Interleukin-2 Therapy Induces CD4 Downregulation, Which Decreases Circulating CD4 T Cell Counts, in African Green Monkeys

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ABSTRACT

African green monkeys (AGMs) are natural hosts of simian immunodeficiency virus (SIV_{AGM}). Because these animals do not develop simian AIDS despite maintaining high viral loads, there is considerable interest in determining how these animals have evolved to avoid SIV disease progression. Unlike nonnatural hosts of SIV, adult AGMs maintain low levels of CD4⁺ T cells at steady states and also have a large population of virus-resistant CD8αα T cells that lack CD4 expression despite maintaining T helper cell functionalities. In recent work, we have shown that homeostatic cytokines can induce CD4 downregulation in AGM T cells *in vitro*. Through administering therapeutic doses of recombinant human interleukin-2 (IL-2) to AGMs, we show here that this mechanism is operative *in vivo*. IL-2 therapy induced transient yet robust proliferation in all major T cell subsets. Within the CD4⁺ T cell population, those that were induced into cycle by IL-2 exhibited characteristics of CD4-to-CD8αα conversion. In all animals receiving IL-2, circulating CD4⁺ T cell counts and proportions tended to be lower and CD4[−] CD8αα⁺ T cell counts tended to be higher. Despite reductions in circulating target cells, the viral load was unaffected over the course of study.

IMPORTANCE

The data in this study identify that homeostatic cytokines can downregulate CD4 *in vivo* and, when given therapeutically, can induce AGMs to sustain very low levels of circulating CD4⁺ T cells without showing signs of immunodeficiency.

African green monkeys (AGMs) are natural hosts of simian immunodeficiency virus (SIV_{agm}) that progress to AIDS very rarely despite maintaining high viral loads in plasma (1). Yet, SIV_{agm} is pathogenic in pigtail macaques when they are experimentally infected, implying a coevolution of SIV_{agm} with its natural host (2, 3). Adult AGMs have low numbers of CD4 T cells and a large population of memory T cells that lack CD4 expression and express CD8α homodimers (4, 5). These CD8αα T cells are uniquely different from classical CD8αβ T cells in that they retain characteristics of CD4 T cells, such as major histocompatibility complex class II (MHC-II) restriction, expression of Foxp3, and production of interleukin-2 (IL-2) and IL-17 (4, 6, 7). We have previously shown that these cells arise from downregulation of CD4 by canonical CD4 T cells (8). Importantly, downregulation of CD4 protects these cells from infection by SIV_{agm} *in vivo* (4, 9). The maintenance of immunological function by cells that are resistant to infection is thought to, in part, underlie the nonprogressive nature of SIV_{agm} infection in AGM.

The exact mechanisms of CD4-to-CD8α conversion in AGMs are not entirely understood, although cellular division is likely important for this process. It is clear that cellular division through antigenic stimuli can induce CD4 downregulation *in vitro* and infection of AGMs with SIV can accelerate CD4-to-CD8α conversion *in vivo* (8). Yet, the conversion of CD4 to CD4[−] CD8α⁺ T cells during infection is not entirely limited to those that are SIV specific, which make up less than 1% of the total T cell pool (4). Moreover, a particular AGM who lacks CD4 T cells can mount an MHC-II-restricted neoantigenic response within the CD4[−] CD8αα⁺ T cell pool, implying that CD4 downregulation can occur through antigen-independent mechanisms *in vivo* (8). Homeostatic T cell division may also contribute to CD4 downregulation, and we have previously observed that AGM T cells downregulate CD4 when stimulated *in vitro* with the common

gamma chain cytokines IL-2, IL-7, and IL-15 (8). It is unknown whether this same process occurs *in vivo* or whether CD4 downregulation can be accelerated with therapeutic intervention.

