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Down-regulation of CD4 by memory CD4+ T cells in vivo renders African green monkeys resistant to progressive SIVagm infection

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Abstract

African green monkeys (genus *Chlorocebus*) can be infected with SIVagm, but do not develop AIDS. This natural host of SIV, like sooty mangabeys, maintains high levels of SIV replication but has evolved to avoid immunodeficiency. Elucidating the mechanisms that allow the natural hosts to co-exist with SIV without overt disease may provide crucial information to understand AIDS pathogenesis. Here we show: (1) many $CD4^+$ T cells from African green monkeys down-regulate CD4 in vivo as they enter the memory pool, (2) down regulation of CD4 by memory T cells is independent of SIV infection, (3) the $CD4^-$ memory T cells maintain functions which are normally attributed to CD4 T cells including production of IL-2, production of IL-17, expression of FoxP3 and expression of CD40L (4) loss of CD4 expression protects these T cells from infection by SIVagm in vivo, and (5) these $CD4^-$ T cells can maintain MHC-II restriction. These data demonstrate that the absence of SIV-induced disease progression in natural hosts species may be partially explained by preservation of a subset of T cells that maintain CD4 T cell function while being resistant to SIV-infection in vivo.

INTRODUCTION

Simian immunodeficiency viruses (SIV) belong to the group of lentiviruses that infect non-human primates (NHP). The lentiviruses that cause immunodeficiencies in humans and Asian macaques originated from cross-species transmission of viruses that naturally infect nonhuman primates in Africa1. Like HIV-1 and HIV-2, all known SIV subtypes use CD4 as a receptor and either CCR5, CXCR4, or, as for SIVrcm, CCR22–4 as a co-receptor. Moreover, both SIV infection of Asian macaques and HIV-1 infection of humans result in chronic infection and the majority of infected individuals progress to AIDS.

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In contrast, after SIV infection natural hosts generally do not progress to AIDS. Since natural hosts of SIV have co-evolved with the virus to avoid disease progression, dissecting the mechanisms underlying the nonprogressive nature of natural SIV infection will lead to a better understanding of the aspects of HIV infection responsible for the progressive nature of the disease in humans5–7. Natural hosts do not avoid disease progression by immunological control of the virus since SIV-infected natural hosts maintain high levels of viremia8–12. Moreover, experimental depletion of $CD8^+$ T cells does not affect plasma viremia13 and natural hosts do not exhibit superior cellular control of viremia compared to HIV-infected humans or SIV-infected rhesus macaques(RM)14. The lentiviruses that infect natural hosts can be pathogenic. SIV infections of AGM and Sooty mangabeys (SM) have been correlated with short life spans of infected cells *in vivo*15–17. Moreover, SIVagm, which naturally infects African green monkeys (AGM), can be used to infect pigtail macaques who subsequently manifest simian AIDS18,19. Isolates of SIVsmm can also cause progressive infection in RM20–23. One fundamental difference between progressive SIV/HIV infection and nonprogressive SIV infection is the absence of immune activation, which is associated with disease progression in HIV-infected individuals24, during the chronic phase of infection in natural hosts9,11,25–28.

Previous studies performed using AGM have reported very low frequencies of $CD4^+$ T cells28. AGM, however, remain disease free despite having low numbers of $CD4^+$ T cells. In two reports, Murayama *et al*, described low frequencies of $CD4^+$ T cells and high frequencies of $CD8^{dim}$ T-cells in healthy adult AGM29,30. They also found the $CD8^{dim}$ T cells could induce antibody production from B-cells *in vitro* and suggested that the $CD8^{dim}$ T-cells might supplement for the lack of $CD4^+$ T-cells29. Here we have studied the frequencies, functionalities and *in vivo* infection frequencies of lymphocyte subsets from 36 AGM, 10 naturally infected, 8 experimentally infected, 11 uninfected adults and 7 uninfected juveniles (Table 1). Our data describe a mechanism by which AGM are able to survive SIVagm infection without succumbing to AIDS.

RESULTS

Decreased frequencies of $CD4^+$ T-cells correlate with increased frequencies of $CD4^-CD8\alpha^{dim}$ T-cells

Loss of $CD4^+$ T-cells is a hallmark of progression to AIDS in humans and Asian macaques. We therefore compared the frequency of $CD4^+$ T-cells in SIVagm-infected and uninfected vervet AGM (one of four subspecies of AGM), HIV-uninfected humans, SIV-uninfected RM and SIVsmm-infected and uninfected SM. We observed three distinct T-cell populations in AGM based upon expression of CD4 and CD8α: $CD4^+$ T-cells, $CD4^-CD8\alpha^{dim}$ T-cells and $CD4^-CD8\alpha^{bright}$ T-cells (Fig. 1a). We analyzed further the phenotypes of each subset based upon expression of CD28 and CD95 (Fig. 1b). The $CD4^+$ and $CD4^-CD8\alpha^{bright}$ T-cells consisted of both memory and naive subsets (Fig. 1b). However, the $CD4^-CD8\alpha^{dim}$ T-cells consisted predominantly of memory T-cells (Fig. 1b). Moreover, adult AGM appeared to have surprisingly low frequencies of $CD4^+$ T-cells and low $CD4^+$ T-cell counts(Table 1). There were significantly lower frequencies of $CD4^+$ T-cells in both SIVagm-infected and uninfected AGM compared to SIVsmm-infected or

uninfected SM, SIV-uninfected RM or HIV-1-uninfected humans (Fig. 1c). In addition, the decrease in $CD4^+$ T-cells was accompanied by an increase in the frequency of $CD4^-CD8\alpha^{dim}$ T-cells (Fig. 1a). Further phenotypic analysis showed that this $CD4^-CD8\alpha^{dim}$ T-cell population lacked expression of CD8β (Fig. 1d). In addition, the frequency of $Ki67^+$ cells was significantly among $CD4^-CD8\alpha^{dim}$ T-cells compared to either the $CD4^+$ or $CD4^-CD8\alpha^{bright}$ subsets, suggesting that the high frequency of $CD4^-CD8\alpha^{dim}$ T-cells was not due to preferential proliferation of$CD4^-CD8\alpha^{dim}$ T-cells *in vivo* **(**Fig. 1e).

The increased frequency of $CD4^-CD8\alpha^{dim}$ T-cells could be a reflection of a mathematical shift in percentages of T-cell populations due to the loss of $CD4^+$ T-cells. However, we found that there was a significant negative correlation between the decrease in $CD4^+$ T-cells and the increase in $CD4^-CD8\alpha^{dim}$ T-cells (Fig. 1f), but not for the $CD4^-CD8\alpha^{bright}$ T-cells (Fig. 1g). Therefore, the increase in the $CD4^-CD8\alpha^{dim}$ T-cell population was directly related to the decrease in $CD4^+$ T-cells, suggesting that some$CD4^-CD8\alpha^{dim}$ T-cells might develop from $CD4^+$ T-cells.

