

comparison of IgG binding levels, MFI values of IgG on rCD4s of the lysate samples are denoted above. IgG, positive IgG control; H, rCD4 lysate from an HIV-1-seronegative healthy donor. (d) Three-dimensional reconstitution confocal micrographs of Igs (Qdot655, green) and CD4 (Cy2, red) in rCD4s from an HIV-1<sup>+</sup> Pt. (e, f) Representative time course of FACS (e) and calculated half-life of sICs (f) in purified rCD4s from an HIV-1<sup>+</sup> Pt. Bar, SD. (g) Percentage of Ig<sup>+</sup> cells in purified HIV-1<sup>+</sup> Pt rCD4s without (none) or with 10 min of 0.05% trypsinization (Tryp). (h) Percentage of Ig<sup>+</sup> cells in purified HIV-1<sup>+</sup> Pt rCD4s before (0 h), after 58 h of culture (58 h), or 58 h of culture with exposure to HIV-1<sup>+</sup> Pt serum (58 h+Pt-serum). (i) Changes in percentages of IgM<sup>+</sup> or IgG<sup>+</sup> rCD4s in blood, plasma VL, and CD4 lymphocyte counts during ART in the four HIV-1<sup>+</sup> Pts. Two patients discontinued therapy after substantial suppression of VLs (left panels). HIV-1 RNA levels in two other patients were suppressed to undetectable levels for approximately 2 yr with ART (right panels). (j, k) Summary of the percentages (k) and representative FACS (j) of Igs on purified HIV-1<sup>+</sup> Pt rCD4s before and after 6 h of PMA (0.3 ng/ml) exposure. (l) Fluorescence and DIC images of purified HIV-1<sup>+</sup> Pt rCD4s that were stained with anti-Ig Abs (Cy2, green) and goat polyclonal anti-CD4 (Cy3, red) before and after 6 h of PMA exposure. Data in d and l are representative of five independent experiments. doi:10.1371/journal.pone.0086479.g002

reduction in sICs in purified peripheral rCD4s from six patients was  $21.76 \pm 5.62$  h ( $\pm$  SD) (**Fig. 2E and F**). rCD4s should contain a certain number of cells in stages beyond G<sub>0</sub>. Therefore, the turnover of VRs and/or sICs in purified rCD4s may be much faster than in qCD4s; taking this into account, the calculated half-life of sICs on the patients' rCD4s roughly matched the turnover of sICs in qCD4s. More importantly, trypsin treatment to remove trypsin-sensitive cell surface molecules (e.g., CD4) significantly reduced the level of sICs (**Fig. 2G**). Similarly, once sIC levels were reduced in rCD4s, the levels were not restored by exposing the cells to Pt serum (**Fig. 2H**), suggesting that rCD4s from HIV-1<sup>+</sup> Pts either do not allow attachment of cICs or do not express surface epitopes for auto-Abs in Pt serum. Collectively, sICs on the patients' rCD4s consisted of cell-bound molecules with similar kinetics to gp120-Igs.

### Longitudinal Cohort Analysis Reveals that Cell-bound HIV-1 or Related Molecules are Involved in the Formation of sICs on Resting CD4<sup>+</sup> T Cells in Vivo

To further characterize whether sICs on patients' rCD4s are linked to cell-bound HIV-1 molecules, we examined the levels of sIC<sup>+</sup> rCD4s in the peripheral blood of antiretroviral therapy (ART)-experienced HIV-1<sup>+</sup> Pts with longitudinal follow-up samples. Eleven individuals with asymptomatic chronic HIV-1 infection were examined. All 11 Pts with asymptomatic chronic HIV-1 infection were followed on an outpatient basis for >3 years (yrs) and were either treated with ART to complete suppression (< 50 RNA copies/ml) or untreated. Means of  $78.53 \pm 7.37\%$  and  $43.89 \pm 21.73\%$  ( $\pm$  SD) of rCD4s in blood from 11 chronic HIV-1<sup>+</sup> Pts stained positive with anti-IgG and anti-IgM Abs, respectively. However, in the 7 subjects for whom treatment led to complete suppression (<50 RNA copies/ml) of plasma VL for 3 yrs, the percentages of sIC<sup>+</sup> rCD4s were significantly reduced, with means of  $15.28 \pm 13.36\%$  ( $\pm$  SD) (vs.  $79.87 \pm 6.46\%$  ( $\pm$  SD), = before treatment),  $P < 0.0001$  and  $4.71 \pm 2.49\%$  ( $\pm$  SD) (vs.  $46.25 \pm 29.29\%$  ( $\pm$  SD), = before treatment),  $P = 0.0045$  of rCD4s in blood positive for IgG and IgM, respectively. In contrast, in the four HIV-1<sup>+</sup> Pts who remained untreated for 3 yrs, the number of sIC<sup>+</sup> rCD4s in blood significantly increased, with means of  $89.75 \pm 8.53\%$  ( $\pm$  SD) (vs.  $73.75 \pm 9.03\%$  ( $\pm$  SD), before),  $P = 0.036$  and  $63.21 \pm 16.18\%$  ( $\pm$  SD) (vs.  $42.75 \pm 13.45\%$  ( $\pm$  SD), before),  $P = 0.0091$  of rCD4s positive for IgG and IgM, respectively.

In **Fig. 2I**, four representative chronic HIV-1<sup>+</sup> Pts who had frequent peripheral blood sampling for CD4 or viral RNA testing are shown. After initiating ART, plasma virus became undetectable (<50 RNA copies/ml) within 200 days in two subjects (**right panels**). In these two subjects, both IgG<sup>+</sup> and IgM<sup>+</sup> rCD4s gradually decreased in the peripheral blood; however, it required approximately 2 yrs for the percentages of IgG<sup>+</sup> and IgM<sup>+</sup> rCD4s to reach less than approximately 10%. In contrast, in the two subjects with treatment interruption, the percentages of both IgG<sup>+</sup> and IgM<sup>+</sup> rCD4s promptly increased (**left panels**). Although the

change in frequency of IgG<sup>+</sup> and IgM<sup>+</sup> rCD4s in blood was relatively slow compared with the change in plasma VLs, the frequencies in peripheral blood correlated to plasma VLs (**Fig. 2I**). Therefore, these results collectively indicate that at least cell-bound HIV-1 or related molecules are involved in the formation of sICs on rCD4s *in vivo*. Interestingly, the percentage of both IgG<sup>+</sup> and IgM<sup>+</sup> rCD4s appears to be inversely correlated to the number of CD4<sup>+</sup> T cells in peripheral blood (**Fig. 2I**, compare closed squares or closed diamonds with open triangles).

### sICs are Attached to Surface CD4 on Resting CD4<sup>+</sup> T Cells from HIV-1-infected Subjects

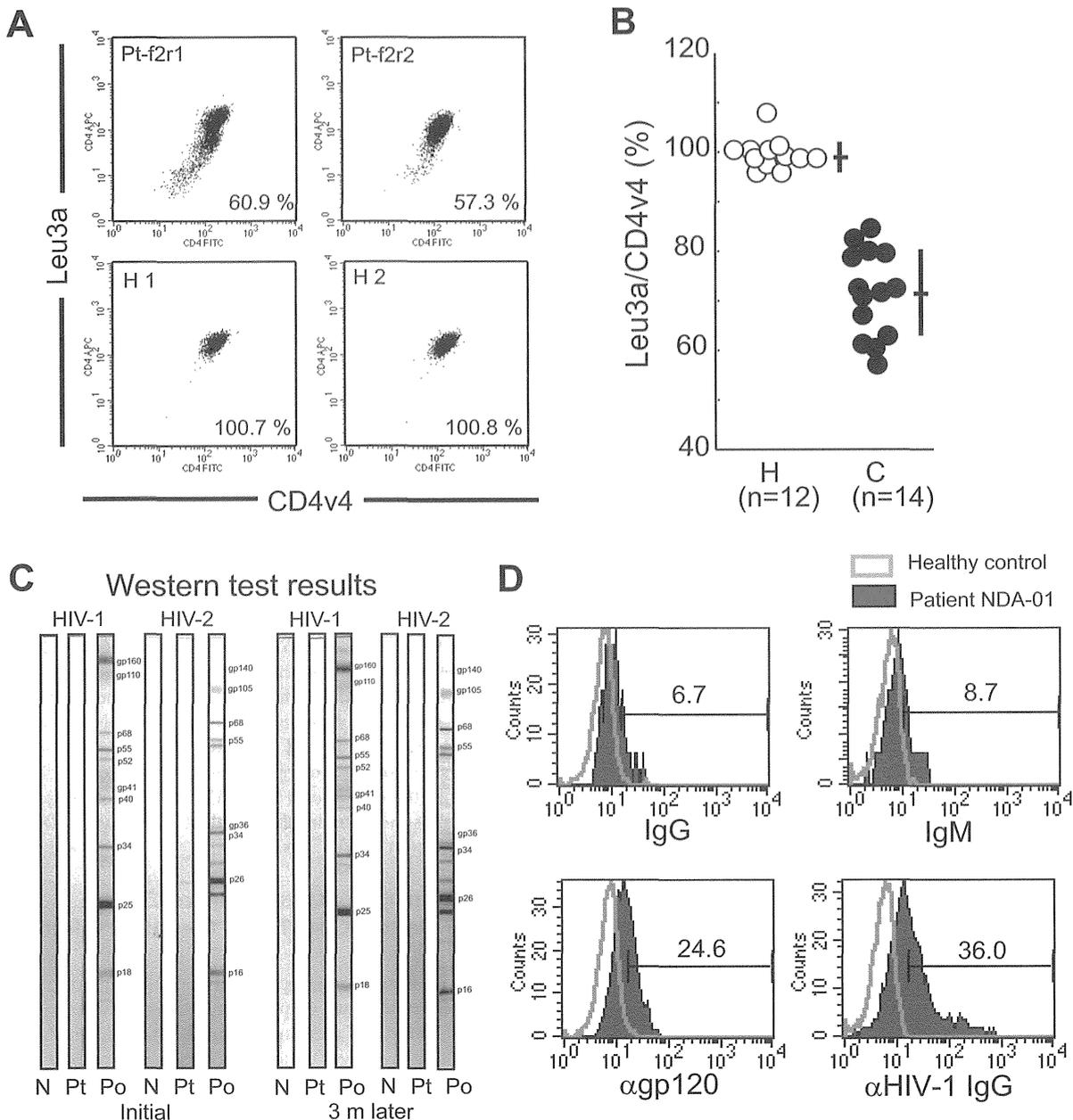
Next, we investigated whether colocalized sICs and CD4 were molecularly linked. To examine this possibility, rCD4s purified from HIV-1<sup>+</sup> Pts were exposed to phorbol myristate acetate (PMA) to induce CD4 internalization and determine whether sICs could co-mobilize with CD4. After 6 h of PMA stimulation, CD4 and most of the sICs had disappeared from the cell surface as determined by FACS (**Fig. 2J and K**). Confocal microscopy revealed that sICs colocalized with surface CD4 were rapidly co-internalized into the cells after 6 h of PMA stimulation (**Fig. 2L**). Collectively, sICs were molecularly linked to surface CD4 on rCD4s purified from HIV-1<sup>+</sup> Pts.

### The gp120-binding Domains of Surface CD4 are Occupied on Resting CD4<sup>+</sup> T Cells from HIV-1-infected Subjects

To further confirm whether gp120 was actually bound to CD4 on the patients' rCD4s, we employed two Abs, namely, gp120-blocking anti-CD4 mAb Leu3a and the mAb CD4-v4, which does not block the binding of gp120 to CD4. When we compared the MFIs of Leu3a with those of CD4-v4, the MFIs of Leu3a were always significantly lower than those of CD4-v4 in peripheral rCD4s from HIV-1<sup>+</sup> Pts but not from healthy controls. This finding suggests that the gp120-binding domains of surface CD4 molecules were occupied in rCD4s from HIV-1<sup>+</sup> Pts (**Fig. 3A and 3B**, percentages of Leu3a/CD4v4 from healthy donors and HIV-1<sup>+</sup> Pts are  $100.1 \pm 3.51\%$  and  $70.61 \pm 9.09\%$  ( $\pm$  SD), respectively;  $P < 0.0001$ ; see also in **Fig. S3 left** for the correlation between the concentration of gp120 exposed to qCD4s and the degree of blocking from Leu3a binding to CD4).

