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IMPAIRED T LYMPHOCYTE RESPONSES IN OLDER MACAQUES: POSSIBLE IMPLICATIONS FOR LENTIVIRAL DISEASE PROGRESSION

by

Sopitsuda Bunnag

A Thesis Submitted in

Partial Fulfillment of the

Requirements for the Degree of

Master of Science

in Biomedical Sciences

at

The University of Wisconsin-Milwaukee

May 2015

ABSTRACT

IMPAIRED T LYMPHOCYTE RESPONSES IN OLDER MACAQUES: POSSIBLE IMPLICATIONS FOR LENTIVIRAL DISEASE PROGRESSION

by

Sopitsuda Bunnag

The University of Wisconsin-Milwaukee, 2015
Under the Supervision of Professor Wail M. Hassan

Lentiviral infections of humans and rhesus macaques result in acquired immunodeficiency almost invariably. Yet the duration between the initial infection and the onset of generalized failure of the immune system varies between subjects, in both organisms. Furthermore, acquiring the infection at an older age tends to accelerate disease progression, but mechanisms underlying the latter phenomenon have not been elucidated. It is widely accepted that the events that take place during the very early stages of infection play a critical role in determining disease progression. During this brief period, a fierce competition between viral virulence mechanisms and host immune defenses takes place. I hypothesize that critical immune responses, such as those associated with better outcome in primate lentiviral infections, are lost in rhesus macaques at older age. If true, the loss of these critical immune responses at the early, fate-determining stages of infection would explain rapid progression among those who acquire the infection at older age. Immunological parameters that have been associated with better outcome in primate lentiviral infections include multifunctional T lymphocyte responses, robust proliferative capacity, and production of interleukin 2 (IL-2) (16). Investigating how these immunological parameters change as the animals advance in age

may help us predict the possible mechanisms underlying rapid progression in older macaques, and by extension people.

In this study, macaques of advanced age (21 – 29 years) were compared to young adult animals (3 – 7 years). I tested T cell qualities that have been linked to better outcome in primate lentiviral infections in the two age groups. Although most previous studies were based on studied carried out using infected animals, I tested uninfected animals. The rationale is that the observed divergence in infection outcome must have resulted from pre-infection, inherent differences between the two age groups. Post-infection studies can help identify protective responses in an immunologically protected group, while pre-infection studies provide an opportunity to define intrinsic differences in an unaltered immune system that might have resulted in the divergent outcome after infection. Since in primate lentiviral infections the younger population is not protected (since the role is susceptibility in humans and macaques), I do not expect to identify a truly protective immune profile by examining post-infection responses. In fact, some of the potentially important responses can be masked by infection-induced impairments of the immune system. For this reason, I decided to focus this study on pre-infection qualities of T cells.

Due to the large number of variables involved in the current study, I used principal components analysis (PCA) to identify the most discriminatory immunological parameters between the two age groups. PCA was used to enable the simultaneous evaluation of multiple parameters, which provides an advantage over univariate statistical analysis. Since PCA, as well as other multivariate methods, are scarcely used in immunology, which contributes to the novelty of this study. Here, I show that the

lentiviral-relevant immune responses, particularly on simian immunodeficiency virus (SIV) I have tested are generally more robust in younger animals compared to animals of advanced age. Younger animals produced more IL-2 in most of the T cell subsets upon both mass and antigen-specific stimulations. Moreover, higher frequencies of multiple cytokine producing cells were also observed in the young group, mainly in $CD4^+$ T cell subsets upon mass stimulation and after exposure to certain antigen-specific stimulants. The data shows an indication of impaired T cell responses in older rhesus macaques that are likely to impact disease progression in primate lentiviral infections. I also show that immunological parameters such as the production of multiple cytokine producing $CD4^+$ and $CD8^+$ T cell subsets were the most important phenotypes in segregating the two age groups, highlighting the potential importance of these immunological qualities for protective immunity.

This study should lay the grounds for the use of multivariate data analysis, particularly PCA, in immune profiling. This approach can potentially be applied to a wide variety of potentially critical areas in HIV or human immunodeficiency virus research, ranging from studying elite controllers to clinical trials, and from studying one arm or tissue of the immune system to studying multiple at once. Therefore, I hope this study will provide new insights to guide future research and ultimately contribute to our understanding of the correlates of immune protection in primate lentiviral infections, particular HIV infection.

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ACKNOWLEDGEMENTS

I am using this opportunity to express my sincere appreciation and thanks to my advisor, Dr. Wail M. Hassan who has supported me throughout the program. I am thankful for his aspiring guidance, constructive criticism and useful advices during my research. I would also like to express my sincerely gratitude to my committee members, Dr. Dean Nardelli and Dr. Anthony Azenabor for sharing their tremendous support throughout my research. I would especially like to thank the staff at the Fluorescence Activated Cell Sorting Core facility at UW-Milwaukee for providing me with access to the flow cytometry machine. For financial support, I thank the College of Health Sciences for funding my research, and the Department of Health Sciences for providing me with UW-Milwaukee Chancellor's Fellowship awards. I also like to express my deepest appreciation to all the members in my lab, Nader Mahdi, Sahar Alhakeem and Cathy Stalvig for their help and support during this project. Last but not least, I would like to thank my family, my parents Suradej and Soodruethai Bunnag for encouraging and inspiring me to follow my dreams. I am especially grateful to my parents, who supported me emotionally and financially. This journey would not have been possible without you two.

Introduction

Lentivirinae is a subfamily of retroviruses (family: *Retroviridae*) that includes the human immunodeficiency viruses (HIV-1 and HIV-2), as well as viruses that infect a variety of nonhuman primate. HIV-1 has been the cause of one of the worst pandemic known to mankind. Infection with lentiviruses typically results in a chronic, progressive disease due, in part, to virus ability to evade host immune mechanisms. HIV has the ability to undergo rapid mutations and the ability to target immune cells, mainly $CD4^+$ T cells causing a decline in $CD4^+$ cell counts and leaving the immune system in a destructed state (5). This will eventually lead to the terminal stage of the infection, which is known as acquired immunodeficiency syndrome (AIDS). HIV has been responsible for 25 million deaths worldwide over the past three decades and more than 35 million people are currently living with the infection (5). Out of all people living with HIV, 97% reside in low- and middle-income countries, mainly in Sub-Saharan Africa (5). In the year 2008 alone, 2 million people died due to HIV/AIDS, and an additional 2.7 million were newly infected (5).

Even though a cure for HIV has not been defined, the severity of the disease can be overcome through the use of different therapies such as antiretroviral therapy (ART), including highly active antiretroviral therapy (HAART) regimens. These therapies are capable of reducing viral loads, extending life expectancy, and improving quality of life for HIV-infected persons. Yet, none of therapies currently available is curative. Therefore, the only intervention that can feasibly control the pandemic is the invention of a protective vaccine and this invention still remains as the ultimate goal of HIV/AIDS research.

Until today, a clear understanding of correlates of immune protection (COIP) needed for rational vaccine design is still not well defined. COIP is measurable immunological parameters that correlate with protection from the disease. The lethal course of HIV infection made experimental infections in humans unethical and unpractical, which puts a limit on the search for protective immunological parameters associated with this infection. Evidence has been gathered from past studies of humans with atypical disease progression. However, the identification of specific mechanisms underlying the acceleration of the disease progression is not well defined and this identification will likely provide new insights into the disease immunopathogenesis and immune protection. In HIV infection, protection is limited to a rare population of long-term nonprogressors (LTNP) and elite controllers (Ec) who are able to control plasma viral loads to low and undetectable levels, respectively. There are many factors that may contribute to the rate of disease progression, including the patient's age at initial infection, plasma viral loads at peak viremia during acute infection, viral set point, viral fitness, and the extent of immune activation. Both qualitative and quantitative impairments in immune function are caused by HIV infection. These impairments ultimately lead to an elevated risk of opportunistic infections at the terminal stage of the disease, or AIDS (5).

Multiple disease progression patterns have been reported in rare populations of both humans and nonhuman primates, but due to the ethical issues of human trials, study of lentiviral infections in nonhuman primates is commonly practiced. Rhesus macaque (RM) or *Macaca mulatta* model has been researched extensively to understand simian immunodeficiency virus (SIV) and eventually HIV. The course of SIV infection in RM is

very similar to that of humans, which involves a sharp decline in SIV viral load during the acute infection while $CD8^+$ T lymphocyte-mediated cytotoxic response to virus-infected cells (6). Then a chronic phase follows, which is defined by the deletion of $CD4^+$ T lymphocytes and eventually leading to the progression of AIDS-like illness or simian AIDS (SAIDS).

In this study, I investigated the lentiviral-relevant aspects of the immune response that are associated with better outcomes of HIV/SIV in uninfected young adult and aged rhesus monkeys. Immunological parameters that are relevant to the disease protection have been widely researched. These parameters include, but may not be limited to the frequencies of multifunctional T cells, multifunctional T cell responses and high-level production of Interlukin-2 (IL-2) by T cells. I based my study on past findings with evidences that acquiring HIV infection in humans at 40 years of age or older increases the risk of rapid progression compared to those that acquired the infection at earlier adult age (7). I reasoned that the higher risk for rapid progression must have resulted from underlying pre-existing weaknesses associated with immunosenescence or the aging of the immune system. Therefore, the central hypothesis of this proposal is that the immunological parameters of T cells that are associated with better outcomes in primate lentiviral infections (i.e. HIV and SIV) are more pronounced in younger animals than they are in older animals since critical immune responses in primate lentiviral infections are expected to be lost in rhesus macaques at older age.

Many studies have published data comparing immune responses that are associated with better outcomes of this disease in different groups of infected subjects. However, to the best of my knowledge the aspects of multiple immunological responses

of stimulated T-cells from uninfected rhesus macaques have not been directly compared in aged and young adult populations. This study of the unadulterated, pre-infection immune system will provide an opportunity to define intrinsic differences, which may offer some explanations for the divergent outcome after infection. Exploring lentiviral-relevant immunological differences that distinguish between subjects with different progression patterns is likely to provide new insights into disease pathogenesis and provide more information to define COIP.

There are many parameters that can be used to evaluate relevant immune responses such as the multifunctionality, proliferative capacity, epitope recognition and more. I decided to peruse this research with the focus on the three cytokines IL-2, interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) produced by T cells in terms of frequency, absolute numbers, and mean fluorescence intensity (MFI). The application of multivariate data analysis will enable the identification of immunological parameters that best discriminate between the young and old age groups. Since many variables are being tested in this study, the use of Principal Component Analysis (PCA) as the multivariate analysis is necessary. This method involves data reduction, which is used to condense multiple variables into fewer principal components (PC). PCs are ranked according to the amount of variance they explain. Although in PCA, the number of PCs that can be extracted from a dataset is equal to the number of measured variables, only the PCs that explain most of the data variance are retained. This enables the visualization of data points in terms of two or three PCs, instead of a large number of variables. In other words, given a high dimensional (more than these parameters) data set, PCA enables plotting the data in two- or three-dimensional space based on the top

PCs, without losing much information. A powerful characteristic of PCA is that it does not take into account predefined groups, thus, all data-point groups visualized on PC plots are formed naturally (i.e. solely based on the data without any optimization). Finally, once groups are identified on the plots, the contribution of the original variables to each PC can be found, which can be used to identify the most discriminatory variables.

In the remainder of the background section, I will summarize immune alterations linked to immunosenescence in humans and nonhuman primates and aspects of the immune response that have been linked to better outcome in lentiviral infections.

Immune dysfunction and aging

The negative impacts that aging has on the performance of the immune system is believed to be influenced by chronic exposure and interactions with foreign antigens, environmental changes and stress (17). As the function of the immune system declines, the susceptibility to viral and bacterial infections will intensify (18). Multiple lines of evidence from research done in the 1960s suggested that changes in the T-cell compartment are the main contributors to the age-dependent decline in immune function. T-cell functions are found to be altered due to aging include T-cell receptor (TCR) signaling, response to vaccines, cell proliferation, and cytokine production. Deterioration of T-cell function may result in reduced activation and cytokine secretion, specifically the secretion of IL-2 from memory T-cells (18). Decreased IL-2 secretion contributes to the reduction of T-B cell interactions, which may lead to an impaired humoral immune response (1).

Age-related thymic involution, which might be the result of hormonal changes, also contributes to a decline in the production of functional T cells. Studies have shown

that the growth of the thymus is terminated after puberty and by that period the thymus will weigh about 25-30 grams (19). In a healthy person, the rate of thymus tissue loss is approximately 1% per year (19).

Multiple reports provided evidence in support of the clear role of diminished $CD4^+$ and $CD8^+$ T cell numbers in age-dependent immune dysfunction (20). The observation that the relative abundance of naïve and memory T cells is preserved until age 65 years led to the hypothesis that naïve T-cells in adults are generated by proliferation of existing cells, and not from maturation in the thymus (20, 21). It has also been shown that the diversity of naïve and memory T-cells remained constant up to age 65 and will start to decline between 65 and 70 years of age (20). Non-human primates such as Rhesus macaques also experience a greater morbidity and mortality from infectious diseases due to the advancement of age (23). T cell subsets in RM resemble those in humans, which includes naïve (N), effector cells (EC), central memory (CM) and effector memory (EM). These subsets can be distinguished according to the expression of surface markers similar to humans. As aging progresses, RM experience the loss of naïve T cells due to a combination of different age related changes in T cell activities such as the decrease in hematopoietic stem cell in bone marrow, decrease in T cell migration to the thymus and the accelerated conversion of naïve T cells to memory T cells (23). The decrease in the ratio of $CD4^+$ and $CD8^+$ is another hallmark of immunosenescence in RM. This is due to the negative correlation between age and $CD8^+$ T cells and the positive correlation between $CD4^+$ T cells and aging (23). Aged populations in both humans and monkeys are, therefore, more prone to infections and tend to take longer to recover from them.

Aspects of T lymphocyte immune responses with relevance to HIV disease outcome

Innate immunity is very crucial in the initiation of adaptive immune responses, which can be further divided into cell-mediated and humoral immune responses involving T-lymphocytes and B-lymphocytes, respectively. T-lymphocytes are classified into smaller subpopulations of CD4 helper T cells and CD8 cytotoxic T cells differentiated by molecular markers and functionalities. T-cell development takes place exclusively in the thymus. As CD4 and CD8 T cells mature they leave the thymus and enter secondary lymphoid organs where they recognize their cognate antigen in the context of major histocompatibility complex (MHC) class II and class I molecules on antigen-presenting cells. Following antigen exposure, $CD4^+$ and $CD8^+$ T cells undergo differentiation thorough various stages. Starting from naïve cells, they proliferate and differentiate to form memory cells under the influence of certain cytokines (12). Protective memory is mediated by effector memory T cells that migrate to inflamed peripheral tissues and display immediate effector function, whereas reactive memory is mediated by central memory T cells which have little or no effector function, but can proliferate and differentiate to effector cells in response to antigenic stimulation. As naive T cells differentiate into memory and effector T cells they acquire the ability to proliferate in response to homeostatic signals. Central memory T cells produce mainly IL-2, but after further antigenic stimulations, they will produce large amounts of IFN-γ. Effecter memory cells however, display characteristic sets of adhesion molecules that are required for homing to inflamed tissues and are characterized by rapid effector function through the production of IFN-γ (13*)*. Memory T cells may survive for a long time in lymphoid organs and peripheral tissues. They are easily activated and can perform

immediate effector functions or undergo activation and clonal expansion in lymphoid organs to mount a secondary immune response if interacted with the same antigen in the future (13). There are many factors that can affect the magnitude T cell activation such as concentration of an antigenic peptide, affinity of TCR toward the antigen, and inflammatory stimuli. Unlike memory T cells, most effector T cells will disappear after the antigenic agent is eliminated. Effector T cells differentiate into two major subtypes of cells known as T_h1 and T_h2 cells, which have the ability to generate different types of cytokines. However the main cytokines produced by effector T cells are IFN-γ, IL-2, and TNF-α.

Most of the cytokine secretions by T-cells are correlated with the rate of viral replication in terms in HIV/SIV infection (24) and research evidences show that the role of $CD8^+$ T-cells is crucial in controlling HIV/SIV replication, including the frequency of replication, epitope recognition and functional quality (25). Studies showed that HIV infected LTNP possess HIV-specific $CD8^+$ T-cell responses with enhanced functionality through the measurement of five specific $CD8^+$ T-cell responses (degranulation, IFN-γ, M1Pb, TNF-α and IL-2 secretions) (25). These HIV specific $CD8^+$ T-cells are equipped with unique functional patterns with different profiles between LTNP and rapid progressors. HIV specific $CD8^+$ T-cells in LTNP have the capacity to produce TNF-α and IL-2 and at the same time maintaining other functions which give the cells characteristics of multifunctionality. However the expression of HIV specific $CD8^+$ T-cells responses in rapid progressors are limited to IFN-γ with low production of TNF-α and even less IL-2 (25). Moreover, LTNP are also known to have the ability to maintain a healthy level of multifunctional HIV specific $CD4^+$ T cells when compared to rapid progressors (25). The

low maintenance of $CD4^+$ T-cells along with HIV specific $CD8^+$ T-cells in the progressors helps explain why little improvement is observed during an initial treatment due to the limited HIV specific $CD4^+$ T-cell pool. During the early course of infection, HIV specific $CD4^+$ T-cell responses are impaired and this is a hallmark of the progressive course of infection (26). However, in Ec subjects, the proliferative capacity of HIV specific $CD4^+$ T-cells is well maintained (27). They are able to produce more IL-2 in cohort with other functions giving them polyfunctional quality. The progression of AIDS can be measured by assessing the "Quality of $CD4^+$ T-cell responses" during infection (16). This quality is defined by the ability of a T cell to produce multiple functional molecules, such as cytokines and enzymes, which led to describing these cells as multifunctional T cells (16). One of the most important aspects of characterizing the potential of T cells is the observation of the magnitude of T cell responses. These responses can be represented by the frequency of specific effector functions such as the secretion of cytokines through the antigen specific stimulation. T cells as a whole, display high heterogeneity and are capable of eliciting a wide range of functions including, the ability to proliferate, organize immune responses by chemo-attractant secretions, eradicate infected cells via cytokine production and many more. Because of the characteristic of the heterogeneity, certain subset of T cells can be protective against certain diseases. This can be presume that the most effective protective immune response from T cells against any infections comes from a certain subset that are expressing a unique combination of functions. The combination of these functions can be defined as "The quality of T cells". Series of recent studies have shown that better quality of T cells

is critical in mounting a protective immune response and has been shown to correlate with disease non-progression.

Cytokine secretions are commonly measure upon stimulation of T cells in order to predict T cell quality. The secretion of IFN-γ has been extensively used as a parameter to assess cellular immune response against infections through its role in clearance of many infectious agents. IFN-γ is known as a "canonical" cytokine of Th1 response and it is responsible for the clearance of bacterial, viral and fungal infections (15). However, some studies have shown that the assessment of IFN-γ secretion alone is not enough to define the correlate of immune protection. This is when the measurement of TNF-α is added to the study for a more accurate analysis of an enhanced protective immune response (16). TNF-α is able to aide in the killing of intracellular bacteria, viruses and parasites. The last cytokine to be included in the study for the analysis of T cell quality is IL-2, which plays a main role in promoting the expansion of $CD4^+$ and $CD8^+$ T-cells, rendering the amplification of the effector T cells (15). IL-2 also has the capability to enhance $CD8^+$ T cells memory capacity and effector functions (16). These three cytokines are commonly used to identify the immune responses elicited by T cells against infections that challenge the T cells such as HIV/SIV. Ferre et al showed that HIV-Gag specific $CD4^+$ T cell responses (which can be measured by the production of IFN-γ, TNF-α and IL-2) were well maintained in subjects with on going antiretroviral therapy (ART) (27). HIV $CD4^+$ specific epitopes are also believed to play important roles in the progression of HIV. Studies from Vingerts et al demonstrated that HIV specific $CD4^+$ T-cells in Ec have higher functional avidity upon the observation of the strong responses of HIV-Gag p24 peptide with multiple HLA class II alleles. Studies concluded with subjects with

abundant HLA class II (B27 and B57) alleles are capable of producing robust HIV specific $CD4^+$ T-cell responses as well as maintaining polyfunctional $CD4^+$ T-cells (27). However, further studies must be performed to determine a clear linkage between HLA alleles and HIV specific $CD4^+$ T-cells as well as HIV specific $CD8^+$ T-cells.

In order to have a clear understanding of the immune responses and the quality of T cells, each subset of $CD4^+$ and $CD8^+$ were examined in this study, which include, naïve cells, effector cells, central memory cells and effector memory cells. Both $CD4^+$ and $CD8^+$ T cells have the ability to differentiate and proliferate into memory cells and effector cells upon contact with specific antigen. Two main populations of memory T cells exist; central memory (CM) and effector memory cells (EM). CM cells screen for the presence of specific antigens, are concentrated in lymphoid tissues, and secrete large amount of IL-2. EM cells act as the first line of defense in the peripheral tissues and mainly produce effector cytokines such as IFN-γ and TNF-α (15). In HIV infection, a rapid loss of $CD4^+$ T cells is one of the hallmarks of the infection, which is frequently accompanied by chronic immune activation. The latter has been shown to associate with faster disease progression (33).

The subsets of T-cells can be classified based on the molecules presented on the surface (surface markers). These markers are often used to associate cells with certain immune functions. Examples of the a few markers that are essential markers for the study of T cell compartment include CD3, CD4 , CD8, CD28, CD40, CD45RA, CCR4, CCR7, CXCR3, CXCR4 and many more. However, this study will base the classification of T-cell subsets on the constructed panel for the staining of surface markers, which includes, CD3, CD4, CD45RA, and CCR7. CD3 is a common marker for distinguishing T cells

from other cells and it is a part of T cell receptor complex. CD4 is a T helper cell lineage marker, which is the initiator of an early phase of T cell activation. CD45RA is expressed on naïve T cells, as well as the effector cells in both $CD4^+$ and $CD8^+$ T cells (14). However, with an increase in the antigen experience, the expression of CD45RA will be lost. Central and effector memory T cells will gain the expression of CD45RO instead (15). The expression of CCR7 aide in the differentiation between T cells with effector function that can migrate to inflamed tissues and T cells that requires secondary stimulus prior to the acquisition of the effector functions. A clear understanding of immunologic memory is very critical for the study of vaccine development, infectious disease, and immune reconstitution (14).

Hypothesis and specific aims

Since age was shown to influence disease progression in HIV infection, there is a possibility that there might be underlying age-dependent immunological alterations to explain this phenomenon of accelerated disease progression in the older population. However, clear descriptions of age-dependent alterations and mechanisms underlying these immunological parameters in rhesus macaques have not been reported.

I hypothesize that critical immune responses or the immunological phenotypes, such as those associated with better outcome in primate lentiviral infections, are lost in rhesus macaques at older age. Those critical immune responses associated with better outcome in primate lentiviral infections include multifunctional T cells, high IL-2 secretion, and high proliferative capacity (16). It is well established that the early events that take place early during acute infection play a crucial role in determining the course of lentiviral infections in primates. This work would provide initial evidence to explain

age-dependent differences in progression patterns and define the immunological parameters that distinguish between the two age groups. These results would also be beneficial in planning of future experiments using the most popular primate model in HIV/AIDS research (the rhesus macaque). More over, this will provide researchers with information to help them choose proper age groups to include in their experiments as well as contributing to the understanding of the correlates of immune protection in primate lentiviral infections.

Specific aim 1. Identification of potential differences in T-cell cytokine production patterns in aged and young rhesus macaques after mass stimulation:

a. Comparing the patterns of IL-2, IFN-γ, and TNF-α secretion, including the frequencies of single cytokine-producing cells and multifunctional cells, after staphylococcal enterotoxin B (SEB) stimulation and phorbol myristate acetate and ionomycin (PMA/I) stimulations. SEB stimulates a large number of cells via the T-cell receptor, while in the case of PMA/I, masses of T cells are stimulated by activating protein kinase C and inducing calcium release, while bypassing the T-cell receptor altogether. This will allow the examination of large numbers of cells and will address the potential of T cells regardless of their T cell receptor specificity.
b. Comparing the expression level of the different cytokines in the two age groups after mass stimulation. Mean fluorescence intensity (MFI) will be used as a measure of expression by individual cells, and will be compared between the two age groups for all three cytokines.

c. Multivariate data analysis will be used to identify the most important parameters in discriminating between the two age groups. I will use principal component analysis (PCA) for this purpose. PCA is used to reduce the number of variables into fewer principal components (PC) and identifies the PCs and variables that contribute the most to the variance in a data set.

Specific aim 2. Identification of potential differences in T-cell cytokine production patterns in aged and young rhesus macaques after antigen-specific stimulation:

a. Comparing cytokine production patterns as mentioned above, but after stimulation with SIV envelop and Gag peptides and Cytomegalovirus (CMV) peptides. Cell frequencies, absolute numbers and MFI will be studied.
b. Multivariate data analysis will be used to identify the most discriminating parameters as discussed under the previous specific aim.

Specific aim 3. Comparing $CD4^+$ and $CD8^+$ T cell proliferative capacity in aged and young rhesus macaques.

a. Comparing the proliferative capacity of T cells after Anti-CD3, SIVgag and CMV peptides stimulation, using Ki-67 antibody staining. Ki-67 stains actively proliferating cells and, thus, the frequency of Ki-67 positive cells is commonly used to evaluate the proliferative capacity of immune cells.

Materials and methods

Flow cytometry

Reagents

PMA and I were purchased from Sigma-Aldrich, St. Louise, MO; staphylococcal enterotoxin B (SEB) from Toxin Technology, Inc., Sarasota, FL; purified, unconjugated anti-CD3, anti-CD3 (PerCp.Cy5.5), anti-CD4 (PE.CF594), anti-CD8 (PE.Cy5), anti-CD14 (PE), anti-CD20 (BV605), anti-CCR7 (PE), anti-CD95 (PE), anti-IL-2 (FITC), anti-IFN-γ (APC), anti-TNF-α (PE.Cy7), anti-IL-2 (FITC), anti-TNF-α (PE.Cy7), anti-IFN-γ (APC), and anti-Ki-67 (PE.Cy7) from BD Biosciences, Franklin Lakes, NJ; anti-CD28 and anti-CD49d from Biolegend, San Diego, CA; and Violet LIVE/DEAD fixable dead cell staining kit from Lifetechnologies, Carlsbad, CA. SIV_{mac239} Env peptide set (6883), SIV_{mac239} Gag peptide set (6204), and HCMV pp65 peptide set (12014) were obtained through the NIH AIDS Reagent program, division of AIDS, NIAID, NIH.

Whole blood samples were collected from rhesus macaques housed at the Wisconsin National Primate Research Center (Madison, WI). Animal care and sample collection were done in accordance with the University of Wisconsin Institutional Animal Care and Use Committee guidelines and the NIH "Guide to the Care and Use of Laboratory Animals." Blood mononuclear cells (PBMC) were purified using gradient centrifugation and were stored in liquid nitrogen until used. Prior to experiments, frozen PBMCwere thawed in a 37°C water bath and washed with RPMI 1640 to remove freezing medium. Additionally, cells were revived by incubation in growth medium (RPMI 1640 containing 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin) at 37°C in 5% carbon dioxide atmosphere for 18 hours.

In vitro stimulation

Incubated revived cells were harvested and centrifuge at 350 xg for 5 minutes. After discarding the supernatant, cells were treated with phorbol myristate acetate and Ionomycin (PMA/I) or cell stimulation with the final concentration of 50 ng/mL and 500 ng/mL respectively. SEB were use to stimulate the cells with the total of 10ug of SEB. A total of 2.5 ug of SIV antigen and peptide were used to stimulate PBMC. Env peptides matching SIV_{mac239} sequence were used to stimulate T lymphocytes through their antigen receptors and super antigen, SEB, was used to stimulate large number of cells. The stimulated cells were be incubated for 12-15 hours. A total of 2.5% of Brefeldin A (GolgiPlug™) was added 1 hour after stimulation. Brefeldin A is a fungal metabolite, which can interfere with protein secretion and inhibit protein transport from the endoplasmic reticulum to the Golgi apparatus upon incubation. It has the ability of enhancing intracellular cytokine staining signals by blocking the transport processes during cell activation. However, cells were incubated up to 12 days with Anti-CD3, SIVgag and CMV peptides for the detection of Ki-67 expression and the medium and stimulants were replenished on day 7 of the incubation. Cells were washed with Wash/Stain after incubation via centrifugation at 350 xg for 5 minutes and removing the supernatant and are then ready to be tested for surface markers of interest.

Staining for flow cytometry

Cells were stained with appropriately titrated fluorescently labeled monoclonal antibodies. Violet LIVE/DEAD staining antibodies were diluted in 1:1000 dilutions with PBS. Cells were first stained with 0.1 uL of violet LIVE/DEAD fixable dead cell staining kit (Lifetechnologies) followed by antibody staining for surface markers and intracellular

staining markers. Staining of cells took place in a dark for 20 minutes at room temperature. Cells were washed with 1 mL of Wash/Stain and centrifuged at 350 xg for 5 minutes. Prior to intracellular staining, cells were fixed and washed twice. Cells were then resuspended in 1 mL of PBS and the data were analyzed by flow cytometry using the appropriate excitation and detection channel

Data collection

Flow cytometry data were collected using a BD FACSAria III cytometer. Flow cytometry data were analyzed using FlowJo (Treestar, Inc., Ashland, OR). Multivariate data analysis was done using Bionumerics (Applied Maths, Austin, TX). To facilitate studying a data set characterized by a large number of variables, PCA reduces the number of variables by condensing them into fewer PCs. PCs are ranked according to their contribution to the variance in the data set. The PC with the largest contribution to data variance is the most discriminative PC. PCs can be ranked according to their contribution to variance and then plotted into two- or three-dimensional graphs. The contribution of each variable to each PC can then be examined to identify the most discriminative variables. I will use this method to identify the immunological parameters that best discriminate between young and aged RM monkeys.

Methods of flow cytometry

Controls and panel development

Compensation with ArC™ cmine reactive beads

In multi-color flow cytometry, more than 6 (often 8 or more) colors are used to label different proteins in the cell. Each one of the color is read by a dedicated channel and a detector/photomultiplier tube (PMT) assembly. With many colors used, some of the

channels may overlap with neighboring channels and, therefore, inaccurately contribute to the readings taken at these channels. For this reason, compensation controls are included in every run. Compensation controls are samples (cells or beads) stained with single color, each of which will read at all channels used in the run. This will enable the investigator to generate a record of the erroneous contribution of each color to all channels in the panel other than its own. These erroneous signals are then subtracted electronically from the readings obtained using all samples. The ArC™ Amine Reactive Compensation Bead Kit (Lifetechnologies) contains the ArC™ reactive beads and the ArC™ negative beads. The incubation with any reactive dye will provide distinct positive and negative populations of beads and this information can be used to setup the compensation. A drop of ArC™ reactive beads is added into appropriately labeled tubes. Prepared fluorescent monoclonal antibodies were added to the corresponding amount of antibodies (same as those used to treat cells) into the ArC™ reactive beads. Beads were incubated for 30 minutes at room temperature away from light. After incubation beads were centrifuged for 5 minutes at 300 xg with 1mL of PBS or buffer. Supernatant were carefully removed and bead pellets were suspended with 0.5 mL of staining buffer. One drop of corresponding ArC™ negative beads was added to each labeled tube. The tubes were well vortexed prior to the analysis by flow cytometey.

Fluorescence minus one control

In this study, multi-parametric flow cytometry was used. This technology involves the simultaneous use of multiple fluorophores, some of which may have partially overlapping emission spectra. This inherent difficulty in the technology is routinely overcome by performing compensation controls. Compensation controls are

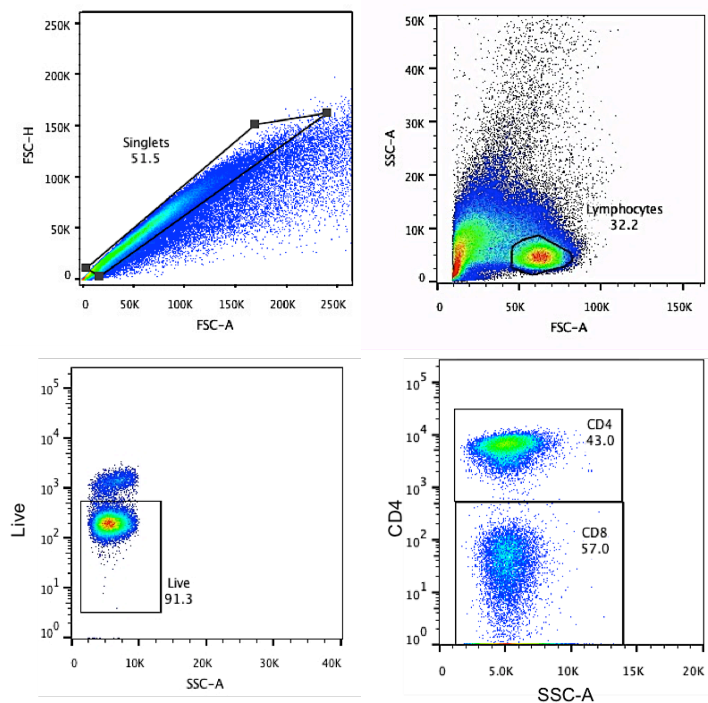
composed of samples each stained with one antibody, in addition to an unstained control. These samples are used to calibrate the cytometer by defining the background fluorescence in each of the channels used in a given experiment (using the unstained control) and the bleed through from each channel into all others, if any. This makes it possible to electronically subtract the bleed through from the reading taken later for the actual samples. Fluorescence Minus One control (FMO) is a type of control in which samples are stained with panels identical to the experimental panel, except that each is missing a single conjugated antibody. This enables the operator to monitor the amount of bleed through into the missing color, as well as the effect of removing each of the color one at a time on all other colors. After electronic compensation, both types of effects should be negligible for all colors in the panel.

Antibody titration

The titration of antibodies is one of the most critical steps to ensure that the data acquired are accurate with a precise concentration of antibody. The overuse of antibody may cause an increase in the background, which will reduce the ability to distinguish the positive population. However, the low amount of antibody will also decrease the positive signals. A precise amount of antibody will yield the optimal separation between positive and negative populations as well as allowing minimum increase in background fluorescence from the flow cytometer as well as limiting a significant degree of nonspecific binding. The issue with nonspecific antibody binding can lead to an inaccuracy in the data. Nonspecific antibody binding is expected to occur when the total number of antibody molecules greatly exceeds the number of target antigens and this may occur in both positive and negative population of the cells.

Gating strategies

The application of optimal gating was used to achieve accurate results of cell assessment. Many factors needed to be considered and incorporated into the gating strategy to yield accurate results and these factors include, exclusion of debris, exclusion of dead cell, utilization of negative controls, and utilization of appropriate concentration of staining markers. Forward scatter (FSC) and side scatter (SSC) were used to eliminate any debris and non lymphocytes. LIVE/DEAD® Fixable Dead Cell Stain kit was used to distinguish between viable and non-viable cells. Live/Dead fluorescence and side scatter (SSC) were used to eliminate the non-viable cells. Once the non-viable cells and debris have been eliminated, further specific analysis can be carried out by selecting specific fluorescence markers that were tagged in the cells.



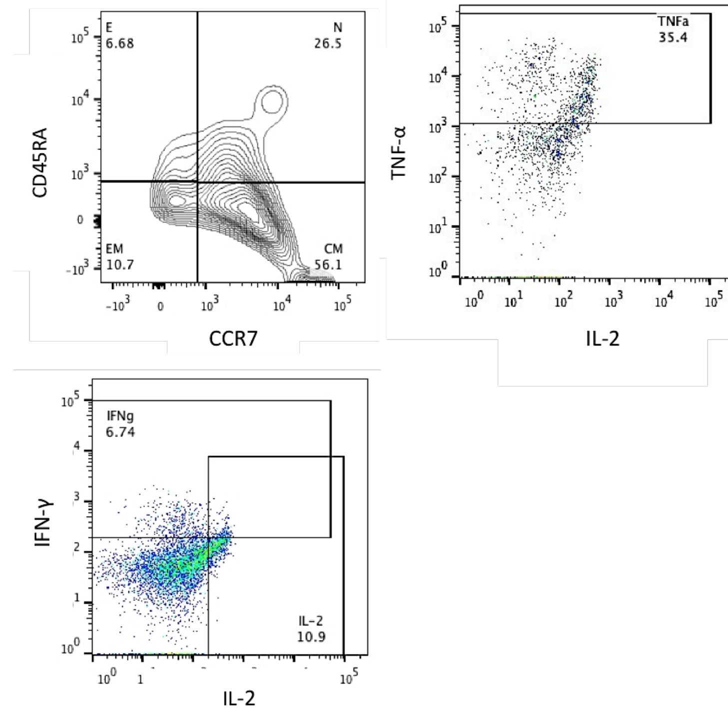


Figure 1 Gating strategy for the analysis by FlowJo: The steps of gating identifies unique functional subsets of CD4⁺ and CD8⁺ T cells as well as the cytokine secretions based on the fluorescence staining of live cells and on the expression of other markers, such as CD3, CD4⁺ , CD8⁺ , CD4⁺ 5RA, CCR7, IFN-γ , IL-2, and TNFα

Results

1. Younger rhesus macaques showed greater T cell responses after mass stimulation with PMA/I or SEB

1a. Percentage of cytokine production from CD4⁺ and CD8⁺ T cell subsets:

The activation or the stimulation of cells plays a critical role in the pathogenesis of many diseases. Studies in the past had demonstrated a growing consensus that a generalized T-cell activation plays a central role in the pathogenesis of HIV/AIDS involving the rate of disease progression. Moreover, acquiring HIV infection in humans at 40 years of age or older increases the risk of rapid progression compared to acquiring the infection at earlier adult age (7). To investigate and compare the potential of T cells

between two age groups (young and old) regardless of their specificity and explore the parameters that are known to be related to better outcomes in HIV/SIV infection, I stimulated PMBC from 10 young and 10 old RM with PMA/I or SEB. The analysis of the pattern of cytokine production upon PMA/I will provide information about the overall potential of the cells in un-altered immune system of pre-infected RM regardless of the T cell receptor specificity because PMA/I have the ability to stimulate T cells by bypassing the T-cell receptor altogether. But SEB has the ability to stimulated mass of T cells through the interaction with T cell receptors. So the immune responses seen upon SEB stimulation will move towards a more realistic process of the immune activation by going through the interaction with T-cell receptors. However SEB stimulated immune response is still considered to be non-specific.

