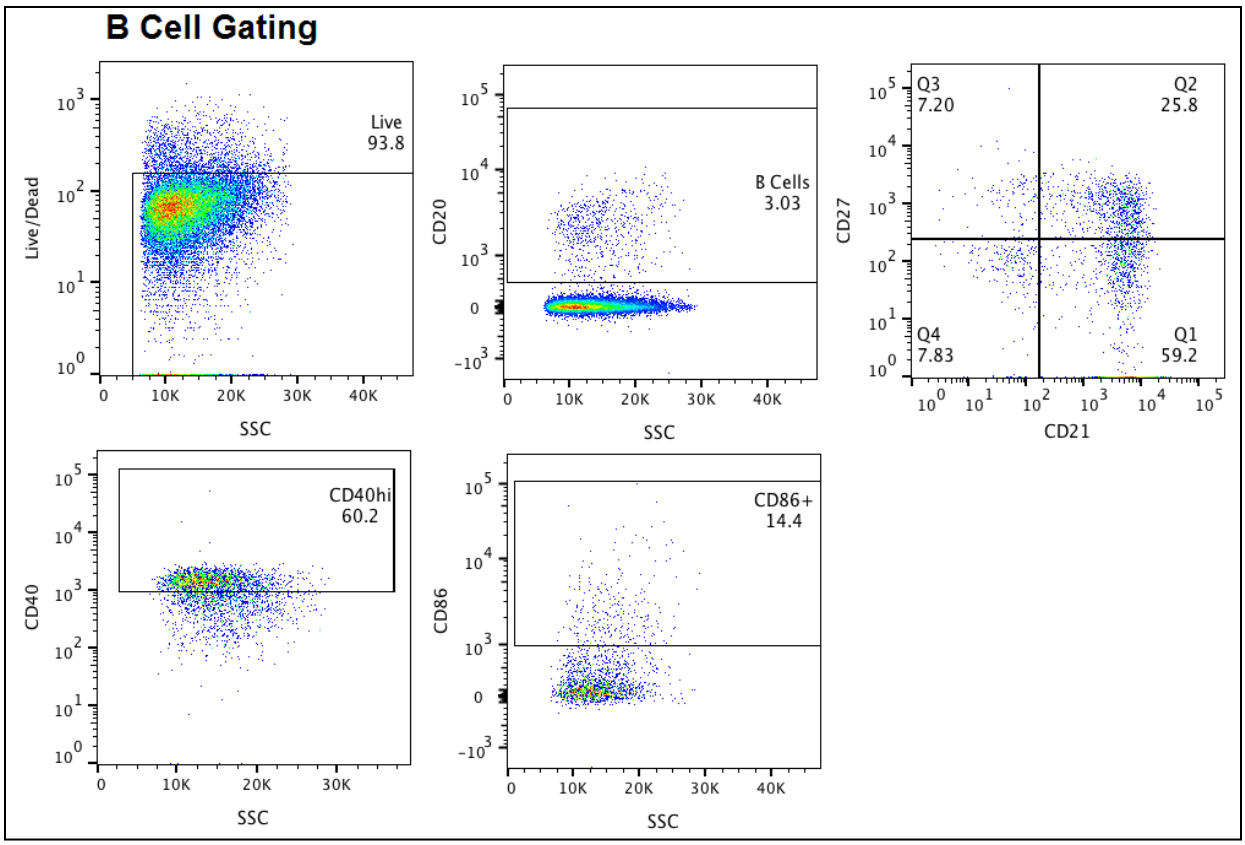
Representative PBMC Flow Cytometry Gating for Rhesus Macaque Lymphocytes

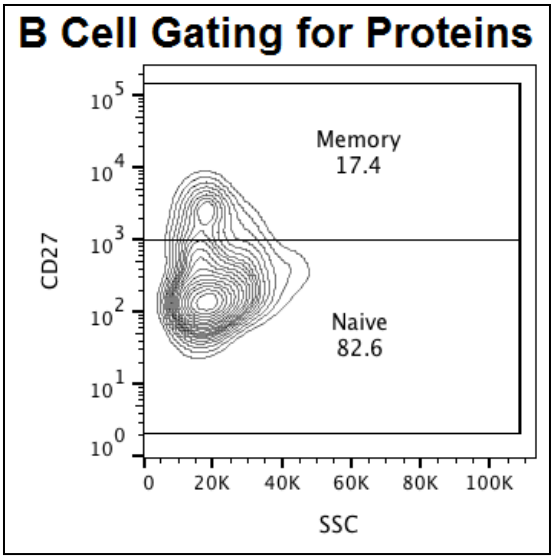
(Left) Gating for singlets with side-scatter (Middle) Gating for singlets with forward-scatter (Right) Gating for lymphocytes



APPENDIX B

Representative Rhesus Macaque B Cell Gating with Activation Surface Markers CD40 and CD86 from Lymphocyte Gating in Appendix A (Top) and B Cell Gating from Live Lymphocytes for BCR Signaling Protein Analysis (Bottom)

