Thrombocytopenia Is Strongly Associated With Simian AIDS in Pigtail Macaques

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Summary: Simian AIDS has a variable time course and presentation making it difficult to define disease effects of progressive simian immunodeficiency virus (SIV) infection. We commonly observed thrombocytopenia (TCP) associated with progressive SIV infection of pigtail macaques (*Macaca nemestrina*). We therefore analyzed the relationship between platelet counts, viral load (VL), and CD4 T-cell levels in 44 unselected macaques with chronic SIV infection. Persistent TCP was observed in 70% of pigtail macaques infected with SIV_{mac251} for up to 77 weeks in the absence of clinically significant bleeding. The presence of TCP correlated with higher SIV plasma VLs and depressed total and memory CD4 T cells. TCP was more common in macaques requiring euthanasia for incipient AIDS than macaques that survived to the end of the studies, although VL and CD4 T-cell decline were stronger independent predictors of AIDS-free survival. There was however no clear correlation between the development of TCP and immune activation as measured by plasma soluble CD14. We conclude that TCP is a useful end point to analyze SIV studies in pigtail macaques.

Key Words: AIDS, macaque, platelets, SIV

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INTRODUCTION

With the failure of recent attempts to successfully vaccinate human subjects against HIV, there have been increasing calls for more insightful studies of macaque models of HIV infection.[1] Several Asian macaque species (*Macaca mulatta*, *Macaca nemestrina*, and *Macaca fascicularis*) develop an AIDS-like illness after inoculation with various strains of simian immunodeficiency virus (SIV). The conduct of pathogenesis and vaccine studies in macaques (and humans) is however complicated by the marked variability in disease course. Much of the variable disease course of HIV in humans and SIV in macaques can be accounted for by genetic factors such as major histocompatibility complex alleles and coreceptor polymorphisms.[2,3] Large numbers of outbred macaques are often required to adequately power studies to define protection against disease, and stratification of groups for protective alleles known is typically warranted.[4,5]

Depletion of CD4 T cells and elevated levels of plasma viral load (VL) are very strong surrogate markers for HIV-1 or SIV-induced opportunistic infections or cancers.[6] Depletion of the central memory subset of CD4 T cells even more strongly correlates with disease progression.[7,8] Recent studies highlight the role of immune activations caused by translocation of gut microbial products in contribution to CD4 T-cell depletion and immunodeficiency.[9]

Despite an improved understanding of the development of immunodeficiency caused by HIV-1 in humans and SIV in macaques, the development of diverse opportunistic infections and cancers can be delayed even when very few CD4 T cells are present and VL is high. This confounds analyses that rely on disease end points. Furthermore, most animal ethics committees rightly discourage disease end points where robust surrogate markers are available. Additional simple surrogate markers of disease progression would be useful to guide macaque SIV studies.

Thrombocytopenia (TCP) is a common complication of human HIV-1 infection, sometimes occurring relatively early during the disease course. HIV-induced TCP is distinct from classical autoimmune TCP, although HIV-induced TCP likely has at least a component of autoimmune pathogenesis, with the development antibodies to various platelet molecules described.[10] TCP in the setting of HIV infection responds well to antiretroviral therapy, implicating effects of HIV replication in its etiology. TCP has also been described during the course of SIV infection in macaques,[11–13] including neuropathogenic infections,[14] although studies formally analyzing TCP in large numbers of SIV-infected macaques are surprisingly limited.

We recently conducted 2 sizeable SIV_{mac251} infection studies in a total of 44 pigtail macaques (*M. nemestrina*).[15,16] We observed a high incidence of TCP and therefore studied the relationship between the development of TCP and simian AIDS.

METHODS

Macaques and SIV Infection

Two large recently published pigtail macaque SIV infection studies were analyzed. The first study involved 12 pigtail macaques, 6 of which were vaccinated with a Kunjin

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virus–like particle replicon expressing SIV Gag and RT and 6 controls received a similar Kunjin replicon expressing HIV-1 Gag.[16] All 12 macaques were challenged intravenously with SIV_{mac251} (40 TCID50), kindly supplied by Pal as previously described.[17] No SIV-specific immune responses were induced by vaccination, and no overall protection from challenge was observed in the Kunjin vaccine study. The animals were followed for up to 23 weeks after SIV infection.

