TABLE 1. PATIENT CHARACTERISTICS

| | *Diagnosis (month)* | *Sex* | *Age* | *VL* | *CD4* | *CD8* | *Treatment period (month)* |
|---|---|---|---|---|---|---|---|
| 19 LVL | | | | | | | |
| *S70* | *9* | *M* | *47* | *53* | *481* | *746* | |
| *T16* | *60* | *F* | *41* | *240* | *492* | *804* | |
| *O12* | *61* | *M* | *32* | *450* | *559* | *1,187* | |
| *S33* | *125* | *M* | *52* | *470* | *400* | *888* | |
| *K2* | *60* | *M* | *30* | *510* | *316* | *871* | |
| *M3* | *160* | *F* | *34* | *730* | *444* | *859* | |
| *S81* | *6* | *M* | *32* | *1,700* | *381* | *1,475* | |
| *Y1* | *131* | *M* | *36* | *1,700* | *358* | *753* | |
| *F4* | *101* | *M* | *32* | *2,000* | *404* | *699* | |
| *E6* | *13* | *M* | *29* | *2,100* | *348* | *649* | |
| T24 | 30 | M | 46 | 400 | 455 | 469 | |
| O16 | 23 | M | 36 | 1,100 | 362 | 812 | |
| F9 | 65 | M | 35 | 1,100 | 517 | 1,066 | |
| K11 | 33 | M | 30 | 1,200 | 521 | 1,137 | |
| F1 | 41 | M | 35 | 1,600 | 424 | 1,151 | |
| H25 | 6 | M | 43 | 1,700 | 749 | 821 | |
| K16 | 61 | F | 23 | 2,000 | 560 | 1,049 | |
| A10 | 32 | M | 25 | 3,400 | 586 | 1,603 | |
| T26 | 28 | M | 36 | 3,600 | 449 | 864 | |
| median | 41 | | 35 | 1,200 | 449 | 868 | |
| 16 HVL | | | | | | | |
| *S60* | *22* | *M* | *36* | *35,000* | *321* | *486* | |
| *H24* | *23* | *M* | *35* | *42,000* | *462* | *907* | |
| *F13* | *22* | *M* | *34* | *51,000* | *520* | *831* | |
| *O29* | *9* | *M* | *21* | *56,000* | *314* | *880* | |
| *Y24* | *14* | *M* | *25* | *58,000* | *381* | *2,023* | |
| *K54* | *10* | *M* | *24* | *82,000* | *386* | *1,056* | |
| *K43* | *11* | *M* | *29* | *110,000* | *361* | *507* | |
| *S78* | *5* | *M* | *48* | *260,000* | *492* | *1,441* | |
| *K46* | *15* | *M* | *47* | *280,000* | *454* | *1,579* | |
| *S55* | *26* | *M* | *56* | *500,000* | *427* | *510* | |
| T37 | 3 | M | 30 | 25,000 | 254 | 626 | |
| K33 | 24 | M | 39 | 27,000 | 314 | 832 | |
| M11 | 58 | M | 38 | 33,000 | 228 | 908 | |
| O17 | 24 | M | 33 | 66,000 | 494 | 632 | |
| T35 | 4 | M | 20 | 78,000 | 434 | 1,133 | |
| S5 | 49 | M | 62 | 85,000 | 516 | 1,029 | |
| median | 18.5 | | 35 | 62,000 | 407 | 894 | |
| 15 Tx | | | | | | | |
| *U5* | *22* | *M* | *42* | *30* | *308* | *581* | *22* |
| *S19* | *82* | *M* | *35* | *30* | *382* | *533* | *22* |
| *T18* | *70* | *M* | *40* | *40* | *508* | *747* | *66* |
| *T8* | *39* | *M* | *42* | *50* | *423* | *491* | *51* |
| *K4* | *73* | *M* | *31* | *50* | *480* | *718* | *71* |
| *Y17* | *33* | *M* | *36* | *50* | *365* | *508* | *31* |
| *I9* | *90* | *M* | *50* | *50* | *406* | *643* | *80* |
| *I5* | *59* | *M* | *44* | *50* | *466* | *668* | *56* |
| *N11* | *119* | *M* | *39* | *67* | *440* | *886* | *117* |
| *N17* | *60* | *M* | *29* | *67* | *382* | *571* | *46* |
| K24 | 150 | M | 54 | 40 | 610 | 805 | 149 |
| N5 | 72 | M | 37 | 45 | 633 | 706 | 41 |
| O9 | 61 | M | 41 | 50 | 335 | 629 | 72 |
| Y5 | 81 | M | 36 | 110 | 753 | 605 | 79 |
| S15 | 113 | M | 38 | 130 | 814 | 1,048 | 111 |
| median | 72 | | 39 | 50 | 440 | 643 | 66 |

Italics indicates the patients used for phenotype and activation/exhaustion status of T cells.
VL, viral load; LVL, low viral load; HVL, high viral load; Tx, treatment experienced.

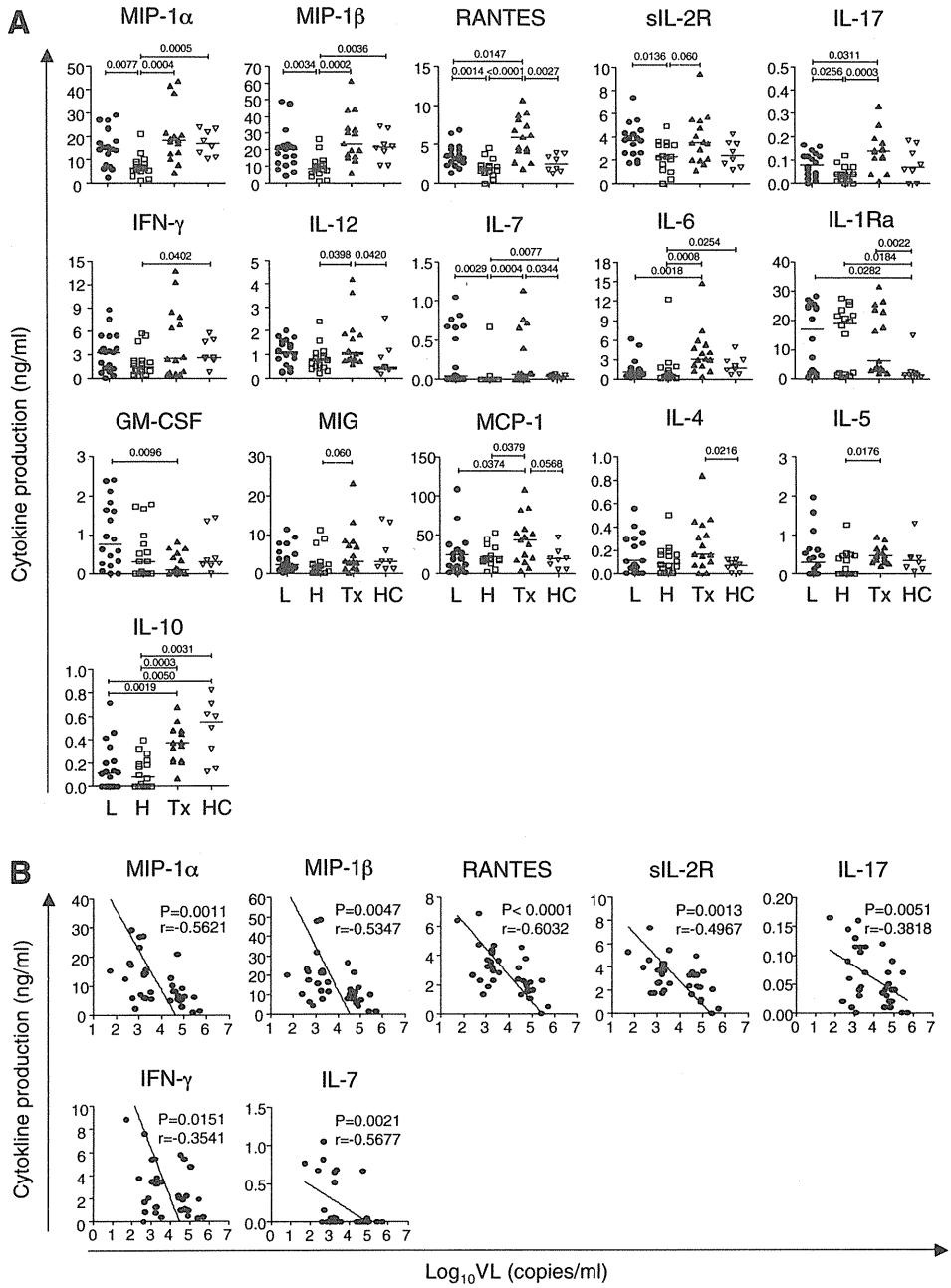


**FIG. 1.** Multiple cytokine production of phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMCs) in chronic HIV-1-infected subjects and healthy individuals. **(A)** Comparison of cytokine production from PHA-stimulated PBMCs. L, LVL subjects (●), H, HVL subjects (□), Tx, HIV-1-infected subjects with prolonged antiretroviral therapy (ART) (▲), HC, healthy control (□). The horizontal bars indicate the median value. Differences between groups were tested for statistical significance by the Mann–Whitney *U* test. **(B)** Correlation between cytokine production and viral load. Correlation analysis was performed with Spearman's rank correlation to determine correlations between variables.

PHA requires accessory cells such as monocytes and macrophages,[23,24] these data indicate that $CD4^+$ and $CD8^+$ T cells are the sources of MIP-1α, MIP-1β, RANTES, IFN-γ, and sIL-2R production, and that only $CD4^+$ T cells are the source of IL-17.

$CD4^+$ T cells were classified into subsets based on cytokine secretion.[14,16] We found VL-associated reductions in levels of IFN-γ and IL-17 levels, which are typical cytokines secreted by Th1 and Th17 cells, respectively. In contrast, as shown in Fig. 1A, the LVL and HVL groups had comparable levels of IL-4, IL-5, and IL-13, which are associated with a Th2-type response, and IL-10, which is produced by regulatory T cells (Treg) (Fig. 1A). These results suggest that $CD4^+$ T cell dysfunction in HVL may occur in a type-specific manner, especially in Th1 and Th17 cells.

To determine which types of T cells could secrete MIP-1α, MIP-1β, and RANTES under our experimental conditions, we examined the expression pattern of MIP-1α/MIP-1β/RANTES, IFN-γ, and IL-17 by intracellular cytokine staining (ICS) after nonspecific T cell stimulation. Production of MIP-1α/MIP-1β/RANTES occurred in IFN-γ-expressing $CD4^+$ T cells, particularly in the subset of cells that expressed high levels of IFN-γ (Fig. 2B left). IL-17 was also produced in $CD4^+$ T cells, but was secreted by a different $CD4^+$ T cell subset. In $CD8^+$ T cells, most IFN-γ-expressing cells produced MIP-1α/MIP-1β/RANTES, and IL-17 was not produced at all (Fig. 2B right). Thus, our assays showed that MIP-1α, MIP-1β, and RANTES are secreted from Th1-type $CD4^+$ T cells and $CD8^+$ T cells, and that IL-17-secreting cells (Th17 cells) are clearly distinct. These data suggest that cytokine production by T