### Direct gp120 Detection on Resting CD4<sup>+</sup> T Cells from an HIV-1-seronegative Chronically HIV-1-infected Subject

We had the opportunity to directly examine cell-bound gp120 in rCD4s purified from a patient whose anti-env Ab levels were below the limit of detection of a conventional clinical western blotting test (**Fig. 3C**, see the results of the western blotting test at initial admission and 3 months later). We assumed that if gp120 were attached to rCD4s *in vivo*, the attached gp120 would not be or be only weakly coated with anti-env Abs in such a Pt. In other words, the epitopes for anti-env Abs would only be loosely



**Figure 3. rCD4s from HIV-1<sup>+</sup> Pts are coated with gp120.** (a) Representative FACS data from rCD4s purified from healthy controls (H1, H2) or chronic asymptomatic patients (Pt-f2r1, Pt-f2r2) stained with Leu3a and CD4v4 (numbers in FACS plots indicate percentages of MFIs of Leu3a/CD4v4). (b) Summary of results of percentages of MFIs of Leu3a/CD4v4 in purified rCD4s from healthy controls (H) and chronic asymptomatic patients (C; CD4 counts:  $420 \pm 84.6$  ( $\pm$  SD); IgG<sup>+</sup> rCD4s:  $75.5 \pm 12.6\%$  ( $\pm$  SD)). (c, d) Detection of cell-bound gp120 on rCD4s in a patient with low anti-gp120 Ab levels. (c) Western blot test results for the HIV-1<sup>+</sup> Pt (NDA-01) at initial admission and three months after. N, negative control; Pt, patient serum; Po, positive control. HIV-1 infection was defined as detectable amounts of plasma HIV-1 RNA ( $1.5 \times 10^5$  copies/ml at initial admission), a positive antibody test (HIV1/2 ELISA), and low CD4<sup>+</sup> T cell counts (38 cells/ $\mu$ l). Plasma HIV-1 env and gag region sequences (38 revealed that the patient was infected with a clade B HIV-1. (d) FACS data from rCD4s stained with anti-IgG (upper left), anti-IgM (upper right), anti-gp120 (rabbit anti-gp120 antiserum) (lower left), or purified IgG from pooled serum from HIV-1<sup>+</sup> Pts (lower right). doi:10.1371/journal.pone.0086479.g003

occupied. Therefore, if such rCD4s were directly stained with anti-env Abs or purified IgG *in vitro*, we could directly detect gp120 on the cell surface. As expected, the rCD4s were only weakly positive for sICs (Fig. 3D, upper panels). However, when stained with an anti-env Ab or a mixture of purified IgG from HIV-1<sup>+</sup> Pts, a significant portion of the rCD4s stained positive (Fig. 3D, lower panels). Therefore, attachment of gp120 to the surface of rCD4s was demonstrated directly in a patient whose anti-env Ab levels

were below the limit of detection. Collectively, these results clearly demonstrate that sICs on rCD4s in HIV<sup>+</sup> Pts link to cell-bound gp120.

#### sIC<sup>+</sup> Resting CD4<sup>+</sup> T Cells Activate Phagocytosis by Macrophages

We next investigated the pathological role of sICs on rCD4s. To this end, we examined whether sICs bound to rCD4s could trigger

Fc-mediated effector systems. We first examined whether sICs that formed *in vitro* on HIV-1/gp120-pre-exposed qCD4s could trigger ADCP by autologous macrophages (Fig. 4A–E). As expected, qCD4s exposed to medium, HIV-1<sup>+</sup> Pt serum, or HIV-1 or gp120 alone did not trigger phagocytosis by macrophages (Fig. 4A, left and middle panels, Fig. 4B, Fig. S5A, and Movie S1). In contrast, sICs that formed *in vitro* on HIV-1- or gp120-pre-exposed qCD4s triggered 84.5% and 43.0% of the macrophages to phagocytose more than one qCD4, respectively (Fig. 4A, right panels, Fig. 4B, Fig. S5B, Fig. S6, and Movie S2). The percentage of macrophages that phagocytosed qCD4s increased in proportion to the MFIs of sICs on qCD4s (Fig. 4C). In contrast, regardless of the usage of heat-inactivated (HI) serum or non-HI serum to form sICs on gp120-pre-exposed qCD4s, there was no difference in the levels of macrophage phagocytosis (Fig. 4B). Because heat inactivation eliminates the function of complement, phagocytosis of sIC<sup>+</sup> qCD4s should be predominantly induced through Fc-mediated pathways. Our time course study and live cell imaging of phagocytosis revealed that the attachment and engulfment of sIC<sup>+</sup> qCD4s by macrophages started immediately after coculture began, and phagocytosis of sIC<sup>+</sup> qCD4s finished within 1.5 to 3 h (Fig. 4D, E, and Fig. S5B). As shown using TUNEL staining, apoptosis of sIC<sup>+</sup> qCD4s became noticeable only after phagocytosis was completed (Fig. 4D). Therefore, the formation of sIC on gp120-exposed rCD4 was not sufficient for inducing cell death, and the induction of phagocytosis of sIC<sup>+</sup> qCD4s was not related to apoptotic changes in the plasma membrane. After 7 h of coculture, apoptosis had occurred in 92% of the ingested qCD4s, and the apoptotic cells were rapidly digested (Fig. 4E, note that the percentage of macrophages containing qCD4s decreased from 82% to 32%).

We next examined whether sICs formed *in vitro* on HIV-1/gp120-pre-exposed qCD4s could trigger ADCC by autologous NK cells. As reported previously [1,15–18], significant cell death was observed when sICs formed *in vitro* on HIV-1- or gp120-pre-exposed qCD4s were cocultured with NK cells in the presence of HIV-1<sup>+</sup> Pt serum (Fig. 4F and G). The number of apoptotic qCD4s increased in proportion to the MFIs of sICs on qCD4s (Fig. 4G). Moreover, as reported previously [42–44], the ADCC response mediated by NK cells was enhanced by IL-2 or IL-15 exposure (Fig. 4G).

We then investigated whether purified rCD4s from HIV-1<sup>+</sup> Pts could trigger Fc-mediated effector systems [45]. Allogeneic macrophages did not phagocytose rCD4s from healthy control subjects; however, a significant number of allogeneic macrophages phagocytosed rCD4s from more than one HIV-1<sup>+</sup> Pt (Fig. 5A and B). Therefore, sIC<sup>+</sup> rCD4s were sufficient to trigger an ADCP reaction to autologous macrophages.

Finally, we sought to determine whether monocytes from HIV-1<sup>+</sup> Pts maintained the capacity to induce Fc-mediated phagocytosis. The phagocytic activities of freshly isolated macrophages from chronic HIV-1<sup>+</sup> and healthy control subjects were measured directly using Fc-OxyBurst assays [46]. As shown in Fig. 5C and D, the phagocytic activities of freshly isolated macrophages from chronic, asymptomatic HIV-1<sup>+</sup> Pts were significantly higher ( $151.25 \pm 56.19$  vs.  $99.25 \pm 14.2$  ( $\pm$  SD),  $p < 0.001$ ) than those from the controls. These results collectively suggest that sIC<sup>+</sup> rCD4s *in vivo* may be destroyed and removed by macrophages or NK cells through ADCP or ADCC, respectively.

### Frequencies and Numbers of sIC<sup>+</sup> Resting CD4<sup>+</sup> T Cells in Blood Increase after Spleen Removal

Finally, we performed a longitudinal analysis of sIC<sup>+</sup> rCD4 levels in peripheral blood from an HIV-1-infected hemophilic

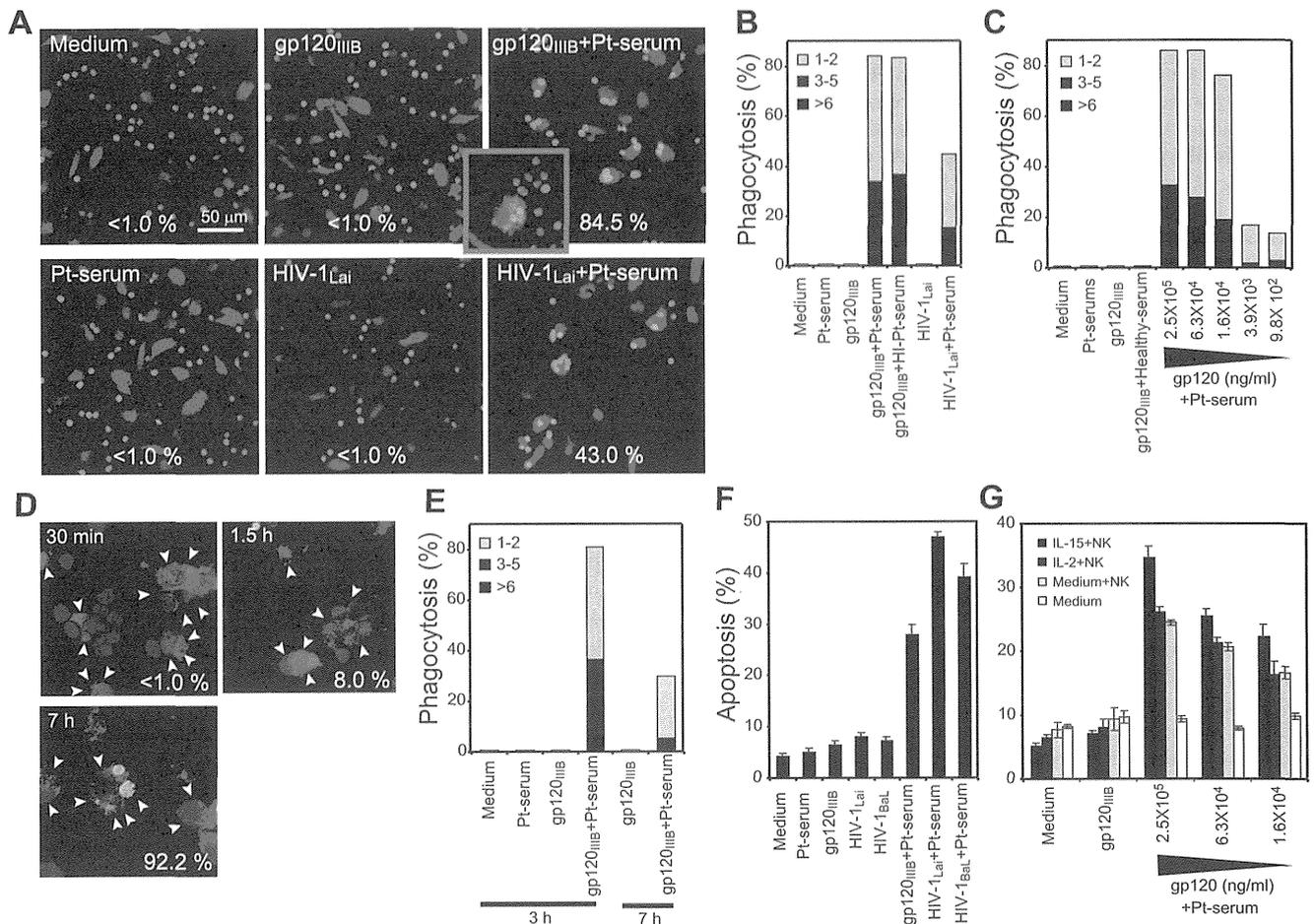
individual who underwent a splenectomy during the course of ART. When ART was discontinued due to side effects, the percentages and numbers of both IgG<sup>+</sup> and IgM<sup>+</sup> rCD4s rapidly increased in peripheral blood (Fig. 6) as shown in the previous section. Thereafter, ART with a different regime was initiated. Approximately 300 days after treatment, the plasma VL became undetectable (<50 RNA copies/ml), and the percentage and number of both IgG<sup>+</sup> and IgM<sup>+</sup> rCD4s gradually declined. However, the patient required a splenectomy for the treatment of a severe, uncontrolled epidural hemorrhage caused by immune thrombocytopenic purpura. Immediately after removal of the spleen, the percentages of IgG<sup>+</sup> and IgM<sup>+</sup> rCD4s increased to 11% and 22%, respectively, and the actual numbers of IgG<sup>+</sup> and IgM<sup>+</sup> rCD4s were markedly elevated from 15/ $\mu$ l and 5/ $\mu$ l to 82/ $\mu$ l and 55/ $\mu$ l, respectively, whereas VL remained undetectable. These results strongly suggest that substantial numbers of sIC<sup>+</sup> rCD4s are trapped or eliminated from circulation in the spleen.

## Discussion

The presence of Ig<sup>+</sup> CD4<sup>+</sup> T cells in the blood of HIV-1<sup>+</sup> Pts has been reported [26–28]; however, these studies examined the percentages of Ig<sup>+</sup> CD4<sup>+</sup> T cells utilizing FACS or related techniques alone. In this study, we first sought to determine whether peripheral blood rCD4s in HIV-1<sup>+</sup> Pts are truly coated with IgG and/or IgM. We utilized biotinylated anti-IgG and/or anti-IgM F(ab')<sub>2</sub> Abs to prevent the non-specific surface binding of Abs through the Fc portion. Furthermore, we simultaneously measured Ig expression levels in rCD4s purified from HIV-1<sup>+</sup> Pts or healthy individuals by FACS and immunoblotting. We confirmed that the levels of surface Ig on rCD4s detected by MFIs of anti-IgG by FACS approximately paralleled the levels of IgG detected by immunoblotting (Fig. 2C). Thus, we confirmed that peripheral rCD4s from HIV-1<sup>+</sup> Pts are truly coated with Igs. In addition, utilizing confocal microscopy, we found that Igs colocalized with surface CD4 on rCD4s from HIV-1<sup>+</sup> Pts (Fig. 2D) and co-mobilized with CD4 when inducing CD4 internalization by PMA exposure (Fig. 2J–L). Collectively, we demonstrated that Igs are attached to surface CD4 on peripheral rCD4s from HIV-1<sup>+</sup> Pts.