The second study involved 32 pigtail macaques in a therapeutic peptide-based vaccine study.[15] All 32 naive macaques were infected intravenously with SIV_{mac251} (40 TCID50) and had high levels of SIV viremia during acute infection. All 32 animals were placed on dual antiretroviral therapy (tenofovir and emtricitabine, kindly supplied by Gilead, Foster City, CA) from weeks 3 to 10 after infection. From weeks 4 to 10, 21 of the 32 animals received 4 doses of autologous peripheral blood mononuclear cell pulsed with overlapping SIV peptides as a therapeutic vaccine under antiretroviral cover. Ten animals did not receive vaccines. All 32 animals had their antiretroviral drugs withdrawn at week 10. The vaccinated animals received a booster set of 3 vaccinations without antiretrovirals at weeks 36–42 after infection. The 21 vaccinated animals had a mean 10-fold reduction in virus levels compared with 11 controls. Euthanasia criteria of SIV-infected macaques were preset in agreement with our animal ethics committee and included persistent weight loss (>10%), diarrhea, lethargy, anorexia, or opportunistic infections.

Blood Cell Counts

Serial fresh EDTA-anticoagulated whole blood samples were analyzed within 6 hours of collection for platelet (PLT) counts regularly (1–4 weekly) during the course of SIV infection by an automated coulter counter (ACT-T diff; Beckman-Coulter, Fullerton, CA). Manual PLT counts on fresh blood smears were performed to confirm the presence of normal or reduced levels of platelets. Bone marrow biopsies from the humeral head were taken from 3 animals to assess levels of megakaryocytes; fresh smears were made immediately upon sampling.

VL, CD4 T-Cell Counts, and Soluble CD14 Levels

At each blood sampling, plasma SIV RNA (VL) was quantified by TaqMan real-time polymerase chain reaction as previously described.[17] $CD4^+$ T-cell counts were quantified by flow cytometry by analyzing the proportion of gated lymphocytes staining for CD3 and CD4 as previously described.[18] We also measured levels of soluble CD14 (sCD14) in macaque plasma with a commercial enzyme immunoassay kit according to the manufacturer's instructions (R&D Systems).[9]

Statistical Analyses

Group comparisons used a 2-sided nonparametric Mann–Whitney *U* test for continuous data and Fisher exact test for binary data. Survival analyses used Cox regression analyses.

RESULTS

SIV Infection and the Development of TCP

We conducted 2 large SIV_{mac251} vaccine studies in 44 pigtail macaques in total, using a well-characterized SIV_{mac251} virus stock commonly studied worldwide.[15,16,19] The first study infected 12 animals and the second 32 animals—in the second study, the animals were treated with antiretroviral drugs for 7 weeks between weeks 3 and 10 after infection. All monkeys became persistently infected with SIV_{mac251}. During the course of chronic infection in both studies, we noted the development of TCP in many animals. PLT counts on the day before infection in all 44 monkeys varied between 222 and 625/μL (mean ± SD of 444 ± 87/μL). The development of a PLT count of <150/μL was not observed during acute SIV infection but became prevalent in 31 of the 44 monkeys during chronic infection. The temporal pattern of the development of TCP among the 32 animals in the second study is shown in Figure 1. When PLTs fell to <150/μL, they always remained low thereafter. Examples of the temporal development of TCP in monkeys and its relationship to SIV VL and CD4 T-cell depletion in 6 animals across both studies analyzed are shown in Figures 1B–G. In some animals, the development of TCP immediately preceded a precipitous drop in CD4 T cells. The PLTs in the thrombocytopenic monkeys were typically stable at 50–100/μL. At the last time point sampled in each thrombocytopenic animal, the range was 7–138/μL (mean 76/μL), with only 5 of the 31 animals having PLTs of 7–50/μL. No overt bleeding diatheses were observed, although increased bruising at the sites of venepuncture was observed intermittently in animals with TCP. We analyzed megakaryocytes and platelets in bone marrow smears from 3 SIV-infected macaques. All had normal populations of megakaryocytes and platelets by manual counting within bone marrow (not shown), consistent with peripheral destruction of platelets.

