the matched co-culture were high (Figure 4a). In the presence of AMD3100, the RL activity of the co-culture of 293FT cells expressing NL4-3-derived *env* with N4X4-DSP$_{1-7}$ cells was reduced by 83%. The RL activity of the co-culture of 293FT cells expressing BaL-derived *env* with N4X4-DSP$_{1-7}$ cells was low in the absence of AMD3100 and was not affected significantly by its presence. The RL activity of the co-culture of 293FT cells expressing BaL-derived *env* with N4R5- DSP$_{1-7}$ was reduced by 81% in the presence of maraviroc. The RL activity of the co-culture of 293FT cells expressing NL4-3-derived *env* with N4R5- DSP$_{1-7}$ was low regardless of the presence or absence of maraviroc. The results indicated that DSP-Pheno could be used as an assay for entry inhibitors.

Cell-fusion assay of clinical samples

To evaluate assay performance using clinical samples, we selected plasma samples from 101 treatment-naïve, HIV-1-positive patients, whose infection with clade B viruses had been confirmed (data not shown). The patient population was classified into two groups based on CD4 T cell count. The low CD4 group consisted of 57 patients with CD4 T cell counts <350 cells/μl; median 228 (range 2–350) cells/μl, and median viral load was 4.77 (range 2.97–6.62) log 10 copies/ml (Figure 5a and b). The high CD4 group consisted of 44 patients with CD4 cell counts >350 cells/μl; median 442 (range 351–843) cells/μl, and median viral load was 4.04 (range 1.60–5.41) log 10 copies/ml. The viral load differences

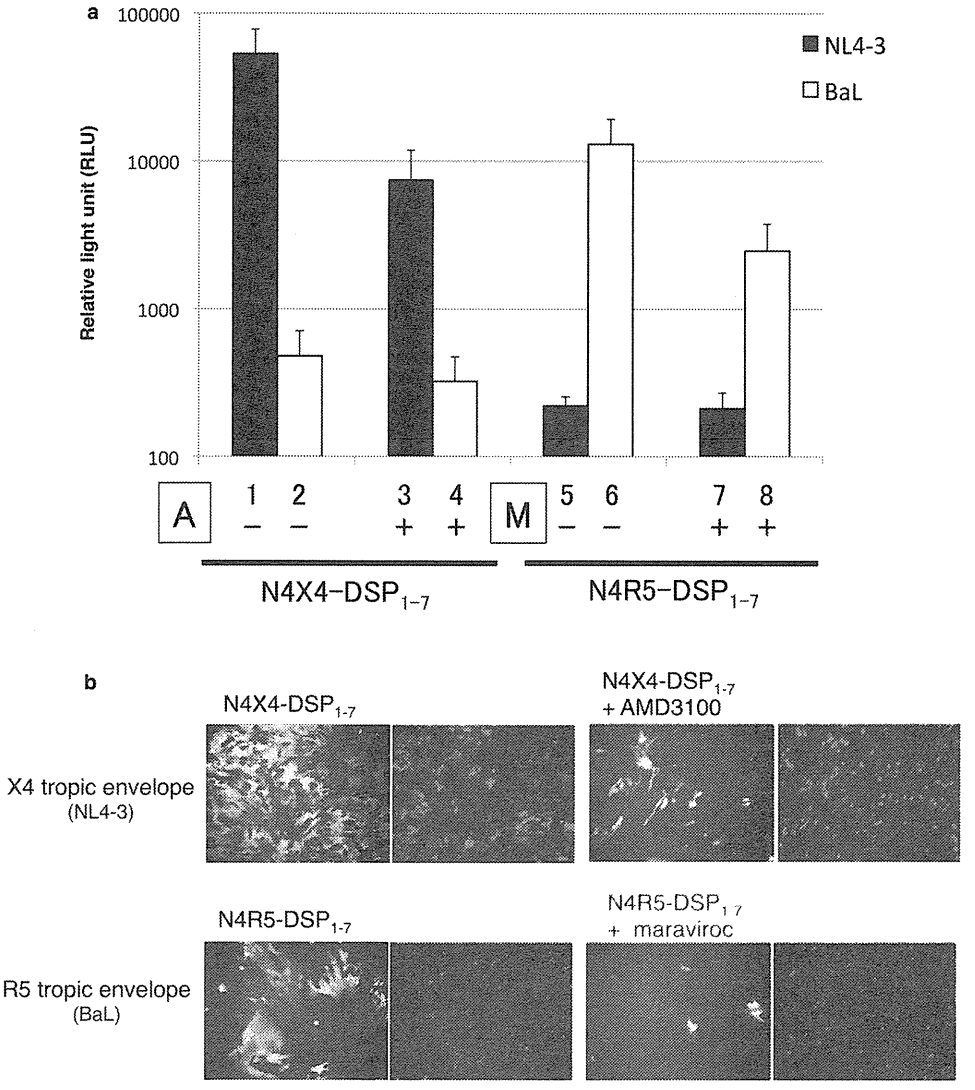

Figure 4. Inhibition of cell fusions by entry inhibitors. Two μM/well of CXCR4 inhibitor AMD3100 or CCR5 inhibitor maraviroc were added into N4X4-DSP$_{1-7}$ and N4R5-DSP$_{1-7}$ cells 90 minutes prior to cell-fusion assay using env derived from reference strains.(a) RL activities. Columns show the mean RLU ± SD from 5 independent experiments. Black columns, RL activities of env derived from X4 reference strain (NL4-3); white columns, RL activities of env derived from R5 reference strain (BaL). Results from X4-indicator (N4X4-DSP$_{1-7}$) (lanes 1–4) and R5-indicator (N4R5-DSP$_{1-7}$) (lanes 5–8). A, AMD3100; M, maraviroc. Presence or absence of inhibitor indicated by + or −, respectively. (b) GFP activities. Green fluorescence in the left panel of each pair shows successful cell fusions; red spots in the right panels show the successful transfection. Reference strains, indicator cells and inhibitors used are shown in the figure.

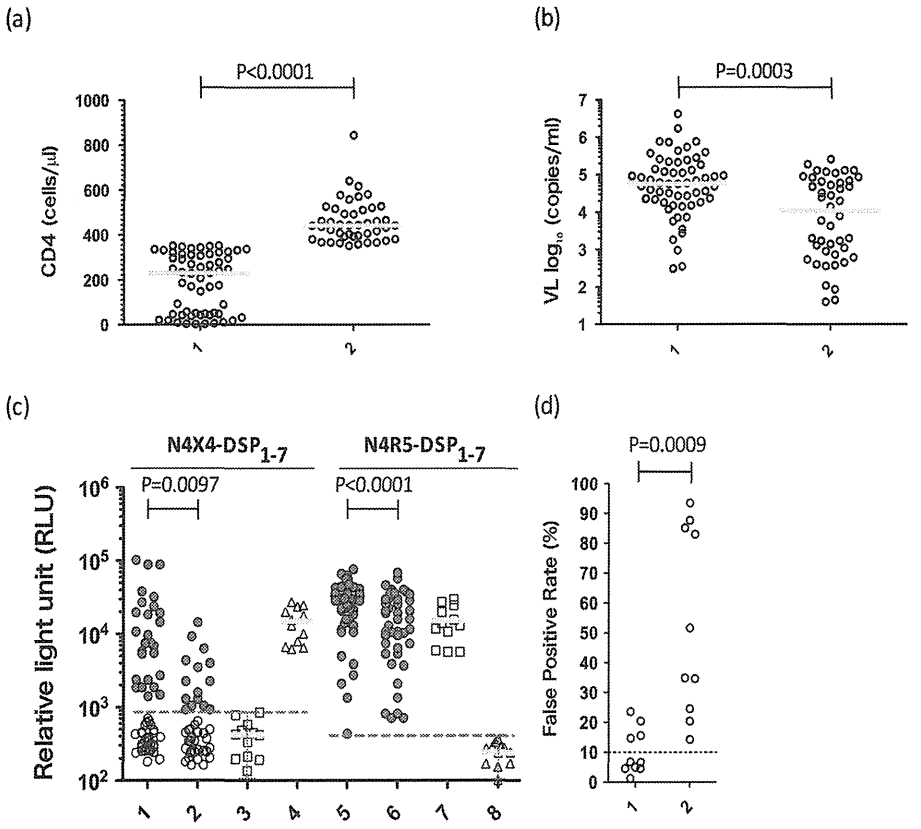

Figure 5. DSP-Pheno and Geno2Pheno on clinical samples. Patients were assigned to one of the two groups based on CD4+ T cell counts. Horizontal green bars indicate the median value. (a) CD4 counts of the patients. Lane 1, Fifty-seven patients with CD4 < 350 cells/μl, median = 228 (range 2–350) cells/μl. Lane 2, Forty-four patients with CD4 > 350 cells/μl, median 442 (range 351–843) cells/μl. (b) Viral load of each group. Lane 1, CD4 < 350 group, median viral load = 4.77 (range 2.97–6.62) log10 copies/ml. Lane 2, CD4 < 350 group, median viral load = 4.04 (range 1.60–5.41) log 10 copies/ml. (c) Mean luciferase activities of the patients' plasma samples. Lanes 1 and 5, CD4 < 350 group; lanes 2 and 6, CD4 > 350 group; lanes 3 and 7, R5 controls (BaL); lanes 4 and 8, X4 controls (NL4-3). Dashed red lines are the cut-off value, that is, the mean value + 2SD based on 3 three determinations in 12 independent experiments for each combination of negative control and indicator cell. (d) Geno2Pheno [co-receptor] analysis of representative samples. Lane 1, 10 samples from dual/X4 [N4X4-DSP$_{1-7}$ (+), N4R5-DSP$_{1-7}$ (+)] group by DSP-Pheno; Lane 2, 10 samples from R5 [N4X4-DSP$_{1-7}$ (−), N4R5-DSP$_{1-7}$ (+)] group by DSP-Pheno. For dual/X4 and R5 group, five patients each from CD4 < 350 and CD4 > 350 groups were chosen. Dashed line indicates the cut-off value as 10% of FPR.

between the two groups were statistically significant by the Mann–Whitney *U* test ($p < 0.001$). Aliquots of viral envelope DNA from each plasma sample were used to construct pRE11-envbulk for transfection into 293FT cells. The plasma viral load necessary for the assay was roughly 3.00 log 10 copies/ml for subtype B viruses, although we could amplify the env gene in a patient with 1.60 log 10 copies/ml.

