# Ⅲ. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

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# Ⅳ. 研究成果の刊行物・別刷

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LETTERS

# Adaptation of HIV-1 to human leukocyte antigen class I

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**The rapid and extensive spread of the human immunodeficiency virus (HIV) epidemic provides a rare opportunity to witness host–pathogen co-evolution involving humans. A focal point is the interaction between genes encoding human leukocyte antigen (HLA) and those encoding HIV proteins. HLA molecules present fragments (epitopes) of HIV proteins on the surface of infected cells to enable immune recognition and killing by $CD8^+$ T cells; particular HLA molecules, such as HLA-B*57, HLA-B*27 and HLA-B*51, are more likely to mediate successful control of HIV infection[1]. Mutation within these epitopes can allow viral escape from $CD8^+$ T-cell recognition. Here we analysed viral sequences and HLA alleles from >2,800 subjects, drawn from 9 distinct study cohorts spanning 5 continents. Initial analysis of the HLA-B*51-restricted epitope, TAFTIPSI (reverse transcriptase residues 128–135), showed a strong correlation between the frequency of the escape mutation I135X and HLA-B*51 prevalence in the 9 study cohorts ($P = 0.0001$). Extending these analyses to incorporate other well-defined $CD8^+$ T-cell epitopes, including those restricted by HLA-B*57 and HLA-B*27, showed that the frequency of these epitope variants ($n = 14$) was consistently correlated with the prevalence of the restricting HLA allele in the different cohorts (together, $P < 0.0001$), demonstrating strong evidence of HIV adaptation to HLA at a population level. This process of viral adaptation may dismantle the well-established HLA associations with control of HIV infection that are linked to the availability of key epitopes, and highlights the challenge for a vaccine to keep pace with the changing immunological landscape presented by HIV.**

The extent to which HIV is evolving at the population level in response to immune selection pressure is under debate[2–6]. Resolving the impact of HLA class I alleles on viral evolution is problematic because it can be obscured by other influences, such as founder effect[6] (polymorphisms present within the early strains establishing the epidemic in a group). In addition, most HLA alleles do not drive significant selection pressure on HIV, a proportion of escape mutations revert to wild type after transmission, and different HLA alleles may drive the identical escape mutation[7].

To test the hypothesis that the frequency of escape mutations in a given population is correlated with the prevalence of the relevant HLA allele in that population, we studied nine distinct cohorts from North America, the Caribbean, Europe, sub-Saharan Africa, Australia and Japan, in which we performed HLA typing, and defined the viral mutations arising within $CD8^+$ T-cell epitopes. We focused initially on a well-characterized mutation, I135X, within the HLA-B*51-restricted epitope, TAFTIPSI (RT 128–135)[8], because it arises in acute infection, non-HLA-B*51 alleles do not also select this mutation[7,9], and it does not revert to Ile 135 after transmission to HLA-B*51-negative subjects[9]. Thus, if highly prevalent HLA alleles drive a high frequency of escape mutations in the population, this would be most obvious in relation to HLA-B*51 and the escape mutant I135X. We then considered an additional 13 well-defined escape mutations, including those known to reduce viral fitness and therefore liable to revert after transmission.

I135X was selected in 205 of 213 (96%) HLA-B*51-positive individuals analysed (Figs 1 and 2, and Supplementary Fig. 1). The I135X variants do not significantly affect viral replicative capacity *in vitro*, other than the rare I135V mutation. This was the only variant observed to revert to wild-type *in vivo* during a 3-year follow-up of 38 HLA-B*51-negative subjects identified during acute HIV infection who carried I135X mutant viruses at transmission (Fig. 1e). The I135X mutants substantially affect HLA binding, and therefore also recognition by $CD8^+$ T cells (Fig. 1f–h). Thus, HIV transmission from HLA-B*51-positive subjects would probably involve transmission of I135X, which would persist in the new host. Newly infected HLA-B*51-positive subjects receiving an I135X mutant would be unable to generate an HLA-B*51-TAFTIPSI-specific response.

To test the hypothesis that the population frequency of I135X is correlated with HLA-B*51 prevalence, HIV sequence and HLA data were collated from the nine study cohorts. One cohort comprised subjects with acute/early HIV infection; the remaining cohorts comprised chronically infected subjects. In all cohorts the odds ratio strongly favoured I135X in the HLA-B*51-positive subjects, even in the acute cohort where I135X was selected sufficiently early to be already over-represented in HLA-B*51-positive subjects (odds ratio 1.65, $P = 0.07$, Fig. 2a). In Japan, where HLA-B*51 is highly

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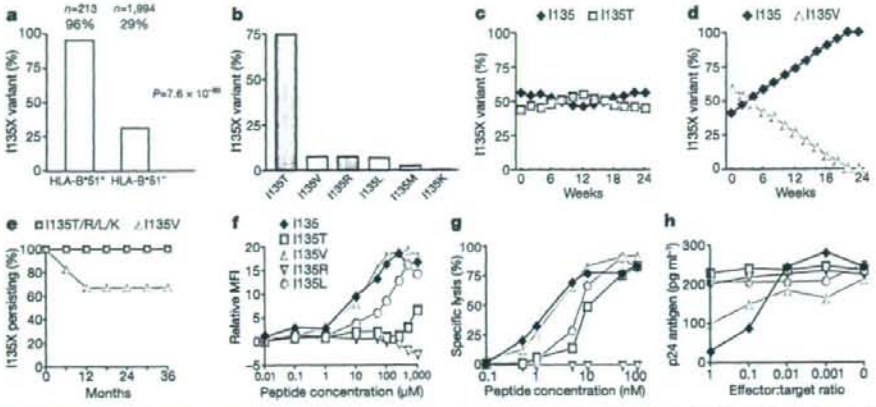


**Figure 1 | Selection and fitness cost of I135X escape variants and recognition by the HLA-B*51–TAFTIPSI (RT 128–135)-specific CD8⁺ T cells.** **a**, Association between I135X and HLA-B*51 in all study cohorts. **b**, Ile 135 variation in HLA-B*51-positive subjects. **c**, **d**, *In vitro* competition assays between NL4-3 wild-type virus and I135X viral variants (I135T (**c**) and I135V (**d**)). I135R and I135L showed no fitness cost (not shown). **e**, Persistence of I135X mutants in 38 HLA-B*51-negative subjects followed from acute infection. **f**, TAFTIPSI variant binding to HLA-B*51 (see Methods). MFI, mean fluorescence intensity. **g**, **h**, Recognition of peptide-pulsed HLA-B*51-matched targets and viral variants by representative TAFTIPSI-specific CD8⁺ T-cell clones.

prevalent[10] (21.9% of the study cohort), the frequency of I135X was >50%, and overall across all cohorts the I135X frequency was strongly correlated with HLA-B*51 prevalence ($P = 0.0001$, Fig. 2b). To control for the possibility that disproportionately more virus sequences from HLA-B*51-positive subjects were analysed, the same analysis comparing I135X frequency in HLA-B*51-negative subjects only was undertaken, with similar findings (Fig. 2c, $P = 0.0006$). These data suggest that HIV may be adapting to HLA-B*51 with respect to the HLA-B*51–TAFTIPSI response in localities where HLA-B*51 is at high prevalence.

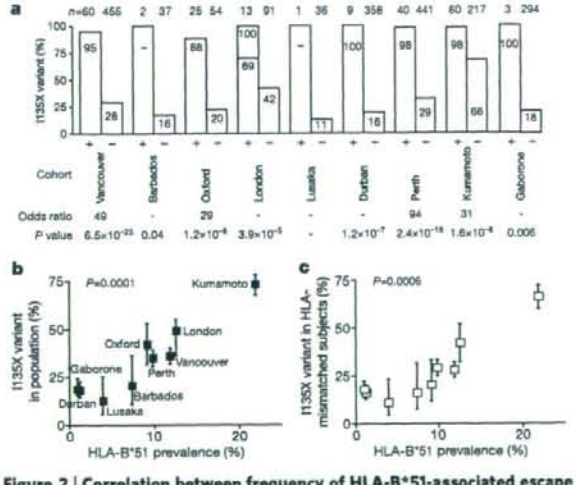


**Figure 2 | Correlation between frequency of HLA-B*51-associated escape mutations and HLA-B*51 prevalence in study cohorts.** **a**, Frequency of I135X mutations within TAFTIPSI (RT 128–135) in HLA-B*51-positive (+) and -negative (−) subjects within nine study cohorts. In the acute cohort (London) 69% of HLA-B*51-positive subjects expressed I135X mutant at enrolment, 100% within 2 years of baseline (Supplementary Fig. 1). **b**, Correlation between frequency of I135X mutation and HLA-B*51 prevalence in the nine study populations. Logistic regression $P = 0.0001$ (Supplementary Table 1). **c**, Correlation between I135X frequency in HLA-B*51-negative subjects and HLA-B*51 prevalence in nine study populations. Error bars represent 95% confidence limits, obtained using a binomial error distribution.

