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Rapid Communication

Low pre-infection levels and loss of central memory CD4+ T cells may predict rapid progression in SIV-infected pigtail macaques

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ABSTRACT

CD4$^+$ T lymphocyte subsets are targeted to different degrees by SIV infection. We studied central memory, effector memory, naïve, and regulatory T cell levels longitudinally in 11 SIV$_{mac251}$-infected pigtail macaques. Depletion of CD28$^+$CD95$^+$ central memory CD4$^+$ T cells, but not other populations, correlated with both SIV viral load and disease progression. A low pre-infection level of central memory CD4$^+$ T cells was also predictive of rapid disease progression. If confirmed in larger studies, our results suggest stratifying macaques for baseline central memory CD4$^+$ T cells would be useful in defining both the pathogenesis of SIV disease and SIV vaccine efficacy.

Introduction

Long-term immunity to viruses is facilitated by the generation and persistence of virus-specific memory T cells. Virus-specific T cells are comprised of both central memory (CM) T cells which are long-lived and provide a continuous source of effector T cells and effector memory (EM) T cells which possess immediate effector functions capable of clearing virus. In human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) the ability to generate and sustain CM and EM CD4$^+$ T cells is impeded by direct infection and killing of memory CD4$^+$ T cells (Groot et al., 2006; Mattapallil et al., 2005; Nishimura et al., 2007; Okoye et al., 2007; Wang et al., 2007). Although several markers have been used to define CM T cells (Okoye et al., 2007) the combination of monoclonal antibodies (mAbs) CD28 and CD95 effectively discriminates CD4$^+$ naïve and memory T cell subsets in rhesus macaques (Pitcher et al., 2002; Sun et al., 2007). It was recently reported that preservation of CD28$^+$CD95$^+$ CM CD4$^+$ T cells during chronic infection is strongly associated with survival but not control of viremia in vaccinated rhesus macaques challenged with SIV (Letvin et al., 2006). Subsequent studies of unvaccinated SIV-infected rhesus and cynomolgus macaques have shown a positive correlation between loss of CM CD4$^+$ T cells and viral load (Karlsson et al., 2007a; Nishimura et al., 2007; Sun et al., 2007). Thus, CM CD4$^+$ T cell counts may function as a useful surrogate marker for the effectiveness of SIV or HIV vaccines. However, the relationship between loss of CM CD4$^+$ T cells and control of viremia or survival has not been assessed in the pathogenic model of SIV infection of pigtail macaques (*Macaca nemestrina*).

In recent years, there has been increased interest in CD25$^+$ FoxP3$^+$ regulatory CD4$^+$ T cells (Tregs). However, it is unclear whether Tregs dampen potentially useful virus-specific immune responses (Estes et al., 2006) or limit harmful T cell hyperactivation (Legrand et al., 2006) since depletion of Tregs *in vitro* enhances the ability to detect HIV-specific T cell immunity (Aandahl et al., 2004; Kinter et al., 2004) yet Treg depletion in HIV infection is also associated with immune activation (Eggena et al., 2005). The comparative role of Tregs and memory T cell subsets in controlling SIV disease progression has not been defined in pigtail macaques. Low surface expression of the IL-7 receptor (CD127) has recently been used to define Tregs that express intracellular FoxP3$^+$ in humans (Liu et al., 2006; Seddiki et al., 2006). The CD25$^+$CD127^{low} phenotype is also characteristic of Tregs in rhesus macaques although in contrast to humans, CD3$^+$CD4$^+$CD25$^+$ cells in this species are uniformly CD127^{low} and FoxP3$^+$ (Hartigan-O'Connor et al., 2007). Whether this phenotype is also applicable to pigtail macaque Tregs is not known.

We undertook a longitudinal prospective analysis of the memory, naïve and Treg CD4$^+$ T cell populations to examine changes in specific CD4$^+$ T cell subsets and their relationship to control of viremia and survival in a pathogenic model of SIV infection of pigtail macaques. We also sought to determine whether pre-infection baseline levels of CM CD4$^+$ T cells or Tregs in peripheral blood could serve as surrogate markers for survival or disease progression in SIV infection.