A cohort study using peripheral blood samples showed that the percentages of Ig<sup>+</sup> rCD4s from HIV-1<sup>+</sup> Pts positively correlated with plasma VLs, suggesting that ICs were formed via HIV-1-related molecules on the cell surface (Fig. 2A and I). HIV-1 virions circulate in HIV-1<sup>+</sup> Pt serum as cICs [47]. Some reports have suggested that peripheral Ig<sup>+</sup> rCD4s may be linked to nonspecific attachment of cICs to the cell. Furthermore, the production of auto-Abs against peripheral rCD4s in HIV-1<sup>+</sup> Pts has also been reported [27]. When B cells, which express both CRs and Fc $\gamma$  receptors, were exposed to patient serum, sICs formed in an quantity that was relatively proportional to VL, whereas when qCD4s, which do not express CRs or Fc $\gamma$  receptors, were exposed to patient serum, no sICs formed on the cells (Fig. S4). Additionally, we excluded the possibility that HIV-1<sup>+</sup> Pt serum contains auto-Abs (Fig. S4E left column). Importantly, once Ig is lost from the surface of HIV-1<sup>+</sup> Pt rCD4s after sustainable cell culture, no sICs formed on the surface even when exposed to patient serum (Fig. 2H), suggesting that rCD4s from HIV-1<sup>+</sup> Pts do not express molecules that bind to cICs or self-antigens that react with certain Abs in Pt serum. Collectively, we have excluded the possibility that sICs are formed due to HIV-1<sup>+</sup> Pt serum containing sufficient levels of auto-Abs or cICs.

Next, to clarify whether gp120 binds to CD4 molecules on rCD4s *in vivo* in HIV-1<sup>+</sup> Pts, we showed that the gp120-binding



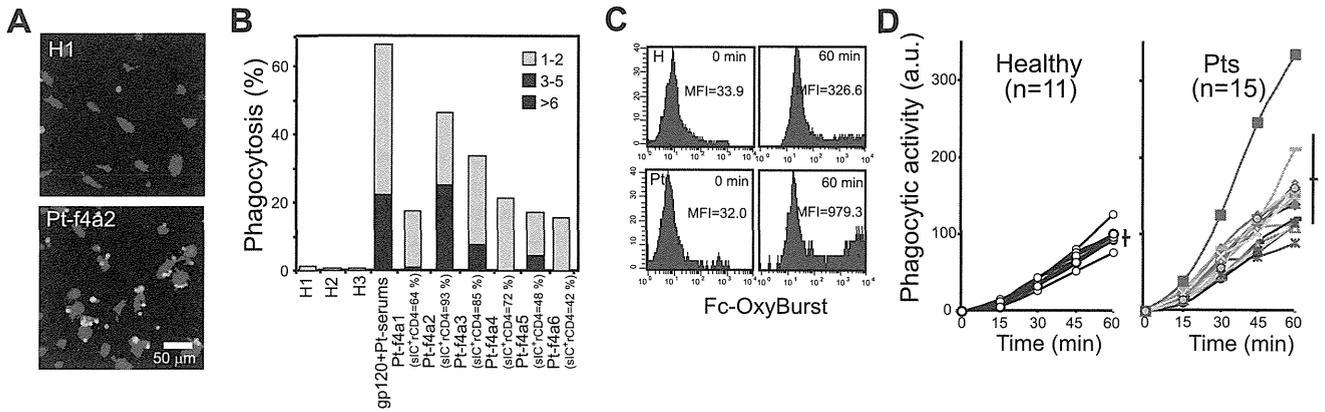
**Figure 4. sICs on qCD4s trigger Fc-mediated effector systems.** (a–e) Autologous macrophages phagocytose qCD4s with sICs. Orange-CMTMR-labeled macrophages (red) cocultured with CFSE-labeled autologous qCD4s (green) exposed to the indicated concentrations of gp120<sub>IIIb</sub>, HIV-1<sub>Lai</sub>, or medium followed by incubation with HI patient serum (Pt-serum), non-HI patient serum, or medium for 1 h before coculture. (a–c) Confocal micrographs of representative data (a) and summary of phagocytosis assays (b, c) shown as percentages of macrophages containing 1–2, 3–5, or >6 qCD4s. The numbers in (a) denote percentages of macrophages containing at least one qCD4; inset shows a macrophage containing >10 qCD4s. (d) Time course of TUNEL assay on sIC<sup>+</sup> qCD4s that were phagocytosed by macrophages. Confocal images of macrophages (arrowheads, red), TUNEL<sup>+</sup> (green) and cell nuclei (Topro-3, blue). The numbers indicate the percentage of TUNEL<sup>+</sup> phagocytosed qCD4s/total phagocytosed qCD4s. (e) Summary of the time course of phagocytosis assays. (f) Summary of apoptotic qCD4s in the NK cell-mediated ADCC assay. CFSE-labeled NK cells incubated with autologous qCD4s (2:1), which were exposed to the indicated concentrations of gp120<sub>IIIb</sub>, gp120<sub>Bal</sub>, HIV-1<sub>Lai</sub>, HIV-1<sub>Bal</sub>, or medium. (g) Summary of effects of IL-2 (50 ng/ml) or IL-15 (20 ng/ml) treatment on NK cell-mediated ADCC. Bars, SD. The data presented here are representative of at least three independent experiments. doi:10.1371/journal.pone.0086479.g004

domain of CD4 was occupied in rCD4s from HIV-1<sup>+</sup> Pts (Fig. 3A and B), thereby indirectly demonstrating that gp120 is attached to CD4 on rCD4s *in vivo*. Furthermore, we showed that anti-env Abs directly bound to rCD4s from an HIV-1-infected individual whose anti-gp120 Ab levels were below the sensitivity of a conventional western blotting test (Fig. 3C and D). Collectively, we conclude that sICs on rCD4s in HIV-1<sup>+</sup> Pts result from CD4-bound gp120. However, we can hypothesize that cell-bound gp120 could reflect the production of HIV-1 in rCD4s. In this respect, it is well established that direct infection of rCD4s does not lead to productive infection [32,48], instead, resulting in a labile state known as preintegration latency. Therefore, gp120 attached to CD4 is not linked to HIV-1 production by rCD4s.

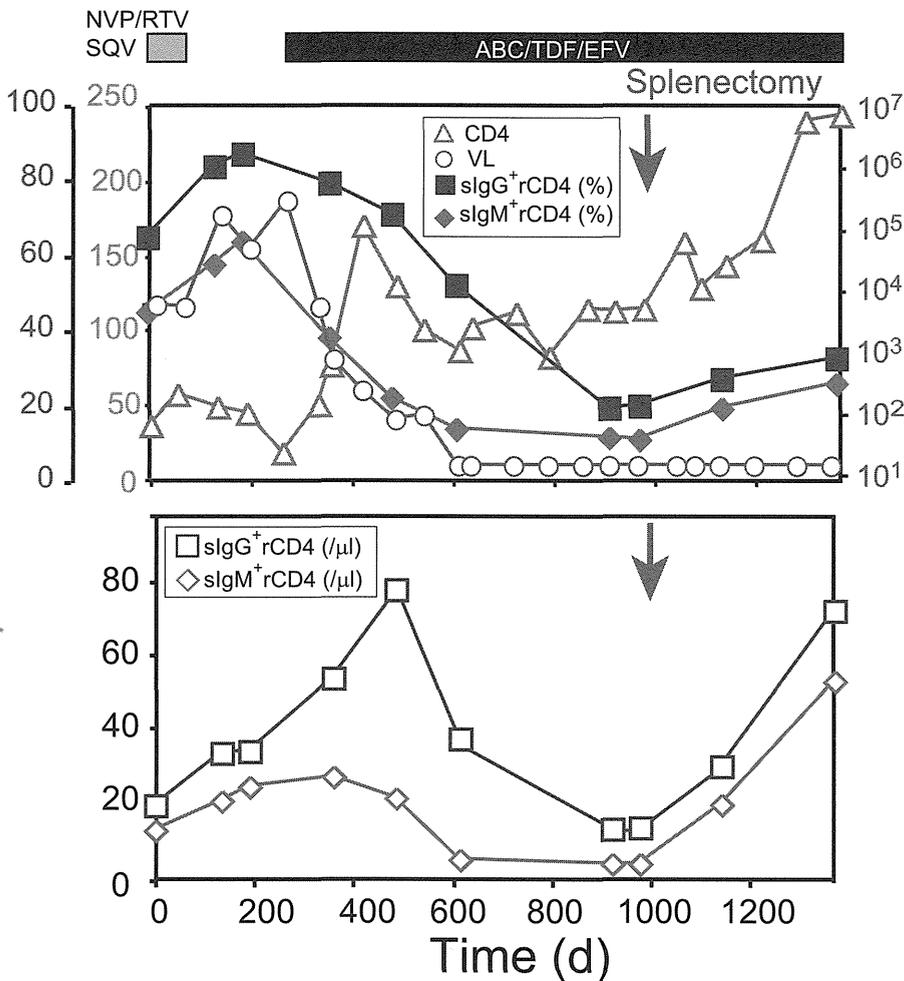
In contrast, *in vitro* culture of purified rCD4s from HIV-1<sup>+</sup> Pts revealed that a 50% reduction in sICs on rCD4s required approximately 20 h (Fig. 2E and F) due to the slow turnover of VRs on rCD4s (Fig. 1, Fig. S1 and S2). Therefore, the half-life of sICs on rCD4s is much longer than the duration on CD4<sup>+</sup> T

cells recirculating between LNs and the peripheral blood [7]. Before sICs disappear from the surface, rCD4s may be continuously exposed to gp120 and/or HIV-1 at high concentrations in the lymphoid organs [5]. Thus, the levels and percentages of sIC<sup>+</sup> rCD4s may become equilibrated to the levels of virus production and/or anti-HIV-1 Abs in the lymphoid organs.

To clarify the pathological effects of sICs on rCD4s, we demonstrated here that sIC<sup>+</sup> rCD4s produced *in vitro* or isolated from HIV-1<sup>+</sup> Pts ultimately induced ADCP and ADCC by autologous macrophages (Fig. 4A–E, Fig. 5A and B, Fig. S5B, Fig. S6, and Movie S2) and NK cells (Fig. 4F and G), respectively. Furthermore, the phagocytic activities of monocytes as measured directly using Fc-OxyBurst assays on freshly isolated monocytes from HIV-1<sup>+</sup> Pts were even stronger in the healthy donors (Fig. 5C and D). Therefore, these results suggest that sIC<sup>+</sup> rCD4s in peripheral blood may be destroyed and removed from circulation at a constant rate.



**Figure 5. Patients' sIC<sup>+</sup> rCD4s trigger Fc-mediated effector systems.** (a, b) Representative confocal micrographs (a) and a summary of phagocytosis assays (b) of purified rCD4s from healthy donors (H) or patients (Pt) with allogeneic macrophages from a healthy donor. Arrowheads indicate the macrophages containing qCD4s. (c, d) Representative FACS data (c) and summary of Fc-OxyBurst assays (d). Macrophages from healthy donors (Healthy) or chronic asymptomatic donors (Pts) were incubated with Fc-OxyBurst immune complexes (Molecular Probes) (10 mg/ml). The relative quantities of superoxide generated by macrophages were measured every 15 min using FACS. Bars, SD. doi:10.1371/journal.pone.0086479.g005



**Figure 6. Frequencies and numbers of sIC<sup>+</sup> rCD4s in the blood increase after spleen removal.** Percentages of sIgG<sup>+</sup> and sIgM<sup>+</sup> rCD4s in the blood, plasma VL, and CD4 lymphocyte counts (upper), or absolute numbers of sIgG<sup>+</sup> and sIgM<sup>+</sup> rCD4s (lower) in a hemophiliac HIV-1<sup>+</sup> Pt who had undergone splenectomy. Patient interrupted therapy with nevirapine, ritonavir, and saquinavir due to side effects and then initiated therapy with abacavir, tenofovir, and efavirenz. The arrow indicates the day on which the splenectomy was performed. doi:10.1371/journal.pone.0086479.g006

We found that the percentages of sIC<sup>+</sup> rCD4s in HIV-1<sup>+</sup> Pts were inversely correlated with the number of CD4<sup>+</sup> T cells in the blood (**Fig. 2I**). Furthermore, we found that in an HIV-1-infected individual whose VL became undetectable with ART, the percentage and number of sIC<sup>+</sup> rCD4s in blood gradually decreased but promptly increased after splenectomy (**Fig. 6**). Therefore, we can hypothesize that sIC<sup>+</sup> rCD4s may be destroyed and removed from circulation by macrophages or NK cells in the spleen or other lymphoid organs. Indeed, splenomegaly is a common symptom of both acute and chronic HIV-1 infection [49]. Furthermore, we found that approximately 100% of patients' IgM<sup>+</sup> rCD4s were also coated with iC3b complement fragments, known as C3 opsonization (**data not shown**), suggesting that cell-bound IgM is capable of fixing complement and that IgM<sup>+</sup> iC3b<sup>+</sup> rCD4s may induce stronger ADCC activity by macrophages than IgG<sup>+</sup> rCD4s.