Additional evidence that I135X is accumulating in Japan comes from the observation that only 3 of 14 (21%) HLA-B*51-negative Japanese haemophiliacs infected in 1983 carried I135X, compared with 30 of 43 (70%) HLA-B*51-negative subjects infected between 1997 and 2008 ($P = 0.002$). Furthermore, HLA-B*51 does not protect against disease progression in Japanese subjects infected between 1997 and 2008, whereas HLA-B*51-positive haemophiliacs infected in 1983 had lower viraemia levels and higher CD4 counts than HLA-B*51-negative haemophiliacs (Supplementary Fig. 2). These data are consistent with fewer HLA-B*51-positive subjects targeting TAFTIPSI during 1997–2008, owing to a population-level increase in the HLA-B*51 I135X escape mutation over this 14–25-year period.

To investigate HIV adaptation to other HLA alleles, we initially examined other escape mutations shown previously to persist stably after transmission[5,7]. We selected the three non-reverting Gag polymorphisms that, from analysis of 673 study subjects in Durban, South Africa[7], were most strongly associated with the relevant restricting allele ($P < 10^{-6}$ after phylogenetic correction), namely, S357X, D260X and D312X within epitopes restricted, respectively, by HLA-B*07 (GPSHKARVL, Gag 355–363), HLA-B*35 (PPIPVGDIY, Gag 254–262) and HLA-B*44 (AEQATQDVKNW, Gag, 306–316). In addition, we analysed a non-reverting I31V variant (LPPIVAKEI, Int 28–36) previously hypothesized to increase in relation to population HLA-B*51 prevalence[5]. These additional polymorphisms show a similar relationship to that between I135X and HLA-B*51, overall showing a strongly significant correlation between variant frequency and prevalence of the restricting HLA allele (Figs 3 and 4a, and Supplementary Fig. 3).

The spectrum of HLA-associated polymorphisms also includes mutations reducing viral fitness[1]. These either revert to wild type after transmission, or persist in the presence of compensatory mutations. We extended these analyses to include epitopes restricted by HLA-B*27 and HLA-B*57, alleles strongly associated with successful immune control of HIV[11,12]. The mutations analysed themselves are associated with precipitating loss of immune control[13–16] and all inflict a documented viral fitness cost, either demonstrated by *in vitro* fitness studies and/or *in vivo* reversion[7,14,17–21] (data not shown for V168I).

Again, a strong correlation between escape mutant frequency and prevalence of the restricting HLA allele was observed (Figs 3c–f and 4b, and Supplementary Fig. 3; overall, for these nine variants affecting viral fitness, $r = 0.69$, $P < 0.0001$). Unexpectedly, this correlation

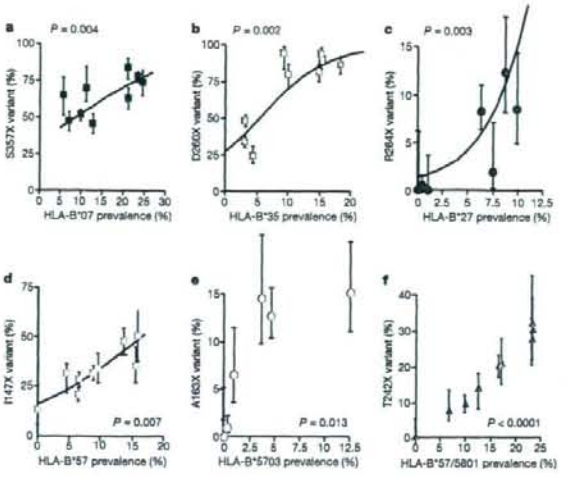


**Figure 3 | Correlation between frequency of HIV sequence variant and HLA prevalence for six additional well-characterized epitopes.** *P* values calculated after logistic regression analysis as shown (calculations after linear regression analysis are shown in Supplementary Table 1). **a**, Frequency of the S357X mutation within the HLA-B*07-restricted epitope GPSHKARVL (Gag 355–363). **b**, Frequency of the D260X mutation within the HLA-B*35-restricted epitope PPIPVGDIY (Gag 254–262). **c**, Frequency of the R264X mutation within the HLA-B*27-restricted epitope KRWIILGLNK (Gag 263–272). **d**, Frequency of the I147X mutation within the HLA-B*57-restricted epitope ISPRTLNAW (Gag 147–155). **e**, Frequency of the A163X mutation associated with the HLA-B*5703-restricted epitope KAFSPEVIPMF (Gag 162–172). **f**, Frequency of the T242X mutation within the B*57/5801-restricted epitope TSTLQEQIAW (Gag 240–249). Error bars represent 95% confidence limits, obtained using a binomial error distribution.

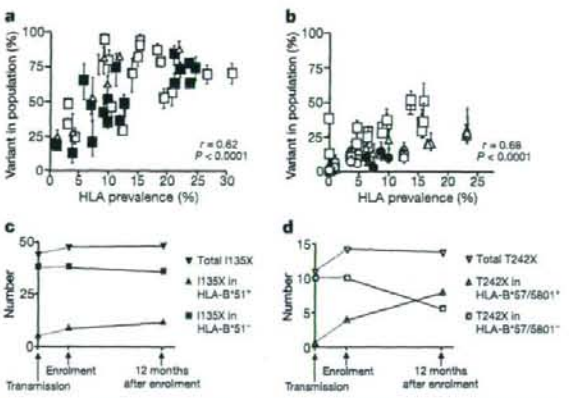


**Figure 4 | Correlation between HIV variant frequency and HLA prevalence for all epitopes studied.** **a**, Correlation between HLA prevalence and the five stable, non-reverting variants (symbols in Figs 2 and 3, and Supplementary Fig. 3; grey triangles, I31V; green squares, D312X). **b**, Eight variants demonstrated to reduce viral fitness (see text, Fig. 3 and Supplementary Fig. 3; turquoise triangles, L268X; yellow squares. A146X; sky-blue squares, V168I; yellow circles, I247X). **c**, **d**, Data from acute London cohort. **c**, Number of HLA-B*51-positive and HLA-B*51-negative subjects carrying the non-reverting I135X variant. The percentage of I135X in HLA-B*51-negative subjects at enrolment (42%) assumed the percentage of I135X in all subjects at transmission (I135X frequency in HLA-B*51-positive subjects at enrolment was 69%, $P = 0.07$). **d**, The reverting HLA-B*57/5801-restricted T242X mutation. T242X frequency in HLA-B*57/5801-negative subjects at enrolment was 7%, versus 33% in HLA-B*57/5801-positive subjects ($P = 0.01$). Error bars represent 95% confidence limits, obtained using a binomial error distribution.

remained significant even when comparing HLA prevalence with variant frequency in the HLA-mismatched population ($r = 0.40$, $P = 0.0004$). As anticipated, non-reverting variants such as I135X accumulate at the population level, but even rapidly reverting[18,20] mutations such as T242N can accumulate, if the selection rate exceeds the reversion rate (Fig. 4c, d).

Although frequency of the analysed HIV polymorphisms and HLA prevalence were strongly correlated overall, some anomalies were observed. For example, despite a 0% prevalence of HLA-B*57 in Japan[10], 38% of the Japanese cohort had the HLA-B*57-associated A146X variant. One potential explanation might be A146X selection by non-HLA-B*57 Japanese alleles. Analysing Gag sequences from Japanese study subjects, we observed a strong association between A146P and HLA-B*4801 ($P = 0.00035$), and then that A146P is indeed selected in HLA-B*4801-positive subjects (Supplementary Fig. 4a, b). We defined a novel HLA-B*4801-restricted epitope (Gag 138–147), showing also that A146P is an escape mutant (Supplementary Fig. 4c–f). These data illustrate that more than one HLA allele can drive the selection of a particular escape mutant (Supplementary Fig. 5). Also, in populations where HIV-specific $CD8^+$ T-cell responses are incompletely characterized, the influences of locally prevalent HLA alleles on HIV sequence variation are unknown.

These data show a strong correlation between HLA-associated HIV sequence variation and HLA prevalence in the population ($r = 0.69$, $P < 0.0001$, Supplementary Fig. 6), suggesting that the frequency of the studied variants is substantially driven by the HLA-restricted $CD8^+$ T-cell responses. Non-reverting variants[5,7], as well as those previously shown to arise at a fitness cost[7,14,16–21], were studied. The latter constitute approximately 55–65% of HLA-associated polymorphisms[7,20]. This current analysis included epitopes whose role in HIV immune control is unknown, as well as those believed to contribute significantly to containment of HIV[4,7,13–19]. Analysis of well-characterized epitopes only also served to limit potential confounding influences of epitope clustering (selection of the same variant by different HLA alleles) and of founder effect. Either would be capable of obscuring a true HLA effect on population variant frequency.

The HLA-B*57-associated A146X mutation illustrates the complexity that may result from epitope clustering. A146X is selected by at least six distinct HLA alleles (Supplementary Fig. 5). A true correlation existing between mutation frequency and individual HLA allele prevalence might thus be obscured by selection of the same mutation by other alleles.

Founder effect also has an undoubted influence on population frequencies of particular polymorphisms[6]. Phylogenetic correction of sequence data excludes founder effect as a confounder[6,7,9], and the highly significant associations between the presence of particular HLA alleles and all 14 HIV polymorphisms studied, persisting after phylogenetic correction (Supplementary Table 3), provide compelling evidence that the effects observed here are substantially HLA-driven. The large numbers of study subjects in these current studies reduce the likelihood of genuine HLA associations with HIV amino acid polymorphisms being obscured by founder effects. The relative impact of HLA and founder effect on variant frequency is harder to quantify, and is likely to differ substantially between particular populations.