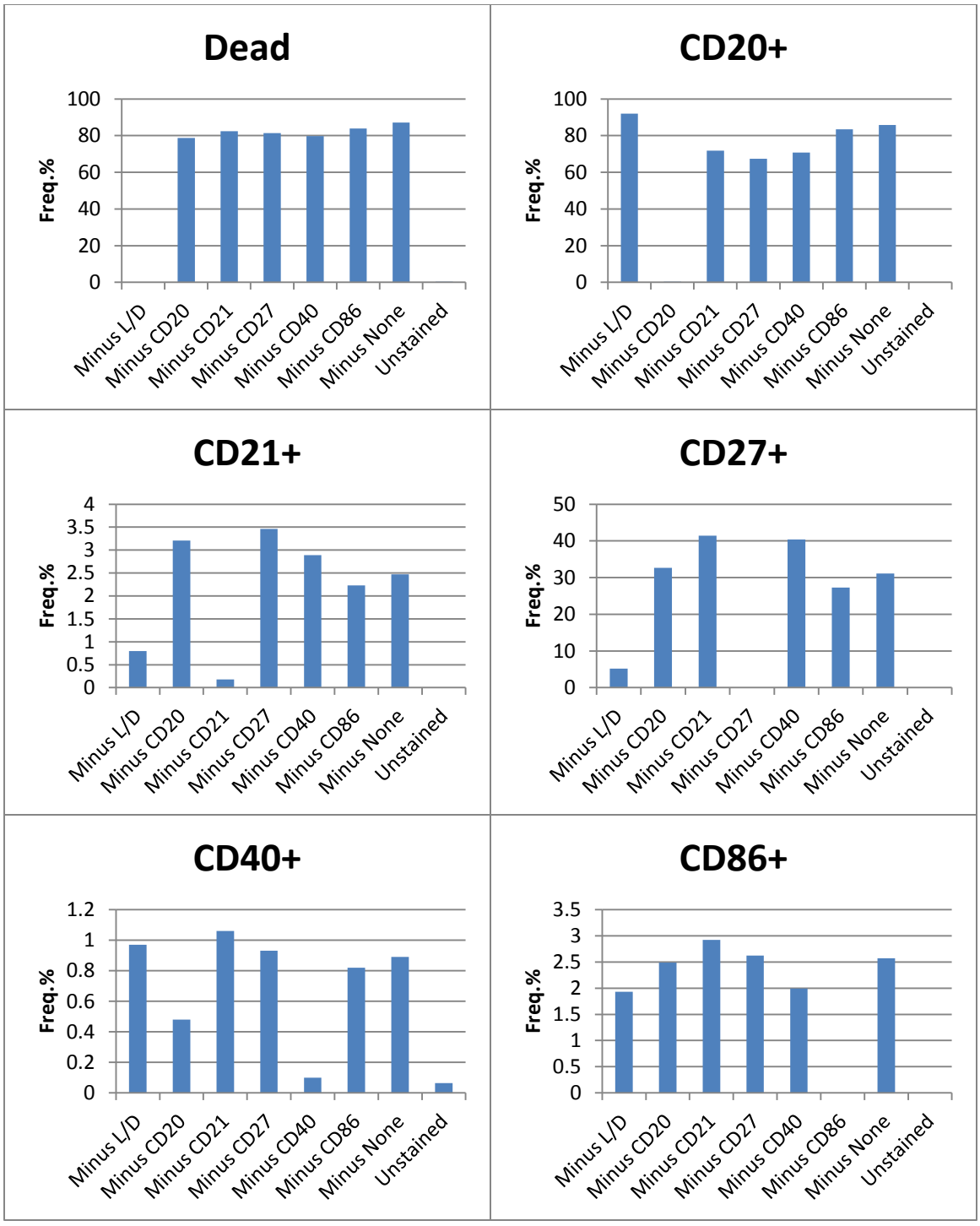




APPENDIX C

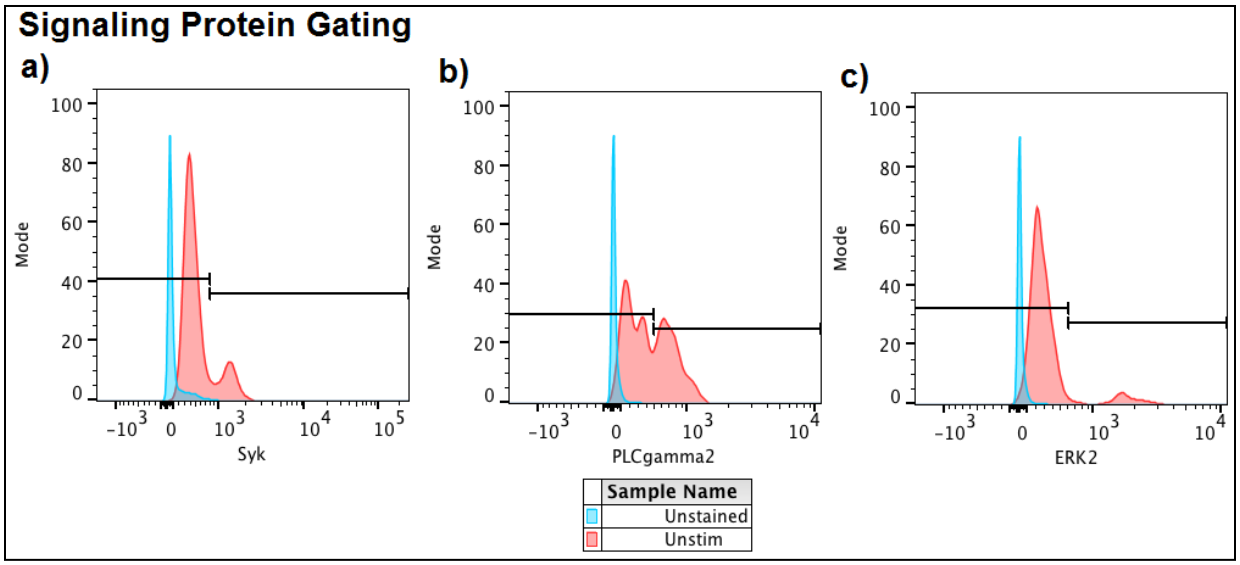
FMO Control Results for B Cell Surface and Activation Markers

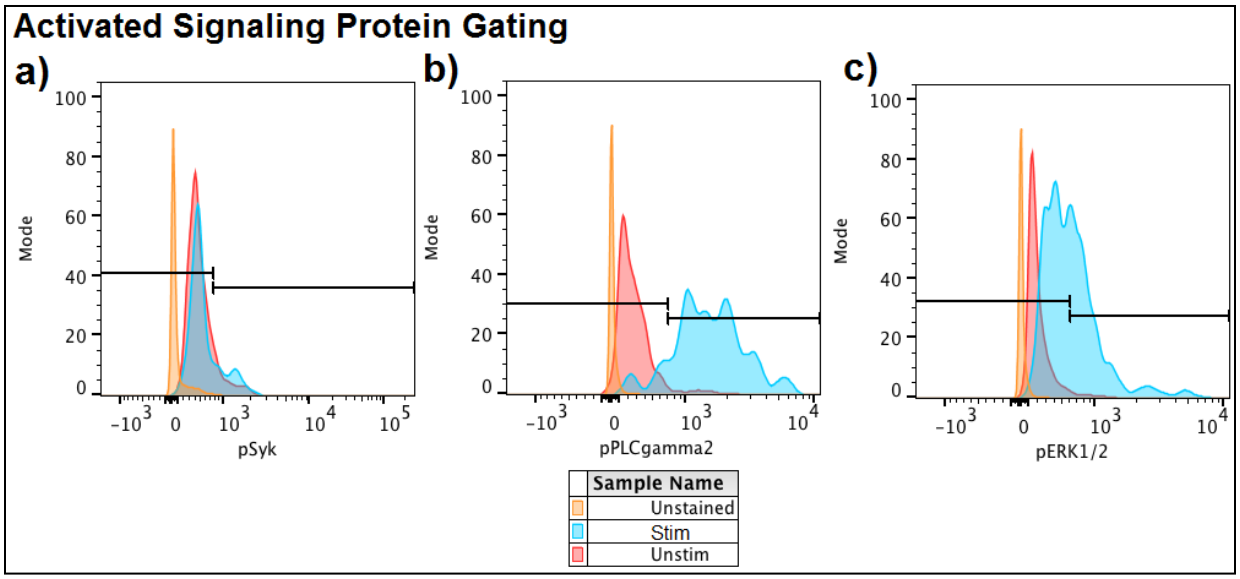
My analysis from a fluorescence-minus-one control done on 5/02/2015 using anti-IgG/IgM antibody-stimulated RM PBMC sample for 48 hr. after revival. Populations were gated on live lymphocytes, with the exception of the Dead population. The lower populations of CD21+ and CD27+ cells within the minus Live/Dead control can be attributed to the population of dead cells that could not be excluded.


Dead
Freq.%
100
80
60
40
20
0
Minus L/D
Minus CD20
Minus CD21
Minus CD27
Minus CD40
Minus CD86
Minus None
Unstained
CD20+
Freq.%
100
80
60
40
20
0
Minus L/D
Minus CD20
Minus CD21
Minus CD27
Minus CD40
Minus CD86
Minus None
Unstained
CD21+
Freq.%
4
3.5
3
2.5
2
1.5
1
0.5
0
Minus L/D
Minus CD20
Minus CD21
Minus CD27
Minus CD40
Minus CD86
Minus None
Unstained
CD27+
Freq.%
50
40
30
20
10
0
Minus L/D
Minus CD20
Minus CD21
Minus CD27
Minus CD40
Minus CD86
Minus None
Unstained
CD40+
Freq.%
1.2
1
0.8
0.6
0.4
0.2
0
Minus L/D
Minus CD20
Minus CD21
Minus CD27
Minus CD40
Minus CD86
Minus None
Unstained
CD86+
Freq.%
3.5
3
2.5
2
1.5
1
0.5
0
Minus L/D
Minus CD20
Minus CD21
Minus CD27
Minus CD40
Minus CD86
Minus None
Unstained

APPENDIX D

Total Signaling Protein-High Gating (Top) and Activated Signaling Protein Gating (Bottom) for Syk (a), PLCγ2 (b), and ERK (c)