I assessed the percentages and absolute numbers of the cytokine producing cells, as well as the mean fluorescence intensity (MFI) of each cytokine. Boolean gating analysis was used to analyze the multifunctional cells by observing the cytokine production in various combinations. The subsets of both $CD4^+$ and $CD8^+$ including naïve cells, effector cells, central memory cells and effector memory cells, were classified by the surface markers of CCR7 and CD45RA as mentioned in the method section. The results were evaluated by measuring the frequency of the cytokine producing cells after the subtraction of background fluorescence. Control samples, which were the un-stimulated cells, provided values for background fluorescence.

As shown in Figure 1A a significant difference was observed in the percentage of multifunctional effector $CD4^+$ T cell producing IL-2 and TNF-α when compared between the young and the old with a higher percentage of multifunctional $CD4^+$ effector T cells

in the younger population. However no significant differences were found between the two age groups in other cytokine combinations after PMA/I stimulation. Figure 1B is shown for the purpose of comparison between the two stimuli of the same subset of $CD4^+$ under the same condition but with SEB stimulation, however no significant differences were found in the multifunctional $CD4^+$ effector cells upon SEB stimulation (Figure 1B).

In Figure 2A, significant differences (*P-value < 0.05) between two age groups were observed in each of the percentage of single cytokine producing central memory $CD4^+$ T-cells (IFN-γ , IL-2, and TNF-α) after PMA/I stimulation. The percentages of multifunctional CM $CD4^+$ T-cell (IFN-γ^+ TNF-α^+) between the two groups was significantly higher in the young. No significant differences were shown in SEB stimulated group (Figure 2B and 2D).

The percentage of IL-2 producing effector memory (EM) $CD4^+$ T-cells are both significantly higher in the young population in both PMA/I (Figure 3A) and SEB stimulation (Figure 3B) with *P-value <0.05 and *** P-value < 0.000, respectively. Conversely, the percentage of multifunctional EM $CD4^+$ T cells (IL-2^+ TNF-α^+) was significantly higher in the young upon SEB stimulation (Figure 3D). The same pattern of significance was observed in the percentage of IL-2 secretion by $CD8^+$ effector cells that were stimulated by SEB (Figure 4B) and $CD8^+$ CM cells upon PMA/I stimulation (Figure 5A). No significant differences were observed in the rest of the subsets and the frequencies of cytokine production (not shown).

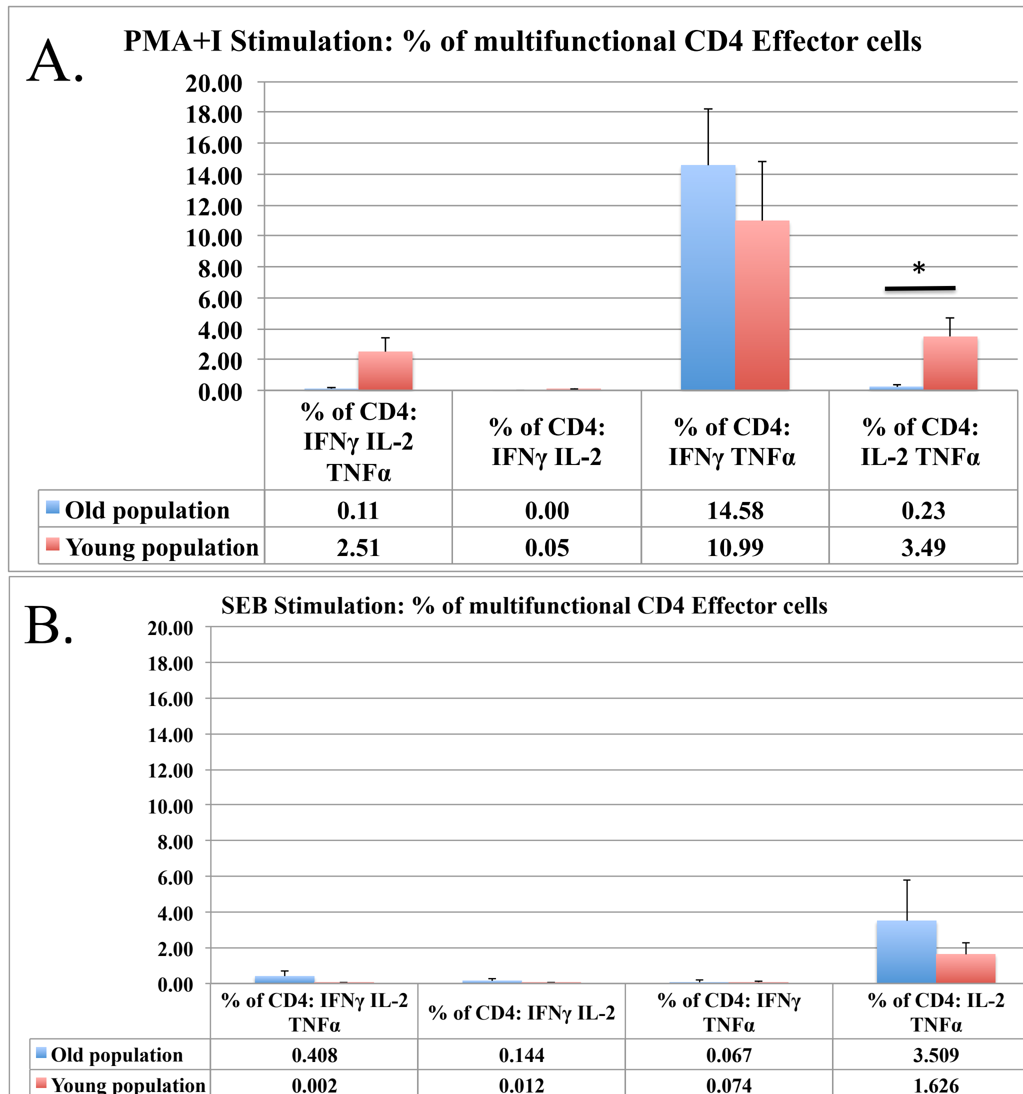
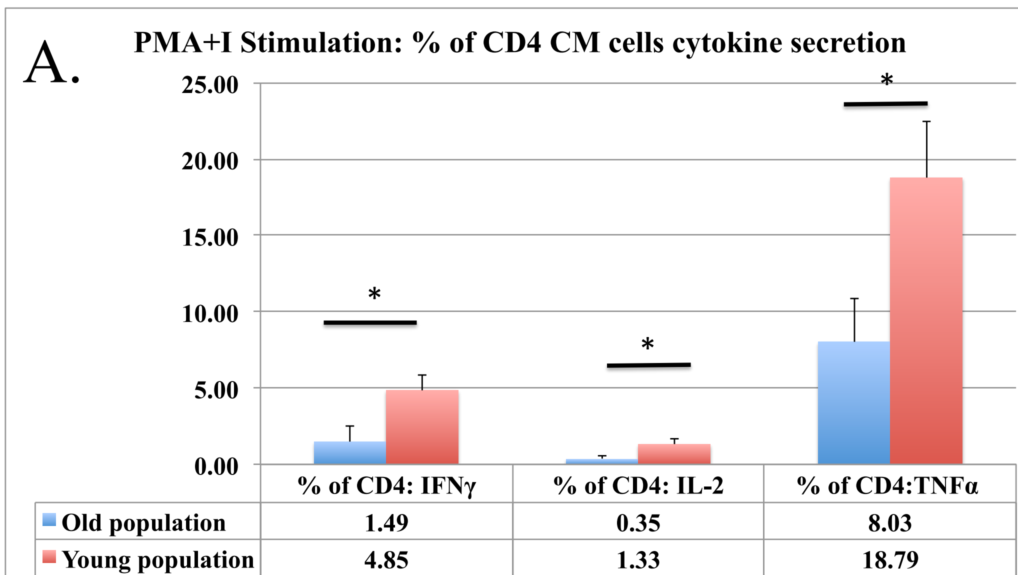
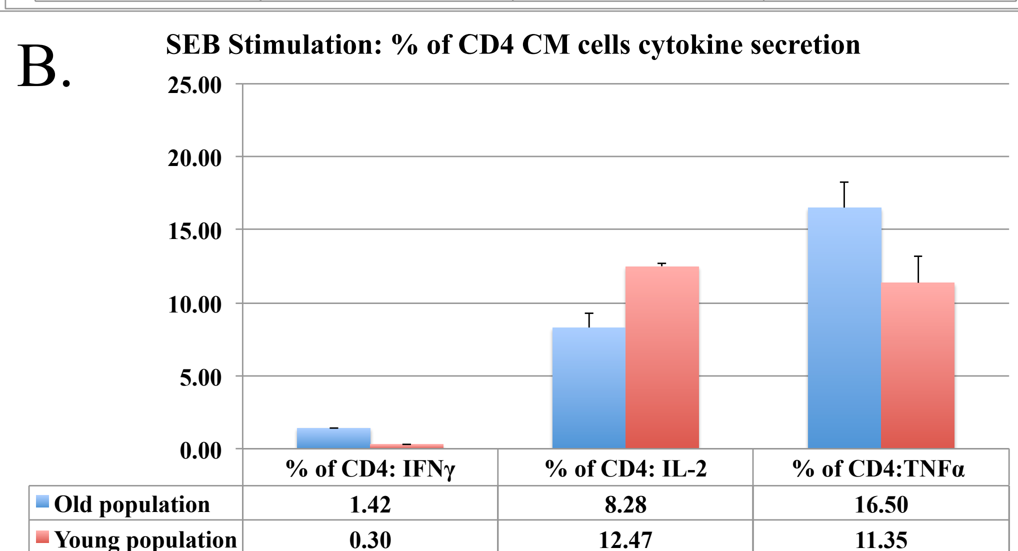


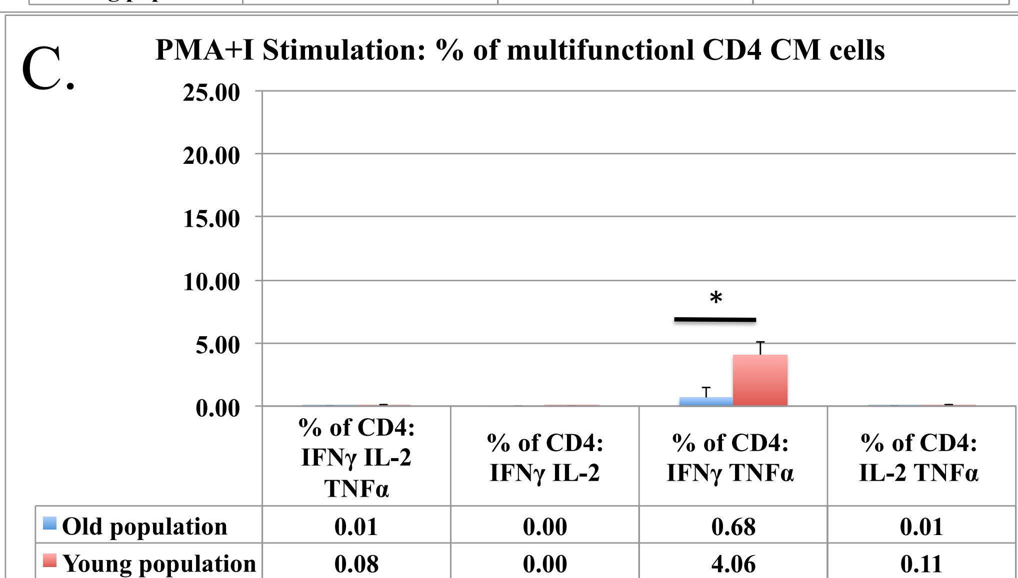
Figure 2 Percentages of multifunctional CD4$^+$ effector cells cytokine production. (A) Percentage of multifunctional CD4$^+$ effector cells after PMA/I stimulation PBMC of 10 young and 10 old RM were stimulated with PMA/I and they stained with specific fluorescence markers to indicate the subsets and the cytokine production (B) Percentage of multifunctional CD4$^+$ effector cells after SEB stimulation PBMC of 10 young and 10 old RM were stimulated with SEB. Data are presented as the percentages of multifunctional CD4$^+$ effector T cells. Multifunctional cells are classified as cells that are capable of producing more than one cytokine at a time.



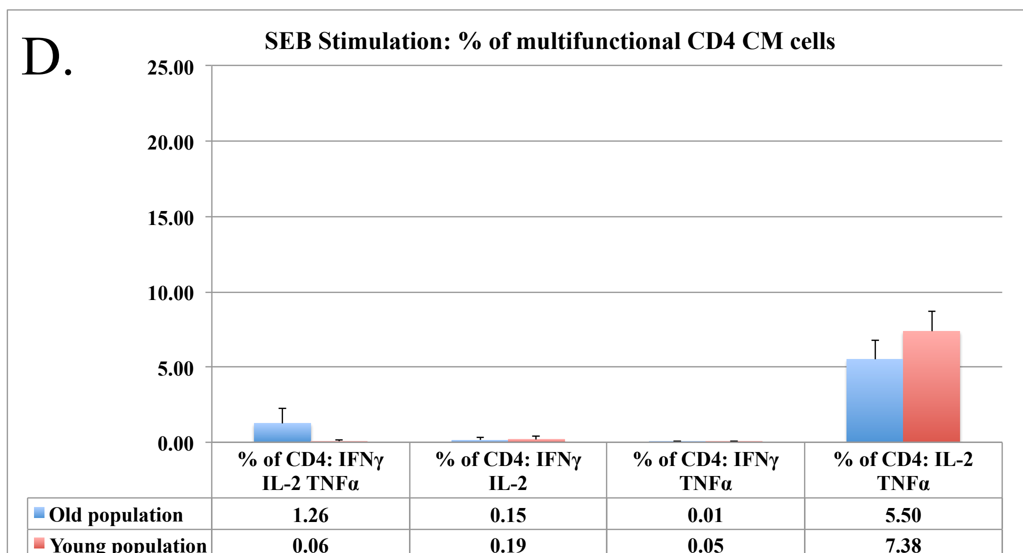
	% of CD4: IFNγ	% of CD4: IL-2	% of CD4:TNFα
Old population	1.49	0.35	8.03
Young population	4.85	1.33	18.79



	% of CD4: IFNγ	% of CD4: IL-2	% of CD4:TNFα
Old population	1.42	8.28	16.50
Young population	0.30	12.47	11.35

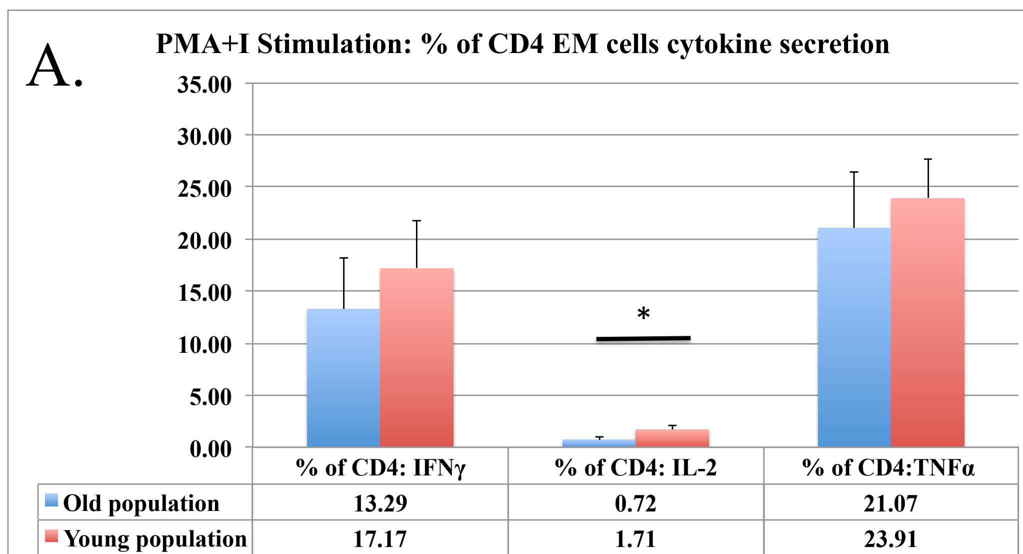


	% of CD4: IFNγ IL-2 TNFα	% of CD4: IFNγ IL-2	% of CD4: IFNγ TNFα	% of CD4: IL-2 TNFα
Old population	0.01	0.00	0.68	0.01
Young population	0.08	0.00	4.06	0.11



	% of CD4: IFNγ IL-2 TNFα	% of CD4: IFNγ IL-2	% of CD4: IFNγ TNFα	% of CD4: IL-2 TNFα
Old population	1.26	0.15	0.01	5.50
Young population	0.06	0.19	0.05	7.38

Figure 3 Percentages of cytokine production in central memory (CM) CD4^{+} T-cell and multifunctional central memory CD4^{+} T-cell. (A) Percentage of cytokine secretion from CM CD4^{+} after PMA/I stimulation. (B) Percentage of cytokine secretion from CM CD4^{+} T cells after SEB stimulation. (C) Percentage of multifunctional CM CD4^{+} T cells after PMA/I stimulation. (D) Percentage of multifunctional CM CD4^{+} T cells after SEB stimulation



	% of CD4: IFNγ	% of CD4: IL-2	% of CD4:TNFα
Old population	13.29	0.72	21.07
Young population	17.17	1.71	23.91

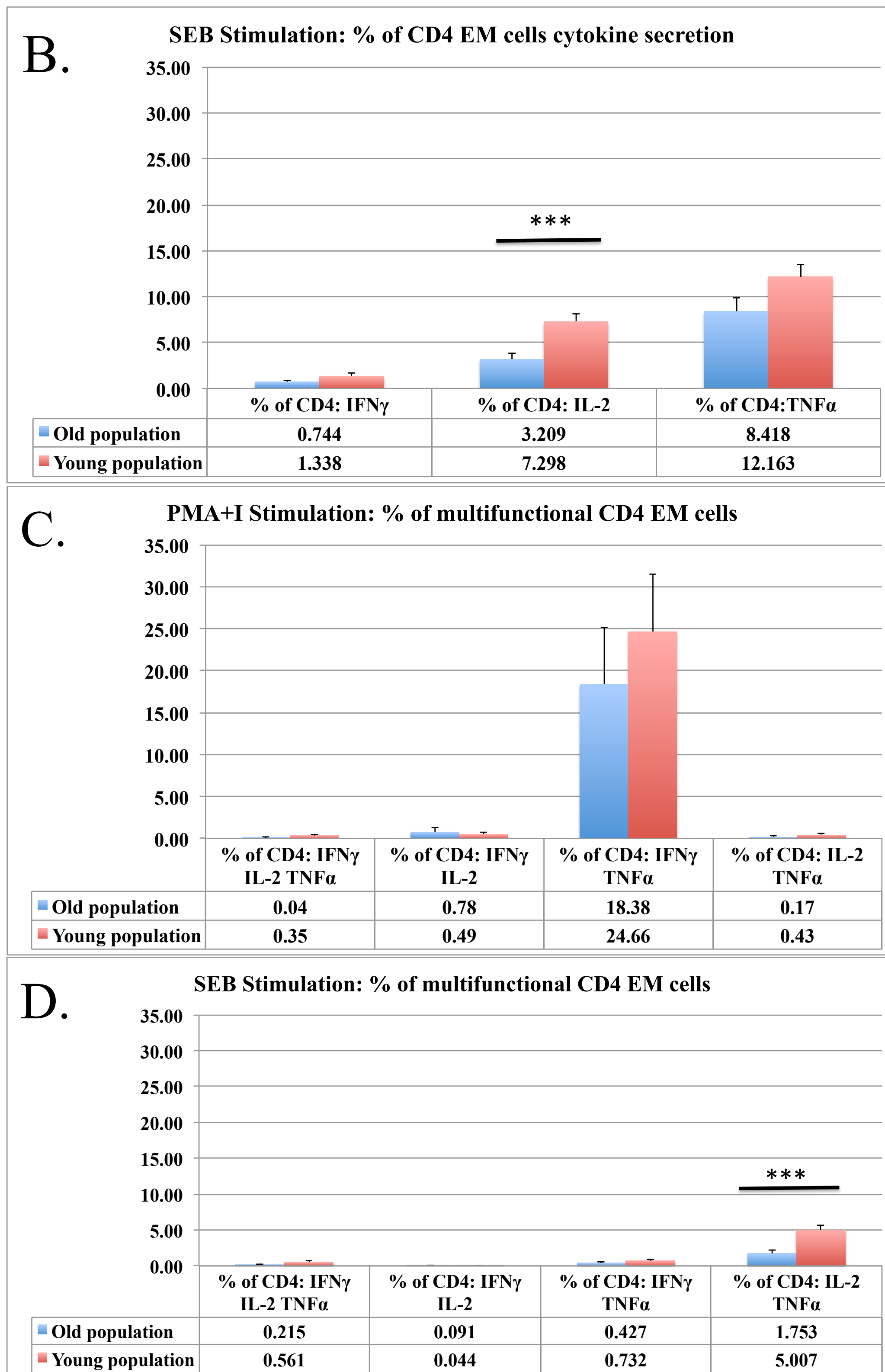
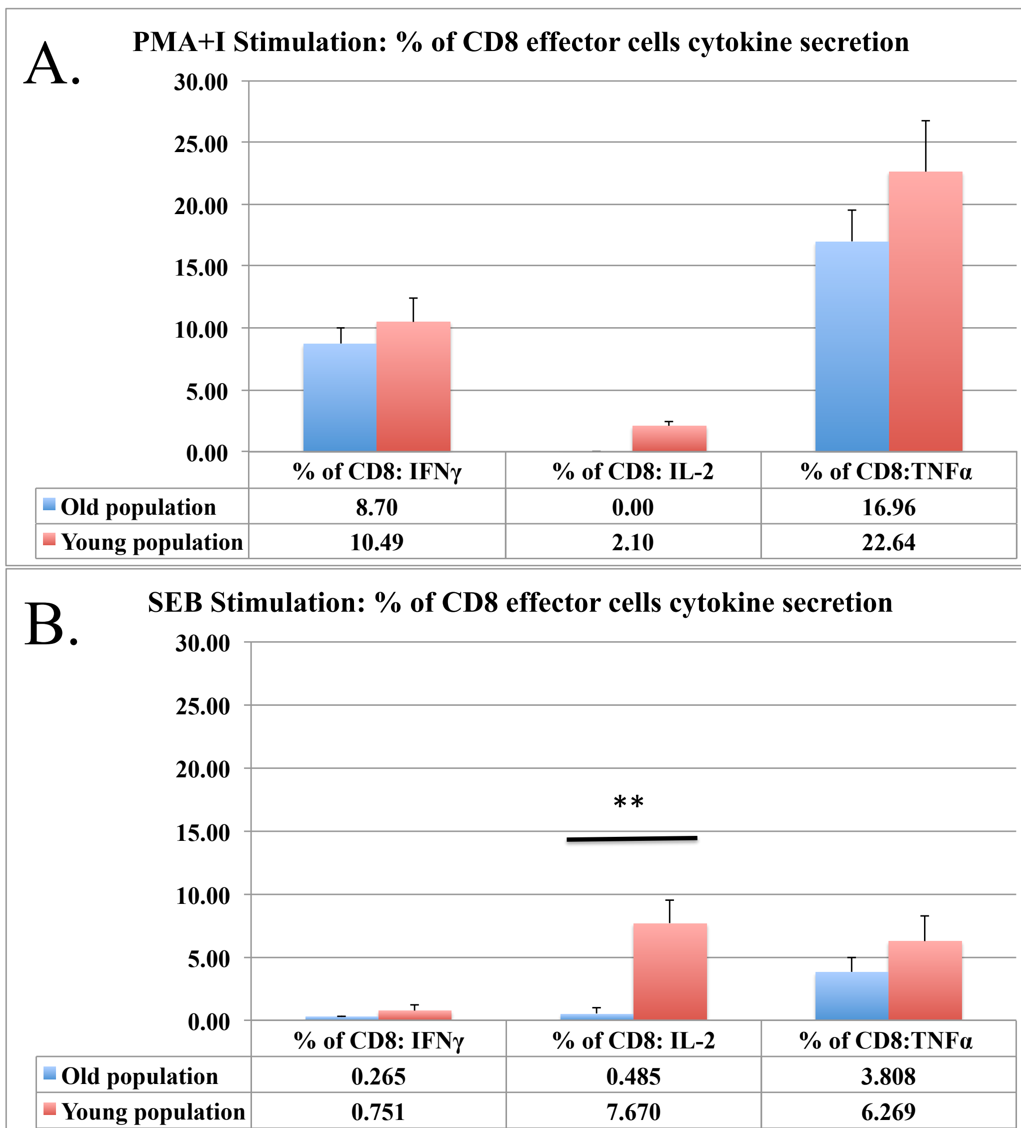


Figure 4 Percentages of cytokine production in effector memory (EM) CD4^{+} T-cell and multifunctional EM CD4^{+} T-cell. (A) Percentage of cytokine secretion from CM CD4^{+} T cells after PMA/I stimulation. (B) Percentage of cytokine secretion from EM CD4^{+} T cells after SEB

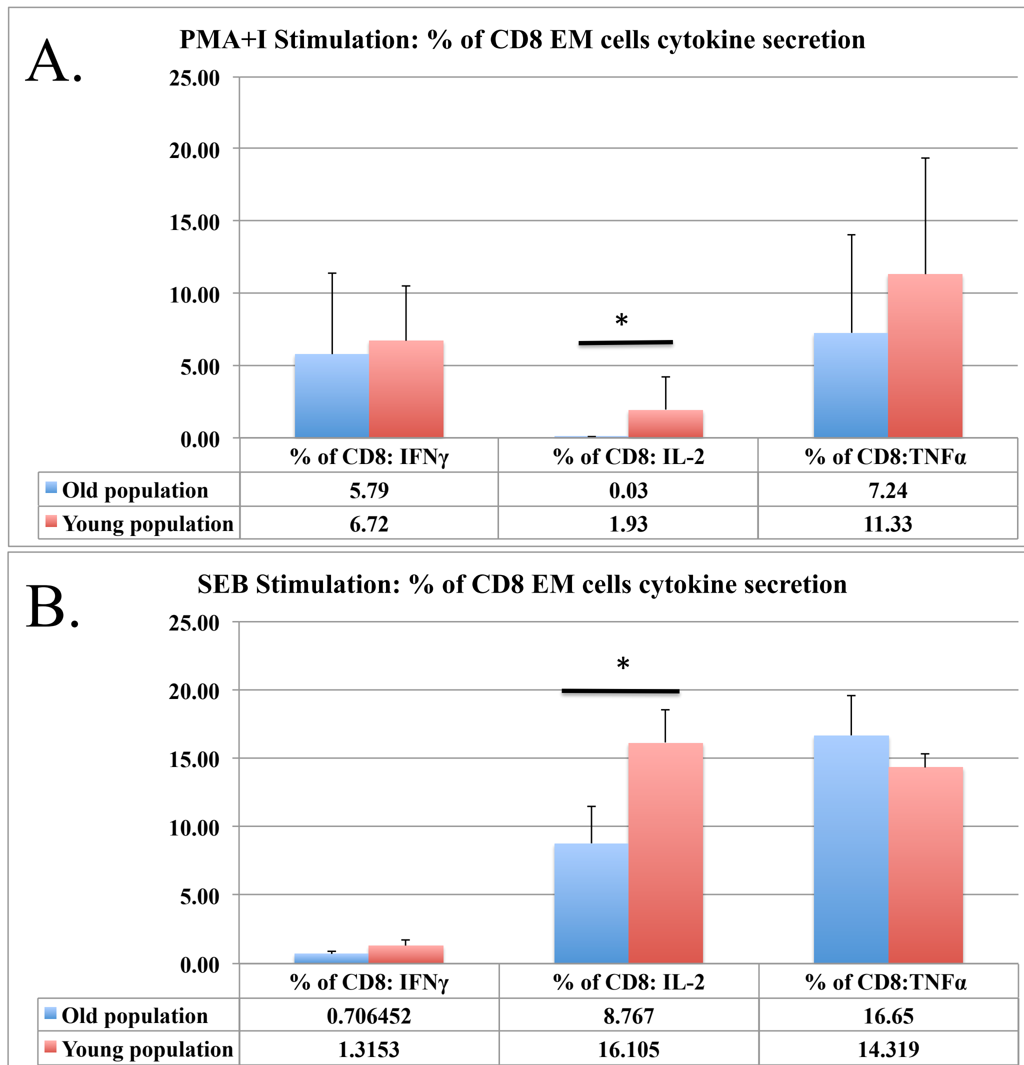
stimulation. (C) Percentage of multifunctional EM CD4$^+$ T cells after PMA/I stimulation. (D) Percentage of multifunctional EM CD4$^+$ T cells after SEB stimulation



	% of CD8: IFNγ	% of CD8: IL-2	% of CD8:TNFα
Old population	8.70	0.00	16.96
Young population	10.49	2.10	22.64

	% of CD8: IFNγ	% of CD8: IL-2	% of CD8:TNFα
Old population	0.265	0.485	3.808
Young population	0.751	7.670	6.269

Figure 5 Percentages of cytokine production in effector CD8$^+$ T-cells. (A) Percentages of cytokine production in effector CD8$^+$ T-cells after PMA/I stimulation. (B) Percentages of cytokine production in effector CD8$^+$ T-cells upon SEB stimulation.



	% of CD8: IFNγ	% of CD8: IL-2	% of CD8:TNFα
Old population	5.79	0.03	7.24
Young population	6.72	1.93	11.33

	% of CD8: IFNγ	% of CD8: IL-2	% of CD8:TNFα
Old population	0.706452	8.767	16.65
Young population	1.3153	16.105	14.319

Figure 6 Percentages of cytokine production in EM CD8$^+$ T-cells. (A) Percentages of cytokine production in EM CD8$^+$ T-cells upon PMA/I stimulation. (B) Percentages of cytokine production in EM CD8$^+$ T-cells upon SEB stimulation.

1b. Absolute number of cytokine producing CD4$^+$ and CD8$^+$ T cell subsets:

The analysis of the absolute cell count is an accurate way of identifying the pattern of cytokine producing based on the complete blood count (CBC) from each specific animal. CBC is a hematology laboratory test that provides important basic information about the host immune system. T lymphocyte count is an important parameter in HIV/AIDS clinical tests and related experimental researches. In this analysis, I analyzed the absolute numbers of cytokine-producing CD4$^+$ or CD8$^+$ T cells

based on the complete blood count (CBC) and the percentages of each cytokine producing cells from each animal. The result of the absolute cell count is presented as the number of cells per microliter. The data with significant differences for the absolute number of the cytokine producing cells will not correspond entirely with the data for the percentages of cytokine production since CBC data for the lymphocyte count of each animal was taken into account for the calculations of the absolute cell count and the CBC values will vary from animal to animal. The data for the absolute cell count should provide information on the quantity of cells that are able to secrete certain cytokines. The table below (Table 1) shows an example of the lymphocyte count per mm^3 from the CBC for each animal used in this experiment. The number of T cells, $CD4^+$ and $CD8^+$ were calculated by taken together the CBC data and the analysis of cells via FlowJo software.

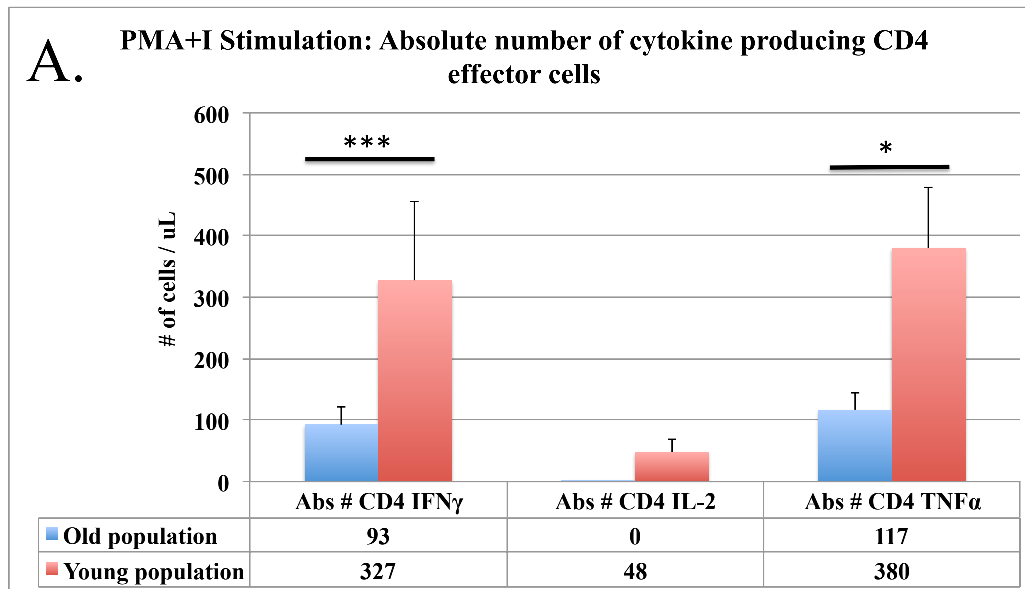
Data from CBC

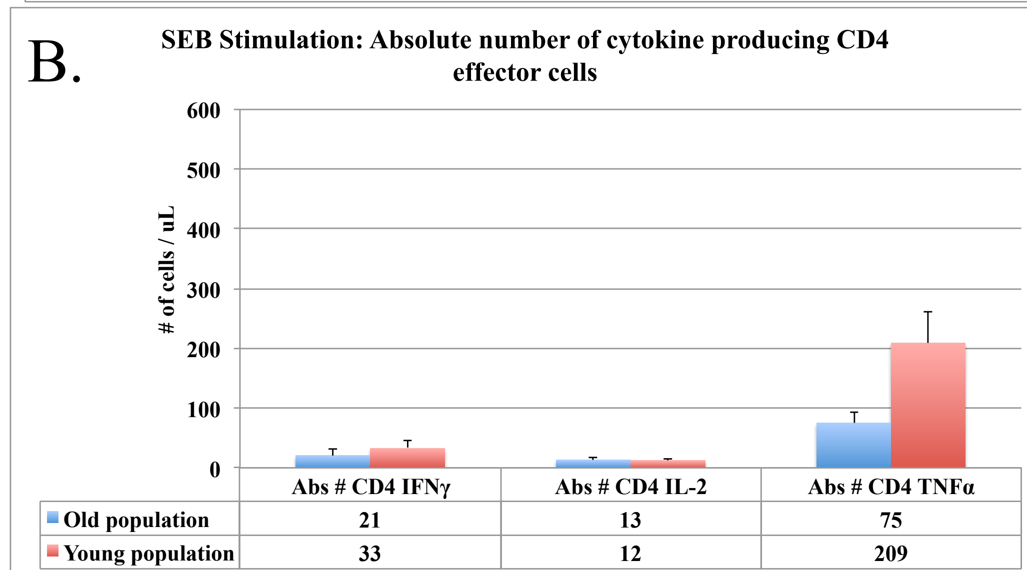
	# of Lymphocytes	# of T cells	# of CD4	# of CD8
Old #1	1870	1099	644	749
Old #2	2500	1741	1216	1048
Old #3	2360	1333	884	874
Old #4	2090	1218	830	760
Old #5	3700	2654	1480	1821
Old #6	3010	2043	1153	1268
Old #7	1460	897	627	575
Old #8	1380	791	476	490
Old #9	2310	1364	800	906
Old #10	3360	1950	1115	1160
Young #1	2250	1609	986	887
Young #2	2751	1720	977	1153
Young #3	4211	2374	1649	1517
Young #4	5424	3522	2196	2042
Young #5	2544	1628	1121	1141
Young #6	5151	2933	1962	1858
Young #7	4910	3517	2101	2449
Young #8	3411	2449	1583	1439
Young #9	7112	4245	2915	2938
Young #10	3498	2422	1344	1405

Table 1: CBC data for the lymphocyte count of 10 young and 10 old RM that were used in the experiment. The number of lymphocytes represent cells per mm^3. The total number of T cells along with the count of $CD4^+$ and $CD8^+$ cells were calculated based on the analysis from FlowJo software.