The homeostatic cytokine IL-2 is an autocrine cell growth factor that binds the high-affinity IL-2 receptor, CD25, to promote T cell proliferation, differentiation, and survival (10). T regulatory (Treg) cells that express high levels of CD25 are particularly responsive to this cytokine, and they depend on IL-2 for their homeostasis (11, 12). Recombinant IL-2 has been given therapeutically in large clinical trials to human immunodeficiency virus (HIV)-infected patients on antiretroviral therapy to boost reconstitution of CD4 T cells (13). Yet despite a significant and sustained increase in CD4 T cell counts, no clinical benefit was observed compared to patients treated with antiretroviral therapy alone (13). IL-2 therapy has also been used in untreated SIV-infected rhesus macaques, yet here too, administration did not improve prognoses (14).

Given these previous studies using IL-2 to expand CD4 T cells

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TABLE 1 Characteristics of study animals^a

Animal ID	DOB	Date inoculated	Inoculation route	SIV strain
AG7	12/3/01	11/19/06	IV	SIVagm90
AG13	1/14/02	11/19/06	IV	SIVagm90
AG26	9/25/06	Born to SIV ⁺ parents	NA	NA
302	2/27/01	4/22/07	IV	SIVagm90

^a DOB, date of birth. NA, not applicable. Dates are in the month/day/year format.

in HIV/SIV-infected individuals coupled with our previous studies demonstrating that IL-2 can cause proliferation and downregulation of CD4 by CD4 T cells in AGM T cells *in vitro*, we hypothesized that IL-2 administration could lead to cellular proliferation and downregulation of CD4 in AGMs *in vivo*. This, we hypothesized, might lead to decreased target cells for SIV and corresponding decreases in plasma viremia. Thus, we treated 4 AGMs with 8 rounds of recombinant human IL-2 and asked whether administration could drive these animals to CD4 downregulation with concomitant reduction in viremia. We find that proportions and absolute numbers of circulating conventional CD4 T cells tended to be lower in all 4 animals and absolute numbers of circulating conventional CD8 $\alpha\alpha$ T cells that lacked CD4 expression tended to be higher. Interestingly, viremia remained relatively unchanged over the course of administration. Thus, a very limited number of CD4 T cells may be sufficient to support ongoing viral replication in AGMs, similar to what is seen in sooty mangabeys (15).

MATERIALS AND METHODS

Ethics. This study was carried out in strict accordance with the recommendations described in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health, the Office of Animal Welfare, and the U.S. Department of Agriculture (16). All animal work was approved by the NIAID Division of Intramural Research Animal Care and Use Committees (IACUC) in Bethesda, MD (protocols LMM-12 and LMM-6). The animal facility is accredited by the American Association for Accreditation of Laboratory Animal Care. All procedures were carried out under ketamine anesthesia by trained personnel under the supervision of veterinary staff, and all efforts were made to maximize animal welfare and to minimize animal suffering in accordance with the recommendations of the Weatherall report on the use of nonhuman primates (17). Animals were housed in adjoining individual primate cages, allowing social interactions, under controlled conditions of humidity, temperature, and light (12-h light/12-h dark cycles). Food and water were available *ad libitum*. Animals were monitored twice daily and fed commercial monkey chow, treats, and fruit twice daily by trained personnel.

Animals. We housed a total of 4 vervet African green monkeys (*Chlorocebus pygerythrus*) (AGMs). The AGMs included 3 SIV⁺ adults that were infected intravenously with the SIVagm90 strain. A 4th AGM was born to SIV⁺ parents yet maintained undetectable viral loads at the time of study. All relevant animal information is summarized in Table 1.

Interleukin-2 administration. Recombinant human interleukin-2 was purchased under the commercial name Proleukin (Prometheus Laboratories, San Diego, CA) at the NIH Clinical Center Pharmacy. Proleukin vials were reconstituted in sterile water, and animals were injected subcutaneously twice daily for 5 days at 600,000 units per kilogram of body weight. The IL-2 dosage was previously estimated from a maximum nontoxic dose of 300,000 to 600,000 U/kg body weight defined for humans (18). Previous reports have shown that these concentrations are well-tolerated in rhesus macaques (19). Proleukin administration was performed for a total of 8 rounds with 56 days between rounds.