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Results and discussion

SIV-infected, untreated pigtail macaques have variable disease progression

We examined naïve and memory CD4$^+$ T cell subsets in 12 SIV$_{mac251}$-infected pigtail macaques from a recently completed vaccine study (Kent et al., 2008). Six of the 12 animals were vaccinated with a recombinant Kunjin virus expressing SIV Gag, the other 6 were unvaccinated controls. No vaccine related immune responses were induced and disease progression following challenge with SIV$_{mac251}$ was identical between the 2 groups (Kent et al., 2008). As expected, the 12 animals had variable success in controlling SIV replication in the absence of treatment and were grouped into rapid, conventional and slow progressor (RP, CP and SP, respectively) groups based on a composite criteria of survival time and mean set point viral load 5–23 weeks post-infection (p.i.) (Figs. 1a,b and Table 1). We identified three SP (5612, 5831 and 6158). Animal 5612 reduced viral load to below the detection threshold (<1500 copies/mL) while 5831 and 6158 remained healthy with low but detectable viremia. All 3 SP survived more than 1 year following SIV infection.

Table 1
Progressor status of 12 SIV$_{mac251}$-infected pigtail macaques

Group	Animal	CD4$^+$ T cell count (×10^3/μl)		Viral load (log$_{10}$ RNA copies/ml)		Time of euthanasia (weeks p.i.)
		Baseline	Nadir	Peak	Mean (weeks 5–23)	
Rapid progressor	5807	1.866	0.970	6.81	5.85	16
	5773	3.329	1.232	7.29	5.76	19
	6115	2.839	0.582	7.05	6.25	19
	6267	1.593	0.602	8.09	7.78	19
Conventional progressor	3C7D	3.618	1.008	6.77	5.68	23
	3117	2.350	0.726	6.98	5.82	23
	6258	2.013	0.376	7.36	6.07	23
	6288	1.972	0.446	6.78	5.24	23
	6361	1.428	0.230	6.85	5.25	23
Slow progressor	5612	1.857	1.047	6.59	3.45	n/a
	5831	3.047	1.343	6.09	4.89	n/a
	6158	2.409	1.094	7.02	4.87	n/a

There were 4 RP (5773, 5807, 6115 and 6267) who were unable to control viral replication and were euthanized with incipient AIDS 16 to 19 weeks post-infection. The remaining 5 animals (3C7D, 3117,