The consequence of HIV adapting to certain $CD8^+$ T-cell responses is unknown. For non-reverting polymorphisms such as HLA-B*35-associated D260E, the variant approaches fixation, because even at population frequencies of 90%, D260E is still significantly selected in HLA-B*35-positive subjects (Supplementary Fig. 7b). Important questions relevant to vaccine design include the extent and rate of sequence change in populations. Relevant factors include the selection rate in subjects expressing the HLA allele, the reversion rate in HLA-mismatched subjects, the population HIV

transmission rate, and HLA allele prevalence. Models would need to include factors such as the selection of compensatory mutations to slow reversion rates, and antiretroviral therapy access that would slow transmission rates.

HLA adaptation to certain $CD8^+$ T-cell responses may also alter currently established HLA associations with slow disease progression. Data here suggest that, whereas 25 years ago HLA-B*51 was protective in Japan[11,12], this is no longer the case (Supplementary Fig. 2). The apparent increase in I135X frequency in Japan over this time supports the notion that HLA-B*51 protection against HIV disease progression hinges on availability of the HLA-B*51-restricted TAFTIPSI response. However, whether this is the case remains unknown.

For HLA-B*27 and HLA-B*57, there is more clear-cut evidence that their association with HIV control depends on the Gag-specific epitopes presented and analysed here[4,7,13–15,18,19]. For each of the HLA-B*27- and HLA-B*57-associated Gag mutations studied, an *in vitro* fitness cost or *in vivo* reversion has been observed. A strong correlation between variant frequency and HLA prevalence even for rapidly reverting variants can be explained, either by mutant acquisition exceeding reversion rate (Fig. 4D), or by selection of compensatory mutations slowing or halting reversion altogether. The clearest example of the latter is the HLA-B*27-associated R264K mutation, 'corrected' by S173A[19]. Compensatory mutations are also well described for the HLA-B*57-associated Gag mutations[14,18]. These data suggest that the escape mutations in these HLA-B*27- and HLA-B*57-restricted epitopes are accumulating over time. Several studies have now demonstrated that transmission of viruses encoding escape mutants in the critical Gag epitopes to individuals expressing the relevant MHC class results in failure to control viraemia[2,21,22]. The accumulation at the population level of these escape mutations in HLA-B*27 and HLA-B*57 Gag epitopes is therefore likely to reduce the facility of these alleles to slow HIV disease progression.

The longer-term consequences of this process for immune control of HIV are unknown. Loss of currently immunodominant epitopes would promote subdominant $CD8^+$ T-cell responses, which can be more effective[23,24]. Also, the adapted virus provides new epitopes that can be presented, potentially with beneficial effects. In hepatitis C virus, for example, HLA-A*0301 holds a particular advantage, but only against the specific strain of virus responsible for the Irish outbreak[25]. In HIV, HLA-B*1801 is associated with high viraemia in C clade but not in B clade infection[10,11,26]; the opposite applies to HLA-B*5301.

Thus, the data presented here, showing evidence that the virus is adapting to $CD8^+$ T-cell responses, some of which may mediate the well-established associations (HLA-B*57, HLA-B*27 and HLA-B*51) with immune control of HIV, highlight the dynamic nature of the challenge for an HIV vaccine. Important questions to be addressed include the speed and extent of sequence change, particularly in Gag, the most effective target for $CD8^+$ T-cell responses[1,7,13,21]. The induction of broad Gag-specific $CD8^+$ T-cell responses may be a successful vaccine strategy, but such a vaccine will be most effective if tailored to the viral sequences prevailing, and thus may need to be modified periodically to keep pace with the evolving virus. Moreover, the strong associations between certain HLA class molecules, such as HLA-B*57, HLA-B*27 and HLA-B*51, and slow disease progression may decline as the epidemic continues, particularly where these HLA alleles are highly prevalent, and where HIV transmission rates are high.

## METHODS SUMMARY

Overall 2,875 subjects were studied, from 9 previously established study cohorts. These cohorts comprised subjects from North America, the Caribbean, Europe, sub-Saharan Africa, Australasia and Asia. All subjects were antiretroviral-therapy-naive. Apart from the London acute cohort ($n = 142$), all cohorts comprised chronically infected subjects. The 14 variants studied are well-defined escape mutations within well-characterized $CD8^+$ T-cell epitopes, and included those persisting after transmission and likely to have little effect on viral fitness ($n = 5$), as well as those shown previously to reduce viral fitness ($n = 9$). Autologous HIV-1 sequences, and HLA class I types, were determined for all study subjects. The replicative capacity of I135X variants selected within the HLA-B*51-restricted epitope TAFTIPSI (RT 128–135) was assessed via *in vitro* competition assays and also via longitudinal follow-up of HLA-B*51-negative subjects infected acutely with I135X variants. Polymorphism frequency in the study cohorts was compared with prevalence of the relevant HLA molecule in the study cohort using a logistic regression model taking into account the different numbers of study subjects in each cohort. Demonstration of an HLA allele driving escape at Gag146 in the Japanese cohort was undertaken first by identification of an association between HLA-B*4801 and A146P, subsequent definition of an HLA-B*4801-restricted $CD8^+$ T-cell response to a novel epitope Gag 138–147 (LI10), and finally demonstration that A146P reduced viral recognition by LI10-specific $CD8^+$ T cells.

**Full Methods** and any associated references are available in the online version of the paper at www.nature.com/nature.

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**Supplementary Information** is linked to the online version of the paper at www.nature.com/nature.

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**Author Information** Accession numbers for newly determined viral sequences are included in Supplementary Information. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to P.G. (philip.goulder@paediatrics.ox.ac.uk).

## METHODS

**Study subjects.** The study cohorts have been described more fully elsewhere[3,7,9,13,14,18,20,21,27]. All comprise chronically infected and highly active antiretroviral therapy (HAART)-naive study subjects, with the exception of the London acute cohort ($n = 142$), who were enrolled immediately after seroconversion between 1999 and 2004, and 54 subjects enrolled during acute infection in Japan between 1997 and 2008. Viral sequences in all 2,679 chronically infected study subjects (all of whom were HAART-naive) were determined from time points after 2000, with the exception of 9 study subjects in the Japanese chronic cohort (1998–99) and all of the British Columbia cohort (1996–99). Sequencing data were obtained from 566 study subjects in the British Columbia cohort, 53 study subjects in the Barbados cohort, 106 in the Oxford cohort, 673 in the Durban cohort, 226 in the Lusaka cohort (chronically infected subjects enrolled between 2005–08), 481 study subjects in the Perth cohort, 277 chronically infected subjects in the Kumamoto cohort, 297 in the Gaborone cohort, and 142 subjects in the acute London cohort. An additional cohort in Japan comprised 117 haemophiliacs who were infected before 1985, the majority of which were believed to have been infected in 1983, and who were enrolled and followed up in out-patient clinics since 1997. These haemophiliacs are all now on HAART except for 4 HAART-naive subjects.

**HLA-associated HIV amino acid polymorphisms studied.** Variants studied that were shown to reduce viral fitness comprised polymorphisms within the HLA-B*27-restricted Gag epitope KRWIILGLNK (Gag 263–272; R264X and L268X) and mutations in three HLA-B*57-restricted Gag epitopes: ISPRTLNAW (ISW9, Gag 147–155), KAFSPEVIPMF (KF11, Gag 162–172) and TSTLQEQIAW (TW10, Gag 240–249). T242X is strongly selected by HLA-B*5801 in addition to HLA-B*57 subtypes[7,14,17]. The HLA-B*57-associated polymorphisms at residues Gag 146, 147 and 248 are selected by all HLA-B*57 subtypes, whereas Gag 163, 165, 166 and 247 are only selected by the HLA-B*5703 subtype (refs 7, 18 and H.C., unpublished data).

**Statistics.** Polymorphism frequency in the study cohorts was compared with prevalence of the relevant HLA molecule in the study cohort using a logistic regression model. To take account of the different numbers of study subjects in each cohort, appropriate confidence limits for the mutation frequencies were calculated, using the Adjusted–Wald method for binomial variables[28]. Logistic regression was calculated by GLMStat (http://www.glmstat.com) using a binomial error distribution and a logit link function. In addition, the Spearman's rank correlation coefficient was calculated in the context of a linear regression model (data shown in Supplementary Tables 1 and 2).

**HLA class I typing.** Because HLA typing was not undertaken consistently to four-digit resolution in all cohorts, two-digit HLA types only were used for these analyses, with the exception of the HLA-B*5703-associated polymorphisms (the Barbados and Oxford cohorts being excluded from these latter analyses as HLA-B*57 subtyping data were not available). Genomic DNA samples were initially typed to an oligo-allelic (two-digit) level using Dynal RELITM reverse SSO kits for the HLA-A, HLA-B and HLA-C loci (Dynal Biotech). Refining the genotype to the allele level was performed using Dynal Biotech sequence-specific priming (SSP) kits in conjunction with the previous SSO type. HLA phenotypic frequencies were determined from the HIV-infected study cohorts themselves.