Absolute cell counts. Absolute cell counts were calculated from flow cytometry frequencies and complete blood count (CBC) absolute lymphocyte counts at days 7, 14, 21, 30, and 53 after the start of each round of IL-2 administration.

Flow cytometry. Cellular frequency and activation status were determined through *ex vivo* staining of isolated peripheral blood mononuclear cells (PBMCs). Cells were washed once with cold phosphate-buffered saline (PBS) and incubated with the Live/Dead fixable Aqua dead cell stain (Invitrogen, Carlsbad, CA) for 5 min at room temperature. Cells then were stained with the fluorescently conjugated monoclonal antibody to CCR7 (clone 3D12, conjugated to Cy7PE; BD Biosciences, Carlsbad, CA) and incubated for 15 min at 37°C, after which antibodies to CD3 (clone SP34-2, conjugated to Alexa Fluor 700; BD Biosciences), CD4 (clone L200, conjugated to Percp Cy5.5; BD Biosciences), CD8 (clone RPA-T8, conjugated to Pacific Blue), CD28 (clone 28.2, conjugated to energy-coupled dye [ECD]; Beckman Coulter, Brea, CA), CD95 (clone DX2, conjugated to Cy5-phycoerythrin [Cy5-PE]; BD Biosciences), HLA-DR (clone L243, conjugated to H7-allophycocyanin [H7-APC]; BD Biosciences), CD25 (clone 2A3, conjugated to brilliant violet 605; BD Biosciences), and $\alpha\beta$ 7 (conjugated to APC; NIH AIDS Reagent Program) were added and incubated for an additional 30 min at 4°C. Cells were washed once with cold PBS, fixed for 1 h with 1 $\times$ fixation/permeabilization solution (eBioscience, San Diego, CA) at 4°C, and subsequently permeabilized with 1 $\times$ permeabilization buffer (eBioscience). For intracellular staining, cells were incubated at 4°C for 30 min with monoclonal antibodies directed against ki67 (clone B56, conjugated to fluorescein isothiocyanate [FITC]; BD Biosciences) and Foxp3 (clone 236A, conjugated to PE; eBioscience). After intracellular staining, cells were washed in 1 $\times$ permeabilization buffer (eBioscience) and fixed in 1% paraformaldehyde solution (Electron Microscopy Sciences, Hatfield, PA). Flow cytometric acquisition was performed on a BD Fortessa (BD Biosciences) flow cytometer, and data were analyzed with Flowjo software (v9.8.3; Tree Star Inc., Ashland, OR).

Quantitation of plasma viral RNA levels. Viral RNA levels in plasma were determined by real-time reverse transcription-PCR (ABI Prism 7700 sequence detection system; Applied Biosystems, Foster City, CA) as previously reported, using reverse-transcribed viral RNA in plasma samples from SIVagm-inoculated African green monkeys (20).

Statistics. Given the low number of samples used in this study, we used the paired Student *t* test to compare means among different time points. This statistical test has been used in other settings with a similar number of animals and in most cases results in type I error rates close to the 5% nominal value (21, 22).

RESULTS

Robust responses to recombinant human IL-2 in AGMs. Unlike what occurs in Asian macaques, humans, or mice, 4 distinct T cell populations can be defined by CD4 and CD8 expression in AGMs (4). While CD4⁺ T cells are present at low frequencies in adult AGMs (4), the majority of T cells lack CD4 surface expression and either do not express CD8, are CD8 α ^{dim} T cells that have downregulated CD4 expression postthymically, or are classical CD8⁺ T cells that express the CD8 α and β chains (Fig. 1A). We have previously shown in AGM T cells that cytokines that induce homeostatic T cell proliferation, such as IL-2, IL-7, and IL-15, induce CD4 downregulation *in vitro* (8). To determine if the same mechanism is operative *in vivo*, we administered recombinant human IL-2 to 4 adult AGMs. A total of eight rounds of IL-2 were given at 56-day intervals, and the frequency, absolute numbers, and phenotypes of circulating T cell populations in AGMs were determined at various time points relative to IL-2 administration. In each round of administration, IL-2 therapy induced transient increases in absolute numbers of circulating CD4⁺, CD4⁺ CD8⁺, CD8 α ⁺, and CD8 α β ⁺ T cells, which were most apparent 7 days after the start of IL-2 administration and normalized after 56 days