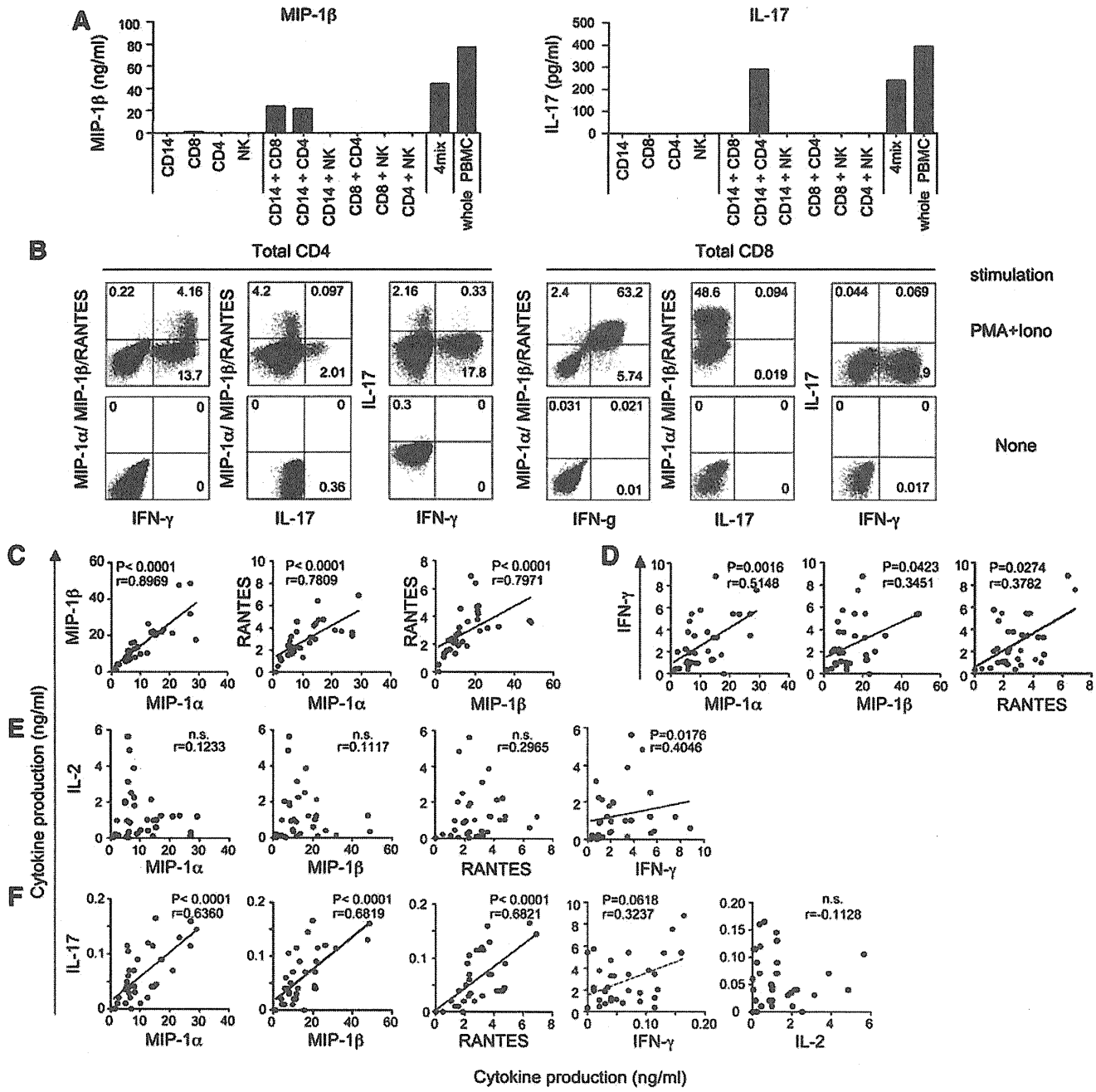


FIG. 2. Identification of cytokine-producing cells and relationships between cytokines. **(A)** Cytokine production in cell fractions from PBMCs. The results of MIP-1$\beta$ and IL-17 production are shown. Fractionated $CD4^+$, $CD8^+$, $CD14^+$, and NK ($CD56^+CD16^+$) cells from PBMCs in healthy individuals were cultured separately or cocultured for 48 h after PHA stimulation. The experiment was repeated twice with PBMCs from different donors. **(B)** Representative flow cytometric analysis of intracellular cytokine staining for MIP-1$\alpha$, MIP-1$\beta$, RANTES, IFN-$\gamma$ (Th1 cytokine), and IL-17 after PMA/ionomycin stimulation in PBMCs of healthy individual. **(C–F)** Correlation between each cytokine production in treatment-naive HIV-1-infected subjects. MIP-1$\alpha$, MIP-1$\beta$, and RANTES production **(C)**, IFN-$\gamma$ production and MIP-1$\alpha$, MIP-1$\beta$, or RANTES production **(D)**, IL-2 production and MIP-1$\alpha$, MIP-1$\beta$, RANTES, or IFN-$\gamma$ production **(E)**, IL-17 production and MIP-1$\alpha$, MIP-1$\beta$, RANTES, IFN-$\gamma$, or IL-2 production **(F)** are shown. Correlation analysis was performed with Spearman's rank correlation to determine correlations between variables.

cells from the HVL group is dysfunctional, specifically in some of the Th1-related cytokines and in IL-17.

We next analyzed the correlation between production of Th1 cytokines (IFN-$\gamma$ and IL-2), MIP-1$\alpha$/MIP-1$\beta$/RANTES, and IL-17 in treatment-naive HIV-1 subjects. The levels of MIP-1$\alpha$, MIP-1$\beta$, and RANTES showed strong positive correlations to each another (Fig. 2C), and correlations between IFN-$\gamma$ and each of them were also significant (Fig. 2D). However, IL-2, another typical Th1 cytokine, did not show any significant correlation with MIP-1$\alpha$, MIP-1$\beta$, and RANTES (Fig. 2E). Surprisingly, we found strong correlations between IL-17 production and MIP-1$\alpha$/MIP-1$\beta$/RANTES

levels or IFN-$\gamma$ levels, despite the fact that these cytokines are produced by different cells (Fig. 2F). These data suggest interrelated production of IFN-$\gamma$, MIP-1$\alpha$, MIP-1$\beta$, RANTES, and IL-17 in T cells, but not of IL-2. Moreover, the capacity of T cells to produce these cytokines appears to be affected by HIV-1 VL *in vivo*.

### *Both central and effector memory CD4$^+$ and CD8$^+$ T cells are highly activated and exhausted in HVL subjects*

The mechanism underlying the reduction in levels of specific cytokines in the HVL group could result either from decreased numbers of the cytokine-producing cells or from decreased productive capacity in those cells. We quantitated CD4$^+$ and CD8$^+$ T cells in HIV-1-infected patients (Table 1) and healthy control subjects (data not shown) by FACS. Although the number of CD4$^+$ T cells was significantly higher and the number of CD8$^+$ T cells significantly lower in HC than in HIV-positive patients, the differences in these T cell subsets were not significant between HVL and LVL.

As the number of monocytes seemed to affect T cell stimulation by PHA, we also analyzed monocytes (CD14$^+$ cells) and found there was no quantitative difference between any of the groups (data not shown).

As the cytokine productive capacity of T cells differs according to their differentiation status,[25] we explored the differentiation status of CD4$^+$ and CD8$^+$ T cells. We divided CD4$^+$ and CD8$^+$ T cells into four subsets depending on the expression pattern of CD45RA and CCR7: naive (CD45RA$^+$/CCR7$^+$), central memory (CM; CD45RA$^-$/CCR7$^+$), effector memory (EM; CD45RA$^-$/CCR7$^-$), and effector (CD45RA$^+$/CCR7$^-$) subsets. The proportion of each subset was highly heterogenic between subjects. The HVL and LVL subjects showed no significant differences in distribution of T cell subsets except in the proportion of naive CD8$^+$ T cells (data not shown), which cannot secrete cytokines even following PHA stimuli (Fig. 3A).[26]

To investigate whether there are qualitative differences in T cells between HVL and LVL subjects, we analyzed the expression of CD38, Ki67, Bcl2, PD-1, and CTLA-4 as markers of the activation and exhaustion status of T cells, which seems to affect their capacity to produce cytokines (Fig. 3B). In both CD4$^+$ and CD8$^+$ T cells, CM and EM subsets that mainly secrete these cytokines were highly activated (CD38$^+$, Ki67$^+$, and/or Bcl-2$^-$) in HVL subjects compared to LVL subjects (Fig. 3C). Especially in CM subsets of CD4$^+$ T cells, the frequency of exhausted cells (PD-1$^+$ and CTLA-4$^+$) was also significantly higher in HVL subjects compared to LVL subjects ($p < 0.05$ for both comparisons). EM subsets in CD4$^+$ T cells and CM and EM subsets in CD8$^+$ T cells also tended to be highly exhausted, although these differences were statistically insignificant in HVL subjects. These data indicate that memory CD4$^+$ and CD8$^+$ T cells, but not naive and effector subsets, are highly activated and exhausted in HVL subjects.

### *Poor cytokine production is directly correlated with activation/exhaustion status in memory T cells*

As exhausted memory CD8$^+$ T cells fail to produce effector cytokines, such as IL-2, IFN-$\gamma$, and TNF-$\alpha$, upon antigen stimulation,[27,28] we analyzed the relationship between the expression level of activation/exhaustion markers (CD38, Ki67, Bcl2, PD-1, and CTLA-4) on memory CD4$^+$ and CD8$^+$ T cells and the reduced production of cytokines seen in HVL subjects in response to PHA stimulation. The proportions of PD-1$^+$ and CD38$^+$ cells in CM subsets were inversely correlated with the capacity to produce MIP-1$\alpha$, MIP-1$\beta$, RANTES, IFN-$\gamma$, and IL-17 (Fig. 4, and data not shown). In the EM subsets, proportions of PD-1$^+$ and CD38$^+$ cells, but not of CTLA4$^+$ cells, were inversely correlated with cytokine production. These data suggest that the compromised productive capacity of Th1-related and IL-17 cytokines is directly associated with persistent activation and exhaustion in memory T cells.

### *Cytokine production capacity is recovered soon after ART initiation, but memory CD8$^+$ T cells remain activated and exhausted even after prolonged viral suppression by ART*

To explore whether the low cytokine production in HVL subjects is a cause or a consequence of high viral load, we compared cytokine production in subjects whose VL had been suppressed by ART for a prolonged period (>22 months) and whose CD4 count was at a similar level to that of HVL and LVL subjects (Tx subjects). In these subjects, production of the cytokines that were decreased in HVL subjects (MIP-1$\alpha$, MIP-1$\beta$, RANTES, IFN-$\gamma$, sIL-2R, IL-7, and IL-17) was significantly higher than in HVL subjects, and production of MIP-1$\alpha$, MIP-1$\beta$, sIL-2R, IL-7, and IFN-$\gamma$ was at a similar level to that seen in LVL and HC subjects (Fig. 1A). Production of RANTES and IL-17 was higher in subjects with long-term viral suppression than in the other groups.

To clarify the relationship between VL and cytokine production capacity, we performed a similar analysis in subjects with dramatic reductions in VL due to recent ART initiation. We measured cytokine production from PBMCs isolated from blood drawn from six HIV-1-infected subjects within 1–2 months after starting ART, when VL had undergone dramatic reduction (mean VL = 440 copies/ml, range 63 to 1100) (Fig. 5A). The levels of cytokines MIP-1$\alpha$, MIP-1$\beta$, RANTES, and IL-7 produced after PHA stimulation were comparable to those seen in subjects with long-term suppression from ART (Fig. 5B). These data indicate that dysfunction of these cytokine production in individuals with high VL is reversible and is recovered soon after the VL reduction.

We also analyzed the activation and exhaustion status of CD4$^+$ and CD8$^+$ T cells in treatment-experienced (Tx) subjects (Fig. 3C). With the sole exception of CTLA-4 expression on the EM subset in CD8$^+$ T cells, proportions of activated (CD38$^+$, Ki67$^+$, and Bcl-2$^-$) cells and exhausted (PD-1$^+$ and CTLA-4$^+$) cells within both memory CD4$^+$ and CD8$^+$ T cell populations were significantly lower in Tx subjects compared to HVL subjects (Fig. 3C).