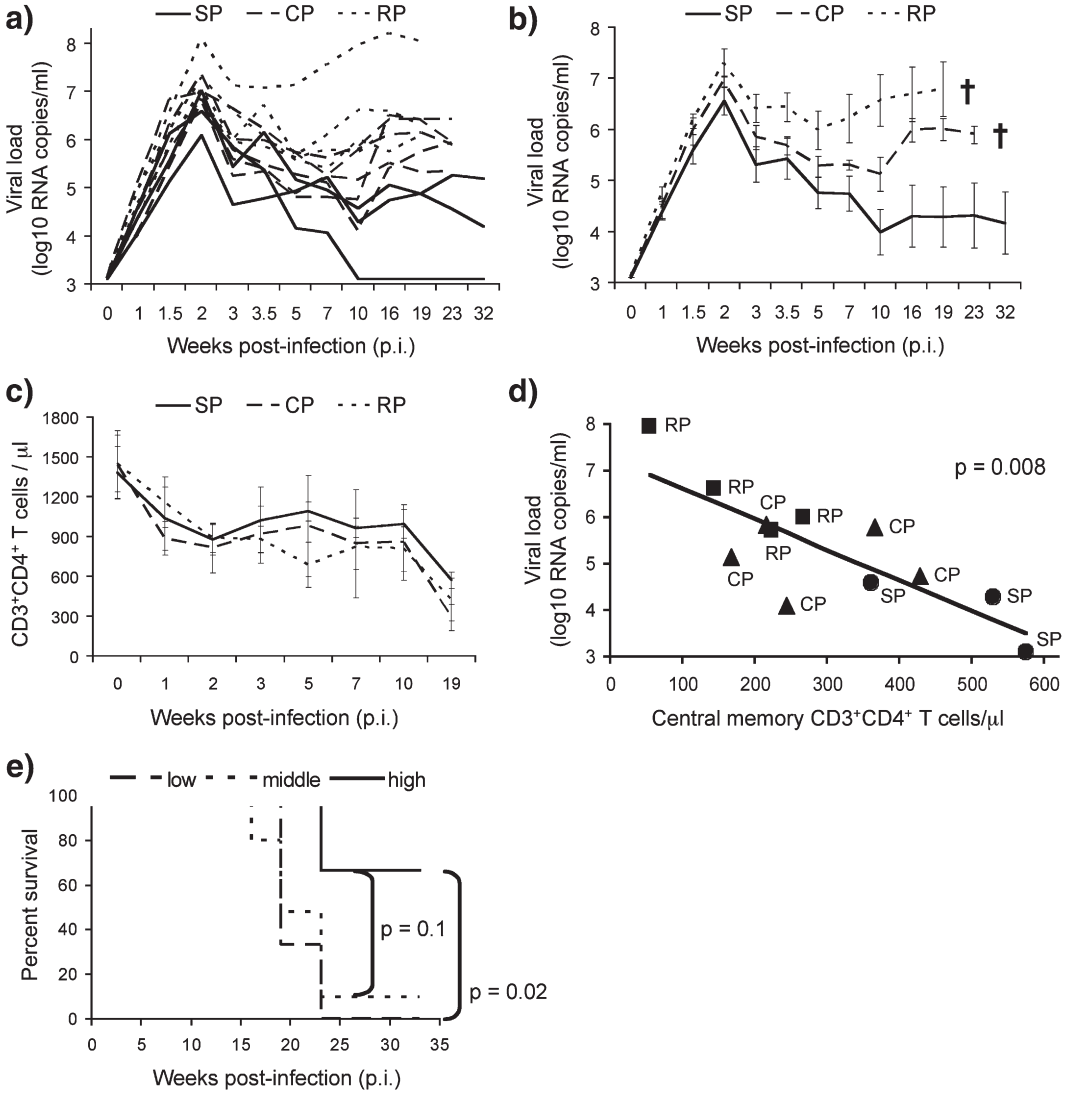

Fig. 1. Analysis of viral load, survival and CD4$^+$ T cell counts in SIV-infected, untreated pigtail macaques. Longitudinal analysis of (a) individual viral loads and mean (±SE) of (b) viral load and (c) CD3$^+$CD4$^+$ T cell counts in slow (SP), conventional (CP) and rapid progressor (RP) macaques; † denotes all animals in group deceased. (d) Correlation between central memory (CD28$^+$CD95$^+$) CD3$^+$CD4$^+$ T cell counts and plasma viral load 10 weeks p.i. (two-tailed *p*-value) and (e) Kaplan Meier survival graph for SIV-infected pigtail macaques divided into terciles (based on central memory (CD28$^+$CD95$^+$) CD3$^+$CD4$^+$ T cells/ml [low<200; middle 200–400; high>400] (Log rank test *p*-values).

6258, 6288 and 6361) had generally intermediate levels of VL and progressive SIV disease and required euthanasia, primarily with weight loss, diarrhoea and lethargy at 23 weeks p.i (between that of RP and SP animals). The criteria for euthanasia were set prospectively and included weight loss (>10%), loss of appetite, diarrhoea, clinical disease consistent with infection or malignancy and/or reduced activity in the setting of consistently elevated SIV viral loads. Animal 6258 was not studied for longitudinal memory CD4 T cell subsets analyses due to poor separation between memory and naïve subsets based on CD95 expression during acute SIV infection (data not shown).