Some $CD4^-CD8\alpha^{dim}$ T-cells develop from $CD4^+$ memory T-cells

Since the frequencies of the $CD4^-CD8\alpha^{dim}$ T-cells negatively correlated with the frequencies of $CD4^+$ T-cells (Fig. 1f), we hypothesized that some of the $CD4^-CD8\alpha^{dim}$ T-cells may have developed from $CD4^+$ T-cells. Phenotypic analysis of the $CD4^+$ T-cells from SIVagm-infected and uninfected AGM illustrated that the $CD95^+$ memory $CD4^+$ T-cells down-regulated CD4 (Fig. 2a) and up-regulated CD8α (Fig. 2b) compared to naive $CD4^+$ T-cells. Fluorescence minus one analysis for CD8α was also performed to confirm that the $CD4^+$ T-cells expressed CD8α (Fig 2c). The median fluorescent intensity (MFI) of CD4 was significantly lower in the memory subset of $CD4^+$ T-cells compared to naive $CD4^+$ T-cells (Fig. 2d) while the MFI of CD8α was significantly increased in the memory $CD4^+$ T-cells over naive (Fig. 2e). Therefore, we hypothesized that upon stimulation $CD4^+$ T-cells could transition to become $CD4^-CD8\alpha^{dim}$ T-cells.

To study down-regulation of CD4 *in vitro*, we CFSE labeled and mitogenically stimulated peripheral blood mononucelear cells (PBMC) from several AGM and, for comparison, pigtail macaques. We then studied expression of CD4 and proliferation by flow cytometry after 5, 7, and 9 days in culture (Fig. 3a–b and Supplemental Fig. 1). We found that while AGM $CD4^+$ T-cells lost CD4 expression, $CD4^+$ T-cells from pigtail macaques maintained CD4 expression with cell division (Fig. 3a). It is a possibility that the CD4 down-regulation in AGM was transient, however, we did not see up-regulation of CD4 by day 9 (Supplemental Fig. 1). To confirm this finding, we also sorted naive $CD4^+$ T-cells (>99% pure) prior to stimulation and found that after stimulation they became $CD4^-$(Supplemental Fig. 1).

Analysis for CD4 mRNA within sorted $CD4^+$, $CD4^-CD8\alpha^{dim}$, $CD4^-CD8\alpha^{bright}$ T-cells and monocytes revealed that CD4 mRNA was only detected within $CD4^+$ T-cells (Fig. 3c). Taken together, these data suggested that AGM memory $CD4^+$ T-cells could become $CD4^-CD8\alpha^{dim}$ T-cells. If our hypothesis was correct, AGM would have concomitant decreases in the overall frequencies of total $CD4^+$ T-cells with increases in memory $CD4^+$ T-cells. Indeed, we found a significant negative correlation between the frequencies of

memory $CD4^+$ T-cells and the total frequency of$CD4^+$ T-cells (Fig. 3d). Hence as AGM accumulate memory $CD4^+$ T-cells, the overall frequency of $CD4^+$ T-cells decreases.

We reasoned that if some $CD4^+$ T-cells were becoming $CD4^-CD8\alpha^{dim}$ T-cells upon stimulation into the memory pool, then animals with very little antigenic experience should have frequencies of $CD4^+$ T-cells comparable to those of healthy RM and humans (Fig. 1c). Therefore, we measured the frequencies of T-cell subsets from PBMC obtained directly *ex vivo* from juvenile AGM (all less than 2 years old). In a total of seven juvenile AGM, the median frequency of $CD4^+$ T-cells was 59% (39.6%–64.4%, Fig. 3e). These frequencies were comparable with those from non-immunocompromised RM and humans and were higher than the frequencies of $CD4^+$ T-cells in adult AGM ($P<0.0006$). Furthermore, after we vaccinated the juvenile AGM with the standard influenza vaccine we observed an influenza-specific T-cell response from the $CD4^-CD8\alpha^{dim}$ T-cells to the MHC-II restricted antigen (Supplemental Fig. 2c). Taken together, these observations strongly suggest that many of the $CD4^-CD8\alpha^{dim}$ T-cells developed from memory $CD4^+$ T-cells.

While we do not know the ages of many of the AGM, as they were imported from Tanzania, in the few animals from which we do have this information, there was no correlation between age and the frequency of $CD4^+$ T-cells (data not shown). However, it is likely that age may be a factor in accumulation of $CD4^-CD8\alpha^{dim}$ T-cells as older animals are exposed to more antigens *in vivo*.

$CD4^-CD8\alpha^{dim}$ T-cells preserve $CD4^+$ T-cell function while evading SIV infection

To elucidate the functions of the $CD4^-CD8\alpha^{dim}$ T-cells, we obtained PBMC from SIVagm-infected and uninfected adult AGM. Since it seemed apparent that many of the $CD4^-CD8\alpha^{dim}$ T-cells developed from $CD4^+$ T-cells, we examined these T-cells for functions generally attributed to $CD4^+$ T-cells. We found that upon stimulation with staphylococcal enterotoxin B (SEB), the $CD4^-CD8\alpha^{dim}$ T-cells could produceIL-2 and IL-17 (Supplemental Fig. 2 and Fig. 4a) and express CD40 ligand (CD40L) (Supplemental Fig. 2 and Fig. 4a). CD40L is typically expressed by activated $CD4^+$ T-cells and results in enhancement of antigen presentation and induces B-cell class switching31. Additionally, a portion of the $CD4^-CD8\alpha^{dim}$ T-cells expressed the transcription factor FoxP3, thought to be predominantly expressed by regulatory $CD4^+$ T-cells (Supplemental Fig. 2 and Fig. 4a)32. Hence the $CD4^-CD8\alpha^{dim}$ T-cell subset included T-cells that performed functions normally restricted to $CD4^+$ T-cells. While the overall frequencies of memory $CD4^+$ T-cells that could perform each function was significantly higher compared to the $CD4^-CD8\alpha^{dim}$ T-cells(Fig. 4a), the total frequency of $CD4^-CD8\alpha^{dim}$ T-cells was significantly greater than the overall frequency of $CD4^+$ T-cells. We therefore calculated the relative number of $cytokine^+$, $CD40L^+$, and $FoxP3^+$ T-cells for each T-cell subset (Fig. 4b). For IL-2, IL-17, and FoxP3 the relative number of $CD4^-CD8\alpha^{dim}$ T-cells was significantly greater than those of the $CD4^+$ and $CD4^-CD8\alpha^{bright}$ T-cell subsets. The relative number of $CD40L^+$ $CD4^-CD8\alpha^{dim}$ T-cells was equal to that of $CD4^+$ T-cells. These observations suggest that there were actually greater numbers of T-cells performing $CD4^+$ T-cell functions without actually expressing CD4.