Relationship Between TCP, SIV VL, and CD4 T-Cell Depletion

The temporal relationship between the development of TCP and CD4 T-cell depletion and higher SIV VLs shown in Figures 1B–G suggested that there might be an overall correlation between TCP, SIV VL, and CD4 T-cell levels. We therefore analyzed the macaque studies for correlations between these variables. We restricted the analyses to the postacute infection period in the absence of antiretroviral therapy because high-peak VLs occur during acute infection before the onset of slowly progressive simian AIDS (Fig. 1).

There was a correlation between lower PLTs and higher VL during chronic SIV infection and to a lesser extent declining CD4 T cells (Figs. 2A, B). There was a distinct grouping of samples with PLTs <200/μL; the majority of samples with low PLTs had high VLs, and many of the samples with lower CD4 T-cell levels had low platelet levels. We therefore also analyzed mean VLs and CD4 T-cell counts of samples with PLT counts of either less than or greater than 200/μL. There was a significantly higher (1.2 $\log_{10}$ copies/mL difference) mean VL and lower CD4 T-cell counts in all samples with <200/μL (Fig. 2C). By comparison, there were

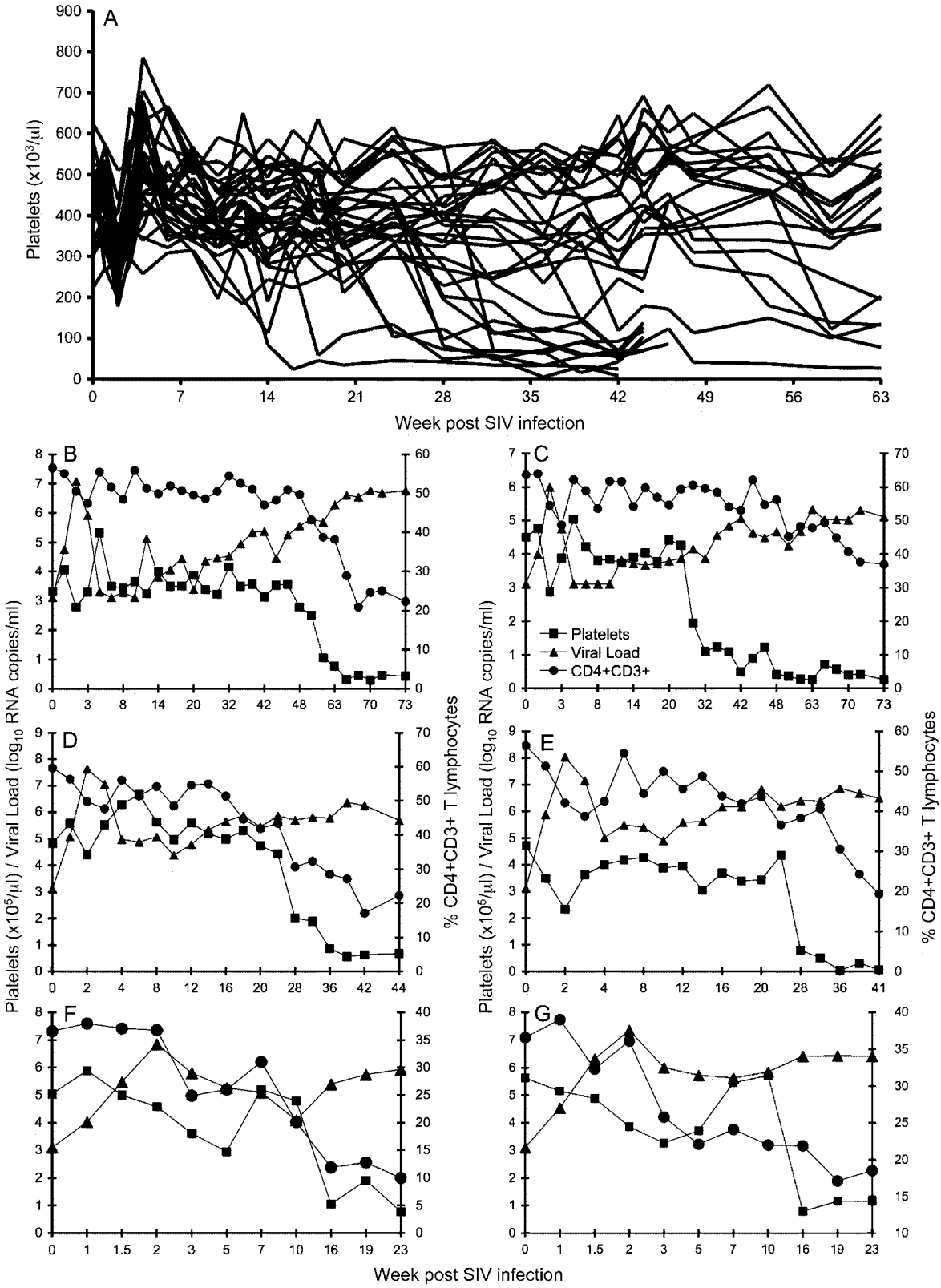

FIGURE 1. Development of TCP in pigtail macaques. A, Serial PLT counts in all 32 animals in a therapeutic vaccine study. B–F, Temporal relationship of TCP, SIV VL, and peripheral CD4 T-cell counts in 6 animals, 4 from the therapeutic vaccine study, where the animals received antiretroviral therapy at weeks 3–10 (B–E), and 2 from an SIV infection study without antiretroviral treatment (F and G).