CD4 molecules on CD4<sup>+</sup> T cells play an important role in forming the immunological synapse between CD4<sup>+</sup> T cells and antigen-presenting cells [50]. However, the attachment of ICs to CD4 molecules could interfere with normal immunological synapse formation between CD4<sup>+</sup> T cells and antigen-presenting cells and suppress the full activation of CD4<sup>+</sup> T cells. Therefore, our findings here can also be extended to explain the reduced immune function of CD4s in HIV-1<sup>+</sup> Pts. However, future studies are needed to confirm this possibility.

In **Fig. 7**, we summarized our hypothesis of the mechanisms of sIC formation on rCD4s and their effects on the dynamics of rCD4 circulation. In our model, the length of time that sICs remain on rCD4s was extremely long compared with CD4s that are circulating between the LNs; as a result, rCD4s continue to be exposed to high concentrations of HIV-1 in the lymphoid organs. Therefore, the percentages and levels of sICs on rCD4s equilibrate to HIV-1 production in the lymphoid organs. However, sIC<sup>+</sup> rCD4s are also subject to immunological pressure from both macrophages and NK cells. Therefore, the percentages and levels of sICs on rCD4s were also at equilibrium with the degree of immunological pressure. Collectively, the percentages and levels of sICs on rCD4s in blood appear to reflect a complex interplay between the levels of virus production in lymphoid tissues, the levels of anti-env Abs, the rate of sIC removal from the cell surface, the duration of repeated exposures to HIV-1/gp120 or ICs, and the degree of immunological elimination and trapping of sIC<sup>+</sup> rCD4s from peripheral circulation. Because ART may not dramatically influence sIC turnover rates on rCD4s, the levels of anti-env Abs, and the duration of CD4<sup>+</sup> T cell circulation among lymphoid tissues, changes in the percentage of sIC<sup>+</sup> rCD4s in the blood after initiation of ART may reflect the level of virus production in lymphoid tissues and the degree of immune pressure on sIC<sup>+</sup> rCD4s. Importantly, our hypothesis here is highly consistent with the previously proposed mathematical model [9] that suggests that the effects of HIV-1 (e.g., induction of LN accumulation and cell death after entering the LNs) on resting T lymphocytes can explain the depletion of CD4<sup>+</sup> T cells from the peripheral blood during HIV-1 infection.

When we analyzed the percentages of sIC<sup>+</sup> rCD4s in blood, sIC<sup>+</sup> rCD4s were detectable in peripheral blood after approximately 2 yrs of complete suppression of plasma VL (**Fig. 2A and I**). Effective ART has been shown to rapidly reduce the levels of both plasma VL and HIV-1-producing cells to undetectable levels; however, HIV-1 replication continues in LNs in patients with undetectable plasma VLs after ART [48,51,52]. If we assume that ART treatment does not significantly change the degree of immunological pressure on sIC<sup>+</sup> rCD4s in our model, the number of sIC<sup>+</sup> rCD4s should mainly reflect residual viral production in

LNs. Therefore, monitoring the percentage of sIC<sup>+</sup> rCD4s in peripheral blood may be a promising tool to examine residual virus replication in patients with undetectable plasma virus levels under ART.

More importantly, sIC<sup>+</sup> rCD4s in blood were only found in HIV-1<sup>+</sup> Pts; we did not find sIC<sup>+</sup> rCD4s in healthy donors or any patients with other viral and bacterial infections and autoimmune diseases (**data not shown**). Therefore, detection of sIC<sup>+</sup> rCD4s in the blood in itself can be used as a marker to confirm the diagnosis of HIV-1 infection. Furthermore, our results suggest that monitoring the Ig subclasses of sICs or the percentage of sIC<sup>+</sup> rCD4s may also be useful for determining the stage and progression of HIV-1 infection (**Fig. 2A**) because during acute infection, sICs largely consisted of IgM, and the percentage of sIC<sup>+</sup> rCD4s gradually increased during the follow-up period (approximately 3 yrs) (**Fig. 2A**). Therefore, it is logical to use Ig<sup>+</sup> rCD4s levels in blood as an independent clinical marker for easily confirming a diagnosis of HIV-1 infection, for identifying clinical stages, and for evaluating residual virus production under ART. Additionally, because sICs were mainly formed by anti-env Abs with non-neutralizing activity, the presence of sIC<sup>+</sup> rCD4s may demonstrate that non-neutralizing anti-env Abs play a detrimental role in uninfected rCD4s. Therefore, preventing the induction of non-neutralizing anti-env Abs by vaccination may facilitate efficient immune responses against HIV-1 infection.

The results of the experiments we describe here were obtained using a limited number of clinical samples. Therefore, it is essential to perform detailed studies in the future utilizing a larger number of samples to answer the following questions: 1, Do sICs on rCD4s influence the dynamics of rCD4s *in vivo*? 2, Are sICs on rCD4s destroyed by ADCC and APCP *in vivo*? 3, As a result of affecting immunological synapse formation, do sICs on rCD4s induce anergy or suppress the full activation of rCD4s? 4, Can sICs on rCD4s be used for confirming the diagnosis of HIV-1 infection, for identifying the clinical stage, and for evaluating residual virus production under ART?

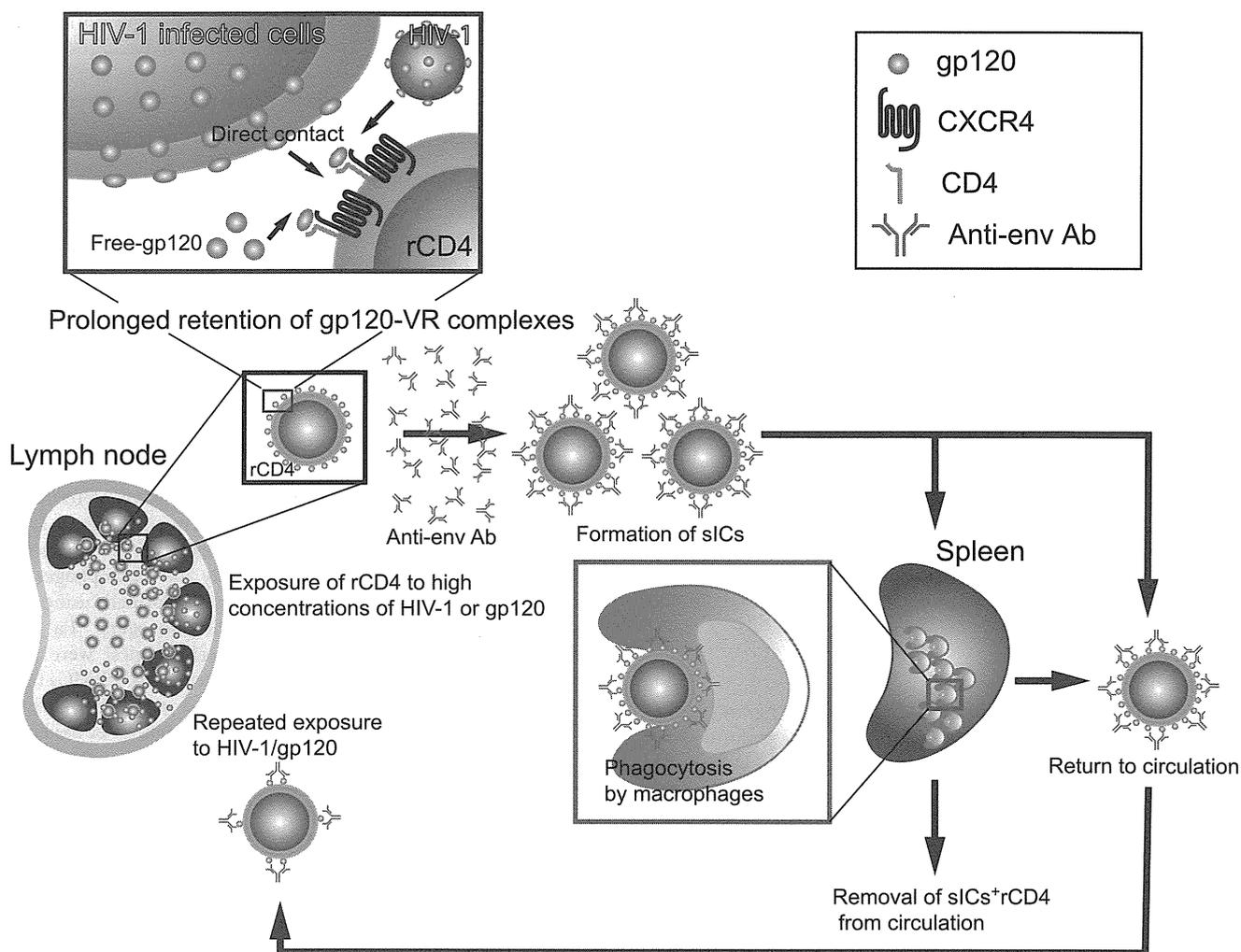
## Materials and Methods

### Reagents

Chemicals, Abs, and recombinant cytokines were purchased from Sigma (St. Louis, MO), BD (San Diego, CA), and R&D Systems (Minneapolis, MN), respectively, unless otherwise specified. Purified gp120<sub>IIIIB</sub> and recombinant gp120<sub>BaL</sub> were obtained from Advanced Biotechnologies, Inc. (ABI, Columbia, MD) and the NIH AIDS Research and Reference Program, respectively. Aldritiol-2 (AT-2)-inactivated HIV-1<sub>Lai</sub>, HIV-1<sub>BaL</sub>, and clinical isolates were prepared as previously described [53,54].

### Subjects and Research Ethics

PBMCs were collected from 49 ART-naive, HIV-1-infected individuals, 46 HIV-1-infected individuals undergoing ART and 54 HIV-1-seronegative individuals to study VR and surface Ig dynamics and to perform phagocytosis assays. The HIV-1<sup>+</sup> individuals were classified as having acute or chronic asymptomatic infections. Acute infection was defined as having less than 14 d of symptoms of acute HIV-1 infection with the presence of HIV-1 RNA in the plasma and seroconversion by HIV-1/2-reactive western blots during follow-up. Chronic asymptomatic HIV-1 infection was defined as being seropositive for >1 yr with CD4 counts >250/μl without any symptoms of opportunistic infections. One HIV-1-seronegative healthy individual served as the negative control. Here, aviremic individuals were those with a plasma VL of <50 HIV-1 RNA copies/ml. This study was approved (IMCJ-



**Figure 7. Schematic figure summarizes the causes and consequences of sIC<sup>+</sup> rCD4s.** rCD4s continuously travel between the blood stream and LNs over a period of approximately 1 d. Because a large proportion of HIV-1 is produced in the LNs, the target T cells that migrate to the LNs are exposed to high concentrations of HIV-1, gp120, or ICs as well as anti-env Abs. Prolonged retention of gp120-VR complexes on rCD4s causes the retention of sICs in a manner that reflects the levels of HIV-1 exposure in the LNs. sIC<sup>+</sup> rCD4s are removed from circulation through ADCP or ADCC by macrophages or NK cells, respectively. The sIC<sup>+</sup> rCD4s that are not removed from circulation remigrate to the LNs to be exposed to a high concentration of gp120/HIV-1. The percentages and amounts of sICs on rCD4s in the blood reflect a balance of five factors, namely, the levels of virus production in lymphoid tissues, the levels of anti-env Abs, the turnover dynamics of sICs on rCD4s, the duration of repeated exposure by continuous migration to the lymphoid organs, and the levels of immunological elimination of sIC<sup>+</sup> rCD4s.  
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H14-60) by the National Center for Global Health and Medicine Ethical Committee, and written informed consent was obtained from every subject.