$CD4^+$ T-cells are restricted by MHC-II, while $CD8^+$ T-cells are restricted to MHC-I. Therefore, to test further our hypothesis that the $CD4^-CD8\alpha^{dim}$ T-cells were acting as $CD4^+$ T-cells, we screened PBMC from adult AGM for T-cell responses to a cytomegalovirus (CMV) whole antigen preparation. Presentation of this antigen requires processing through MHC-II33. We found one AGM that had T-cells responsive to CMV (AG731) and the entire CMV-specific T-cell response was restricted to $CD4^-CD8\alpha^{dim}$ T-cells(Fig. 4c and Supplemental Fig. 2b,c). To confirm that these CMV-specific $CD4^-CD8\alpha^{dim}$ T-cells were restricted by MHC-II we stimulated the T-cells in the presence of blocking antibodies specific to either MHC-II or MHC-I and measured production of cytokines. Blocking with anti-MHC class-II antibody decreased the frequency of responding CMV-specific $CD4^-CD8\alpha^{dim}$ T-cells by more than two-thirds, while blocking MHC-I had virtually no effect on the ability of CMV-specific $CD4^-CD8\alpha^{dim}$ T-cells to respond (Fig. 4c). Hence $CD4^-CD8\alpha^{dim}$ T-cells can be restricted by MHC-II in AGM.

Vervets are one of four subspecies of AGM along with sabeus, tantalus and grivets. To determine whether the phenomena which we observed were specific to vervet AGM, we examined T-cell populations in 6 adult sabeus AGM. Indeed, we found the presence of $CD4^-CD8\alpha^{dim}$ T-cells. Similar to the vervets, the $CD4^-CD8\alpha^{dim}$ T-cells from sabeus were able to perform CD4 functions (Supplementary Fig. 3). Confirmation of this phenomenon occurring in a second subspecies of AGM suggest that down-regulation of CD4 is characteristic of all AGM.

Since the $CD4^-CD8\alpha^{dim}$ T-cells have many functional characteristics of $CD4^+$ T-cells, wenext determined which lymphocytes were infected by SIVagm *in vivo*. We flow cytometrically sorted individual subsets of lymphocytes from eighteen SIVagm-infected AGM and measured the *in vivo* infection frequency by quantitative real time PCR for SIVagm DNA. We sorted naive and memory $CD4^+$ T-cells, memory $CD4^-CD8\alpha^{dim}$ T-cells, memory $CD4^-CD8\alpha^{bright}$ T-cells, memory $CD4^-CD8\alpha^-$ T-cells and monocytes. We found that memory $CD4^+$ T-cells were the primary target for SIVagm *in vivo* (Fig. 5). Consistent with infection frequency patterns of HIV34, we also found that SIVagm could infect naive $CD4^+$ T-cells, but memory $CD4^+$ T-cells were preferentially infected. Importantly, $CD4^-CD8\alpha^{dim}$ T-cells, which we have shown can develop from $CD4^+$ T-cells and maintain functions of $CD4^+$ T-cells, were only very rarely, if ever, infected by SIVagm *in vivo* (Fig. 5). In 78% of the SIVagm-infected AGM we detected no viral DNA within the $CD4^-CD8\alpha^{dim}$ T-cell subset. Cell numbers were often limiting and it was conceivable that, in some cases, we did not detect any viral DNA due to a small number of sorted cells in each PCR. In samples in which no viral DNA was detected, we reported values calculated as 1/2 the lower limit of detection (closed circles), these values are based upon the number of sorted cells. In the few (22%) AGM where we did detect viral DNA within the $CD4^-CD8\alpha^{dim}$ T-cell subset the infection frequencies were very low (<0.01%). Taken together, these data suggest that $CD4^-CD8\alpha^{dim}$ T-cells preserve $CD4^+$ T-cell function while evading SIV infection *in vivo* and in turn, these findings suggest a mechanism by which AGM are able remain disease free despite SIV infection.

DISCUSSION

We have shown that AGM, regardless of infection with SIVagm, have decreased numbers of total $CD4^+$ T-cells that correlates with an increased population of $CD4^-CD8\alpha^{dim}$ T-cells. We demonstrated that some of these $CD4^-CD8\alpha^{dim}$ T-cells developed from memory $CD4^+$ T-cells. This conclusion is strengthened by the observation that juvenile AGM, who have very low frequencies of memory T-cells *in vivo*, have high frequencies of $CD4^+$ T-cells and low frequencies of $CD4^-CD8\alpha^{dim}$ T-cells and that *in vitro* stimulation of naive $CD4^+$ T-cells results in down-regulation of CD4 and up-regulation of CD8α. In addition, we have demonstrated that in adult AGM some of these $CD4^-CD8\alpha^{dim}$ T-cells, upon stimulation, exhibit functions generally attributed to $CD4^+$ T-cells. The $CD4^-CD8\alpha^{dim}$ T-cells can be restricted by MHC-II. Despite the fact many of the T-cells in this population likely developed from $CD4^+$ T-cells and maintain the ability to perform functions attributed to $CD4^+$ T-cells, they are able to evade infection by SIVagm. These data provide a mechanism by which AGM are able to survive chronic SIVagm infection without progression to simian AIDS.

Our proposed mechanism underlying the nonpathogenic nature of SIVagm infection could contribute to the lack of immune activation seen in the natural hosts of SIV. Indeed, preservation of $CD4^+$ T-cell function may well contribute to the lack of immune activation in AGM and SM28,35,36. Additionally, there is a significantly lower frequency of $CD4^+CCR5^+$ T-cells in natural hosts of SIV when compared to non-natural hosts16,37, suggesting that the lack of CCR5 expression results in decreased homing to sites of inflammation, thereby preventing activation and inflammation. This decrease in activated T-cells could reduce the number targets for SIV. Taken together, these data may suggest that in natural hosts, the virus may be preferentially targeting macrophages. However, we have found no SIVagm DNA within highly purified monocytes, consistent with previous reports that SIVagm preferentially replicates in lymphocytes during chronic infection16,28. Also, previous comparative studies between pathogenic HIV infection of humans or SIV infection of Asian macaques and SIV infection of SM or AGM have shown that while both SM and AGM lose $CD4^+$ T-cells from the gastrointestinal tract during the acute phase of infection, these animals do not manifest immune activation11,28,36. Microbial translocation, which causes immune activation in the chronic phase of HIV and SIV infection of Asian macaques38–45, does not occur in the chronic phase of SIV infection of either AGM or sooty mangabeys28,42. The functionality of mucosal $CD4^-CD8\alpha^{dim}$ T-cells, which is present at high frequencies in AGM28, should be assessed for a role in preventing microbial translocation and immune activation in SIVagm-infected AGM.