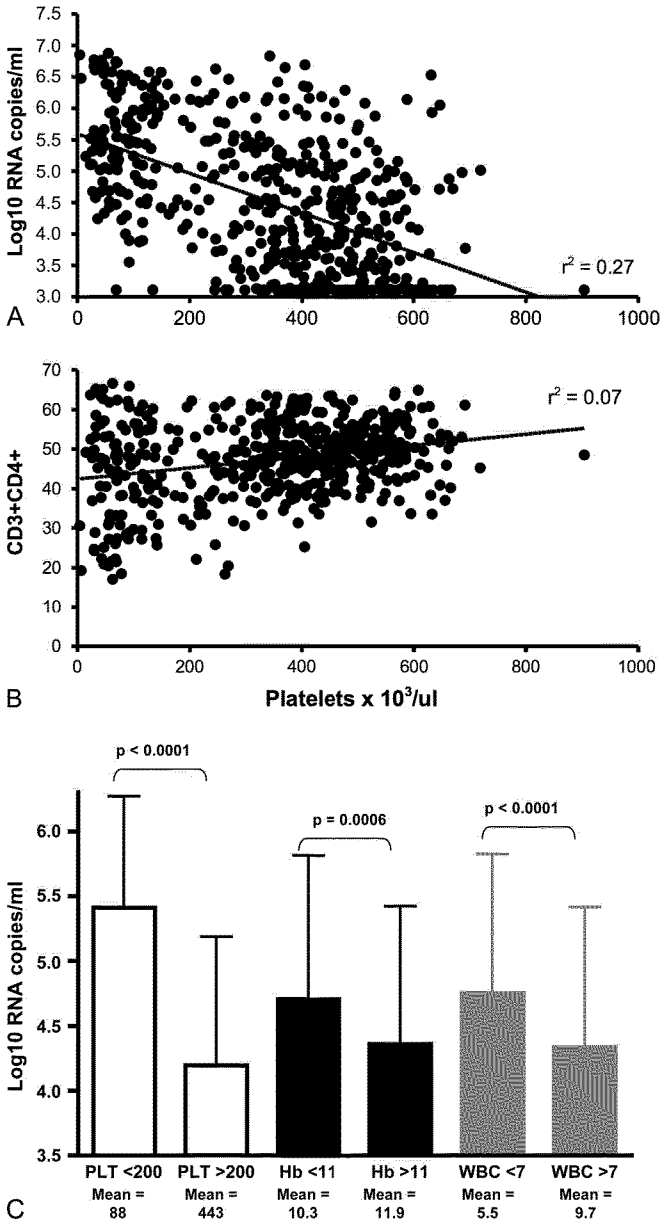

FIGURE 2. Relationship of TCP to SIV VL and CD4 T cells. PLT counts were plotted against concurrent SIV VL (A) and peripheral $CD3^+$ $CD4^+$ T-cell counts (B) in the 44 SIV-infected macaques. Samples were correlated from weeks 16 to 77 in the therapeutic vaccine study of 32 macaques that received antiretroviral therapy from weeks 3 to 10 and from weeks 7 to 23 in the 12 macaques infected with SIV without antiretroviral therapy. C, Levels of SIV VL in samples with low platelets (<200/μL), hemoglobin (<11 g/dL), and total white cell ($<7 \times 10^3$/μL) counts from the 32 animals in the therapeutic SIV vaccine study (weeks 16–77) in comparison to animals with higher levels of platelets, hemoglobin, and white cells. The bar represents the mean ± SD of the VL; *P* values are from a nonparametric 2-sided Mann–Whitney *U* test. The mean level of each group is shown below the legend.

significant, but much less marked, differences in SIV VL in macaques with lower hemoglobin and total white cell count levels during chronic SIV infection (0.3 $\log_{10}$ copies/mL differences). We also analyzed the relationship between $CD28^+$ $CD95^+$ central memory CD4 T cell and TCP because central memory CD4 T cells are primary infected by SIV and have been more strongly associated with simian AIDS than total CD4 T cells. From week 0 (time of SIV infection) to week 28 after infection, there was a mean fall in central memory CD4 T cells of 21% in animals that developed TCP before week 63 compared with a mean fall of 13% in animals that did not develop TCP ($P = 0.056$).

In the therapeutic vaccine study of 32 animals, 21 received SIV peptide vaccinations and 11 did not. The vaccinated animals had mean 0.9 $\log_{10}$ lower VLs during chronic infection. Although our primary goal was to analyze the relationship between TCP and SIV infection across a large number of macaques, we also analyzed the development of TCP by vaccine group. Six of the 11 control macaques developed substantial TCP (PLT < 100/μL) by week 63 after SIV infection compared with only 6 of 21 vaccinated macaques.