Total, baseline and nadir CD4$^+$ T cell counts do not account for different disease progression phenotypes

We first examined whether variable disease outcomes in the 11 untreated pigtail macaques was due to differences in total CD4$^+$ T cells and CD4 subset counts. CD4$^+$ T cell subsets were delineated by flow cytometry in fresh whole blood samples. A gradual depletion of total CD4$^+$ T cells was observed in all animals, but there was no statistical difference between the three groups (nonparametric Mann Whitney ranksum $p>0.4$, Fig. 1c). Group baseline (week 0) total CD4$^+$ T cell counts were not statistically different and nadir CD4$^+$ T cell counts, defined as the lowest measured CD4$^+$ T cell count, were only marginally different between the SP and CP groups ($p=0.04$, two-tailed t-test) (Table 1).

Preservation of central memory CD4$^+$ T cells in SIV-infected pigtail macaques is associated with survival and control of viremia

The lack of a strong association between total CD4$^+$ T cells and disease progression of SIV-infected pigtail macaques, suggested that examining subsets of CD4$^+$ T cells may reveal depletion of memory CD4$^+$ T cells as reported for rhesus and cynomolgus macaques (Karlsson et al., 2007a; Sun et al., 2007). We therefore examined central memory (CM), effector memory (EM) and naive T cell subsets in serial blood samples pre- and post-infection based on well-validated CD28 and CD95 expression patterns (Picker et al., 2004; Pitcher et al., 2002). To assess the interplay between CD4$^+$ T cell subsets and disease progression in SIV infection, we examined longitudinal data of memory and naïve CD4$^+$ T cells in the 11 SIV-infected pigtail macaques. There was a highly significant inverse correlation between CD28$^+$CD95$^+$ CM CD4$^+$ T cells and viral load [$p=0.008$ (two-tailed), $R^2=0.647$] during chronic SIV infection [Fig. 1d]. We also analyzed the groups by terciles of CM CD4$^+$ T cells (low <200, middle 200–400 and high >400 CM CD4+ T cells/μl) and observed that preservation of CM CD4$^+$ T cells in the highest tercile was associated with survival in untreated macaques [Fig. 1e]. Thus, preservation of CM CD4$^+$ T cells was associated with both survival and control of viremia in SIV-infected pigtail macaques.

Rapid progressors have low CM CD4$^+$ T cell counts pre- and post-SIV infection

Since there was a strong relationship between post acute CM CD4$^+$ T cell concentration and the outcome of SIV infection, we extended this analysis for the duration of SIV infection. Longitudinal analysis of CD4$^+$ T cell subsets showed no difference in naïve and EM CD4$^+$ T cell counts between SP, CP and RP throughout acute and chronic SIV infection (nonparametric Mann Whitney ranksum p-values>0.14) [Figs. 2a and b]. However, there was a trend towards overall retention of CM CD4$^+$ T cell counts over time in SP compared with CP or RP (nonparametric Mann Whitney ranksum p-values; both $p=0.03$) [Fig. 2c]. Interestingly, we also noted higher naïve CD4$^+$ T cell and lower CM CD4$^+$ T cell levels pre-infection in the RP group, as shown in the week 0 timepoint of Figs. 2a and c.