We next examined the activation/exhaustion status of memory CD4$^+$ and CD8$^+$ cells in Tx subjects compared to uninfected control subjects to determine whether the T cell status can revert to normal status after prolonged viral suppression by ART. The Tx and HC groups did not differ significantly in expression levels of markers in memory CD4$^+$ T cells (Fig. 3C). In contrast, the two groups differed significant in the activation/exhaustion status of memory CD8$^+$ T cells, with higher levels of Ki67, PD-1, and/or CTLA-4 expression and lower levels of Bcl-2 expression, in Tx subjects compared

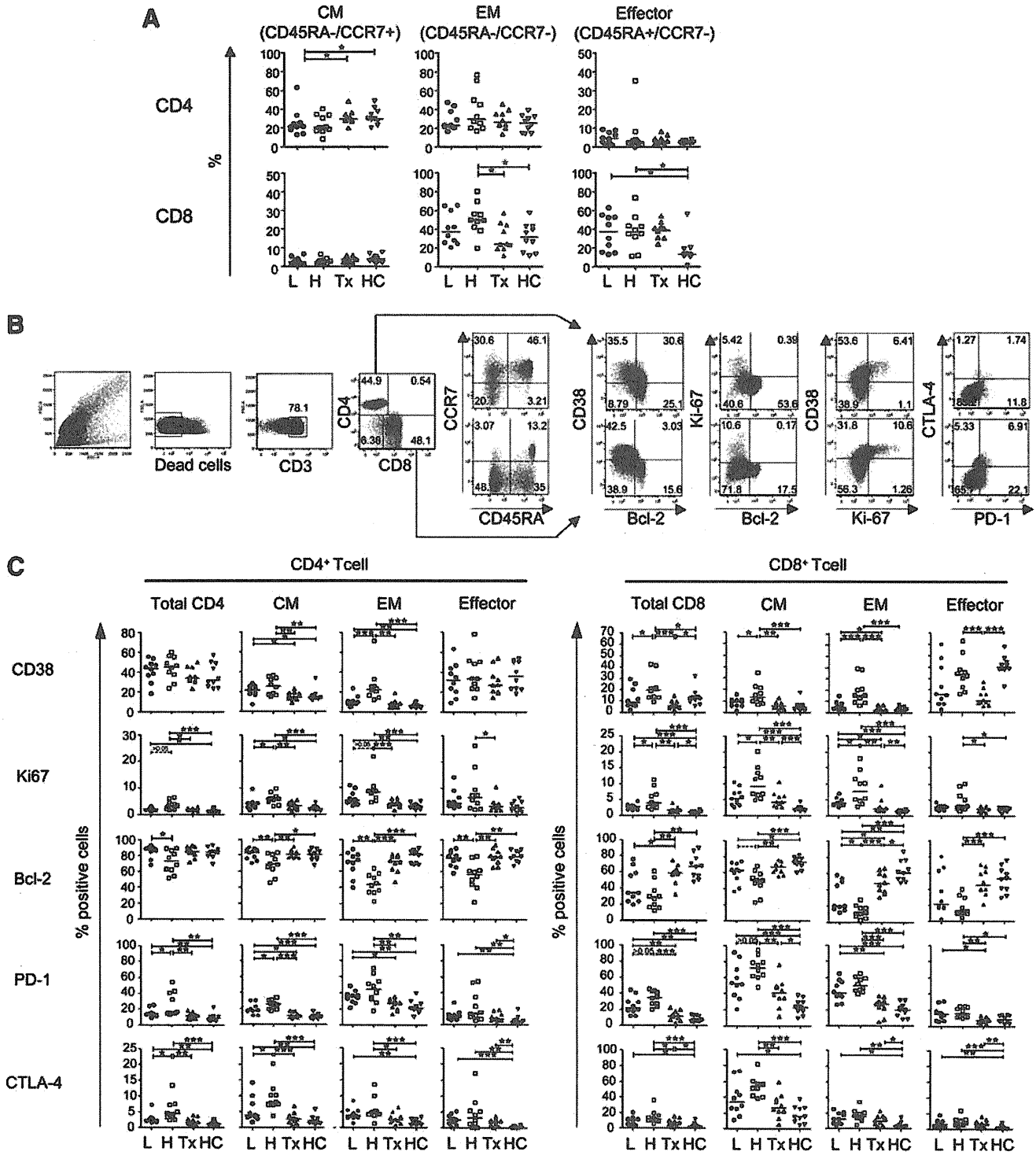


**FIG. 3.** Differentiation and activation/exhaustion status of $CD4^+$ and $CD8^+$ T cells in HIV-1-infected subjects. **(A)** Comparison of the frequency of $CD4^+$ and $CD8^+$ T cell subsets. The percentages of central memory (CM; $CD45RA^-/CCR7^+$), effector memory (EM; $CD45RA^-/CCR7^-$), and effector ($CD45RA^+/CCR7^-$) subsets in $CD4^+$ and $CD8^+$ T cells are shown. **(B)** Representative flow cytometric analysis of activation (CD38, Ki67, and Bcl2 expression) and exhaustion (PD-1 and CTLA-4 expression) status in $CD4^+$ and $CD8^+$ T cells. **(C)** Comparison of the activation/exhaustion status in $CD4^+$ and $CD8^+$ T cell subsets. L, LVL subjects (●), H, HVL subjects (□), Tx, HIV-1-infected subjects with prolonged ART (▲), HC, healthy control (□). The horizontal bars indicate the median value. Differences between groups were tested for statistical significance by the Mann–Whitney *U* test. $*p = 0.01$ to 0.05, $**p = 0.001$ to 0.01, $***p < 0.001$ (Mann–Whitney test).

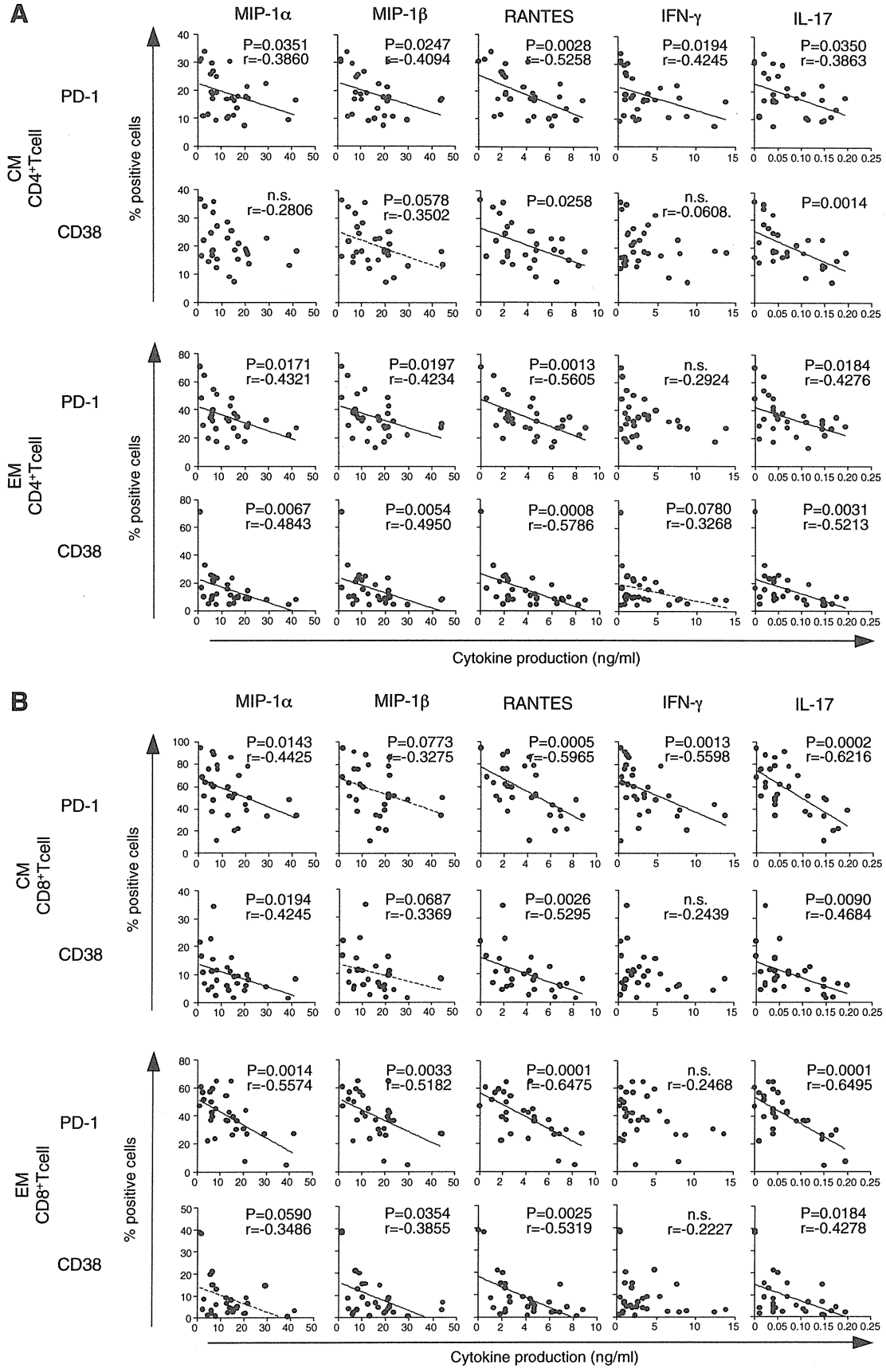


**FIG. 4.** Correlation between activation/exhaustion status in memory T cell subsets and cytokine production. Each panel indicates the relationship between the frequency of PD-1 or CD38 expressing cells in central memory (CM) and effector memory (EM) T cells and each cytokine production. The results of $CD4^+$ T cells and $CD8^+$ T cells are shown in **(A)** and **(B)**, respectively. Correlation analysis was performed with Spearman's rank correlation to determine correlations between variables.

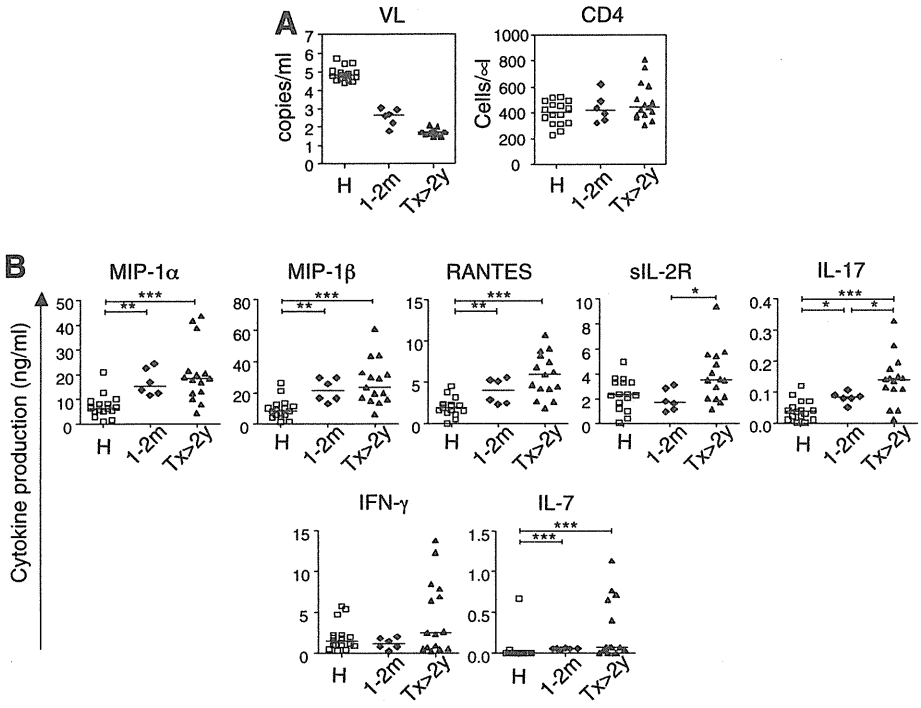


FIG. 5. Rapid recovery of cytokine production after initiation of antiretroviral therapy. **(A)** Viral load and CD4 count of each group. **(B)** Comparison of cytokine production by PHA-stimulated PBMCs. H, HVL subjects (□), 1–2 months, subjects 1–2 months after starting ART (□), Tx > 2 years, subjects with prolonged ART (▲). $^{*}p = 0.01$ to 0.05, $^{**}p = 0.001$ to 0.01, $^{***}p < 0.001$ (Mann–Whitney test).

to HC subjects. These data suggest that although suppression of HIV-1 replication by ART dramatically improves the cytokine production capacity of T cells to a normal level, memory $CD8^+$ T cells, but not memory $CD4^+$ T cells, remain somewhat activated even after prolonged viral suppression.

## Discussion

Despite intensive research, it remains unclear how HIV-1 can cause the collapse of the host immune system and development of AIDS after chronic infection. In this study, we demonstrate that high HIV-1 viral load associates with skewed T cell dysfunction in cytokine production, independently of CD4 T cell count. Diminished cytokine production in subjects with high VL is specific for some Th1-related cytokines (MIP1-α, MIP-1β, RANTES, and IFN-γ), IL-17, IL-7, and sIL-2R, and is associated with activation and exhaustion status in both $CD4^+$ and $CD8^+$ T cells, especially in memory subsets. The dysfunctional production of these cytokines in HVL subjects appears to be reversible, with recovery occurring after VL reduction by ART.