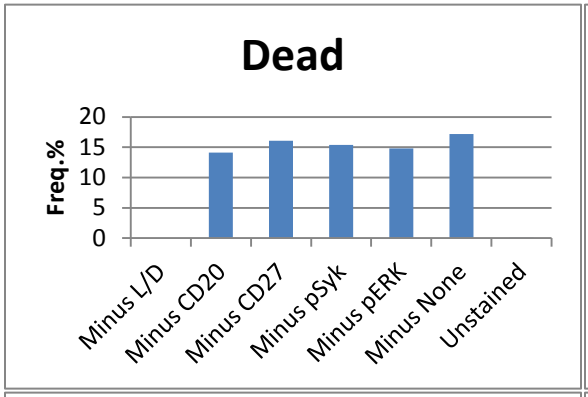


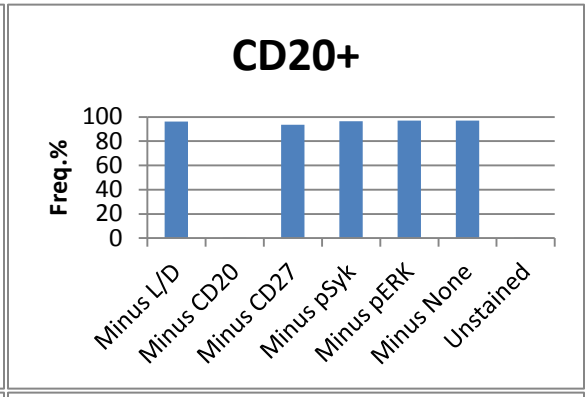


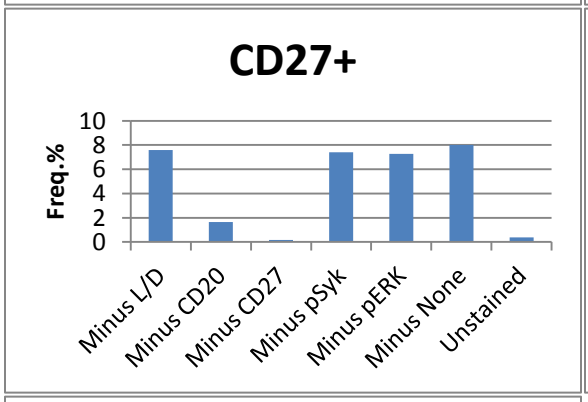
APPENDIX E

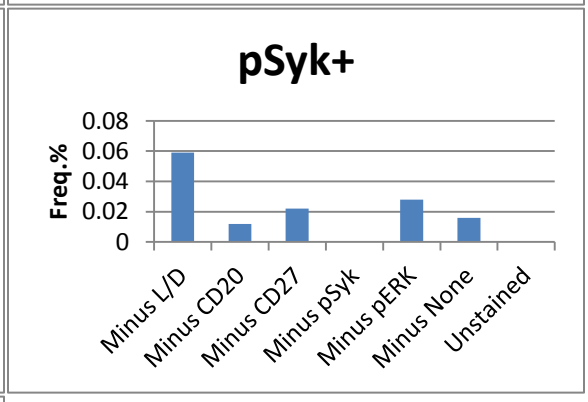
FMO Control Results for B Cell Signaling Protein Panel With pSyk and pERK

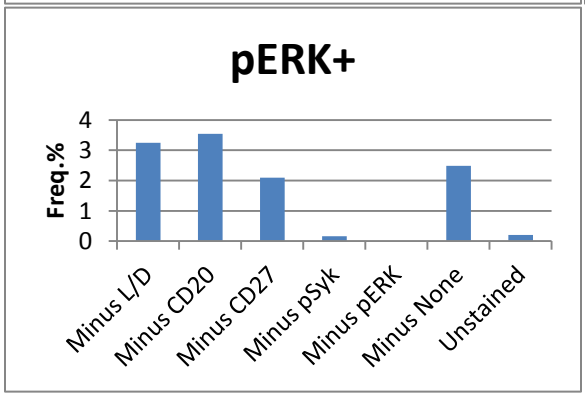
My analysis from a fluorescence-minus-one control done on 5/02/2015 using anti-IgG/IgM antibody-stimulated RM PBMC sample as described in the methods section. Populations were gated on live lymphocytes, with the exception of the Dead population.







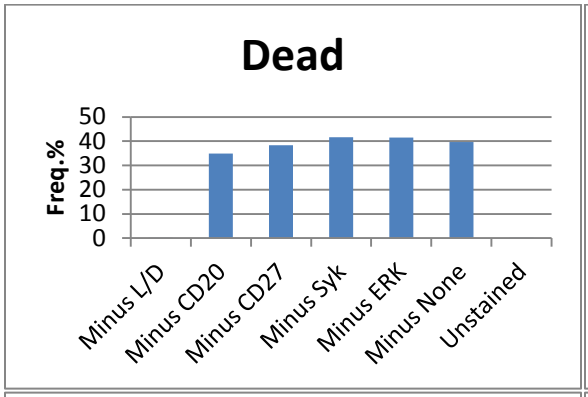


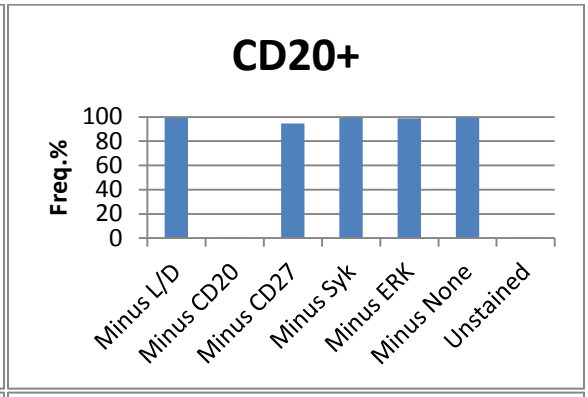


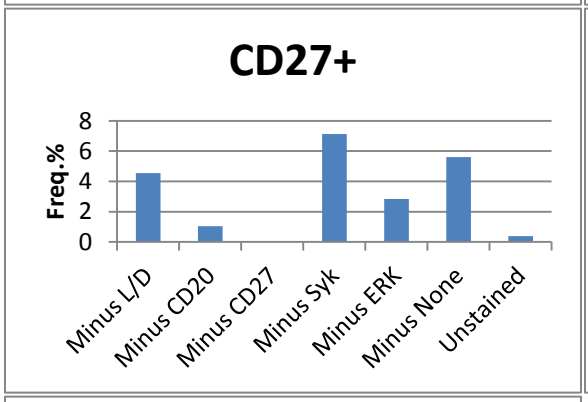
APPENDIX F

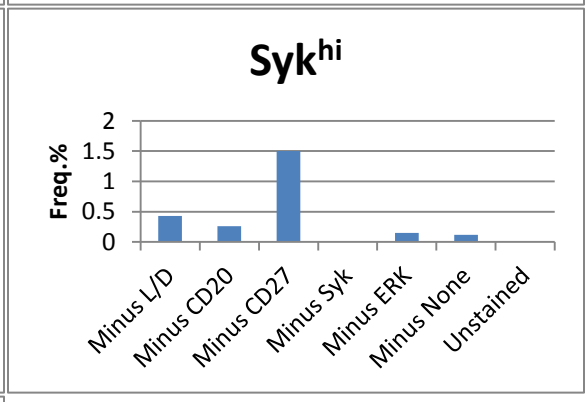
FMO Control Results for B Cell Signaling Protein Panel With Total Syk and ERK

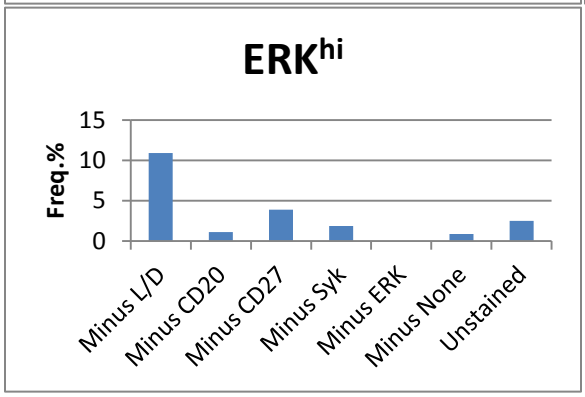
My analysis from a fluorescence-minus-one control done on 5/02/2015 using RM PBMC as described in the methods section. Populations were gated on live lymphocytes, with the exception of the Dead population.