The first significant difference that was observed in the data for the absolute cell count was from the group treated with PMA/I stimulation which showed a significantly higher count for IFN-γ and TNF-α production in $CD4^+$ effector cells from the younger RM with *P-value < 0.05 (Figure 6A) while no significant differences were seen in the same subset with SEB treatment (Figure 6B and 6D). In the multifunctional population of $CD4^+$ effector cells, cytokine production with a the absolute cell count for IFN-γ^+ IL-2^+ TNF-α^+ producing cells was significantly higher in the younger population (Figure 6C). As for the subset of $CD4^+$ central memory (CM) cells, younger population showed significantly higher absolute count of each cytokines producing cells (IFN-γ , IL-2, and TNF-α) (Figure 7A) as well as IFN-γ^+ IL-2^+ producing multifunctional $CD4^+$ CM cells upon PMA/I stimulation (Figure 7C). Upon SEB treatment, the absolute number of IL-2 producing $CD4^+$ CM cells were siginificantly higher in the young population with **p-value < 0.01 (Figure 7B) but no significant differences were seen in the $CD4^+$ CM multifunctional cell count (Figure 7D). The next subset with significant differences between the two age groups is the cell count for $CD4^+$ effector memory (EM) subset with significantly higher cell count for each IL-2 and TNF-α producing cells in the younger population upon both PMA/I (Figure 8A) and SEB stimulation (Figure 8B). Furthermore, the multifunctional $CD4^+$ EM cells producing all three cytokines IFN-γ^+ IL-2^+ TNF-α^+ showed a significant difference between the two age groups in upon both PMA/I (Figure 8C) and SEB treatment (Figure 8D). However, another significant difference was also seen in the multifunctional $CD4^+$ EM cells that secrete IL-2^+ TNF-α^+ upon SEB stimulation (Figure 8D). For the subset of $CD4^+$ naïve cells, the only observable

significant difference was in the cell count for IFN-γ secreting cells upon PMA/I stimulation (Figure 9A) and non upon SEB treatment (Figure 9B).





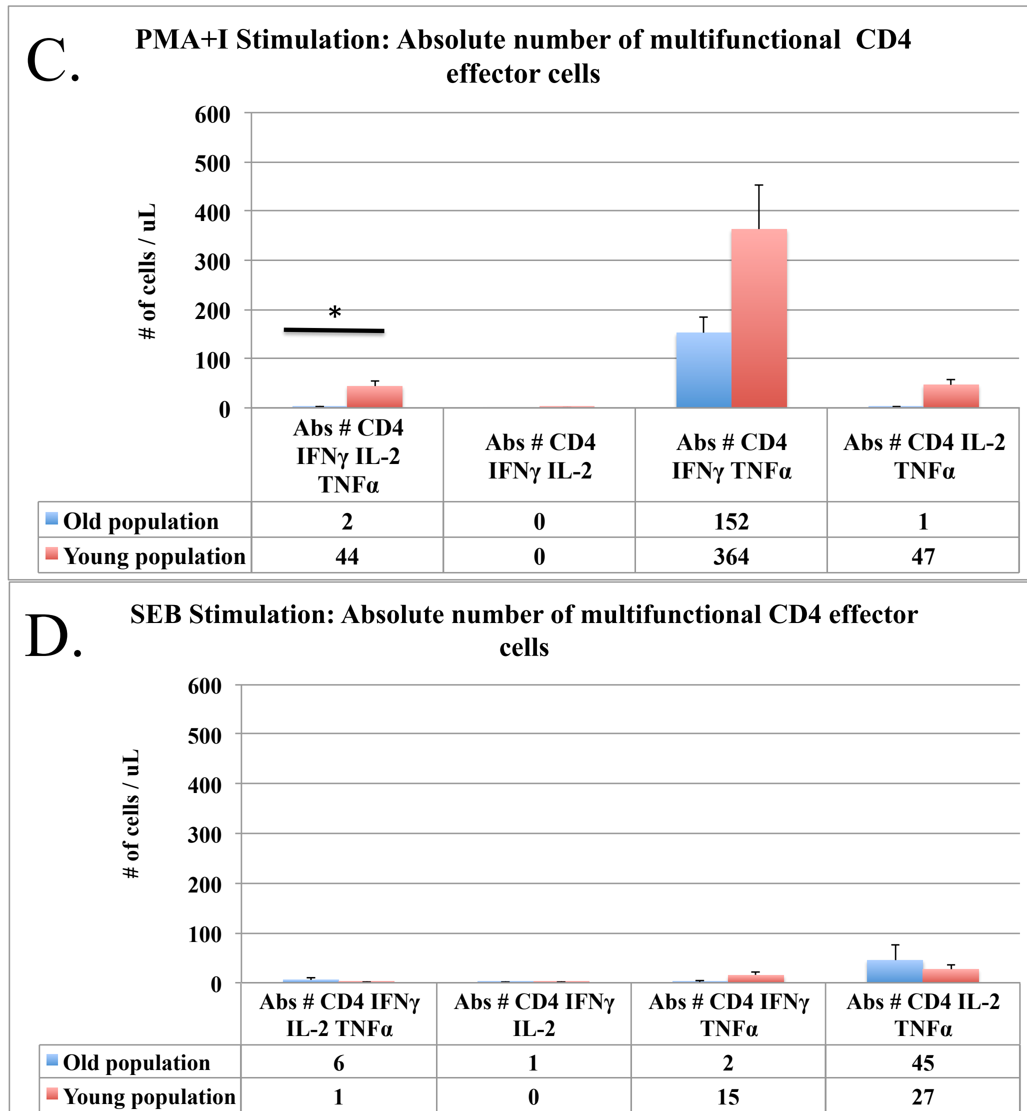
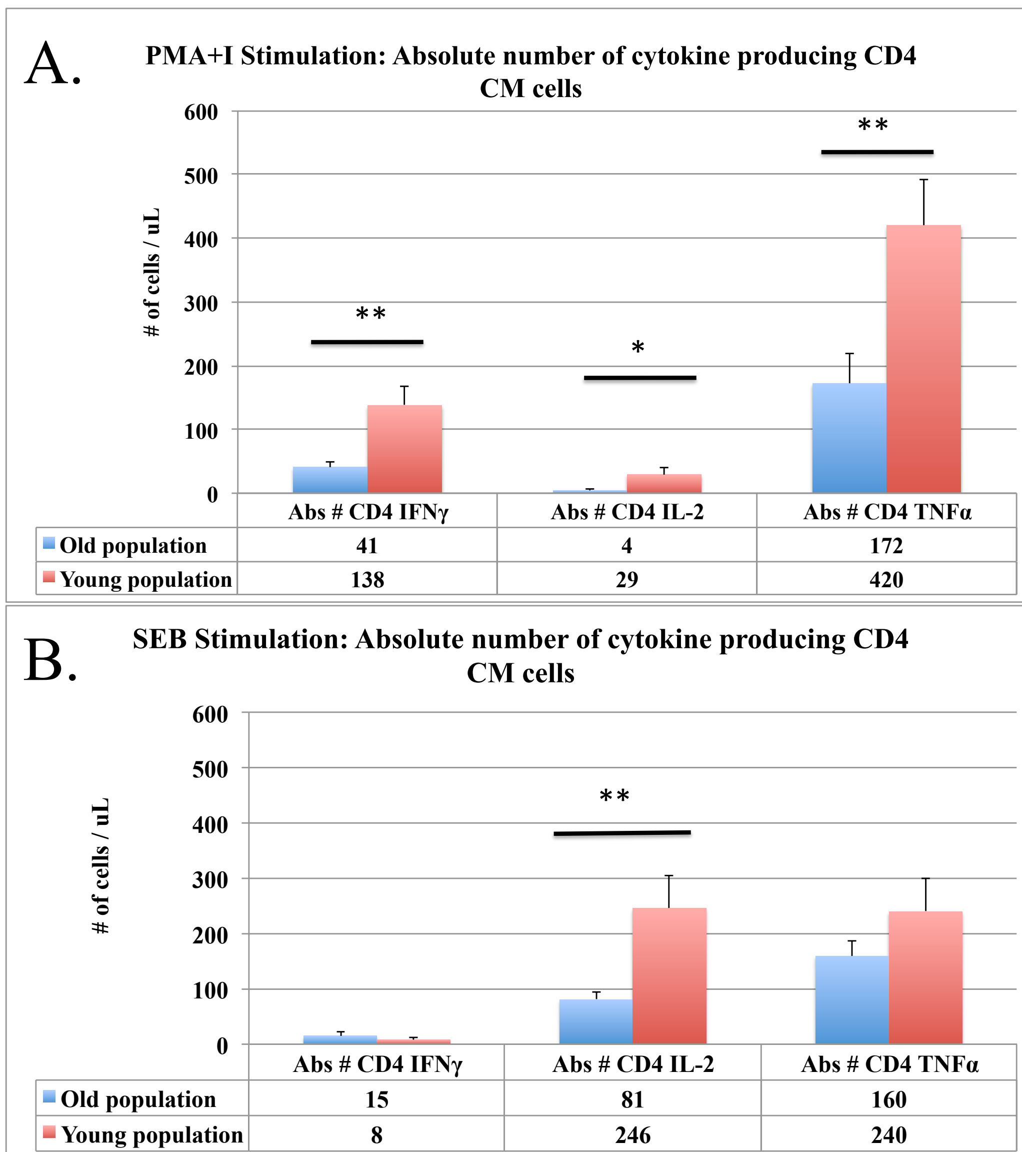
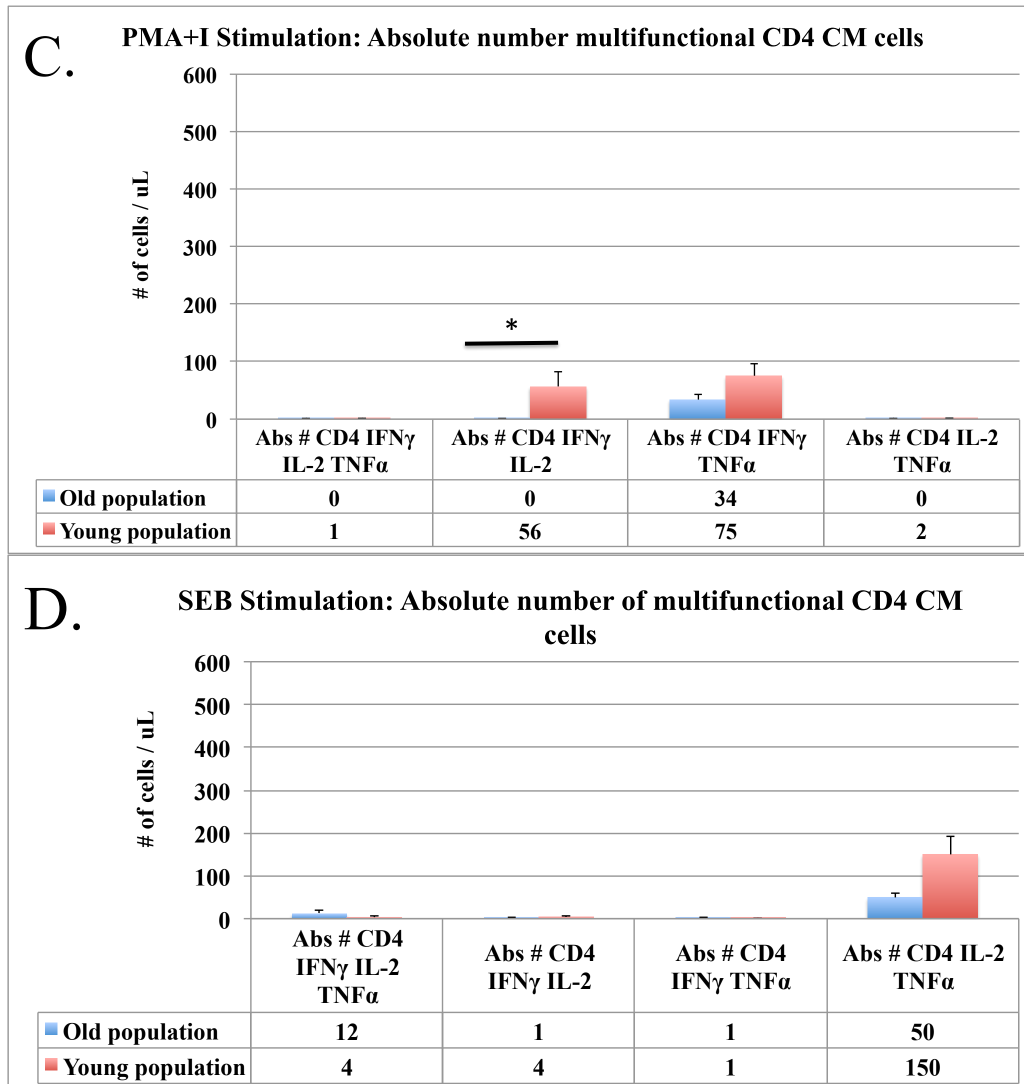


Figure 7: Absolute number of cytokine producing CD4$^+$ effector cells after (A) PMA/I stimulation and (B) SEB stimulation. (C) Absolute number of multifunctional CD4$^+$ effector T cells upon PMA/I stimulation and (D) SEB stimulation.

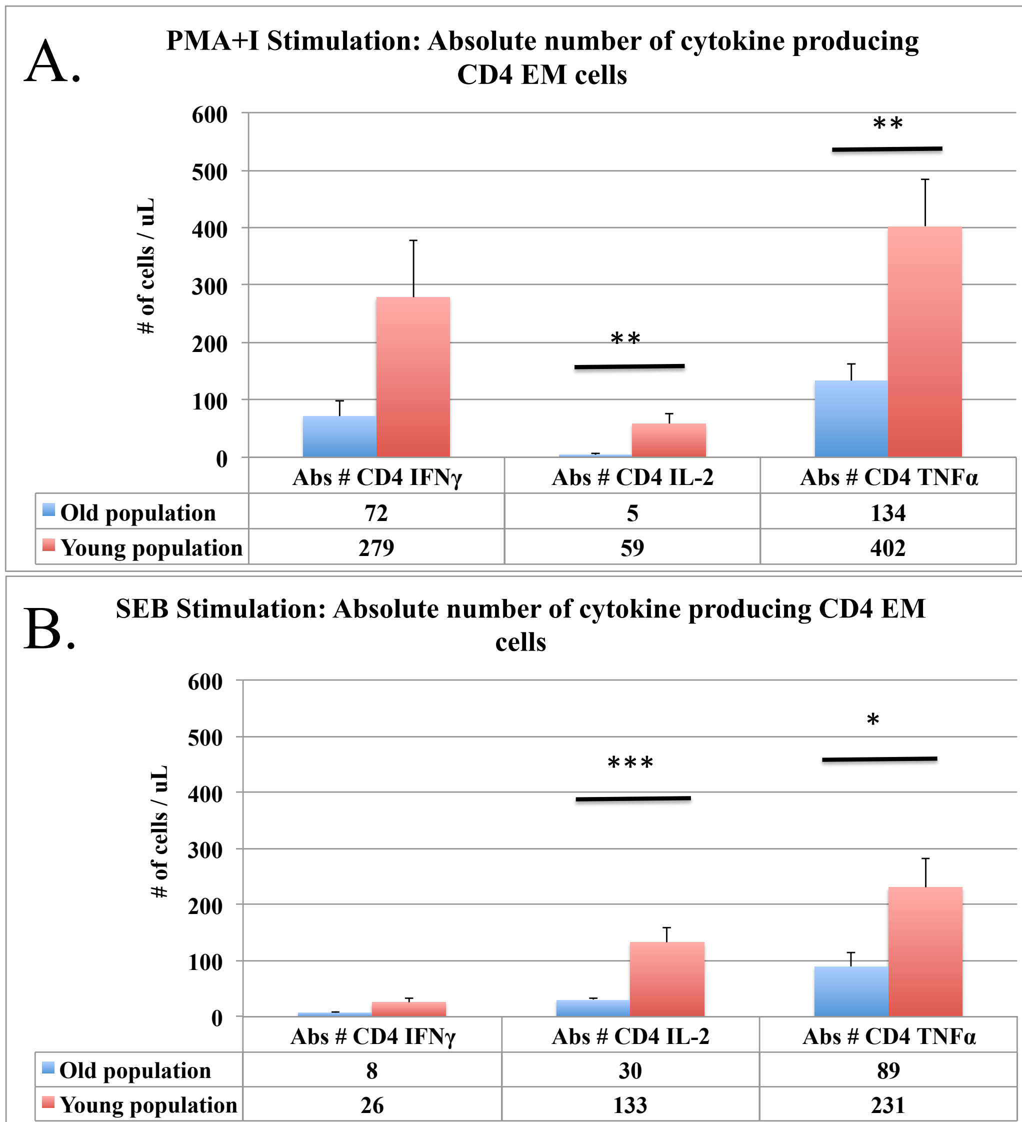

A.
PMA+I Stimulation: Absolute number of cytokine producing CD4 CM cells
# of cells / uL
600
500
400
300
200
100
0
**
*
**
Abs # CD4 IFNγ
Abs # CD4 IL-2
Abs # CD4 TNFα
Old population
41
4
172
Young population
138
29
420
B.
SEB Stimulation: Absolute number of cytokine producing CD4 CM cells
# of cells / uL
600
500
400
300
200
100
0
**
Abs # CD4 IFNγ
Abs # CD4 IL-2
Abs # CD4 TNFα
Old population
15
81
160
Young population
8
246
240

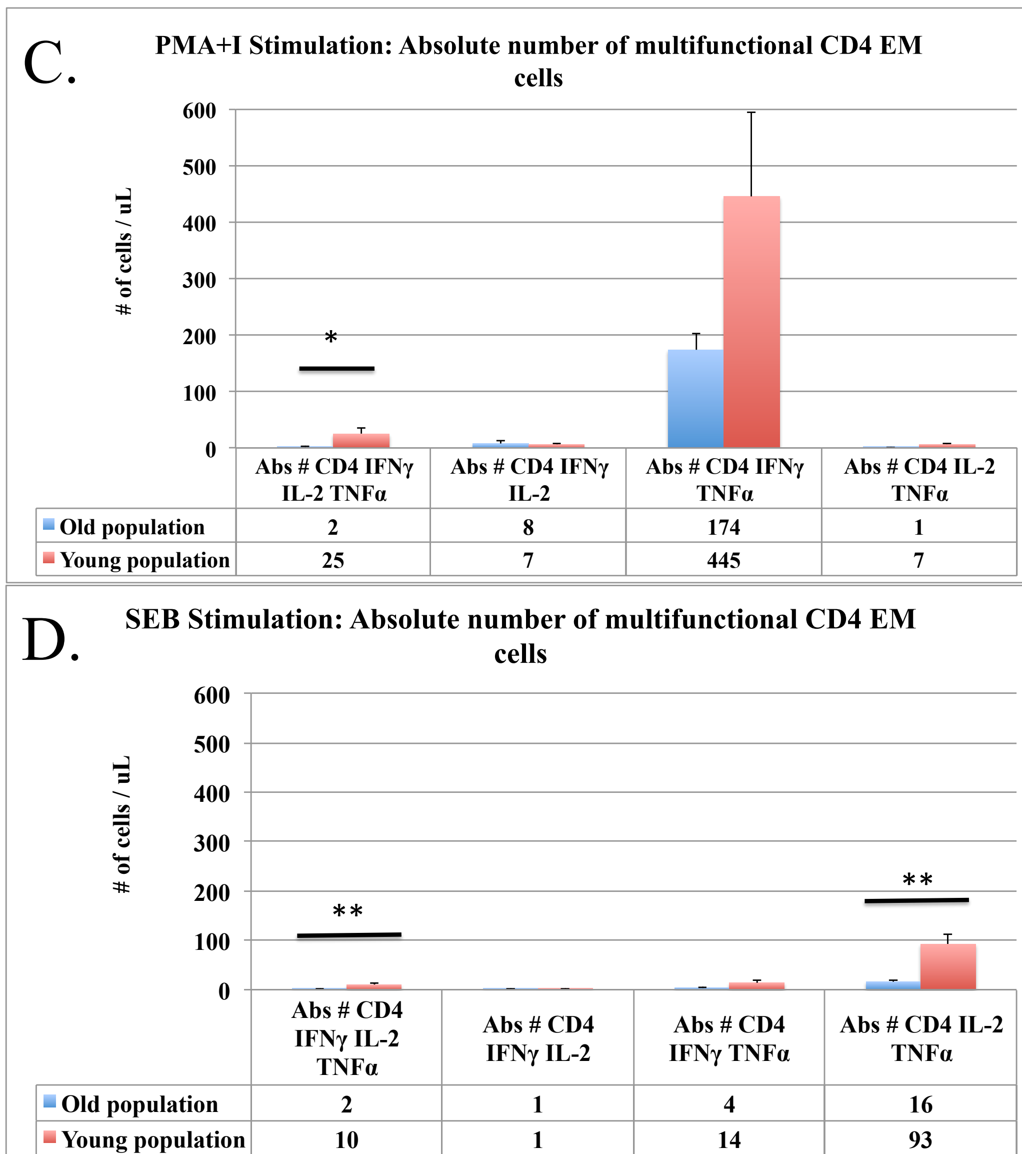


C. PMA+I	Abs # CD4 IFNγ IL-2 TNFα	Abs # CD4 IFNγ IL-2	Abs # CD4 IFNγ TNFα	Abs # CD4 IL-2 TNFα
Old population	0	0	34	0
Young population	1	56	75	2

D. SEB	Abs # CD4 IFNγ IL-2 TNFα	Abs # CD4 IFNγ IL-2	Abs # CD4 IFNγ TNFα	Abs # CD4 IL-2 TNFα
Old population	12	1	1	50
Young population	4	4	1	150

Figure 8: Absolute number of cytokine producing Central Memory (CM) $CD4^+$ T-cells and multifunctional CM $CD4^+$ T-cells. (A) Absolute number of cytokine producing CM $CD4^+$ T-cell upon PMA/I stimulation and (B) SEB stimulation. (C) Absolute number of multifunctional CM $CD4^+$ T cells upon PMA/I stimulation and (D) SEB stimulation.


A.
PMA+I Stimulation: Absolute number of cytokine producing CD4 EM cells
# of cells / uL
600
500
400
300
200
100
0
**
**
Abs # CD4 IFNγ
Abs # CD4 IL-2
Abs # CD4 TNFα
Old population
Young population
72
5
134
279
59
402
B.
SEB Stimulation: Absolute number of cytokine producing CD4 EM cells
# of cells / uL
600
500
400
300
200
100
0
***
*
Abs # CD4 IFNγ
Abs # CD4 IL-2
Abs # CD4 TNFα
Old population
Young population
8
30
89
26
133
231



	Abs # CD4 IFNγ IL-2 TNFα	Abs # CD4 IFNγ IL-2	Abs # CD4 IFNγ TNFα	Abs # CD4 IL-2 TNFα
Old population	2	8	174	1
Young population	25	7	445	7

	Abs # CD4 IFNγ IL-2 TNFα	Abs # CD4 IFNγ IL-2	Abs # CD4 IFNγ TNFα	Abs # CD4 IL-2 TNFα
Old population	2	1	4	16
Young population	10	1	14	93

Figure 9: Absolute number of cytokine producing EM CD4$^+$ T-cells and multifunctional EM CD4$^+$ T-cells. (A) Absolute number of cytokine producing EM CD4$^+$ T-cells upon PMA/I stimulation and (B) SEB stimulation. (C) Absolute number of multifunctional EM CD4$^+$ T-cells upon PMA/I stimulation and (D) SEB stimulation

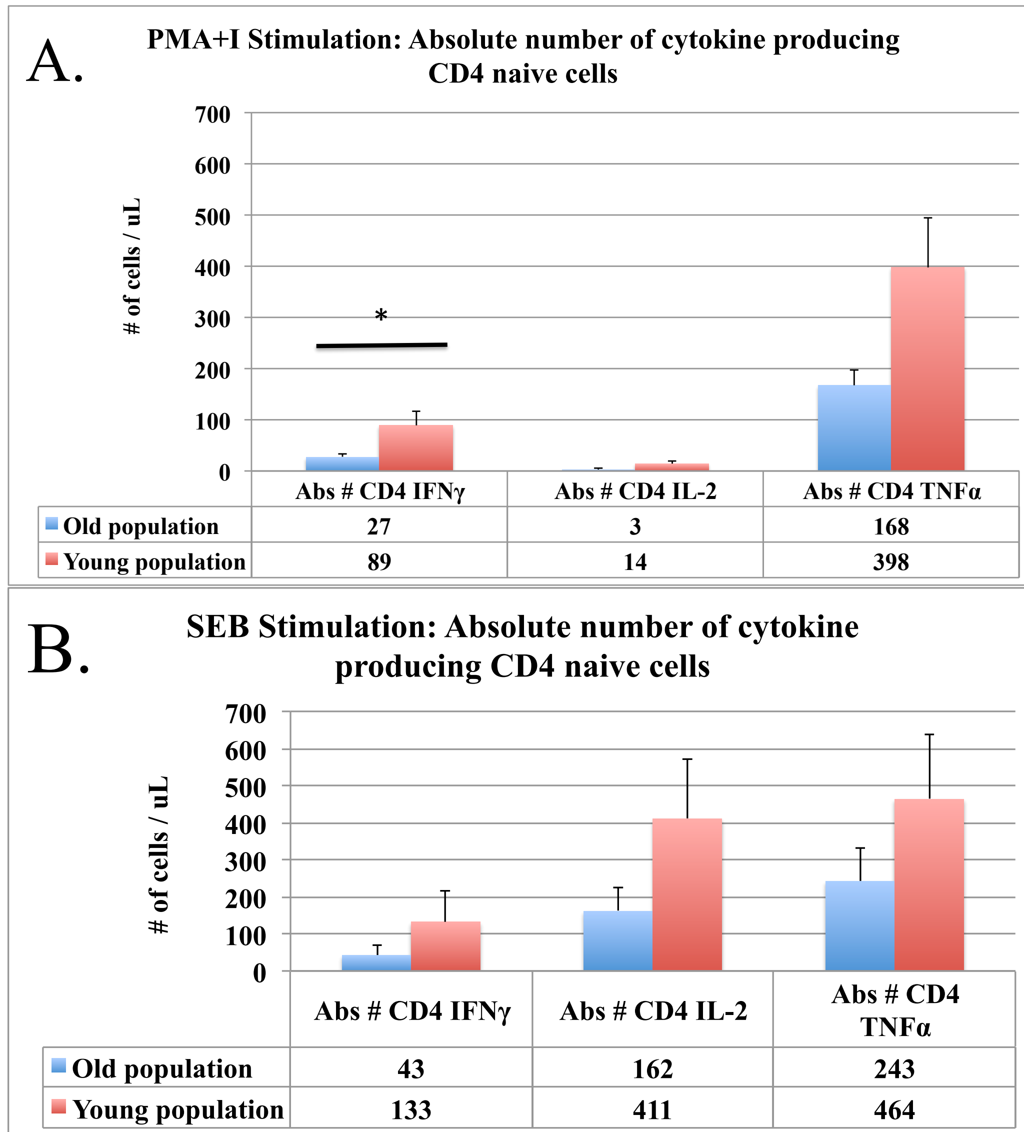
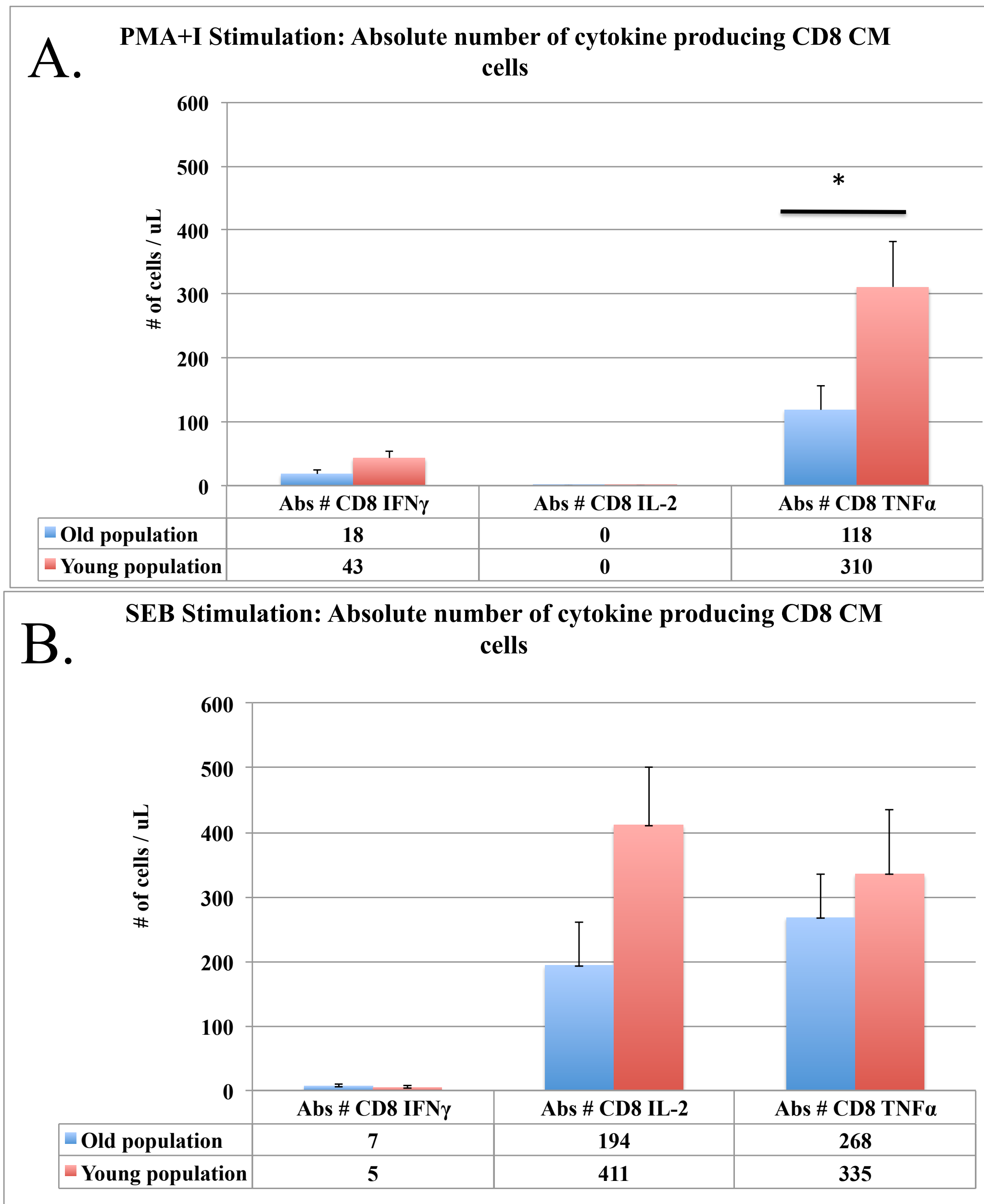


Figure 10: Absolute number of CD4^{+} naive T cells after (A) PMA/I stimulation and (B) SEB stimulation

In CD8^{+} T-cell subset, the significant differeces were only obsereved in the subsets of CM, EM and naive CD8^{+} T cells. The significant difference seen in the subset of CD8^{+} CM cells falls in the absolute cell count of TNF-α producing cells upon PMA/I stimulation (Figure 10A) and again no differences were seen upon SEB stimulation (Figure 10B). For CD8^{+} EM cells, younger population had a significantly higher absolute cell count for IL-2 producing cell in both PMA/I treated group (Figure 11A) as well as

SEB treated group (Figure 11B). Moreover, samples treated with PMA/I also showed a significant difference in the absolute cell count of TNF-α producing $CD8^+$ EM cells (Figure 11A). As for $CD8^+$ naïve cells, younger population had a significantly higher absolute cell count for TNF-α producing cell, again in both PMA/I (Figure 12A) and SEB stimulation (Figure 12B).



	Abs # CD8 IFNγ	Abs # CD8 IL-2	Abs # CD8 TNFα
Old population	18	0	118
Young population	43	0	310

	Abs # CD8 IFNγ	Abs # CD8 IL-2	Abs # CD8 TNFα
Old population	7	194	268
Young population	5	411	335

Figure 11: Absolute number of cytokine producing CM $CD8^+$ T-cells. (A) Absolute number of cytokine producing CM $CD8^+$ T-cells upon PMA/I stimulation and (B) SEB stimulation

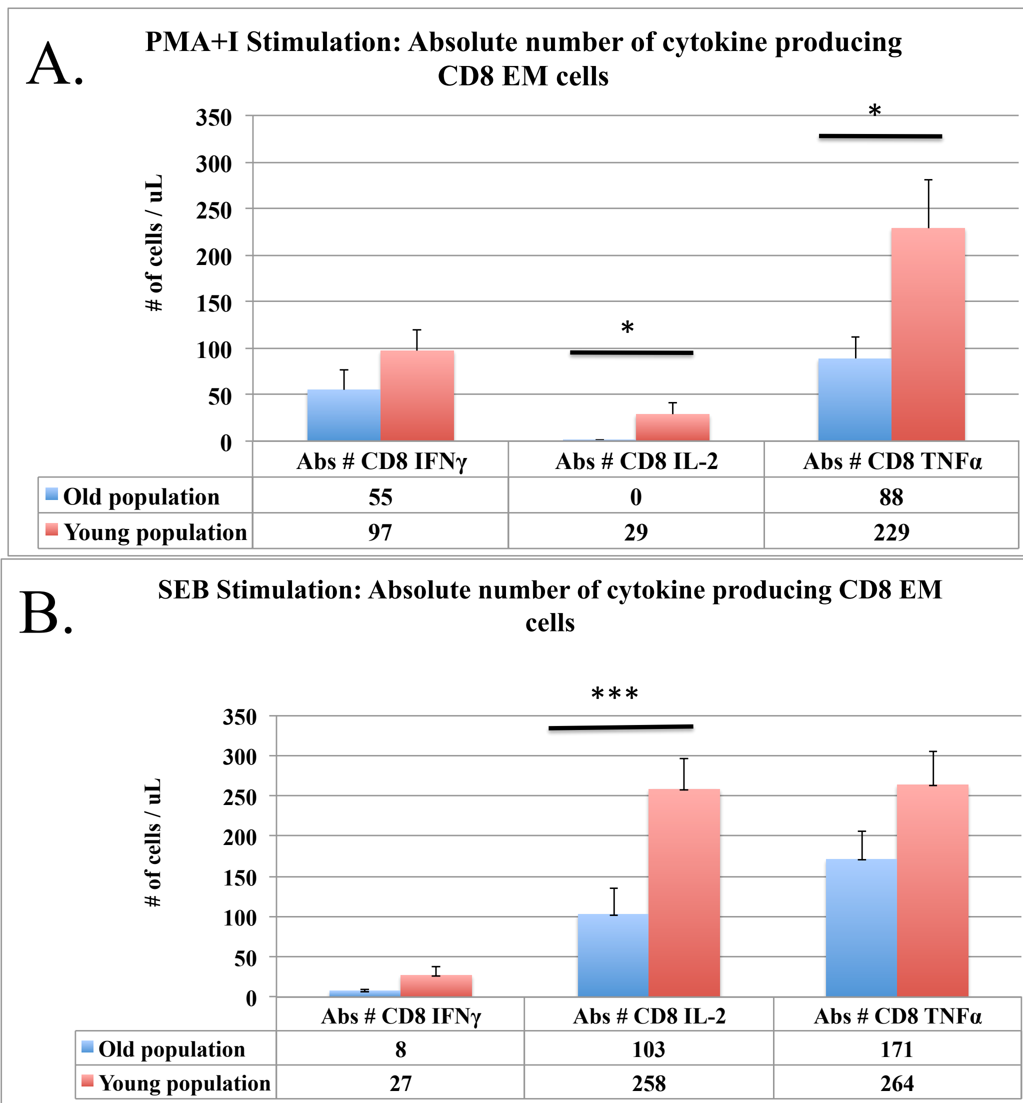


Figure 12: Absolute number of cytokine producing EM $CD8^+$ T-cells after (A) PMA/I stimulation and (B) SEB stimulation

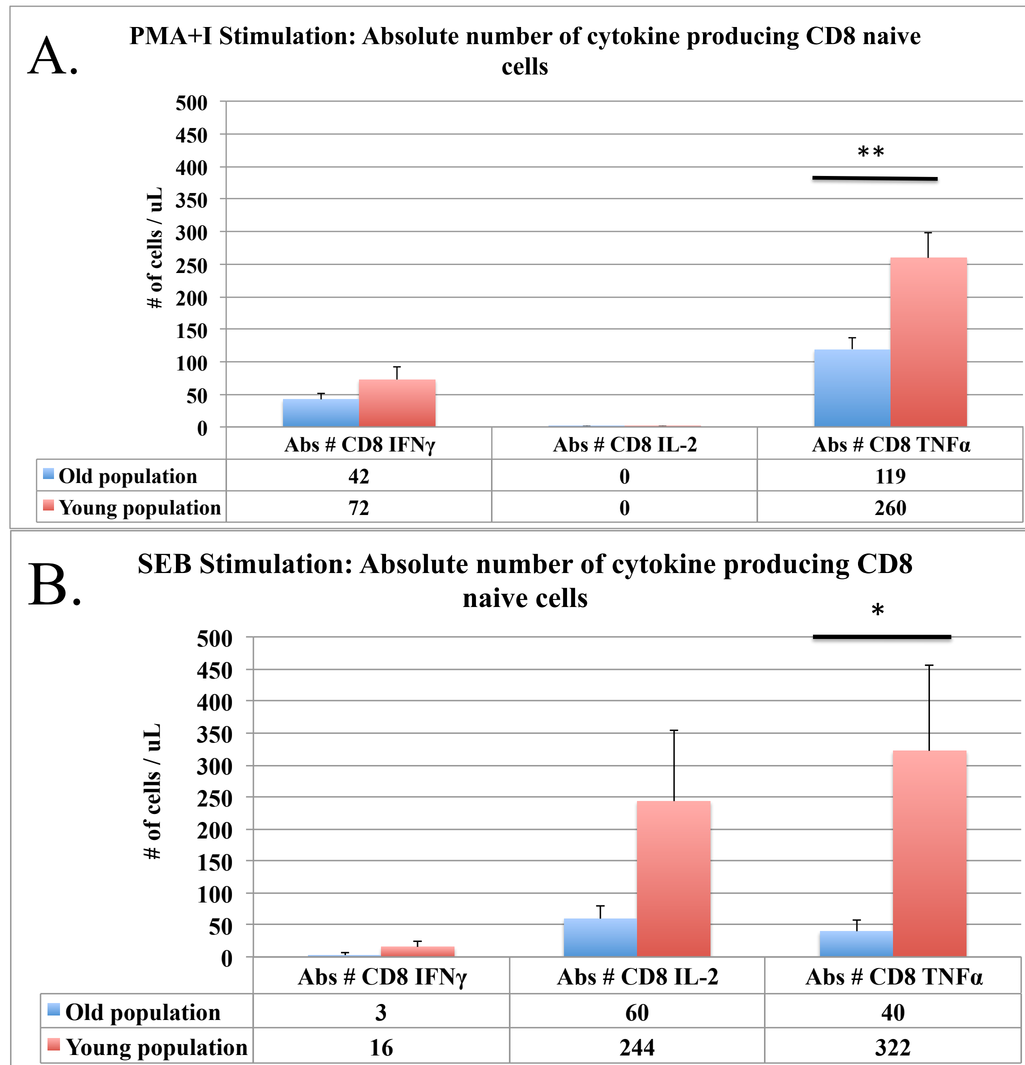
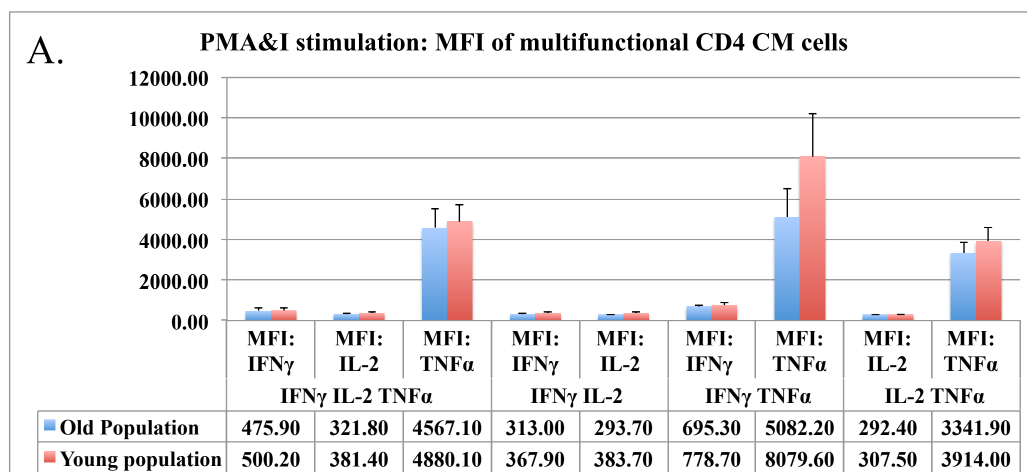


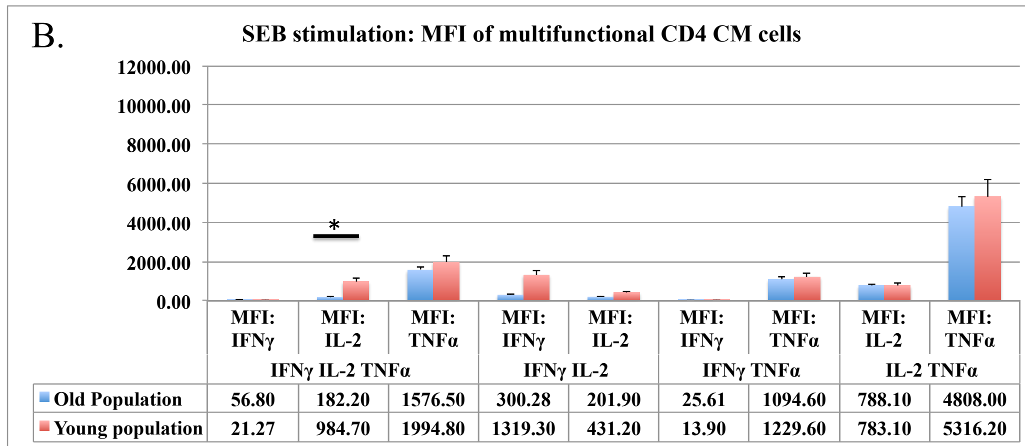
Figure 13: Absolute number of cytokine producing CD8^{+} naive T-cells after (A) PMA/I stimulation and (B) SEB stimulation

1c. Mean Fluoresence Inentsity (MFI) of cytokine producing CD4^{+} and CD8^{+} T cell subsets

Mean Fluoresence Inentsity (MFI) is the fluorescence intensity or the brightness of each event in average that represents the expression quantity of the chosen parameter on each event. In this paper, MFI is used to quantify the measurement of the cytokine production per cell. All of the MFI values were retrieved from FlowJo (Treestar, Inc.). MFI were evaluated for both single-cytokine secreting cells and for multifunctional cells.