In this study, we tried to find as many cytokines as possible that differ between LVL and HVL subjects. For this reason, we used a strong stimulus and long incubation times to show the results clearly. The 48-h culture period is long enough to allow expression both of late-response genes and of secondary response genes that may be induced following the primary response.

Production of MIP-1α, MIP-1β, and RANTES was dramatically reduced in HVL subjects and showed a close inverse correlation with plasma VL (Fig. 1). As the natural ligands of HIV-1 coreceptor CCR5, MIP-1α/MIP-1β/RANTES are potent inhibitors of CCR5-tropic HIV-1 (R5-HIV-1) infection.[29] Physiologically, these chemokines also play a key role in induction of cellular immune responses by recruiting $CCR5^+$ Th1 lymphocytes to the infectious site *in vivo*.[30–33] In the case of HIV-1 infection, decreased production of these chemokines seems to favor both viral expansion and reduced migration of effector T cells *in vivo*. In recent studies, a high copy number of CCL3L1 (one of the genes encoding MIP-1α) combined with a low CCR5 expression genotype was associated with low VL in HIV-1-infected subjects,[34,35] suggesting that CCL3L1-CCR5 genotypes may be able to modify the clinical course of HIV-1 infection.

In our study plasma VL affected the ability of T cells to produce IFN-γ, one of the cytokines that defines Th1 cells, and IL-17, which is a Th17-type cytokine. However, no effect was seen on Th2-type cytokines (IL-4, IL-5, and IL-13) or IL-10. Interferon-γ, MIP-1α, MIP-1β,and RANTES are produced by Th1 cells (Fig. 2B), which preferentially express CCR5,[30] and Th17 cells are known to express CCR5 in peripheral blood.[36,37] However, Th2 cells do not express CCR5.[30] Transcription of these cytokines in T cells may be influenced by CCR5 signaling. Large amounts of R5-HIV-1 or the Env protein might persistently trigger the signaling pathway by binding to CCR5, thereby causing reductions in levels of specific cytokines in chronically HIV-1 subjects.

In our experiments, MIP-1α, MIP-1β, and RANTES were produced by IFN-γ-expressing cells in subsets of $CD4^+$ and $CD8^+$ T cells, and IL-17 was produced by a different subset of $CD4^+$ T cells. Surprisingly, IL-17 production was strongly correlated with MIP-1α, MIP-1β, RANTES, and IFN-γ production even though the producer cells are different (Fig. 2F). This correlation might reflect a general ability of Th1 and Th17 cells to produce cytokines. However, IL-2 production was not correlated with MIP-1α, MIP-1β, RANTES, and IFN-γ production, despite the fact that IL-2 should be produced by the same IFN-γ-producing cells (Fig. 2C–F). Critical regions of IFN-γ promoter (i.e., consensus GATA motif and essential functional motif) are not found in the IL-2 promoter region, but are found in the MIP-1α and MIP-1β promoters.[38] In addition, the same sequence in the promoter region of IFN-γ,

MIP-1α, and MIP-1β was found in the IL-17 promoter.[39] Interferon-γ, MIP-1α, MIP-1β, RANTES, and IL-17 production in T cells may be coordinately regulated, and the productive capacity of these cytokines appears to be affected by HIV-1 VL in a similar fashion. Alternatively, we measured cytokine production 48 h after PHA stimulation in this study. The period is long enough to develop sequential reactions occurring in response to primary reaction. As IFN-γ is known as an early-response gene and has the potential to affect multiple immune responses,[40] the production of MIP-1α, MIP-1β, RANTES, and IL-17, but not IL-2 may depend on the amount of IFN-γ as the primary response. Further studies are required to elucidate the mechanism by which IL-17 production is correlated with MIP-1α, MIP-1β, RANTES, or IFN-γ production. The IFN-γ pathway protects against intracellular pathogens through cellular immunity, and IL-17 provides protection against extracellular pathogens and fungal infections.[41,42] Although their target pathogens differ, IL-17 regulates the Th1 immune response through IL-17 receptor-expressing dendritic cells (DC) and macrophages.[43] These data suggest that Th1-type and Th17-type immune responses are closely related, and that their interaction is crucial for immune protection.

In a pathogenic SIV infection model, the loss of Th17 cells in the gastrointestinal tract dampens the intestinal mucosal barrier, resulting in microbial translocation, which in turn induces systemic immune activation.[44–47] In SIV infection the loss of Th17 cells in intestinal mucosa and in PBMCs is inversely correlated with plasma VL.[48] In this study, we observed a strong inverse correlation between IL-17 production and the proportion of activated and exhausted memory T cells. Our results suggest that not only the number of IL-17-producing cells but also the quality of those cells may account for the dysfunction of the Th17-type immune response in HVL subjects.

During chronic HIV-1 infection expression of the inhibitory coreceptors PD-1 and CTLA-4 on total T cells (not only HIV-1-specific T cells) is associated with plasma VL and CD4 count.[11,12] In this study, we found that the proportions of PD-1$^+$, CTLA4$^+$, and CD38$^+$ cells in total memory subsets of CD4$^+$ and CD8$^+$ T cells were inversely correlated with the ability of T cells to produce MIP-1α, MIP-1β, RANTES, IFN-γ, sIL-2R, and IL-17 in response to PHA stimulation (Fig. 4). It has been reported that PD-1 expression depends on the status of activation markers such as CD38 and on the differentiation stage of T cells.[49,50] Other studies have shown that blocking the pathway of the PD-1/PD-L1 interaction augments the cytokine production capacity of HIV-1-specific CD4$^+$ and CD8$^+$ T cells *in vitro*.[11,51] In our study, prolonged virus suppression by ART resulted in cytokine production capacities returning to normal (Fig. 1A). Memory subsets of CD4$^+$ T cells were no longer activated and exhausted (Fig. 3C), although memory subsets of CD8$^+$ T cells remained slightly activated/exhausted. These data suggest that activation and/or exhaustion of T cells is directly associated with the ability to produce these specific cytokines and that the impairment in T cell function is reversible.

Our study is the first to show that the T cell impairment in high VL subjects is specific for production of some of Th1-type and Th17-type cytokines, and that production of these cytokines is strongly correlated with one another. In subjects with high VL, a vicious cycle occurs, as T cells increasingly lose the capacity to produce these important cytokines. Notably, we also found that subjects who maintain a low VL, yet who are not "elite controllers," are capable of producing normal levels of these cytokines. These findings could be useful in guiding the development of new therapies focusing on immune control to reduce T cell activation in chronic HIV-1 infection.

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## Author Disclosure Statement

No competing financial interests exist.

## References

1. Mellors JW, Munoz A, Giorgi JV, *et al.*: Plasma viral load and CD4+ lymphocytes as prognostic markers of HIV-1 infection. Ann Intern Med 1997;126:946–954.
2. Grossman Z, Meier-Schellersheim M, Paul WE, and Picker LJ: Pathogenesis of HIV infection: What the virus spares is as important as what it destroys. Nat Med 2006;12:289–295.
3. Liu Z, Cumberland WG, Hultin LE, Prince HE, Detels R, and Giorgi JV: Elevated CD38 antigen expression on CD8+ T cells is a stronger marker for the risk of chronic HIV disease progression to AIDS and death in the Multicenter AIDS Cohort Study than CD4+ cell count, soluble immune activation markers, or combinations of HLA-DR and CD38 expression. J Acquir Immune Defic Syndr Hum Retrovirol 1997;16:83–92.
4. Silvestri G, Sodora DL, Koup RA, *et al.*: Nonpathogenic SIV infection of sooty mangabeys is characterized by limited bystander immunopathology despite chronic high-level viremia. Immunity 2003;18:441–452.
5. Hirsch VM: What can natural infection of African monkeys with simian immunodeficiency virus tell us about the pathogenesis of AIDS? AIDS Rev 2004;6:40–53.
6. Betts MR, Nason MC, West SM, *et al.*: HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. Blood 2006;107:4781–4789.
7. Freel SA, Lamoreaux L, Chattopadhyay PK, *et al.*: Phenotypic and functional profile of HIV-inhibitory CD8 T cells elicited by natural infection and heterologous prime/boost vaccination. J Virol 2010;84:4998–5006.
8. Nemes E, Bertoncelli L, Lugli E, *et al.*: Cytotoxic granule release dominates gag-specific CD4+ T-cell response in different phases of HIV infection. AIDS 2010;24:947–957.
9. Brenchley JM, Karandikar NJ, Betts MR, *et al.*: Expression of CD57 defines replicative senescence and antigen-induced apoptotic death of CD8+ T cells. Blood 2003;101:2711–2720.
10. Palmer BE, Blyveis N, Fontenot AP, and Wilson CC: Functional and phenotypic characterization of CD57+ CD4+ T cells and their association with HIV-1-induced T cell dysfunction. J Immunol 2005;175:8415–8423.
11. Day CL, Kaufmann DE, Kiepiela P, *et al.*: PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. Nature 2006;443:350–354.
12. Kaufmann DE, Kavanagh DG, Pereyra F, *et al.*: Upregulation of CTLA-4 by HIV-specific CD4+ T cells correlates with