TCP and Simian AIDS

Although TCP correlated with higher VLs and lower CD4 T-cell counts, we also assessed whether there was a relationship between TCP and euthanasia for incipient AIDS. We euthanized SIV-infected animals with persistent weight loss, loss of appetite, lethargy, and/or opportunistic infections. The 32 animals in the second trial were grouped according to whether they had developed persistent TCP (<150/μL) by 63 weeks after SIV infection (1 year after withdrawal of antiretroviral therapy in this study). Of the 14 animals that developed TCP, all 14 were euthanized before week 77, whereas 11 of the 18 animals without TCP survived to week 77. There was a significant survival benefit for animals that did not develop TCP (Fig. 3). Development of PLT counts of <100/μL by week 63 were even more predictive of simian AIDS (11 of 12 animals with platelets <100/μL died vs 1 of 20 without). The mean time of TCP (<100/μL) before euthanasia was 11 weeks (range 4–24 weeks).

Because survival, VL, and CD4 T-cell counts were all associated with TCP, we therefore analyzed to what degree TCP (PLT <200 μL) was independently predictive of AIDS-free survival using a multivariate analysis (Table 1). Although TCP was strongly predictive of survival in the univariate analysis (hazard ratio 6.0, $P = 0.002$), TCP was less predictive of survival in a multivariate analysis taking into account VL and CD4 T-cell decline and did not reach statistical significance (hazard ratio 2.8, $P = 0.087$).

Immune Activation and TCP

Recent studies suggest a strong link between immune activation induced by translocation bacterial products across the gut and progressive HIV-1 and SIV disease in humans and macaques.[9,20] Bacterial lipopolysaccharide activates monocytes to shed the CD14 molecule, and the level of sCD14 in plasma is a strong surrogate marker for immune activation.[9] The temporal occurrence of TCP suggested the possibility that immune activation might be involved in etiology of TCP, and