We used the laboratory strain, BaL as the R5 control and NL4-3 as the X4 control to define the cut-off values. We examined BaL on N4X4-DSP$_{1-7}$ cells and NL4-3 on N4R5-DSP$_{1-7}$ cells. We defined the cut-off value tentatively as the mean value + 2SD based on 3 determinations in 12 independent experiments for each combination of negative control and indicator cell (red dashed line in Figure 5c). As expected, both combinations showed stably low RL activities, with cut-off values of 876 for N4X4-DSP$_{1-7}$ cells and 397 RLU for N4R5-DSP$_{1-7}$ cells.

Samples from all patients gave positive RL signals on R5 indicator cells (N4R5-DSP$_{1-7}$) in the fusion assay, which suggested that the bulk of virus in each patient was able to use CCR5 as the co-receptor (Figure 5c, lanes 5 and 6). Median RLU value of the low CD4 group was significantly higher than that of the high CD4 group on R5 indicator cells ($p < 0.0001$). Median RLU value of the low CD4 group was also higher significantly on X4 indicator cells ($p = 0.0097$) and 26/57 (46%) of low CD4 cases versus 15/44 (34%) of high CD4 cases gave positive RL signals (Figure 5c, lanes 1 and 2). Higher fusion activities on both indicator cells are compatible with higher viral loads in patients with lower CD4 T cell counts and may suggest more dual or X4 tropic (dual/X4) viruses in this group of patients.

To compare the result with conventional GTA, we selected 10 samples each from dual/X4 [N4X4-DSP$_{1-7}$ (+), N4R5-DSP$_{1-7}$ (+)] and R5 [N4X4-DSP$_{1-7}$ (−), N4R5-DSP$_{1-7}$ (+)] cases. Env V3 nucleotide sequences from pRE11-envbulk plasmids were subjected to the Geno2Pheno [co-receptor]. R5-representative samples showed significantly higher FPR than dual/X4-representative samples ($p = 0.0009$) (Figure 5d). DSP-Pheno and Geno2Pheno gave concordant results in 10/10 R5 and 6/10 dual/X4 samples (Figure 5d).

Although there were four samples with discordant result in dual/X4 samples, FPR of these samples were low (range: 14.7–23.6%).

Discussion

We developed a quick, safe and sensitive HIV-1 PTA utilizing double split proteins (DSP-Pheno) and validated the specificity of the assay using laboratory strains with known co-receptor usage. We recognize several limitations of this preliminary study, but the results nevertheless are promising. We assayed bulk envelope genes amplified from plasma from HIV-1-infected patients, rather than cloned envelope genes, and our sample only included subtype B HIV-1. Future studies are necessary to demonstrate the usefulness of the DSP-Pheno.

One caveat of the DSP-Pheno assay is that it is a cell-fusion system, and cell–cell fusion may differ in significant details from virus–cell fusion. For example, recent studies have shown that HIV-1 virions carry fewer surface glycoproteins than previously assumed [24]. The DSP-Pheno assay uses neuroglyoma cell-derived NP-2 cell lines with overexpressed CD4 and co-receptors. Although these NP-2-derived cell lines have been characterized extensively [16,17], some unknown cell surface molecules may be involved in the fusion process. The DSP-Pheno assay is a gag-free system and requires only the assembly of reporter proteins pre-formed in the fusion partner, but infection by a retrovirus requires that the entire gag particle pass through the fusion pore. Careful comparison between DSP-Pheno and in-house pseudoviral assay or GTA using clonal clinical isolates is under way.

GFP portion is necessary as a module of DSP to compensate weak self-association of split RL [15]. Although RL would be more suitable for quantitative assay, GFP may prove single clear positive fusion in the sample with very low RL readout. This feature of DSP-Pheno incorporating two different assays may be useful for certain scientific purposes.

Although several issues remain to be clarified, DSP-Pheno has multiple advantages over the conventional pseudoviral PTA: (i) the turnaround time for DSP-Pheno is short, with results available in as few as 5 days, starting from patients' plasma; (ii) DSP-Pheno is a virus-free assay that does not require a special biosafety facility, making it particularly appealing for in-house use; and (iii) the RL assay in DSP-Pheno has high sensitivity and specificity and compares favourably with the best pseudoviral PTA published in the detection of minor X4 populations using laboratory strains. Trofile™ (Monogram Biosciences Inc., CA, USA) is currently the only commercially available PTA approved for clinical use, and the latest version, "Enhanced Trofile™," detects X4 minor populations present in concentrations as low as 0.3% [25]. A pseudoviral PTA described by Soda and colleagues had 1% detection threshold for X4 viruses [16]. Although the RL assay in DSP-Pheno could detect X4 laboratory strains present in concentrations as low as 0.3%, further studies are needed to apply the assay for the clinical use. DSP-Pheno may also be useful for the comparison of with GTA to improve the algorithm for the co-receptor usage of non-B subtypes.

Conclusions

We described a new cell-fusion-based, high-throughput PTA for HIV-1, which would be suitable for in-house studies. Equipped with a two-way reporter system, RL and GFP, DSP-Pheno is sensitive and offers a short turnaround time. Although maintenance of cell lines and laboratory equipment for the assay is necessary, it provides a safe assay system without infectious viruses. With further validation against other conventional analysis, DSP-Pheno may prove to be a useful laboratory tool. The assay may be useful especially for the research on non-B subtype HIV-1 whose co-receptor usage has not been studied much.

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Competing interests

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For the remaining authors, there are no competing interests.

Authors' contributions

PT, NH and AI planned the experimental design. PT and NH did the experiments. NK, ZM and HH provided the materials. ZM, HH, AK-T and GFG and joined the discussion. TF, TK, HN, MK and AI were responsible for the patient care and provided clinical information. PT, NH and AI wrote the article. PT and NH contributed equally to the work.

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Effect of Maraviroc Intensification on HIV-1-Specific T Cell Immunity in Recently HIV-1-Infected Individuals

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Abstract

Background: The effect of maraviroc on the maintenance and the function of HIV-1-specific T cell responses remains unknown.

Methods: Subjects recently infected with HIV-1 were randomized to receive anti-retroviral treatment with or without maraviroc intensification for 48 weeks, and were monitored up to week 60. PBMC and *in vitro*-expanded T cells were tested for responses to the entire HIV proteome by ELISpot analyses. Intracellular cytokine staining assays were conducted to monitor the (poly)-functionality of HIV-1-specific T cells. Analyses were performed at baseline and week 24 after treatment start, and at week 60 (3 months after maraviroc discontinuation).

Results: Maraviroc intensification was associated with a slower decay of virus-specific T cell responses over time compared to the non-intensified regimen in both direct *ex-vivo* as well as *in in-vitro* expanded cells. The effector function profiles of virus-specific $CD8^+$ T cells were indistinguishable between the two arms and did not change over time between the groups.

Conclusions: Maraviroc did not negatively impact any of the measured parameters, but was rather associated with a prolonged maintenance of HIV-1-specific T cell responses. Maraviroc, in addition to its original effect as viral entry inhibitor, may provide an additional benefit on the maintenance of virus-specific T cells which may be especially important for future viral eradication strategies.

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Introduction

Maraviroc is an antiretroviral agent that blocks HIV-1 entry by binding the virus' coreceptor CCR5. Given its molecular target, maraviroc treatment may modulate the natural expression and function of CCR5, and negatively affect chemotaxis and effector function of Th1-type $CD4^+$ T cell and memory $CD8^+$ T cells. Maraviroc may have additional immunomodulatory effects by blocking the binding of the natural ligands of CCR5 (MIP-1α, MIP-1β and, RANTES), yet little data exist on how maraviroc may interfere with the cellular host immunity, especially the one directed against HIV-1.

While CCR5 deficiency (in the form of a 32 base-pair homozygous deletion) can mediate resistance to HIV-1 infection [1–3], it also has the potential to impair control of other viral infections, such as West Nile virus (WNV), both in mouse and humans [4,5]. In particular, murine T cells lacking CCR5 expression have been shown to secrete lower amounts of IL-2 compared to $CCR5^+$ T cells, and a similar phenotype has been observed in T cells from humans expressing the CCR5-:32 mutation [6]. Furthermore, $CD8^+$ T cell exhaustion during chronic Lymphocytic choriomeningitis virus (LCMV) infection is more severe in the absence of RANTES, one of the natural CCR5 ligands [7]. Thus, although CCR5-:32 homozygosity does not

seem to negatively affect humans, blocking its function by agents like maraviroc may negatively affect immune responses, including T cell responses to HIV-1.

In previous clinical trials, treatment with maraviroc has been shown to result in more extensive increases in CD4 counts in treatment-naïve and -experienced subjects, though the mechanisms involved remain unknown [8–11]. In addition, some studies have indicated that adding maraviroc to suppressive combination antiretroviral treatment (cART) reduces markers of immune activation [12–15]. Also, *in vitro* exposure to maraviroc decreases some markers of immune activation on T lymphocytes [16]. While these findings suggest that maraviroc may have beneficial effects on global host immune status, maraviroc has also been found to increase T cell activation both in gut and peripheral blood [17]. Thus, it is still controversial whether maraviroc has net immunological benefits or disadvantages on host cellular immune responses. In addition, the impact of maraviroc on antigen-specific T cell responses, especially towards HIV-1-derived antigens, has not been assessed, despite its potential implications with regards to immune interventions, particularly therapeutic vaccination in maraviroc treated subjects. To address these issues, we analyzed in a longitudinal study the effects of cART versus maraviroc–intensified cART on the maintenance (breadth, magnitude and specificity) of HIV-1-specific T cell responses, their differentiation potential and their polyfunctionality.