**Sequencing of viral RNA and proviral DNA.** Viral sequencing of *gag* and *pol* from plasma RNA and proviral DNA was undertaken, using primers as previously described[7,9]. PCR products were sequenced directly or they were cloned by using a TOPO TA cloning kit (Invitrogen) and then sequenced. Sequencing was done with a Big Dye terminator v1.1. cycle sequencing kit (Applied Biosystems) and analysed by an ABI PRISM 310 genetic Analyser.

**Competitive HIV-1 replication assay.** Freshly prepared H9 cells ($3 \times 10^5$) were exposed to the mixtures of paired virus preparations (300 blue cell-forming units) each of NL-432 versus mutant virus (I135T, I135V, I135R and I135L)), to be examined for their replication ability for 2 h, washed twice with PBS, and cultured as described previously[29]. On day 1, one-third of infected H9 cells were harvested and washed twice with PBS, and the proviral HIV-1 reverse transcriptase gene was sequenced (0 week). Every 7 days, the supernatant of the virus culture was transmitted to new uninfected H9 cells. The cells harvested at the end of every other passage (that is, at 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 weeks) were subjected to direct DNA sequencing of the HIV-1 reverse transcriptase gene, and the viral population change was determined by the relative peak height on the sequencing electrogram. The persistence of the original amino acid substitution was confirmed for all infectious clones used in this assay.

**HLA-B*5101 stabilization assay.** Binding of HIV-1-derived peptides to HLA-B*5101 was measured as previously described by using RMA-S-B*5101 cells[8].

**Assays to determine recognition of peptide-pulsed or virus-infected targets.** C1R and .221 cells expressing HLA-B*5101 or HLA-B*4801 were generated as previously described[30]. All cells were maintained in RPMI 1640 medium supplemented with 10% FCS and 0.15 mg ml$^{-1}$ hygromycin B. Cytotoxicity of CD8$^+$ T cells for C1R-B*5101 cells pre-pulsed with peptide measured by the standard $^{51}$Cr release assay was as previously described[8]. .221-B*4801 and .221 cells infected with NL4-3 or NL4-3 A146P mutant virus were used as target cells for intracellular cytokine staining assay.

**Generation of the NL4-3 A146P mutant virus.** The p82-2 plasmid containing the A146P mutation[4] was digested with BssHII and ApaI. The BssHII–ApaI 1.3-kb fragment was purified and then ligated into the same site of BssHII–ApaI-digested pNL-432 plasmid. To obtain pNL-432 including the A146P mutant (pNL-432 A146P), 293T cells were transfected with pNL-432 A146P using Lipofectamine 2000 (Invitrogen). Supernatants from transfected 293T cell cultures were stored at −80 °C.

**Generation of CD8$^+$ T-cell clones and peptide-specific CD8$^+$ T-cell lines.** Cytotoxic T lymphocyte (CTL) clones were generated from HIV-1-specific bulk-cultured T cells by limiting dilution as previously described[8]. Peptide-specific CD8$^+$ T-cell lines were generated by stimulating peripheral blood mononuclear cells (PBMCs) from the HLA-B*4801-positive HIV-1-seropositive individual KI-092 with the NI11 (NLQGQMVHQAI) peptide and then culturing them for 2 weeks[8]. Cytotoxicity of CD8$^+$ T cells for target cells pre-pulsed with peptide measured by the standard $^{51}$Cr release assay was as previously described[8].

**Suppression assay of HIV-1 replication by HIV-1-specific CTLs.** The ability of HIV-1-specific CTLs to suppress HIV-1 replication was examined as previously described[30].

**Intracellular cytokine staining assays.** PBMCs from HIV-1-infected individuals were stimulated with the desired peptide (1 μM) and cultured for 12–14 days. These cultured PBMCs were assessed for IFN-γ-producing activity as previously described[30].


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# High frequency and proliferation of $CD4^+FOXP3^+$ Treg in HIV-1-infected patients with low CD4 counts

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**The frequency of Treg is reported to be higher in patients with chronic HIV type 1 (HIV-1) infection and $CD45RA^+$ Treg exist in normal adults. In this study, we found a lower absolute number (15 cells/μL) but a higher proportion (16.2%) of $FOXP3^+$ cells (Treg) in the $CD4^+$ population in treatment-naïve HIV-1 patients with low CD4 (<200 cells/μL) counts than in those with high CD4 counts (34 cells/μL and 9.3%) or healthy adults (48 cells/μL and 7.5%). In HIV-1 patients, $CD45RA^+CCR7^+$, $CD45RA^-CCR7^+$, and $CD45RA^-CCR7^-$ subsets were identified in the Treg population, and the proportion of $CD45RA^-CCR7^-$ Treg was higher (57.9%) in patients with low CD4 than high CD4 counts (38.3%). Treg were in a high proliferation state especially in patients with low CD4 counts. HIV viral load correlated positively with the Treg proliferation rate and the proportion of $CD45RA^-CCR7^-$ Treg. Furthermore, the proliferation of Treg correlated positively with the $CD45RA^-CCR7^-$ Treg proportion but negatively with Treg numbers. Successful antiretroviral therapy resulted in a limited increase in Treg numbers, but their frequency was reduced in 1–2 months due to a rapid rebound of $FOXP3^-$ $CD4^+$cells. Our results suggest that HIV-activating Treg may be a reason for the high frequencies of Treg and $CD45RA^-CCR7^-$ Treg in the peripheral blood of late-stage HIV-1-infected patients.**

**Key words:** Cell proliferation · HIV · Immune regulation · Treg

Supporting Information available online

## Introduction

HIV type 1 (HIV-1) infection is characterized by a progressive loss and dysfunction of $CD4^+$ T cells [1, 2]. With regard to reduced T-cell functions, accumulating evidence suggests that the balance between the immune suppression function of natural Treg cells and the effector functions of other types of lymphoid cells influences the magnitude of immune reactions in various types of infections, e.g. those caused by *Leishmania major*, *Shistosoma mansonia*, and hepatitis C virus [3–7]. FOXP3 is not only a specific marker but also a critical lineage specification factor for Treg [8–11]. Treg are considered mainly as $CD45RA^-$ cells. However, recent studies have shown that $CD45RA^+$ cells also exist among immune-suppressing $CD25^+CTLA4^+CD4^+$ T cells in adults [12, 13].

The local interaction between Treg and other T cells plays an important role in immune suppression and the local density of Treg determines the course of immune responses to infections [4, 7, 14]. Thus, Treg can be both detrimental and beneficial to the host in response to pathogens [5, 7]. For example, in HIV-infected patients, $CD4^+CD25^+$ Treg have been reported to be proportionally increased, decreased, or highly increased in tonsils, their numbers to correlate with HIV viral load, and to exhibit suppression activity [15–23]. Furthermore, antiretroviral

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therapy (ART) has been reported to have either a negative or no influence on Treg or expression of FOXP3 [18, 23]. In HIV-1-infected individuals, immunodeficiency is often considered when the CD4 cell count falls below 200 cells/μL [1]. However, to our knowledge, there is controversy or little information about the absolute number, frequency, and status of homing markers of Treg in HIV-1-infected patients especially in those with low CD4 counts and late-stage AIDS-related diseases or not on ART [24, 25]. Little is known about the dynamic changes of Treg after ART has been introduced.

It is considered that the CCR7 molecule on T cells is an essential trafficking factor for T cells homing to lymphoid tissues as well as an important marker for defining differentiation stage of T cells with CD45RA molecule [26–28].

The present study was designed to investigate Treg in late-stage HIV-1-infected patients with CD4 count $<200$ cells/μL and the early impact of ART on Treg. We used the chemokine receptor CCR7 and CD45RA molecules to characterize distinct population of migratory Treg.

## Results

### High-frequency but low absolute numbers of Treg in HIV-1 patients with low CD4 counts

In this study, we enrolled 95 HIV-1-infected patients and 21 HIV-1-negative Japanese adults as our subjects. Because most AIDS-related diseases occur in HIV-1 patients when their CD4 count decreases to below 200 cells/μL, we classified the patients into two groups, a low CD4 group with a $CD4^+$ T cell count less than 200 cells/μL and a high CD4 group with a $CD4^+$ T cell count not less than 200 cells/μL, for some comparison analysis. Table 1 lists the demographic and clinical characteristics of HIV-1-infected patients and healthy HIV-1-negative controls.

Although FOXP3 expression is considered as the best and most specific marker of Treg, some studies have reported that CD127 and CD25 could distinguish Treg [29, 30]. Accordingly, we first compared the staining of FOXP3 with CD25 and CD127 using PBMC from HIV-1-positive individuals. As shown in Supporting Information Fig. 1A and B, $CD25^+CD127^-$ were a proportion of the CD4 cells. However, gating these cells as Treg seems difficult because of the smear staining of both CD25 and CD127. However, gating FOXP3 in CD4 cells was much easier because of the clear staining of FOXP3. Furthermore, we tested the correlation of the Treg by the two classification markers. Supporting Information Fig. 1C shows a good correlation between the proportion of $FOXP3^+$ and $CD25^+$ $CD127^-$ in CD4 cells in 18 HIV-1 patients. Therefore, in the present study, we considered the $FOXP3^+CD4^+$ cells as Treg, and called $FOXP3^-CD4^+$ cells as conventional $CD4^+$ T cells (Tcon).