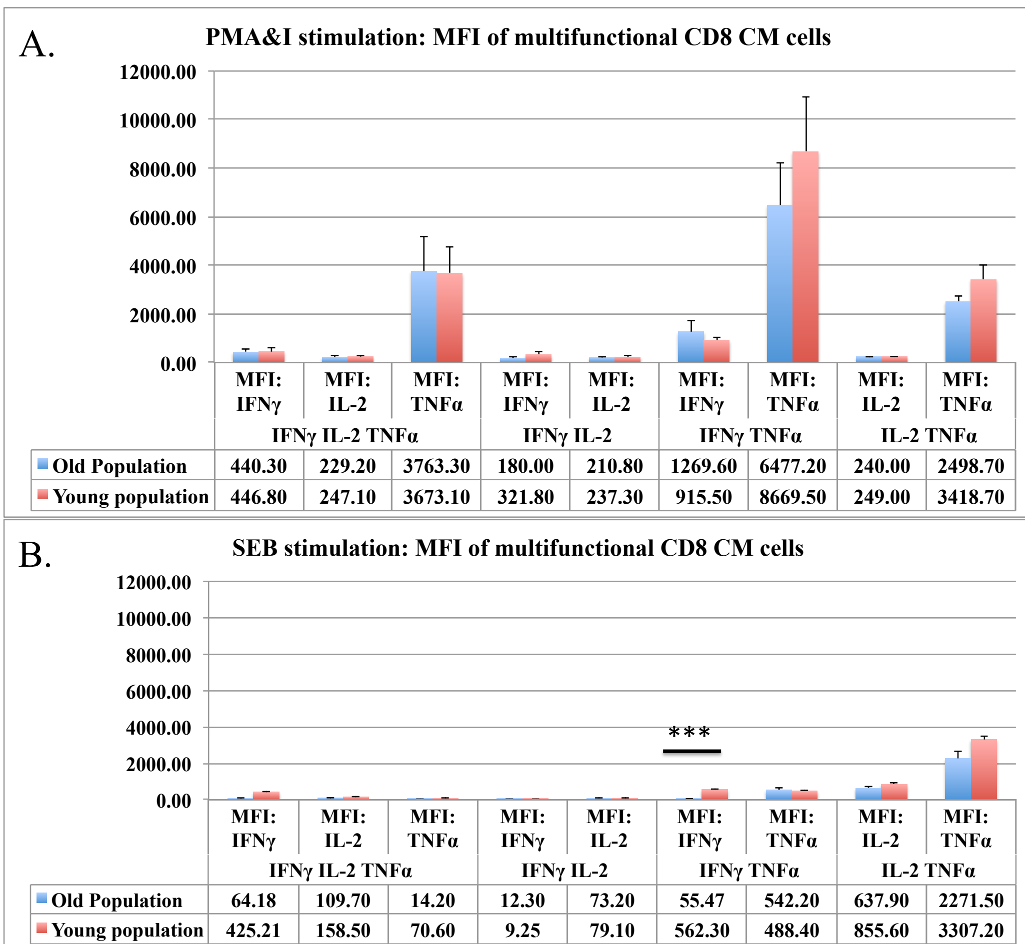
Each cytokine secreted in multifunctional cells or multiple cytokine secreting cells were assessed individually. For example, the MFI values for multifunctional cells secreting all of the three cytokines (IFN-γ^{+} IL-2^{+} TNF-α^{+}) were assessed for IFN-γ , IL-2 and TNF-α individually and the values of each cytokine produced in the multifunctional cells were compared between the two age groups. The significant differences of MFI were detected only in subset of central memory of CD4^{+} and CD8^{+} multifunctional T-cells. Figure 13 compares the MFI of multifunctional CD4^{+} CM cells upon the treatment of PMA/I (Figure 13A) versus SEB (Figure 13B). However a significant difference was found in the values for IL-2 in multifunctional cells secreting IFN-γ^{+} IL-2^{+} TNF-α^{+} and that were treated with SEB (Figure 13B). Similarly for CD8^{+} CM cells (Figure 14), the significant difference was observed in the SEB treated group but with this subset, the significant difference was found in the IFN-γ of multifunctional cells secreting IFN-γ^{+} TNF-α^{+} (Figure 14B). Again no significant differences were found in PMA/I stimulated group of CD8^{+} CM cells (Figure 14A).





	IFNγ IL-2 TNFα			IFNγ IL-2		IFNγ TNFα		IL-2 TNFα	
	MFI: IFNγ	MFI: IL-2	MFI: TNFα	MFI: IFNγ	MFI: IL-2	MFI: IFNγ	MFI: TNFα	MFI: IL-2	MFI: TNFα
Old Population	56.80	182.20	1576.50	300.28	201.90	25.61	1094.60	788.10	4808.00
Young population	21.27	984.70	1994.80	1319.30	431.20	13.90	1229.60	783.10	5316.20

Figure 14: MFI of cytokine producing multifunctional CD4^{+} CM T-cells upon (A) PMA/I stimulation and (B) SEB stimulation.



	IFNγ IL-2 TNFα			IFNγ IL-2		IFNγ TNFα		IL-2 TNFα	
	MFI: IFNγ	MFI: IL-2	MFI: TNFα	MFI: IFNγ	MFI: IL-2	MFI: IFNγ	MFI: TNFα	MFI: IL-2	MFI: TNFα
Old Population	440.30	229.20	3763.30	180.00	210.80	1269.60	6477.20	240.00	2498.70
Young population	446.80	247.10	3673.10	321.80	237.30	915.50	8669.50	249.00	3418.70

	IFNγ IL-2 TNFα			IFNγ IL-2		IFNγ TNFα		IL-2 TNFα	
	MFI: IFNγ	MFI: IL-2	MFI: TNFα	MFI: IFNγ	MFI: IL-2	MFI: IFNγ	MFI: TNFα	MFI: IL-2	MFI: TNFα
Old Population	64.18	109.70	14.20	12.30	73.20	55.47	542.20	637.90	2271.50
Young population	425.21	158.50	70.60	9.25	79.10	562.30	488.40	855.60	3307.20

Figure 15: MFI of cytokine producing multifunctional CD8^{+} CM T-cells upon (A) PMA/I stimulation and (B) SEB stimulation.

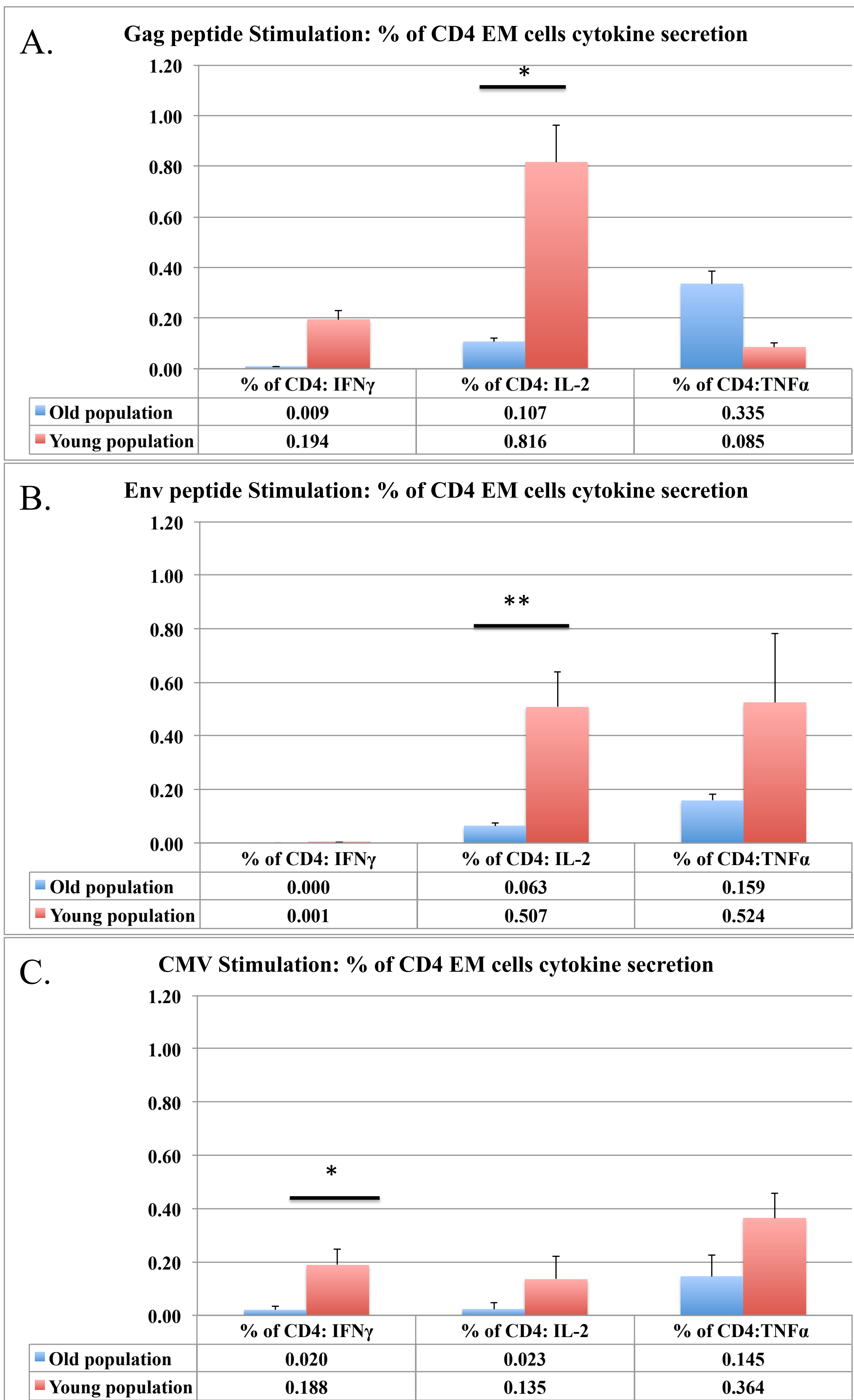
2. Younger rhesus macaques showed greater T cell responses after antigen-specific stimulation (CMV, SIVenv peptide, SIVgag peptide)

2a. Percentage of cytokine production from $CD4^+$ and $CD8^+$ T cell subsets

Apart from observing the immune responses upon generalized T-cell activation, an antigen-specific response was also tested. This observation of antigen specific immune responses is a step closer to those immune responses seen upon the actual infection. Immunological mechanisms involved in protective immunity against lentiviral infections are crucial to the development of an effective vaccine. In the aspect of HIV infection, antigen-specific T cells responses also play a key role during acute and chronic HIV infection. Since test subjects were not infected with SIV, the secretion of specific immune response is not highly anticipated. However, the immune response activated by SIV peptides in these pre-infected subjects are expected to be produced by cross reactivity between the immune cells and SIV peptides. This study will allow the exploration of the ability for pre-infected RM to react to SIV peptides which might be correlated to the immune activation post infection. Antigen-specific responses in $CD4^+$ and $CD8^+$ T cells were then observed in this experiment. To investigate this, PBMC of 20 uninfected RM (10 young and 10 old) were stimulated with SIVgag peptide and SIVenv peptide as well as CMV to test for the potential of the cells to response to different antigen specific stimulants. HIV/AIDS-relevant parameters will be assessed and compared between the two age groups. RM PBMC were treated with 2.5ug/mL of SIVgag peptive, SIVenv peptide and CMV peptide. CMV peptide was used as a control to assess the specificity of the SIV-related peptide upon stimulation. I then assessed the percentages of the cytokine producing cells, absolute numbers of cytokine producing cells and the mean fluorescence intensity (MFI) of each cytokine. Both single-cytokine

secreting cells and multifunctional cells were assessed. Gating procedure and the classification of the T-cell subsets were also tested as mentioned in the previous experiment.

Figure 15 shows the percentages of cytokine production from $CD4^+$ effector memory (EM) cells with significant differences between the two age groups on the production of IL-2 upon both SIVgag (Figure 15A) and SIVenv (figure 15B) treatment. However, a significant difference of IFN-γ producing cells was observed in CMV treatment group. As for the percentages of multifunctional $CD4^+$ EM cells (Figures 15D-15F), young population had significantly higher percentage of IFN-γ^+ IL-2^+ TNF-α^+ producing cells upon stimulation with SIVgag peptide (Figure 15D) and higher percentage of IL-2^+ TNF-α^+ producing cells upon stimulation with CMV peptide (Figure 15F). But no significant differences were seen upon SIVenv peptide stimulation (Figure 15E). The other subset of $CD4^+$ upon antigen-specific stimulation that showed a significant difference was the percentage of IFN-γ -producing naïve $CD4^+$ T-cells upon CMV treatment (Figure 16C) and interestingly, no significant differences were observed in neither SIVgag nor SIVenv peptide stimulated group (Figure 16A-16B).


A.
Gag peptide Stimulation: % of CD4 EM cells cytokine secretion
*

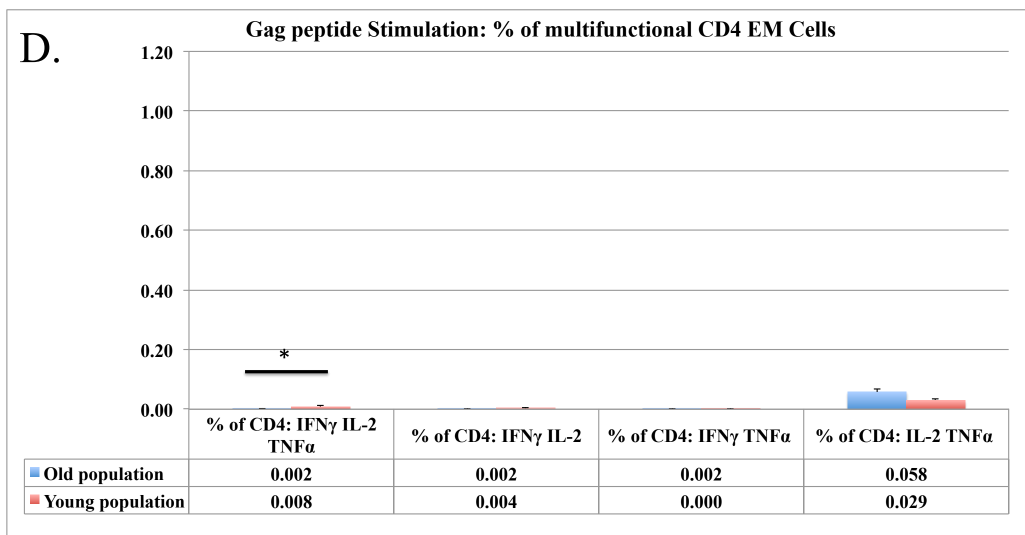
	% of CD4: IFNγ	% of CD4: IL-2	% of CD4:TNFα
Old population	0.009	0.107	0.335
Young population	0.194	0.816	0.085

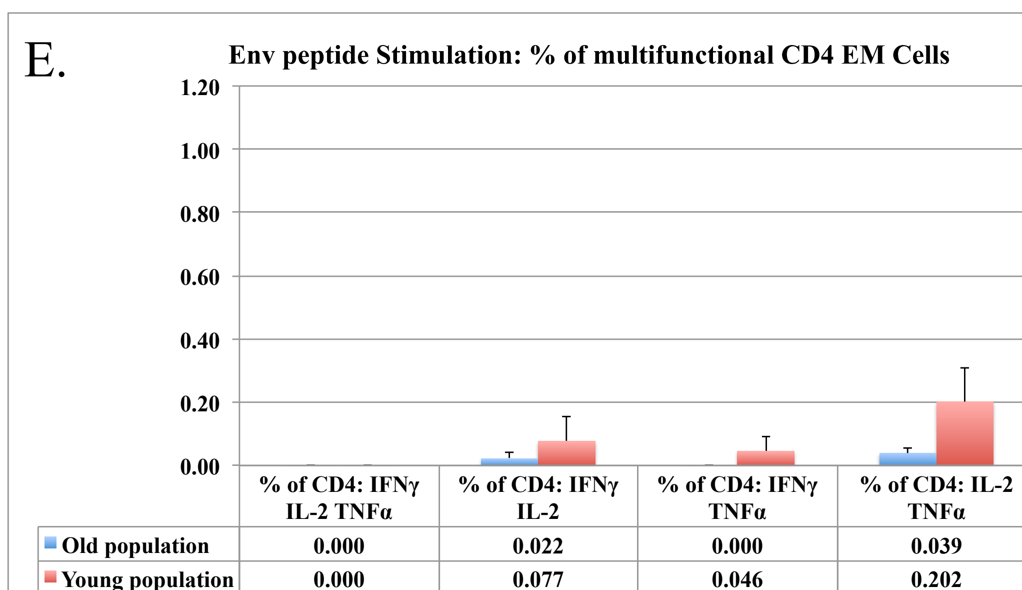
B.
Env peptide Stimulation: % of CD4 EM cells cytokine secretion
**

	% of CD4: IFNγ	% of CD4: IL-2	% of CD4:TNFα
Old population	0.000	0.063	0.159
Young population	0.001	0.507	0.524

C.
CMV Stimulation: % of CD4 EM cells cytokine secretion
*

	% of CD4: IFNγ	% of CD4: IL-2	% of CD4:TNFα
Old population	0.020	0.023	0.145
Young population	0.188	0.135	0.364


D.
Gag peptide Stimulation: % of multifunctional CD4 EM Cells
1.20
1.00
0.80
0.60
0.40
0.20
0.00
*
% of CD4: IFNγ IL-2 TNFα
% of CD4: IFNγ IL-2
% of CD4: IFNγ TNFα
% of CD4: IL-2 TNFα
Old population 0.002 0.002 0.002 0.058
Young population 0.008 0.004 0.000 0.029


E.
Env peptide Stimulation: % of multifunctional CD4 EM Cells
1.20
1.00
0.80
0.60
0.40
0.20
0.00
% of CD4: IFNγ IL-2 TNFα
% of CD4: IFNγ IL-2
% of CD4: IFNγ TNFα
% of CD4: IL-2 TNFα
Old population 0.000 0.022 0.000 0.039
Young population 0.000 0.077 0.046 0.202

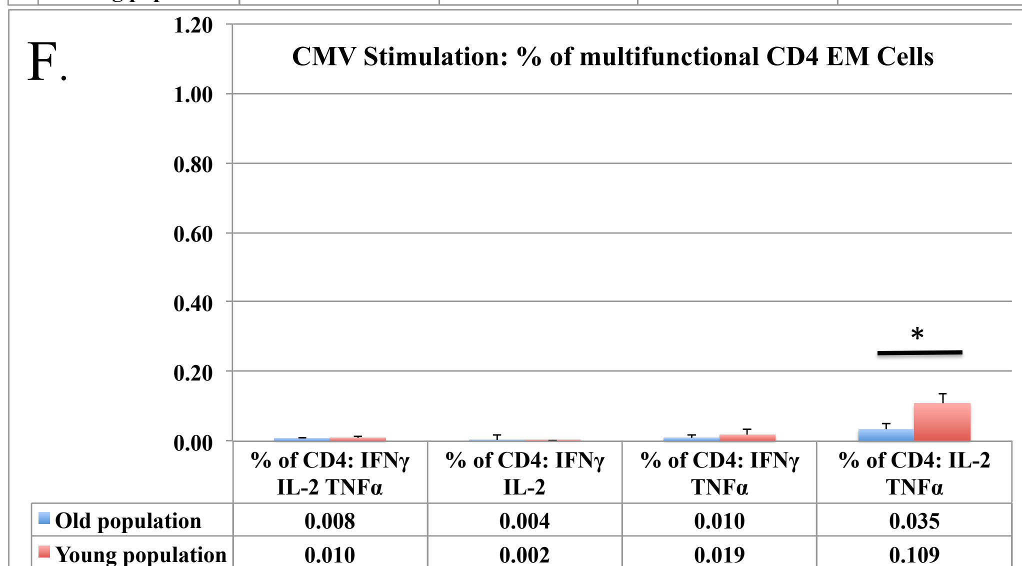
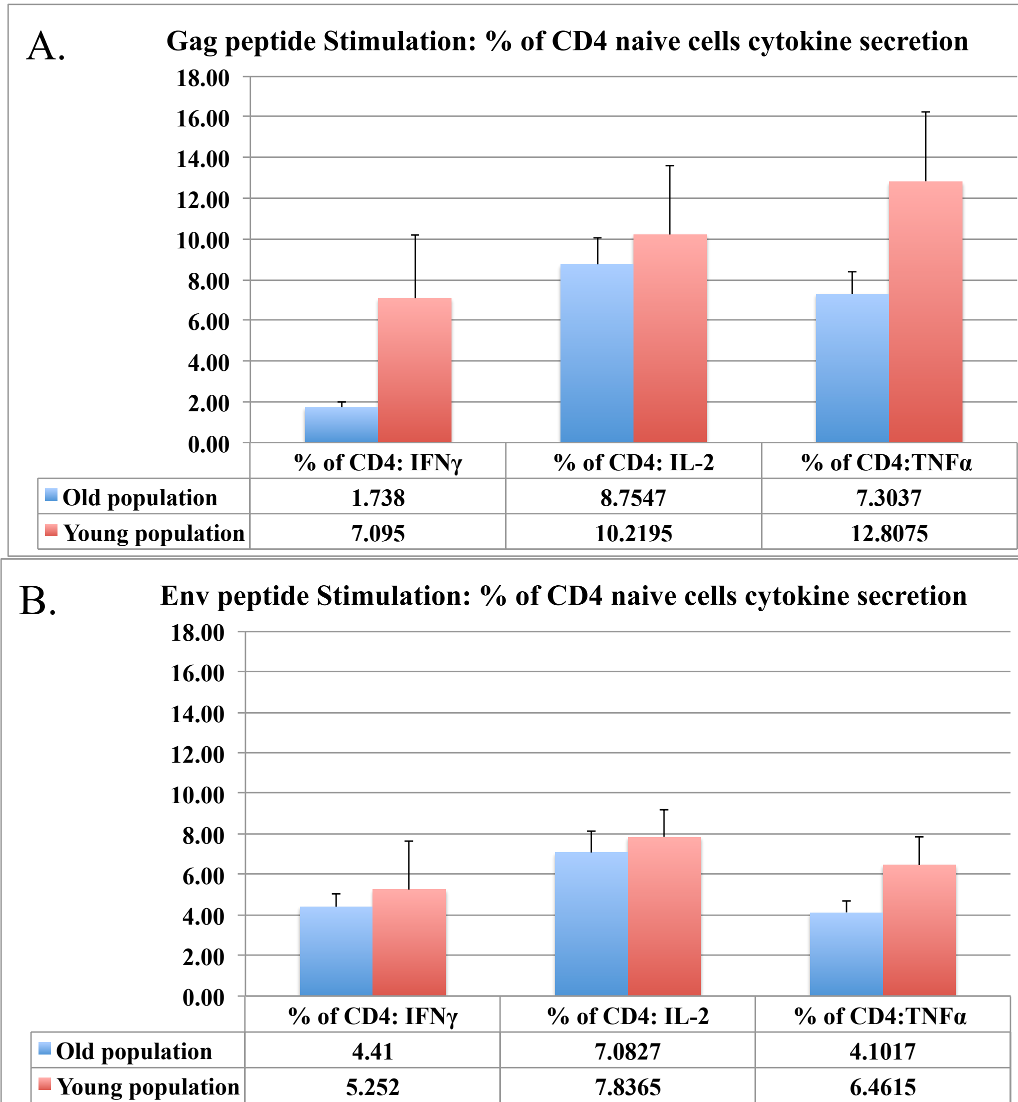
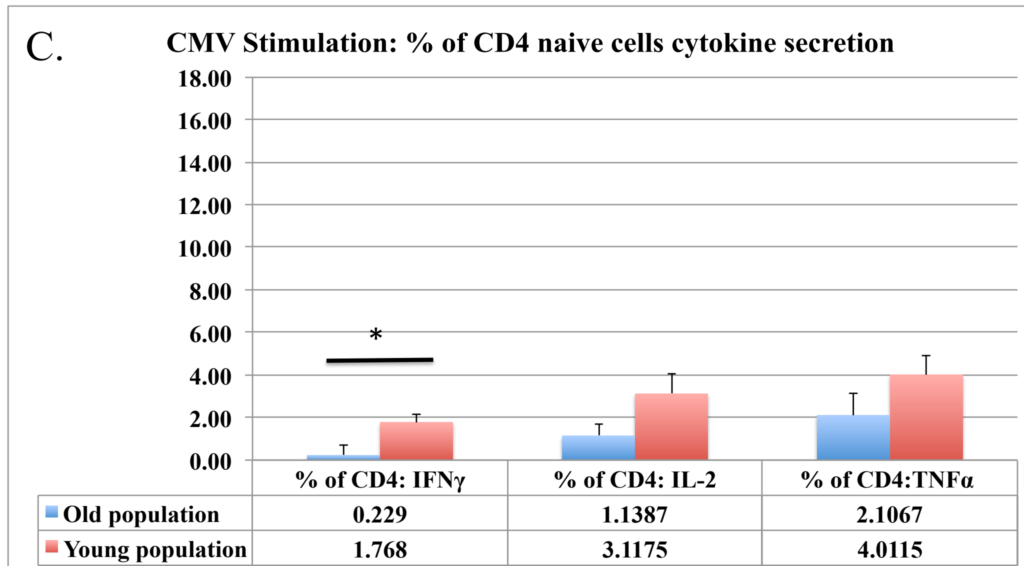

F.
CMV Stimulation: % of multifunctional CD4 EM Cells
1.20
1.00
0.80
0.60
0.40
0.20
0.00
*
% of CD4: IFNγ IL-2 TNFα
% of CD4: IFNγ IL-2
% of CD4: IFNγ TNFα
% of CD4: IL-2 TNFα
Old population 0.008 0.004 0.010 0.035
Young population 0.010 0.002 0.019 0.109

Figure 16: Percentages of cytokine production in effector memory (EM) CD4^{+} T-cells and multifunctional EM CD4^{+} T-cells. (A) Percentage of cytokine secretion from CM CD4^{+} T-cells after SIVgag peptide stimulation. (B) Percentage of cytokine secretion from EM CD4^{+} T-cells after SIVenv peptide stimulation. (C) Percentage of cytokine secretion from EM CD4^{+} T-cells after CMV peptide stimulation (D) Percentage of multifunctional EM CD4^{+} T-cells after SIVgag peptide stimulation, (E) SIVenv peptide stimulation and (F) CMV peptide stimulation.



	% of CD4: IFNγ	% of CD4: IL-2	% of CD4:TNFα
Old population	1.738	8.7547	7.3037
Young population	7.095	10.2195	12.8075

	% of CD4: IFNγ	% of CD4: IL-2	% of CD4:TNFα
Old population	4.41	7.0827	4.1017
Young population	5.252	7.8365	6.4615

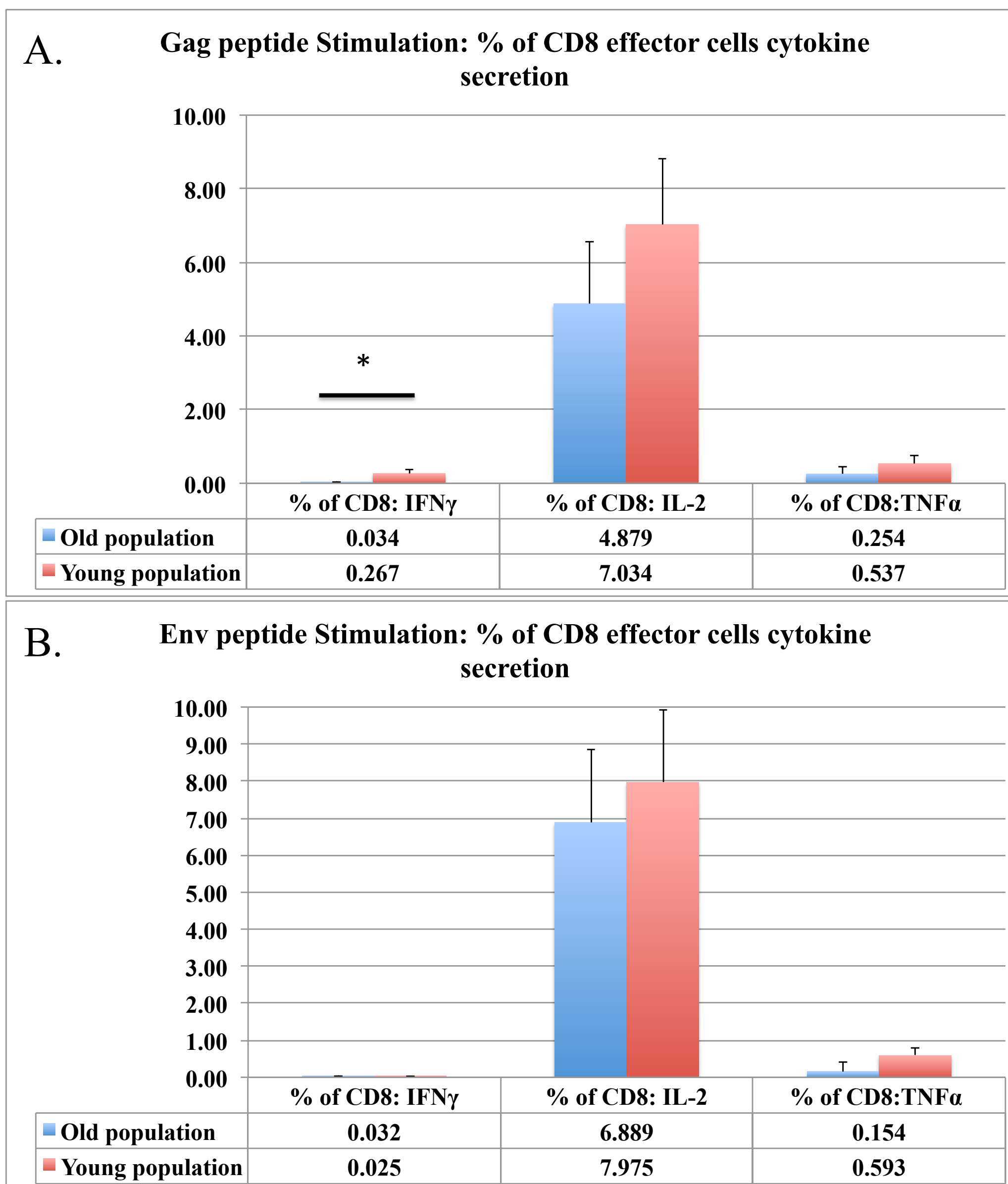


	% of CD4: IFNγ	% of CD4: IL-2	% of CD4:TNFα
Old population	0.229	1.1387	2.1067
Young population	1.768	3.1175	4.0115

Figure 17: Percentages of cytokine production in $CD4^+$ naïve T-cells and multifunctional naive $CD4^+$ T-cells. (A) Percentage of cytokine secretion from $CD4^+$ naïve T-cells upon SIVgag peptide stimulation, (B) SIVenv peptide stimulation and (C) CMV peptide stimulation.

As for the subset of $CD8^+$ T-cells, more significant differences were obsereved in the subsets of $CD8^+$ effector cells (Figure 17), $CD8^+$ CM cells (Figure 18) and $CD8^+$ EM cells (Figure 19). In the subset of $CD8^+$ effector cells, a significant difference was seen in percentage of IFN-γ -producing cells upon SIVgag peptide stimulation (Figure 17A) and another significant difference was observed in the percentage of IL-2-producing $CD8^+$ effector cells upon CMV stimulation (Figure 17C). For the central memory compartment of $CD8^+$, the young population only had a significantly higher percentage of IL-2 secretion upon the treatment with SIVenv peptide (Figure 18B), while no significant differences were found in the groups stimulated with SIVgag peptide (Figure 18A) nor CMV peptide (Figure 18C). The last group is $CD8^+$ EM cells, with a critically higher percentages of IL-2 secreting $CD8^+$ EM cells in the young upon SIVgag peptide stimulation (***p-value <0.000) and as well as upon SIVenv peptide stimulation

(* p-value <0.05) (Figure 19A-19B). Yet, no significant values were found upon CMV stimulated group (Figure 19C).