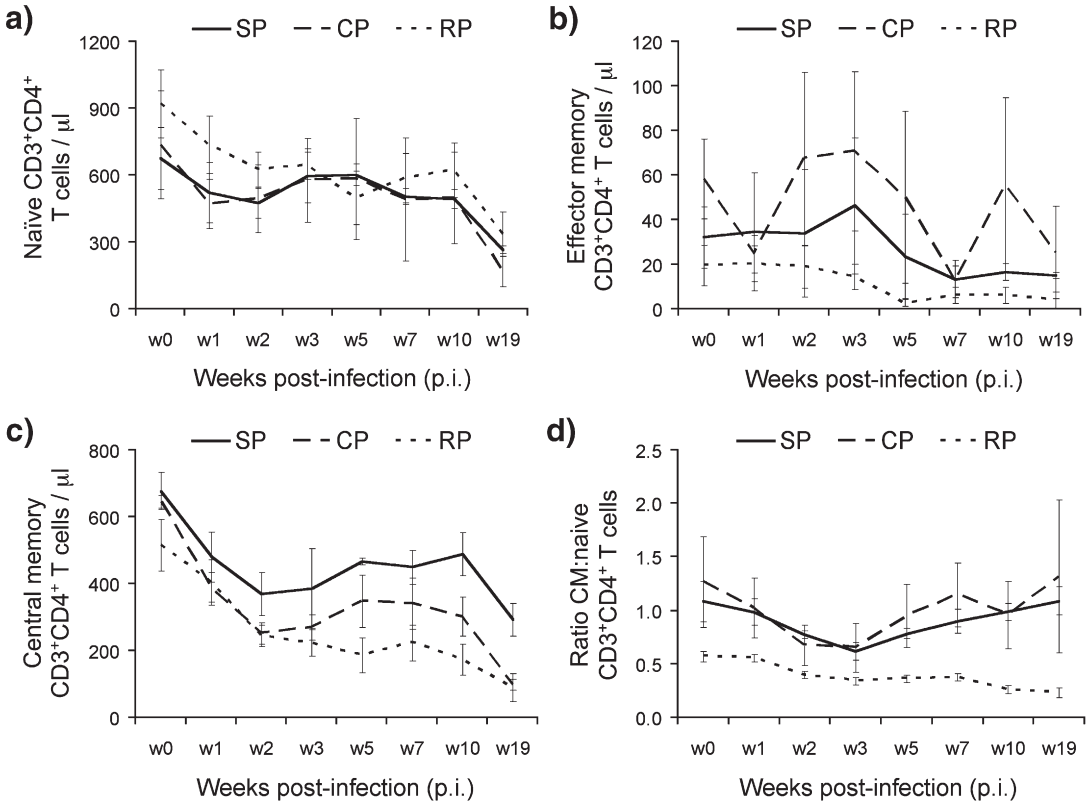

Fig. 2. Longitudinal analysis of memory and naïve CD4$^+$ T cell subsets in SIV-infected, untreated pigtail macaques. Longitudinal analysis of mean (±SE) of (a) naïve (CD28$^+$CD95$^-$), (b) effector memory (CD28$^-$CD95$^+$) and (c) central memory (CD28$^+$CD95$^+$) CD3$^+$CD4$^+$ T cell counts and (d) central memory to naïve CD3$^+$CD4$^+$ T cell ratios in SP, CP and RP macaques.

Low CM:naïve CD4$^+$ T cells ratios are associated with rapid progression

Given the apparent significance of baseline CM CD4$^+$ T cell levels to disease outcome, we compared CM:naïve CD4$^+$ T cell ratios in the 3 groups. The RP's demonstrated uniformly low CM:naïve CD4 T cell ratios which were significantly lower both pre- and post-infection (p=0.04, 0.006 respectively) than CP and SP groups combined [Fig. 2d]. Both SP controllers and CP had a decline in CM:naïve CD4$^+$ T cell ratios during acute infection but were able to restore CM:naïve CD4$^+$ T cell ratios close to pre-infection levels. CM:naïve CD4$^+$ T cell ratios over time between SP and CP groups were almost identical. RP animals, however, experienced a continuous decline in the ratio of CM to naïve CD4$^+$ T cells throughout SIV infection as compared with CP and SP (nonparametric Mann Whitney ranksum p=0.08, 0.03 respectively).