FIG 1 Recombinant human IL-2 induces robust, well-tolerated, proliferative responses in AGM T cells *in vivo*. (A) Representative expression of CD4 and CD8 by adult AGM T cells. (B to F) Absolute numbers (blue lines) and proportions (red lines) of CD4⁺ T cells (B), CD4[−] CD8[−] T cells (C), CD4[−] CD8αα⁺ T cells (D), CD4[−] CD8αβ⁺ T cells (E) over 8 rounds of IL-2 administration. (F) Proportions of CD4⁺, CD4[−] CD8[−], CD4[−] CD8αα⁺, and CD4[−] CD8αβ⁺ T cells that are cycling over 8 rounds of IL-2 administration. (G and H) CD4 and CD8 surface expression on circulating AGM “T regulatory-like” cells (CD25⁺ Foxp3⁺) (G) with their associated proportions and absolute numbers (H). (I) Frequencies of CD25⁺ Foxp3⁺ cells that are ki67⁺; significant expansions of Treg-like cells are denoted by an asterisk (*).

posttreatment (Fig. 1B to E). Changes in the proportions of these T cell populations in AGMs were less dramatic (Fig. 1B to E). Expression of the cell cycle protein ki67 was found to parallel periods of peak T cell expansion induced by IL-2, as ki67 was expressed in some CD4⁺ T cells and much greater proportions of distinct T cell populations that lacked CD4 expression (Fig. 1F). These data indicate that IL-2 may expand T cells that lack CD4 expression in AGMs.

We next assessed the dynamics of T regulatory cells in response to IL-2 therapy, as these cells can respond preferentially to IL-2 compared to other T cell subsets. While expression of Foxp3 can be a general consequence of T cell activation and Foxp3 expression does not always confer regulatory T cell function (23), we do find that a fraction of circulating Treg-like T cells are present in AGMs that coexpress Foxp3 and CD25. Unlike in other species, the majority of Foxp3⁺ CD25⁺ T cells in AGMs do not express CD4 and express low levels of the CD8αα homodimer (Fig. 1G). Absolute numbers of circulating CD25⁺ Foxp3⁺ T cells expanded acutely with each round after the start of IL-2 therapy yet normalized thereafter, and this was apparent in CD25⁺ Foxp3⁺ T cell propor-

tions as well (Fig. 1H). Additionally, IL-2 administration induced ki67 expression in nearly all circulating CD25⁺ Foxp3⁺ T cells at the peak of T cell expansion (Fig. 1I). Taken together, these data suggest that AGMs do not mount a humoral immune response to the recombinant human IL-2 that might hinder its effectiveness.

Cycling in AGM CD4⁺ T cells promotes transition to a CD4[−] CD8αα⁺ memory phenotype. In previous work, we had found that a large proportion of divided CD4⁺ T cells downregulated CD4 expression in response to IL-2 stimulation *in vitro* and that CD4 surface densities were lower for each generation of divided cells (8). As this suggests that cellular division is likely important for CD4 downregulation, we reasoned that those CD4⁺ T cells found to be in cycle immediately after IL-2 administration in AGMs would exhibit some characteristics of memory CD4[−] CD8αα⁺ T cells. We compared CD4 expression in cycling and noncycling CD4⁺ T cells at 7 days after the first round of IL-2 administration and found that surface densities were significantly lower on both T regulatory and non-T-regulatory subsets that were found to be in cycle (Fig. 2A). Surface densities of CD8 on cycling CD4⁺ T cells tended to be higher in both Treg-like and