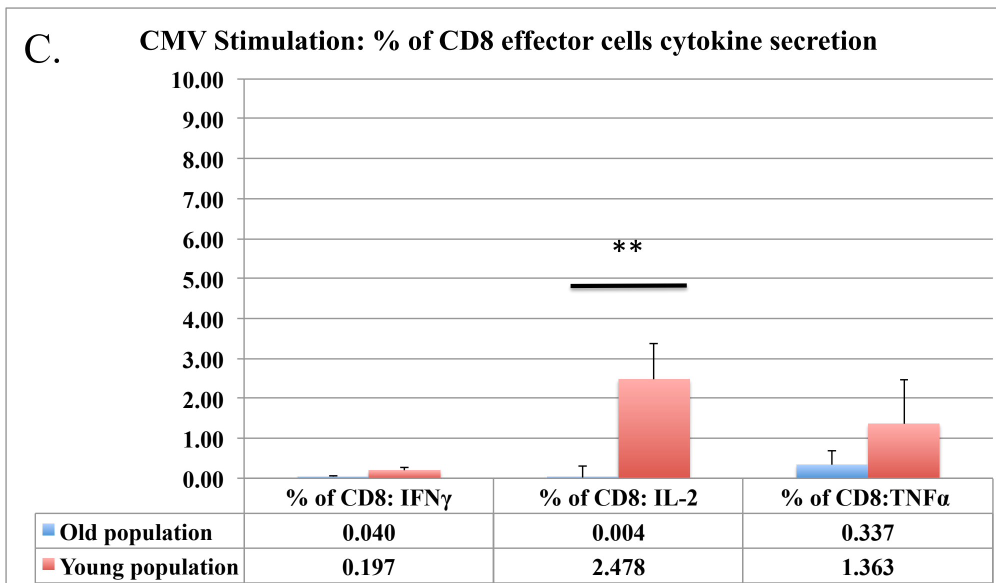
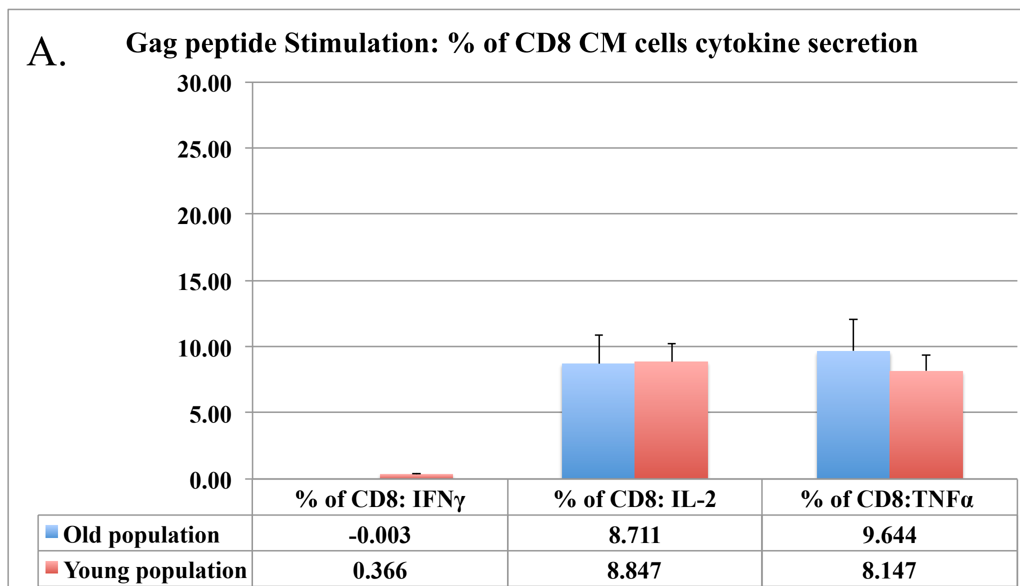
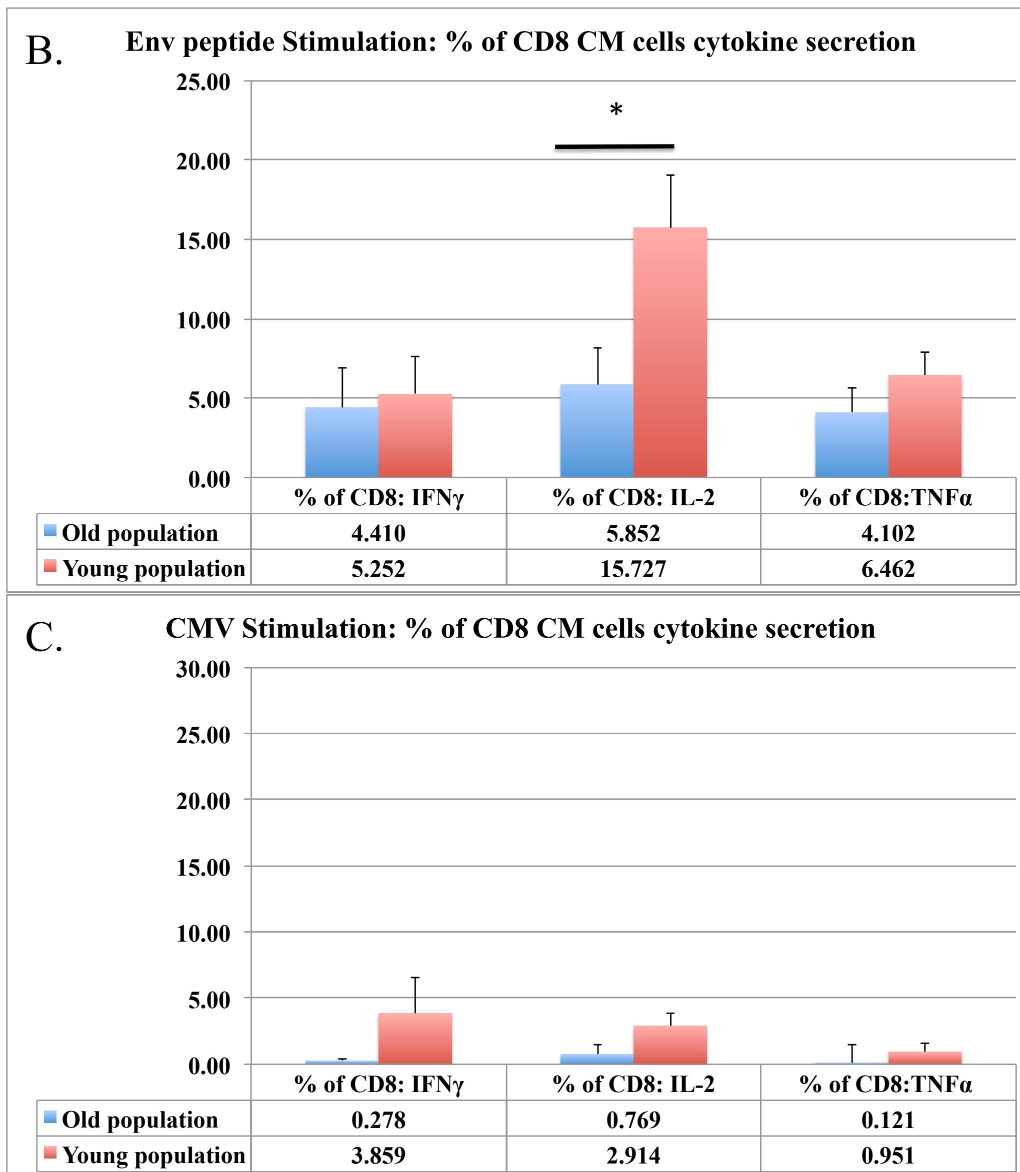


Figure 18: Percentages of cytokine production in CD8^{+} effector T-cells (A) Percentage of cytokine secretion from CD8^{+} effector T-cells upon SIVgag peptide stimulation, (B) SIVenv peptide stimulation and (C) CMV peptide stimulation.

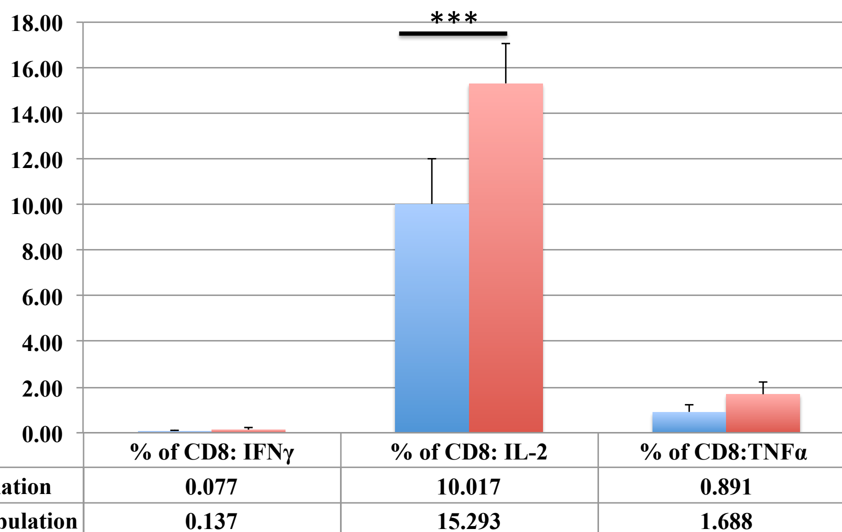




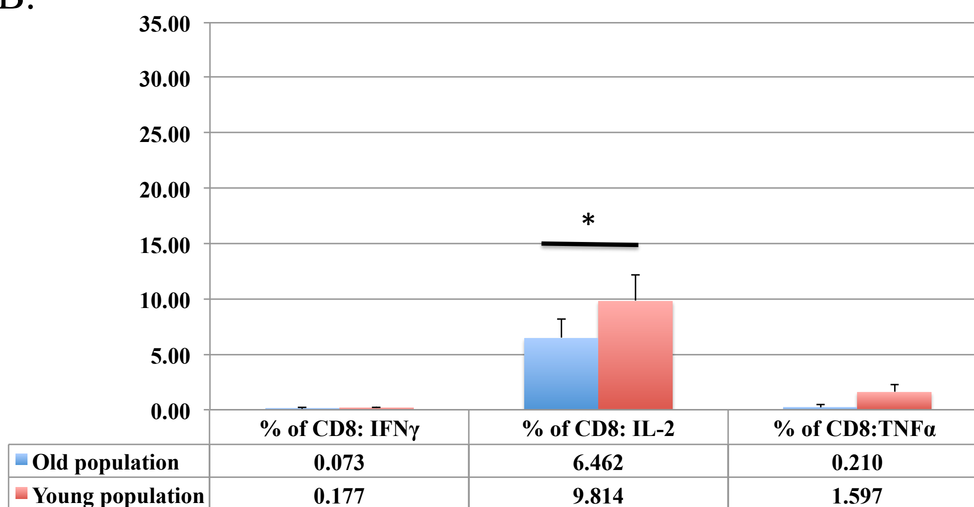
	% of CD8: IFNγ	% of CD8: IL-2	% of CD8:TNFα
Old population	4.410	5.852	4.102
Young population	5.252	15.727	6.462

	% of CD8: IFNγ	% of CD8: IL-2	% of CD8:TNFα
Old population	0.278	0.769	0.121
Young population	3.859	2.914	0.951

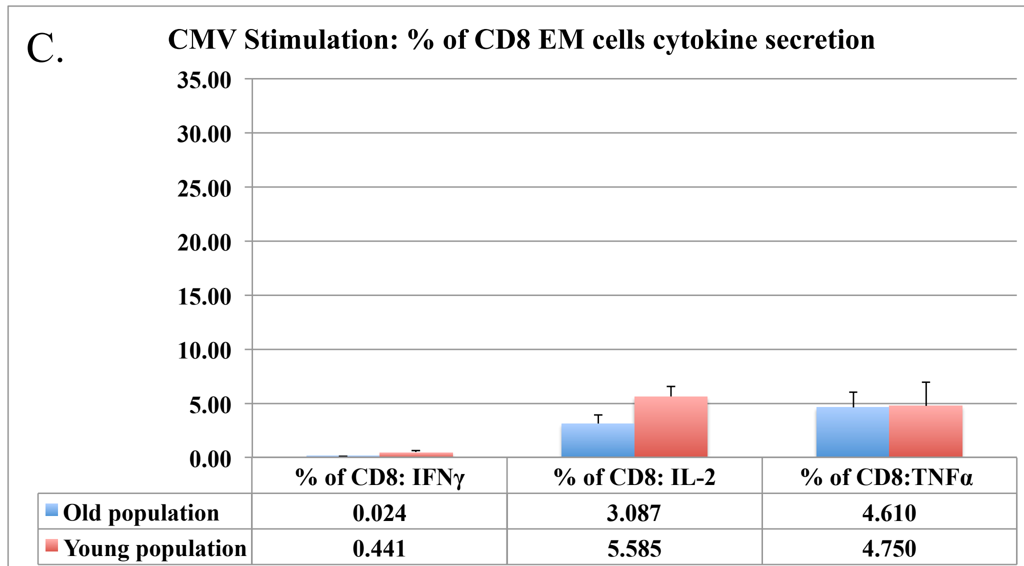
Figure 19: Percentages of cytokine production in CM CD8$^+$ T-cells and multifunctional CM CD8$^+$ T-cells. (A) Percentage of cytokine secretion from CM CD8$^+$ T-cells upon SIVgag peptide stimulation, (B) SIVenv peptide stimulation and (C) CMV peptide stimulation.



	% of CD8: IFNγ	% of CD8: IL-2	% of CD8:TNFα
Old population	0.077	10.017	0.891
Young population	0.137	15.293	1.688



	% of CD8: IFNγ	% of CD8: IL-2	% of CD8:TNFα
Old population	0.073	6.462	0.210
Young population	0.177	9.814	1.597



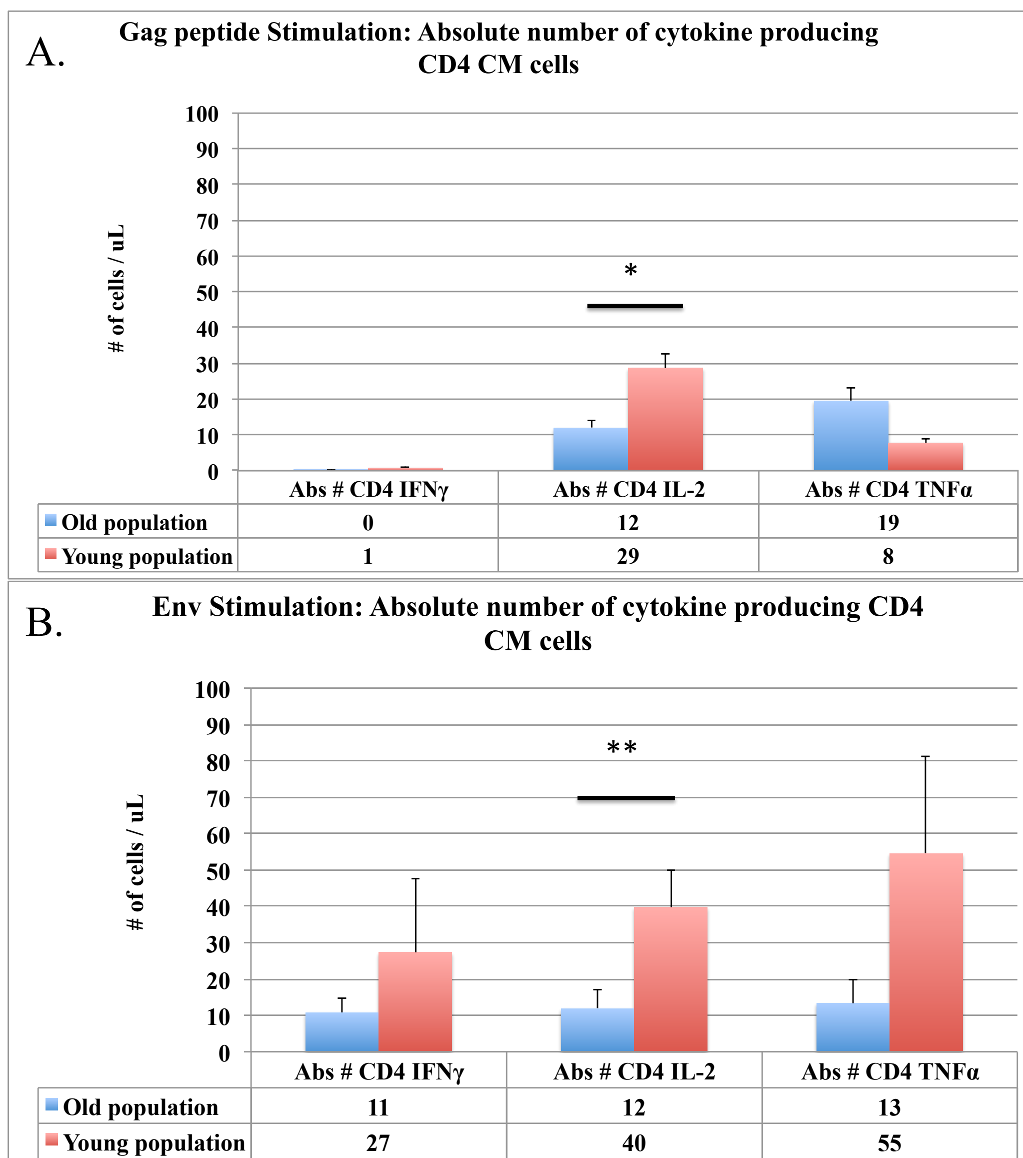
	% of CD8: IFNγ	% of CD8: IL-2	% of CD8:TNFα
Old population	0.024	3.087	4.610
Young population	0.441	5.585	4.750

Figure 20: Percentages of cytokine production in EM CD8^{+} T-cells and multifunctional EM CD8^{+} T-cells. (A) Percentage of cytokine secretion from EM CD8^{+} T-cells upon SIVgag peptide stimulation, (B) SIVenv peptide stimulation and (C) CMV peptide stimulation.

2b. Absolute number of cytokine producing CD4^{+} and CD8^{+} T cell subsets

The protocol for calculaing the absolute number of cytokine producing T cells upon antigen specific stimulation is the same as the one mentioned in the previous section of mass stimulation. Starting with the significant differences in the subset of CD4^{+} CM cells. The significant differences between the two age groups in the absolute count in IL-2 producing cells were seen in all of the treatment groups, SIVgag, SIVenv and CMV peptide stimulations (Figure 20A-C). However, an additional significant difference was seen upon CMV peptide stimulation in the absolute count of TNFα-producing cells (Figure 20C). For CD4^{+} EM T cells subset, significant differences were observed in all of the treatment groups. Upon SIVgag and SIVenv peptide stimulation, younger population showed a significantly higher count in IL-2 secreting cells (Figure 21A-B). On the other hand, significant differences were seen in single cytokine secreting CD4^{+} EM cells that were secreting IFN-γ , IL-2, and TNF-α in the subset treated with CMV

peptide (Figure 21C). The last group of CD4^{+} that showed a significant difference is the CD4^{+} naïve T cells with a significant difference in the count of TNF-α secreting cells upon SIVgag peptide stimulation (Figure 22A). In contrast, no other significant differences were seen upon other peptide treatments (Figure 22B-C). Interestingly, no significant differences were observed in the absolute count of multifunction cells of CD4^{+} and CD8^{+} (not shown).



	Abs # CD4 IFNγ	Abs # CD4 IL-2	Abs # CD4 TNFα
Old population	0	12	19
Young population	1	29	8

	Abs # CD4 IFNγ	Abs # CD4 IL-2	Abs # CD4 TNFα
Old population	11	12	13
Young population	27	40	55

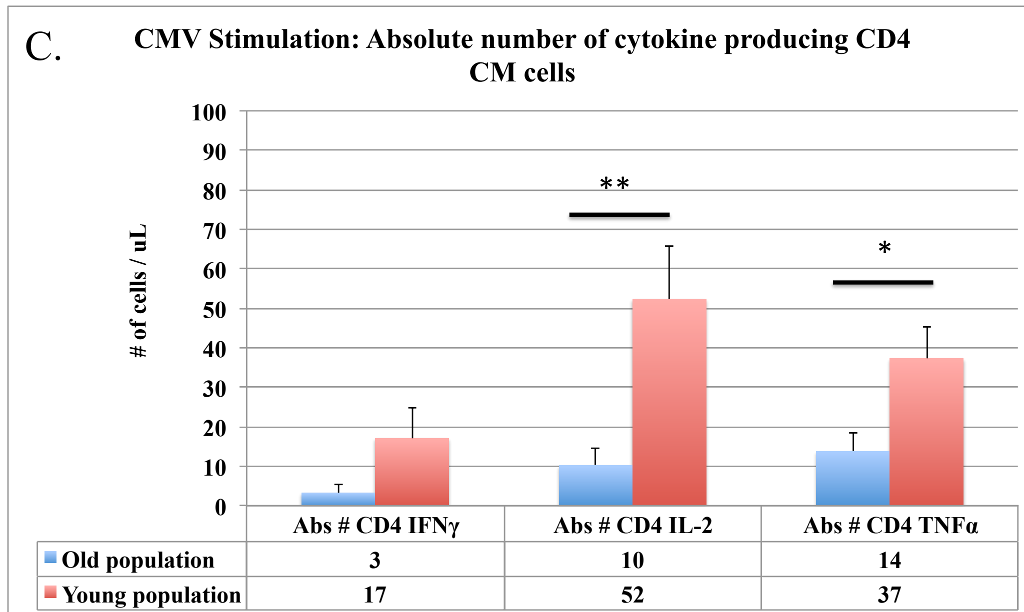
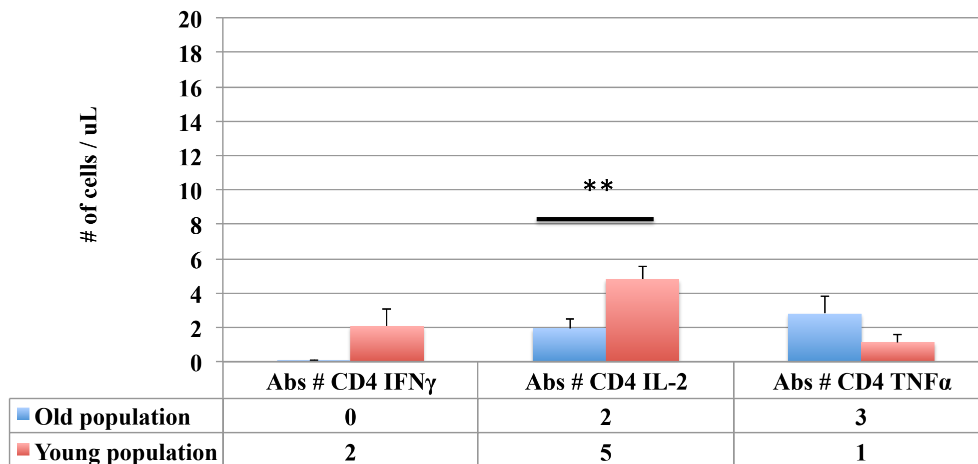
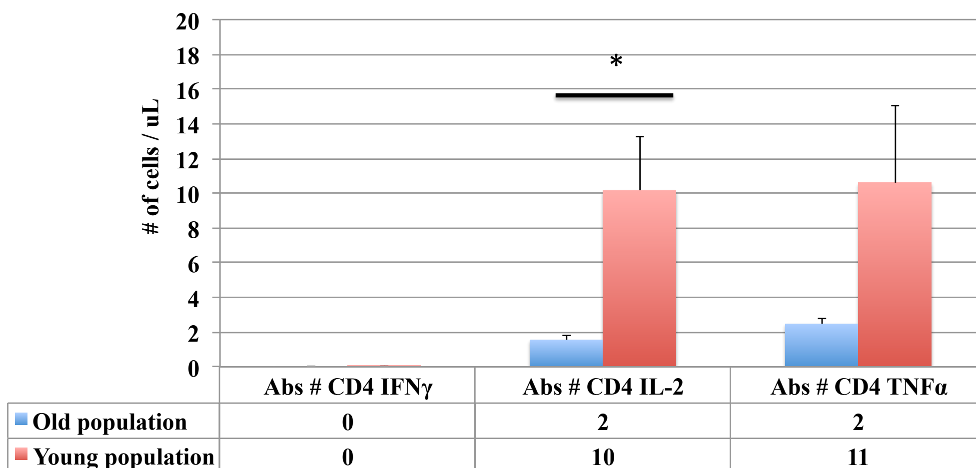


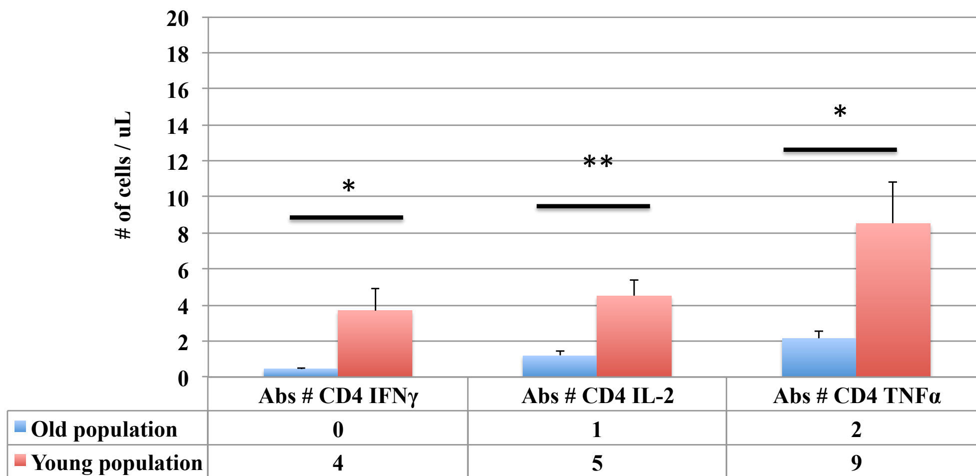
Figure 21: Absolute number of cytokine producing CM $CD4^{+}$ T cells upon (A) SIVgag peptide stimulation (B) SIVenv peptide stimulation and (C) CMV peptide stimulation.



	Abs # CD4 IFNγ	Abs # CD4 IL-2	Abs # CD4 TNFα
Old population	0	2	3
Young population	2	5	1

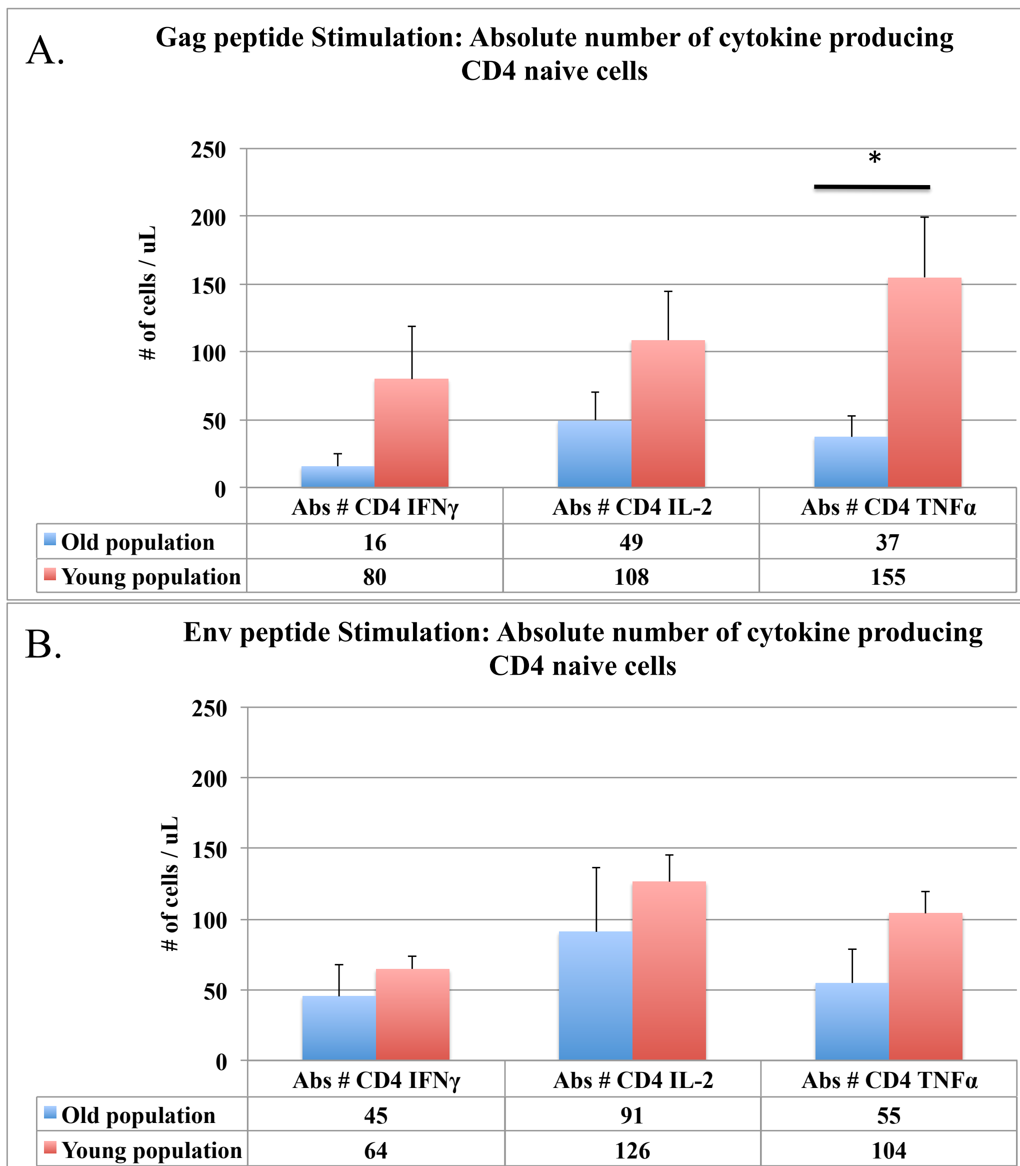


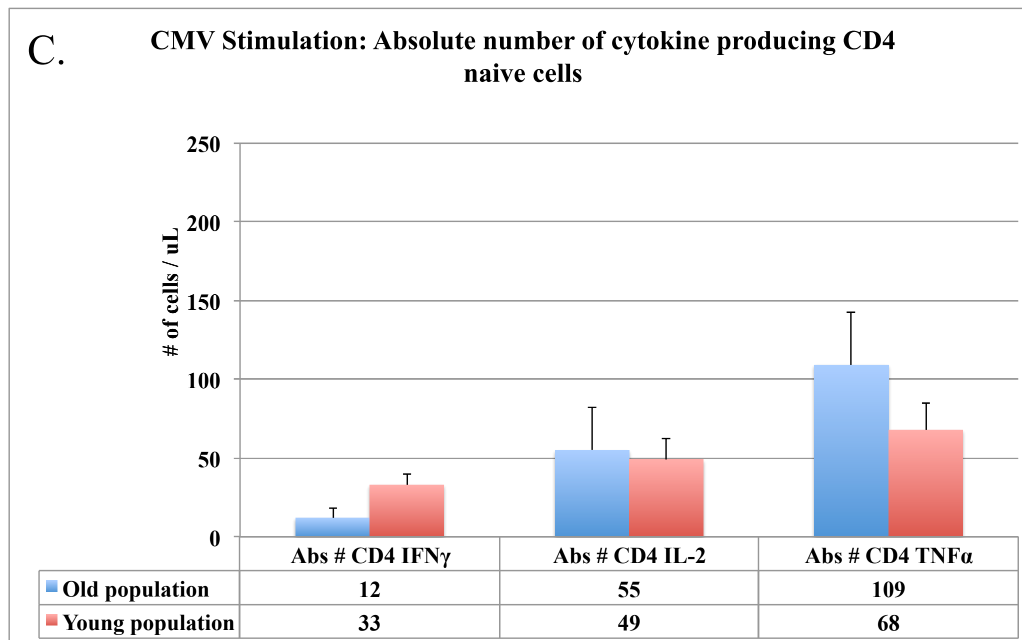
	Abs # CD4 IFNγ	Abs # CD4 IL-2	Abs # CD4 TNFα
Old population	0	2	2
Young population	0	10	11



	Abs # CD4 IFNγ	Abs # CD4 IL-2	Abs # CD4 TNFα
Old population	0	1	2
Young population	4	5	9

Figure 22: Absolute number of cytokine producting EM CD4^{+} T-cells upon (A) SIVgag peptide stimulation (B) SIVenv peptide stimulation and (C) CMV peptide stimulation.



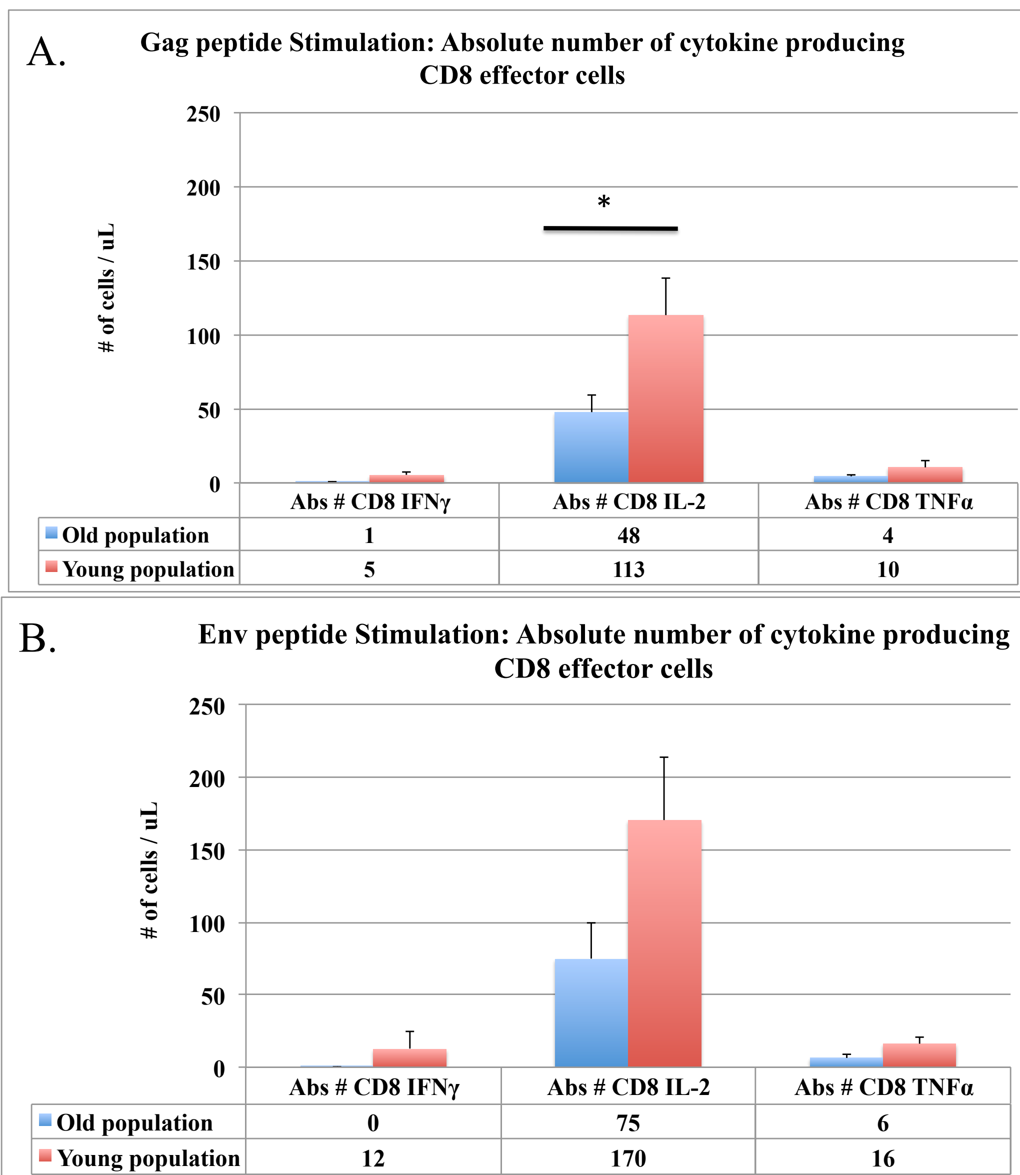


	Abs # CD4 IFNγ	Abs # CD4 IL-2	Abs # CD4 TNFα
Old population	12	55	109
Young population	33	49	68

Figure 23: Absolute number of cytokine producing $CD4^+$ naïve T-cells upon (A) SIVgag peptide stimulation (B) SIVenv peptide stimulation and (C) CMV peptide stimulation.

$CD8^+$ T cells is another set of T cells that were analyzed and the sifinficant differnces were seen in the $CD8^+$ effector T cells, CM and EM subsets. In the subset of $CD8^+$ effector T cells, the siginificant differences between the age groups were seen in the count of IL-2 secreting cells that were treated with SIVgag peptide and CMV peptide (Figure 23A and 23C) but no significant differnces were soon upon SIVenv peptide treatments (Figure 23B). As for $CD8^+$ CM subset, more significant difference were seen across different cytokine secretion. Upon SIVgag peptide stimulation, younger population had a significantly higher count in IFN-γ secreting cells as well as IL-2 secreting cells (Figure 24A) and for the group treated with SIVenv peptide, younger population had a significantly higher count in IL-2 secreting cells and TNF-α secreting cells (Figure 24B). On the other hand, CMV stimulated group showed a significant difference in count in IL-2 secreting cells with ***P-value < 0.000. Lastly, $CD8^+$ EM cells subset upon all three stimulation showed significant differences with younger

population having a high absolute cell count in IL-2 secreting cells (Figure25A-C). Again, no significant differences were detected in the absolute count of any multifunctional cells (not shown).