#### Purification and Isolation of CD4<sup>+</sup> T, B, and NK Cells

The highly purified drCD4s from healthy donors, which were used as representative qCD4 T cells *in vivo*, were purified by negative selection with magnetic beads followed by T cell density gradient separation on a discontinuous Percoll gradient (Pharmacia Biotech, Uppsala, Sweden) as described previously [34]. The rCD4s were purified using the CD4<sup>+</sup> T Cell Isolation Kit II (Miltenyi Biotech, Auburn, CA) followed by magnetic depletion with anti-CD25 and anti-CD69 Abs. CD20<sup>+</sup> IgG<sup>dull</sup> IgM<sup>dull</sup> B cells were isolated using the B Cell Isolation Kit II (Miltenyi Biotech) followed by magnetic depletion with anti-IgG and anti-IgM Abs. NK cells and macrophages were isolated using the NK Cell

Isolation Kit II and the Macrophage Isolation Kit II (Miltenyi Biotech), respectively.

#### Preparation of Activated qCD4s

Purified qCD4s were stimulated for 72 h with plate-bound anti-CD3 Abs (UCTH1:40 mg/ml) and anti-CD28 Abs (Lew-28:20 mg/ml) in RPMI 1640 containing fetal calf serum (FCS) at 37°C in humidified air containing 5% CO<sub>2</sub>.

#### Kinetic Studies and Flow Cytometry

Kinetic studies of CXCR4 and CD4 expression were performed as previously described [30]. Briefly, A3.01 cells, qCD4s, and activated qCD4s were cultured in flat-bottom 96-well microtiter plates (Nalge Nunc, Penfield, NY) (in triplicate) with or without ActD (20 μg/ml), BFA (10 μg/ml), and/or cycloheximide (50 μg/ml). The concentrations of inhibitors used were as previously described [30]. The percent change in surface receptor expression

was calculated from the MFIs (**except for Fig. 1A and B**) or percentages of cells in predetermined gates (**Fig. 1A and B**).

For kinetic studies, qCD4s were incubated on ice with gp120<sub>IIIB</sub> (250 ng/ml), gp120<sub>BaL</sub> (250 ng/ml), or AT-2-inactivated HIV-1<sub>Lai</sub>, HIV-1<sub>BaL</sub>, or an HIV-1 clinical isolate (R5 strain as determined by biological assays) for 30 min in binding buffer (PBS with 10% FCS) and then washed with binding buffer. Aliquots of qCD4s exposed to either gp120 or HIV-1 were cultured with 10% FCS containing medium alone or medium containing 10% HI patient serum (pooled from five HIV-1-seropositive subjects). Cells that were cultured in medium without 10% FCS were stained with rabbit anti-gp120 antiserum (ABI) followed by anti-rabbit IgG-FITC (DAKO, Hamburg, Germany) and fixed with 1.0% paraformaldehyde. Cells that were cultured in patient serum-containing medium were stained with the same serum followed by anti-human IgG-FITC. The effects of T22 or Enf were studied by incubating cells with the drugs in binding buffer for 30 min on ice followed by exposure to gp120 or HIV-1 in binding buffer containing the corresponding drug. The exposed cells were washed thoroughly with binding buffer containing the corresponding drug, subsequently cultured in the absence (for T22 experiments) or presence (for Enf experiments) of drug for the indicated times. The percent change in cell-bound gp120 was calculated from MFIs.

### Confocal Microscopy

Purified qCD4s were incubated with gp120 (250 ng/ml) or AT-2-inactivated HIV-1. Immunofluorescence was performed by serial staining with goat anti-CD4 polyclonal Abs (R&D Systems), Cy3-conjugated secondary Abs (Sigma), rabbit anti-gp120 antiserum (ABI), anti-rabbit IgG-FITC (Dako), biotinylated anti-CXCR4 monoclonal Abs (R&D Systems), and streptavidin-Qdot 605 (Life Technologies, Carlsbad, CA), in that order. The cells were then fixed with 4% paraformaldehyde. sICs were visualized by staining purified rCD4s with goat anti-CD4 polyclonal Abs, Cy3-conjugated secondary Abs, biotinylated F(ab')<sup>2</sup> anti-human Igs (Life Technologies), and streptavidin-Qdot 525, in that order. Multicolor confocal and DIC images with a 512×512 resolution were acquired using a Zeiss LSM510 system with a Plan-Apochromatic 63×1.4 NA oil immersion DIC objective (Carl Zeiss, Oberkochen, Germany) using multi-track scanning.

### sIC Analysis

For phenotypic analysis, PBMCs purified using Ficoll-Paque were stained with anti-CD3-PerCP, anti-CD4-APC, anti-CD25-PE, anti-CD69-PE, and biotin-F(ab')<sup>2</sup> anti-human IgG (BioSource) or biotin-F(ab')<sup>2</sup> anti-human IgM (Life Technologies), in that order. After washing, the cells were stained with streptavidin-FITC. PBMCs from an HIV-1-seronegative donor were simultaneously stained as a negative control. For longitudinal analyses of sICs, purified PBMCs were cryopreserved at -80°C, and each sample set from the patient was labeled simultaneously.

### Western Blotting, IgG Purification, and HIV-1 RNA in Situ Hybridization

Whole-cell lysates derived from gp120 (250 ng/ml)- or HIV-1-pulsed qCD4s or rCD4s purified from HIV-1-seropositive or healthy individuals were subjected to SDS-PAGE and were transferred to polyvinylidene difluoride membranes and blotted with antibodies against gp120 (Life Technologies), p24 (Life Technologies), or human-IgG (Dako) after blocking with TBST/5% milk. Proteins were visualized using the SuperSignal West Pico Chemiluminescent Kit (Thermo Fisher Scientific, Waltham, MA)

and Biomax-MR film (Kodak, Rochester, NY). IgG purification and flow cytometry-based HIV-1 RNA *in situ* hybridization were performed using the Melon-Gel IgG Spin Purification kit (Thermo Fisher Scientific) and ViroTect (Invirion, Oak Brook IL), respectively.

### Macrophage Ab-dependent Cellular Phagocytosis (ADCP), TUNEL, Fc-OxyBurst, and Chemotaxis Assays

Macrophages were cultured in X-VIVO 10 (Lonza, Zurich, Switzerland) containing 10% heat-inactivated human serum AB (Lonza). Macrophages were collected on day 5 of culture and labeled with Orange-CMTMR (5 nM) (Life Technologies). Target qCD4s were coated with gp120 (250 ng/ml unless otherwise specified) or AT-2-inactivated HIV-1 at 4°C for 1 h, washed thoroughly, and exposed to HI- or non-HI-patient serum for 1 h at 37°C. After labeling with CFSE, qCD4s were incubated with Orange-CMTMR-labeled macrophages (5:1) in X-VIVO 10 in glass bottom dishes (*Matsunami Glass*, Osaka, Japan). After fixation, the number of macrophages containing qCD4s was determined from three-dimensional reconstructions generated using an LSM 510 system. The percentage of macrophages that phagocytosed rCD4s was determined using approximately 500 macrophages per experiment. The TUNEL reaction was performed using the FragEL-DNA fragmentation detection kit (Oncogene, La Jolla State, CA). Fc-OxyBurst assays (Life Technologies) were performed according to the manufacturer's instructions. PBMCs from one HIV-1-seronegative individual were used as the standard for calculating the percent change and relative quantities of oxidative species generated by macrophages as follows: relative O<sup>•</sup> production =  $(F_{\text{sample}} - F_{\text{min-sample}}) / (F_{\text{ref}} - F_{\text{min-ref}})$  where  $F_{\text{min-sample}}$  and  $F_{\text{min-ref}}$  are background MFIs in the patient sample and control, respectively. qCD4 chemotaxis activity was determined using 5- $\mu$ m microchemotaxis plates (NeuroProbe, Gaithersburg, MD) as described [34].

### Macrophage ADCP Time-lapse Microscopy

For time-lapse microscopy, two-color confocal and DIC images were collected every 30 s with a Zeiss LSM 510 system with a Plan-Neofluar 40×1.3 NA oil immersion DIC objective (Carl Zeiss).

### NK Cell ADCC Assays

Purified qCD4s were coated with gp120 (250 ng/ml unless otherwise specified) or AT-2-treated HIV-1. Highly purified CFSE-labeled (3 nM) NK cells were incubated for 48 h with gp120/HIV-1-coated qCD4s in 10% serum from HIV-1-seropositive subjects, and cytotoxicity was determined by PI labeling (100  $\mu$ g/ml). Cytokine effects were studied by culturing purified NK cells for 42 h with or without IL-2 (50 ng/ml) or IL-15 (10 ng/ml) in complete medium with 10% FCS. After the cells were labeled with CFSE, ADCC assays with gp120-coated qCD4s were performed.

### Supporting Information

**Figure S1 Rapid turnover of CXCR4 on A3.01 T lymphoma cells and activated qCD4s.** (a) The schematic summarizes the inhibitory activities of the indicated compounds. (b) Effect of inhibitors on CXCR4 expression on A3.01 and activated qCD4s. (c) After T22 exposure, anti-CXCR4 mAb (12G5) binding to CXCR4 was assessed by FACS. Percent recovery was calculated using MFIs. Bars indicate SD. Data are representative of three independent experiments. (EPS)

**Figure S2 Turnover of cell-bound gp120 or HIV-1 on qCD4s or anti-CD3 Ab-exposed qCD4s.** (a) Representative FACS data. (b) The quantity of cell-bound p24 on HIV-1<sub>Lai</sub>-pulsed qCD4s was assessed by immunoblotting with anti-p24 Abs. qCD4s were pulsed with HIV-1<sub>Lai</sub> in the presence or absence of Enf. Numbers indicate cell-bound p24 relative to cell-bound p24 at 0 h. Data are representative of three independent experiments. (EPS)

**Figure S3 HIV-1 patient serum contains sufficient levels of anti-gp120 Abs to form sICs on qCD4s.** Summary of the percentages of Leu3a/CD4v4 (left) and the MFIs of sICs on qCD4s exposed to the indicated concentrations of the gp120 (right). gp120<sub>IIIb</sub> was incubated at various concentrations with qCD4s, which were then stained with Leu3a and CD4v4 or serum from HIV-1<sup>+</sup> patients. (EPS)

**Figure S4 sICs in the serum of viremic HIV-1<sup>+</sup> Pts are sufficient to form sICs on B cells but not on resting CD4<sup>+</sup> T cells.** (a, b) Summary of the percentages (a) and representative FACS data (b) of IgM<sup>+</sup> or IgG<sup>+</sup> sICs or IgM<sup>+</sup> sIC formation on purified CD20<sup>+</sup> IgG<sup>dull</sup> IgM<sup>dull</sup> B cells after exposure to serum from a healthy control donor or HIV-1<sup>+</sup> Pts with various VLs. (c, d) Summary of the percentages (d) and representative FACS data (c) of fluorescence-based HIV-1 RNA *in situ* hybridization in B cells exposed to serum from a healthy control donor or HIV-1<sup>+</sup> Pts with various VLs. Plasma VLs are indicated next to the HIV-1<sup>+</sup> Pt numbers. (e) Summary of the percentages of sIg<sup>+</sup> rCD4s in gp120-pulsed or non-pulsed qCD4s that were exposed to serum (**gp120+serum or Serum**) or the percentages of sIg<sup>+</sup> rCD4s in non-pulsed qCD4s that were exposed to purified IgG (100 mg/ml) (**IgG**) from a healthy control or HIV-1<sup>+</sup> Pts with various VLs. (EPS)

**Figure S5 Time-lapse microscopy of phagocytosis of gp120-coated qCD4s and sIC<sup>+</sup> qCD4s by macrophages.** (a, b) Representative time-lapse image sequence of phagocytosis of gp120-coated qCD4s (a) and sIC<sup>+</sup> qCD4s (b) by macrophages.

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The color overlay images show macrophages (Orange-CMTMR, red) and qCD4s (CFSE, green). Schematic figures and trajectories of qCD4s (various colors) and macrophages (red) are also shown. (EPS)

**Figure S6 Three-dimensional images of phagocytosis of sIC-coated qCD4s by macrophages.** Data show 3D image reconstruction of deconvoluted stacks through X-Y-Z projections of fluorescence confocal micrographs of phagocytosis assays at 3 h. The color overlay images show macrophages (Orange-CMTMR, red) and qCD4s (CFSE, green). (EPS)

**Table S1 Percentage of expression of CR and FcγRII in B and CD4<sup>+</sup> T cells from patients and controls.** (DOCX)

**Movie S1 Time-lapse microscopy of phagocytosis of gp120-coated qCD4s by macrophages.** The color overlay images show macrophages (Orange-CMTMR, red) and qCD4 (CFSE, green). (AVI)

**Movie S2 Time-lapse microscopy of phagocytosis of sIC<sup>+</sup> qCD4s by macrophages.** The color overlay images show macrophages (Orange-CMTMR, red) and qCD4 (CFSE, green). (AVI)

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## Author Contributions

Conceived and designed the experiments: YS. Performed the experiments: YS. Analyzed the data: YS. Contributed reagents/materials/analysis tools: YS HG NT SO. Wrote the paper: YS.