In the next step, we investigated the frequency and absolute number of Treg in HIV-1-infected individuals without an ART history and compared them with those of healthy Japanese adults. Figure 1A and B shows FOPX3 expression in $CD4^+$ cells. As shown in Table 2, the proportion of Treg in CD4 cells was $16.2\pm2.6\%$ in HIV-1 patients with a low CD4 count and

**Table 1.** Demographic and clinical characteristics of subjects

| | Group[a] | | |
|---|---|---|---|
| | A (low CD4) | B (high CD4) | H (healthy) |
| *Characteristics* | | | |
| Numbers | 27 | 68 | 21 |
| Age (years, range) | 39 (21–65) | 38 (21–67) | 38 (21–60) |
| Gender (male:female) | 27:0 | 16:1 | 3:4 |
| CD4 count (cells/μL, SD) | 102 (58) | 383 (164) | 650 (178) |
| LogVL (SD) | 5 (0.6) | 4.2 (0.7) | N/A |
| AIDS-related diseases[b] (*n*, %) | 23 (85) | 11 (16) | N/A |
| Months of $HIV^+$ (range)[c] | 12.3 (0–97) | 21 (0–124) | N/A |
| *Numbers for tests* | | | |
| Frequency and subsets of Treg[d] | 20 | 39 | 21 |
| Ki67 staining *versus* FOXP3[e] | 11 | 24 | 5 |
| CCR7FOXP3 *versus* CD25[f] | 3 | 16 | |
| CD127CD25 *versus* FOXP3[g] | 6 | 12 | |

[a] Low CD4: $<200$ cells/μL; high CD4: $\geq200$ cells/μL.
[b] AIDS-related diseases included: candida, herpes simplex virus infection, tuberculosis, pneumocystis jirovici pneumonia, lymphoma (kaposis sarcoma), *etc.*
[c] Months between the date of the first time of consulting the hospital and the date of blood collected.
[d] Table 2 and Fig. 1.
[e] Figure 2 and Supporting Information Fig. 2.
[f] Figure 1C.
[g] Supporting Information Fig. 1.

 

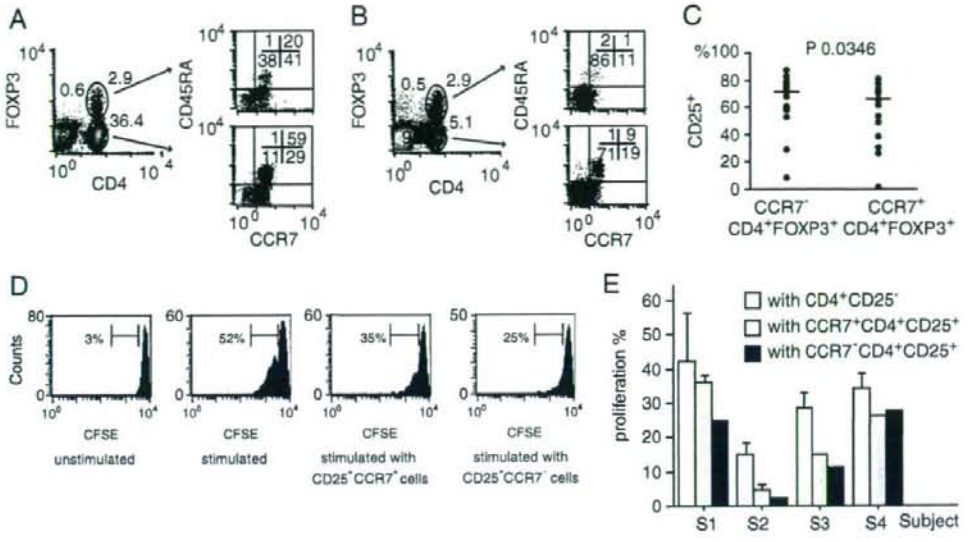


**Figure 1.** Subsets of Treg in healthy adults and HIV-1-infected patients. (A) Staining of a healthy adult. (B) Staining of an HIV-1-infected patient with low CD4 count. FOXP3 was mainly found in $CD4^+$ T cells both in healthy adults and HIV-1 patients. Treg ($FOXP3^+CD4^+$) cells could be subdivided into $CD45RA^+CCR7^+$, $CD45RA^-CCR7^+$, and $CD45RA^-CCR7^-$ subsets, similar to Tcon ($FOXP3^-CD4^+$, conventional CD4 cells). (C) In HIV-1 patients, the proportion of $CD25^+$ among $CCR7^-$ Treg was higher than that among $CCR7^+$ Treg ($p<0.05$, $n=19$). (D) A representative proliferation of $CD4^+CD25^-$ responder cells cultured with $CCR7^-CD25^+CD4^+$, $CCR7^+CD25^+CD4^+$ cells, or unlabeled $CD25^-CD4^+$ cells stimulated by anti-CD3 mAb with autologous APC (the data are derived from healthy control). (E) $CCR7^+$ and $CCR7^-$ Treg suppression of responder cells in four subjects. S1–S3: healthy subjects, S4: HIV-1-positive patient (the error bars show duplicate or triplicate tests). Horizontal bars represent median values and $p$ value represents comparison result from Wilcoxon-signed rank test.

$9.3\pm0.5\%$ in patients with a high CD4 count. The absolute counts of Treg in low CD4 and high CD4 groups were $15\pm3$ and $34\pm2$ cells/μL, respectively. In healthy adults, the mean CD4 count was 650 cells/μL, and the frequency of Treg among $CD4^+$ cells was $7.5\pm0.5\%$ with a mean absolute number of $48\pm4$ cells/μL. Therefore, HIV-1 patients with low CD4 counts had a lower absolute count but a significantly higher frequency of Treg than HIV patients with high CD4 and healthy controls.

## High proportion of $CD45RA^-CCR7^-$ Treg in HIV-1 patients with low CD4

Considering the distinct homing potentials and effector functions, CD4 T cells could be subdivided into three subsets, namely naïve ($CD45RA^+CCR7^+$), central memory ($CD45RA^-CCR7^+$), and effector memory ($CD45RA^-CCR7^-$) cells, based on their surface marker and cytokine secretion [26]. Given that local interaction of Treg and Tcon plays an important role in immune suppression and the local number and/or density of Treg reflects immune suppression, we next investigated whether Treg have the same characteristic phenotype as Tcon. Figure 1A shows that Treg could be divided into three subsets, similar to Tcon, based on CD45RA and CCR7 staining in healthy controls. Interestingly, the proportion of each subset of Treg was different compared with the respective subsets of Tcon (Table 2). In healthy adults, the proportion of $CD45RA^-CCR7^-$ Treg ($39.7\pm2\%$) was higher than $CD45RA^-CCR7^-$ Tcon cells ($15.6\pm1.2\%$), but the proportion of $CD45RA^+CCR7^+$ Treg ($19.3\pm1.6\%$) was lower than $CD45RA^+CCR7^+$ Tcon cells ($45.8\pm2.4\%$).

In HIV-1-infected patients, the staining patterns of intracellular FOXP3 and surface CD4, CD45RA, and CCR7 were similar to those in healthy controls (Fig. 1A and B). Figure 1B shows a high proportion of $CD45RA^-CCR7^-$ Treg in a representative patient with a low CD4 count. As shown in Table 2, the proportion of $CD45RA^-CCR7^-$ Treg in the low CD4 group ($57.9\pm4.2\%$) was significantly higher than in the high CD4 ($38.3\pm1.8\%$) or control groups ($39.7\pm2\%$). In contrast, the proportion of $CD45RA^-CCR7^+$ Treg in patients with low CD4 counts was significantly lower than in those with high CD4 counts and the control groups. In all subject groups, the proportions of $CD45RA^-$ cells in Treg were higher than in Tcon. Moreover, we found that in HIV-1-infected patients, the proportion of $CD25^+$ in $CCR7^-$ Treg ($64\pm19\%$) was higher than in $CCR7^+$ Treg ($58.8\pm21\%$, Fig. 1C).