Materials and Methods

Study design

The present study was performed as sub-study of the Maraviboost study (ClinicalTrials.gov Identifier: NCT00808002). The Maraviboost study was a multi-center, randomized, open-label, phase III clinical trial. The main goal of the parental clinical trial was to assess whether intensification with maraviroc in recently HIV-1 infected patients with standard triple therapy could accelerate the decay of the HIV-1 reservoir [18]. Thirty subjects recently infected with CCR5-tropic HIV-1 (subtype B) were recruited and randomized into 2 groups (n = 15 each), one being treated with triple therapy consisting of Raltegravir (RAL) plus Tenofovir (TDF)/Emtricitabine (FTC) alone while the second group received additionally maraviroc (MVC) intensification for the first 48 weeks in the trial. The primary end point of the main study was week 48, but patients were followed until week 72 if possible. Frozen PBMC from pre-defined time points before starting cART (baseline, BL), 24 weeks after study initiation, and 12 weeks after maraviroc discontinuation (week 60), were analyzed in the present study. One patient without maraviroc intensification, who dropped out the study because of adherence problem, was excluded from the analysis. Three patients (01028, 01039, 23012) were lost at week 24 (n = 1) or 36 (n = 2), respectively. All patients received RAL plus TDF/FTC after week 48 except 4 patients (01021, 01031, 01034, 01043), who changed their anti-HIV drug regimen. Of the 29 individuals, peripheral blood mononuclear cells (PBMC) from at least one time point were available for 13 patients with maraviroc intensification (MVC arm) and 14 patients without MVC intensification (Control, CNT arm, Table 1). The study was approved by the ethics committee of Hospital Germans Trias i Pujol, Badalona, Spain. All patients gave their written informed consent before enrolling in the study.

Flow cytometry for T cell phenotype analysis

PBMC were thawed and rested overnight at 37°C in RPMI1640 supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100μg/ml streptomycin, and 2 mM glutamine (R10). The following day, the cells were stained with LIVE/DEAD Fixable Dead Cell Stain Kits (Invitrogen), washed and stained with the following antibodies: anti-CD3-APC-Cy7, anti-CD4-V450, anti-CD8-PE-Cy7, anti-CD45RA-APC (BD Biosience), and anti-CCR7-PE (e-BioScience). The cells were washed and fixed with 1% Formaldehyde in PBS. All data were collected on a BD LSR II flow cytometer (BD Biosience) and analyzed using FlowJo 8.7.7 (TreeStar).

Peptides

A set of 410 overlapping-peptides (OLPs) was used to screen for HIV-specific T-cell responses [19]. The peptides spanned all HIV-1 proteins and were based on the HIV clade B consensus sequence of 2001, available at the Los Alamos National Laboratory HIV immunology database. For ELISpot analyses, peptides were used in a matrix layout of 6–12 peptides per pool for comprehensive screening as previously described [19]. Reconfirmations of all positive wells in the matrix screen were performed the following day on a single-peptide base. For multi-functional analysis by flow cytometry, peptide pools were used that contained peptides spanning either full-length Gag, Protease, RT, IN, gp120, gp41, or Nef. Peptides spanning Tat, Rev, Vif, Vpr, and Vpu were combined into one peptide pool (accessory proteins peptide pool. "Acc").

IFN-γ ELISpot assay using ex-vivo PBMC and in-vitro expanded T cells

Thawed PBMC were rested for 3 hrs at 37°C in R10. If sufficient PBMC were recovered, thawed cells were used directly in IFN-γ ELISpot assays (11 and 7 samples at baseline, 6 and 7 samples at week 24, and 8 and 7 samples at week 60 in the CNT and MVC arm, respectively). In addition, 1×10^6 thawed cells were stimulated with an anti-CD3 monoclonal antibody and cultured for 2–4 weeks in R10 supplemented with 50 U/ml of recombinant IL-2 [20]. Before use in ELISpot assays, the expanded cells were washed twice with R10 and incubated overnight at 37°C in the absence of IL-2. Per well, 75,000–100,000 cells were used and peptides were added as in the direct ex-vivo assay. Thresholds for positive responses were defined as 1) at least five spots (50–66 SFC/10^6 PBMC) per well, 2) as responses exceeding the mean of negative wells plus 3 standard deviation and 3) responses exceeding three times the mean of negative (no peptide) wells; whichever was the highest. For reconfirmation ELISpot, the remaining cells and cells from negative wells from initial matrix screens were recycled as previously described [20].

Flow cytometric analysis of $CD8^+$ T cell function

Thawed PBMC were rested overnight at 37°C in R10. The following day, costimulatory antibodies (anti-CD28 and anti-CD49d at 1μg/ml; BD Biosciences) and monensin (GolgiStop; BD Bioscience) were added, and cells were stimulated with the different peptide pools (5μg/ml per peptide) as indicated. A negative (no peptide) and a positive control (phorbol-12-myristate-13-acetate (PMA at 10 ng/ml and ionomycin, 1μM) were included in each assay. Following incubation for 6 hrs, the cells were washed with PBS containing 1% FCS and the fluorescent reactive dye (Invitrogen) for dead cells was added. Cells were washed again, and stained with anti-CD3-V450, anti-CD8-PerCP, and anti-CD107a-PE (BD BioScience). Following washing, the cells were fixed and permeabilized using Fix & Perm cell permeabilization reagents (Invitrogen). The cells were then stained with anti-MIP-1β-FITC, anti-IL-2-PE-Cy7, anti-IFN-γ-APC (BD Bioscience). Data were collected on a BD LSR II flow cytometer (BD

Table 1. Characteristics of participants.

patient ID	age	Estimated duration from infection	baseline			week24			week60		
			VL	CD4	CD8	VL	CD4	CD8	VL	CD4	CD8
	(year-old)	(months)	(copies/ml)	(cells/μl)	(cells/μl)	(copies/ml)	(cells/μl)	(cells/μl)	(copies/ml)	(cells/μl)	(cells/μl)
Control group (n = 14)											
01022	28	6.2	61,000	606	873	50	843	731	50	741	606
01025	32	5.8	[a]36,000	287	691	[b]50	446	809	50	432	527
01028	26	3.1	490,000	366	1,893	56	518	900	[c]not determined		
01030	21	4.0	19,000	297	1,124	50	435	464	50	650	650
01032	32	4.5	[a]200,000	372	1,830	200	478	956	50	580	825
01036	42	5.1	40,000	273	1,034	50	352	641	50	429	617
01037	40	3.2	[a]26,000	589	1,326	50	533	999	50	556	641
01039	26	8.6	[a]63,000	450	1,125	50	654	1,162	[c]not determined		
01040	50	2.8	1,500,000	379	2,245	[b]230	601	631	50	620	589
01044	35	3.8	[a]540,000	454	1,127	50	962	1,683	50	396	834
23010	26	4.7	[b]1,091	629	1,204	[b]50	624	559	50	649	593
23012	38	4.0	10,738	656	579	50	688	615	[c]not determined		
23013	39	4.1	30,210	492	1,624	94	644	984	50	624	635
23019	32	6.5	8,497	620	1,033	[b]50	688	899	50	543	688
median	32	4.3	38,000	452	1,126	50	613	854	50	580	635
(itnterquartile range)	(26–39)	(3.7–5.9)	(16,935–272,5000)	(349–610)	(993–1,676)	(50–65)	(470–688)	(627–989)	(50–50)	(432–649)	(593–688)
MVC intensified group (n = 13)											
01021	39	5.1	46,000	649	1,750	50	954	1,371	[c]not determined		
01027	32	6.6	120,000	558	888	50	767	684	50	941	811
01034	33	4.6	12,000	310	496	55	285	498	[c]not determined		
01035	35	4.2	320,000	384	1,024	50	706	1,169	50	984	1,312
01041	34	2.0	160,000	619	1,695	50	1,034	853	50	542	383
01042	33	2.3	[a]140,000	280	1,595	50	602	1,228	50	654	782
01043	37	2.3	320,000	617	1,163	50	679	928	[c]not determined		
01045	49	1.1	[a]470,000	639	408	60	1,077	661	50	832	407
23005	28	5.4	5,666	421	666	[b]50	572	717	[b]50	500	521
23007	26	2.2	149,556	641	1,124	50	770	1,183	50	839	896
23011	31	5.8	11,081	454	1,473	[b]50	680	1,214	50	843	1,448
23015	42	8.2	54,216	283	2,384	61	492	1,638	50	743	1,974
23016	35	6.8	51,478	397	719	50	653	642	50	515	480
median	34	4.6	120,000	454	1,124	50	680	928	50	788	797

Table 1. Cont.

patient ID	age	Estimated duration from infection	baseline			week24			week60		
			VL	CD4	CD8	VL	CD4	CD8	VL	CD4	CD8
	(year-old)	(months)	(copies/ml)	(cells/μl)	(cells/μl)	(copies/ml)	(cells/μl)	(cells/μl)	(copies/ml)	(cells/μl)	(cells/μl)
(tinterquartile range)	(31–38)	(2.3–6.2)	(29,000–240,000)	(347–629)	(693–1,645)	(50–53)	(587–863)	(673–1,221)	(50–50)	(535–868)	(462–1,346)
P value			*0.5541*	*0.6623*	*0.5603*	*0.5925*	*0.1263*	*0.2541*		*0.0378*	*0.6472*

a: data from the closest previous timpoint for VL, CD4, CD8. The gap was 14–35 days.
b: not analyzed in this study because of sample limitation.
c: not determined because of lost patients.
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Biosience) and analyzed using FlowJo 8.7.7 (TreeStar). After gating for each effector function, a Boolean gate platform was used to create the full array of possible combinations and SPICE software (version 5.22) was used to analyze the polychromatic flow cytometry data. We applied a threshold for positive responses using negative values distribution after background subtraction (i.e. unstimulated cultures), as previously described [21].