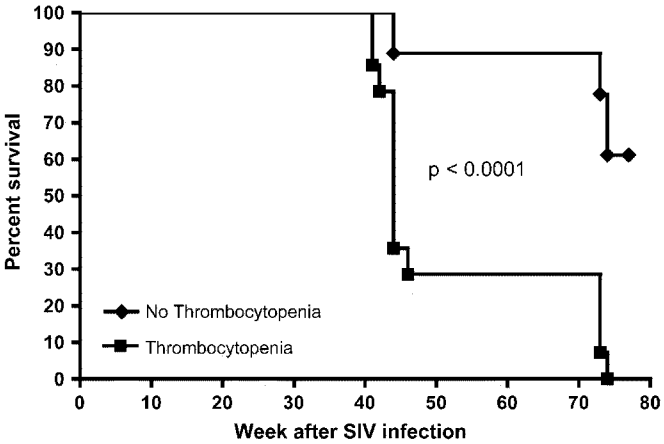

FIGURE 3. Survival analysis of SIV-infected pigtail macaques with TCP. The 32 animals in the therapeutic SIV vaccine study were classified as having persistent TCP (2 or more consecutive values of <150/μL) by week 63 of infection or not and survival analyzed. *P* value represents Cox regression analysis.

we therefore analyzed plasma sCD14 levels using a commercial enzyme immunoassay assay in SIV-infected macaques developing TCP.

We studied sCD14 levels before and 16–36 weeks after SIV infection in 10 macaques that developed TCP (Fig. 4A). There was no significant overall rise is sCD14 levels across the group, although some animals had a modest rise from baseline levels. We went on to serially measure sCD14 levels in 3 macaques that developed TCP over the course of SIV infection. There was no temporal rise in sCD14 levels in association with the development of TCP (Fig. 4B). Furthermore, a cross-sectional analysis of the total cohort of 44 SIV-infected animals demonstrated no clear correlation between the development of TCP and sCD14 levels (Fig. 4C). These results suggest that the development of TCP is not tightly linked to sCD14 levels.

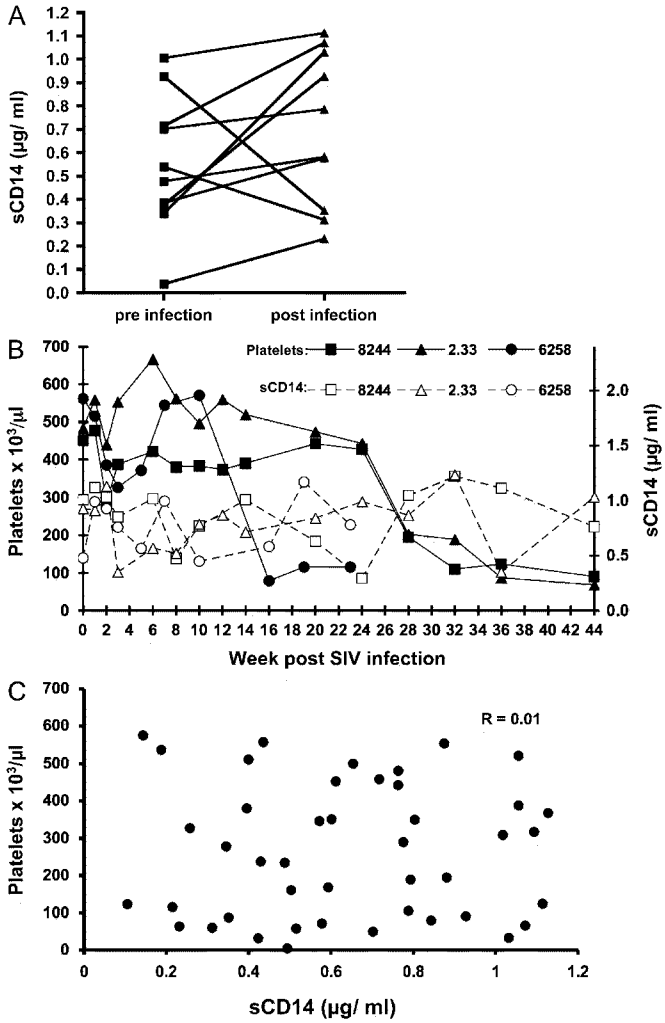

FIGURE 4. Relationship of sCD14 to TCP. Plasma sCD14 was measured by enzyme immunoassay: (A) before and 16–36 weeks after SIV infection in 10 animals, (B) serially in 3 animals that developed TCP, and (C) in a cross-sectional analysis of all 44 SIV-infected macaques where plasma was studied at week 16 in the 12 animal study of SIV infection and week 36 in the 32 animal study, a therapeutic SIV vaccine study.