disease progression and defines a reversible immune dysfunction. Nat Immunol 2007;8:1246–1254.
13. D'Souza M, Fontenot AP, Mack DG, *et al.:* Programmed death 1 expression on HIV-specific CD4+ T cells is driven by viral replication and associated with T cell dysfunction. J Immunol 2007;179:1979–1987.
14. Mosmann TR and Coffman RL: TH1 and TH2 cells: Different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol 1989;7:145–173.
15. Barrat FJ, Cua DJ, Boonstra A, *et al.:* In vitro generation of interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. J Exp Med 2002;195:603–616.
16. Harrington LE, Hatton RD, Mangan PR, *et al.:* Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat Immunol 2005;6:1123–1132.
17. Charlton B and Lafferty KJ: The Th1/Th2 balance in autoimmunity. Curr Opin Immunol 1995;7:793–798.
18. Clerici M and Shearer GM: A TH1→TH2 switch is a critical step in the etiology of HIV infection. Immunol Today 1993;14:107–111.
19. Thobakgale CF, Streeck H, Mkhwanazi N, *et al.:* CD8(+) T cell polyfunctionality profiles in progressive and nonprogressive pediatric HIV type 1 infection. AIDS Res Hum Retroviruses 2011;27:1005–1012.
20. Hammarstrom S, Hammarstrom ML, Sundblad G, Arnarp J, and Lonngren J: Mitogenic leukoagglutinin from Phaseolus vulgaris binds to a pentasaccharide unit in N-acetyllactosamine-type glycoprotein glycans. Proc Natl Acad Sci USA 1982;79:1611–1615.
21. Janossy G, Greaves MF, Doenhoff MJ, and Snajdr J: Lymphocyte activation. V. Quantitation of the proliferative responses to mitogens using defined T and B cell populations. Clin Exp Immunol 1973;14:581–596.
22. Scott MG and Nahm MH: Mitogen-induced human IgG subclass expression. J Immunol 1984;133:2454–2460.
23. Ceuppens JL, Baroja ML, Lorre K, Van Damme J, and Billiau A: Human T cell activation with phytohemagglutinin. The function of IL-6 as an accessory signal. J Immunol 1988;141:3868–3874.
24. Unanue ER and Allen PM: The basis for the immunoregulatory role of macrophages and other accessory cells. Science 1987;236:551–557.
25. Sallusto F, Lenig D, Forster R, Lipp M, and Lanzavecchia A: Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature 1999;401:708–712.
26. Agarwal S, Viola JP, and Rao A: Chromatin-based regulatory mechanisms governing cytokine gene transcription. J Allergy Clin Immunol 1999;103:990–999.
27. Wherry EJ and Ahmed R: Memory CD8 T-cell differentiation during viral infection. J Virol 2004;78:5535–5545.
28. Wherry EJ, Blattman JN, Murali-Krishna K, van der Most R, and Ahmed R: Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. J Virol 2003;77:4911–4927.
29. Cocchi F, DeVico AL, Garzino-Demo A, Arya SK, Gallo RC, and Lusso P: Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells. Science 1995;270:1811–1815.
30. Bonecchi R, Bianchi G, Bordignon PP, *et al.:* Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. J Exp Med 1998;187:129–134.
31. Ferbas J, Giorgi JV, Amini S, *et al.:* Antigen-specific production of RANTES, macrophage inflammatory protein (MIP)-1alpha, and MIP-1beta in vitro is a correlate of reduced human immunodeficiency virus burden in vivo. J Infect Dis 2000;182:1247–1250.
32. Siveke JT and Hamann A: T helper 1 and T helper 2 cells respond differentially to chemokines. J Immunol 1998;160: 550–554.
33. Taub DD, Conlon K, Lloyd AR, Oppenheim JJ, and Kelvin DJ: Preferential migration of activated CD4+ and CD8+ T cells in response to MIP-1 alpha and MIP-1 beta. Science 1993;260:355–358.
34. Dolan MJ, Kulkarni H, Camargo JF, *et al.:* CCL3L1 and CCR5 influence cell-mediated immunity and affect HIV-AIDS pathogenesis via viral entry-independent mechanisms. Nat Immunol 2007;8:1324–1336.
35. Gonzalez E, Kulkarni H, Bolivar H, *et al.:* The influence of CCL3L1 gene-containing segmental duplications on HIV-1/AIDS susceptibility. Science 2005;307:1434–1440.
36. Gosselin A, Monteiro P, Chomont N, *et al.:* Peripheral blood CCR4+ CCR6+ and CXCR3+ CCR6+ CD4+ T cells are highly permissive to HIV-1 infection. J Immunol 2010;184: 1604–1616.
37. Lim HW, Lee J, Hillsamer P, and Kim CH: Human Th17 cells share major trafficking receptors with both polarized effector T cells and FOXP3+ regulatory T cells. J Immunol 2008; 180:122–129.
38. Fernandez N, Renedo M, Garcia-Rodriguez C, and Sanchez Crespo M: Activation of monocytic cells through Fc gamma receptors induces the expression of macrophage-inflammatory protein (MIP)-1 alpha, MIP-1 beta, and RANTES. J Immunol 2002;169:3321–3328.
39. Dodon MD, Li Z, Hamaia S, and Gazzolo L: Tax protein of human T-cell leukaemia virus type 1 induces interleukin 17 gene expression in T cells. J Gen Virol 2004;85:1921–1932.
40. Penix L, Weaver WM, Pang Y, Young HA, and Wilson CB: Two essential regulatory elements in the human interferon gamma promoter confer activation specific expression in T cells. J Exp Med 1993;178:1483–1496.
41. Acosta-Rodriguez EV, Rivino L, Geginat J, *et al.:* Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. Nat Immunol 2007;8:639–646.
42. Ouyang W, Kolls JK, and Zheng Y: The biological functions of T helper 17 cell effector cytokines in inflammation. Immunity 2008;28:454–467.
43. Lin Y, Ritchea S, Logar A, *et al.:* Interleukin-17 is required for T helper 1 cell immunity and host resistance to the intracellular pathogen Francisella tularensis. Immunity 2009;31: 799–810.
44. Brenchley JM, Price DA, Schacker TW, *et al.:* Microbial translocation is a cause of systemic immune activation in chronic HIV infection. Nat Med 2006;12:1365–1371.
45. Raffatellu M, Santos RL, Verhoeven DE, *et al.:* Simian immunodeficiency virus-induced mucosal interleukin-17 deficiency promotes Salmonella dissemination from the gut. Nat Med 2008;14:421–428.
46. Brenchley JM, Paiardini M, Knox KS, *et al.:* Differential Th17 CD4 T-cell depletion in pathogenic and nonpathogenic lentiviral infections. Blood 2008;112:2826–2835.
47. Brenchley JM and Douek DC: HIV infection and the gastrointestinal immune system. Mucosal Immunol 2008;1:23–30.
48. Cecchinato V, Trindade CJ, Laurence A, *et al.:* Altered balance between Th17 and Th1 cells at mucosal sites predicts

AIDS progression in simian immunodeficiency virus-infected macaques. Mucosal Immunol 2008;1:279–288.
49. Appay V, van Lier RA, Sallusto F, and Roederer M: Phenotype and function of human T lymphocyte subsets: Consensus and issues. Cytometry A 2008;73(11):975–983.
50. Sauce D, Almeida JR, Larsen M, *et al.:* PD-1 expression on human CD8 T cells depends on both state of differentiation and activation status. AIDS 2007;21:2005–2013.
51. Trautmann L, Janbazian L, Chomont N, *et al.:* Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction. Nat Med 2006;12: 1198–1202.

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Original article

# Huwe1, a novel cellular interactor of Gag-Pol through integrase binding, negatively influences HIV-1 infectivity

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**Abstract**

Integration, an indispensable step for retrovirus replication, is executed by integrase (IN), which is expressed as a part of a Gag-Pol precursor. Although mechanistic detail of the IN-catalyzed integration reaction is well defined, numerous evidence have demonstrated that IN is involved in multiple steps of retrovirus replication other than integration. In this study, Huwe1, a HECT-type E3 ubiquitin ligase, was identified as a new cellular interactor of human immunodeficiency virus type 1 (HIV-1) IN. The interaction was mediated through the catalytic core domain of IN and a wide-range region of Huwe1. Interestingly, although depletion of Huwe1 in target cells did not affect the early phase of HIV-1 infection in a human T cell line, we found that infectivity of HIV-1 released from the Huwe1 knockdown cells was significantly augmented more than that of virus produced from control cells. The increase in infectivity occurred in proviral DNA synthesis. Further analysis revealed that Huwe1 interacted with HIV-1 Gag-Pol precursor protein through an IN domain. Our results suggest that Huwe1 in HIV-1 producer cells has a negative impact on early post-entry events during the next round of virus infection via association with an IN region of Gag-Pol.


*Keywords:* HIV-1; MoMLV; Integrase; Huwe1; Gag-Pol precursor protein

## 1. Introduction

An infecting retroviral virion contains genomic RNA together with viral proteins that are required for completing the early phase of infection. Following entry into the host cell, a copy of viral DNA is synthesized from the viral genome by its reverse transcriptase (RT) and, subsequently, integrated into chromosomal DNA to form provirus by the preintegration complex (PIC). This integration step is essential for retroviral replication and is catalyzed by a viral enzyme, integrase (IN). Retroviral IN consists of three structurally and functionally distinct domains called the N-terminal domain (NTD), catalytic core domain (CCD), and C-terminal domain (CTD) [1]. CCD is highly conserved amongst retroviral INs and contains the triad of conserved amino acids that comprise Asp, Asp, and Glu residues termed the D, D-35-E motif. This domain possesses a key role during the integration reaction [1]. A well-conserved motif is also

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found in the NTD, which comprises two His and two Cys residues (HHCC motif). HHCC motif is a zinc-binding motif that contributes to the multimerization and catalytic function of IN. In contrast, CTD is the least conserved of the domains amongst retroviral INs but makes nonspecific contacts with DNA [1]. Retroviral integration catalyzed by IN proceeds in well-characterized 3′-processing and strand transfer steps; these chemical reactions are reproducible *in vitro* with recombinant IN and DNA substrates, demonstrating that IN alone is sufficient to carry out the DNA breakage and joining reactions [1,2].

IN is expressed as a C-terminal part of the Gag-Pol polyprotein from integrated provirus and incorporated into the virion during the late phase of infection. After assembly, viral protease (PR) cleaves the Gag-Pol precursor as well as the Gag precursor to yield a mature virion [3]. In retroviral genomic RNA, Gag and Pol proteins are encoded by overlapping open reading frames (ORF), and Gag-Pol is generated by either a ribosomal frameshifting during translation of *gag* or a read-through suppression on the *gag* termination codon. These translation mechanisms result in intracellular synthesis of Gag-Pol proteins at 10- to 20-fold-lower than Gag proteins [3]. Although the synthesis of Gag suffices to produce virus-like particles, incorporation and processing of Gag-Pol is integral to the morphogenesis of virions [3,4], indicating an important role of the Gag-Pol precursor in the formation of infectious virions during retroviral replication.

As mentioned above, the enzymatic activities of retroviral IN in integration reactions have been clearly defined by numerous *in vitro* biochemical studies using recombinant IN protein and oligonucleotide DNA substrates [1]. However, many mutation studies on the *IN* gene of infectious HIV-1 molecular clones have demonstrated that the IN gene also has roles on multiple steps of virus replication in addition to integration. This pleiotropic effect of IN is characterized by a defect in uncoating, reverse transcription, nuclear import, viral gene expression, virion precursor protein processing, and virion morphology [5–11]; however, the mechanism for the pleiotropic effect of IN gene is still poorly understood.

A number of cellular proteins have been identified as binding partners for human immunodeficiency virus type 1 (HIV-1) IN [2,12] and Moloney murine leukemia virus (MoMLV) IN [13]. In the case of HIV-1, some of the cellular interactors have been reported to assist in facilitating the integration reaction in virus-infected cells [2]. In particular, lens epithelium-derived growth factor (LEDGF) is a critical factor that acts as a chromosomal receptor that tethers IN and viral DNA to host DNA, thereby promoting integration during HIV-1 infection [12]. On the other hand, several lines of evidence demonstrate that other cellular partners for IN such as integrase interactor 1 (INI1), Gemin2, von Hippel-Lindau binding protein 1, transportin 3, and nucleoporin 153 appear to be implicated in steps other than the integration process during HIV-1 infection [2,14–18]. Overexpression or small interfering RNA (siRNA)-mediated depletion of these factors influence stages of reverse transcription, nuclear import, gene expression, and virion production during HIV-1 replication cycle [14,15,17–19]. Although exact roles of the IN-binding proteins in retroviral infection remain to be completely elucidated, these data suggest that a protein–protein interaction between retroviral IN and its cellular partner would be a primary basis for the pleiotropic effects that have been found from studies with IN mutant viruses.

In the present study, we identified Huwe1 (HECT, UBA, and WWE domain containing 1), a HECT (homologous to E6-AP carboxyl terminus)-domain ubiquitin ligase, as a novel cellular interactor of HIV-1 IN. The interaction was mediated through the CCD of HIV-1 IN and a wide-range region of Huwe1. Interestingly, Huwe1 also interacted with the IN portion of the HIV-1 Gag-Pol precursor protein. In addition, our study showed that Huwe1 appeared to modulate HIV-1 infectivity in virus producer cells, which influenced the step of proviral DNA synthesis in the next round of infection.

## 2. Materials and methods

### 2.1. Cells

NIH3T3 cells, 293T cells, and TZM-bl cells (a HeLa cell line expressing CD4, CXCR4, and CCR5 and containing an integrated *lacZ* reporter gene under the control of an HIV-1 LTR) [20] were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (FCS) and antibiotics. MT-4 cells were maintained in RPMI 1640 supplemented with 10% FCS and antibiotics.

### 2.2. Construction of expression vectors for MoMLV and HIV-1 IN

To generate a plasmid expressing TAP tag-fused MoMLV IN (pCeMM-GS-MoMLV IN), MoMLV IN cDNA was amplified by PCR from pNCA [21] and inserted into pCeMM-NTAP(GS)-Gw [22]. For construction of a plasmid expressing C-terminal HA-tagged MoMLV IN, the MoMLV IN cDNA was cloned into pCTAP-A (Stratagene) and, subsequently, an intron sequence of human *β-globin* gene [23] and HA tag sequence were inserted into the vector. The plasmid expressing C-terminal V5-tagged HIV-1 IN and its domain mutants were described previously [23].

### 2.3. Tandem affinity purification and mass spectrometry analysis

NIH3T3 cells ($7 \times 10^5$ cells) were transfected with 50 μg of pCeMM-GS-MoMLV-IN or empty vector by the calcium phosphate method, collected 48 h after transfection, and lysed in TAP lysis buffer (20 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 150 mM KCl, 0.2% NP-40, 1 mM DTT, protease inhibitors). Cell debris was removed by centrifugation, and 200 μl of IgG Sepharose 6 Fast Flow (GE Healthcare) was added to lysate and incubated at 4 °C for 2 h. Beads were washed with lysis buffer twice and, subsequently, with TAP washing buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.5 mM EDTA, 0.2% NP-40, 1 mM DTT) once. Proteins were eluted in 300 μl of TAP washing buffer containing 30 U of tobacco etch virus (TEV) protease (AcTEV, Invitrogen) at 4 °C. The sample was then

incubated with 100 μl of Streptavidin Sepharose High Performance (GE Healthcare) at 4 °C for 2 h. The beads were washed with TAP washing buffer containing protease inhibitors five times. Finally, protein complexes were eluted in 2 ml of TAP washing buffer containing 5 mM biotin (Pierce) and protease inhibitors, and concentrated. Mass spectrometric identification of proteins was performed as previously described [24].