Many other cellular populations, such as NK cells, γδ T-cells and NKT-cells, express the CD8αα homodimer. Indeed, some of the $CD4^-CD8\alpha^{dim}$ T-cells from AGM express the γδ TCR (data not shown). Moreover, many of the $CD28^-$ memory T-cells within the $CD4^-CD8\alpha^{dim}$ T-cell subset express granzyme B (data not shown), not typically expressed by $CD4^+$ T-cells. Therefore, it is clear that not all of the $CD4^-CD8\alpha^{dim}$ T-cells were originally $CD4^+$ T-cells. While AGM have significantly higher frequencies of $CD4^-CD8\alpha^{dim}$ T-cells compared to humans, Bolassel *et al*, recently showed that slow progressing HIV-1-infected individuals have a significantly higher frequency of

$CD4^{-}CD8\alpha^{dim}$ T-cells compared to chronically HIV-infected individuals46. This finding suggests a similar phenomenon to what we describe here could slow disease progression in HIV-infected humans.

While $CD4^{-}CD8\alpha^{dim}$ T-cells have previously been studied, it has not been demonstrated that memory $CD4^{+}$ T-cells down-regulate CD4 and up-regulate CD8α. How these T-cells are able to perform functions without the CD4 molecule is unclear. Importantly, Rahemtulla *et al*, demonstrated normal development and function of lymphoid organs, B cells and $CD8^{+}$ T-cells in CD4 knockout mice. However, antibody production, IL-2 secretion, and MHC-II restricted responses were significantly abrogated47. The authors subsequently showed that $CD4^{-}CD8^{-}$ T-cells in CD4 knockout mice could partially compensate for the lack of CD4 T-cells48, similar to what we observe in AGM. CD4 has been shown to be important for initiating the downstream kinase signaling that results in T-cell activation49. It is possible that in AGM $CD4^{-}CD8\alpha^{dim}$ T-cells sufficient lck phosphorylation occurs, or alternative signals exist, but the actual mechanism(s) underlying the switch from $CD4^{+}$ to $CD4^{-}CD8\alpha^{dim}$ remains unclear. Indeed, previous studies have reported genetic differences between certain regulatory elements from AGM compared to other primates50. One possibility is that a change may occur in the methylation states of the enhancer and silencer regions in the genome51. Also, differential expression of the MAZR protein, a protein suppressor of the CD8α enhancer region52, may also exist.

It is not unprecedented that changes in the frequencies of T-cells within individual subsets occur in lentiviral infections. For example, while SM generally maintain healthy $CD4^{+}$ T-cell counts during chronic SIVsmm infection, infection with a dual tropic (CXCR4/CCR5) strain of SIVsmm resulted in the loss of $CD4^{+}$ T-cells, but preservation $CD4^{-}CD8^{-}$ T-cells53. Moreover, one report identified several naturally SIV-infected SM with very low frequencies of $CD4^{+}$ T-cells26. The functions and ontogeny of T-cells in these SIVsmm-infected SM infected are currently under investigation but could represent a similar phenomenon.

Natural hosts have co-evolved with SIV to avoid disease progression, though the mechanisms by which this occurs may diverge since most SM maintain healthy frequencies of $CD4^{+}$ T-cells. In AGM, we show that this co-evolution with SIVagm has occurred, in part, by the development of $CD4^{-}CD8\alpha^{dim}$ T-cells from memory $CD4^{+}$ T-cells. Additionally, African lions, who remain disease free after infection with feline immunodeficiency virus, maintain a high frequency of $CD8\beta^{dull}$ T-cells regardless $CD4^{+}$ T-cell loss54. Hence, down-regulation of CD4 may be associated with lack of disease progression in multiple immunodeficiency lentiviral infections.

We have identified and characterized a mechanism by which AGM are able to survive chronic SIVagm infection. In non-natural hosts of immunodeficiency lentiviruses depletion of $CD4^{+}$ T-cells leads to AIDS. However, SIVagm-infected AGM maintain immune responses, remain healthy, and live normal life spans. We provide evidence for the conversion of $CD4^{+}$ T-cells to $CD4^{-}CD8\alpha^{dim}$ T-cells, which likely plays a key role in the lack of clinical signs of AIDS in AGM. Once the mechanism by which the $CD4^{+}$ T-cells are

able to convert to $CD4^{-}CD8\alpha^{dim}$ T-cells has been understood, interventions aimed at mimicking this phenomenon could be developed for preventative and therapeutic trials.

METHODS

Animals

Eighteen SIVagm-infected vervet AGM (*Chlorocebus pygerythrus)*, 11SIVagm-uninfected vervet AGM, 7SIVagm-uninfected juveline (less than 2 years old) vervet AGM, and 12 SIV-negative rhesus macaques (*Macaca mulatta)* were housed at Bioqual (Rockville, MD) and 6SIV-infected sabaeus AGM (*C. sabeus*) were housed at the Tulane National Primate Center (Convington, LA). SIVsmm-infected and uninfected SM were housed at the Yerkes National Primate Center (Atlanta, GA). All animals were housed in accordance with the National Research Council Guide for the Care and use of Laboratory Animals, and all protocols were approved by the relevant institutional animal care and use committees. Ten of the SIV^{+} AGM vervets, were infected in the wild and eight were experimentally infected with 50 or 1000 $TCID_{50}$ of SIVagm90 intravenously (Table 1). Virus was isolated as previously described10. AG11 and AG15 are seropositive for SIVagm.

Human Subjects

Five HIV-1 uninfected subjects were recruited at the National Institutes of Health. The subjects all gave informed consent prior to entry into this study and all studies were approved by the institution's IRB.

Flow cytometry

For intracellular cytokine staining (ICS), PBMC were incubated overnight at 37°C with media alone, 1μg of Staphylococcal enterotoxin B (SEB) (Sigma, St. Louis, MO), or 1μg of cytomegalovirus (CMV) whole antigen (Microbix Biosystems, Toronto, Ontario, Canada) in the presence of 0.5μg each of monoclonal antibodies to CD28 (CD28.2, Beckman Coulter, Fullerton, CA) and CD49d (9F10, BD Bioscience, San Jose, CA) and 1 μg/ml Brefeldin A (Sigma, St. Louis, MO). For some experiments PBMC were pretreated for 1 hour at 37°C with antibodies against MHC-I (G46-2.6, BD Bioscience) or MHC-II (TU39, BD Bioscience). After stimulation, cells were washed twice and incubated with Live/Dead fixable aqua dead cell stain (Invitrogen, Carlsbad, CA). Cells were then stained for the surface markers using monoclonal antibodies to CD3 (SP34-2, BD Bioscience), CD4 (L200, BD Bioscience), CD8 (RPA-T8, BD Bioscience), and CD95 (DX2, BD bioscience). Cells were washed and permeabilized with Cytofix/Cytoperm buffer (BD Bioscience). Cells were then intracellularly stained with fluorescent-conjugated monoclonal antibodies to IFNγ (4S.B3, BD Bioscience), IL-17 (eBio64DEC17, eBioscience, San Diego, CA), IL-2 (MQ1-17H12, BD Bioscience), CD40L (TRAP1, BD Bioscience), or Ki67 (B56, BD Bioscience). Cells were incubated at 4°C for 20 minutes. Cells were washed and then fixed with a 1% paraformaldehyde solution (Electron Microscopy Sciences, Hatfield, PA).