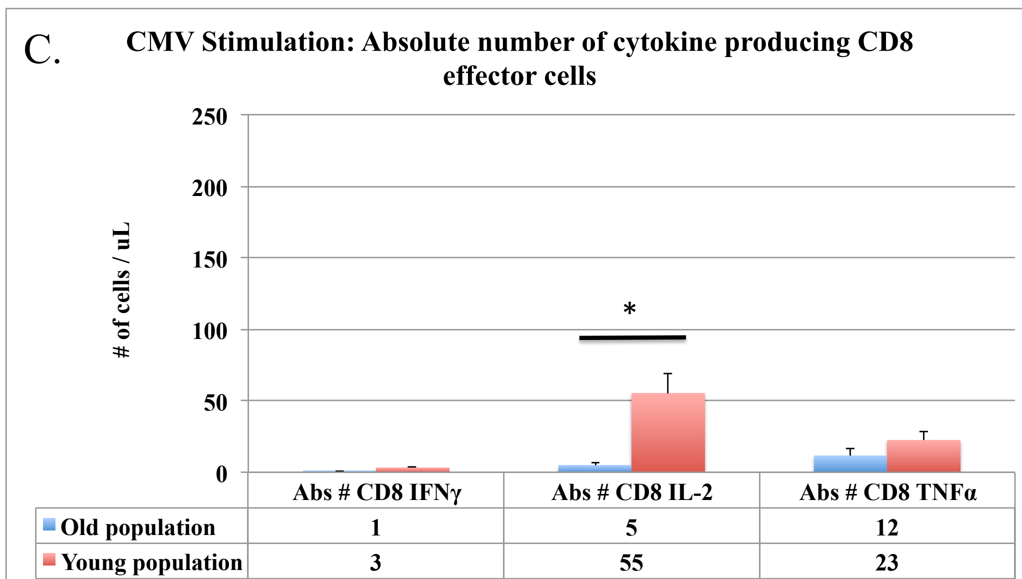
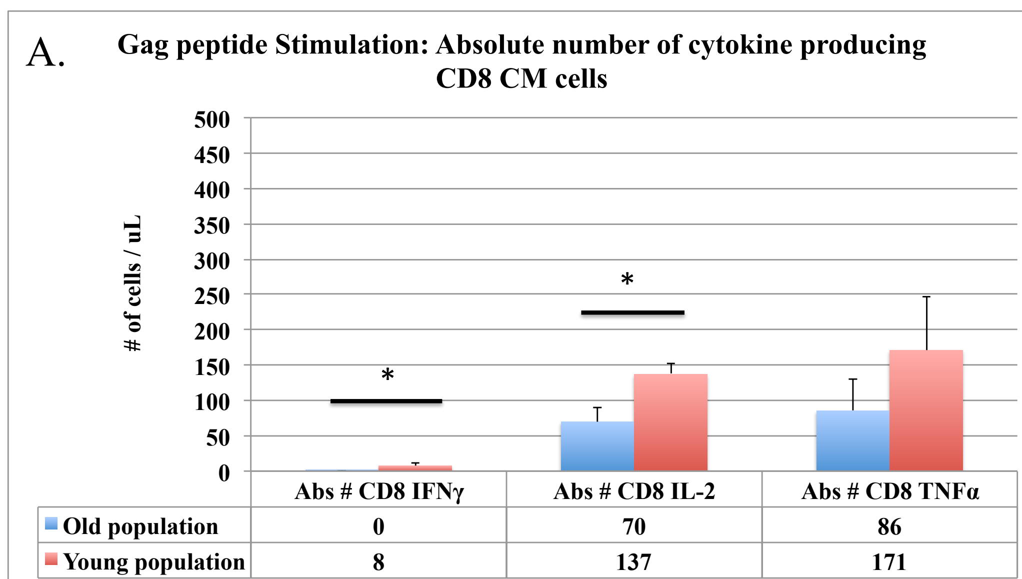
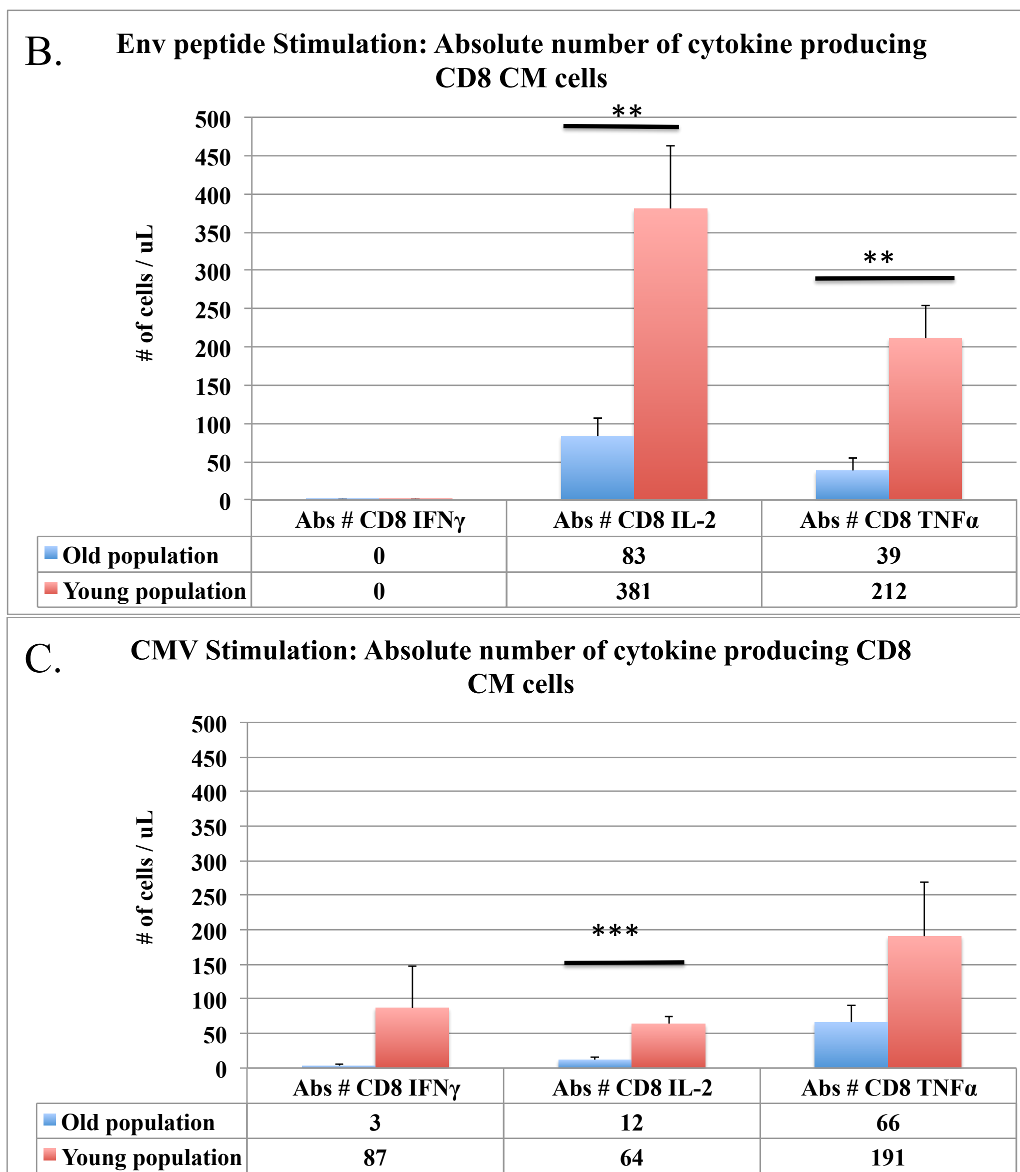


Figure 24: Absolute number of cytokine producting effector CD8+ T-cells upon (A) SIVgag peptide stimulation (B) SIVenv peptide stimulation and (C) CMV peptide stimulation.





	Abs # CD8 IFNγ	Abs # CD8 IL-2	Abs # CD8 TNFα
Old population	0	83	39
Young population	0	381	212

	Abs # CD8 IFNγ	Abs # CD8 IL-2	Abs # CD8 TNFα
Old population	3	12	66
Young population	87	64	191

Figure 25: Absolute number of cytokine producing CM CD8$^+$ T-cells upon (A) SIVgag peptide stimulation (B) SIVenv peptide stimulation and (C) CMV peptide stimulation.

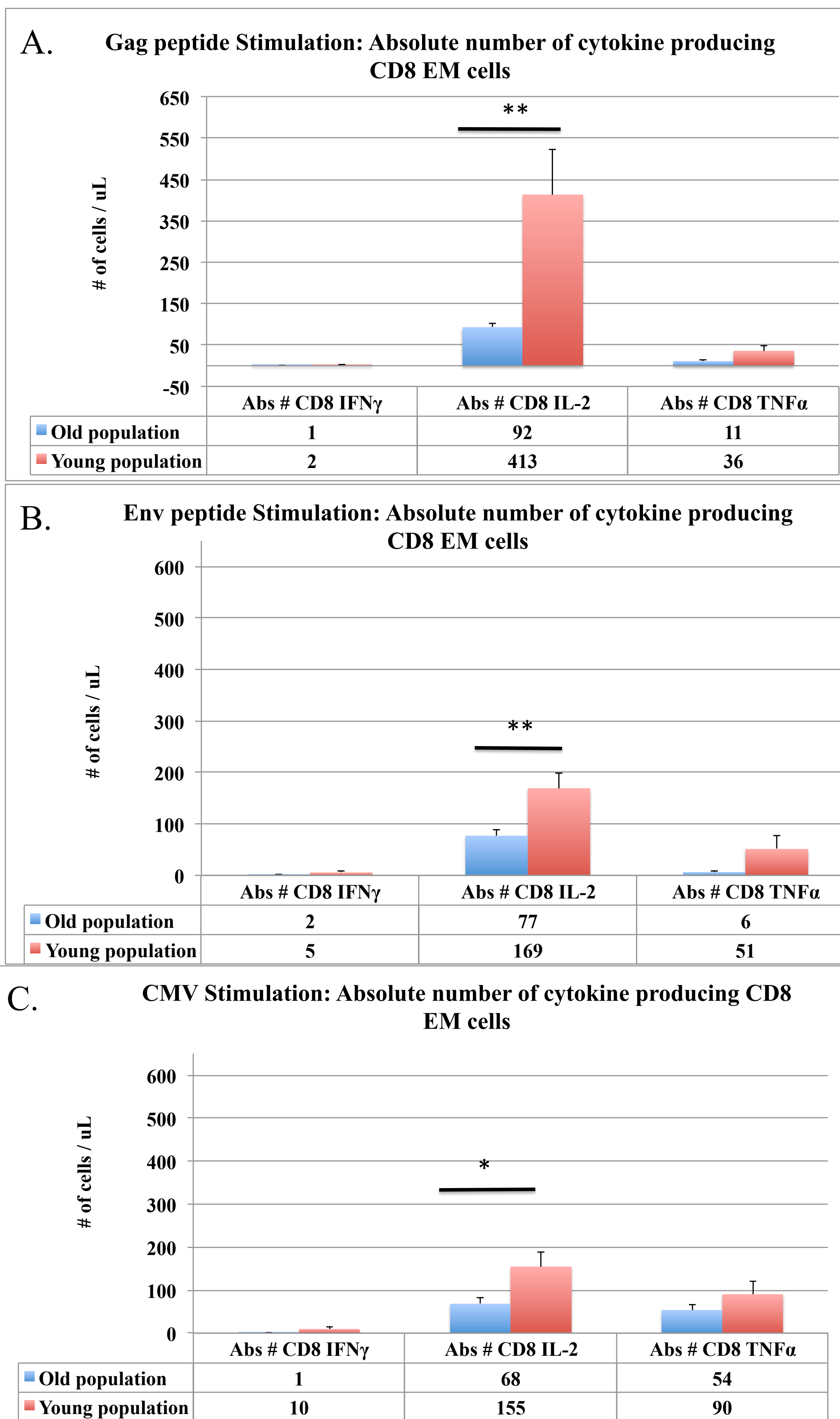
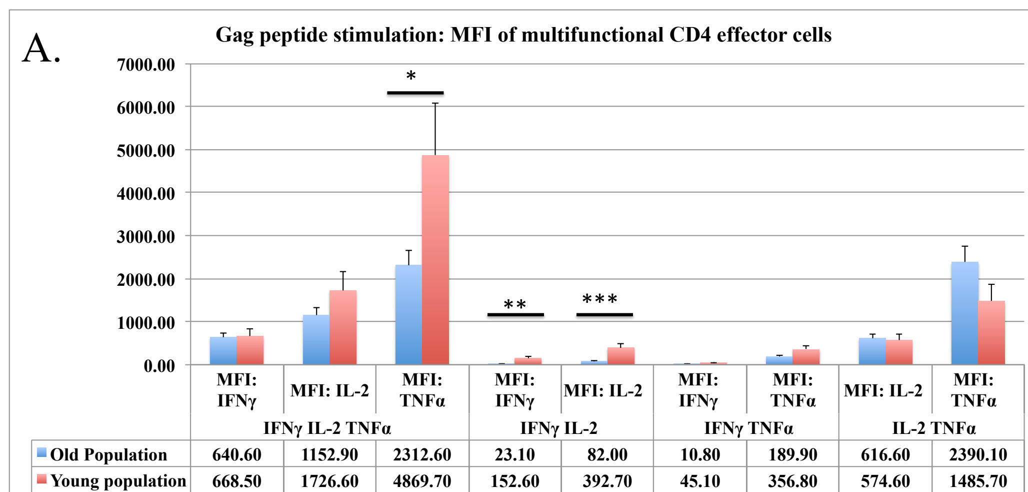

A.
Gag peptide Stimulation: Absolute number of cytokine producing CD8 EM cells
# of cells / uL
650
550
450
350
250
150
50
-50
**
Abs # CD8 IFNγ
Abs # CD8 IL-2
Abs # CD8 TNFα
Old population
1
92
11
Young population
2
413
36
B.
Env peptide Stimulation: Absolute number of cytokine producing CD8 EM cells
# of cells / uL
600
500
400
300
200
100
0
**
Abs # CD8 IFNγ
Abs # CD8 IL-2
Abs # CD8 TNFα
Old population
2
77
6
Young population
5
169
51
C.
CMV Stimulation: Absolute number of cytokine producing CD8 EM cells
# of cells / uL
600
500
400
300
200
100
0
*
Abs # CD8 IFNγ
Abs # CD8 IL-2
Abs # CD8 TNFα
Old population
1
68
54
Young population
10
155
90

Figure 26: Absolute number of cytokine producting EM CD8$^+$ T-cells upon (A) SIVgag peptide stimulation (B) SIVenv peptide stimulation and (C) CMV peptide stimulation.

2c. Mean Fluoresence Intensity (MFI) of of cytokine producing CD4$^+$ and CD8$^+$ T cells subsets

The last analysis for the antigen specific is the MFI analysis. The first observable significant difference appeared in the floresence of TNF-α in multifunctinal effector CD4$^+$ T cells screting IFN-γ$^+$ IL-2$^+$ TNF-α$^+$ upon SIVgag peptide stimulation (Figure 26A) and SIVenv peptide stimulation (Figure 26B). Furthermore, in the group treated with SIVgag peptide, the younger population also showed a higher MFI value in the florescence of both IFN-γ and IL-2 in the multifunctinal effector CD4$^+$ T cells screting IFN-γ$^+$ IL-2$^+$ (Figure 26A). However, no significant differences were seen with CMV stimulation (Figure 26C). Figure 27 shows the MFI data for the multifunctional CD4$^+$ CM cells with significant differences between the two age groups in the flouresence intensity of IFN-γ and IL-2 from the multifunctinal CD4$^+$ CM T cells screting IFN-γ$^+$ IL-2$^+$ upon SIVgag peptide stimulation (Figure 27A) as well as CMV peptide stimulation (Figure 27C). Another set of significant difference was observed upon SIVenv peptide stimulation in the fluorescence intensity of IFN-γ from the multifunctinal CD4$^+$ CM T cells screting all three cytokines IFN-γ, IL-2 and TNF-α (Figure 27B). Interestingly, in the subset of CD4$^+$ EM cells, the only significant differences were seen in the group stimulated with SIVgag peptide which falls showed a significantly higher MFI value in the younger population in the fluorescence intensity of IFN-γ and IL-2 from the multifunctional CD4$^+$ EM T cells screting IFN-γ and IL-2 (Figure 28A). No other significant differences were seen in SIVenv peptide nor CMV peptide treated groups (Figure 28B-C). However, in the subset of naïve CD4$^+$ Tcells, both single cytokine

secreting cells and multifunctional cells had significant differences between the two groups with a higher MFI in the young population in both SIVgag and SIVenv peptide stimulation. Upon SIVgag stimulation, all of the significant differences of MFI were observed in each cytokine (IFN-γ , IL-2 and TNFα) in the single cytokine producing $CD4^+$ naïve cells (Figure 29A) but for SIVenv peptide stimulated group, the significant differences was seen only in IL-2 secreting cells (Figure 29B). As for multifunctional $CD4^+$ naïve cells, again SIVgag treatment group showed a significantly higher MFI value in the younger population in the fluorescence intensity of IFN-γ , IL-2 and TNFα from the multifunctional $CD4^+$ naive T cells screting all three cytokines (IFN-γ , IL-2 and TNFα) (Figure 29D). While SIVenv treated group showed significant difference only in the fluorescence intensity of TNFα in the same type of multifunctional cell which is the multifunctional $CD4^+$ naive T cells screting all three cytokines (IFN-γ , IL-2 and TNFα) (Figure 29E). No significant difference was observed in CMV stimulation (Figure 29C, 29F).



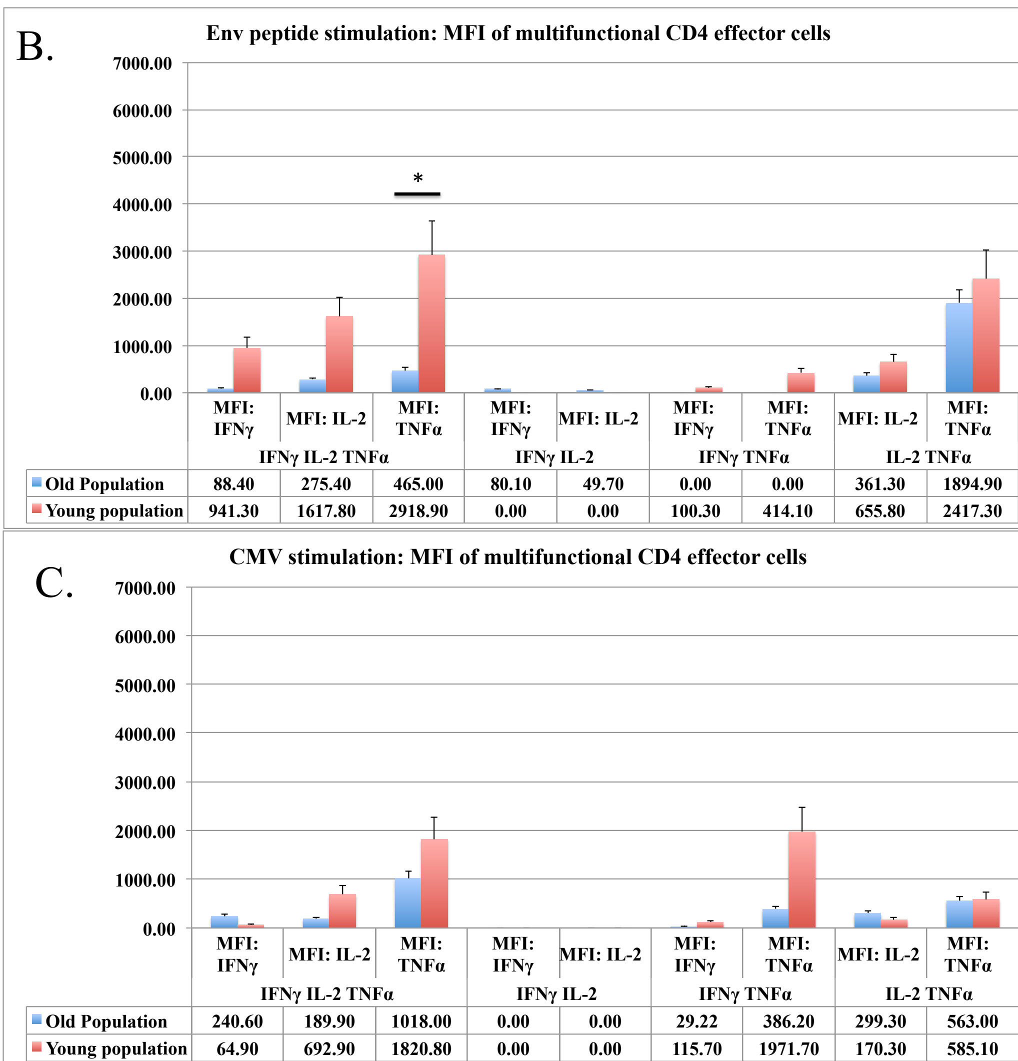
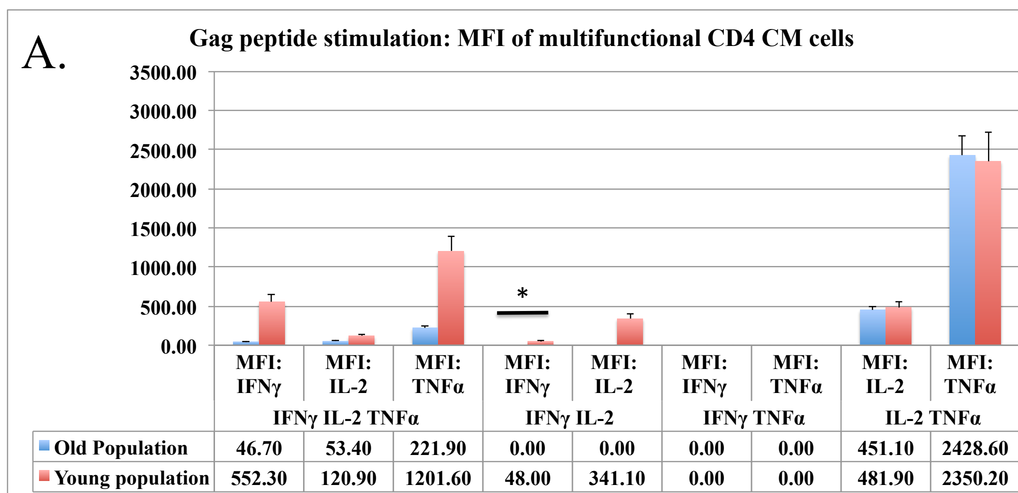


Figure 27: MFI of cytokine producing multifunctional CD4$^+$ effector cells upon (A) SIVgag peptide stimulation (B) SIVenv peptide stimulation and (C) CMV peptide stimulation.



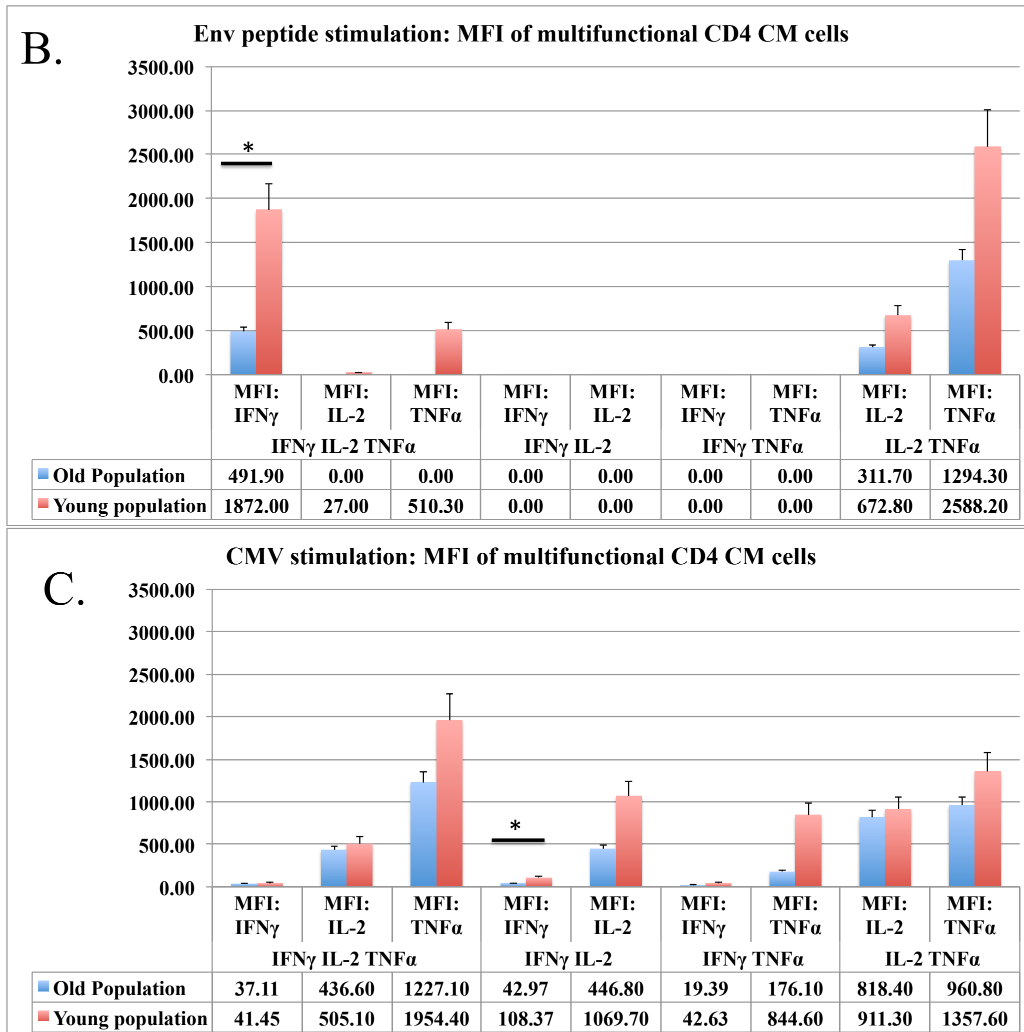
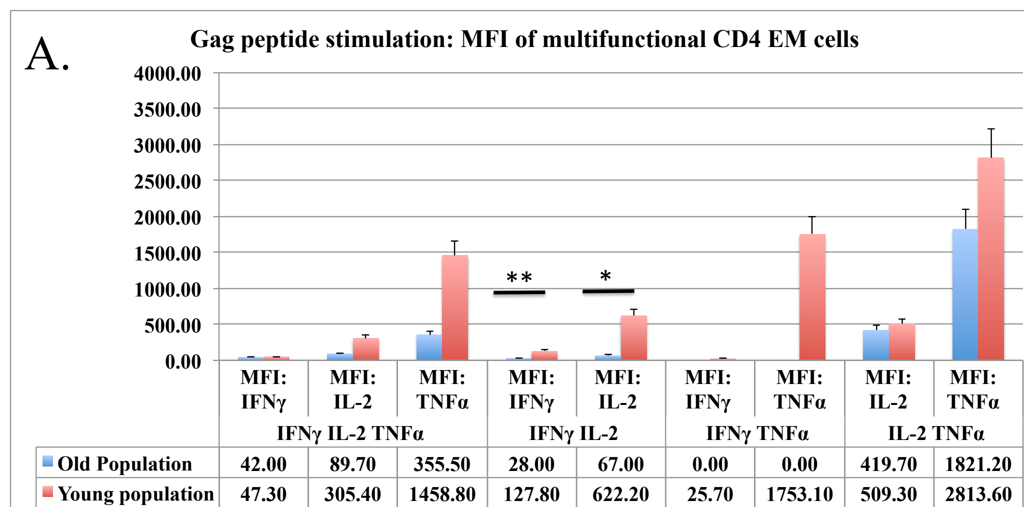


Figure 28: MFI of cytokine producing multifunctional CM CD4^{+} T-cells upon (A) SIVgag peptide stimulation (B) SIVenv peptide stimulation and (C) CMV peptide stimulation.



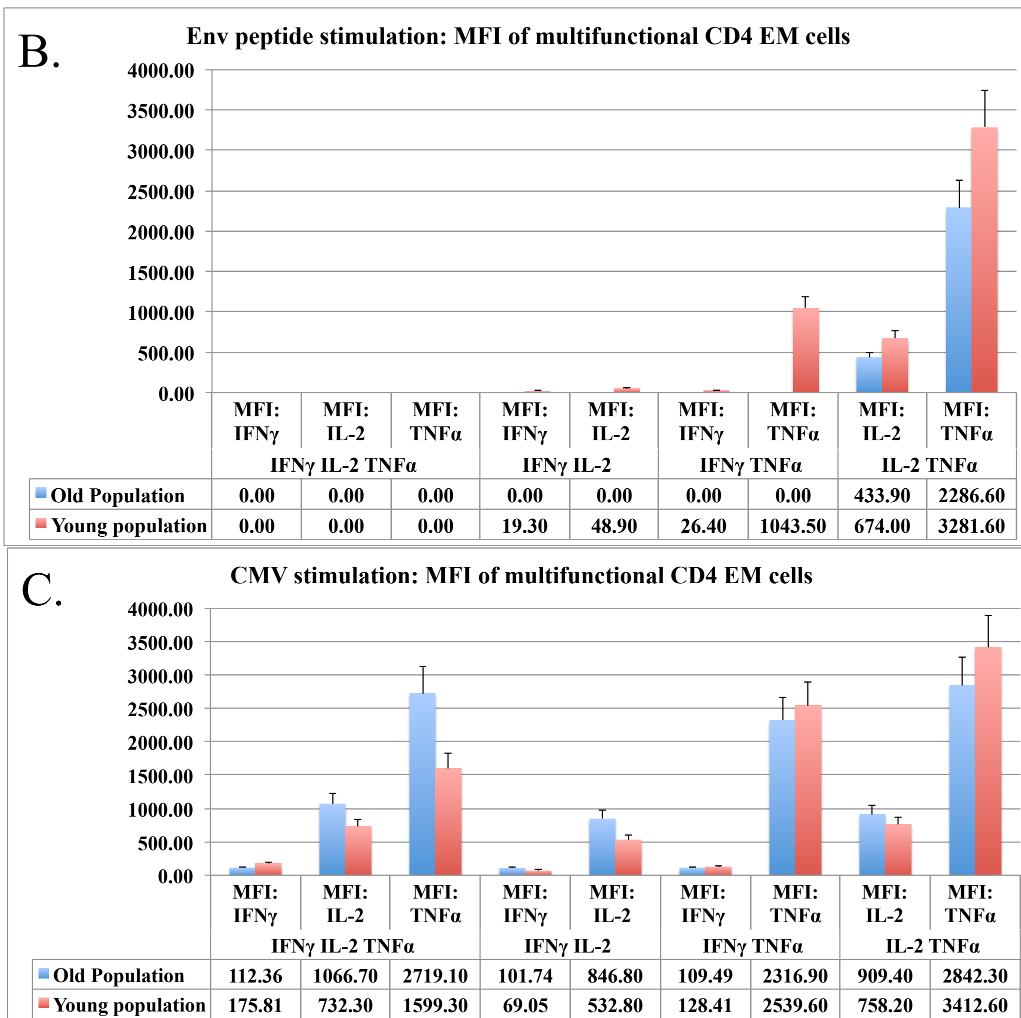
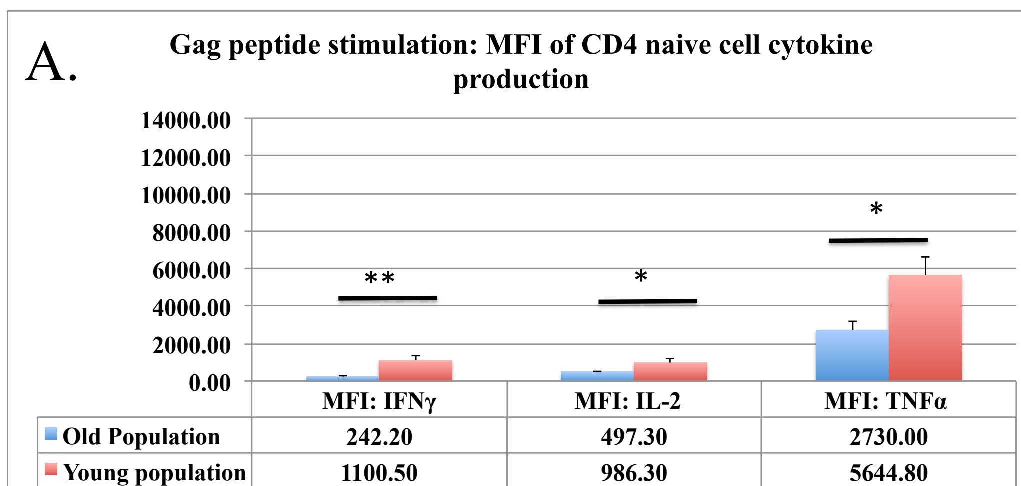
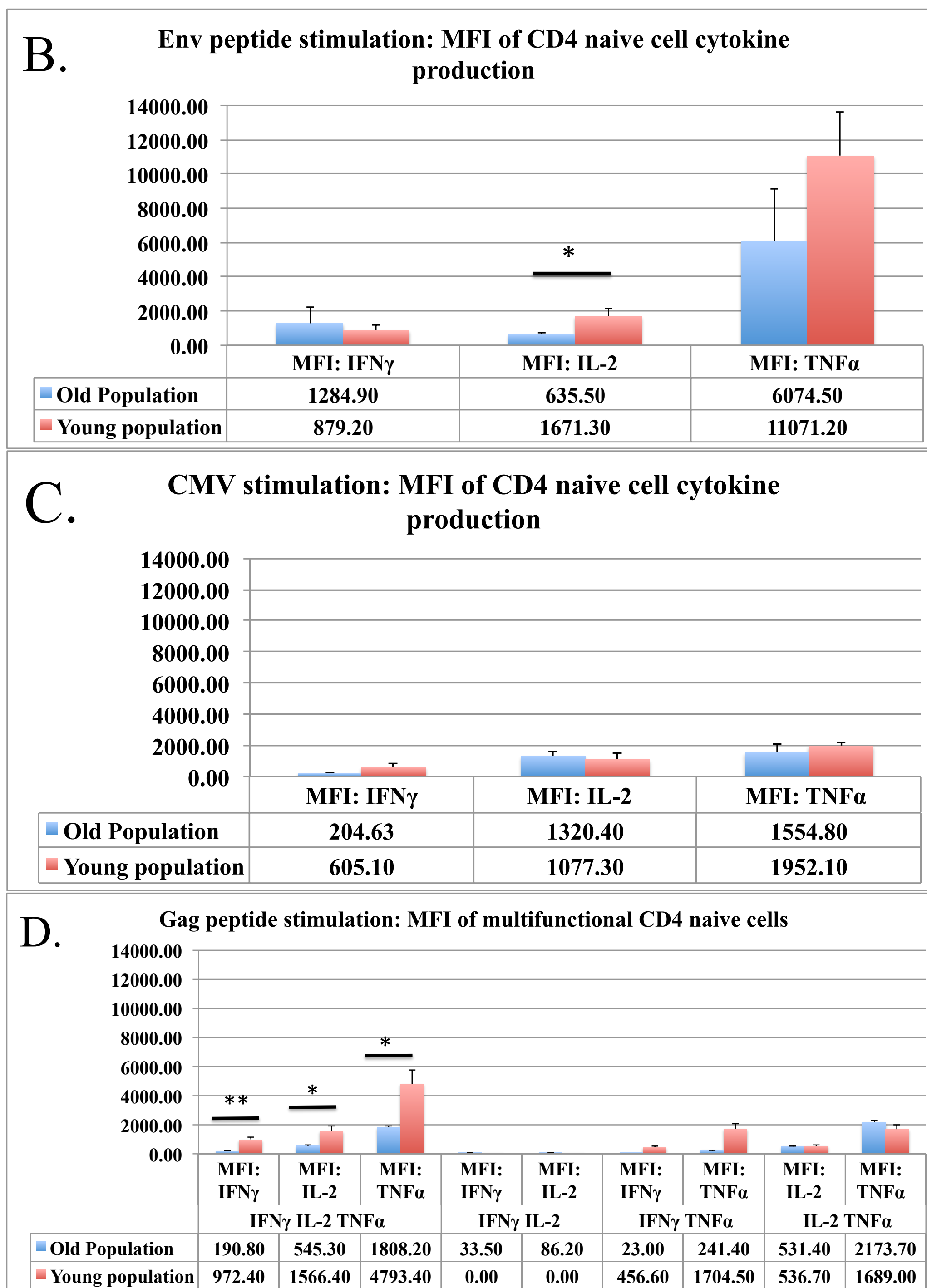


Figure 29: MFI of cytokine producing multifunctional EM CD4^{+} T-cells upon (A) SIVgag peptide stimulation (B) SIVenv peptide stimulation and (C) CMV peptide stimulation.