### 2.4. Construction of expression vectors for Huwe1

To generate an ORF of murine Huwe1 (13137 bp), cDNA covering amino acid position 1–3567 (fragment 1), 3214–7178 (fragment 2), 5487–8907 (fragment 3), 8531–10850 (fragment 4), and 10732–13137 (fragment 5) were amplified by RT-PCR from total the RNA of NIH3T3 cell and subcloned into pFLAG-CMV-2 (Sigma). Fragments 2–5 were excised from each plasmid by digestion with appropriate restriction enzymes and sequentially inserted into pFLAG-CMV-2 encoding fragment 1. For truncation mutants, Huwe1 cDNA containing position 2394–3617 was obtained from a plasmid encoding fragments 1–4 and cloned into the pFLAG-CMV-2. Similarly, cDNA positions 4005–4378 (HECT) was excised from a plasmid encoding fragment 5 and cloned into the pFLAG-CMV-2. Vectors encoding fragments 1 and 2 were used for expression of Huwe1 position 1–1072 and 1072–2394, respectively.

### 2.5. Immunoprecipitation assay

293T cells were transfected with a plasmid expressing either HA-tagged MoMLV IN or V5-tagged HIV-1 IN by the calcium phosphate method. Cells were collected at 48 h after transfection and lysed in TAP lysis buffer. After centrifugation, supernatant was incubated with 10 μg of anti-α-tubulin mouse monoclonal IgG (DM1A, Sigma) or anti-Huwe1 rabbit polyclonal IgG (Novus Biologicals) in presence of 20 μl of Protein G Sepharose Fast Flow (GE Healthcare) for 4 h. Beads were then washed with TAP lysis buffer 3 times. In additional immunoprecipitation assay, FLAG-tagged Huwe1 was co-expressed with V5-tagged HIV-1 IN in 293T cells, and subjected to immunoprecipitation assay using 5 μg of anti-FLAG mouse monoclonal IgG (M2, Sigma).

### 2.6. Western blotting analysis

Samples were lysed in SDS buffer [25], resolved by SDS-PAGE gels, and transferred to Immobilion P Transfer Membrane (Millipore). Primary antibodies used were anti-α-tubulin mouse monoclonal IgG (DM1A, Sigma), anti-Huwe1 rabbit polyclonal IgG (Novus Biologicals), anti-V5 mouse monoclonal IgG (Invitrogen), and anti-FLAG mouse monoclonal IgG (M2, Invitrogen). Horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG (Cell Signaling) was used as a secondary antibody. Detection of HA-tagged protein was carried out using HRP-conjugated anti-HA rat monoclonal IgG (3F10, Roche). For immunoprecipitation analysis, TrueBlot ULTRA HRP anti-mouse or anti-rabbit IgG (eBioscience) was used as a secondary antibody.

### 2.7. Generation of Huwe1 knockdown cell line and HIV-1 infection experiments

By using the BLOCK-iT Pol II miR RNAi Expression Kit (Invitrogen), synthesized oligonucleotide containing microRNA (miRNA) sequence against human Huwe1 ORF (nucleotide position 10854–10874) or no specific gene (control miRNA [Invitrogen]) was cloned into a lentiviral vector, CSII-CMV-MCS-GATEWAY-IRES-hrGFP, in which the multiple cloning site of CSII-CMV-MCS-IRES-hrGFP [26] had been replaced with a Gateway cloning system reading frame cassette. Infectious lentiviral vectors pseudotyped with vesicular stomatitis virus G (VSV-G) were produced from 293T cells [25]. MT-4 cells were exposed to the lentiviral vectors and enriched for hrGFP positive cells using the FACSAria cell sorting system (BD Bioscience).

Preparation of virus stocks of HIV-$1_{NL4-3}$ and a HIV-1-based vector bearing a luciferase gene (HIV-Luc), and the determination of $p24^{CA}$ concentration in virus solutions and the 50% tissue culture infectious dose ($TCID_{50}$) of HIV-$1_{NL4-3}$ stock were performed as described previously [27,28].

In single-round infection experiments, $3 \times 10^5$ Huwe1 knockdown or control MT-4 cells were exposed to HIV-Luc containing 5 ng of $p24^{CA}$ at 37 °C for 2 h and cultured after washing twice. Cells were harvested at 48 h post-infection and subjected to a luciferase activity assay [27]. To measure the level of viral DNA synthesis, HIV-$1_{NL4-3}$ containing 100 ng of $p24^{CA}$ were added to $1 \times 10^6$ cells, and incubated at 37 °C for 2 h. After three times washing, cells were cultured in fresh medium. Total DNA was isolated 24 h after infection and subjected to quantitative PCR with HIV Tat/Rev specific primers [29].

To analyze HIV-1 production from virus-infected cells, $3 \times 10^5$ Huwe1 knockdown or control MT-4 cells were infected with HIV-$1_{NL4-3}$ at a MOI of 0.001 at 37 °C for 2 h. Cells were washed twice and cultured with 1.5 ml of fresh medium. Culture supernatant was harvested at 3 days after infection and 100 μl of the virus solution was added to $1 \times 10^5$ TZM-bl cells. β-galactosidase activity in the TZM-bl cells was measured 48 h after infection [30] and normalized for $p24^{CA}$ concentration in culture supernatant of MT-4 cells.

### 2.8. Electron microscopy analysis

HIV-1-infected cells were fixed with 2% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.2 at 4 °C for 90 min. Fixed cells were washed six times with the same buffer, further fixed with 2% osmium tetroxide in the same buffer at 4 °C for 90 min, contrast-enhanced with 1% tannic acid (Mallinckrodt) in same buffer, treated with 1% sodium sulfate in the same buffer, and dehydrated in a graded ethanol series. The samples were then embedded in Plain Resin (Nisshin EM, Japan). Ultrathin sections made with an ultramicrotome (Sorvall MT-5000, Du Pont) were doubly-stained with uranyl acetate and lead citrate, and observed under a transmission electron microscope (H-7650, Hitachi, Japan).

### 2.9. Quantification of HIV-1 DNA

Virus solution (100 μl) derived from HIV-1-infected Huwe1 knockdown and control cells were added to 1 × $10^6$ MT-4 cells, and incubated at 37 °C for 2 h. After washing twice, cells were cultured in fresh medium and harvested at 3, 6, and 24 h post-infection. Total DNA was purified using DNeasy Blood & Tissue Kit (Qiagen). Quantitative PCR for RT products and semiquantitative *Alu*-LTR PCR for integrated DNA were performed as described previously [31]. Heat-inactivated (65 °C, 30 min) virus was used as a negative control for infection.

### 2.10. Assay for HIV-1 Gag-Pol protein

An HIV-1 molecular clone expressing HA-tagged Gag-Pol was generated by the deletion of frameshift signal and the in-frame insertion of the HA tag sequence into the C-terminus of the *pol* gene of pNL4-3 bearing an inactive form of PR (Haraguchi and Morikawa, unpublished) [32]. Similar molecular clone expressing HA-tagged Gag-Pol/ΔIN was created by the addition of an HA tag at the C-terminus of RT and the insertion of a premature termination codon at the junction of RT-IN (Haraguchi and Morikawa, unpublished). FLAG-tagged Huwe1 and HA-tagged Gag-Pol were co-expressed in 293T cells, and cell lysate was subjected to immunoprecipitation using an anti-FLAG antibody.

### 2.11. Statistical analysis

Student's *t* test was used to determine statistical significance. *P* values lower than 0.05 were considered significant.

## 3. Results

### 3.1. Identification of new cellular interactors of retroviral IN

In order to identify novel cellular proteins that interact with retroviral IN, we employed a tandem affinity purification (TAP) procedure. TAP is a generic two-step affinity purification protocol for the isolation of TAP tag-fused proteins together with associated proteins under physiological conditions [22]. In this study, the TAP tag consisted of two IgG binding units and a streptavidin-binding peptide, which were separated by a TEV protease cleavage site [22]. N-terminal TAP tag-fused MoMLV IN was expressed transiently in NIH3T3 cells and recovered from cell extracts by two sequential purification steps [22]. Purified proteins were visualized by silver staining after SDS-PAGE. As shown in Fig. 1A, TAP-tagged MoMLV IN (lane 2), but not the TAP tag alone (lane 3), specifically co-purified with a 55 kDa and a >200 kDa proteins. Mass spectrometry analysis identified the 55 kDa band as a mixture of α- and β-tubulins, which exhibit similar molecular weight, and the >200 kDa band as

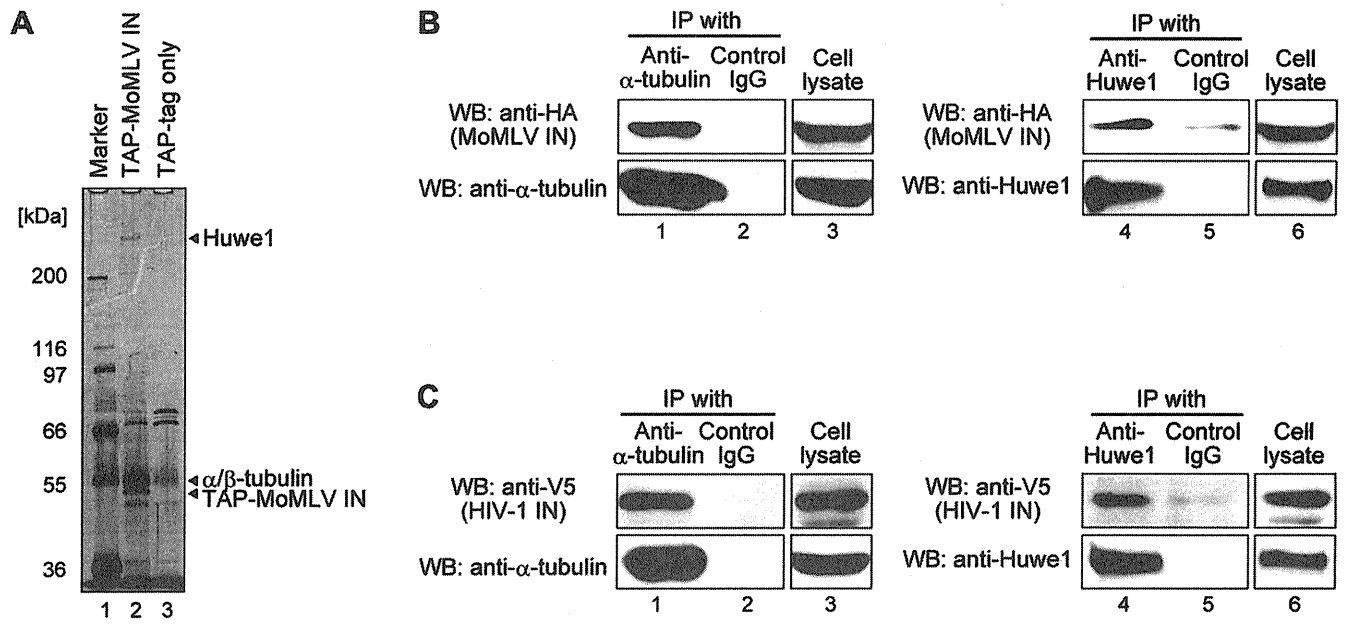


Fig. 1. Identification of tubulin and Huwe1 as cellular interactors of retroviral INs. (A) Tandem affinity purification for MoMLV IN. TAP tag-fused MoMLV IN (lane 2) was expressed in NIH3T3 cells, and protein complexes containing the TAP tag-fused IN were purified from cytoplasmic extract by two-step affinity purification procedure using IgG Sepharose and streptavidin Sepharose beads. Proteins were visualized by silver staining on a 10–20% SDS-PAGE gel, excised from the gel, and analyzed by MALDI-TOF/MS. As a control experiment, TAP tag was expressed and subjected to the tandem affinity purification (lane 3). Lane 1 shows molecular weight marker. (B and C) Interactions of endogenous α-tubulin and Huwe1 with MoMLV and HIV-1 IN. HA-tagged MoMLV IN (B) or V5-tagged HIV-1 IN (C) was expressed in 293T cells and co-immunoprecipitated (IP) with anti-α-tubulin (lane 1), anti-Huwe1 (lane 4), and control (lanes 2 and 5) antibodies. MoMLV IN and HIV-1 IN in the immunoprecipitates were detected by western blotting analysis (WB) using anti-HA (B) and anti-V5 antibodies (C), respectively.