For proliferation, PBMC or flow cytometrically sorted naive CD4 T-cells were stained with 0.125 μM carboxyfluorescein succinimidyl ester (CFSE, Invitrogen) and then stimulated with 1μg/mL concanavalin A (Sigma), 1μg/mL staphylococcal enterotoxin B (Sigma), or

anti-CD3/anti-CD28 microbeads at a 1:4 cell to bead ratio (generous gift from Joern E. Schmitz and Roland Zahn) for 5, 7, and 9 days. Cells were then labeled with fluorescent antibodies directed towards CD3, CD4, and CD8 (BD Bioscience).

For analysis of FoxP3 expression fresh PBMCs were surface stained and then permeabilized using FoxP3 permeabilization solution (eBioscience). Cells were intracellularly stained for FoxP3 (PCH101, eBioscience). Cells were washed and then fixed using a 1% PFA solution. All flow cytometry samples were run on a FACSAria (BD Bioscience) using FACSDiva software (BDBioscience) and data were analyzed using FlowJo (Tree Star, Ashland, OR).

Polymerase chain reaction

Quantitative real time PCR—Cell populations were sorted flow cytometrically and were lysed using 25 μL of a 1:100 dilution of proteinase K (Roche, Indianapolis, IN) in 10mM Tris buffer. Quantitative PCR was performed using 5 μL of cell lysates per reaction. Reaction conditions were as follows: 95°C holding stage for 5 minutes, and 40 cycles of 95°C for 15 seconds followed by 60°C for 1 minute. The Taq DNA polymerase kit (Invitrogen) was used. The sequence of the forward primer for SIVagm is GTCCAGTCTCAGCATTTACTTG. The reverse primer sequence is CGGGCATTGAGGTTTTTCAC. The probe sequence is CAGATGTTGAAGCTGACCATTTGGG. For cell number quantitation monkey albumin was measured as previously described55. The PCR machine used was the StepOne Plus (Applied Biosystems, Foster City, CA) and the analysis was performed using StepOne software (Applied Biosystems).

Reverse transcription PCR—Viable cell populations were sorted flow cytometrically and mRNA was isolated using Oligotex Direct mRNA Mini Kit (Qiagen, Valencia, CA) by following the manufacturer's protocol. Following cDNA synthesis with random hexamers and Superscript II RNase H- reverse transcriptase transcripts of CD4 were amplified by PCR using the forward primer TCGGATTGACTGCCAACTCTG and reverse primer AAGGCGAGCGGGAAGGAGAA. Reaction conditions were as follows: 95°C holding stage for 5 minutes, and 40 cycles of 95°C for 15 seconds followed by 60°C for 1 minute. To control for the amount of mRNA, a second PCR was performed using primers for albumin (as above).

Statistics

All statistical analyses were performed using Prism software (GraphPad, La Jolla, CA). Statistically significance was based upon P values less than 0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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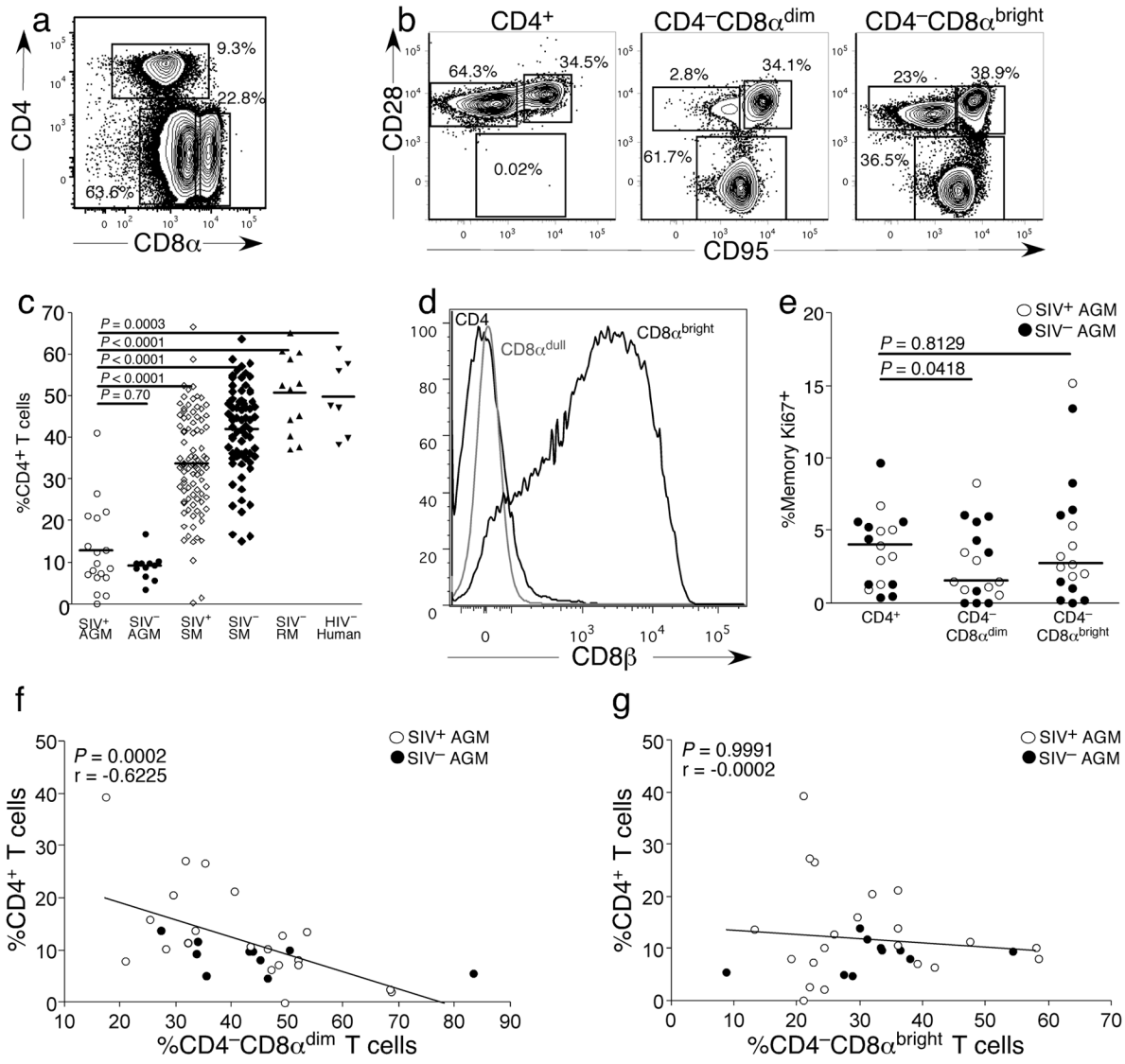