DISCUSSION

We observed a correlation between TCP and high-SIV VLs, depletion of total and central memory CD4 T cells, and the development of AIDS requiring euthanasia in pigtail macaques. VL and CD4 T-cell decline primarily predict AIDS-free survival, with TCP failing to independently predict survival in a multivariate analysis. Interestingly, the TCP was mild in most macaques (generally 50–100/μL) and not associated with overt bleeding. The onset of TCP occurred a mean of 11 weeks before euthanasia with simian AIDS. There were adequate numbers of megakaryocytes and platelets in bone marrow suggesting peripheral destruction, as described for HIV in humans.[10]

There is surprisingly little information on TCP in macaque models of SIV infection, with generally case reports or small series described[11] and 1 larger study of an association of TCP with SIV-induced neuropathogenesis, also in pigtail macaques.[14] Whether the lack of literature on TCP in SIV-infected macaques relates to poor observation of PLT

TABLE 1. Predictors of AIDS-Free Survival

	HR	95% Confidence Interval	*P*	*P* Trend for CD4 Decline
Univariate				
TCP* (PLT < 200)	6.0	1.9 to 18.7	0.002	0.003
% CD4 T cell, 42.1–48*	0.55	0.19 to 1.6	0.272	
% CD4 T cell, 48.1–53	0.11	0.01 to 0.90	0.04	
% CD4 T cell, >53.1	0.14	0.03 to 0.65	0.012	
VL (>4.5 $\log_{10}$ copies)*	11.4	2.6 to 50.3	0.001	
Multivariate (adjusted for CD4 and VL)				
TCP (PLT < 200)	2.8	0.86 to 9.2	0.087	

HR, hazard ratio.
*PLT count ≥200, CD4 T-cell count ≤42%, and VL ≤4.5 log copies per milliliter were the reference values for the respective HRs.

counts in prior studies, the differing susceptibility to disease across the various Asian macaque species, or other genetic factors is not clear. Nonetheless, the pigtail macaques we have studied had a remarkably high incidence of TCP (70%) that clearly correlated with surrogate markers and disease end points of SIV. In light of these observations, we now carefully follow PLT counts as a marker of progressive SIV disease in all studies. A caveat to the relationship between TCP and simian AIDS in the variable timing and presentation of diseases necessitating euthanasia. Although we prospectively set euthanasia criteria with our ethics committee, the diverse nature of signs of disease and the desire to avoid any suffering of the animals make this analysis inherently variable. Interestingly, we observed a clustering of deaths in animals with TCP soon after weeks 42 and 71, which were shortly after boosting immunization without antiretroviral therapy in this study. We recently reported that immunizations in these SIV-infected macaques without antiretroviral therapy result in a transient rise in VL, presumably due to the activation of a population of CD4 T cells.[21] This rise in VL may have accelerated the development of complications of simian AIDS.

The high incidence of TCP in pigtail macaques affords an opportunity to study the pathogenesis of this TCP in more detail. We were surprised that a useful marker of immune activation, sCD14, did not correlate strongly with the development of TCP. Future studies of additional markers of microbial translocation and immune activation (eg, lipopolysaccharide levels, CD38 expression on lymphocytes) on even larger numbers of animals may uncover subtle clues to the pathogenesis of TCP in SIV-infected macaques. Assessment of autoimmunity, including antiplatelet antibodies, is also clearly suggested by these studies, although reliable assays are not yet available for macaque samples to our knowledge. Further studies of bone marrow samples, including development of reagents to stain and assess function of macaque megakaryocytes, are also a priority for future studies. The high incidence of TCP in this model also enables future opportunities to study interventions to prevent and treat TCP.

In summary, we describe a clear relationship between TCP and SIV infection as demonstrated by associations between SIV VL, CD4 T cells, and euthanasia. This provides an additional marker to analyze for progressive SIV infection in pigtail macaques. Furthermore, our work establishes a primate model for TCP that should prove useful in dissecting the pathogenesis of HIV-induced TCP and potentially improving treatments for TCP.

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