$CD45RA^+$ Treg have been reported to show suppressive function [12]. Based on the finding of a high proportion of $CCR7^-$ Treg in patients with a low CD4 count (Table 2), and considering that $CCR7^+$ cells tend to home to lymphoid tissues whereas $CCR7^-$ cells tend to move to peripheral tissues, we next investigated whether there is any difference in the suppressive activity between $CCR7^+$ and $CCR7^-$ Treg. The results showed

**Table 2.** Comparison of Treg and Tcon in healthy persons and HIV-1-infected patients[a)]

| | Healthy (H) | HIV-1(+)ART(–) | | p value | | |
|---|---|---|---|---|---|---|
| | | CD4<200 (A) | CD4≥200 (B) | A versus B | A versus H | B versus H |
| Number of subjects | 21 | 20 | 39 | | | |
| Lymphocytes (cells/μL) | 1718 (381) | 1028 (447) | 1661 (579) | <0.0001 | <0.0001 | NS |
| CD4 (cells/μL) | 650 (178) | 108 (58) | 395 (195) | <0.0001 | <0.0001 | <0.0001 |
| CD4 (%) | 38.4 (8.6) | 11.4 (7.6) | 20.5 (8.5) | 0.0001 | <0.0001 | <0.0001 |
| Treg (cells/μL) | 48 (16) | 15 (11) | 34 (14) | <0.0001 | <0.0001 | 0.0008 |
| Treg (%) | 7.5 (2.4) | 16.2 (11.8) | 9.3 (3.4) | 0.0137 | 0.0004 | 0.0464 |
| Treg (%) | | | | | | |
| $CCR7^+$ | 57 | 40.1 | 59.6 | 0.0001 | 0.0029 | NS |
| $CD45RA^+CCR7^+$ | 19.3 | 13.4 | 21.1 | 0.0109 | 0.0504 | NS |
| $CD45RA^-CCR7^-$ | 39.7 | 57.9 | 38.3 | 0.0001 | 0.0006 | NS |
| $CD45RA^-CCR7^+$ | 37.7 | 26.7 | 38.5 | 0.0005 | 0.0057 | NS |
| $CD45RA^-$ | 77.4 | 84.6 | 76.8 | 0.0131 | 0.0419 | NS |
| Tcon (%) | | | | | | |
| $CCR7^+$ | 81.3 | 55.8 | 74.8 | 0.0178 | 0.0035 | NS |
| $CD45RA^+CCR7^+$ | 45.8 | 31.9 | 41.1 | NS | 0.0217 | NS |
| $CD45RA^-CCR7^-$ | 15.6 | 36.8 | 22.1 | 0.0283 | 0.0035 | 0.04 |
| $CD45RA^-CCR7^+$ | 35.5 | 23.9 | 33.7 | 0.0048 | 0.0045 | NS |
| $CD45RA^-$ | 51.1 | 60.7 | 55.8 | NS | NS | NS |
| p Value (Treg versus Tcon) | | | | | | |
| $CCR7^+$ | <0.0001 | 0.0187 | <0.0001 | | | |
| $CD45RA^+CCR7^+$ | <0.0001 | 0.0001 | <0.0001 | | | |
| $CD45RA^-CCR7^-$ | <0.0001 | 0.0004 | <0.0001 | | | |
| $CD45RA^-CCR7^+$ | NS | NS | 0.005 | | | |
| $CD45RA^-$ | <0.0001 | <0.0001 | <0.0001 | | | |

[a)] Data are means (SD). NS: not significant. CD4<200, CD4≥200: 200 cells/μL. Mann–Whitney U-test was used for comparison between groups (A versus B, A versus H, B versus H). Wilcoxon-signed rank test was used for comparison in group (Treg versus Tcon).

that both $CCR7^+$ and $CCR7^-$ $CD25^+CD4^+$ cells suppressed the proliferation of responder cells (Fig. 1D). The suppressive activity was observed in three healthy controls and one HIV-1 patient (Fig. 1E), although no difference was found in the suppression function between the $CCR7^+$ and $CCR7^-$ Treg.

The above results demonstrated the existence of $CD45RA^+CCR7^+$, $CD45RA^-CCR7^+$, and $CD45RA^-CCR7^-$ Treg subsets, similar to Tcon. The proportion of $CCR7^+$ Treg was lower than $CCR7^+$ Tcon cells in both healthy controls and HIV-1 patients. However, the proportion of $CD45RA^-CCR7^-$ Treg was higher than $CD45RA^-CCR7^-$ Tcon, particularly in patients with low CD4 count.

## High proliferation of Treg correlates with HIV-1 viral load

Immune cells are activated in HIV-infected patients and such activation is linked to CD4 cell depletion [31]. To determine the mechanism of the high frequency of Treg and $CD45RA^-CCR7^-$ Treg in advanced HIV patients, we stained CD4 cells for the proliferation markers Ki67 in 24 patients (including 11 patients with low CD4 counts and 13 patients with high CD4 counts) and five healthy controls. Figure 2A shows that there was no difference between gating the Ki67 in Treg and Tcon in a healthy control and an HIV-1-infected person. As shown in Fig. 2, the proportions of Ki67-stained cells among Treg in low CD4, high CD4, and control groups (41.7, 24.5, and 22.3%, respectively) were higher than those in Tcon cells (18.1, 11.8, and 7.4%, respectively) (Fig. 2B). The expression of Ki67 in both Treg and Tcon cells was higher in patients with low CD4 counts than in those with high CD4 counts and healthy controls. Furthermore, in the 24 HIV-1-infected patients assessed for Ki67, HIV-1 viral load showed a positive correlation with the frequency of Ki67 in Treg and the proportion of $CD45RA^-CCR7^-$ in Treg. However, the CD4 count showed a negative correlation with the frequency of Ki67 in Treg (Fig. 2C). Moreover, the frequency of Ki67 in Treg correlated negatively with the Treg count and the proportion of $CD45RA^+CCR7^+$ in Treg, but positively with the proportion of $CD45RA^-CCR7^-$ in Treg (Fig. 2D). The same correlation was also observed in Tcon cells (Supporting Information Fig. 2).

## ART reduces the frequency of Treg

In HIV-1-infected patients, ART can effectively reduce the HIV viral load and improve CD4 counts. In highly active ART-treated patients, a depleted or normalized Treg was observed in

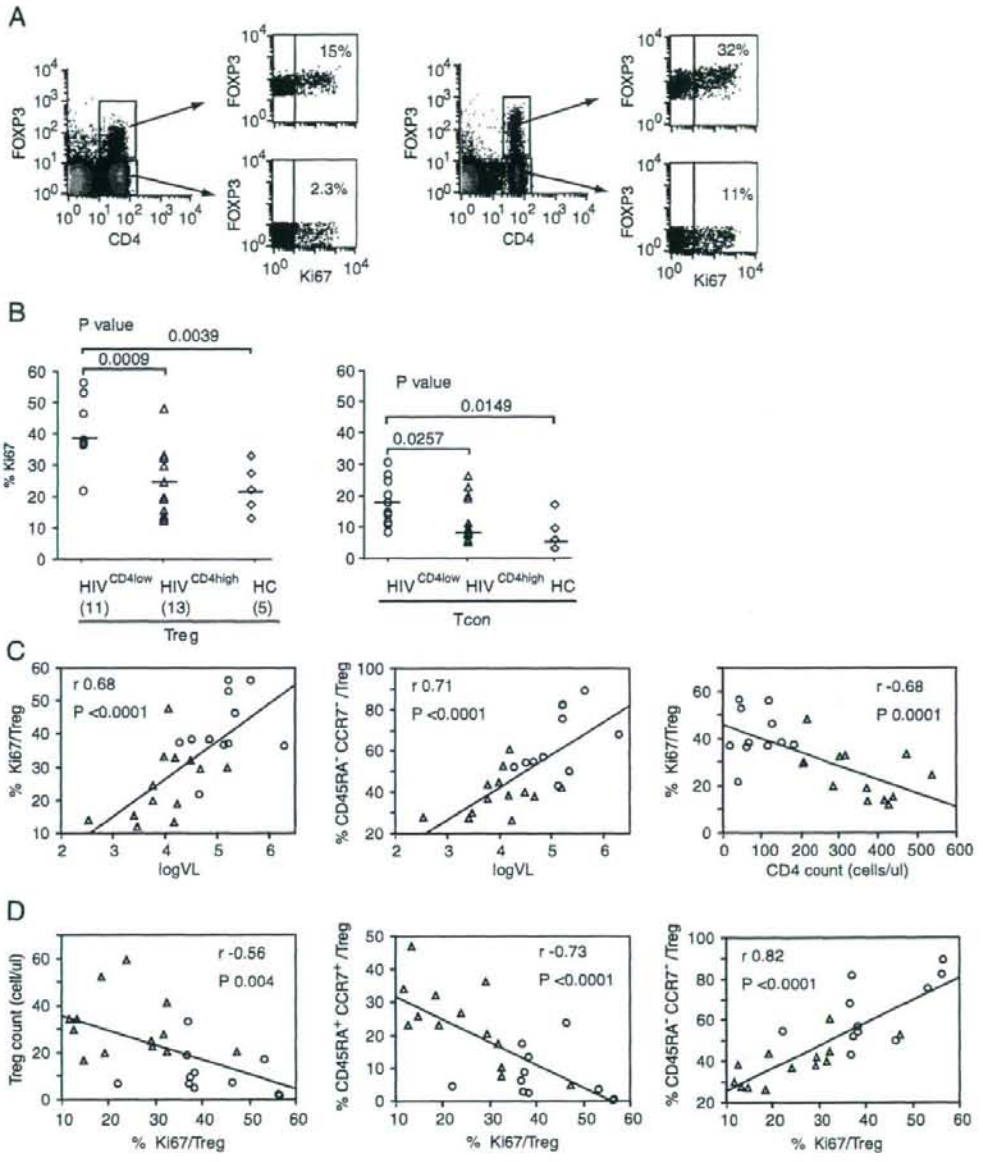


**Figure 2.** Ki67 staining and high proliferation rate of Treg is associated with viral load. (A) Gating of Ki67 in FOXP3$^+$ and FOXP3$^-$ CD4$^+$ cells in a healthy control (left panel) and an HIV-1-infected person (right panel). (B) Proportion of Ki67-positive Treg (left panel) is higher than that of Ki67-positive Tcon cells (right panel) in healthy controls (HC), HIV-1-infected patients with low CD4 count (HIV$^{CD4low}$) and HIV-1-infected patients with high CD4 count (HIV$^{CD4high}$) (numbers in parentheses represent the number of subjects tested). The percentages of Ki67-positive Treg and Tcon cells in the low CD4 group are higher than those in the high CD4 group and healthy control, respectively. (C) HIV-1 viral load shows a positive correlation with the percentage of Ki67 in Treg (left panel) and the proportion of CD45RA$^-$CCR7$^-$ Treg (middle panel). The CD4 count shows a negative correlation with the percentage of Ki67 in Treg (right panel). (D) The percentage of Ki67 in Treg shows correlation negatively with Treg count (left panel) and the proportion of CD45RA$^+$CCR7$^+$ Treg (middle panel) but positively with the proportion of CD45RA$^-$CCR7$^-$ Treg (right panel). Horizontal bars represent median values and $p$ values represent results from Wilcoxon-signed rank test. Simple regression was used for correlation analysis.