Figure 1. Phenotypic analysis of T-cell populations in vervet African green monkeys

(**a**) Phenotype of T-cells in adult AGM. (**b**) Phenotype of individual subsets of T-cells in adult AGM. (**c**) Comparison of percent of $CD3^+$ T-cells that express CD4 in peripheral blood in SIV^+ and SIV^- adult AGM, adult SIV^+ and SIV^- SM, adult SIV^- RM, and HIV^- adult humans. (**d**) Characterization of $CD4^-CD8\alpha^{dim}$ T-cells as $CD8\alpha\beta^-$ by analysis of CD8β expression. Cells were gated on live $CD3^+$ small lymphocytes and analyzed for both CD4 and CD8β expression. (**e**) Ki67 expression by different subsets of memory T-cells. (**f**) Negative correlation between the frequencies of $CD4^+$ T-cell and $CD4^-CD8\alpha^{dim}$ T-cells in adult AGM. (**g**) Correlation of the frequency of $CD4^+$ T-cells and the frequency of $CD4^-CD8\alpha^{bright}$ T-cells in adult AGM. White circles represent SIV-infected AGM and black circles represent uninfected AGM. White diamonds represent SIV-infected SM and black diamonds prepresent uninfected SM. Black triangles represent SIV-uninfected RM and black squares represent HIV-uninfected humans. A Mann-Whitney U test was performed for **c**. A Spearman rank correlation was calculated for **f** and **g**.

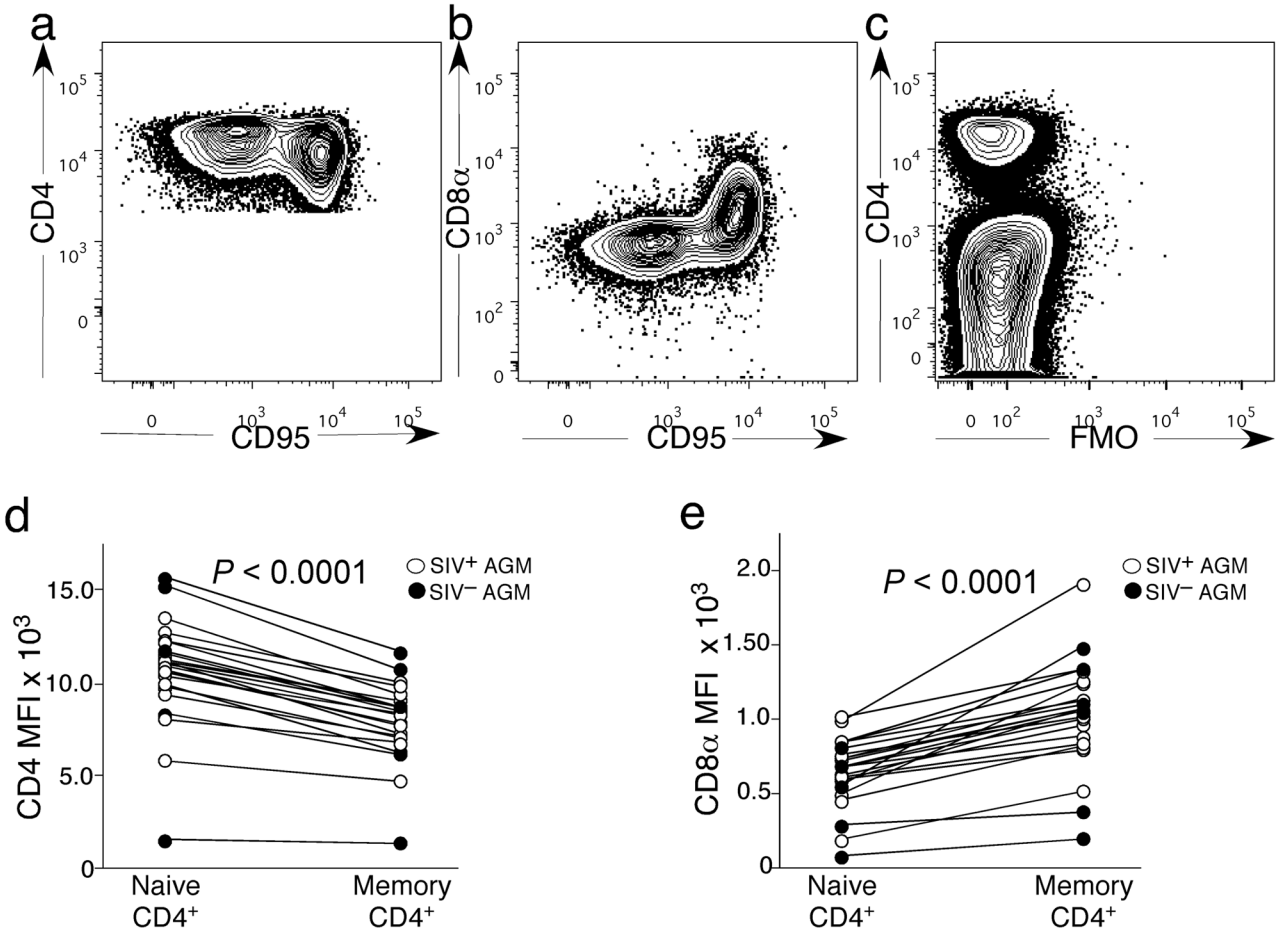

Figure 2. CD8α and CD4 expression by naive and memory CD4$^+$ T-cells

(**a**) Representative staining of CD4$^+$ memory and naive T-cells for CD4. (**b**) Representative staining of CD4$^+$ memory and naive T-cells for CD8. (**c**) Fluorescence minus one control lacking antibodies against CD8α. (**d**) Median fluorescence intensity for CD4 expression in naive and memory CD4$^+$ T-cells. (**e**) Median fluorescence intensity for CD8 expression in naive and memory CD4$^+$ T-cells. A Wilcoxon matched pairs test was performed for **2d–e**.