Treg levels do not predict disease progression

Given the proposed Treg suppression of virus-specific T cell immunity, we sought to examine peripheral CD4$^+$ Tregs over time in the 11 SIV-infected macaques. Intracellular staining for FoxP3 on CD4$^+$CD25$^+$ T cells defines regulatory T cells, but is technically cumbersome and does not allow for subsequent functional analyses. We recently reported a simple surface staining method to define Tregs as CD25$^+$CD127^{lo} CD4$^+$ T cells in humans (Seddiki et al., 2006) and adapted this assay to pigtail macaque T cells. The CD25$^+$CD127^{lo} CD4$^+$ T cell population robustly defined the same CD25$^+$FoxP3$^+$ population of Tregs in all 11 pigtail macaques [Fig. 3a] and is consistent with Treg phenotype observed in rhesus macaques (Hartigan-O'Connor et al., 2007). We then used this assay to surface stain fresh peripheral blood over the course of SIV infection [Fig. 3b]. There was no difference in Treg numbers pre-infection and depletion in total Tregs during the course of SIV infection in all 3 progressor populations. The pattern of rapid decline in Tregs following acute SIV infection with subsequent rebound and then more gradual decline during chronic SIV infection is similar to that reported for Tregs in SIV-infected cynomologus macaques (Karlsson et al., 2007b).

In summary, we evaluated the relationship between memory and Treg subsets of CD4$^+$ T cells with survival and disease progression in SIV-infected pigtail macaques. Preservation of CM CD4$^+$ T cells was associated with survival in pigtail macaques, consistent with previous reports in rhesus macaques (Letvin et al., 2006). In accordance with previous studies of rhesus and cynomolgus macaques (Karlsson et al., 2007a; Sun et al., 2007), we observed a significant association between preservation of CM CD4$^+$ T cells and control of viremia in SIV-infected pigtail macaques. Given the strong associations between viral levels and disease outcome, an association between CM CD4$^+$ T cells and VL is not unexpected.

Our longitudinal analysis of CM, EM, naïve and Treg CD4$^+$ T cell subsets in 12 treatment naïve SIV-infected pigtail macaques showed an unexpected finding of low baseline CM CD4$^+$ T cells in rapid progressors, but not other CD4$^+$ T cell subsets. Low pre-infection levels of CM CD4$^+$ T cells predicting SIV disease has not been reported in rhesus macaques although it is unclear whether this has been carefully studied in the rhesus-SIV model (Letvin et al., 2006; Okoye et al., 2007). In our experience pigtail macaques progress more rapidly to AIDS in comparison to rhesus macaques (Batten et al., 2006). We speculate that the more rapid progression in the pigtail macaque model may make animals with lower pre-infection levels of CM CD4$^+$ T cells more vulnerable to rapid progression in comparison to models with slower disease progression. Further analyses on even larger cohorts of SIV-infected macaques are clearly warranted. Depletion of already low CM CD4$^+$ T cell levels might predispose to disease progression and, if confirmed in larger studies, should be taken into account in randomizing macaques for pathogenesis and vaccine studies.

Materials and methods

Animals and viruses

Twelve SIV$_{mac251}$-infected pigtail macaques (*M. nemestrina*) examined in this study were part of a recently completed failed Kunjin replicon SIV vaccine trial (Kent et al., 2008) approved by the University of Melbourne animal ethics committee. The SIV$_{mac251}$ (kindly supplied by Drs N. Miller and R. Pal) was injected IV at 40 TCID50. Plasma SIV RNA levels were determined by real-time PCR as previously described (Fernandez et al., 2007). Prospective criteria for euthanasia for incipient AIDS, agreed with our animal ethics committee, were weight loss (>10%), loss of appetite, persistently reduced activity, chronic diarrhoea, or clinical disease consistent with infection or malignancy.

Phenotyping of CD4$^+$ T cell subsets

Staining of CD4$^+$ T cell subsets was performed on fresh whole blood collected in sodium heparin vacuette tubes. 250 μl of whole blood was stained for 1 h at room temperature with a monoclonal antibody cocktail containing either i) anti-CD3 allophycocyanin (APC) clone SP34-2, anti-CD4 phycoerythrin-Cy7 (PE-Cy7) clone SK3, anti-CD28