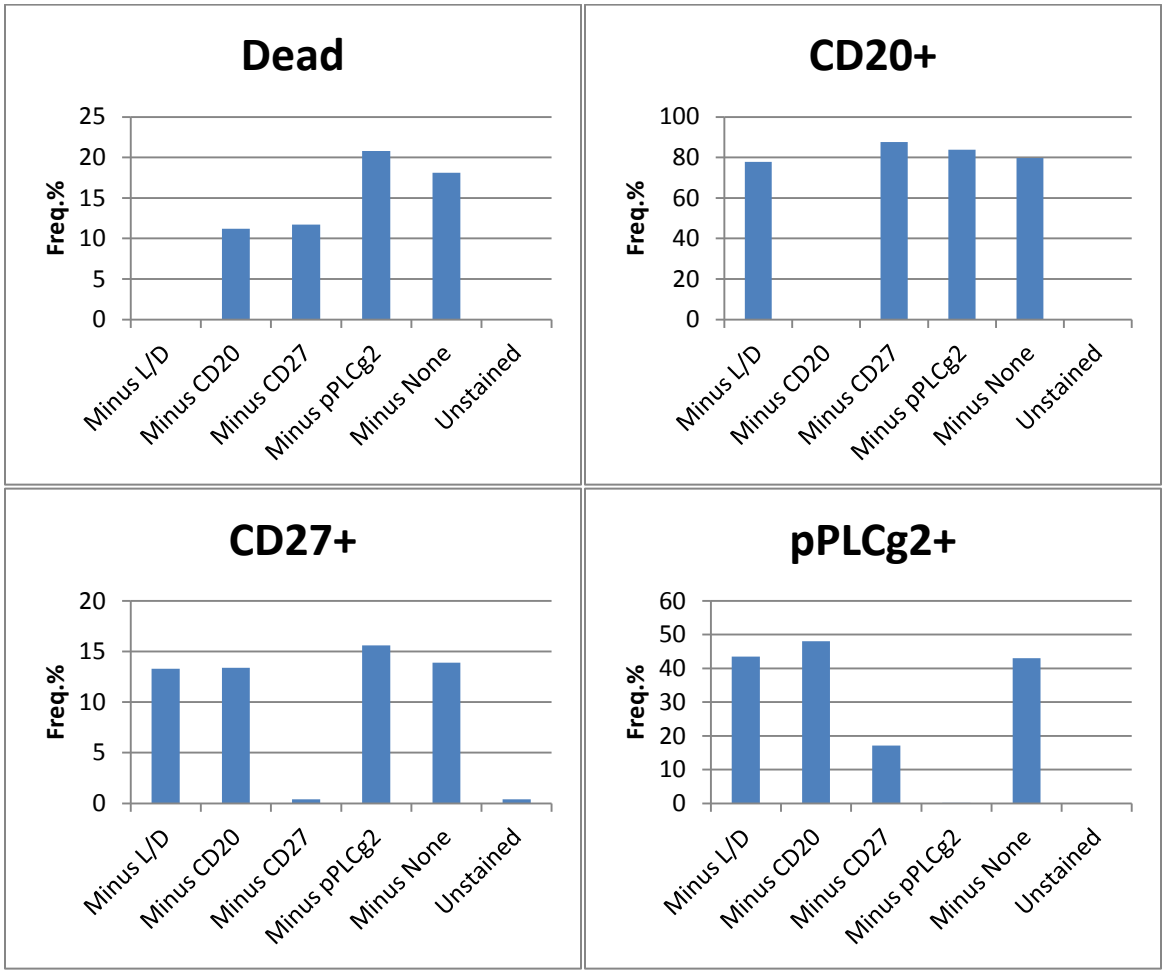




APPENDIX G

FMO Control Results for B Cell Signaling Protein Panel With pPLCg2

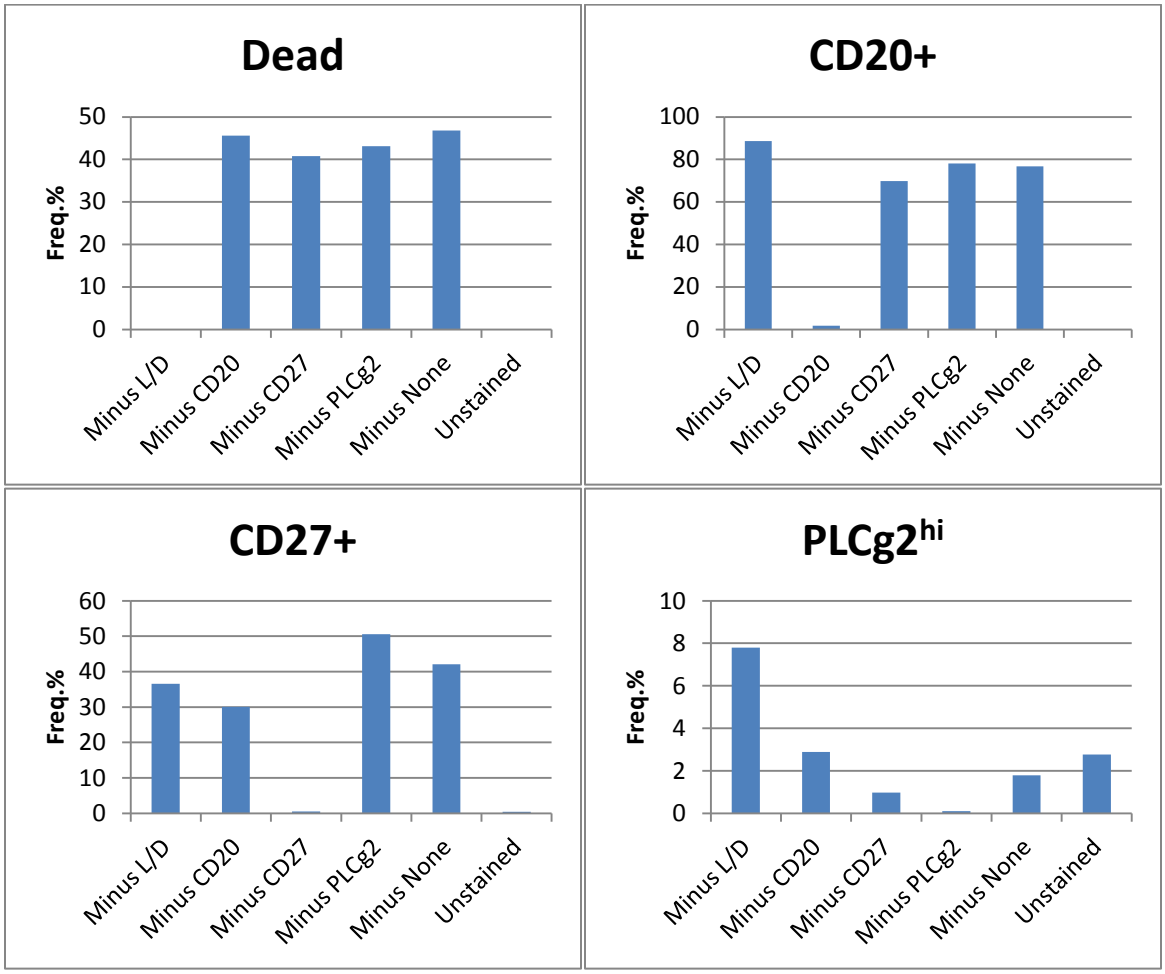
My analysis from a fluorescence-minus-one control done on 5/02/2015 using anti-IgG/IgM antibody-stimulated RM PBMC as described in the methods section. Populations were gated on live lymphocytes, with the exception of the Dead population.