Statistical analyses

Statistical analyses were performed using Graph Pad Prism 5.0. The results are given as medians and interquartile range (IQR) as indicated. Mann-Whitney test and Wilcoxon matched paired test were used for unpaired and paired comparisons, respectively. For multiple comparison analysis, we performed Bonferroni correction. Correlations between *ex-vivo* and *in-vitro* ELISpot data were analyzed by using Spearman's rank correlation coefficient, and linear regression analysis.

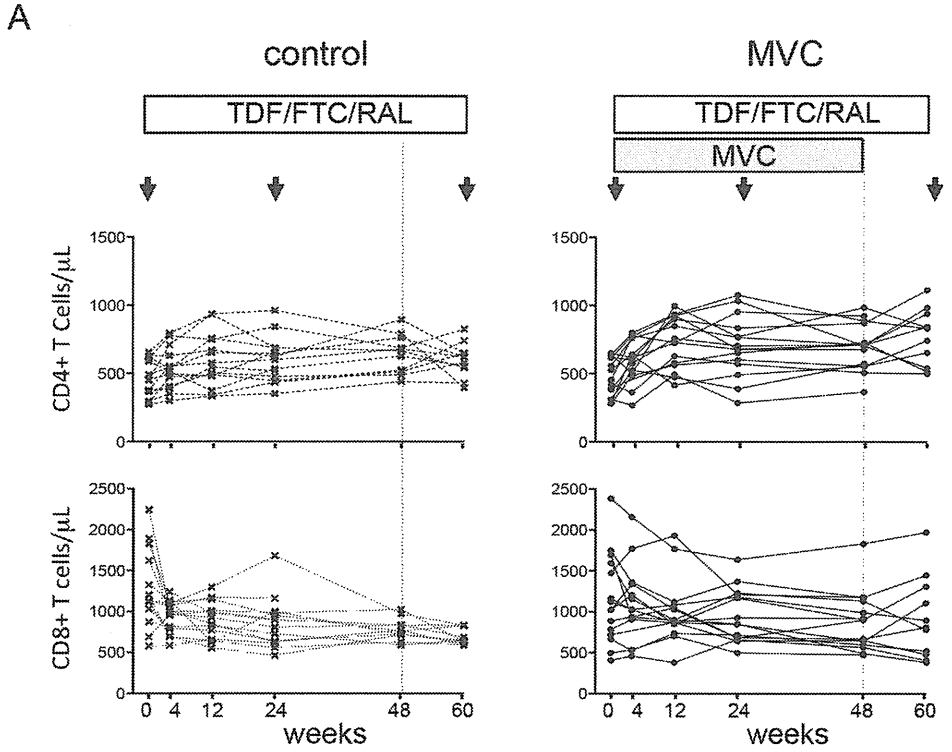
Results

Changes in $CD4^+$ and $CD8^+$ T cell count and their differentiation status

HIV-1-specific T cell responses are known to decrease upon cART initiation, although not all responses and specificities may show similar decay kinetics [22,23]. To determine whether maraviroc-intensified cART would lead to an equally rapid or even faster decay of global T cell responses to HIV-1, longitudinal changes in the breadth and magnitude of total HIV-1-specific T cell responses were compared between the maraviroc and control study arms at week 24 and week 60, i.e. 12 weeks after stopping maraviroc intensification. As previously reported, plasma viral load decreased under the limits of detection within the first 4-week cART in most patients [18]. $CD4^+$ T cell counts showed higher increases in the MVC subjects at week 12 and were significantly elevated in the MVC arm at week 60 when compared to the control subjects ($p = 0.0378$, Table 1 and ref [18]). At the same time, the decay in $CD8^+$ T cells was significantly slower in MVC subjects than in the control subjects (Fig. 1A and [18]) To examine whether these effects on $CD4^+$ and $CD8^+$ T cell counts were associated with a modulation of T cell differentiation markers, the expression of CD45RA and CCR7 was assessed over time and compared between the two groups. The data show that the frequency of effector memory (EM, $CD45RA^-/CCR7^-$) $CD8^+$ T cells was significantly decreased in both study arms at week 24 and week 60 compared to baseline, possibly reflecting the strong reduction in viral loads in both arms upon cART initiation (Fig. 1B). No significant changes for any other $CD4^+$ or $CD8^+$ T cell subset was observed, neither over time nor between study arms. These data indicate that maraviroc does not affect T cell differentiation during and after maraviroc intensification and that the different kinetics of $CD4^+$ and $CD8^+$ T cell counts between the arms are not reflected by gross alterations in differentiation markers.

Maraviroc intensification is associated with maintenance of HIV-1-specific T cell responses

To assess whether the effect of maraviroc intensification on cell homeostasis affected the magnitude, breadth and specificity of the HIV-1-specific T cell response, we performed IFN-γ ELISPOT assay on PBMC from individuals in both arms of the study using a 18-mer overlapping peptide (OLP) set covering the full HIV-1 proteome [19]. At baseline, the median magnitude of HIV-1-specific T cell responses in all patients was 2,708 SFC/10^6 PBMC (range 395–13,860), with a median breadth of 6 (range 2–15)


A
control
MVC
TDF/FTC/RAL
TDF/FTC/RAL
MVC
CD4+ T Cells/µL
CD8+ T cells/µL
weeks
weeks

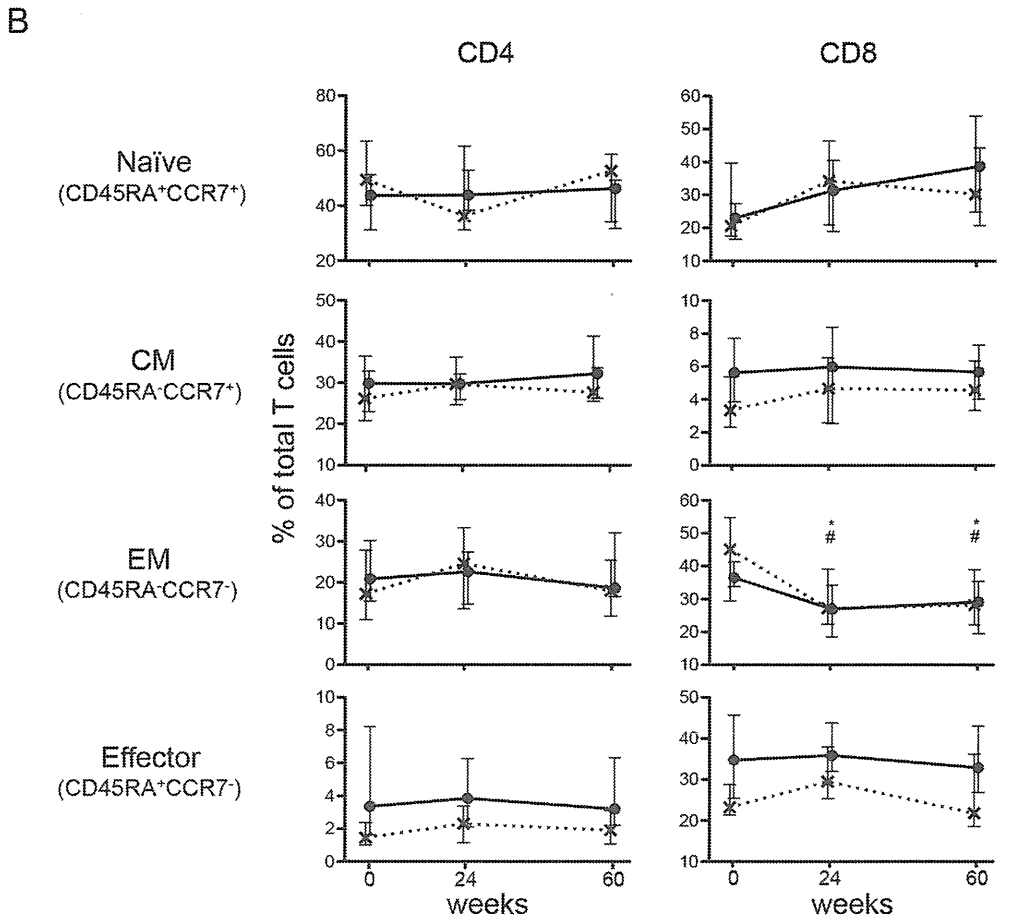

B
CD4
CD8
Naïve
(CD45RA+CCR7+)
CM
(CD45RA-CCR7+)
EM
(CD45RA-CCR7-)
Effector
(CD45RA+CCR7-)
% of total T cells
weeks
weeks

Figure 1. Differentiation status in CD4$^+$ and CD8$^+$ T cells. A. Changes of CD4$^+$ and CD8$^+$ T cell count in each subject. B. The proportion of naïve (CD45RA$^+$/CCR7$^+$), central memory (CM, CD45RA$^-$/CCR7$^+$), effector memory (EM, CD45RA$^-$/CCR7$^-$), and Terminal effector memory (T_{EMRA}, CD45RA$^+$/CCR7$^-$) cells among CD4$^+$ and CD8$^+$ T cells in the control (cross and hatched line) and MVC arm (circle and solid line). The median and interquartile range (vertical line) are shown. Stars (control) and hatches (MVC arm) above the lines indicate significant differences relative to baseline values ($p< 0.05$).
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responses per individual (Fig 2, left hand panels). The magnitude and the breadth in this cohort were considerably lower than that of chronically infected patients reported previously but in line with described breadth of responses in early, untreated HIV-1 infection [19,24]. No significant difference was observed in magnitude and breadth of HIV-1-specific response between the arms at any time point (Fig. 2, right panels). When we assessed changes in the virus-specific response in each arm, the magnitude of the HIV-1-specific response in the control arm was significantly reduced by week 24 (median 454 SFC/10^6 PBMC (range 27–7584), $p = 0.0042$) and even more so by week 60 (median 115 SFC/10^6 PBMC (range 0–1,475), $p = 0.0043$, Fig 2A). In contrast, subjects in the MVC arm did not show a significant reduction until week 60 when their median magnitude was still more than 5-fold higher than responses in the control arm (median 691 SFC/10^6 PBMC (range 0–3,535), Fig 2A). Similarly, the breadth of response was reduced over time as well, with significant reductions seen by week 60 in the control arm but not in the maraviroc intensified group (Fig 2B). There was no difference between the arms in regards to protein specificity of the HIV-1-specific CD8$^+$ T cells that remained at 24 and 60 weeks after starting cART (data not shown).