PBMC and mucosal tissue [23, 32]. To investigate the impact of ART on Treg, we checked the dynamic change in Treg, their proliferation state, and subsets in nine patients until 9 months after commencement of ART (Fig. 3). The plasma viral load decreased sharply soon after commencement of ART (Fig. 3A). Associated with the decrease in viral load was a rise in the $CD4^+$ count especially in the first 2 months of ART. The CD4 count increased more than 100 cells/μL average in the first month (Fig. 3B). The absolute count of Treg increased in the first month but decreased to some extent thereafter (Fig. 3C); the frequency of Treg decreased rapidly to normal levels within 1–2 months of commencement of ART in all patients (Fig. 3D). On the other hand, the change in the proportion of Ki67 among Tcon and Treg showed a complex pattern. The proportion of Ki67 among Tcon cells increased in the first month of treatment and then decreased and fluctuated on a small scale thereafter (Fig. 3E). However, in the first 1–2 months of ART, the proportion of Ki67 among Treg decreased but maintained high levels until 9 months of ART (Fig. 3F). There was no significant change in each subset in both Treg and Tcon (Fig. 3G and H). However, the $CD45RA^-CCR7^-$ subset still accounted for a high proportion, especially in Treg (Fig. 3G and H, the right panels). The detailed change of each item in each patient is shown in Supporting Information Fig. 3. These results suggest that after initiation of ART, the slow change in the absolute number of Treg and the rapid rebound of Tcon counts resulted in a rapid normalization of the frequency of Treg in HIV-1 patients.

## Discussion

Regulation of the immune response is important in maintaining self-tolerance. However, in individuals with immunodeficiency, such as patients with HIV infection, severe immune suppression may contribute to progression of AIDS. Previous studies reported activation of the immune system in HIV-1-infected patients and indicated that human $CD4^+CD25^{high}FOXP3^+$ Treg cells are derived through rapid turnover of memory populations *in vivo* [31, 33, 34].

In the present study, we found that untreated HIV-1-infected patients with low CD4 counts have a high frequency of Treg and $CD45RA^-CCR7^-$ Treg. Cell proliferation was higher in Treg than Tcon cells, especially in HIV-1 patients with low CD4 counts. In these patients, both Tcon and Treg showed a high proliferation state, particularly about 40% Treg were Ki67-positive. Ndhlovu *et al.* [22] reported that $FOXP3^+CD127^{lo}$ $CD4^+$ T cells in PBMC showed a strong negative correlation with T-cell activation during the early chronic stage of HIV infection. In our study, we also found a negative correlation between the proliferating frequency of Treg and Treg absolute count. However, we found that the proliferation of Treg correlated positively with the proportion of $CD45RA^-CCR7^-$ Treg. Furthermore, HIV viral load showed a positive correlation with both Treg proliferation and the proportion of $CD45RA^-CCR7^-$ Treg. These results suggest that HIV infection may activate Treg and result in an increased proportion of $CD45RA^-CCR7^-$ among Treg. On the other hand, Epple *et al.* [32] reported that the frequency and absolute counts of mucosal Treg were highly increased in untreated HIV patients. This finding may be considered another reason for our results because $CCR7^+$ lymphocytes tend to home to lymph nodes and lymphoid tissues. Therefore, we consider that in HIV-infected patients, HIV could simultaneously activate the differentiation of Treg as well as stimulate $CCR7^+$ Treg homing to lymph nodes and lymphoid tissues. These two effects of HIV on Treg result in the high frequency of Treg and a high proportion of $CD45RA^-CCR7^-$ Treg in peripheral blood in patients with low CD4 counts.

ART has been a great success in controlling HIV replication and aiding the recovery of CD4 T cells. However, data about its impact on Treg, especially in detail, are rare. In the current study, we observed that with the rapid decrease in viral load was a robust rebound of Tcon 1–2 months after ART initiation; however, the number of Treg increased in some patients but was almost unchanged in others. The unbalanced change in Tcon and Treg resulted in the frequency of Treg decreasing precipitously to normal levels in the first 1–2 months of therapy. Although the viral load decreased to a very low level in a short period after ART introduction, the proliferative state of Tcon and Treg did not decrease significantly. On the contrary, both Tcon and Treg maintained a high proliferation level, especially Treg. Moreover, the three subsets, *i.e.* $CD45RA^+CCR7^+$, $CD45RA^-CCR7^+$, and $CD45RA^-CCR7^-$ in Tcon and Treg did not show a robust change till 9 months. The results suggest that the recovery of phenotypes needs a much longer period, even if they can recover after ART.

Chase *et al.* [23] observed Treg depletion in highly active ART-treated HIV-1 patients but not in elite suppressors. Here, we did not observe depletion of Treg counts after ART introduction, but we indeed noticed a rapid normalization of the Treg frequency. As we know, to do the suppression assay *in vitro*, an appropriate ratio of Treg to responder cells is needed for observing significant suppression. Considering the suppressive function of both $CCR7^+$ and $CCR7^-$ Treg, we think that the high frequency of Treg, but not the low absolute number of Treg, provides a much better suppressive marker in treatment-naïve HIV-1 patients with low CD4 counts. On the other hand, ART may induce some improvement of the immune suppression because it could reduce the frequency of Treg.

In summary, our results of high frequencies of Treg and $CD45RA^-CCR7^-$ Treg, which tend to migrate to non-lymphoid tissues, in untreated HIV-1 patients with low CD4 counts, emphasize the potential role of Treg in immune deficiency in late-stage HIV-1 infection. Furthermore, anti-HIV treatment could result in a rapid rebound of conventional T cells but not a robust improvement of Treg within 9 months after ART initiation. The different response of Treg and Tcon to ART leads to a rapid decrease in the frequency of Treg. Recently, immune reconstitution syndrome (IRS) is becoming an important problem in HIV treatment. Most IRS occurs in 1–3 months after commencement

 