APPENDIX H

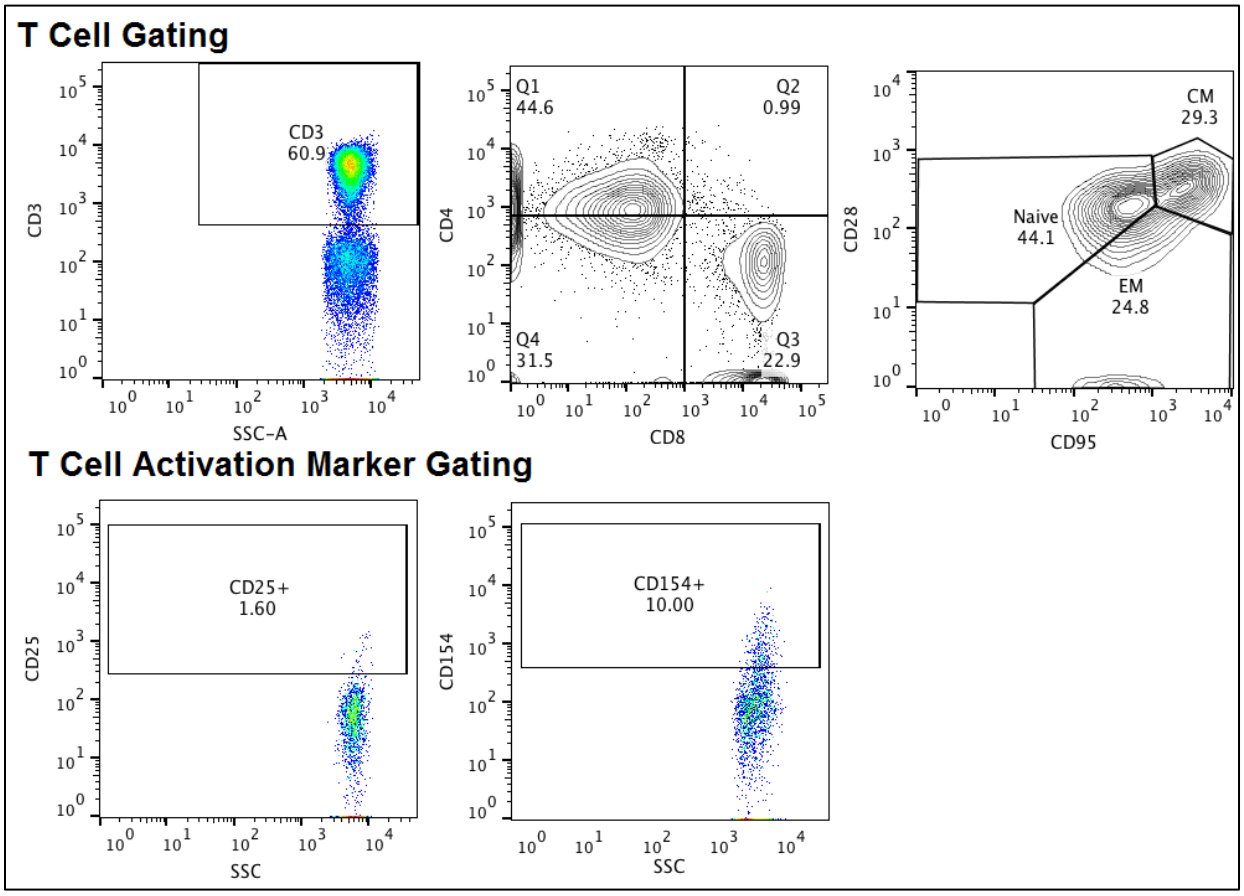
FMO Control Results for B Cell Signaling Protein Panel With Total PLCg2

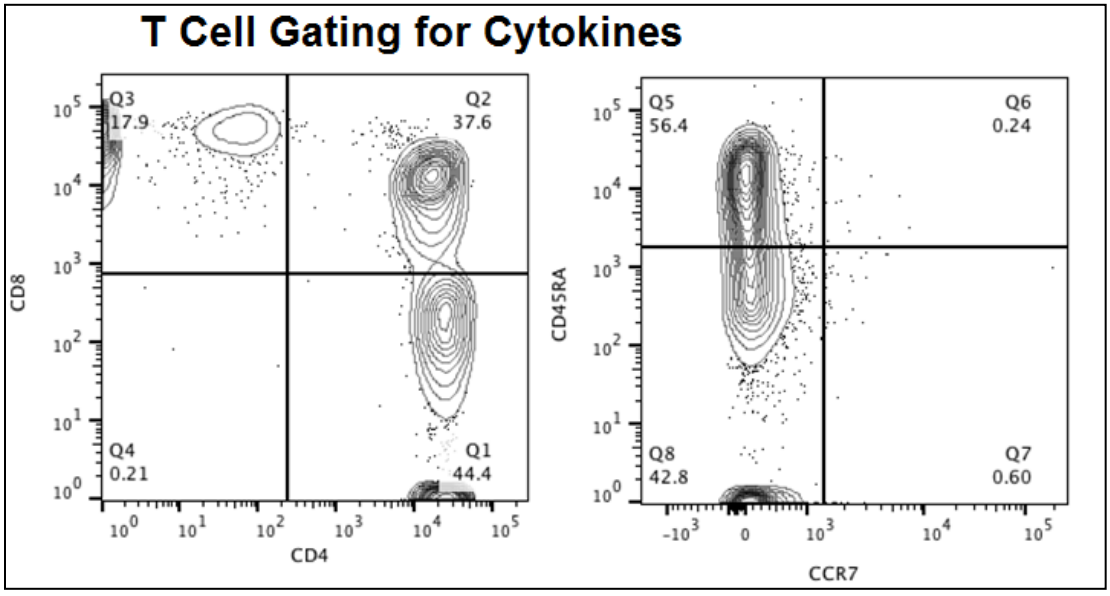
My analysis from a fluorescence-minus-one control done on 5/02/2015 using RM PBMC as described in the methods section. Populations were gated on live lymphocytes, with the exception of the Dead population.



APPENDIX I

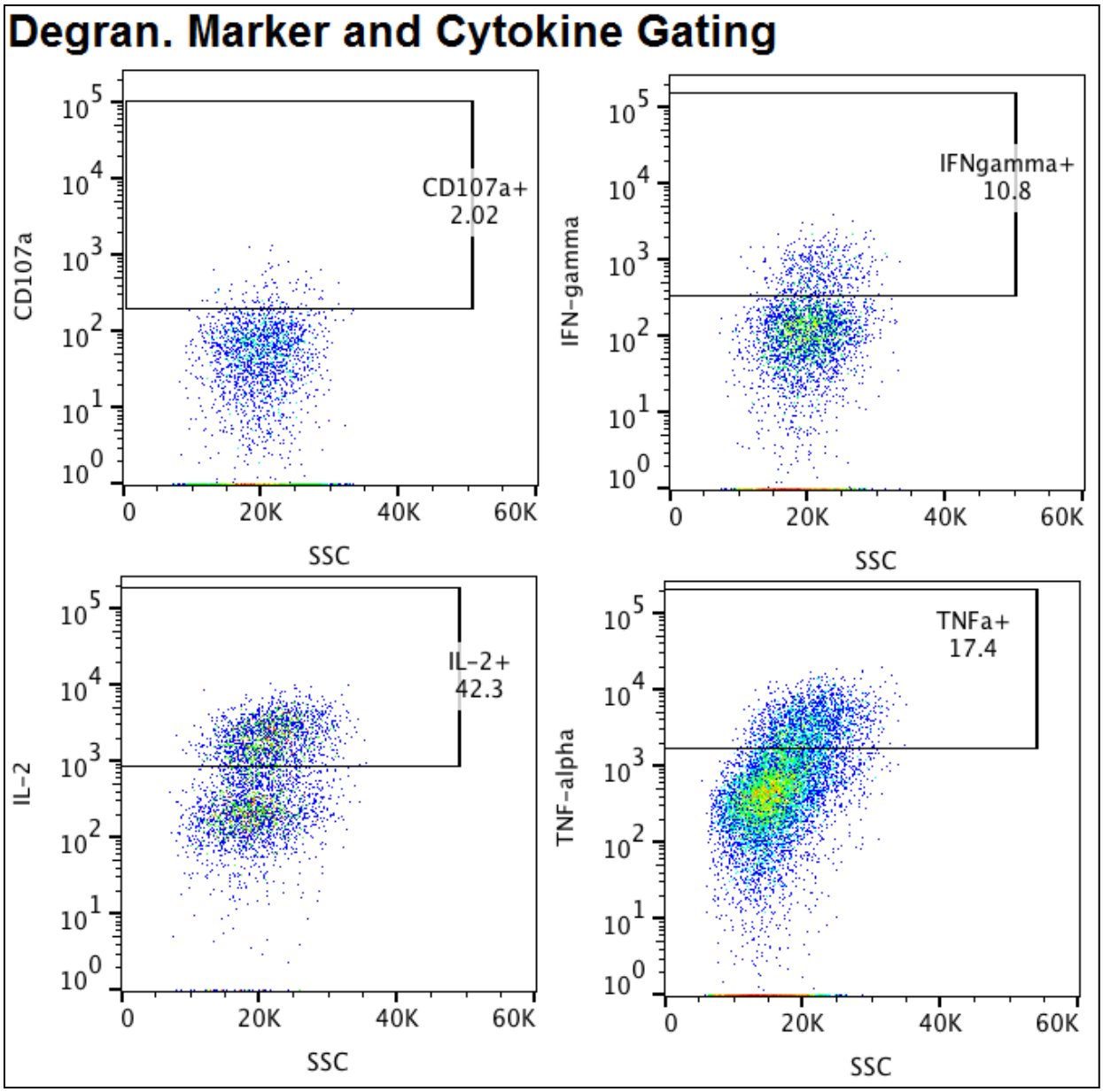
Representative T Cell Subpop. Gating (from Live Lymphocytes) for Surface Activation Markers CD25 and CD154 (Top) and T cell Subpop. Gating Strategy (from Live Lymphocytes) for Cytokine Analysis (Bottom)