FIG 2 Evidence of CD4-to-CD8 $\alpha\alpha$ conversion in cycling CD4 $^{+}$ T cells. Surface densities of CD4 (A), CD8 α (B), and the memory marker CD95 (C) were assessed in CD25 $^{-}$ Foxp3 $^{-}$ and CD25 $^{+}$ Foxp3 $^{+}$ CD4 $^{+}$ T cells that were induced into cell cycle by IL-2 and compared to those CD4 $^{+}$ T cells not in cycle. Black circles correspond to SIV $^{+}$ AGMs, and the red circle denotes the SIV $^{-}$ AGM used in this study. Correlation of circulating CD4 $^{+}$ T cells and CD4 $^{-}$ CD8 $\alpha\alpha$ $^{+}$ T cells that are not cycling (D) or are in cell cycle (E) in 4 adult AGMs over the course of IL-2 therapy. Surface densities of CD4, CD8, and CD95 were compared by the Student t test. A Spearman rank correlation was calculated for panels D and E.

FIG 3 IL-2 therapy tends to decrease the size of the CD4⁺ T cell pool in AGMs. Proportions (A) and absolute numbers (B) of circulating CD25⁻ Foxp3⁻ T cells that were CD4⁺, CD4⁻ CD8 $\alpha\alpha$ ⁺, or CD4⁻ CD8 $\alpha\beta$ ⁺ were compared just prior to the start of IL-2 therapy (day -5) to 53 days after the 8th round of IL-2 administration in 4 adult AGMs. Proportions and absolute numbers of CD25⁻ Foxp3⁻ T cells that were CD4⁺, CD4⁻ CD8 $\alpha\alpha$ ⁺, or CD4⁻ CD8 $\alpha\beta$ ⁺ were compared in a similar fashion in these animals. Closed circles represent SIV⁺ AGMs. The open circle represents the one SIV⁻ AGM used in this study. A Student *t* test was used for all comparisons.

non-Treg subsets, yet these did not reach statistical significance (Fig. 2B). Cycling CD4⁺ T cells acquired some characteristics of a memory phenotype and CD95 was dramatically upregulated in cycling Treg and non-Treg populations (Fig. 2C). These results suggest that in AGMs, homeostatic signals can induce a memory phenotype on CD4⁺ T cells and that cell cycle entry may be important for this process.

Because IL-2 appeared to drive cycling CD4 T cells to become more “CD8 α -like,” we reasoned that the disappearance of CD4⁺ T cells would be related to increases in the CD4⁻ CD8 $\alpha\alpha$ ⁺ T cell compartment. Throughout the course of the study, we found a significant negative correlation between noncycling CD4⁺ T cells and CD8 $\alpha\alpha$ ⁺ T cells, yet this relationship was less apparent in CD4⁺ T cells and CD8 $\alpha\alpha$ ⁺ T cells that were found to be in cycle (Fig. 2D). Thus, CD4-to-CD8 α conversion may be a transitory process that is driven by cycling, yet once CD4 T cells divide, they stably downregulate CD4 expres-

sion and upregulate CD8 α . These data are in accordance with previous work demonstrating a lack of CD4 gene transcripts in mature CD4⁻ CD8 $\alpha\alpha$ ⁺ T cells (4).

Intermittent IL-2 administration tends to reduce CD4 T cell numbers in AGMs. In many species, CD4⁺ T cells that divide through homeostatic stimuli such as IL-2 largely retain their phenotype. AGMs are unique in that homeostatic cytokine-induced proliferation *in vitro* induces CD4⁺ T cells to acquire a phenotype resembling that of memory CD8 $\alpha\alpha$ T cells that lack CD4 expression. Thus, while IL-2 therapy durably increases CD4⁺ T cell counts in many species, we hypothesized that AGMs would exhibit CD4⁺ T cell losses and concomitant rises in CD8 $\alpha\alpha$ ⁺ T cell numbers over the course of treatment. After 8 rounds of IL-2, frequencies of CD4⁺ T cell subsets that lacked CD25 and Foxp3 expression tended to be lower whereas classical CD25⁻ Foxp3⁻ CD8 $\alpha\beta$ T cells were significantly higher (Fig. 3A). When we assessed the absolute numbers of these same CD25⁻ Foxp3⁻ T cell