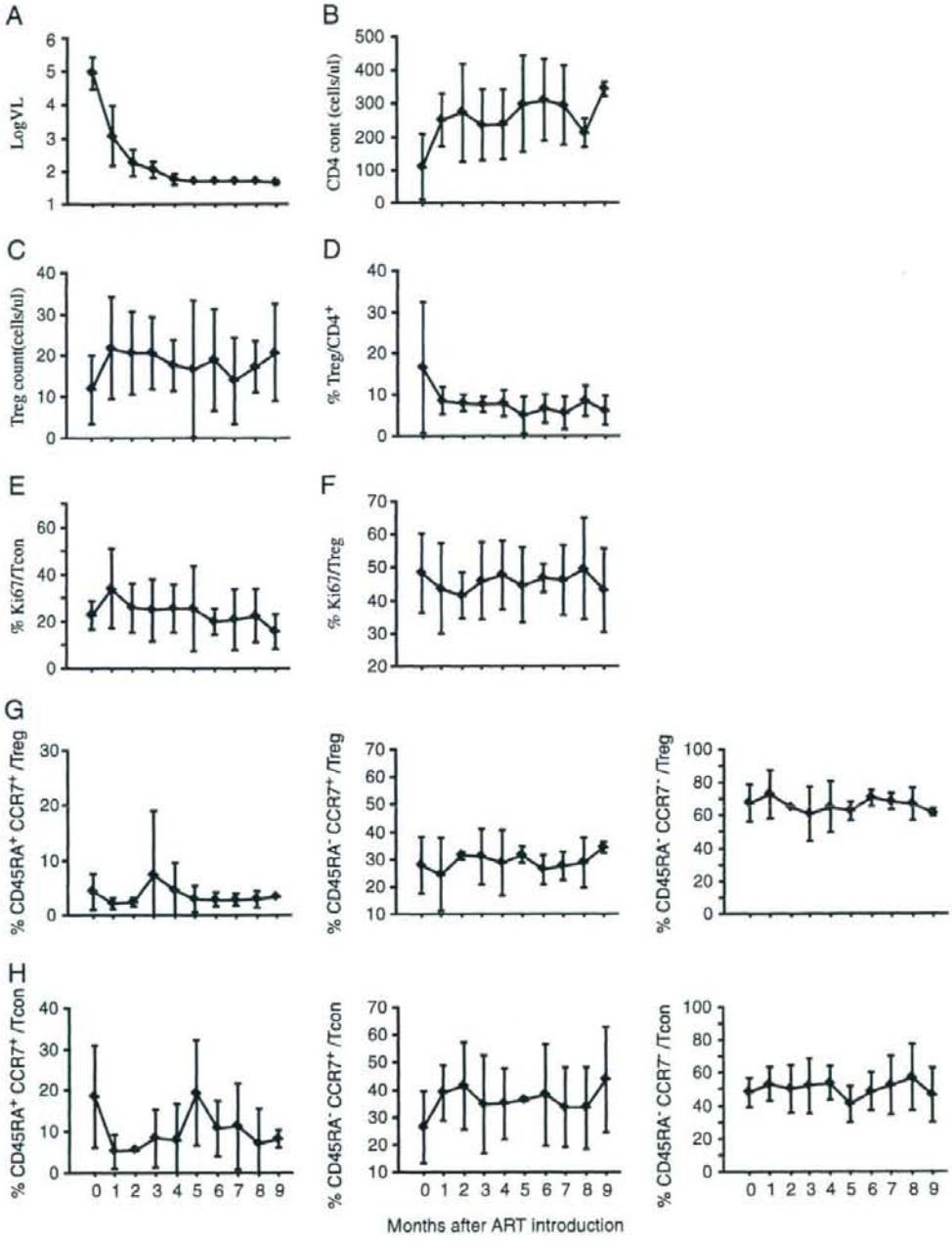


**Figure 3.** Serial changes in Treg and Tcon cells after commencement of ART. Commencement of ART resulted in rapid reduction in HIV viral load (A) and increase in $CD4^+$ cell count (B). Treatment caused increase in the absolute number of Treg in the first month, then fluctuated slightly thereafter (C), but resulted in a sharp decrease in their percentages in 1 month (D). The proportion of Ki67-positive Tcon increased in the first month but decreased in some extent thereafter (E), while the proportion of Ki67-positive Treg showed some change but still retained a high level at 9 months of commencement of ART (F). At 9 months after ART started, the recovery of the proportion of $CD45RA^+CCR7^+$ Treg (G, left panel) and Tcon (H, left panel) seems very slow, while the proportion of $CD45RA^-CCR7^+$ Treg (G, middle panel) and Tcon (H, middle panel) increased in some extent. However, the proportion of $CD45RA^-CCR7^-$ Treg (G, right panel) and $CD45RA^-CCR7^-$ Tcon (H, right panel) showed a small-scale change, but $CD45RA^-CCR7^-$ Treg maintained a high proportion till 9 months. (A–F) was from nine patients, while (G–H) was from six of them. Vertical bars represent mean ± 1SD.

 

of ART. Thus, we suppose that the unbalanced improvement of conventional CD4 cells and Treg after commencement of ART might be a factor for IRS. However, this issue needs more investigation.

## Materials and methods

### Subjects

The subjects were 95 HIV-1-infected patients who have not received any ART and gave written consent before enrollment in this study at the AIDS Clinical Center, International Medical Center of Japan, Tokyo. Nine patients who started ART were followed up for investigation of the impact of ART on Treg. Twenty-one HIV-1-negative adults were recruited as healthy controls. The demographic and clinical characteristics of the subjects are listed in Table 1. HIV-1 viral load was quantified by AMPLICOR HIV-1 MONITOR Test (Roche Diagnostics).

### Cell preparation

PBMC were prepared from blood samples collected into EDTA-containing tubes by Ficoll-paque gradient centrifugation. Ki67 staining and evaluation of the ART-treated patients were carried out using cryopreserved PBMC.

For suppression assay, $CD4^+$ cells were isolated from freshly prepared PBMC by using $CD4^+$ T-cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to instructions provided by the manufacturer. $CD4^+$ cells were separated by anti-CD25 mAb (PE) and anti-PE Multisort Kit (Miltenyi) into $CD25^-$ and $CD25^+$ cells. After microbeads release, $CD25^+$ cells were sorted into $CCR7^+$ and $CCR7^-$ cells by using anti-CCR7 mAb (FITC, mouse IgG2a, R&D Systems, Minneapolis, MN) and Rat Anti-Mouse IgG2a+b Microbeads (Miltenyi). The $CD4^+$ $CD25^-$ cells were labeled by 2 μM 5-6-CFSE as responder cells in the suppression assay. Unlabeled $CD4^+CD25^-$ cells were used as non-Treg for cell number control. PBMC that were depleted of $CD3^+$ cells by CD3 MicroBeads (Miltenyi) and irradiated with 3000 rad were used as APC.

### Cell staining and flow cytometry

Freshly isolated PBMC were surface stained and also stained intracellularly for FOXP3 (PE/APC labeled, clone PCH101, eBioscience, San Diego, CA) and other markers. The stained cells were analyzed on Becton Dickinson FACSCalibur with CellQuest software (BD Bioscience, San Jose, CA). The monoclonal antibodies used in these staining procedures included anti-CCR7-FITC, anti-CD4-perCP, anti-CD25-PE, anti-CD45RA-APC/perCP, anti-Ki67-PE (BD PharMingen, San Diego, CA), and anti-CD127-FITC (eBioscience).

### *In vitro* suppression assay

In a 96-well, round-bottom plate coated with anti-CD3 mAb (0.25–0.5 μg/mL), $5 \times 10^4$ CFSE-labeled $CD4^+CD25^-$ cells were seeded and followed by adding autologous APC ($2.5 \times 10^4$). For testing Treg suppression, the same number of $CD4^+CD25^+$ $CCR7^+$ or $CCR7^-$ cells was added as regulatory cells. In control wells, the same number of unlabeled non-Treg $CD4^+CD25^-$ cells was added in order to adjust cell numbers in each well. After 3–4 days culture in an incubator at 37°C under 5% $CO_2$, the cells were harvested and analyzed on FACSCalibur. Live cells were gated and the dilution of CFSE was measured as proliferation of responder cells.

### Statistical analysis

Data are expressed as mean ± SD. Differences between groups or stratified groups were examined for statistical significance using Mann–Whitney *U*-test and Wilcoxon-signed rank test. Simple linear regression was used for correlation analysis. All analyses were conducted using the StatView software (version 5.0). A $p$ value of $<0.05$ was considered statistically significant.

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*Abbreviations:* **ART:** antiretroviral therapy · **HIV-1:** HIV type 1 · **IRS:** immune reconstitution syndrome · **Tcon:** conventional CD4$^+$ T cells

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Original article

# HLA-A*2402-restricted HIV-1-specific cytotoxic T lymphocytes and escape mutation after ART with structured treatment interruptions

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**Abstract**

Although a limited duration of immune activation of structured treatment interruptions (STIs) has been reported, the immune escape mechanism during STIs remains obscure. We therefore investigated the role of three immunodominant cytotoxic T lymphocyte (epitopes) in 12 HLA-A*2402-positive patients participating longitudinally during the clinical study of early antiretroviral treatment (ART) with five series of structured treatment interruptions (STIs). The frequency of HLA-A*2402-restricted CTLs varied widely and a sustained CTL response was rarely noted. However, a Y-to-F substitution at the second position in an immunodominant CTL epitope Nef138-10 (Nef138-2F), which was previously demonstrated as escape mutation, was frequently detected in seven patients primarily and emerged in the remaining five patients thereafter, and the existence of escape mutations was correlated with high pVL levels early in the clinical course. These findings suggest that escape mutation in the immunodominant CTL epitope may be one of the mechanisms to limit HIV-1-specific immune control in STIs.


*Keywords:* Structured treatment interruptions; Cytotoxic T lymphocyte; HLA-A*2402; Escape variant

## 1. Introduction

Structured treatment interruption (STI) is considered one of the immune stimulatory interventions for HIV-1 infection, based on the hypothesis that viral rebound during treatment interruption might induce HIV-specific immune responses [1–3]. Since the 1999 case report of the early-treated patient who achieved sustained viral suppression without highly antiretroviral therapy (HAART) after two occasional treatment interruptions [1], the STI strategy has been studied in various clinical settings [4–7]. Because cytotoxic T lymphocytes (CTLs) play a critical role in the control of HIV-1 replication and HIV-specific CD4+ T-cell response is important to maintain effective HIV-1-specific CTLs [8-11], early treatment that can preserve HIV-1-specific-CD4+ T cells is considered to have the greater impact on STI in early infection than in chronic infection [11–13]. However, the majority of previous STI trials revealed the limitation of immune activation with risk of viral resistance [4,14,15] and the mechanisms of viral control failure in STI strategy have remained unclear.

Viral mutation in immunodominant epitopes is one of the obstacles to HIV-1 vaccine development [16–21]. Since HIV-1-specific T-cell responses are restricted by HLA alleles, its escape variant can be transmitted and adopted in populations sharing some dominant HLA alleles [19–21]. In Japan where HLA-A*2402 is the most frequent HLA class I allele with 70% prevalence, HLA-A*2402-restricted CTLs and its immunodominant epitopes have been extensively assessed [22]. Nef138-10, which has been proved previously as an HLA-A*2402-restricted CTL epitope provoking strong cytolytic activity [22], is one of the immunodominant CTL epitopes in HLA-A*2402-positive Japanese patients [21,22].

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