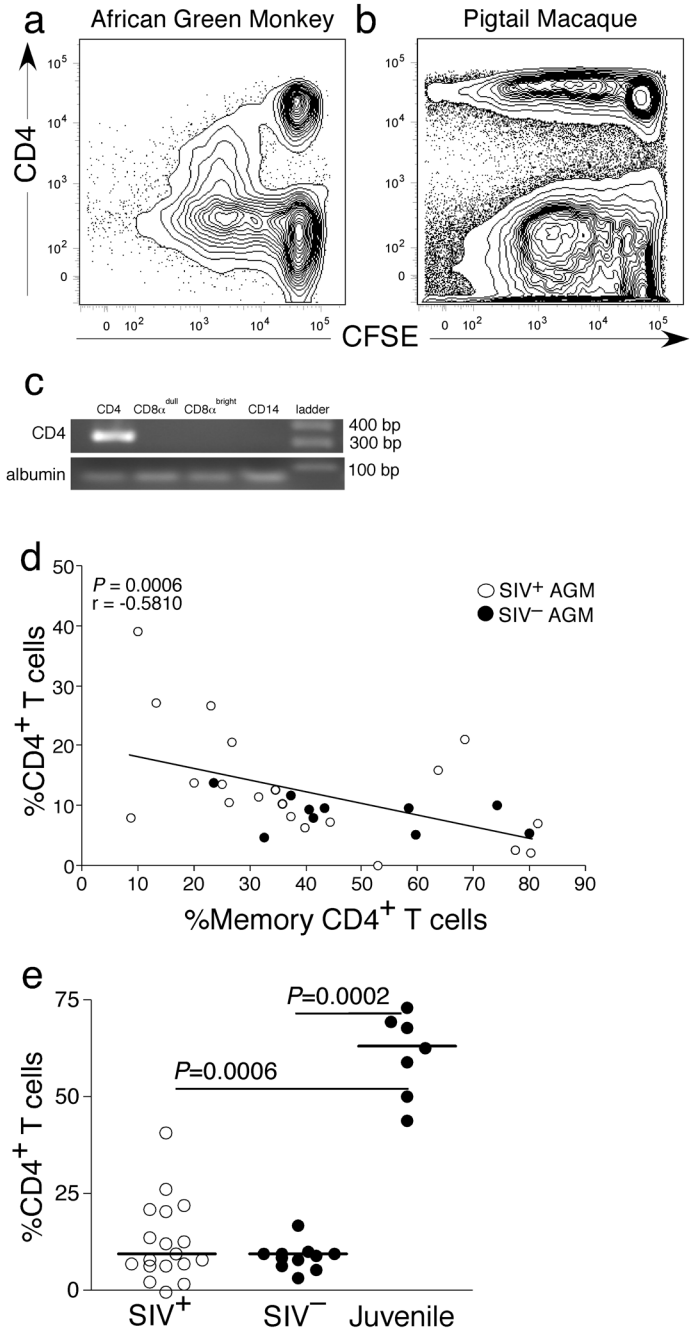

Figure 3. CD4^{-}CD8α^{dim} T-cells can develop from memory CD4^{+} T-cells

(**a**) Down-regulation of CD4 by stimulated *in vitro* PBMC from AGM after 5 days of stimulation with SEB. (**b**) Maintenance of CD4 expression by stimulated PBMC from pigtail macaques *in vitro* after 5 days of stimulation with SEB. (**c**) CD4 mRNA expression in CD14^{+}, CD4^{-}CD8α^{dim}, CD4^{-}CD8α^{bright}, or CD4^{+} lymphocyte subsets of an adult AGM. (**d**)Negative correlation between the frequency of memory CD4^{+} T-cells and the frequency of the total CD4^{+} T-cells in adult AGM. (**e**) Comparison of percent CD4^{+} T-cells in peripheral blood in SIV^{+} and SIV^{-} adult and SIV^{-} juvenile AGM. White circles represent

SIV-infected AGM and black represent uninfected AGM. A Spearman rank correlation was calculated for **3c**. A Mann Whitney U test was performed for **3e**.

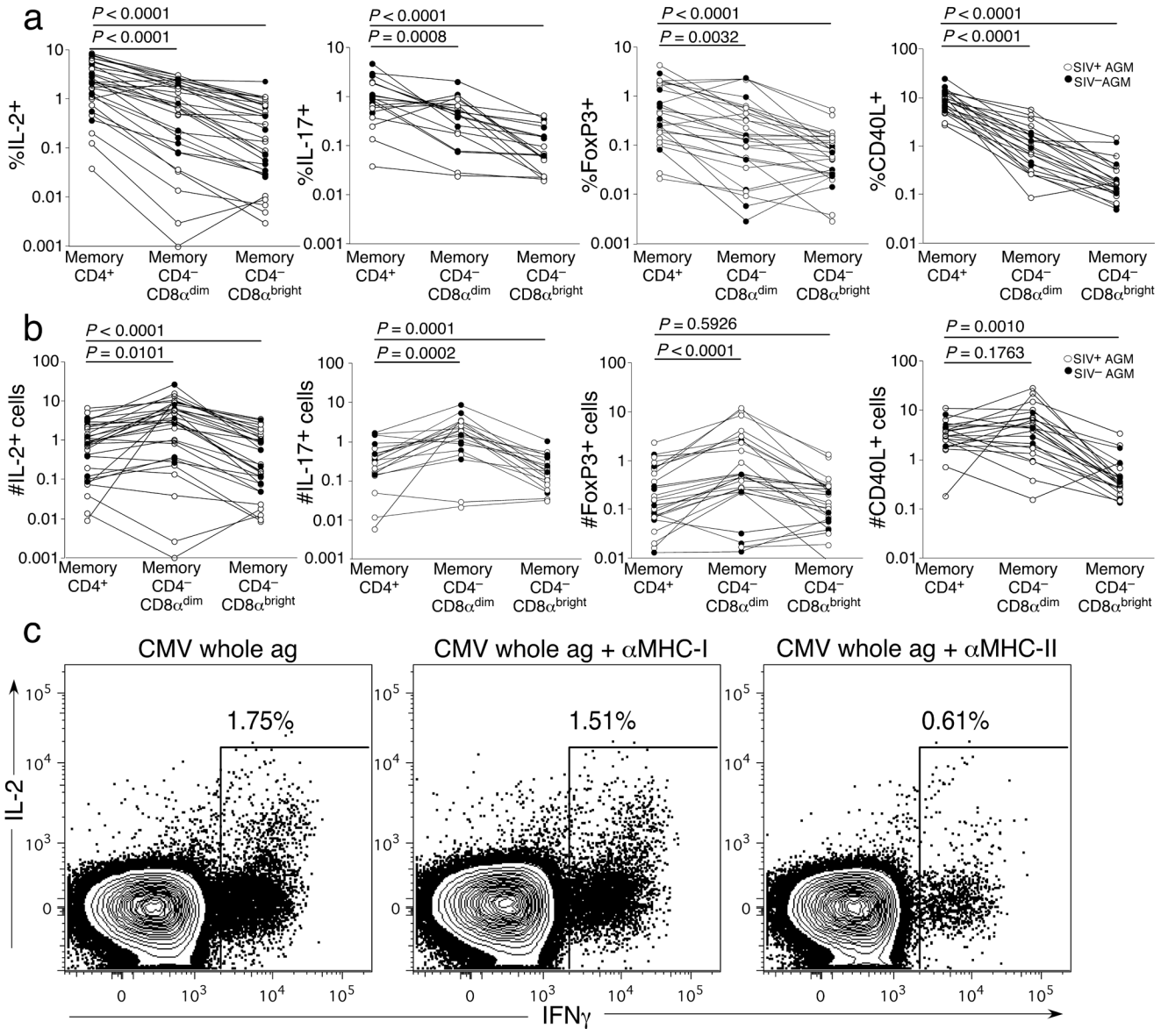

Figure 4. CD4⁻CD8α^{dim} T-cells can preserve CD4^{+} T-cell function

(**a**) Comparison of the frequency of memory CD4^{+}, CD4^{-}CD8α^{dim}, and CD4^{-}CD8α^{bright} T-cells performing various functions: IL-2 and IL-17 production, and FoxP3 and CD40L expression. (**b**) Comparison of the relative numbers of memory CD4^{+}, CD4^{-}CD8α^{dim}, and CD4^{-}CD8α^{bright} T-cells from different memory T-cell subsets performing various functions: IL-2 and IL-17 production, and FoxP3 and CD40L expression (**c**) Responses of CD4^{-}CD8α^{dim} T-cells to CMV whole antigen in the presence and absence blocking antibodies to MHC-II or MHC-I. White circles represent SIV-infected AGM and black represent uninfected AGM. A Wilcoxon matched pairs test was performed for **a–b**.