	MFI: IFNγ	MFI: IL-2	MFI: TNFα
Old Population	1284.90	635.50	6074.50
Young population	879.20	1671.30	11071.20

	MFI: IFNγ	MFI: IL-2	MFI: TNFα
Old Population	204.63	1320.40	1554.80
Young population	605.10	1077.30	1952.10

	IFNγ IL-2 TNFα			IFNγ IL-2		IFNγ TNFα		IL-2 TNFα	
	MFI: IFNγ	MFI: IL-2	MFI: TNFα	MFI: IFNγ	MFI: IL-2	MFI: IFNγ	MFI: TNFα	MFI: IL-2	MFI: TNFα
Old Population	190.80	545.30	1808.20	33.50	86.20	23.00	241.40	531.40	2173.70
Young population	972.40	1566.40	4793.40	0.00	0.00	456.60	1704.50	536.70	1689.00

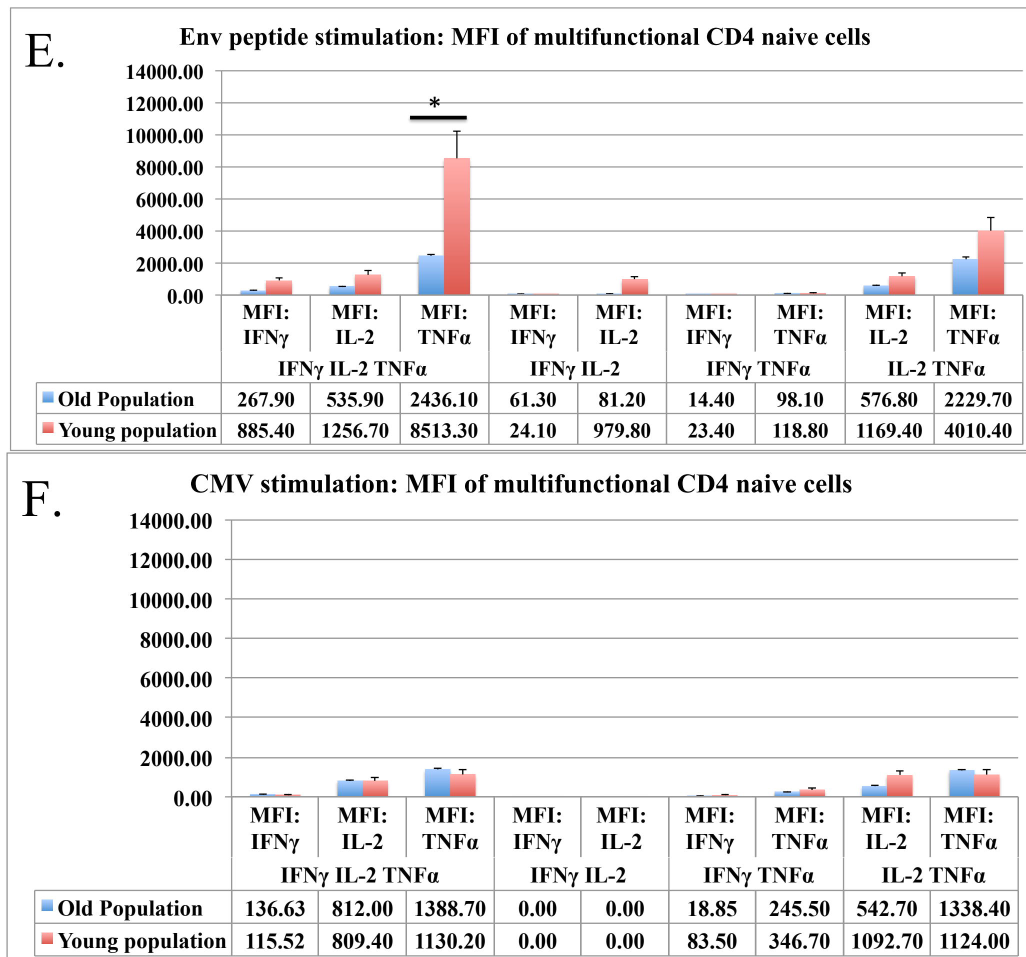
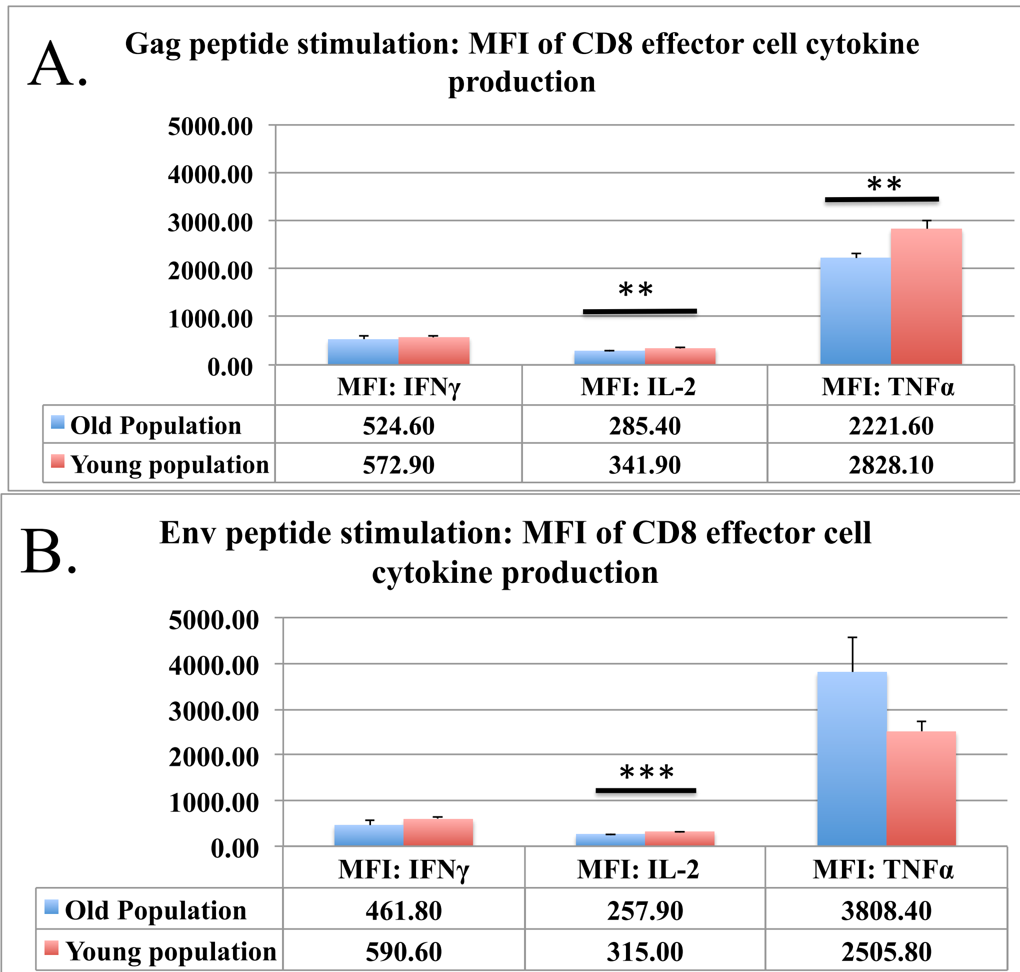


Figure 30: MFI of cytokine producing CD4+ naive T-cells. (A) MFI of cytokine producing CD4+ naive T-cells upon SIVgag peptide stimulation, SIVenv peptide stimulation and (C) CMV peptide stimulation. (D) MFI of of cytokine producing multifunctional CD8+ naive T-cells upon (D) SIVgag peptide stimulation, (E) SIVenv peptide stimulation and (F) CMV peptide stimulation.

The subset of CD8+ T cells showed less significant values of MFI data when compared to CD4+ T cells under the same antigen specific stimulants with no significant differences seen in under the stimulation of CMV peptide (Figure 30C, 31C). The significant differences of MFI between the two age groups is seen in the subset of CD8+ effector cells from both SIVgag and SIVenv peptides stimulation. Upon SIVgag peptide stimulation, young population had a significantly higher MFI values of IL-2 and TNFα from single cytokine secreting cells (Figure 30A) while the same CD8+ subset under SIVenv peptide stimulation had the significant difference only in the MFI value of IL-2

from single cytokine secreting cells (Figure 30B). The last CD8^{+} subset with significant difference in the MFI data is the CD8^{+} EM cells, with a significant difference in the MFI of IL-2 in from single cytokine secreting cells upon SIVgag peptide stimulation (Figure 31A) and significant differences in the MFI of both IL-2 and TNFα from single cytokine secreting cells under SIVenv peptide stimulation (Figure31B).



	MFI: IFNγ	MFI: IL-2	MFI: TNFα
Old Population	524.60	285.40	2221.60
Young population	572.90	341.90	2828.10

	MFI: IFNγ	MFI: IL-2	MFI: TNFα
Old Population	461.80	257.90	3808.40
Young population	590.60	315.00	2505.80

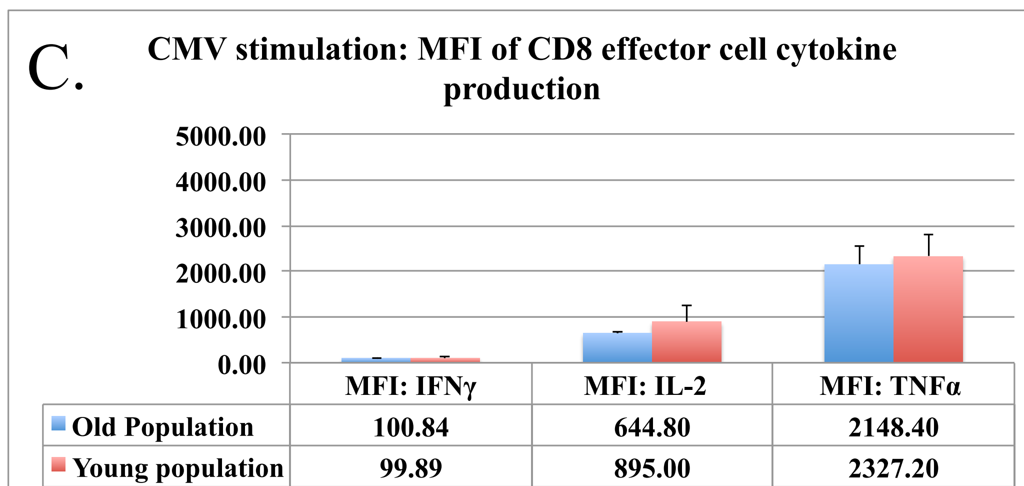


Figure 31: MFI of cytokine producing CD8^{+} effector cells upon (A) SIVgag peptide stimulation, SIVenv peptide stimulation and (C) CMV peptide stimulation.

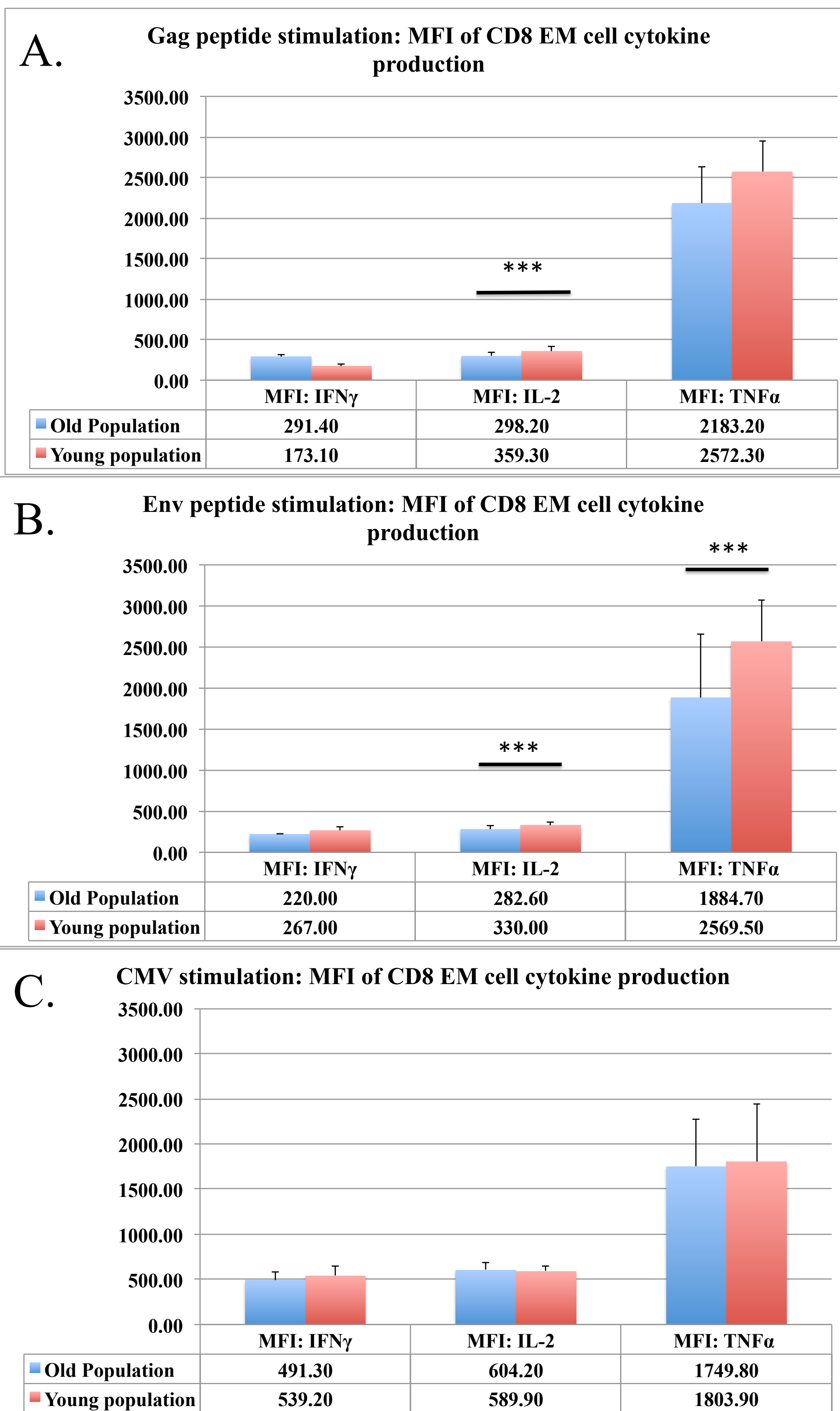

A.
Gag peptide stimulation: MFI of CD8 EM cell cytokine production
3500.00
3000.00
2500.00
2000.00
1500.00
1000.00
500.00
0.00
***
MFI: IFNγ
MFI: IL-2
MFI: TNFα
Old Population
291.40
298.20
2183.20
Young population
173.10
359.30
2572.30
B.
Env peptide stimulation: MFI of CD8 EM cell cytokine production
3500.00
3000.00
2500.00
2000.00
1500.00
1000.00
500.00
0.00
***
***
MFI: IFNγ
MFI: IL-2
MFI: TNFα
Old Population
220.00
282.60
1884.70
Young population
267.00
330.00
2569.50
C.
CMV stimulation: MFI of CD8 EM cell cytokine production
3500.00
3000.00
2500.00
2000.00
1500.00
1000.00
500.00
0.00
MFI: IFNγ
MFI: IL-2
MFI: TNFα
Old Population
491.30
604.20
1749.80
Young population
539.20
589.90
1803.90

Figure 32: MFI of cytokine producing EM CD8$^+$ T-cells upon (A) SIVgag peptide stimulation, SIVenv peptide stimulation and (C) CMV peptide stimulation.

3. Proliferative capacity of T cells upon stimulation with Anti-CD3, SIVSIVgag peptide and CMV peptide using Ki-67 antibody staining.

The proliferative capacity of T-cells was determined in this part of the experiment by observing the expression of Ki-67 in T cell subsets of CD4$^+$ and CD8$^+$. Ki-67, a nuclear antigen expressed in the G_1, G_2, S, and M phases but not the G_0 phase of the cell cycle, which makes it a useful index of cell prolifreation (32). Intracellular staining for the Ki-67 antigen, which enabled accurate measurement of proliferating cells in response different stimulants. In this experiment, I treated the samples with 3 different conditions with 1 control group. Stimulants used in this study include, Anti-CD3, SIVgag and CMV peptides. Two costimulatory signals were provided by anti-CD28 and anti-CD4$^+$ 9d antibodies. All of these stimulants have the same mode of activation, which is the formation of complex with the TCR. The use of antibodies against the CD3 complex is a specific stimulus for activating T cells and it is commonly used as a T cell activator. Anti-CD3 antibodies have the ability to provide an initial activation signal but require the addition of costimulatory antibodies to provide the stimulus for robust proliferation. An important role of costimulation is to prolong the survival of activated T cells (The role of the CD28 receptor during T cell responses to antigen). SIVgag was chosen as one of the stimulants because Gag-specific immune response is one of the relevant immunological factors that is associated with HIV/SIV viral control.

PBMC from a total of 6 young and 6 old uninfected RM were used in the experiment. The surface markers, CD3, CD4$^+$ and CD8$^+$ were used to classify CD4$^+$ and CD8$^+$ into 4 subsets including CD4$^+$, CD8$^+$, CD4$^+$ CD8$^+$ and CD4$^-$ CD8$^-$ T cells.

3a. Percentages of Ki-67 expression in CD4$^+$ and CD8$^+$ T cells

The first set of data demonstrated the percentages of Ki-67 expression in different subsets of CD4$^+$ and CD8$^+$ T cells. Figure 32 shows graphs comparing the expression of Ki-67 of the two age groups in different subsets of T cells upon three different stimulants. Figure 32A shows an interesting result of a significantly higher percentage of Ki-67 expression in CD4$^+$ T cells when compared to the young. However, upon SIVgag peptide stimulation (Figure 32B), an opposite effect was observed. Young population showed a significantly higher percentage of Ki-67 expression in CD4$^+$ T cells. No significant differences of Ki-67 expression were observed in samples treated with CMV peptide.

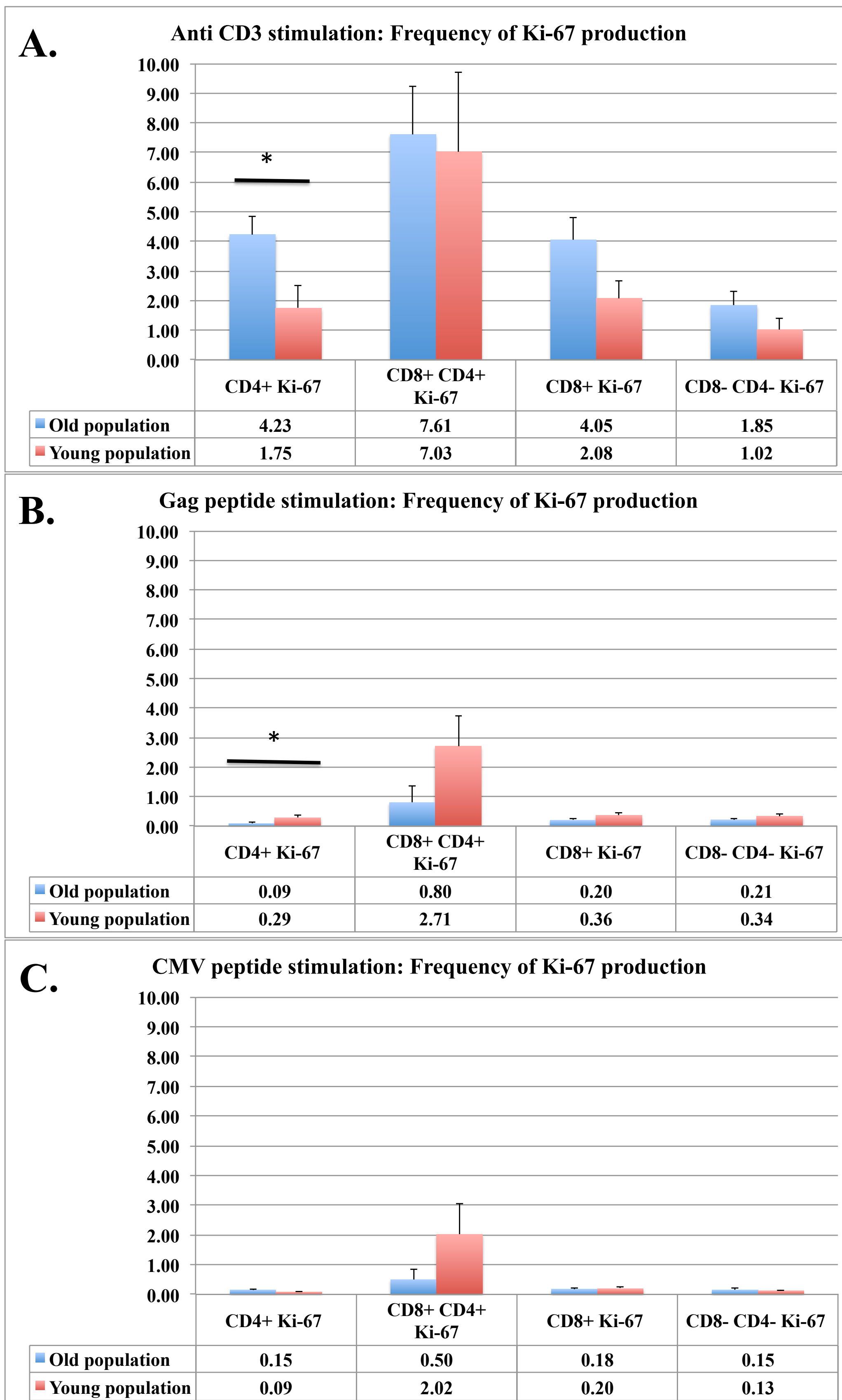
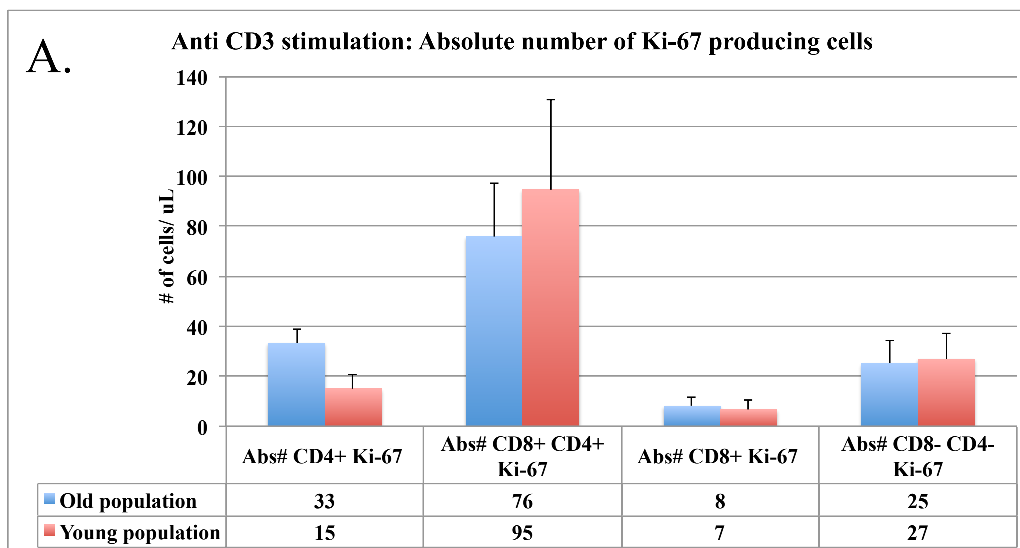

A.
Anti CD3 stimulation: Frequency of Ki-67 production
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
*
CD4+ Ki-67
CD8+ CD4+ Ki-67
CD8+ Ki-67
CD8- CD4- Ki-67
Old population
Young population
4.23
7.61
4.05
1.85
1.75
7.03
2.08
1.02
B.
Gag peptide stimulation: Frequency of Ki-67 production
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
*
CD4+ Ki-67
CD8+ CD4+ Ki-67
CD8+ Ki-67
CD8- CD4- Ki-67
Old population
Young population
0.09
0.80
0.20
0.21
0.29
2.71
0.36
0.34
C.
CMV peptide stimulation: Frequency of Ki-67 production
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
CD4+ Ki-67
CD8+ CD4+ Ki-67
CD8+ Ki-67
CD8- CD4- Ki-67
Old population
Young population
0.15
0.50
0.18
0.15
0.09
2.02
0.20
0.13

Figure 33: Percentages of Ki-67 expression in $CD4^+$ and $CD8^+$ T-cell subsets upon (A) Anti-CD3 stimulation, (B) SIVSIVgag peptide stimulation and (C) CMV peptide stimulation.

3b. Absolute number of Ki-67 expressing $CD4^+$ and $CD8^+$ T cells

The principal of a calculating the absolute numbers of Ki-67 expressing T cells follows the same protocol as mentioned in the first and second specific aims. Figures 33 A-C provided the data for the absolute number of cells that were expressing Ki-67. The overall picture shows a higher absolute cell count in the young population except in the absolute cell count of Ki-67 expression in $CD4^+$ T cells after Anti-CD3 stimulation. However, no significant differences were found in any of the treatment group for this part of the analysis.



	Abs# CD4+ Ki-67	Abs# CD8+ CD4+ Ki-67	Abs# CD8+ Ki-67	Abs# CD8- CD4- Ki-67
Old population	33	76	8	25
Young population	15	95	7	27

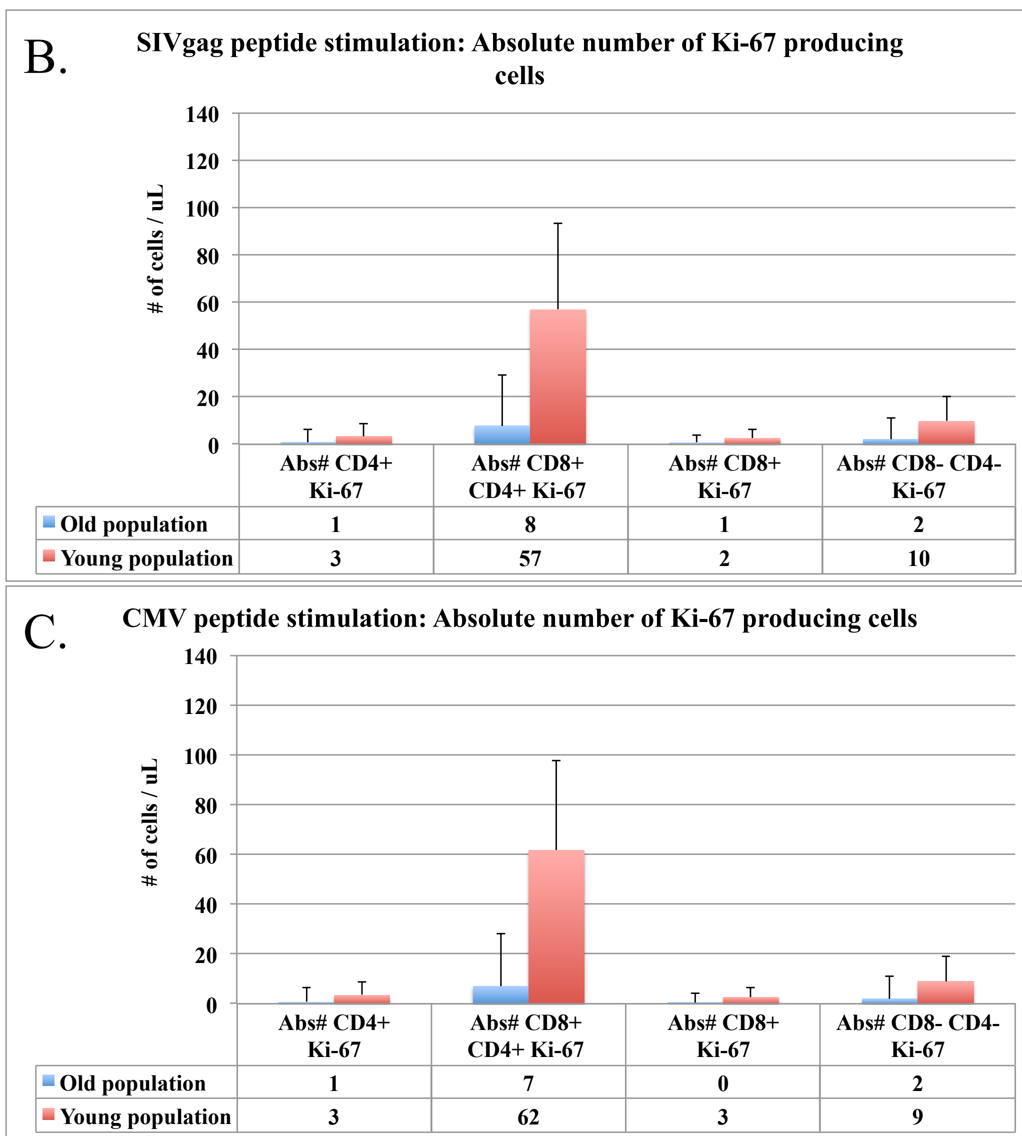


Figure 34: Absolute number of Ki-67 expression in $CD4^+$ and $CD8^+$ cells upon (A) Anti-CD3 stimulation, (B) SIVSIVgag peptide stimulation and (C) CMV peptide stimulation.

Percentages of cytokine production								
		Single cytokine production			*Double cytokine production*			*Triple cytokine production*
Stimulants	**T cell subsets**	**IFNγ**	**IL-2**	**TNFα**	**IFNγ IL-2**	**IFNγ TNFα**	**IL-2 TNFα**	**IFNγ IL-2 TNFα**
PMA&I	CD4 Effector cells	-	-	-	-	-	Y > O (*)	-
	CD4 CM cells	Y > O(*)	Y > O(*)	Y > O(*)	-	Y > O(*)	-	-
	CD4 EM cells	-	Y > O(*)	-	-	-	-	-
	CD4 Naïve cells	-	-	-	-	-	-	-
	CD8 Effector cells	-	-	-	-	-	-	-
	CD8 CM cells	-	-	-	-	-	-	-
	CD8 EM cells	-	Y > O(*)	-	-	-	-	-
	CD8 Naïve cells	-	-	-	-	-	-	-
SEB	CD4 Effector cells	-	-	-	-	-	-	-
	CD4 CM cells	-	-	-	-	-	-	-
	CD4 EM cells	-	Y > O (***)	-	-	-	Y > O (***)	-
	CD4 Naïve cells	-	-	-	-	-	-	-
	CD8 Effector cells	-	Y > O (**)	-	-	-	-	-
	CD8 CM cells	-	-	-	-	-	-	-
	CD8 EM cells	-	Y > O (*)	-	-	-	-	-
	CD8 Naïve cells	-	-	-	-	-	-	-
SIVgag	CD4 Effector cells	-	-	-	-	-	-	-
	CD4 CM cells	-	-	-	-	-	-	-
	CD4 EM cells	-	Y > O (*)	-	-	-	-	Y > O (*)
	CD4 Naïve cells	-	-	-	-	-	-	-
	CD8 Effector cells	Y > O (*)	-	-	-	-	-	-
	CD8 CM cells	-	-	-	-	-	-	-
	CD8 EM cells	-	Y > O (***)	-	-	-	-	-
	CD8 Naïve cells	-	-	-	-	-	-	-
SIVenv	CD4 Effector cells	-	-	-	-	-	-	-
	CD4 CM cells	-	-	-	-	-	-	-
	CD4 EM cells	-	Y > O (**)	-	-	-	-	-
	CD4 Naïve cells	-	-	-	-	-	-	-
	CD8 Effector cells	-	-	-	-	-	-	-
	CD8 CM cells	-	-	-	-	-	-	-
	CD8 EM cells	-	Y > O (*)	-	-	-	-	-
	CD8 Naïve cells	-	-	-	-	-	-	-
CMV	CD4 Effector cells	-	-	-	-	-	-	-
	CD4 CM cells	-	-	-	-	-	-	-
	CD4 EM cells	Y > O (*)	-	-	-	-	Y > O (*)	-
	CD4 Naïve cells	Y > O (*)	-	-	-	-	-	-
	CD8 Effector cells	-	Y > O (**)	-	-	-	-	-
	CD8 CM cells	-	-	-	-	-	-	-
	CD8 EM cells	-	-	-	-	-	-	-
	CD8 Naïve cells	-	-	-	-	-	-	-

Table 2: Summary table of the percentages of cytokine production upon mass stimulations and antigen specific stimulations. Y=Young population; O=Old population. Value of significance: P < 0.05 *, P < 0.01 **, P < 0.001 *.**

Absolute count of cytokine production								
		Single cytokine production			*Double cytokine production*			*Triple cytokine production*
Stimulants	**T cell subsets**	**IFNγ**	**IL-2**	**TNFα**	**IFNγ IL-2**	**IFNγ TNFα**	**IL-2 TNFα**	**IFNγ IL-2 TNFα**
PMA&I	CD4 Effector cells	Y > O (***)	-	Y > O (*)	-	-	-	Y > O (*)
	CD4 CM cells	Y > O (**)	Y > O (*)	Y > O (**)	Y > O (*)	-	-	-
	CD4 EM cells	-	Y > O (**)	Y > O (**)	-	-	-	Y > O (*)
	CD4 Naïve cells	Y > O (*)	-	-	-	-	-	-
	CD8 Effector cells	-	-	-	-	-	-	-
	CD8 CM cells	-	-	Y > O (*)	-	-	-	-
	CD8 EM cells	-	Y > O (*)	Y > O (*)	-	-	-	-
	CD8 Naïve cells	-	-	Y > O (**)	-	-	-	-
SEB	CD4 Effector cells	-	-	-	-	-	-	-
	CD4 CM cells	-	Y > O (**)	-	-	-	-	-
	CD4 EM cells	-	Y > O (***)	Y > O (*)	-	-	Y > O (**)	Y > O (**)
	CD4 Naïve cells	-	-	-	-	-	-	-
	CD8 Effector cells	-	-	-	-	-	-	-
	CD8 CM cells	-	-	-	-	-	-	-
	CD8 EM cells	-	Y > O (***)	-	-	-	-	-
	CD8 Naïve cells	-	-	Y > O (*)	-	-	-	-
SIVgag	CD4 Effector cells	-	-	-	-	-	-	-
	CD4 CM cells	-	Y > O (*)	-	-	-	-	-
	CD4 EM cells	-	Y > O (**)	-	-	-	-	-
	CD4 Naïve cells	-	-	Y > O (*)	-	-	-	-
	CD8 Effector cells	-	Y > O (*)	-	-	-	-	-
	CD8 CM cells	Y > O (*)	Y > O (*)	-	-	-	-	-
	CD8 EM cells	-	Y > O (**)	-	-	-	-	-
	CD8 Naïve cells	-	-	-	-	-	-	-
SIVenv	CD4 Effector cells	-	-	-	-	-	-	-
	CD4 CM cells	-	Y > O (**)	-	-	-	-	-
	CD4 EM cells	-	Y > O (*)	-	-	-	-	-
	CD4 Naïve cells	-	-	-	-	-	-	-
	CD8 Effector cells	-	-	-	-	-	-	-
	CD8 CM cells	-	Y > O (**)	Y > O (**)	-	-	-	-
	CD8 EM cells	-	Y > O (**)	-	-	-	-	-
	CD8 Naïve cells	-	-	-	-	-	-	-
CMV	CD4 Effector cells	-	-	-	-	-	-	-
	CD4 CM cells	-	Y > O (**)	Y > O (*)	-	-	-	-
	CD4 EM cells	Y > O (*)	Y > O (**)	Y > O (*)	-	-	-	-
	CD4 Naïve cells	-	-	-	-	-	-	-
	CD8 Effector cells	-	Y > O (**)	-	-	-	-	-
	CD8 CM cells	-	Y > O (***)	-	-	-	-	-
	CD8 EM cells	-	Y > O (*)	-	-	-	-	-
	CD8 Naïve cells	-	-	-	-	-	-	-

Table 3: Summary table of the absolute count of cytokine prodcuing cells upon mass stimulations and antigen specific stimulations.