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## Clinical Importance of Hyper-Beta-2-Microglobulinuria in Patients With HIV-1 Infection on Tenofovir-Containing Antiretroviral Therapy

### To the Editors:

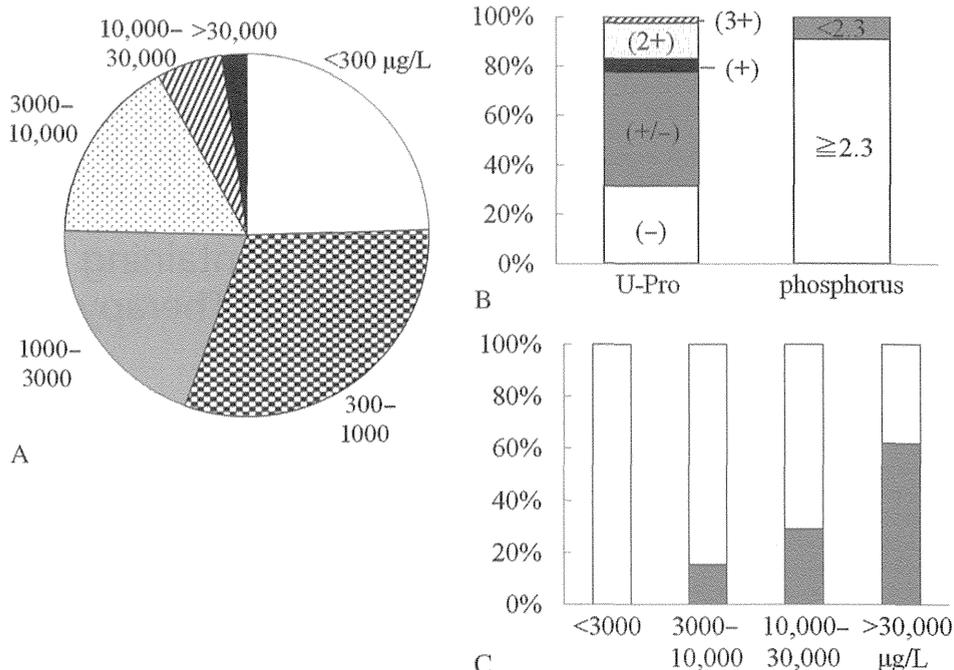
A single-tablet once-daily regime of elvitegravir (EVG), cobicistat (COBI), tenofovir disoproxil fumarate (TDF), and emtricitabine (FTC) showed non-inferior safety and efficacy compared with 2 current first-line regimens recommended for antiretroviral treatment (ART)-naive HIV-1-infected patients,<sup>1,2</sup> and it is now listed as an alternative regimen in the ART guidelines.<sup>3,4</sup> Although TDF is considered to be nephrotoxic, the assessment of renal dysfunction based on the monitoring of serum creatinine may be somewhat difficult during EVG/COBI/TDF/FTC-ART, because COBI inhibits the active tubular secretion of creatinine, resulting in an increase in serum creatinine and a reduction in estimated creatinine clearance without actually affecting glomerular function.<sup>5</sup> Therefore, routine monitoring of not only serum creatinine but also urinary protein, and periodic monitoring of serum phosphorus in high-risk patients, are recommended during the EVG/COBI/TDF/FTC-ART in the guidelines and prescribing information.<sup>3,6</sup> These recommendations seem to be reasonable because tubular dysfunction usually precedes the decline in the glomerular filtration rate in TDF-induced nephrotoxicity. How-

ever, specific markers of renal tubular function may be more sensitive.<sup>7,8</sup> We identified previously urinary  $\beta_2$ -microglobulin (U- $\beta_2$ MG) as a useful marker for monitoring TDF-induced tubular dysfunction, and we currently measure it routinely in TDF-treated patients.<sup>9,10</sup> To address whether the measurement of U- $\beta_2$ MG enables the detection of TDF-induced tubular dysfunction, we compared the values of U- $\beta_2$ MG in TDF-treated patients with those of urinary protein and serum phosphorus. We also determined the clinical significance of hyper- $\beta_2$ -microglobulinuria in patients with TDF-induced tubular dysfunction.

Urinary protein and U- $\beta_2$ MG levels, measured by the dip stick method and latex agglutination turbidimetric immunoassay, respectively, were available from the medical record of 943 patients who had been on TDF-containing ART for >3 months in the Outpatient Clinic of AIDS Clinical Center, National Center for Global Health and Medicine, Tokyo, Japan, between 2010 and 2012. The distribution of the peak value of U- $\beta_2$ MG in each patient is shown in Figure 1A. U- $\beta_2$ MG was persistently within the normal range (<300  $\mu\text{g/L}$ ) in only 231 patients (24.5%), but transiently or persistently abnormal in the other patients (75.5%), indicating that at least mild tubular dysfunction is common in TDF-treated patients. Severe tubular dysfunction (U- $\beta_2$ MG >10,000  $\mu\text{g/L}$ ) was observed in 76 patients (8.1%). Their urine protein measured at the same time is shown in the left bar graph of Figure 1B. Proteinuria (+, 2+, and 3+) was observed in 17 patients (22.4%) only, indicating that the dipstick method is not sufficiently sensitive to detect tubular dysfunction associated with TDF use. Serum phosphorus values were measured in 55 patients with a peak U- $\beta_2$ MG of >10,000  $\mu\text{g/L}$  (Fig. 1B, right bar). Of these, only 5 (9.1%) patients had hypophosphatemia, indicating that serum phosphorus is not a useful marker for TDF-caused tubular dysfunction. Considered together, measurements of urinary protein and serum phosphorus levels do not seem sufficient for monitoring TDF-treated patients, and measurement of markers specific for renal tubular dysfunction may be necessary.

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**FIGURE 1.** A, Peak levels of U-β2MG in tenofovir-treated patients. Data are the distribution of peak levels of U-β2MG in 943 patients treated with tenofovir for >3 months. B, Urinary protein and serum phosphorus levels in patients with severe renal tubular damage associated with tenofovir use. Urinary protein in 76 and serum phosphorus in 55 patients with U-β2MG of >10,000 μg/L are shown. C, Tenofovir withdrawal rate. Nine hundred forty-three tenofovir-treated patients were divided into 4 groups according to the peak level of U-β2MG, and the rate of tenofovir withdrawal in each group is shown. U-Pro, urinary protein.



Next, to define the clinical significance of U-β2MG, we divided the patients based on the peak value of U-β2MG and analyzed the rate of TDF discontinuation associated with renal damage. TDF-containing ART was discontinued during the observation period in none of 711 patients with U-β2MG persistently <3000 μg/L, suggesting that U-β2MG level <3000 μg/L is not clinically significant in TDF-treated patients (Fig. 1C). The peak values of U-β2MG were >3000 and <10,000 μg/L (3000–10,000) in 156 TDF-treated patients, and TDF was discontinued because of renal damage in 24 (15.4%) patients. The peak U-β2MG levels were >10,000 and <30,000 μg/L (10,000–30,000) in 55 TDF-treated patients, and TDF was discontinued in 16 (29.1%) of these patients. These data suggest that the close monitoring of tubular dysfunction and renal damage is necessary when U-β2MG increases beyond 10,000 μg/L. In the remaining 21 patients, the peak U-β2MG levels were >30,000 μg/L (>30,000), and TDF was withdrawn in 13 patients (61.9%) of them, indicating that TDF withdrawal should be considered when U-β2MG is ≥30,000 μg/L.

TDF-related renal damage is more common in Japanese patients than in whites, because low body weight is one

of the risk factors for TDF-related nephrotoxicity.<sup>11,12</sup> Considering that TDF use has expanded in Asia and Africa where patients have lower body weights compared with whites, appropriate monitoring methods for TDF-induced renal damage should be recognized globally. Measurement of not only serum creatinine and urinary protein but also renal tubular damage-specific markers, such as U-β2MG, is recommended.

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## Reply

### To the Editors:

We appreciate the interest from Carrieri et al and their comments regarding our article about the effect of different alcohol consumption levels on HIV surrogate markers in the Swiss HIV Cohort Study (SHCS).<sup>1</sup> Carrieri et al conducted a similar longitudinal analysis using data from the French ANRS APROCO-COPILOTE CO-08 cohort. In contrast to our study, they found in 1108 individuals with 11 years of follow-up that low alcohol consumption levels were associated with higher CD4 cell counts, whereas alcohol abstinence and higher levels of alcohol consumption were associated with lower CD4 counts. No significant association between alcohol consumption and virological failure was found in both cohorts.

Some important issues raised merit a few comments.

Follow-up period was indeed shorter in our study (median follow-up 2.5 years) compared with that in the French cohort (median follow-up 6.9 years). This time difference could possibly explain why in the SHCS no influence of alcohol on CD4 cell counts was observed because the toxic effect of alcohol needs time to evolve. However, despite the shorter follow-up period, we had a larger sample size ( $n = 2982$  vs.  $n = 1108$  in the French cohort) and our cohort also has an excellent follow-up where individuals are

asked about alcohol consumption every 6 months and only very few missing data are recorded (<1%). Carrieri et al did not specify the frequency of the alcohol assessments and the level of missing data in the French cohort.

Most importantly, we would like to highlight that the 2 studies cannot be compared one to one because different baselines were used. Individuals in our study were antiretroviral therapy (ART) naïve and initiated first ART. The French cohort, however, used “protease inhibitor initiating date” as baseline and only 45% were ART naïve at baseline. This could cause a problem in model fitting because individuals at different stages of ART treatment have different trajectories for both HIV viral load and CD4 cell counts. In particular, the definition for virological failure used in both studies would only be appropriate in the ART initiation period. As a consequence, individuals in the French cohort were more heterogeneous with a longer period since HIV diagnosis (3.8 years vs. 1.0 years) and more AIDS events (21% vs. 13%). Furthermore, the French cohort had more injecting drug users (17% vs. 7%) and individuals co-infected with hepatitis C virus (22% vs. 10%). One could argue that individuals in the French cohort were sicker and more often at risk of severe alcohol drinking and therefore of nonadherence to ART. However, we do not have information on treatment interruption and adherence to ART in the French cohort.

Furthermore, in the French cohort, different categories for alcohol consumption were used from what we used. We applied the categories of the World Health Organization<sup>2</sup>: low consumption was <20 g/d for women and <40 g/d for men, moderate 20–40 g/d for women and 40–60 g/d for men, and severe >40 g/d for women and >60 g/d for men, whereas in the French study, low alcohol consumption was <10 g/d for both men and women, moderate 10–30 g/d for women and 10–40 g/d for men, and severe >30 g/d for women and >40 g/d for men, respectively. Therefore, if the French definition would be applied to our cohort, there would be fewer individuals categorized in the moderate drinking group in favor of

the high drinking group. Compared with the French study, we had 46% nondrinkers (vs. 19%), 46% light health risk drinkers (vs. 54%), 5% moderate (vs. 21%), and 2% severe health risk drinkers (vs. 6%). Thus, in our cohort, there were significantly more nondrinkers and indeed fewer individuals with severe health risk drinking, limiting possibly the power in the latter category; the same was true for moderate health risk drinkers. But we would also like to rectify the comment made by Carrieri et al that we collapsed the categories of low and moderate health risk drinking, which is not the case. We fused the categories of none and light health risk drinkers, because both of them gave similar results for CD4 cell counts and HIV viral loads in the single analysis.

Besides the alcohol drinking quantity, the drinking pattern also plays an important role. Binge drinking is known to be associated with worse prognosis concerning morbidity and mortality from alcohol-induced problems and adherence to ART.<sup>3,4</sup> Furthermore, the type of the consumed alcohol might also be important in predicting health outcomes.<sup>5</sup> For the French paradox, red wine was shown to be associated with lower cardiovascular mortality, whether this is true for hard liquor as well is unknown.<sup>6,7</sup> As in both cohorts neither the drinking pattern nor the type of the consumed alcohol is known, no conclusions can be drawn.

The U-shaped curve between alcohol consumption levels and CD4 cell counts as shown by Carrieri et al<sup>8</sup> was found by the same research team for cardiovascular mortality, the so-called French paradox. According to our pathophysiological understanding and also in animal models, it is difficult to explain the U-shaped association between alcohol and CD4 cell counts. It is not clear how a postulated cytotoxic effect of alcohol can be protective for CD4 cells at a certain low level, but being toxic below and above this level.<sup>9–11</sup>

Overall, this French study contributes to the ongoing controversy whether alcohol has an influence on HIV surrogate markers or not. Both studies are well and carefully performed and

The authors have no conflicts of interest to disclose.

# Clinical Significance of High Anti-*Entamoeba histolytica* Antibody Titer in Asymptomatic HIV-1-infected Individuals

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**Background.** Anti-*Entamoeba histolytica* antibody (anti-*E. histolytica*) is widely used in seroprevalence studies though its clinical significance has not been assessed previously.