To extend the longitudinal analyses of responses between the intensified and non-intensified arms of the study to additional individuals for whom sample availability was limiting, we performed the same analysis using *in vitro* expanded cells. Aside from including additional individuals into the analyses, this also offered the opportunity to test for potential differences in the proliferative capacities of HIV specific T cells in the two arms. Thawed PBMC were expanded using an anti-CD3 mAb and kept in culture until sufficient cell numbers were reached. The culture time needed between the two study arms was comparable (both arms a median of 19 days), indicating intact proliferative capacities of T cells in maraviroc intensified cART treated individuals. For samples for which direct *ex-vivo* PBMC and *in vitro* expanded cells were tested, the ELISpot results were compared to validate the approach of using *in vitro*, unspecifically expanded cells. Overall, the breadth of responses in expanded cells correlated well with the direct *ex-vivo* results (Fig 3A, $r = 0.78$, $p<0.0001$). The magnitude of responses was generally increased in expanded cells, with later time points (week 24 and 60) showing stronger recovery of responses when compared to unexpanded cells (Fig. 3B). Of note, the correlation between results from direct *ex-vivo* analyses and *in-vitro* stimulated cells became stronger over time ($r = 0.5235$, 0.8455, 0.8720, and $p = 0.0374$, 0.0018, 0.0004 for comparisons at BL, w24, and w60 respectively). No differences were observed in proliferative capacity between the arms These data indicate that in both arms, HIV-1-specific T cell responses showed intact *in-vitro* proliferative capacities after prolonged cART and that in settings with limited sample availability, the *in-vitro* expansion approach produces reliable data [25].

HIV-1-specific T cell responses measured in expanded cells showed a significant decline in their magnitude during first 24 weeks in all subjects together (Fig. 3C, left panel). However, the reduction was generally less than three-fold (median 8,110 SFC/10^6 PBMC in BL and 2,656 SFC/10^6 PBMC in week 24) and thus not as dramatic as in unexpanded cells (median 6.3 fold, 2,708 SFC/10^6 PBMC in BL, and 424 SFC/10^6 PBMC in w24) (Fig. 2A and 3C, left panel). When the longitudinal changes in magnitude and breadth of responses were analyzed for each treatment arm separately, no significant reductions at week 24 and week 60 were noted (Fig 3C, D). However, when *in-vitro* stimulated responses were compared between the two arms, there was a trend that MVC-intensified subjects maintained stronger HIV-1-specific response at week 24 than control individuals (median 1,450 (IQR 277–2,965) in the control arm, 3,957 (1,714–13,018) in MVC, $p = 0.0625$, Fig. 3C, right panel). In addition, the median HIV-specific response was three-fold higher in MVC (median 3,957 (275–4,691)) compared to the control arm (1,114 (2,394–6,882)) until week 60. These data further support the notion that HIV-1-specific T cell responses are maintained for longer at higher levels in subjects with maraviroc intensification compared to individuals receiving non-intensified cART.

Poly-functionality of HIV-1-specific CD8$^+$ T cells is maintained under MVC intensified cART

The ability of HIV-1-specific T-cells to respond to antigenic stimulus with multiple different effector functions has been associated with the relative control of HIV-1 infection [26,27]. Since therapeutic strategies that aim at prolonged treatment interruptions or even viral eradication, will possibly depend on such polyfunctional T cell responses, we assessed the effector functions of HIV-1-specific CD8$^+$ T cells in cART treated subjects with and without maraviroc intensification. To this end, direct *ex-vivo* isolated PBMC were stimulated using peptide pools covering each of the viral proteins and analyzed for the expression of the degranulation marker (CD107a) or the production of intracellular cytokines, including IFN-γ, MIP-1β, and IL-2. The frequency of IFN-γ producing T cell responses correlated well with the data from the *ex-vivo* ELISpot analyses (Fig 4A, $r = 0.8265$, $p<0.0001$). The magnitude of the total HIV-1-specific CD8$^+$ T cell responses with at least one effector function by flow analysis varied widely in baseline samples (0.43% to 16.44% of total CD8$^+$ T cells across arms) and, as expected, was reduced at week 24 and week 60 (Fig. 4B). Although the magnitude of total HIV-specific CD8$^+$ T cells between the arms was comparable at the different time points, a significant reduction in the strength of the ex-vivo response was seen in the control arm but not in MVC arm, as observed in direct *ex vivo* ELISpot analysis (Fig. 1A and 4B). Also, as the reduction in frequency of HIV-specific CD8$^+$ T cell fractions with different cytokine secretion pattern was similar between the two arms, the data indicate that maraviroc intensification does not skew HIV-specific CD8$^+$ T cell function (Fig. 4C). The same was observed when the relative contribution of T cell populations with different numbers of effecter functions to the total HIV-specific CD8 T cell responses was compared between arms and over time (Fig. 4D),in line with previous reports [26,28].

Discussion

Since its development as a HIV entry inhibitor, CCR5 has been used as a target in several clinical studies of HIV infection as well as in other applications, including auto-immune diseases, cancer and transplantation [15,29–34]. Although some results remain still controversial [11–15,17,32] blocking the CCR5 co-receptor is

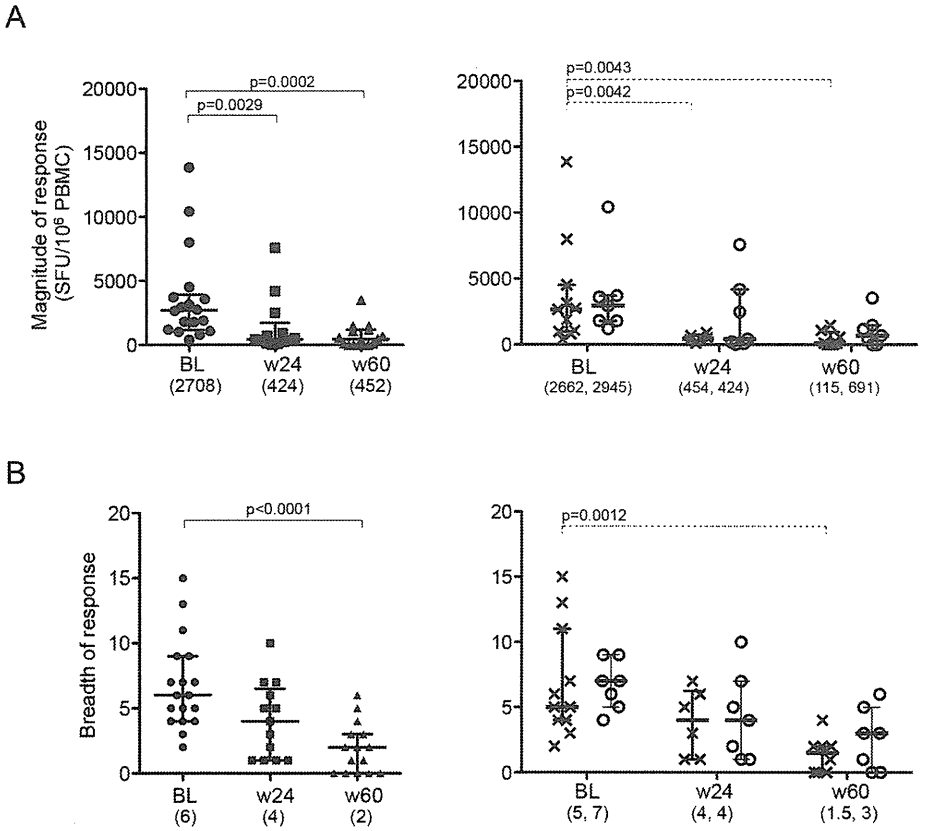

Figure 2. Longitudinal analyses of HIV-1-specific T cell responses in PBMC. The total magnitude (A) and breadth (B) of ELISpot responses at baseline (BL), week 24 (w24) and week 60 (w60) are shown for all subjects together (left panels) and for each study arm separately (right panels, crossed lines for control arm, circles for MVC arm). Horizontal lines represent median values of Spot-forming cells (SFC)/10^6 PBMC and the IQR, respectively. Mann-Whitney test was used in all statistical analysis. Only p values with significance after Bonferroni correction was shown. The numbers in parenthesis below the x-axis represent the median value.
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thought to suppress adverse immune activation and inflammation by blocking the chemotactic activity via its inhibition of CCR5-mediated signals. Due to its potential immune-modulatory properties, maraviroc may thus also affect the HIV-specific immune response, not necessarily only in a beneficial manner. While a number of studies have described effects on total T cell counts, $CD4^+$ and $CD8^+$ T cell kinetics and outcome of vaccination to other pathogens [35–39], no study has, to our knowledge, investigated the effect of MVC on the total HIV-1-specific $CD4^+$ and $CD8^+$ T cell response. In the present study, we investigated the effect of maraviroc intensification on HIV-specific T cell responses in primary HIV-1 infected subjects treated with standard cART or maraviroc intensified regimen. Although there was no gross difference in specific T cell subsets, maraviroc intensification showed extended maintenance of stronger HIV-1-specific T cell responses when compared to non-intensified treatment in PBMCs.