Huwe1 [33] (Fig. 1A). We then confirmed the association by expression of HA-tagged MoMLV IN and subsequent co-immunoprecipitation with an anti-α-tubulin or an anti-Huwe1 antibody. Western blotting of the precipitates revealed that HA-tagged MoMLV IN was recovered by anti-α-tubulin and anti-Huwe1 antibodies (Fig. 1B, lanes 1 and 4), but not by control IgG (Fig. 1B, lanes 2 and 5), indicating specific binding of MoMLV IN to α-tubulin and Huwe1.

We next tested whether tubulin and Huwe1 bind to HIV-1 IN as well. To examine the binding, C-terminal V5-tagged HIV-1 IN was expressed transiently in 293T cells and subjected to co-immunoprecipitation assay using anti-α-tubulin and anti-Huwe1 antibodies. Western blotting analysis of the immunoprecipitates showed that endogenous α-tubulin and Huwe1 also interact with HIV-1 IN (Fig. 1C, lanes 1 and 4). It should be noted that, even when the lysates of MoMLV and HIV-1 IN expressing cells were treated with nucleases, interactions of the IN with α-tubulin and Huwe1 were not disrupted (data not shown), indicating that these interactions were not mediated through nucleic acids. Therefore, tubulin and Huwe1 were identified as the cellular interactors common to both MoMLV and HIV-1 IN.

### 3.2. Interaction domains between HIV-1 IN and Huwe1

Amongst the cellular proteins identified by our TAP procedure, Huwe1 is a novel IN-binding host protein, whereas tubulin has been reported to interact with HIV-1 IN [2]. Thus, we focused on the interaction between HIV-1 IN and Huwe1. To map the binding domain of IN, a series of V5-tagged HIV-1 IN containing truncated domain mutants were co-expressed with FLAG-tagged Huwe1 in 293T cells, and co-immunoprecipitation using an anti-FLAG antibody was performed. As in the case of the immunoprecipitation for endogenous Huwe1 (Fig. 1C), V5-tagged HIV-1 IN was associated with the FLAG-tagged Huwe1 (Fig. 2A, lane 2). A parallel co-immunoprecipitation assay using IN truncation mutants showed that the CCD of HIV-1 IN (Fig. 2A, lane 6), but not the NTD or CTD (Fig. 2A, lanes 4 and 8), was found to be sufficient for the interaction with Huwe1.

We next determined which region of Huwe1 is involved in the interaction with HIV-1 IN CCD. Analysis of the amino acid sequences have revealed that Huwe1 possesses several recognizable domains; ARLD (Armadillo [ARM] repeat like domain) 1 and 2 in the N-terminus, UBA (ubiquitin-associated), WWE, and well-conserved BH3 domains in the middle, and a HECT domain in the C-terminus (Fig. 2B) [34,35]. To understand the interaction region in Huwe1, we constructed plasmids expressing FLAG-tagged Huwe1 mutants encompassing ARLD1/2 (1–1072), UBA, WWE, and BH3 domains (1072–2394), C-terminal uncharacterized domain (2394–3617) alone, or HECT domain alone (Fig. 2B), and transfected them, respectively, into 293T cells together with a plasmid expressing V5-tagged HIV-1 IN CCD. Co-immunoprecipitation using an anti-FLAG antibody showed that the HIV-1 IN CCD associated with all of the truncation mutants of Huwe1 (Fig. 2C, lanes 2–4) except for the HECT domain mutant (Fig. 2C, lane 6). These results indicate that Huwe1 interacts with HIV-1 IN CCD through a broad region spanning 3500 amino acids.

### 3.3. Dispensable role of Huwe1 in early stage of HIV-1 infection

IN is a key component of the PIC [2]. Our preliminary experiment showed that PICs isolated from MoMLV-infected cells were immunoprecipitated by an anti-Huwe1 antibody, indicating the association of Huwe1 with the PIC (data not shown). Taken together with the result of physical interaction of IN and Huwe1, these data raise the possibility that Huwe1 may have a functional role during an early phase of virus replication. In order to assess the role of Huwe1 in HIV-1 infection, endogenous Huwe1 was depleted from a human T cell line, MT-4, by transduction of a lentivirus vector expressing miRNA against the Huwe1 ORF. Specific reduction of endogenous Huwe1 expression was confirmed by western blotting in the Huwe1 miRNA-expressing cells, but not in control miRNA-expressing cells (Fig. 3A). To estimate the efficiency of HIV-1 infection in the knockdown cells, a single-round replication assay was performed [7]. The Huwe1 knockdown and control MT-4 cells were used as target cells for infection by HIV-Luc pseudotyped with NL4-3 envelope (Env), and luciferase activity was measured 48 h after infection. As observed in Fig. 3B, a comparable level of luciferase activity was detected in Huwe1 knockdown and control cells. A similar result was also obtained by the infection with HIV-Luc pseudotyped with VSV-G (data not shown). In addition, when the cells were infected with replication competent HIV-$1_{NL4-3}$, no apparent difference in the amount of newly synthesized viral DNA was observed between Huwe1 knockdown and control cells (Fig. 3C). These results indicate that Huwe1 in target cells is not involved during the early steps of HIV-1 replication, from post-entry until viral gene expression.

### 3.4. Modulatory role of Huwe1 in HIV-1 infectivity

Some cellular interactors of HIV-1 IN have been shown to be involved in multiple steps of virus replication other than integration [14–16]. Thus, we next addressed the potential role of Huwe1 on the post-integrative steps in HIV-1 replication. Huwe1 knockdown and control cells were infected with $HIV_{NL4-3}$ at a MOI of 0.001 for 2 h, and after washing, cells were cultured with fresh medium. When the culture supernatants were analyzed 3 days after infection, equivalent levels of $p24^{CA}$ were detected (Fig. 4A). In electron microscopy, the morphology of virus particles in Huwe1 knockdown cells was indistinguishable from the virus produced in control cells (Fig. 4B), indicating that virus particle production from Huwe1 knockdown and control cells were comparable. However, when the HIV-1 infectivity in the culture supernatants were compared by using TZM-bl indicator cells [30], we found that the infectivity of virus derived from Huwe1 knockdown cells was 2.2-fold higher than that of virus from control cells (Fig. 4C). This enhanced infectivity of HIV-1 was reproducibly observed with a separate preparation of Huwe1 knockdown cells (data not shown).

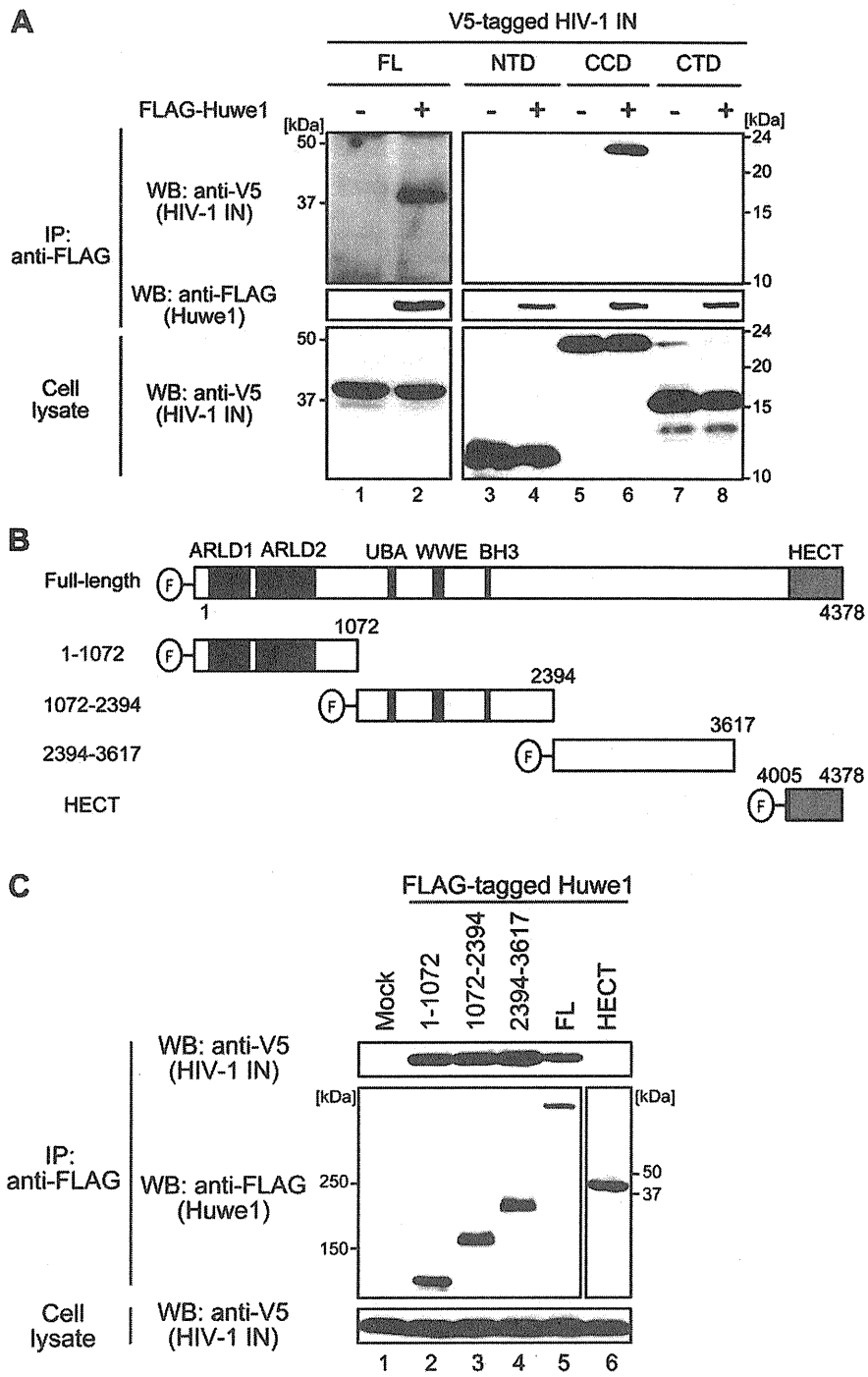


Fig. 2. Interaction domains between HIV-1 IN and Huwe1. (A) Binding domain in HIV-1 IN. FLAG-tagged Huwe1 was co-expressed with full-length (FL), N-terminus domain (NTD), catalytic core domain (CCD), or C-terminus domain (CTD) of V5-tagged HIV-1 IN in 293T cells. Cell lysates were subjected to immunoprecipitation (IP) using an anti-FLAG antibody, and HIV-1 IN and Huwe1 in immunoprecipitates were detected by western blotting analysis (WB) using anti-V5 and anti-FLAG antibodies, respectively. Masses of molecular weight standards are indicated at left (for FL IN) and right (for IN truncation mutants). (B) A schematic representation of Huwe1 used for co-immunoprecipitation assay with HIV-1 IN CCD. N-terminal FLAG (F)-tagged full-length Huwe1 and its truncation mutants (1–1072, 1072–2394, 2394–3617, and HECT) were constructed. ARLD, Armadillo repeat like domain. UBA, ubiquitin-associated domain. HECT, homologues to E6-AP carboxyl terminus domain. (C) Mapping of interaction domain in Huwe1. A series of FLAG-tagged Huwe1 (lane 5) or truncation mutants (lanes 2, 3, 4, and 6) were co-expressed with V5-tagged HIV-1 IN CCD in 293T cells and subjected to co-immunoprecipitation assay (IP) using an anti-FLAG antibody. Immunoprecipitates were analyzed by western blotting analysis (WB) using an anti-V5 (for HIV-1 IN CCD) or an anti-FLAG (for Huwe1) antibody. Masses of molecular weight standards are indicated at left (for Huwe1 1–1072, 1072–2394, 2394–3617, and FL) and right (for Huwe1 HECT).