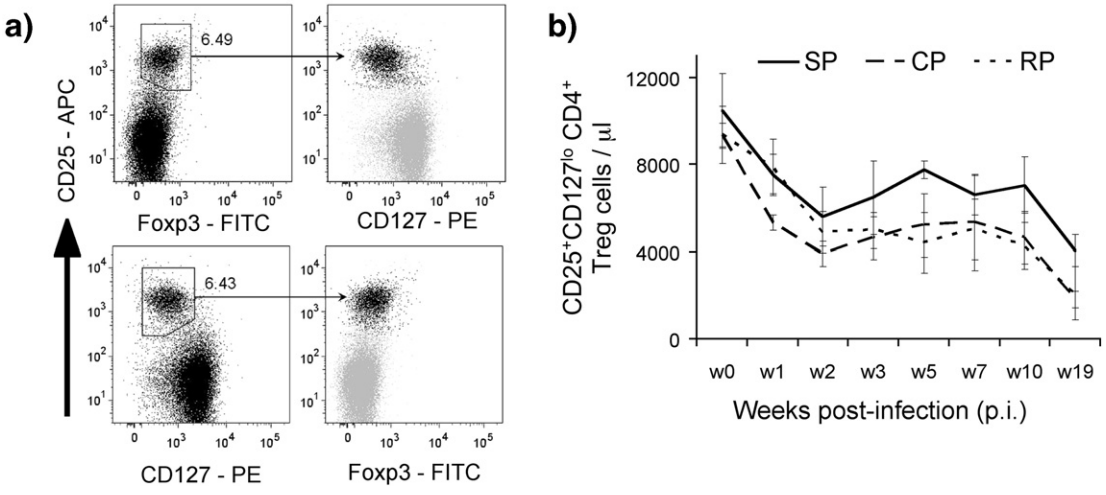

Fig. 3. CD4$^+$ T regulatory (Treg) cell analysis in SIV-infected, untreated pigtail macaques. (a) Co-staining of CD4$^+$ T cells for CD25$^+$ and CD127^{lo} identifies equivalent CD25$^+$Foxp3$^+$ CD4$^+$ Treg cell population; gates drawn based on CD25, CD127 and FoxP3 fluorescence minus one (FMO) controls (performed for all animals and shown for animal 6267). (b) Longitudinal analysis of mean (±SE) of CD25$^+$CD127^{lo} CD4$^+$ Treg cell counts in SP, CP and RP macaques.

peridinin-chlorophyll-protein complex Cy5.5 (PerCP Cy5.5) clone L293 and anti-CD95 fluorescein isothiocyanate (FITC) clone DX2 (all from BD Biosciences) for phenotyping of naïve, CM and EM $CD4^+$ T cells or ii) anti-CD4 (FITC) clone M-T477 (BD Biosciences), anti-CD25 (APC) 2A3 (BD Biosciences) clone L293 and anti-CD127 phycoerythrin (PE) clone R34.34 (Beckman Coulter, Fullerton, CA, USA) for phenotyping of Tregs. After red blood cells were lysed at room temperature for 10 min with 2 ml BD FACS Lysing Solution (BD Biosciences), the cells were washed with 2 ml FACS wash buffer (PBS, 0.5% BSA, 2 mM EDTA, pH 8). For Treg samples requiring intracellular FoxP3 staining, cells were permeabilized at room temperature for 10 min with 0.5 mL BD FACS Permeabilizing Solution 2 (BD Biosciences), washed with 3.5 mL FACS wash buffer then incubated for 1 h at room temperature with anti-human FoxP3 (FITC) clone 206D (BioLegend, San Diego, CA, USA). A final wash with PBS was performed and the cells were fixed with 1% formaldehyde. Fluorescence minus one (FMO) controls for CD25, CD28, CD95, CD127 and FoxP3 were included in each run. Samples were acquired on a BD LSR II flow cytometer (BD Bioscience) within 3 h of staining and compensation was optimized using BD CompBeads (BD Biosciences). At least 50,000 $CD4^+$ T cell events were collected for each sample and subsequent data analyses were performed using FlowJo Version 7.2.2 for Windows (Treestar, Ashland, OR, USA). Statistical analyses used nonparameteric Mann Whitney ranksum tests to compare levels of T cells subsets over time in macaques groups.

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