APPENDIX J

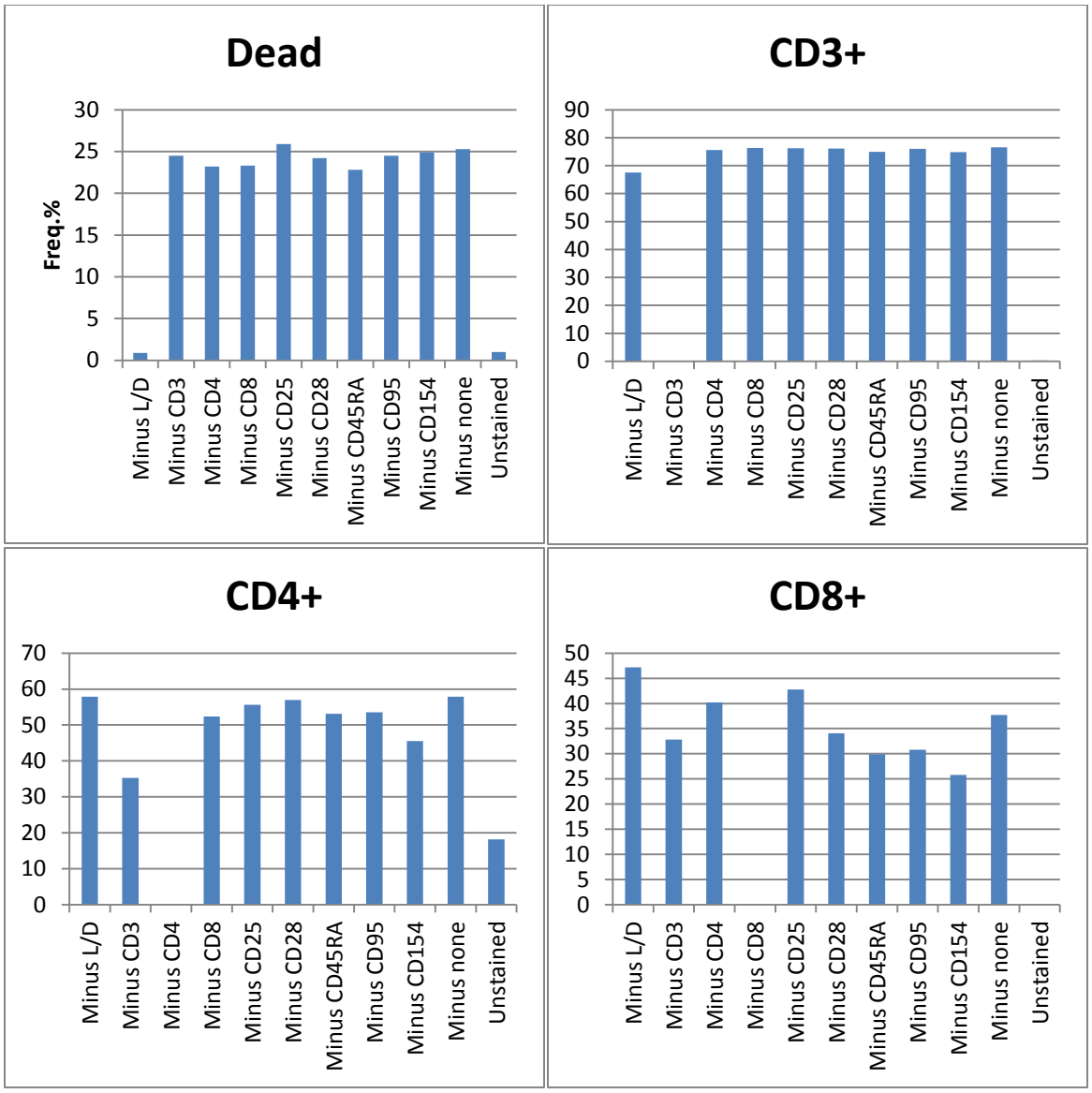
Representative T Cell Degranulation Marker and Cytokine Gating from T Cell Gating in Appendix E

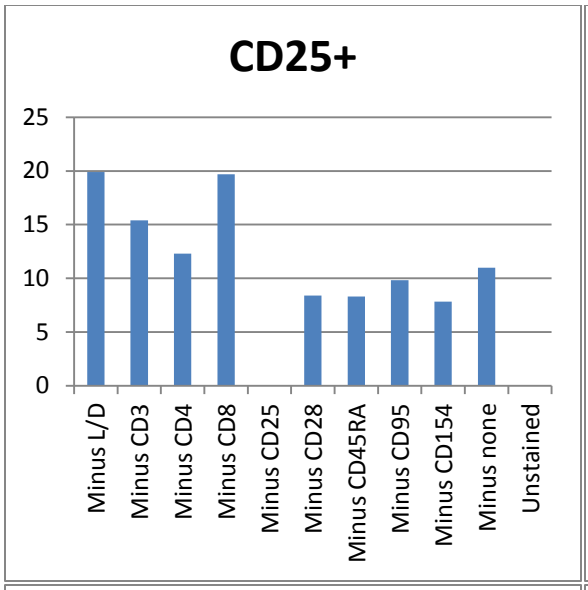


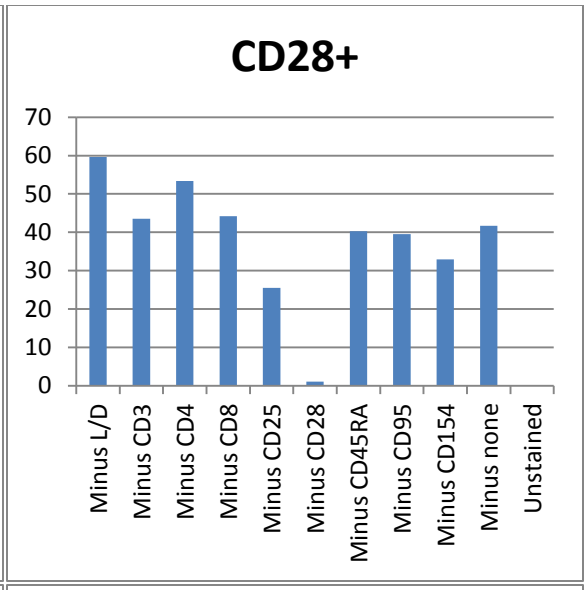
APPENDIX K

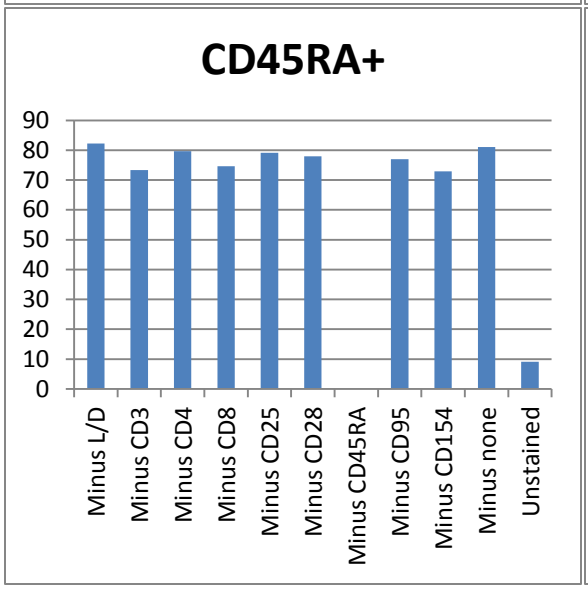
FMO Results for T Cell Surface & Activation Markers