**Methods.** Anti-*E. histolytica* titer was measured at first visit to our clinic (baseline) in 1303 patients infected with human immunodeficiency virus type 1 (HIV-1). The time to diagnosis of invasive amebiasis was assessed by Kaplan-Meier method and risk factors for the development of invasive amebiasis were assessed by Cox proportional-hazards regression analysis. For patients who developed invasive amebiasis, anti-*E. histolytica* titers at onset were compared with those at baseline and after treatment.

**Results.** The anti-*E. histolytica* seroprevalence in the study population was 21.3% (277/1303). Eighteen patients developed invasive amebiasis during the treatment-free period among 1207 patients who had no history of previous treatment with nitroimidazole. Patients with high anti-*E. histolytica* titer at baseline developed invasive amebiasis more frequently than those with low anti-*E. histolytica* titer. Most cases of invasive amebiasis who had high anti-*E. histolytica* titer at baseline developed within 1 year. High anti-*E. histolytica* titer was the only independent predictor of future invasive amebiasis. Anti-*E. histolytica* titer was elevated at the onset of invasive amebiasis in patients with low anti-*E. histolytica* titer at baseline.

**Conclusions.** Asymptomatic HIV-1-infected individuals with high anti-*E. histolytica* titer are at risk of invasive amebiasis probably due to exacerbation of subclinical amebiasis.

**Keywords.** seroprevalence; *Entamoeba histolytica*; HIV-1; anti-*E. histolytica* antibody; amebiasis.

Invasive amebiasis caused by *Entamoeba histolytica* is the second most common cause of parasite infection-related mortality worldwide, accounting for 40 000–100 000 deaths annually [1]. Recently, it was reported that invasive amebiasis is prevalent not only in developing countries where food or water is contaminated with stool, but also in East Asian developed countries (Korea, China, Taiwan and Japan) and Australia as a sexually transmitted infection (STI) [2–4]. On the

other hand, the annual incidence of human immunodeficiency virus type 1 (HIV-1) infection is also on the rise among men who have sex with men (MSM) in these countries [5–8], with resultant growing concern regarding invasive amebiasis in HIV-1-infected MSM [9–14].

Serum anti-*E. histolytica* antibody (anti-*E. Histolytica*) is widely used as an index marker for the presence of amebiasis. It is used not only in developing countries [15–22] but also in developed countries where amebiasis is spreading as an STI [3, 9, 23–26]. Furthermore, the seroprevalence of anti-*E. histolytica* antibody in HIV-1-infected individuals is generally higher than in HIV-1 negative ones [3, 9, 15, 24]. However, only limited information is available on the seroprevalence of amebiasis in Japan [25, 26] despite the increasing number of invasive amebiasis among HIV-1-infected individuals reported lately [27, 28].

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Serum anti-*E. histolytica* antibody is also widely used for the diagnosis of invasive amebiasis based on the high sensitivity and good differentiation ability from other amoeba species, such as *Entamoeba dispar* and *Entamoeba moshkovskii* [29]. However, the primary disadvantage of this method is that it cannot distinguish current infection from past infection. Moreover, anti-*E. histolytica* antibody titer can be elevated even in asymptomatic infected individuals, and seroconversion of anti-*E. histolytica* was reported in the absence of any symptoms in longitudinal follow-up in endemic areas [14]. At present, the pathogenesis of amebiasis in asymptomatic anti-*E. histolytica*-positive individuals remains poorly understood.

In the present study, we found high seroprevalence of anti-*E. histolytica* antibody in HIV-1-infected adult Japanese. Retrospective analysis of these seropositive individuals indicated that those with high anti-*E. histolytica* titer are prone to future invasive amebiasis. These findings highlight the clinical significance of anti-*E. histolytica* positivity and enhance our understanding of the pathogenesis of invasive amebiasis.

## MATERIALS AND METHODS

### Ethics Statement

This study was approved by the Human Research Ethics Committee of our hospital, the National Center for Global Health and Medicine, Tokyo. The study was conducted in accordance with the principles expressed in the Declaration of Helsinki.

### Study Design and Population

The present study was a single-center retrospective cohort study. Our facility is one of the largest core hospitals for patients with HIV-1 infection in Japan, with >3000 registered patients. The study population was HIV-1-infected patients who were referred to our hospital for management of HIV-1 infection for the first time between January 2006 and April 2012.

### Anti-*E. histolytica* Antibody Testing

Indirect fluorescent-antibody (IFA) assay was used for the detection of anti-*E. histolytica* antibody in serum by using a slide precoated with fixed *E. histolytica*. This method can distinguish amebiasis caused by *E. histolytica* from that caused by other amoeba species, such as *E. dispar* and *E. moshkovskii*. The sensitivity and specificity of this method for the detection of *E. histolytica* infection are comparable with other methods, such as counterimmunoelectrophoresis and indirect hemagglutination amebic serology [29, 30]. The commercial kit, Amoeba-Spot IF (bioMerieux SA), is currently approved for the diagnosis of *E. histolytica* infection in Japan. Based on the instructions enclosed with the kit, the biological samples were initially diluted at 1:100 with phosphate-buffered saline (PBS) and then incubated for 30 minutes at room temperature on slides precoated with fixed *E. histolytica*. Then, the slides were washed with PBS

twice, treated with the fluorescent-labeled anti-human antibodies, and incubated for another 30 minutes at room temperature. The slides were washed again, and cover slips with buffered glycerol were placed over the slides. Fluorescence in each slide was examined with fluorescence microscope and compared with negative control slides. Seropositivity was defined as positive response in serum sample diluted at 1:100, and anti-*E. histolytica* titer was determined by the highest dilution for the positive response.

### Development of Invasive Amebiasis in Patients Without History of Nitroimidazole Treatment

Newly registered HIV-1-infected individuals who underwent anti-*E. histolytica* testing at first visit were included in this analysis. Patients were excluded from the follow-up study (1) if they had been treated previously with nitroimidazole (metronidazole or tinidazole) or (2) if they were treated with nitroimidazole at first visit to the clinic. The clinical characteristics and results of serological tests for other STIs, such as syphilis and hepatitis B and C viruses (HBV and HCV), were collected from the medical records. The follow-up period spanned from the time of the first visit to May 2012, unless patients died from other causes during this period, dropped out, or were referred to other facilities.

The diagnosis of invasive amebiasis was based on the medical records of 3 different clinicians and satisfied one of the following 2 criteria, as described elsewhere [12–14]; (1) identification of erythrophagocytic trophozoites in biological specimens (stool or biopsy sample) of HIV-1-infected patients with symptoms of invasive amebiasis, such as fever, tenesmus, and diarrhea, (2) identification of liver abscess by imaging studies in seropositive (titer  $\geq$ 100) patients with symptoms related to invasive amebiasis who showed clinical improvement after nitroimidazole monotherapy. For patients who developed invasive amebiasis during follow-up, we compared anti-*E. histolytica* titer at the time of onset of invasive amebiasis with those at first visit (baseline) and after nitroimidazole therapy.

### Statistical Analysis

The patients' characteristics and results of serological tests on STIs were compared using  $\chi^2$  test or Student *t* test for qualitative or quantitative variables, respectively. The time to the diagnosis of invasive amebiasis was calculated from the date of the first visit of our hospital to the date of diagnosis of invasive amebiasis. Censored cases represented those who died, dropped out, or were referred to other facilities during the follow-up. The time from first visit to the diagnosis of invasive amebiasis was calculated by the Kaplan-Meier method followed by log-rank test to determine the statistical significance. The Cox proportional-hazards regression analysis was used to estimate the impact of anti-*E. histolytica* titer at baseline on the incidence of invasive amebiasis. The impact of basic clinical characteristics,

**Table 1. Characteristics of All Patients Who Underwent Anti-*E. histolytica* Testing (n = 1303)**

	Anti- <i>E. histolytica</i> Negatives (n = 1026)	Anti- <i>E. histolytica</i> Positives (n = 277)	PValue
Age, years (range)	36 (18–77)	37 (19–74)	.06
Japanese nationality, no. (%)	921 (89.8%)	250 (90.3%)	.81
Male sex, no. (%)	960 (93.6%)	272 (98.2%)	.003
MSM, no. (%)	789 (76.9%)	245 (88.4%)	<.001
TPHA test positive, no. (%)	366/1012 (36.2%)	151/275 (54.9%)	<.001
HBV exposure, <sup>a</sup> no. (%)	524/1017 (51.5%)	187/272 (68.8%)	<.001
HCV Ab positive, no. (%)	40/1011 (4.0%)	5/273 (1.8%)	.09
Past history of IA, no. (%)	13 (1.3%)	60 (21.7%)	<.001
Diagnosis of IA at first visit, no. (%)	1 (0.1%)	7 (2.5%)	<.001

Abbreviations: Ab, antibody; Anti-*E. histolytica*, anti *Entamoeba histolytica* antibody; HBV, hepatitis B virus; HCV, hepatitis C virus; IA, invasive amebiasis; MSM, men who have sex with men; TPHA, *Treponema pallidum* hemagglutination.

<sup>a</sup> HBV exposure: HBsAg-positive or HBsAb-positive, and/or HBc-Ab positive.

such as sexuality and serology status of other STIs, was estimated with univariate Cox proportional hazards regression. We also conducted multivariate Cox hazards regression analysis using variables identified in univariate analysis with *P* values of < .20. In all analyses, statistical significance was defined as 2-sided *P* value of < .05. We used the hazard ratio (HR) and 95% confidence interval (95%CI) to estimate the impact of each variable on the development of invasive amebiasis. All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL).

**RESULTS**

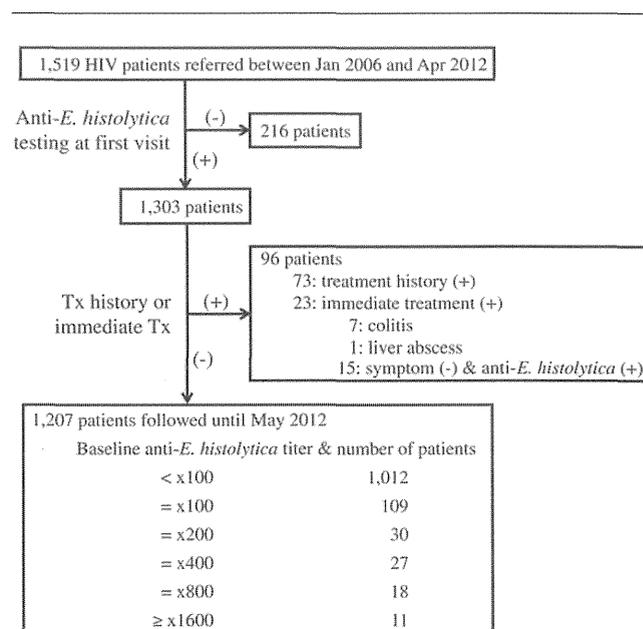
**Clinical Characteristics of Asymptomatic Anti-*E. histolytica*-positive HIV-1-infected Patients**

A total of 1519 patients were referred to our hospital during the study period. Anti-*E. histolytica* testing was conducted in 1303 patients at first visit, including 73 with history of invasive amebiasis, and anti-*E. histolytica* was positive in 277 of these (21.3%). Among the anti-*E. histolytica*-positive individuals, the rates of MSM (88.4%) and those with previous exposure to syphilis (TPHA test positive) (54.9%) and HBV (68.8%) were higher than those of anti-*E. histolytica*-negatives individuals, indicating that sexually active MSM are prone to *E. histolytica* infection among HIV-1-infected individuals in Japan (Table 1). Eight patients were diagnosed with invasive amebiasis at first visit, including 7 cases of amebic colitis and 1 case of amebic liver abscess, and they were treated immediately with metronidazole.

**Incidence of Invasive Amebiasis During Follow-up of HIV-1 Infected Individuals**

To assess the frequency of development of invasive amebiasis in patients free of symptomatic invasive amebiasis and who had not previously received nitroimidazole therapy, we

excluded 96 patients from the analysis, including 73 patients because they had been treated previously for invasive amebiasis, and 23 patients (7 cases of amebic colitis, 1 case of amebic liver abscess, and 15 asymptomatic but anti-*E. histolytica*-positive cases treated preemptively) because they were treated with nitroimidazole at first visit (Figure 1). The remaining 1207 patients, including 195 anti-*E. histolytica*-positive patients (16.2%), were followed-up for median period of 25.3 months (interquartile range: 7.0–47.2). During the follow-up period, 18 patients developed invasive amebiasis (median time to onset: 9.1 months), including amebic appendicitis in 1 patient



**Figure 1.** Flow diagram of patient recruitment process. Abbreviations: Anti-*E. histolytica*, anti-*Entamoeba histolytica* antibody; IA, invasive amebiasis; Tx, treatment.