FIG 4 Circulating proportions of CD4 and CD8 T cells are stable in AGMs not treated with IL-2. Frequencies of circulating nonregulatory (A) or regulatory (B) T cells based on CD4 and CD8 expression were compared in a group of AGMs that did not receive IL-2 over a time period of >1 year. All animals in this cohort were SIV positive. Comparisons were made by paired Student's *t* test.

populations after 8 rounds of IL-2 administration, circulating CD25⁺ Foxp3⁺ CD4⁺ T cell numbers were lower and numbers of CD25⁺ Foxp3⁺, CD4⁺ CD8 $\alpha\alpha$ ⁺, and CD4⁺ CD8 $\alpha\beta$ ⁺ T cells increased, yet these changes did not reach statistical significance (Fig. 3B). When assessing frequencies and absolute numbers of circulating Treg-like cells in AGMs, only absolute numbers of CD25⁺ Foxp3⁺ CD4⁺ CD8 $\alpha\alpha$ ⁺ T cells tended to be higher after 8 rounds of IL-2 (Fig. 3C and D). Taken together, all animals receiving IL-2 exhibited similar trends of decreasing CD4⁺ T cell counts and proportions. Concomitant increases in CD4⁺ CD8 $\alpha\alpha$ ⁺ T cell counts were observed as well. In contrast, proportions of circulating Foxp3⁺ CD25⁺ and Treg-like Foxp3⁺ CD25⁺ CD4⁺, CD4⁺ CD8⁺, CD4⁺ CD8 $\alpha\alpha$ ⁺, and CD4⁺ CD8 $\alpha\beta$ ⁺ T cells did not change over a similar time course in a cohort of AGMs not receiving IL-2 (Fig. 4A and B). Given that only 4 animals were administered IL-2 in this study, it is striking that these differences in the IL-2-treated AGMs approached statistical significance.

Recombinant IL-2 therapy does not reduce viremia in AGMs.

In nonnatural hosts of SIV such as rhesus macaques as well as HIV⁺ humans that are not receiving antiretroviral therapy (20), single or intermittent rounds of IL-2 do not affect the SIV or HIV load (14, 24). Because homeostatic proliferation induces CD4 T cells to downregulate CD4 in AGMs and become resistant to SIV infection, we asked whether the loss of target cells induced by IL-2 might reduce viremia in these animals. Prior to treatment, 3 of the 4 animals displayed persistent viremia whereas the 4th animal was SIV uninfected despite being born to SIV⁺ parents. IL-2 therapy induced transient spikes of viremia that were observed in all 3 of the viremic animals (Fig. 5A); however, no diminishing trends in viral loads were seen after the completion of 8 rounds of therapy (Fig. 5B). These data indicate that viremia persists in animals that are incompletely depleted of CD4 T cells and that viral replication can be sustained in a relatively low number of target cells.

FIG 5 IL-2 therapy does not alter viremia in SIV⁺ adult AGMs. (a) Plasma viral load over the course of IL-2 therapy in SIV⁺ AGMs. (b) Comparison of viral loads just prior to the start of therapy (day -5) and 53 days after the 8th round of IL-2 administration in SIV⁺ AGMs.