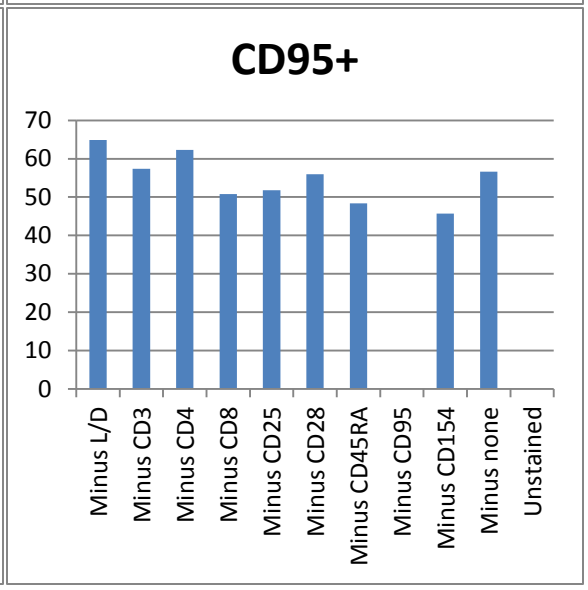
RM PBMC sample rh9033 was revived and stimulated with PMA & I as described in the methods section for T cell surface activation markers. Populations were gated on live lymphocytes, with the exception of the Dead population.

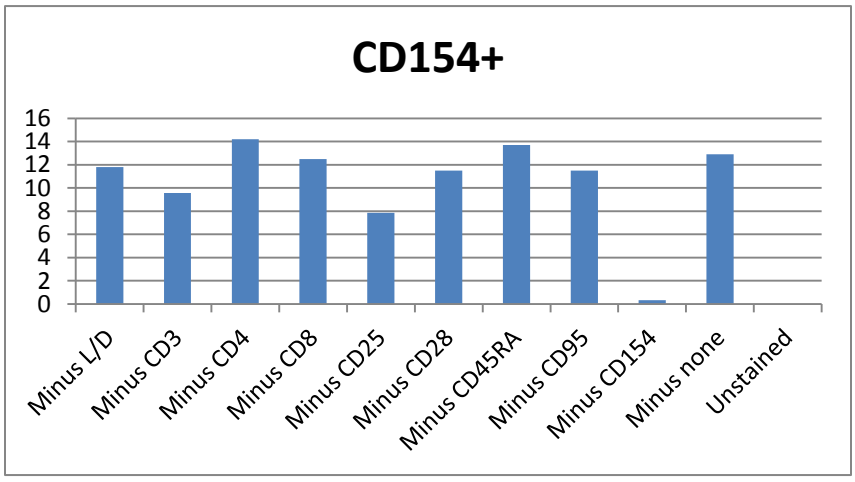

Dead
Freq.%
30
25
20
15
10
5
0
Minus L/D
Minus CD3
Minus CD4
Minus CD8
Minus CD25
Minus CD28
Minus CD45RA
Minus CD95
Minus CD154
Minus none
Unstained
CD3+
90
80
70
60
50
40
30
20
10
0
Minus L/D
Minus CD3
Minus CD4
Minus CD8
Minus CD25
Minus CD28
Minus CD45RA
Minus CD95
Minus CD154
Minus none
Unstained
CD4+
70
60
50
40
30
20
10
0
Minus L/D
Minus CD3
Minus CD4
Minus CD8
Minus CD25
Minus CD28
Minus CD45RA
Minus CD95
Minus CD154
Minus none
Unstained
CD8+
50
45
40
35
30
25
20
15
10
5
0
Minus L/D
Minus CD3
Minus CD4
Minus CD8
Minus CD25
Minus CD28
Minus CD45RA
Minus CD95
Minus CD154
Minus none
Unstained


CD25+
25
20
15
10
5
0
Minus L/D
Minus CD3
Minus CD4
Minus CD8
Minus CD25
Minus CD28
Minus CD45RA
Minus CD95
Minus CD154
Minus none
Unstained


CD28+
70
60
50
40
30
20
10
0
Minus L/D
Minus CD3
Minus CD4
Minus CD8
Minus CD25
Minus CD28
Minus CD45RA
Minus CD95
Minus CD154
Minus none
Unstained


CD45RA+
90
80
70
60
50
40
30
20
10
0
Minus L/D
Minus CD3
Minus CD4
Minus CD8
Minus CD25
Minus CD28
Minus CD45RA
Minus CD95
Minus CD154
Minus none
Unstained

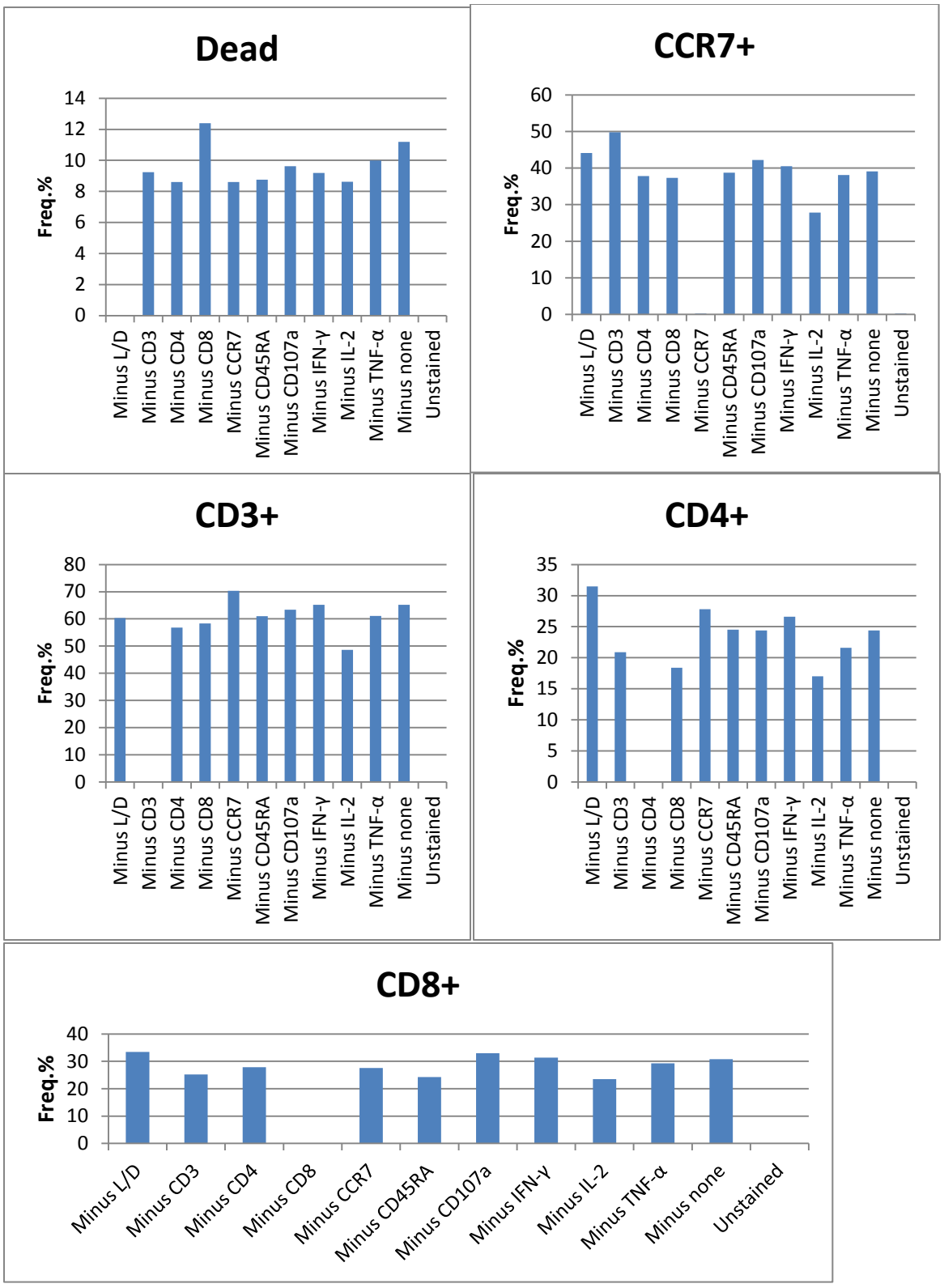

CD95+
70
60
50
40
30
20
10
0
Minus L/D
Minus CD3
Minus CD4
Minus CD8
Minus CD25
Minus CD28
Minus CD45RA
Minus CD95
Minus CD154
Minus none
Unstained