Our data in recently infected and early treated individuals showed that maraviroc intensification accelerated recovery of $CD4^+$ T cell counts and maintained higher $CD4^+$ T cell count after its discontinuation (Table 1 and [18]). As $CD4^+$ T cell help is critical for maintenance of memory $CD8^+$ T cells [40], this early increase of $CD4^+$ T cells may also provide the basis for the extended maintenance of virus-specific T cell responses. Alternatively, the maintenance of higher HIV-1-specific T cell responses in maraviroc intensified subjects may be a reflection of a slower reduction in the total $CD8^+$ T cells in the peripheral blood. This would be in line with clinical data showing that maraviroc intensification increase $CD4^+$ T cells faster and reduce $CD8^+$ T cell slower than non-intensified regimen (Fig. 1A and [18]). In addition, others have recently reported that maraviroc intensification increased $CD8^+$ T cell counts in peripheral blood and decreased $CD8^+$ T cells in rectal tissue in chronically HIV-infected subjects on stable cART [17], suggesting a possible *in vivo* redistribution of T cells by maraviroc. However, the relative changes of total $CD8^+$ T cell counts between control arm and intensified group were less pronounced than the extensive changes in HIV-specific $CD8^+$ T cell frequencies, making it unlikely that a MVC-driven redistribution of virus-specific $CD8^+$ T cells would be the sole driving force behind the prolonged maintenance of these cells in the peripheral blood. Maintenance of virus-specific T cells has also be linked to the availability of cognate antigen[22]. As the reduction in virmeia in both arms was comparable, additional mechanisms may be at work in maraviroc-intensified individuals that lead to extended presence of cells. As shown in previous analyses, not all HIV-specific T cell response contract with the same kinetics and some even expand after cART initiation[23]. As there were no differences in the specificity of HIV-specific T cell response between the two arms in the present study, the mechanism for the maintenance of responses in the MVC intensified group remain unclear. One possibility is that the slower CD4 T cell decline in the intensified arm [18], together with a

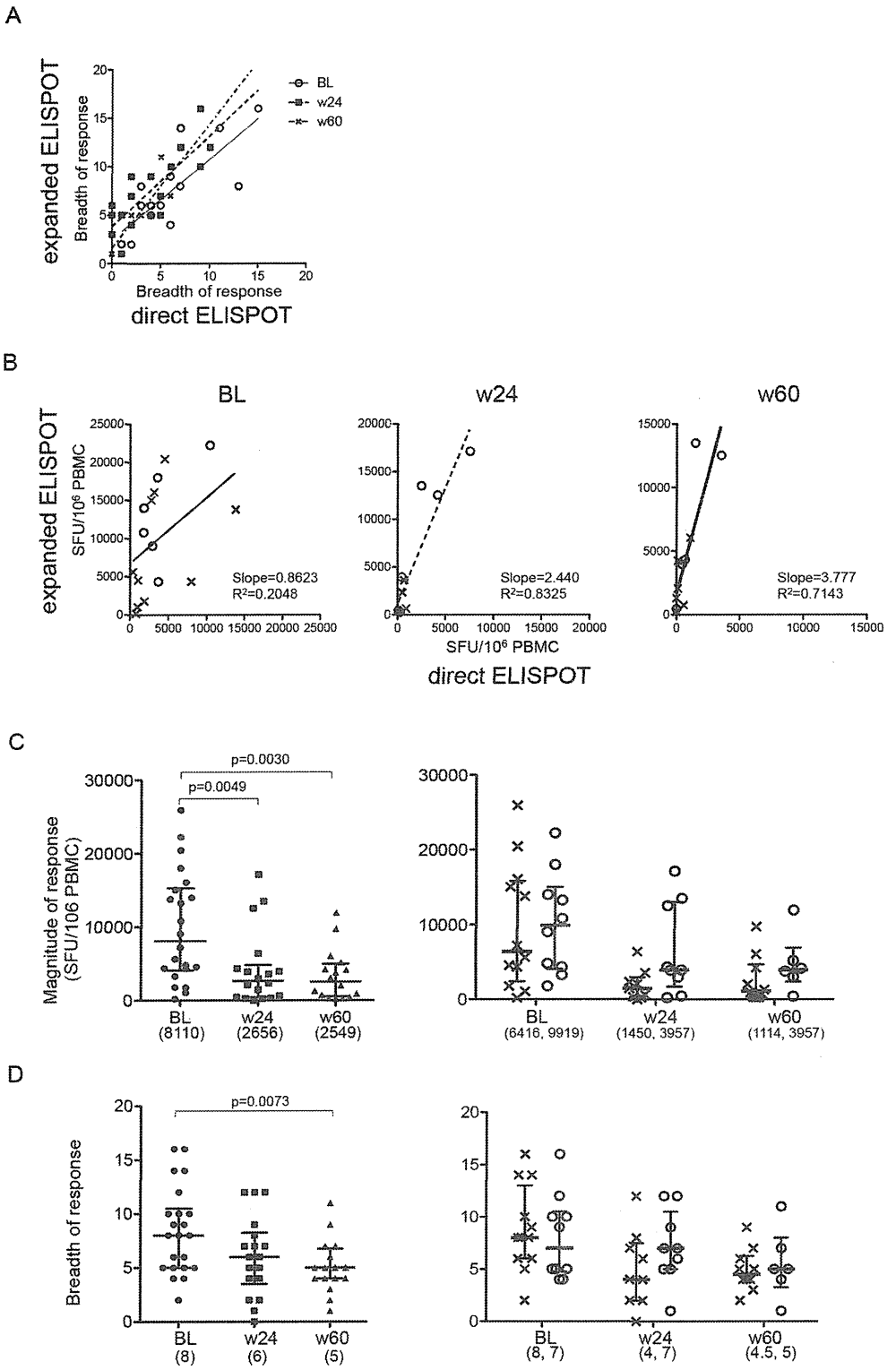

Figure 3. Longitudinal assessment of HIV-specific T cell responses with *in vitro* expanded T cells. A. Relationship of the breadth between responses detected by direct ELISpot and ELISpot using *in vitro* expanded cells. Responses on the x-axes represent the total HIV-1-specific responses in direct ELISpot, and the y-axes indicate total HIV-1-specific responses in expanded ELISpot for samples taken at baseline (circle), week 24 (square), and week 60 (triangle). B. Relationship of the magnitude between direct ELISpot and expanded ELISpot at each time point. cross: control arm, circle: MVC arm. The lines in A and B show linear regression lines. C, D. Changes in magnitude and breadth of total HIV-specific T cell responses in expanded ELISpot are shown as in Figure 2.
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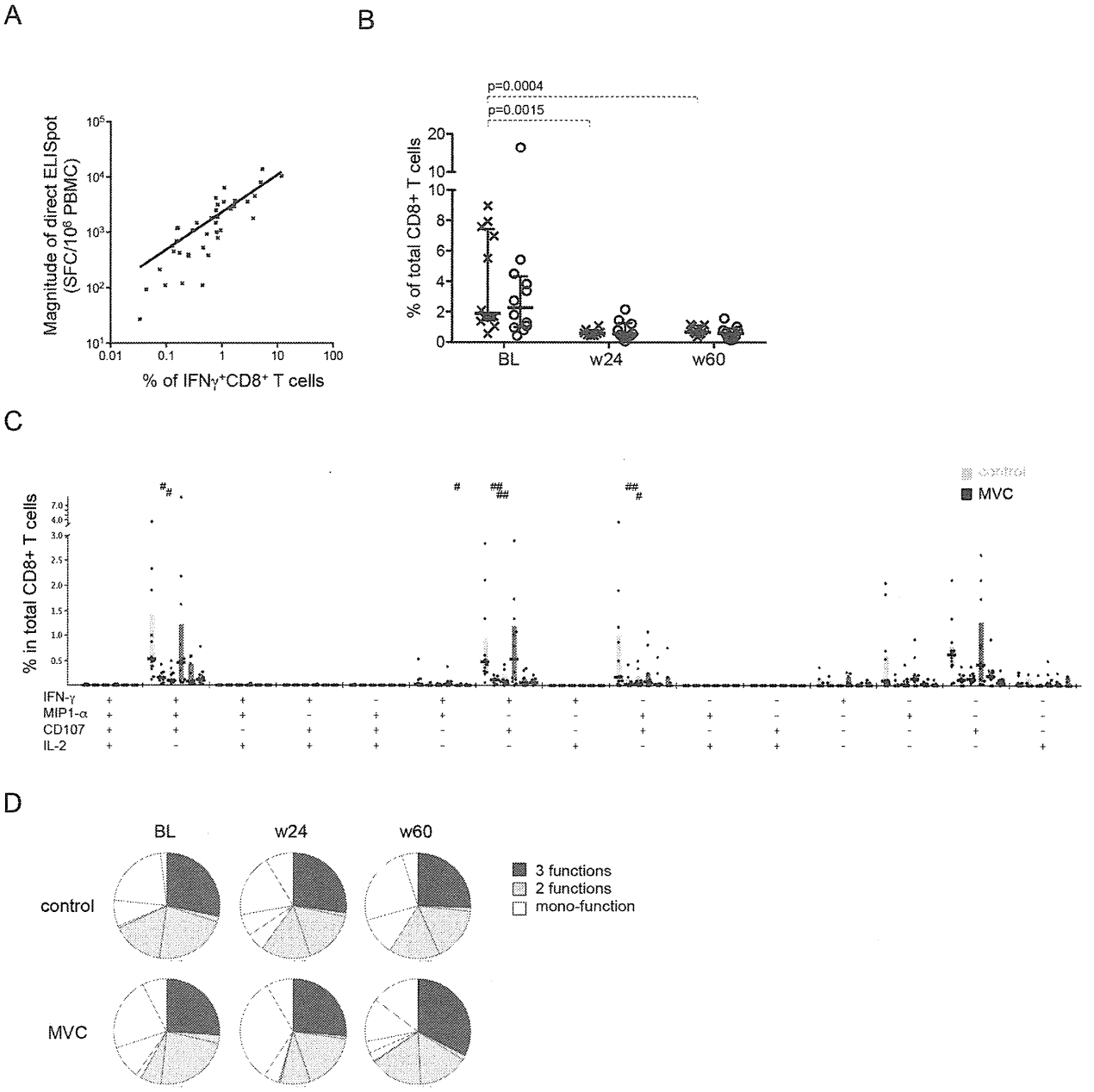

Figure 4. Longitudinal assessment in functional profile of HIV-specific CD8$^+$ T cells during cART with MVC intensification. A. Correlation between the total HIV-specific responses determined by direct ex-vivo ELISpot analysis (as spot-forming cells (SFC)/10^6 PBMC) and by ICS analysis (% of IFN-γ^+ CD8$^+$ T cells). Linear regression line, and correlation coefficient and p-values (Spearman's rank correlation test) are shown. B. The change in total HIV-specific CD8$^+$ T cell frequency over time by ICS analysis. Horizontal lines indicate median values of all positive responses. P values were determined by Mann-Whitney tests and shown if the significance remains after Bonferroni correction. C, D. Effector function profiles of HIV-specific CD8$^+$ T cells over time in controls and MVC treated subjects. (left, baseline; middle, week 24; right, week 60). The median and IQR are indicated by horizontal lines and boxes, respectively. Differences relative to baseline values in each arm were tested for statistic significance by Mann-Whitney tests, and shown as # for $p<0.05$, ## for $p<0.01$.
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reported increased in T cell activation upon maraviroc intensification [17] maintains activated CD8 T cell for longer. Although a number of studies show conflicting data in terms of immune activation [11–15], it is important to note that the present data were generated in early treated subjects, which may yield different results than the analyses in maraviroc intensification during chronic HIV infection.