DISCUSSION

In previous work, we established that homeostatic signals can downregulate CD4 expression on AGM T cells *in vitro* and may be important in driving the generation of the CD8 $\alpha\alpha$ T cell pool, a population that displays functional similarity to CD4 T cells but is resistant to SIV infection (8). Here, we sought to determine whether treatment of AGMs with therapeutic doses of the homeostatic cytokine IL-2 could accelerate CD4 $^{-}$ CD8 $\alpha\alpha$ $^{+}$ T cell conversion and resultantly lower viremia. Although host-derived immune responses against recombinant human IL-2 were not specifically examined in this study, the treatment was generally well tolerated and it is unlikely that strong anti-IL-2 antibodies developed given the continued bioactivity of IL-2 after multiple administrations *in vivo*. We find that recombinant human IL-2 was well tolerated, with no apparent immune response directed against the cytokine despite being administered to AGMs. IL-2 induced a transient yet robust proliferation in all major AGM T cell populations, and within the CD4 T cell population, those that were induced into cycle by IL-2 displayed characteristics of CD4-to-CD8 α T cell conversion. After 8 rounds of administration, exogenous IL-2 tended to reduce the proportions and absolute numbers of circulating CD4 T cells and concomitantly increase the absolute numbers of CD4 $^{-}$ CD8 $\alpha\alpha$ $^{+}$ T cells, whereas these proportions remained constant in a group of AGMs not receiving IL-2. Changes in T cell proportions, however, did not appear to influence viral replication. These data demonstrate that the CD4 $^{-}$ CD8 $\alpha\alpha$ $^{+}$ T cell pool can be generated *in vivo* through homeostatic signals and that relatively few numbers of target cells can sustain viral replication.

It is not entirely surprising that AGMs could be driven toward CD4 depletion experimentally, as this has occurred under natural circumstances in some animals of the Cercopithecini tribe (which includes all species of AGMs and the closely related patas monkeys). One AGM, AG346, has sustained very low numbers of circulating CD4 T cells for several years at steady state and has concomitantly become aviremic, suggesting that a lack of target cells may prevent sustained viral replication in this animal (7). Additionally, a similar report of a patas monkey that maintains very low numbers of CD4 T cells has been described (25). In both of these cases, nearly complete CD4 T cell depletion did not result in immunodeficiency. Similarly, immunodeficiency was not observed in the 4 AGMs treated with IL-2 despite circulating T cell counts that would be AIDS defining in nonnatural hosts. Although IL-2 tended to decrease CD4 T cells over the course of therapy in these animals, there did not appear to be the homeostatic proliferation and increase in numbers of residual CD4 T cells that occurs with rhesus macaques who are depleted of CD4 T cells experimentally (26). Thus, it appears that AGMs can maintain negligible numbers of CD4 T cells, and this can represent a steady state for these animals.

Other primate species who are natural hosts of SIV exhibit similar characteristics of severe CD4 $^{+}$ T cell depletion without showing any clinical signs of AIDS. When infected with SIV, a subset of sooty mangabeys develop a large population of virus-resistant CD4 $^{-}$ CD8 $^{-}$ double negative T cells that maintain many functional qualities of CD4 $^{+}$ T cells (27, 28). The precursors of this double negative population or the signals responsible for their development are not known. However, it is worth noting that MHC-II-restricted immune responses against neoantigens, such

as those of influenza virus, can develop within the CD4 $^{-}$ CD8 $^{-}$ T cell pool, which bear striking similarity to a CD4 T cell helper response (27), suggesting that these T cells can develop independent of antigen exposure, just as the CD4 $^{-}$ CD8 $\alpha\alpha$ $^{+}$ T cell pool can develop in AGMs.

In either species of natural hosts, the uncoupling of adaptive immunity from T cell populations that support viral replication may be important in avoiding disease progression. Other mechanisms unique to AGMs that may contribute to disease nonprogression have also been described, including a robust but transient type I interferon response and non-CCR5 entry pathways of SIVagm (29, 30). Our findings that homeostatic signals can drive CD4 downregulation give key insights on how AGMs can preserve these essential immune functions while still maintaining a diverse T cell repertoire, as one would expect a skewed representation with some T cell clones more represented than others if the CD4 $^{-}$ CD8 $\alpha\alpha$ $^{+}$ T cell pool were driven solely by antigen.