		Single cytokine production			*Double Cytokine production*						*Triple cytokine production*		
MFI					IFNγ IL-2		IFNγ TNFα		IL-2 TNFα		IFNγ IL-2 TNFα		
Stimulants	**T cell subsets**	**IFNγ**	**IL-2**	**TNFα**	**MFI: IFNγ**	**MFI: IL-2**	**MFI: IFNγ**	**MFI: TNFα**	**MFI: IL-2**	**MFI: TNFα**	**MFI: IFNγ**	**MFI: IL-2**	**MFI: TNFα**
PMA&I	CD4 Effector cells	-	-	-	-	-	-	-	-	-	-	-	-
	CD4 CM cells	-	-	-	-	-	-	-	-	-	-	-	-
	CD4 EM cells	-	-	-	-	-	-	-	-	-	-	-	-
	CD4 Naïve cells	-	-	-	-	-	-	-	-	-	-	-	-
	CD8 Effector cells	-	-	-	-	-	-	-	-	-	-	-	-
	CD8 CM cells	-	-	-	-	-	-	-	-	-	-	-	-
	CD8 EM cells	-	-	-	-	-	-	-	-	-	-	-	-
	CD8 Naïve cells	-	-	-	-	-	-	-	-	-	-	-	-
SEB	CD4 Effector cells	-	-	-	-	-	-	-	-	-	-	-	-
	CD4 CM cells	-	-	-	-	-	-	-	-	-	-	Y > O (*)	-
	CD4 EM cells	-	-	-	-	-	-	-	-	-	-	-	-
	CD4 Naïve cells	-	-	-	-	-	-	-	-	-	-	-	-
	CD8 Effector cells	-	-	-	-	-	-	-	-	-	-	-	-
	CD8 CM cells	-	-	-	-	-	Y > O (***)	-	-	-	-	-	-
	CD8 EM cells	-	-	-	-	-	-	-	-	-	-	-	-
	CD8 Naïve cells	-	-	-	-	-	-	-	-	-	-	-	-
SIVgag	CD4 Effector cells	-	-	-	Y > O (**)	Y > O (***)	-	-	-	-	-	-	Y > O (*)
	CD4 CM cells	-	-	-	Y > O (*)	-	-	-	-	-	-	-	-
	CD4 EM cells	-	-	-	Y > O (**)	Y > O (*)	-	-	-	-	-	-	-
	CD4 Naïve cells	Y > O (**)	Y > O (*)	Y > O (*)	Y > O (**)	Y > O (*)	Y > O (*)	-	-	-	-	-	-
	CD8 Effector cells	-	Y > O (**)	Y > O (**)				-	-	-	-	-	-
	CD8 CM cells	-	-	-	-	-	-	-	-	-	-	-	-
	CD8 EM cells	-	Y > O (***)	-	-	-	-	-	Y > O (***)	-	-	-	-
	CD8 Naïve cells	-	-	-	-	-	-	-	-	-	-	-	-
SIVenv	CD4 Effector cells	-	-	-	-	-	-	-	-	-	-	-	Y > O (*)
	CD4 CM cells	-	-	-	-	-	-	-	-	-	Y > O (*)	-	-
	CD4 EM cells	-	-	-	-	-	-	-	-	-	-	-	-
	CD4 Naïve cells	-	Y > O (*)	-	-	-	-	-	-	-	-	-	Y > O (*)
	CD8 Effector cells	-	Y > O (***)	-	-	-	-	-	-	-	-	-	-
	CD8 CM cells	-	-	-	-	-	-	-	-	-	-	-	-
	CD8 EM cells	-	Y > O (***)	Y > O (***)	-	-	-	-	-	-	-	-	-
	CD8 Naïve cells	-	-	-	-	-	-	-	-	-	-	-	-
CMV	CD4 Effector cells	-	-	-	-	-	-	-	-	-	-	-	-
	CD4 CM cells	-	-	-	Y > O (*)	-	-	-	-	-	-	-	-
	CD4 EM cells	-	-	-	-	-	-	-	-	-	-	-	-
	CD4 Naïve cells	-	-	-	-	-	-	-	-	-	-	-	-
	CD8 Effector cells	-	-	-	-	-	-	-	-	-	-	-	-
	CD8 CM cells	-	-	-	-	-	-	-	-	-	-	-	-
	CD8 EM cells	-	-	-	-	-	-	-	-	-	-	-	-
	CD8 Naïve cells	-	-	-	-	-	-	-	-	-	-	-	-

Table 4: Summary table of MFI values of the cytokines upon mass stimulations and antigen specific stimulations.

Prolifeative capacity

Stimulants	T cell subsets	% Ki-67	Abs cell count: KI-67
Anti-CD3	CD4+	Y < O (*)	-
	CD8+	-	-
	CD4+CD8+	-	-
	CD4-CD8-	-	-
SIVgag	CD4+	Y > O (*)	-
	CD8+	-	-
	CD4+CD8+	-	-
	CD4-CD8-	-	-
CMV	CD4+	-	-
	CD8+	-	-
	CD4+CD8+	-	-
	CD4-CD8-	-	-

Table 5: Summary table of percentages, absolute cell count and MFI values of Ki-67 expression upon differnt stimulants.

4. Multivariate Analysis: Principal component analysis

Since this study tested multiple variables, multivariable analysis was applied. Principal component analysis was used as a method of analysis to reduce or condense the variables in the data set into "Principal components" (PC). PC with the largest contribution to the variance of the data will define the important parameters that contribute most to the data set, which can be used to distinguish the immunological parameters that contribute most to the data set and help to identify the parameters that best discriminate between the old and young Rhesus macaques. Tables below show three-dimensional graphs of the three main analyses of $CD4^+$ and $CD8^+$ T-cells, which are the frequencies, absolute cell count and mean fluorescence intensity of the cytokine productions. Correlation coefficients which define each principal component are plotted in the three dimensional graphs. A high correlation between PC and a variable indicates

that the variable is strongly associated with maximum variation in the data set. However the significance of the separation between the two age groups with multiple variables cannot be calculated statistically so the graphs below only show visual displays of the data and the separation of the two sample groups can be visually determined.

Figure 34 demonstrate the frequencies of cytokine production in both total cytokine production multiple cytokine production (Boolean) or $CD4^+$ and $CD8^+$ T cells. The best separation between the two groups is seen in Figure 34C which is the multivariate data analysis of the frequency of cytokine production in $CD8^+$ multiple-cytokine production and in figure 34E which shows the multivariate data analysis of the frequency of total cytokine production of T cells including both $CD4^+$ and $CD8^+$.

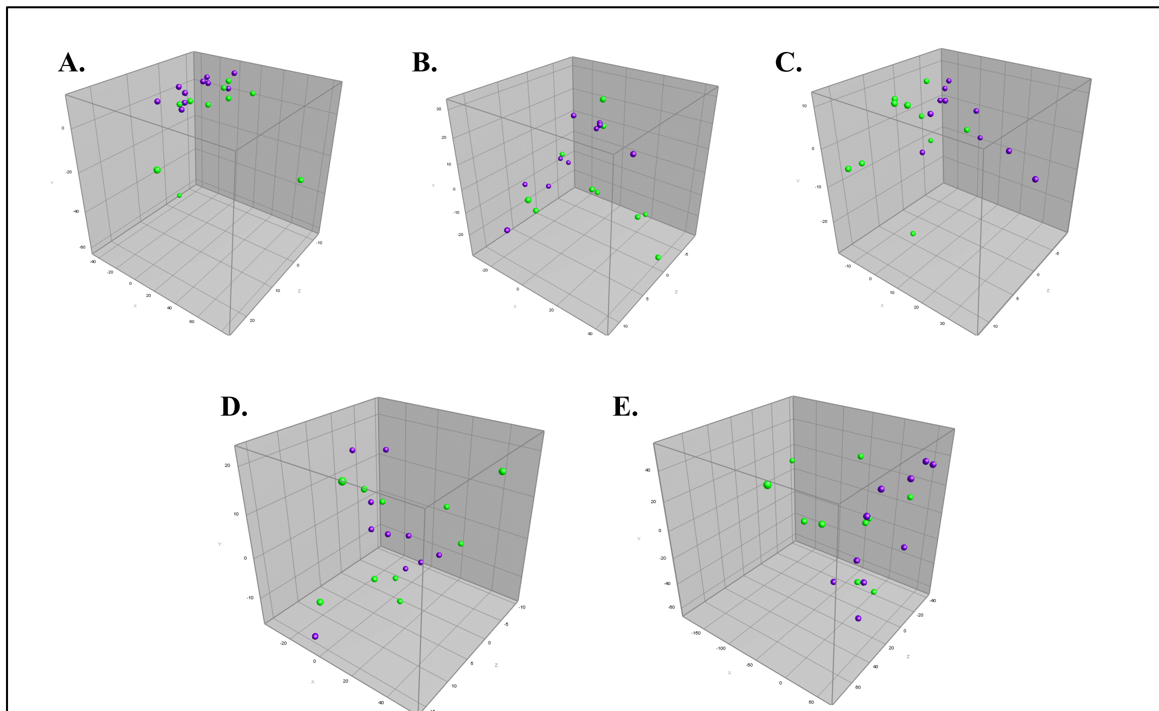


Figure 35 Multivariate data analysis of the frequency of cytokine production in (A) $CD4^+$ multiple-cytokine production, (B) $CD4^+$ total cytokine production, (C) $CD8^+$ multiple-cytokine production, (D) $CD8^+$ total cytokine production and (E) total cytokine production of T cells

However, the clearer separation between the two groups can be seen across the T cell subsets in the multivariate data analysis of the absolute cell count (Figure 35). The best separation of the two groups appear in Figure 35A which demonstrate the analysis of the absolute count of cytokine producing cells in $CD4^+$ multiple-cytokine production as well as figure 35B, which shows the data analysis of the absolute count of total cytokine production in $CD4^+$ cells. Figure 35E, which demonstrate the absolute count of the total cytokine production of T cells also shows a clear clustering of the two groups and this provide a clear visualization of the separation.

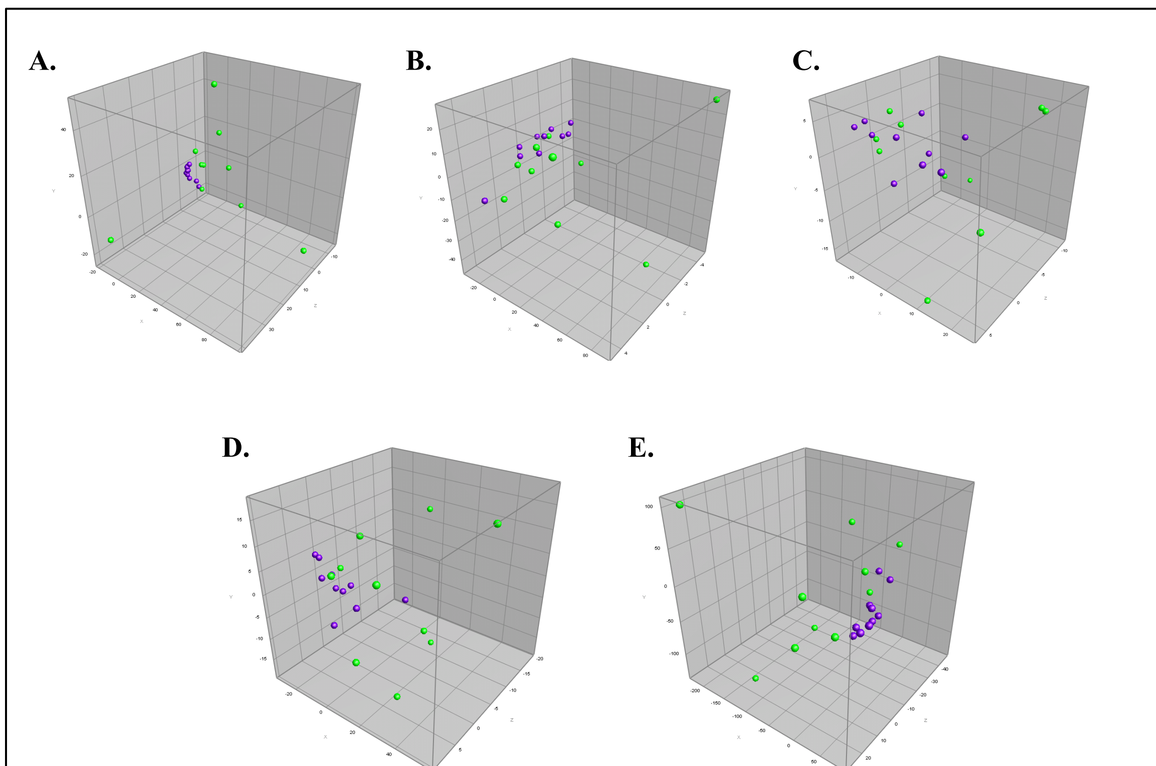


Figure 36 Multivariate data analysis of the absolute count of cytokine producing cells in (A) $CD4^+$ multiple-cytokine production, (B) $CD4^+$ total cytokine production, (C) $CD8^+$ multiple-cytokine production, (D) $CD8^+$ total cytokine production and (E) total cytokine production of T cells

The differentiation between the two groups in the context of MFI is not very clear in the graphs shown in Figure 36. However, Figures 36B and 36C seem to show some separation between the two age groups. The purple dots appear towards the front of the

3D graph while the green dots tend to stay at the back. There two figures display a visual separation of the MFI values of CD4$^+$ total cytokine production (Figure 36B) and CD8$^+$ multiple-cytokine production (Figure 36C).

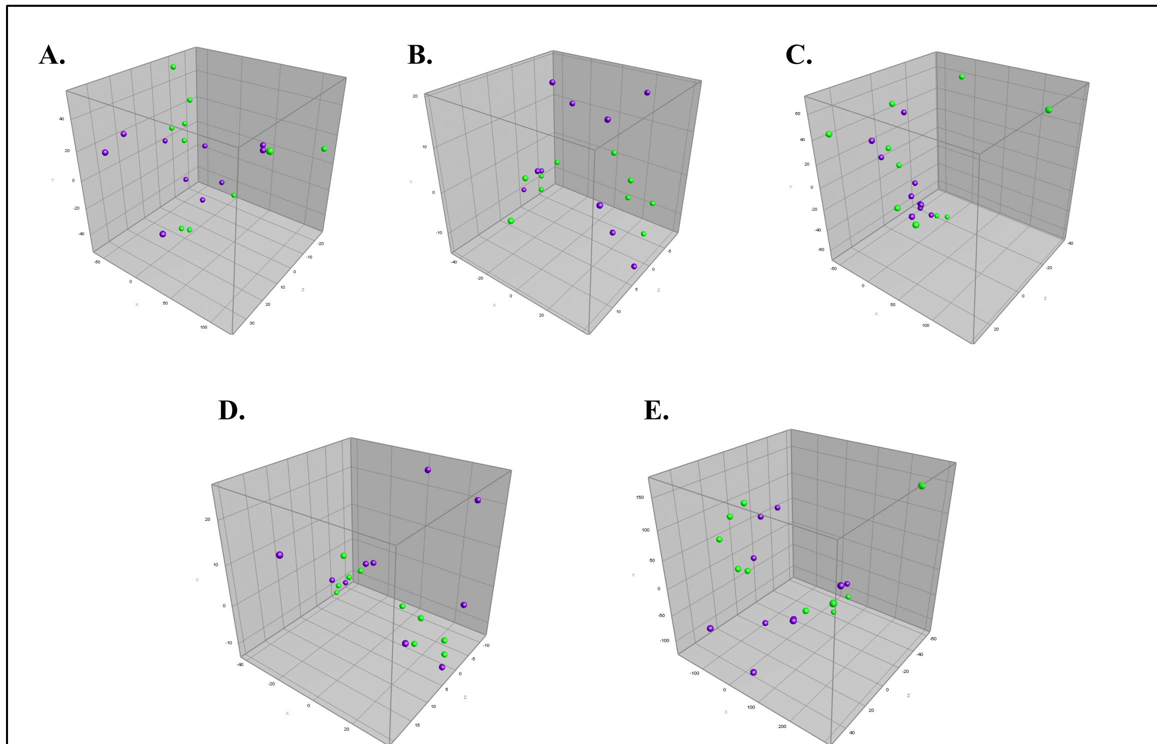


Figure 37 Multivariate data analysis of the Mean Fluorescence Intensity (MFI) of cytokine production in (A) CD4$^+$ multiple-cytokine production, (B) CD4$^+$ total cytokine production, (C) CD8$^+$ multiple-cytokine production, (D) CD8$^+$ total cytokine production and (E) total cytokine production of T cells

Discussion

In this study, T cell responses were compared between rhesus macaques of young adult age and those of advanced age. My interest in the study stems from the fact that acquiring HIV or SIV infection at advanced age is associated with accelerated progression to AIDS. Based on this phenomenon, I postulated that some immune responses that are crucial to limiting viral replication and pathogenesis during acute infection are lacking in older animals. Therefore, I selected certain T cell responses whose associations with better outcomes in HIV/SIV disease have been previously

demonstrated to test my hypothesis. This work has been done using blood samples from uninfected animals. Pre-infection studies provide an opportunity to define intrinsic differences that might have resulted in the divergent outcome in an unaltered immune system. The question targeted in this study was whether or not the younger population will be able to illustrate more potent immune responses associated with better outcomes of the disease when compared to the old population upon treatment with different stimulants and which immunological parameters are most discriminatory between the two groups. To answer this question, PBMC of uninfected old and young RM were treated with different stimulants including mass stimulants and antigen specific stimulants, to test for the potential of the cells and to compare between the two age groups with different disease progression patterns. Moreover, this study also looked for the immunological parameters that distinguish the two age groups. The potential of T cells were determined by the secretion of three cytokines IL-2, IFN-γ and TNF-α in terms of frequency, absolute numbers, and mean fluorescence intensity (MFI).

By stimulating the cells bypassing the T cell receptors using PMA/I, a variety of cells were stimulated, which enables the observation and comparison of the overall potential of cells to mount protective immune response in the two age groups. The result of this study shows a higher potential of T cells in most subsets from the young population through this type of stimulation. Next, the stimulation with SEB was done to determine and compare the ability for cells to mount protective immune responses through the interaction with the superantigen and T cell receptors. As expected, cells from the young RM showed stronger potential to mount protective immune response upon SEB stimulation, especially in the percentage of IL-2 positive cells. The last

stimulation concentrated specifically on the responses towards SIV peptide (pool) stimulations, which brings the focus of the immune response to a specific group. Since the tested subjects are not infected with the virus, I did not expect to see a high secretion of SIV specific immune responses but some cross reactivities between the T cell receptors and SIV peptides was expected. CMV stimulated group was tested along side with SIV peptide stimulation to identify they specificity of the immune responses towards SIV pooled peptides. Once again, the younger subjects showed a higher potential of T cell responses towards SIV peptides. SIV peptides stimulations was considered to be SIV-specific since the pattern of significant differences (in terms of frequencies) for each T cell subsets between the two age groups were similar upon the treatment with SIVgag and SIVenv peptides (Table 1). While the pattern of cytokine production upon CMV peptide did not truly correspond to the SIV peptides.

The overall investigation of cytokine productions in this study shows that the protective immune responses, including the secretion of IL-2, and the abundance of multiple cytokine-secreting cells, were observed more from the younger RM. This finding agrees with the central hypothesis, which stated that critical immune responses, such as those associated with better outcome in primate lentiviral infections are lost in rhesus macaques at older age. This finding corresponds with previous studies on the negative impacts of aging on the magnitude of the immune response. Studies in the past have shown dramatic differences in the quality of the T-cell responses when comparing HIV-infected progressors with LTNPs. One study reported an increase in the frequencies of T cells expressing IL-2 only or both IL-2 and IFN-γ together as well as a higher frequency of IL-2+ TNF-α+ IFN-γ+ (triple cytokine production) in LTNP and HIV

infected patients who received HAART therapy when compared to untreated patients (35, 36). However, most of the significant differences in my study were seen in the secretion of single cytokines rather than the secretion of double or triple cytokine secretions.

According to the summary chart in Table 1, most of the significant differences in the percentage of cytokine production of IL-2 mostly came from the memory subsets of $CD4^+$ and $CD8^+$ T cells, which showed a higher secretion of this cytokine from the young population. According to past studies, IL-2 production and multi-cytokine secretion from the memory subsets of T cells correspond to a better prognosis of the disease progression. Potter et al, showed that HIV controllers or LNTP, are able to maintain potent functional activation in the $CD4^+$ memory cell compartments. This group of people is able to preserve high IL-2 secretions in the memory compartments of T cells (66). One of the reasons that might explain the robust production of IL-2 from the memory compartment of T cells in this study is the faster activation rate of memory T cells. Memory T cells can perform immediate effector functions in peripheral tissues or undergo activation as well as clonal expansion when compared to other subsets of T cells (13). Upon stimulations or contact with specific antigens or peptides, the effector memory cells can achieve effector functions instantly, whereas central memory cells can rapidly proliferate, expand and acquire the effector functions. The production of IL-2 from $CD4^+$ T cells, contribute largely to the clonal expansion and differentiation of $CD8^+$ T cells. IL-2 signals are also able to rescue $CD8^+$ T cells from cell death and provide a robust increase in memory $CD8^+$ T-cell counts as well as encouraging the primary and secondary expansion of $CD8^+$ T cells, which in turn optimizes $CD8^+$ T-cell functions (44). Moreover, Litjens et al. concluded in their studies that memory $CD4^+$ T cells that produce IL-2 is also associated

with the generation of IgG-secreting plasma cells (45). The robust production of IgG induced by IL-2 will be able to provide a stronger protective response.

IL-2 is known for its ability to promote and regulate proliferation, differentiation, expansion and survival of T cells (16, 26). One study demonstrated a significant reduction in IL-2 production in patients with HIV infection, which results in impaired lymphocyte functions, and increased rate of lymphocyte apoptosis (38, 39). The weak or absent proliferative capacity of $CD4^+$ T cell responses is a hallmark of progressive HIV infection. This is largely caused by the loss of functions, in particularly, IL-2 secretion (40). IL-2 can be considered as one of the most important cytokines in relation to a better prognosis of HIV infection. In the mid 1990s, National Institutes of Health (NIH) Clinical Center performed studies to evaluate the potential of IL-2 in HIV infected patients by administering IL-2 via continuous intravenous infusion or subcutaneous injection. As a result, there was a 3-4 folds increase in $CD4^+$ T cell counts that persisted for up to 3 years in patients who received the treatment (41, 42, 43).

IFN-γ is another important cytokine commonly used to determine the potential of T cells. The frequency of this cytokine is also widely used as a parameter to assess vaccine induced responses that are able to mount cellular specific responses against specific infections (16). However, in my study, few significant differences between the two age groups were seen in the percentage of IFN-γ production in all of the treatment conditions. However, if the absolute cell count is taken into consideration, the significant differences in IFN-γ production mainly fall under the group treated with PMA/I in central memory compartment and very few in the antigen specific stimulated groups (Table 2). This phenomenon can be explained with the fact that PMA/I are mass stimulants. They

are small organic compounds, which can diffuse through the cell membrane into the cytoplasm and bypassing TCR signaling, omitting surface receptor stimulation. Due to this ability of PMA/I, non-specific and mass stimulation of cells can be observed upon treatment. However, upon SIVgag peptide stimulation, young population had a significantly higher percentage of IFN-γ production in the $CD8^+$ effector T cell subset but no significant differences were seen in the SIVenv peptide stimulation. When taking absolute numbers into account, the young population had a significantly higher cell count of IFN-γ production in $CD8^+$ CM T cells instead. Studies have also shown that $CD8^+$ T-cell responses against the Gag protein measured by IFN-γ are associated with lower viremia in chronic HIV-1 infection (46,47). However, significant differences in the two age groups of the percentage and the absolute cell count of IFN-γ upon CMV stimulation falls mainly under $CD4^+$ T-cell subset, which differs, completely from SIV specific stimulation. So the treatment of CMV shows a strong potential as a control group to test for SIV antigen activated immune responses in pre-infected RM.

The last cytokine production that was studied in this study is TNF-α. TNF-α is a pro-inflammatory cytokine that promotes death of cancer cells, signals the increase in inflammation and is able to enhance the proliferation of T cells (48). In my study, no significant differences were seen in the percentages of TNF-α secretion in any of the treatment groups except for $CD4^+$ CM cells upon PMA/I stimulation (Table 1). However, when looking at the absolute count of cells producing TNF-α, the significant differences were seen in all of the stimulants and again mostly in the PMA/I treated groups (Table 2). This again can be concluded by the non-specific and mass stimulation ability of PMA/I. Moreover, the young population displayed a significantly higher count of TNF-α

secreting cells in $CD4^+$ EM and $CD8^+$ naïve subsets after SEB treatment, in $CD4^+$ naïve subset in SIVgag treatment, in $CD8^+$ CM subset upon SIVenv treatment and lastly in $CD4^+$ CM and EM subsets upon CMV treatment. The results of TNF-α secreted cell count did not provide any consistent pattern to conclude for any potential ability of cells to mount HIV/SIV protective immunity. Nonetheless, the overall observation shows that the data with significant difference in the production of TNF-α had a high production in the young population, which again agrees with past observations on the deterioration of the immune responses with age. This then explains the reason for the stronger ability of the young RM to secrete cytokines in response to stimulants. Moreover, the data in this study shows lower potential of cells from the old population to secrete significantly higher amount of TNF-α in most of the T- cell subsets. However, past studies had some contradicting observations with TNF-α in relation to HIV/SIV. Some studies showed that TNF-α is a potent inducer of viral gene expression. It is able to activate HIV-1 in chronically infected T cells through the activation and translocation of NF-κB, making the HIV-1 toll-like receptors (LTR) more accessible and resulting in viral transcription (49, 50, 51). This will provide the ability to promote the stimulation of HIV-1 replication in cultured PBMC (52). Another contradictory finding is inhibition of HIV-1 replication by TNF- α by down regulating the expression of CCR5 and inhibiting the entry of CCR5-dependent viruses (53, 54). Further studies will need to be conducted to determine the accurate relationship between HIV/SIV proliferative capacity and TNF-α.

The secretions of double and triple cytokine production (multifunctional cells) were also determined in this study. As expected, very few significant data were observed in this multifunctional part of the analysis due to the fact that multifunctional T cells

make up a small part of the total population. According to my data, the significant differences in percentages of double cytokine production (Table 1), were observed upon PMA/I, SEB, and CMV stimulations. Significant difference of IL-2^+ TNF-α^+ production upon treatment with PMA/I, SEB and CMV were from the memory compartments of $CD4^+$ T-cells. The ability for the younger RM to secrete double cytokine of IL-2^+ TNF-α^+ correspond with previous studies which provided evidences for the better prognosis of HIV infection in combination with the enhanced secretion of IL-2^+ TNF-α^+ by $CD4^+$ multifunctional T cells (35). Interestingly, one significant data was observed in the production of triple cytokine (IFN-γ^+ IL-2^+ TNF-α^+) from the subset of $CD4^+$ EM T-cells upon SIVgag stimulation with a higher production of triple cytokine from the young population. Studies have shown that an increase in the multifunctional $CD4^+$ T-cells is associated with an improved control of HIV upon comparison between HIV infected progressors and LTNPs (16). Evidence revealed that LTNPs and those who received treatments had higher frequencies of IFN-γ^+ TNF-α^+ IL-2^+ or IL-2^+ IFN-γ^+ T cells when compared to the progressors (35, 55, 56). Furthermore, the presence of HIV/SIV antigen tends to drive $CD4^+$ towards the production of single-cytokine which will eventually lead to cell death and the depletion of $CD4^+$ T-cells but in contrast, multifunctional T cells have the ability to resist throughout the infection (16). According to my data of the percentage of multiple cytokine production, it can be concluded that young population of RM has more potential to mount a multiple-cytokine production in the subset of $CD4^+$ EM cells upon SIVgag peptide stimulation, indicating a better chance of mounting a more protective immune response once in contact with the SIV. However, the absolute cell counts of multiple cytokine production show significant data only upon treatment with

mass stimulants (PMA/I and SEB) and mostly from $CD4^+$ memory compartments. The reasons that the significant results from the absolute cell count analysis (Table 2) differs slightly from the significant data seen in the analysis of percentages of cytokine production is because the absolute cell count data is based on the CBC from each specific animal with different CBC values lymphocyte count in each animal. So the results of the absolute cell count measures the quantity of the cells that are capable of mounting protective immune responses but on the other hand, the percentage of the cytokine production measures the quality of the cells to mount each or certain sets of cytokines.

Taken all of the results together, it can be concluded that the multifunctional $CD4^+$ T-cells have more robust responses to stimulants especially in the young population when compared to the old, which demonstrate a higher potential of cells in the pre-infected young population to mount sets of cytokines associated with better outcomes of HIV/SIV infection.

Proliferative capacity of $CD4^+$ T cells was also observed in this study. Low proliferative capacity of $CD4^+$ T-cells is associated with the decrease in the number of $CD4^+$ T cells, which leads to impairment of immune functions in HIV/SIV infection so $CD4^+$ counts is commonly used as one of the predictors of HIV disease (57). Many studies have shown evidences of poor $CD4^+$ T cell proliferative responses following T cell receptor stimulation in HIV-infected patients. $CD4^+$ T cell proliferation responses to HIV antigens is known to be associated with the control of viral replication at all stages of disease (58, 59, 60). The inability of patient cells to proliferate has been associated with decreased production of IL-2 (61) and enhanced susceptibility to apoptosis (62). Age-related impairments of the immune activation has been studied in the past and my

result on the observation of a higher IL-2 production as well as other multiple cytokine secretions from the young population should support my hypothesis that the proliferative capacity in the young population will be greater than the old. Here, I investigated cell proliferation, measured by expression of the Ki-67 nuclear antigen in $CD4^+$ and $CD8^+$ lymphocyte with the subsets of $CD4^+$, $CD8^+$, double positive and double negative, upon three different stimulants (Anti-CD3, SIVgag and CMV peptides). Surprisingly, older population demonstrated a significantly higher percentage of Ki-67 expression in $CD4^+$ T-cells upon stimulation with Anti-CD3 (with the addition of Anti-CD28 and Anti-CD49d as co-stimulatory molecules). However, the opposite affect was seen in the same subset of cells upon SIVgag peptide stimulation, with higher expression of Ki-67 in the young population. But no significant differences were seen in the group treated with CMV peptides nor the absolute counts in all of the conditions. One possible explanation to this phenomenon might be because the immune system of the older monkeys are exposed to more antigens leading to the higher count in the memory compartment of the immune cells. With more exposure to a variety of antigens and with higher proportion of memory cells, this might provide the more opportunity for the older RM to interact with the stimulant (Anti-CD3) causing a higher expression of the proliferative marker. Further analysis on the compartments of T cells comparing the percentages of $CD4^+$ and $CD8^+$ subsets in the young and the old RM was done to support this concept. According to my analysis, the older RM had significantly higher percentages of $CD4^+$ effector memory and central memory cells as well as $CD8^+$ effector memory cells when compared to the young (data not shown). This finding corresponds to previous study by Saule et al which

provided evidences for the positive correlation between age and the amount of memory T cell compartment.

However, further study is needed to confirm this phenomenon possibly a study into a more detail of the surface marker is necessary to confirm this result and to see if there is any up-regulations and down-regulations of certain receptors that are associated with HIV/SIV infection. But the result of higher Ki-67 expression (higher proliferative capacity) in the young population in $CD4^+$ T cells upon SIVgag stimulation supports my hypothesis and these results contribute to the ability of young RM to mount stronger response for a protective immunity towards HIV/SIV infection since high proliferative capacity suggest the preservation of immune cells and less impairments of immune functions.

Mean fluorescence intensity (MFI) of each cytokine was observed in this study. Fluorescence has a property that could potentially be used to generate a signature of cell state. In this study, MFI data were used as measurements of cytokine expression by individual cells. From my study, most of the significant differences of MFI values between the two age groups were seen upon the treatment with SIVgag peptide. Significantly higher MFI values were observed mainly in the double cytokine secretion ($IFN\text{-}\gamma^+$ $IL\text{-}2^+$) of $CD4^+$ subsets in the young population. This result corresponds with previous studies on the multifunctional characteristics of T cells and their association with protective immune response or an improved control of the virus (26). The significantly higher MFI values in the young indicate the ability of the young RMs to mount a higher quantity of the cytokine production per cell especially in $CD4^+$ subsets. Significant differences of MFI values were also detected upon SIVenv peptide treatment

but most of the significant data in this group falls under triple cytokine production in the portion of TNF-α production, in $CD4^{+}$ effector and naïve subsets. However, very few significant differences as observed in groups treated with PMA/I, SEB and CMV peptides. The fact that we are seeing many significant differences in the MFI of different combination of cytokines with a higher degree in the young population shows the greater SIV peptide specific response from the product of cross reaction between T cell receptors and the peptide in the young population when compared to the old. It also shows that each cell from the young has more ability to produce a greater quantity of each cytokine upon stimulation with SIV specific peptides. As for the MFI data for Ki-67 expression, young population had a significantly higher value than the old in $CD8^{+}$ T-cells upon SIVgag stimulation. Again, the fact that young population had a higher capacity of each cell to regulate Ki-67 expression upon SIVgag stimulation provide another evidence for a greater ability for the young to mount a protective immune response that is associated with a better outcome of HIV/SIV infection when compared to the old RM.

Since there are multiple variables and parameters to be considered in this study, the multivariate data analysis via principal component analysis was used as a method to define the important immunological parameters that contribute most to the variance in data set, which further led to the identification of the parameters that best discriminate between the young and old age groups of RM. The contribution of each parameter to the whole data set was defined and plotted on the three-dimensional chart using correlation coefficient defining each component in the data. High correlation values contribute more to the data as well as the contribution to the separation of the two groups as shown on the three-dimensional graphs. Some immunological parameters that best discriminate the

two age groups were defined upon the application of multivariate analysis of the frequency of cytokine production according to the coefficient values (data not shown). Immunological parameters that contribute most to the data and aide in the differentiation of the young and old upon stimulations include, the frequencies of

1. $CD4^+$ naïve cells producing $IL\text{-}2^+$ $TNF\text{-}\alpha^+$ and $IFN\text{-}\gamma^+$ $IL\text{-}2^+$ $TNF\text{-}\alpha^+$
2. $CD4^+$ effector cells producing $IFN\text{-}\gamma^+$ $IL\text{-}2^+$ $TNF\text{-}\alpha^+$
3. $CD4^+$ central memory cells producing IFN-γ
4. $CD8^+$ naïve cells producing $IFN\text{-}\gamma^+$ $TNF\alpha^+$
5. $CD8^+$ effector cells producing $IFN\text{-}\gamma^+$ $TNF\alpha^+$
6. $CD8^+$ effector memory cells producing $IFN\text{-}\gamma^+$ $TNF\text{-}\alpha^+$
7. $CD8^+$ central memory cells producing TNF-α and IFN-γ

Upon multivariate analysis of the absolute cell count of cytokine production Immunological parameters that contribute most to the data include, the absolute cell count of

1. $CD4^+$ effector cells producing $IL\text{-}2^+$ $TNF\text{-}\alpha^+$, $IFN\text{-}\gamma^+$ $IL\text{-}2^+$ $TNF\alpha^+$, IFN-γ, and TNF-α
2. $CD4^+$ naïve cells producing $IFN\text{-}\gamma^+$ $IL\text{-}2^+$ $TNF\text{-}\alpha^+$ and $IL\text{-}2^+$ $TNF\text{-}\alpha^+$
3. $CD4^+$ effector memory cells producing IFN-γ, TNF-α and IL-2
4. $CD4^+$ central memory cells producing IL-2 and IFN-γ
5. $CD8^+$ naïve cells producing $IFN\text{-}\gamma^+$ $TNF\text{-}\alpha^+$ and TNF-α
6. $CD8^+$ effector memory cells producing $IFN\text{-}\gamma^+$ $TNF\text{-}\alpha^+$ and TNF-α,
7. $CD8^+$ effector cells producing $IFN\text{-}\gamma^+$ $TNF\text{-}\alpha^+$ and TNF-α alone,

The multivariate data analysis of the Mean Fluorescence Intensity (MFI) shows the following immunological parameters that best discriminate between the two age groups:

1. Fluorescence Intensity of IFN-γ from IFN-γ^+ IL-2^+ TNF-α, IFN-γ^+ IL-2^+, and IFN-γ^+ TNF-α^+ produced by $CD4^+$ central memory cells, and fluorescence Intensity of TNF-α and IL-2 from $CD4^+$ central memory cells.
2. Fluorescence Intensity of IFN-γ from IFN-γ^+ TNF-α^+ produced by $CD4^+$ effector memory cells, and fluorescence Intensity of TNF-α from $CD4^+$ effector memory cells.
3. Fluorescence Intensity of IFN-γ from IFN-γ^+ TNF-α^+ produced by $CD4^+$ naïve cells.
4. Fluorescence Intensity of IFN-γ from IFN-γ^+ TNF-α^+, and TNF-α alone produced by $CD4^+$ effector cells
5. Fluorescence Intensity of IL-2 from IFN-γ^+ IL-2^+ TNF-α^+ produced by $CD8^+$ effector cells, fluorescence Intensity of TNF-α from IFN-γ^+ IL-2^+ TNF-α^+ produced by $CD8^+$ effector cells and fluorescence Intensity of IFN-γ from IFN-γ^+ TNF-α^+ produced by $CD8^+$ effector cells.
6. Fluorescence Intensity of IFN-γ from IFN-γ^+ TNF-α^+, and TNF-α alone produced by $CD8^+$ naive

Most of the immunological parameters that have high contribution towards the differentiation of the two age groups whether in the analysis of the frequency, absolute cell count or MFI are mostly the multiple cytokine production (double and triple-cytokine production). These results show that the two age groups of RM have observable differences in the production of multiple cytokines or the possession of multifunctional

cells, which is known to be one of the factors associated with better outcomes in HIV/SIV disease. In addition, according to tables 1-3, the data of this study demonstrate that most of the younger group showed significantly higher production of cytokines in terms of frequencies, absolute cell count and MFI when compared to the older sample group.

Conclusion

The overall data from this study clearly demonstrated that upon stimulation with either mass stimulation of SIV specific antigen, the young population of RM provides stronger protective responses in terms of HIV infection. In addition, most of the discriminatory immunological factors that best differentiate between the young and the old mostly fall into the group of multifunctional cytokine production in various subsets of $CD4^+$ and $CD8^+$ T cells, which again is known to provide stronger protective responses towards the infection. The data in my study shows immune impairment as aging progresses, especially in terms of SIV/HIV-relevant parameters. By studying the responses of uninfected RM, it provided us with the information on the potential of the cells of this species that are susceptible to the disease just like human. The underlying potential to mount greater protective immune responses against SIV in the young can be used to confirm the previous studies on the protective immune responses in RM. This finding would provide initial evidence to explain age-dependent differences in progression patterns. These results might also be beneficial in planning of future experiments using the most popular primate model in HIV/AIDS research (the rhesus macaque), and providing researchers with information to help them choose proper age groups to include in their experiments

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