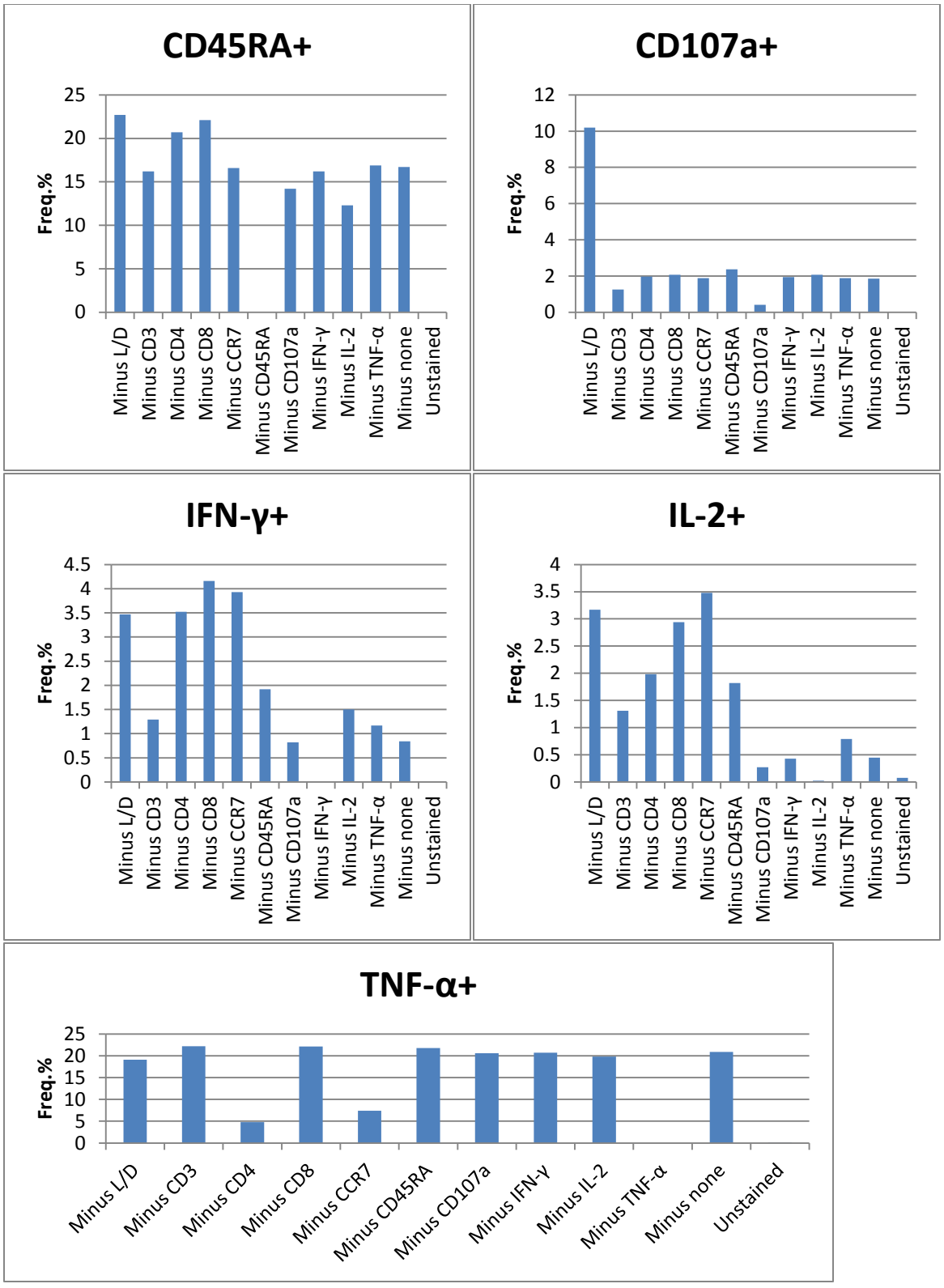

CD154+
16
14
12
10
8
6
4
2
0
Minus L/D
Minus CD3
Minus CD4
Minus CD8
Minus CD25
Minus CD28
Minus CD45RA
Minus CD95
Minus CD154
Minus none
Unstained

APPENDIX L

FMO Results for T Cell Cytokine Markers

RM PBMC sample rh8056 was revived and stimulated with PMA & I as described in the methods section for cytokine analysis. Populations were gated on live lymphocytes, with the exception of the Dead population.


Dead
Freq.%
0
2
4
6
8
10
12
14
Minus L/D
Minus CD3
Minus CD4
Minus CD8
Minus CCR7
Minus CD45RA
Minus CD107a
Minus IFN-γ
Minus IL-2
Minus TNF-α
Minus none
Unstained
CCR7+
Freq.%
0
10
20
30
40
50
60
Minus L/D
Minus CD3
Minus CD4
Minus CD8
Minus CCR7
Minus CD45RA
Minus CD107a
Minus IFN-γ
Minus IL-2
Minus TNF-α
Minus none
Unstained
CD3+
Freq.%
0
10
20
30
40
50
60
70
80
Minus L/D
Minus CD3
Minus CD4
Minus CD8
Minus CCR7
Minus CD45RA
Minus CD107a
Minus IFN-γ
Minus IL-2
Minus TNF-α
Minus none
Unstained
CD4+
Freq.%
0
5
10
15
20
25
30
35
Minus L/D
Minus CD3
Minus CD4
Minus CD8
Minus CCR7
Minus CD45RA
Minus CD107a
Minus IFN-γ
Minus IL-2
Minus TNF-α
Minus none
Unstained
CD8+
Freq.%
0
10
20
30
40
Minus L/D
Minus CD3
Minus CD4
Minus CD8
Minus CCR7
Minus CD45RA
Minus CD107a
Minus IFN-γ
Minus IL-2
Minus TNF-α
Minus none
Unstained


CD45RA+
Freq.%
25
20
15
10
5
0
Minus L/D
Minus CD3
Minus CD4
Minus CD8
Minus CCR7
Minus CD45RA
Minus CD107a
Minus IFN-γ
Minus IL-2
Minus TNF-α
Minus none
Unstained
CD107a+
Freq.%
12
10
8
6
4
2
0
Minus L/D
Minus CD3
Minus CD4
Minus CD8
Minus CCR7
Minus CD45RA
Minus CD107a
Minus IFN-γ
Minus IL-2
Minus TNF-α
Minus none
Unstained
IFN-γ+
Freq.%
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Minus L/D
Minus CD3
Minus CD4
Minus CD8
Minus CCR7
Minus CD45RA
Minus CD107a
Minus IFN-γ
Minus IL-2
Minus TNF-α
Minus none
Unstained
IL-2+
Freq.%
4
3.5
3
2.5
2
1.5
1
0.5
0
Minus L/D
Minus CD3
Minus CD4
Minus CD8
Minus CCR7
Minus CD45RA
Minus CD107a
Minus IFN-γ
Minus IL-2
Minus TNF-α
Minus none
Unstained
TNF-α+
Freq.%
25
20
15
10
5
0
Minus L/D
Minus CD3
Minus CD4
Minus CD8
Minus CCR7
Minus CD45RA
Minus CD107a
Minus IFN-γ
Minus IL-2
Minus TNF-α
Minus none
Unstained