It is worth noting that in our study increases in proportions or absolute numbers of CD4 $^{-}$ CD8 $\alpha\alpha$ $^{+}$ T cells were not always similar in magnitude compared to the loss of CD4 $^{+}$ T cells in some of the animals receiving IL-2. It is possible that expansion of CD4 $^{-}$ CD8 $\alpha\alpha$ $^{+}$ T cells may have occurred at other anatomical sites such as the gastrointestinal (GI) tract, where CD4 $^{-}$ CD8 $\alpha\alpha$ $^{+}$ T cells represent the vast majority of the T cell pool in AGMs (4). Another possibility is that because CD4 $^{-}$ CD8 $\alpha\alpha$ $^{+}$ T cells vastly outnumber the CD4 $^{+}$ T cell pool in AGMs, CD4 $^{-}$ CD8 $\alpha\alpha$ $^{+}$ T cell conversion induced by IL-2 may make statistically insignificant impacts on the size of the existing CD4 $^{-}$ CD8 $\alpha\alpha$ $^{+}$ T cell pool. Nevertheless, we do find evidence of a “CD8 α -like” memory phenotype in cycling CD4 $^{+}$ T cells, and once divided, CD4 $^{-}$ CD8 $\alpha\alpha$ $^{+}$ T cell proportions correlate inversely with CD4 T cells, suggesting that some of the CD4 T cells induced into cycle by IL-2 may go on to become CD4 $^{-}$ CD8 $\alpha\alpha$ $^{+}$ memory T cells.

Interestingly, while IL-2 therapy induced circulating numbers of target cells that were CD4 $^{+}$ to decrease in AGMs, we did not observe any significant changes in viremia in these animals. These findings might contrast to some instances stated earlier whereby parallel decreases in CD4 T cells and viral loads have been observed in some particular animals who are natural hosts of SIV (8). Although we did not assess SIVagm replication at tissue sites, it remains a possibility that IL-2 administration could affect viral loads locally but not systemically. It is worth noting, however, that there are instances where viral load is not always coupled to the quantity of total CD4 $^{+}$ T cells. For example, memory CD4 $^{+}$ T cells represent the main targets of viral replication in both juvenile and adult AGMs, yet juvenile AGMs maintain viral loads similar to those of adults despite being immunologically inexperienced (4, 8). The same is true of SIV $^{+}$ rhesus macaques who are experimentally depleted of CD4 T cells yet maintain viral loads comparable to those of SIV $^{+}$ rhesus macaques with ample numbers of CD4 $^{+}$ T cells (26). In humans, recombinant IL-7 therapy induces cell division predominantly in CD4 $^{+}$ memory T cells (31). Because most viral replication in AGMs occurs in CD4 $^{+}$ memory T cells, IL-7 may affect the dynamics of viral replication differently from IL-2, which preferentially induces T regulatory cells to divide (12).

One limitation of this study is that only a limited number of AGMs were treated with IL-2. Thus, while reductions in circulating CD4 T cells induced by IL-2 approached statistical significance in these 4 animals, this study is underpowered and we cannot statistically conclude that the response to IL-2 is representative of

all AGMs. However, based on previous *in vitro* studies of IL-2-induced CD4 downregulation and the broad T cell receptor (TCR) diversity that exists in the CD4⁺ CD8αα⁺ T cell pool, we can be reasonably certain that larger studies would recapitulate these findings.

We also cannot conclude in this study that CD25⁺ Foxp3⁺ T cells induced by IL-2 therapy are true T-regulatory cells. In HIV-infected humans, IL-2 induced CD25⁺ Foxp3⁺ CD4 T cell expansion, yet these exerted only weak suppressive capability (32, 33). It is worth noting that peaks in Treg-like expansion did not correspond with reduced effector function of conventional T cell subsets in our study (data not shown), arguing that the suppressive function of CD25⁺ Foxp3⁺ T cells induced by IL-2 is limited.

In four AGMs, we have shown that the homeostatic cytokine IL-2 can induce CD4 downregulation *in vivo* and lower CD4⁺ T cell counts. Regardless of whether CD4 downregulation is induced by homeostatic or antigenic signals, there are likely commonalities in the mechanism that drives this phenomenon in AGMs. A better understanding of this mechanism may pave the way for potential therapies in HIV⁺ humans that could uncouple adaptive immune processes to viral replication.

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