Blocking CCR5 signaling *in-vivo* might inhibit migration of memory T cells expressing CCR5 to the site of the cognate antigen, thus preventing these memory T cells to be stimulated properly to acquire effector functions and exert effective anti-viral immunity. In fact, maraviroc has been shown to inhibit chemotactic activity of lymphocytes and monocytes *in vitro* and to reduce the risk of Graft-versus-host disease (GVHD) in patients

with hematologic cancers after allogeneic hematopoietic stem cell transplantation [16,30,41]. CCR5 polymorphisms and gene copy number of CCL3L, encoding one of CCR5's ligands, can affect delayed-type hypersensitivity (DTH) response [42], suggesting that CCR5 is critical for differentiation of $CD8^+$ T cell, the acquisition of effector functions and the ability to traffic to the site of viral replication. However, we didn't observe any difference in T cell differentiation between the arms, and our data using directly isolated PBMC and *in vitro* expanded T cells indicate that the proliferative capacity of HIV-1-specific $CD8^+$ T cells in Maraviroc intensified subjects were not compromised. Furthermore, the effector function profiles were essentially identical between the two treatment arms, suggesting that maraviroc intensification does not negatively affect the quality of HIV-1-specific $CD8^+$ T cells. This is further supported by studies of the effects of maraviroc intensification on response to vaccination and immune profile in HIV-1 infected subjects [35]. Thus, studies of T cell effector function profiles in maraviroc intensified therapy, including their ability to mount HIV epitope-specific DTH reactions [43] may offer interesting insights into how maraviroc can modulate, and potentially improve, anti-viral immunity. In light of recent studies showing reduced viral reservoir sizes in MVC treated individuals [15] and data suggesting that a robust and functional HIV-1-specific $CD8^+$ T cell responses may be required for viral eradication strategies [44], a prolonged maintenance of functionally intact virus-specific T cells could provide the patient with a crucial advantage to further reduce the viral reservoir.

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Author Contributions

Conceived and designed the experiments: CB BM JB JMP AI ALP JML BC. Performed the experiments: AKT. Analyzed the data: IB RE MCP. Contributed reagents/materials/analysis tools: JML IB RE BM JP CM JMM JMG. Wrote the paper: AKT CB. Designed and executed the main Maraviboost clinical trial: MARAVIBOOST investigators.

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Original article

Identification of cross-clade CTL epitopes in HIV-1 clade A/E-infected individuals by using the clade B overlapping peptides

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Abstract

Identification of cross-clade T cell epitopes is one of key factors for the development of a widely applicable AIDS vaccine. We here investigated cross-clade $CD8^+$ T cell responses between clade B and A/E viruses in chronically HIV-1 clade A/E-infected Japanese individuals. $CD8^+$ T cell responses to 11-mer overlapping peptides derived from Nef, Gag, and Pol clade B consensus sequences were at a similar level to those to the same peptides found in clade B-infected individuals. Fifteen cross-clade CTL epitopes were identified from 13 regions where the frequency of responders was high in the clade A/E-infected individuals. The sequences of 6 epitopes were conserved between the clade B and clade A/E viruses whereas 9 epitopes had different amino acid sequences between the 2 viruses. $CD8^+$ T cells specific for the 6 conserved epitopes recognized cells infected with the clade A/E virus, whereas those for 8 diverse epitopes recognized both the clade A/E virus-infected and clade B-infected cells. All of the cross-clade $CD8^+$ T cells specific for conserved and diverse epitopes were detected in chronically HIV-1 clade A/E-infected individuals. These results show that in addition to conserved regions polymorphic ones across the clades can be targets for cross-clade CTLs.

Keywords: Cross-clade; CTL; HLA; Epitope; HIV

1. Introduction

The HIV-1 genome is characterized by genetic diversity wherein distinct HIV-1 clades are expanding not only in different geographical regions but also even in the same locality [1]. HIV-1 clade B is the most prevalent virus in Japan accounting for more than 80% of the patients in this country. CRF01_AE (clade A/E) is the second most prevalent virus, accounting for 6.1% (Sugiura W, unpublished report). In contrast, the clade A/E virus is mainly prevalent in south-east Asian countries including Thailand. An RV144 phase III vaccine trial, which was recently performed in Thailand, demonstrated a partial beneficial effect on HIV-1 infection [2]. In this trial, the recombinant canarypox virus-vectored HIV-1 *gag/pol/env* vaccine (ALVAC-HIV) and the recombinant glycoprotein 120 subunit vaccine (AIDSVAX B/E) were used for priming and boosting, respectively. These vaccines were generated by using genes from both the clade B and A/E viral strains [2] to cover a wider range of potential challenge strains in Thailand, where approximately 80% and 10% of HIV-1-infected individuals are infected with the clade A/E and clade B viruses, respectively [3]. Recent analyses confirmed $CD4^+$ cell-mediated and humoral immune responses in vaccines [3–6]. Thus, this clinical trial also highlighted the importance of the cross-clade immune responses to the clade B and A/E viruses. Although T cell functions in the acute and

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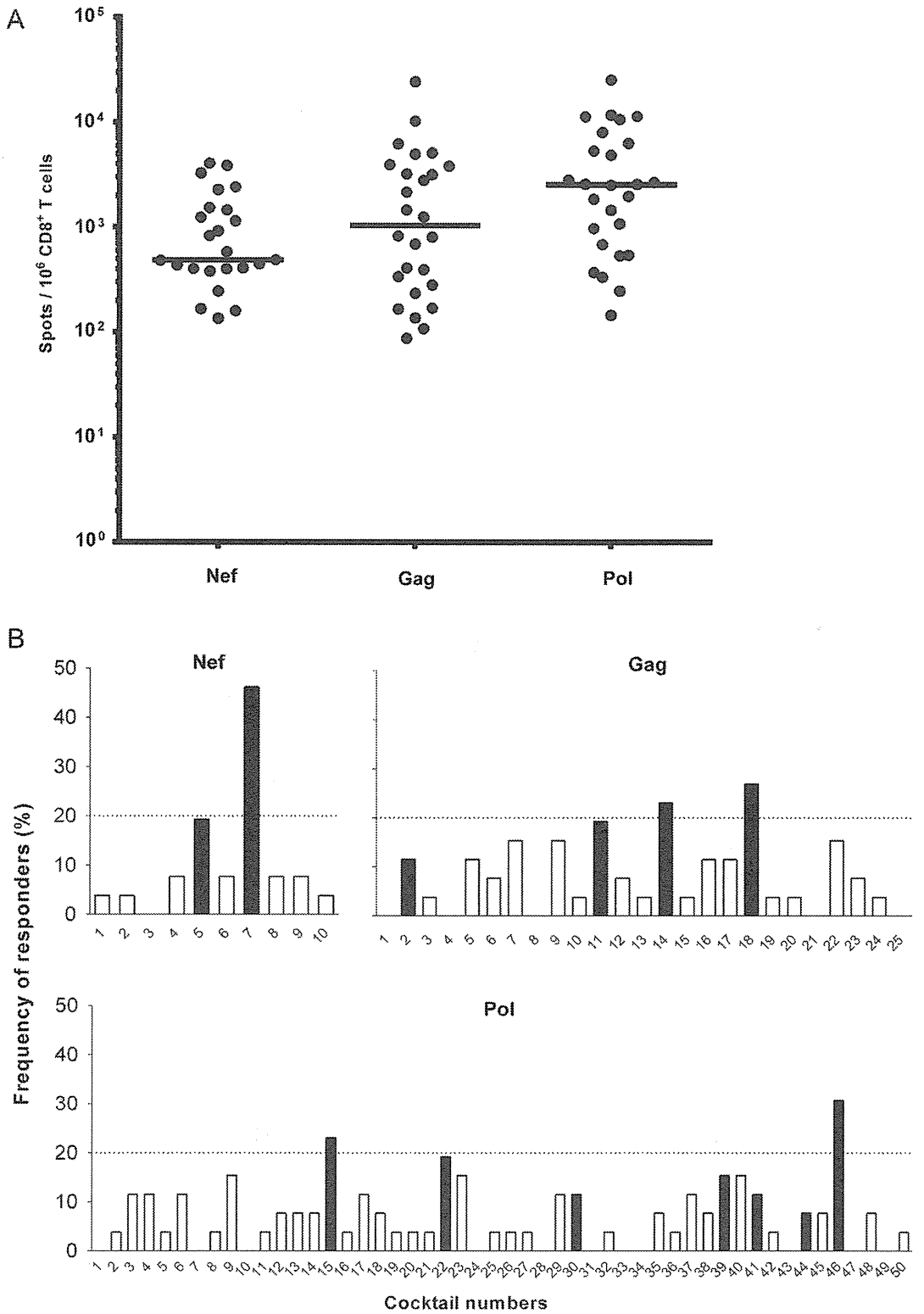
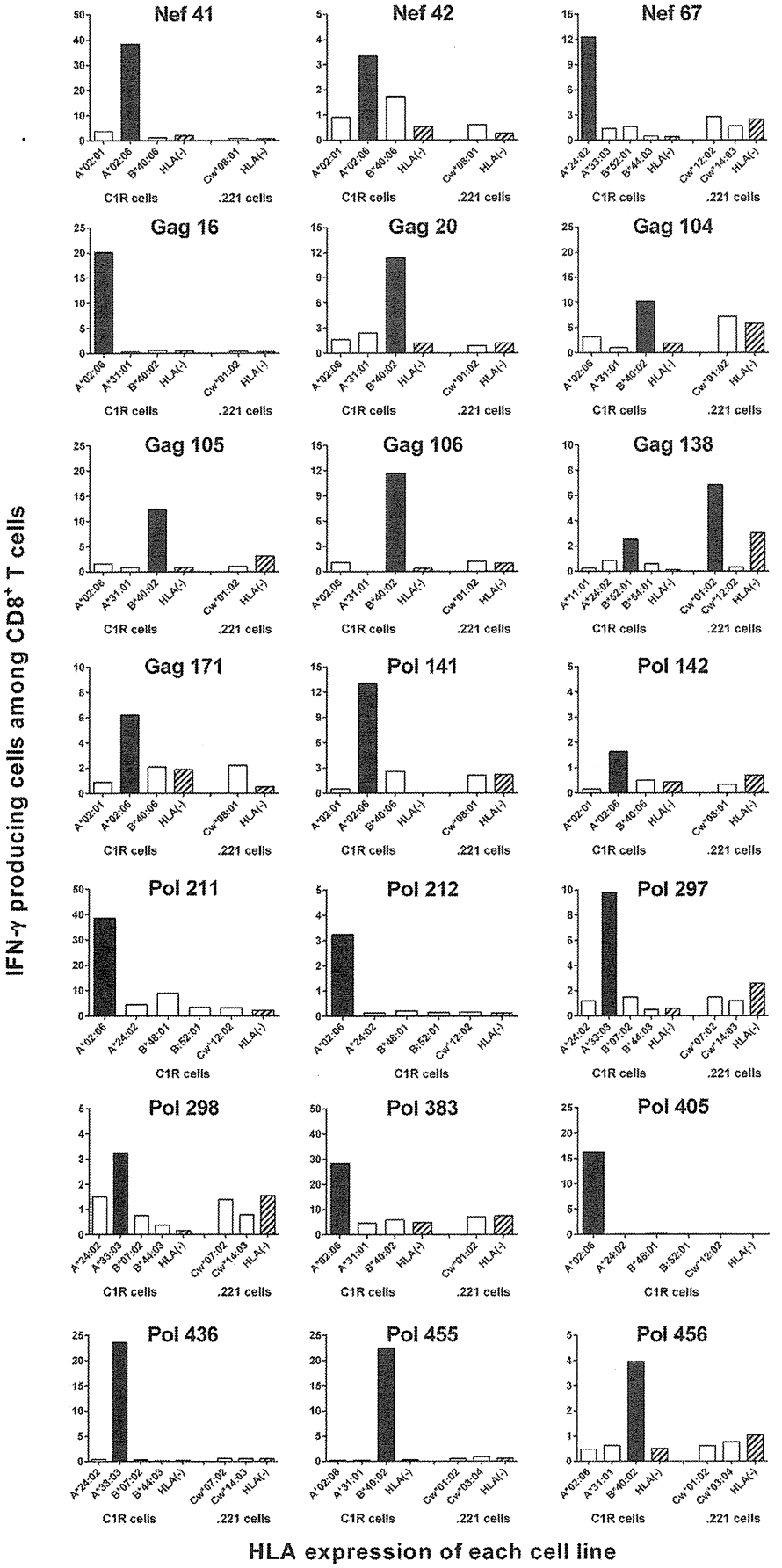