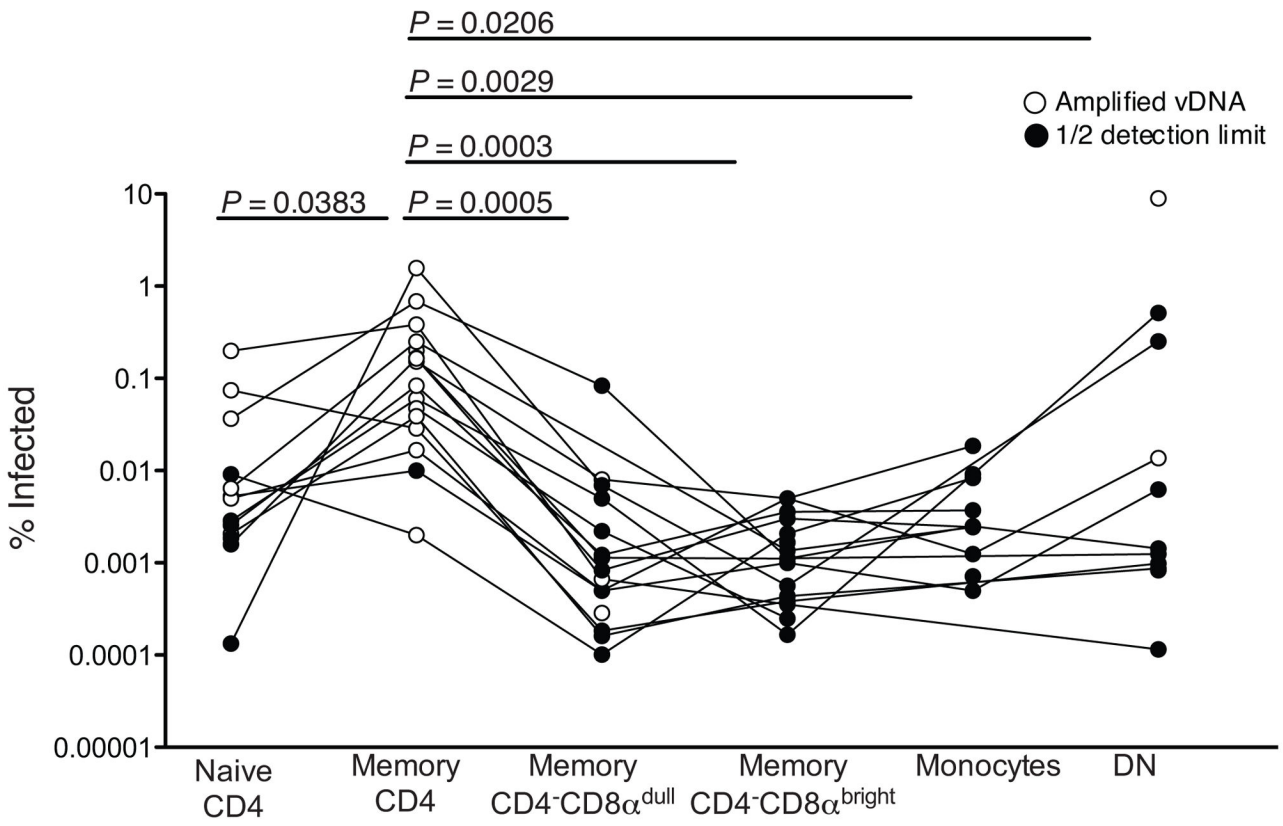

Figure 5. Infection frequency of lymphocyte subsets

Infection frequency of sorted lymphocyte subsets from SIVagm-infected adult AGM as determined by PCR. White circles represen T-cells with detectable viral DNA and black circles represent an undetectable infection frequency reported as one half the lower limit of detection based on the number of cells within each PCR reaction. A Wilcoxon matched pairs test was performed for this analysis.

Table 1

Clinical information of African green monkeys.

Animal	Virus Strain	Infection Dose (TCID)	Plasma Viral Load (/mL)	CD4 T-cells (/μl blood)
AG3	Naturally Infected	NA	29600	341
AG4	Naturally Infected	NA	19600	252
AG5	Naturally Infected	NA	26000	63
AG6	Naturally Infected	NA	1400	314
AG10	Naturally Infected	NA	8000	210
AG11	Naturally Infected	NA	Undetected	156
AG12	Naturally Infected	NA	23600	60
AG15	Naturally Infected	NA	Undetected	495
AG16	Naturally Infected	NA	61100	452
AG17	Naturally Infected	NA	148000	235
AG7	SIVagm90	1000	55800	449
AG8	SIVagmVer1	50	1660	180
AG13	SIVagm90	1000	31500	308
AG19	SIVagmVer1	50	44300	427
AG22	SIVagm90	1000	2066100	185
AG24	SIVagm90	1000	11500	301
AG302	SIVagm90	1000	19700	170
AG346	SIVagm90	1000	2000	17
AG9	Uninfected	NA	NA	289
AG23	Uninfected	NA	NA	821
AG731	Uninfected	NA	NA	129
AG5339	Uninfected	NA	NA	165
AG5387	Uninfected	NA	NA	147
AG5419A	Uninfected	NA	NA	35
AG5419B	Uninfected	NA	NA	67
AG5431	Uninfected	NA	NA	62
AG5441	Uninfected	NA	NA	114
AG5504	Uninfected	NA	NA	55
AG5506	Uninfected	NA	NA	99
AG25	Uninfected juvenile	NA	NA	1625
AG26	Uninfected juvenile	NA	NA	2593
AG28	Uninfected juvenile	NA	NA	2791
AG30	Uninfected juvenile	NA	NA	1531
AG31	Uninfected juvenile	NA	NA	3214
AG32	Uninfected juvenile	NA	NA	2121
AG33	Uninfected juvenile	NA	NA	2798
HK14	Uninfected sabeus	NA	NA	378