**Table 2. Comparison of Clinical Characteristics of Patients With and Without Invasive Amebiasis**

	Amebic Colitis (n = 11)	Extraintestinal IA <sup>a</sup> (n = 7)	Non-IA (n = 1189)	P Value IA vs Non-IA
Age (years), average (SD)	35.9 (12.3)	38.2 (11.0)	37.5 (10.8)	.81
Japanese nationality, no. (%)	10 (90.9)	6 (85.7)	1068 (89.8)	.71
Male sex, no. (%)	11 (100)	7 (100)	1119 (94.1)	.62
MSM, no. (%)	11 (100)	6 (85.7)	929 (78.1)	.15
TPHA test-positive, no. (%)	5 (45.5)	2 (28.6)	451/1175 (38.4)	.91
HBV exposure, <sup>a</sup> no. (%)	6 (54.5)	5 (71.4)	630/1178 (53.5)	.15
HCVAb-positive, no. (%)	0/11 (0)	0/7 (0)	42/1172 (3.6)	1.00
Anti- <i>E. histolytica</i> at baseline, median (IQR)	×100 (<×100–×800)	×400 (×100–×400)	<×100 (<×100–<×100)	<.001
Anti- <i>E. histolytica</i> at the onset of IA, median (IQR)	×800 (×200–×800)	×400 (×100–×800)	...	
Follow-up period, median months (IQR)	7.8 (3.3–25.1)	10.5 (4.9–17.9)	25.5 (7.0–47.3)	

Data were compared using  $\chi^2$  test, Student *t* test, or Mann–Whitney *U* test for qualitative or quantitative variables, respectively.

Abbreviations: Ab, antibody; Anti-*E. histolytica*, anti *Entamoeba histolytica* antibody; HBV, hepatitis B virus; HCV, hepatitis C virus; IA, invasive amebiasis; IQR, interquartile range; MSM, men who have sex with men; SD, standard deviation; TPHA, *Treponema pallidum* hemagglutination.<sup>a</sup>Extraintestinal cases include one case of appendicitis and 6 cases of liver abscess.

(confirmed by identification of erythrophagocytic trophozoites in surgically removed specimen), amebic liver abscess in 6, and amebic colitis in 11 (confirmed by identification of erythrophagocytic trophozoites in stool samples). The median anti-*E. histolytica* titer at baseline was significantly higher among patients who developed invasive amebiasis than that among those who did not, but the other clinical and laboratory parameters were not different between the 2 groups (Table 2). Although no significant differences in the frequency of invasive amebiasis were evident in patients with ×100 ( $P = .77$ ) and ×200 ( $P = .18$ ) anti-*E. histolytica* titers at baseline, compared with negative anti-*E. histolytica* patients (<×100), the frequency was higher in patients with ×400 ( $P < .001$ ), ×800 ( $P = .025$ ), and ≥×1600

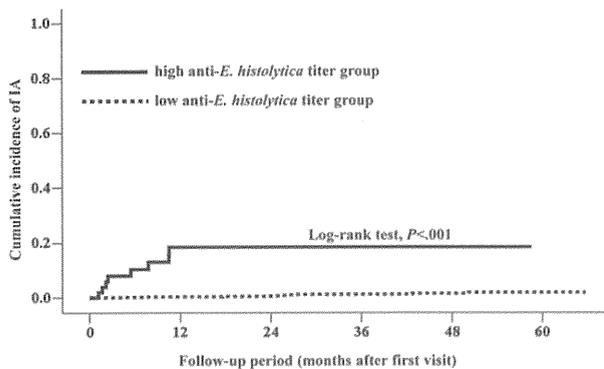
( $P < .001$ ) anti-*E. histolytica* titers at baseline, compared with negative anti-*E. histolytica* patients. Univariate and multivariate analyses also showed that future development of invasive amebiasis correlated only with high titer of anti-*E. histolytica* antibody at baseline (≥×400: Univariate, HR: 20.985, 95% confidence interval [CI], 8.085–54.467; multivariate, HR: 22.079, 95% CI, 7.964–61.215) (Table 3). Furthermore, the risk of development of invasive amebiasis was significantly higher in the high anti-*E. histolytica* titer group (patients with anti-*E. histolytica* titer ≥×400 at baseline) than in the low anti-*E. histolytica* titer group (patients with anti-*E. histolytica* titer ≤×200 at baseline; log-rank test:  $\chi^2 = 80.203$ ,  $P < .001$ , Kaplan-Meier estimate, Figure 2). Moreover, most patients of the high anti-*E. histolytica*

**Table 3. Risk Analysis for Development of Invasive Amebiasis by Cox Proportional Hazard Regression Model**

	Univariate Analysis		Multivariate Analysis	
	HR (95% CI)	P Value	HR (95% CI)	P Value
older age (by 1 y)	0.989 (.947–1.033)	.624		
Japanese nationality	1.334 (.305–5.840)	.702		
Male sex	21.884 (.002–241297.39)	.516		
MSM	4.318 (.573–32.518)	.156	4.048 (.488–33.584)	.195
TPHA test-positive	0.901 (.348–2.335)	.831		
HBV exposure-positive	2.183 (.778–6.124)	.138	1.839 (.644–5.249)	.255
HCVAb-positive	0.047 (.000–2697.344)	.584		
Anti- <i>E. histolytica</i> titer ≥×400	20.985 (8.085–54.467)	<.001	22.079 (7.964–61.215)	<.001

The Cox proportional-hazards regression analysis was used to estimate the impact of anti-*E. histolytica* titer at baseline on the incidence of invasive amebiasis. The impact of basic clinical characteristics, such as sexuality and serology status of other STIs, was estimated with univariate Cox proportional hazards regression. Multivariate Cox hazards regression analysis using variables identified in univariate analysis with *P* values of < .20. In all analyses, statistical significance was defined as *P* value of < .05.

Abbreviations: Ab, antibody; Anti-*E. histolytica*, anti *Entamoeba histolytica* antibody; CI, confidence interval; HBV, hepatitis B virus; HCV, hepatitis C virus; IA, invasive amebiasis; IA, invasive amebiasis; IQR, interquartile range; MSM, men who have sex with men; TPHA, *Treponema pallidum* hemagglutination.



**Figure 2.** Incidence of invasive amebiasis in low and high anti-*E. histolytica* titer groups. Differences in the time from first visit to the diagnosis of invasive amebiasis (IA) between the low anti-*E. histolytica* titer group ( $\leq \times 200$  at baseline) and high anti-*E. histolytica* titer group ( $\geq \times 400$  at baseline) were analyzed by Kaplan-Meier method. Log-rank test was used to determine the statistical significance. Abbreviations: Anti-*E. histolytica*, anti-*Entamoeba histolytica* antibody; IA, invasive amebiasis.

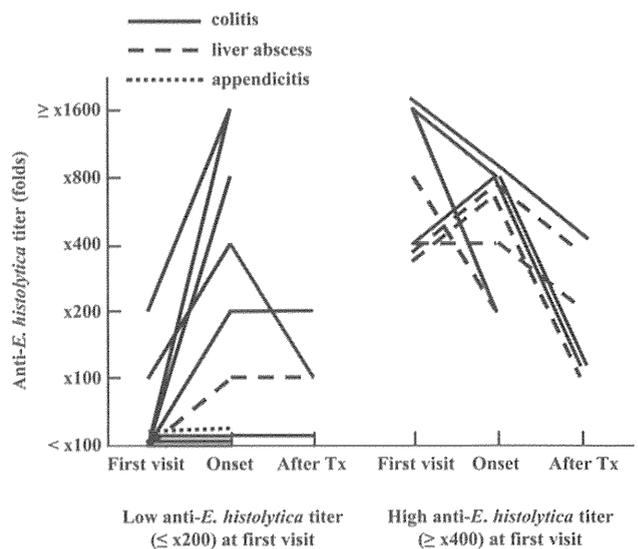
titer group developed invasive amebiasis during the first year of follow-up, whereas those of the low anti-*E. histolytica* titer group developed this complication more lately and new cases of invasive amebiasis were diagnosed throughout the follow-up period.

#### Transitional Changes in Anti-*E. histolytica* Titer Among Patients Who Developed Amebiasis

The median anti-*E. histolytica* titer was significantly higher at the onset of invasive amebiasis than that at first visit in patients with low baseline anti-*E. histolytica* titer ( $\leq \times 200$ ;  $P = .028$ , Wilcoxon signed-rank test) (Figure 3). In contrast, the median anti-*E. histolytica* titers at these 2 time points were not different in patients with high baseline anti-*E. histolytica* titer ( $\geq \times 400$ ;  $P = .18$ , Wilcoxon signed-rank test). Serum samples taken after nitroimidazole treatment (median time from the commencement of treatment 289 days [range 174–841]) were available in 10 patients. Anti-*E. histolytica* titers were lower after the treatment in 7 of the 10 patients, compared with the baseline values. To define the natural decay of anti-*E. histolytica*, we measured serum anti-*E. histolytica* titers at 9 months after study enrollment in 37 patients with high anti-*E. histolytica* titer at baseline but did not develop invasive amebiasis during the study period. The titers were lower, or similar to the baseline in 19 and 15 patients, respectively, whereas the remaining 3 patients showed 2-fold increase in the titer.

## DISCUSSION

In the present study, the seroprevalence of anti-*E. histolytica* antibody among HIV-1-infected patients was 21.3%, which was



**Figure 3.** Anti-*E. histolytica* titer before and after diagnosis of invasive amebiasis. Anti-*E. histolytica* titer at the onset of IA was compared to that at baseline (first visit to the clinic) by Wilcoxon signed-rank test. Anti-*E. histolytica* titers after treatment were measured at 219 days [range: 174–252] and 367 days [272–841] after the completion of treatment of patients with low and high anti-*E. histolytica* titer at first visit, respectively. Abbreviations: Anti-*E. histolytica*, anti-*Entamoeba histolytica* antibody; IA, invasive amebiasis.

much higher than those reported in other developed countries where amebiasis is considered as an STI [3, 9, 23, 24]. In addition, our results showed that sexually active MSM tend to be seropositive for *E. histolytica* infection, in agreement with previous studies from our group [27, 28].

The pathogenesis of amebiasis, such as incubation period after cyst ingestion and the mechanism of spontaneous remission, remains unclear. Although previous study showed anti-*E. histolytica*-positive children were more susceptible to *E. histolytica* infection than their seronegative counterparts [31], the clinical significance of anti-*E. histolytica* seropositivity and its titer in asymptomatic individuals had not been fully assessed. We measured serum anti-*E. histolytica* immunoglobulin M (IgM) levels in 18 patients at the onset of invasive amebiasis [32], but the level was detectable only in 3 patients with amebic colitis and 1 patient with liver abscess. The present study demonstrated that patients with high anti-*E. histolytica* titer ( $\geq \times 400$ ) at first visit developed invasive amebiasis much more frequently than those with low anti-*E. histolytica* titer ( $\leq \times 200$ ). The cumulative risk for invasive amebiasis among patients with high anti-*E. histolytica* titer at baseline rapidly increased during the first one year of follow-up but plateaued thereafter, suggesting that exacerbation of subclinical amebiasis occurs frequently within one year in these patients. On the other hand, the cumulative risk for invasive amebiasis among patients with low anti-*E. histolytica* titer at baseline increased more slowly and

developed at the same pace throughout the follow-up period, suggesting that the invasive amebiasis in these patients represented new infection rather than exacerbation of subclinical infection. The median anti-*E. histolytica* titer at the onset of invasive amebiasis in patients of high anti-*E. histolytica* titer group was not higher than that at first visit, whereas the titer increased at the onset compared with that at baseline in low anti-*E. histolytica* titer group. In addition, uni- and multivariate analyses identified high titer of anti-*E. histolytica* antibody at baseline as the only significant risk factor for future development of invasive amebiasis; seropositivity to other STIs was not a significant factor. These results add support to the aforementioned hypothesis regarding the difference in the pathology of invasive amebiasis between the high and low anti-*E. histolytica* groups. In this study, 15 asymptomatic but anti-*E. histolytica*-positive patients were treated with metronidazole at first visit (excluded from the follow-up analysis study), and none of them developed invasive amebiasis (median follow-up period, 11.7 months), suggesting the potential effectiveness of preemptive therapy for asymptomatic individuals with high anti-*E. histolytica* titer.

In conclusion, our results showed a relatively high prevalence of amebiasis in HIV-1-infected individuals in Japan, and that subclinical amebiasis is common among these individuals. The results emphasize the difficulty of disease control in not only individual patients with amebiasis but also in epidemiological control of this condition due to the long duration of subclinical infection of *E. histolytica*. Anti-*E. histolytica* testing for high-risk individuals could be helpful in early diagnosis of subclinical amebiasis, and early treatment of patients with such infection could prevent the development of invasive amebiasis and the transmission to others in the same community. Further studies to clarify the pathogenesis of invasive amebiasis are warranted.

## Notes

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**Potential conflicts of interest.** All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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