Fig. 1. $CD8^+$ T cell responses of clade A/E-infected individuals to HIV-1 clade B-derived overlapping peptides $CD8^+$ T cell responses to peptide cocktails containing clade B consensus overlapping 11-mer peptides were analyzed by performing the ELISPOT assay using $CD8^+$ T cells from 26 clade A/E-infected individuals. A. Total magnitude of $CD8^+$ T cell responses to the clade B consensus overlapping peptides spanning Nef, Gag and Pol regions. B. Frequency of the responders to each cocktail. Positive response is defined as more than 200 spots. The cocktails for which the frequency of responders was more than 20% or less than 20% are shown as solid bars. In the latter case, at least 1 patient showed a high response (>750 spots).



chronic phases of an HIV-1 infection were well studied in Caucasians infected with the clade B virus and in Africans infected with the clade C virus [7–12], there are only a limited number of studies about the cross-clade reactivity of CTLs [13–17]. However, even in such studies a comprehensive analysis of cross-clade reactivity of the CTLs was not performed.

In the context of HIV vaccine development, it is very important to choose vaccine immunogens capable of eliciting CTLs that can control the variable mutant viruses and exhibit cross-reactivity across the different clade viruses [18,19]. The conserved parts of HIV-1 are good candidates as vaccine antigens [11,12,16,20,21], since they include epitopes conserved among viruses not only in the same clade but also among those clades. Indeed, CTL vaccines containing conserved epitopes have been shown to elicit CTL responses to HIV-1 [22–25]. Although the safety of these consensus CTL vaccines was confirmed in humans [26–32], such vaccines were poorly immunogenic in previous phase I and II trials [27,28,30,31]. Thus more studies on cross-clade effective epitopes will be needed for the development of more potent vaccines.

In the present study, we analyzed cross-clade $CD8^+$ T cells between HIV-1 clade B and A/E viruses in chronically HIV-1 clade A/E-infected Japanese individuals. For this analysis, we sought to identify cross-clade CTL epitopes between the clade B and A/E viruses in the Japanese individuals by using 11-mer overlapping peptides derived from the clade B consensus sequence spanning Nef, Gag, and Pol regions. Thereafter, we analyzed cross-clade $CD8^+$ T cell recognition for epitope peptides between clade A/E and B as well as CTL recognition for cells infected with the clade B or the A/E virus. This is the first comprehensive study to identify cross-clade $CD8^+$ T cells by using overlapping HIV-1 peptides.

2. Materials and methods

2.1. Patient samples

This study was approved by the Ethical Committee of in National Center for Global Health and Medicine and Kumamoto University. Informed consent was obtained from all subjects, according to the Declaration of Helsinki. Plasma and peripheral blood mononuclear cells (PBMCs) were separated from whole blood. HLA types of HIV-infected individuals were determined by standard sequence-based genotyping. HIV-1 subtypes were determined by the sequence results on Pol and Gag, and confirmed by Env sequencing. All samples were collected from the cohort in AIDS Clinical Center, National Center for Global Health and Medicine.

2.2. Sequence of autologous virus

Viral RNA was extracted from plasma samples from HIV-1-infected patients by the use of a QIAamp MinElute virus spin kit (Qiagen). cDNA was synthesized from the RNA by use of the SuperScript III First-Strand Synthesis System for RT-PCR and random hexamers (Invitrogen). Nef, Gag, and Pol regions were amplified by nested PCR using Taq DNA polymerase (Promega). The PCR products were purified by using ExoSAP-IT (GE). All DNA sequencing was performed with a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) and an ABI 3500 Genetic Analyzer.

2.3. Synthetic peptides

We previously designed overlapping peptides consisting of 11-mer amino acids and spanning Gag, Pol, and Nef of HIV-1 clade B consensus sequences. Each 11-mer peptide was overlapped by 9 amino acids [33]. These 11-mer peptides and truncated peptides were synthesized by utilizing an automated multiple peptide synthesizer and purified by high-performance liquid chromatography (HPLC). The purity was examined by HPLC and mass spectrometry. Peptides with more than 90% purity were used in the present study.

2.4. ELISPOT assay

$CD8^+$ T cells were sorted from cryopreserved PBMCs from 26 chronically HIV-1 clade A/E-infected Japanese individuals by using CD8 magnetic beads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The sorted cells were plated in 96-well polyvinylidene plates (Millipore, Bedford, MA) that had been pre-coated with 5 mg/mL anti-IFN-γ mAb 1-D1K (Mabtech, Stockholm, Sweden). The appropriate amount of peptide cocktails including 10 overlapping 11-mer peptides were added in a volume of 50 μL, and then PBMCs were added at 1×10^5 cells/well in a volume of 100 μL. The plates were incubated for 16 h at 37 °C in 5% CO_2 and then washed with PBS before the addition of biotinylated anti-IFN-γ Mab (Mabtech) at 1 mg/mL. After the plates had been incubated at room temperature for 90 min and then washed with PBS, they were subsequently incubated with streptavidin-conjugated alkaline phosphatase (Mabtech) for 60 min at room temperature. Individual cytokine-producing cells were detected as dark spots after a 20-min. reaction with 5-bromo-4-chloro-3-idolyl phosphate and nitro blue tetrazolium by using an alkaline phosphatase-conjugate substrate (Bio-Rad, Richmond, CA, USA). The spot number was counted by using an Eliphoto-Counter (Minerva Teck, Tokyo,

Fig. 2. Identification of HLA restriction of the responses to each 11-mer peptide Peptide-specific $CD8^+$ bulk T cells were induced from PBMCs of the following 6 responders by stimulating the cells with each single peptide. KI-648 for Nef 41, Nef 42, Gag 171, Pol 141, and Pol 142 peptides, KI-632 for Nef 67, Pol 297, Pol 298, and Pol 436 peptides, KI-388 for Gag 16, Gag 20, Gag 104, Gag 105, Gag 106, and Pol 383 peptides, KI-724 for Gag 138 peptide, KI-964 for Pol 211, Pol 212, and Pol 405 peptides, and KI-837 for Pol 455 and Pol 456 peptides. Induced $CD8^+$ bulk T cells were stimulated with the corresponding peptide-prepulsed C1R cells or 0.221 cells expressing each HLA-class I allele molecule. IFN-g production by $CD8^+$ T cells was detected by performing the intracellular cytokine staining (ICS) assay.