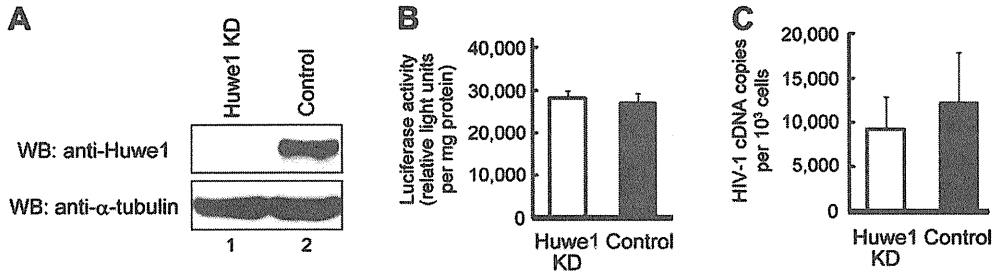


Fig. 3. Effect of Huwe1 depletion in the early stage of HIV-1 infection. (A) Establishment of Huwe1 knockdown (KD) cell. MT-4 cells were transduced with lentivirus vectors expressing miRNA against Huwe1 (lane 1) or control miRNA (lane 2). Expression of endogenous Huwe1 in both cell lines was examined by western blotting analysis (WB) using an anti-Huwe1 antibody. (B) Susceptibility of Huwe1 knockdown cells to single-round HIV-1 infection. Huwe1 knockdown (white bar) and control (black bar) MT-4 cells were infected with NL4-3 Env-pseudotyped HIV-Luc, and luciferase activity was measured 48 h after infection. (C) Level of HIV-1 DNA synthesis. Huwe1 knockdown and control MT-4 cells were infected with HIV-$1_{NL4-3}$, and the amount of newly synthesized viral DNA 24 h after infection was measured by quantitative PCR using HIV Tat-Rev specific primers. Data are expressed as mean value of three independent experiments with error bars indicating standard deviations.

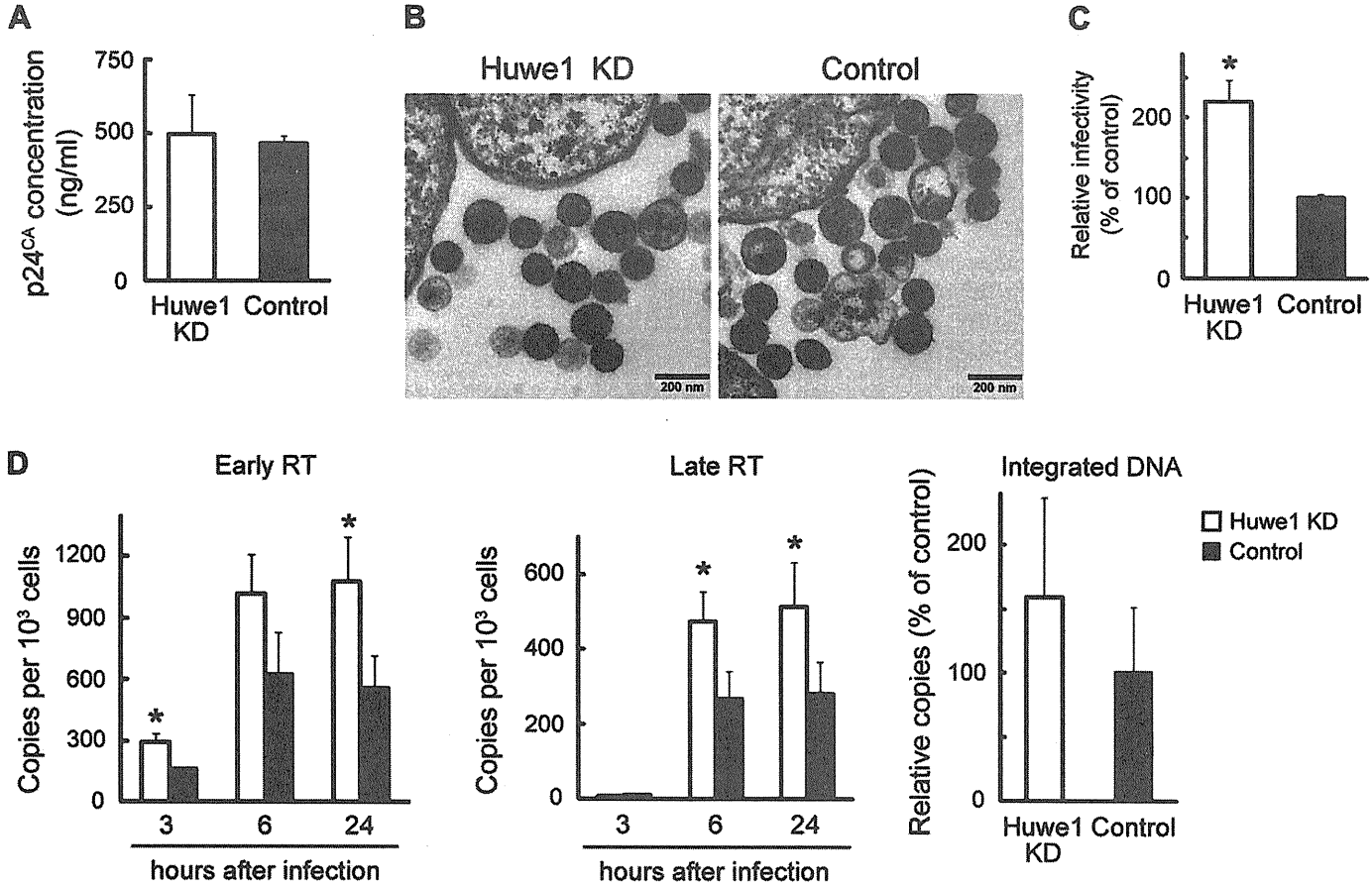


Fig. 4. Enhanced infectivity of HIV-1 produced from the Huwe1 knockdown cells. (A) Level of $p24^{CA}$ concentration in culture supernatants of HIV-1-infected cells. Huwe1 knockdown (KD, open bar) and control (closed bar) MT-4 cells were infected with HIV-$1_{NL4-3}$ at a MOI of 0.001 for 2 h. The culture supernatants were collected 3 days after infection, and concentration of $p24^{CA}$ was measured by ELISA. (B) Electron microscopic analysis of HIV-1 particles. Virus-infected Huwe1 knockdown (left panel) and control (right panel) cells were analyzed by thin section electron microscopy. (C) Infectivity of HIV-1 derived from Huwe1 knockdown and control cells. Culture supernatants were collected 3 days after infection and added to TZM-bl indicator cells. Forty-eight hours after addition, β-galactosidase activity in the TZM-bl cells was measured. Results were normalized by $p24^{CA}$ concentration in the culture supernatants from producer cells. (D) Activity of reverse transcription and integration of HIV-1 derived from Huwe1 knockdown cells. Viruses produced in culture supernatants of Huwe1 knockdown (white bars) and control (black bars) cells were used for infection toward untransduced MT-4 cells. The levels of early and late RT products at point of 3, 6, and 24 h after infection and integrated DNA at point of 24 h after infection were analyzed by quantitative PCR. All data are expressed as mean value of three independent experiments with error bars indicating standard deviations. As for C and D, the $P$ values versus control virus are $<0.05$ by Student's $t$ test (*).

In order to clarify the steps in virus replication responsible for the enhanced infectivity, the culture supernatants from Huwe1 knockdown and control cells were added to parental (untransduced) MT-4 cells, and the efficiency of viral DNA synthesis and integration were measured by quantitative PCR. As shown in Fig. 4D, early and late RT products during the first day of infection were significantly increased in the MT-4 cells infected with Huwe1 knockdown cell-derived HIV-1 (left and middle panels). In parallel with the level of reverse transcription, increased amounts of integrated DNA was also detected in the cells infected with the Huwe1 knockdown cell-derived virus at 24 h after infection (Fig. 4D, right panel). Taken together, these data demonstrate that Huwe1 in virus producer cells has a negative impact on the formation of infectious HIV-1 particles, impeding the next round of infection.

### *3.5. Association of Huwe1 with the Gag-Pol through IN domain*

During formation of HIV-1 particles, IN is expressed from provirus as a part of a 160 kDa Gag-Pol precursor [3]. Enhanced infectivity of HIV-1 produced from Huwe1-depleted cells (Fig. 4) raised the next possibility that Huwe1 associates with IN of Gag-Pol as well, thereby interfering with proper functions of the precursor protein during assembly of infectious virions. In order to test the interaction of Huwe1 with Gag-Pol polyprotein, we used an HIV-1 molecular clone expressing HA-tagged Gag-Pol without production of the Gag protein (Gag-Pol-HA, Fig. 5A). As expected, co-immunoprecipitation analysis revealed the interaction between HA-tagged Gag-Pol and FLAG-tagged Huwe1 in 293T cells (Fig. 5B, lane 2). When we used another molecular clone expressing HA-tagged Gag-Pol lacking the IN region (Gag-Pol-HA/ΔIN, Fig. 5A), FLAG-tagged Huwe1 did not co-immunoprecipitate with the mutant Gag-Pol (Fig. 5B, lane 4). This data indicates that Huwe1 binds to HIV-1 Gag-Pol protein and the interaction is in an IN region dependent manner.

## 4. Discussion

Genetic studies of HIV-1 DNA have demonstrated that certain mutations of the *IN* gene are pleiotropic, and the effects are often observed at stages distinct from integration [5–11]. Similarly, a number of cellular interactors of IN have been reported, with some of the factors appearing to have functional roles in reverse transcription, gene expression, and infectious particle production [12]. For instance, INI1, the first IN-binding protein identified, is reported to have an effect on infectious particle production in the HIV-1 replication cycle through incorporation into virions [16,19]. These evidence indicate that protein–protein interactions between IN and cellular factor(s) would influence multiple steps of retrovirus replication. In this study, we report that Huwe1, a new cellular partner for IN and Gag-Pol, somehow modulates efficient formation of infectious virions on the post-integrative steps in

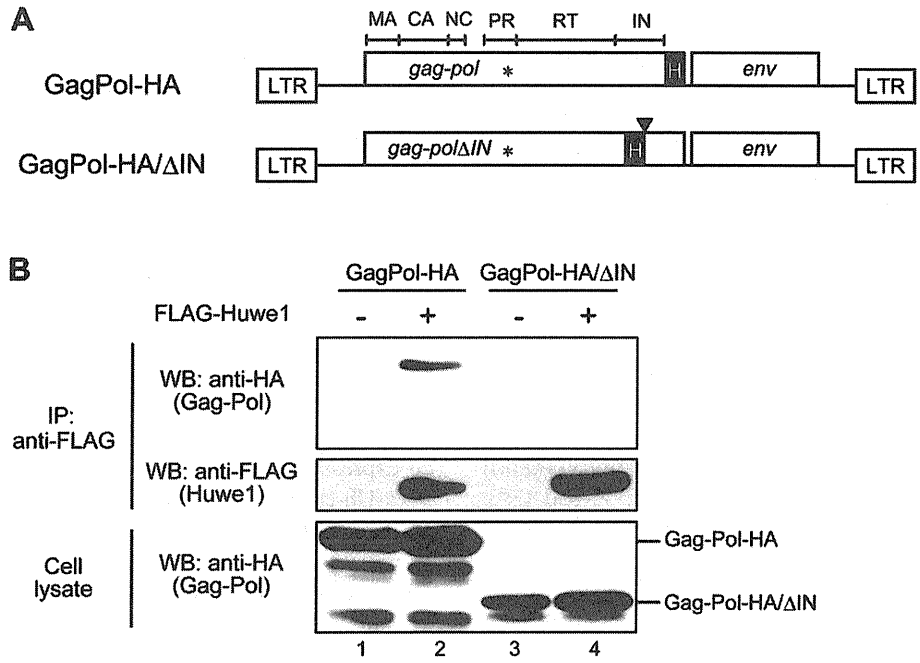


Fig. 5. Interaction of Huwe1 with HIV-1 Gag-Pol protein through IN domain. (A) A schematic representation of HIV-1 molecular clones expressing HA-tagged Gag-Pol polyproteins. HA (H) tag sequence was inserted in the C-terminus of the *pol* gene (Gag-Pol-HA). For the expression of HA-tagged Gag-Pol lacking IN region (Gag-Pol-HA/ΔIN), HA tag sequence (H) and subsequent premature termination codon (arrowhead) were introduced at the junction between RT and IN regions. All constructs were generated from pNL4-3 bearing an inactive PR, in which Asp-25 had been replaced with Ala (asterisks) [32]. (B) Interaction of Huwe1 with Gag-Pol through the IN region. FLAG-tagged Huwe1 were co-expressed with either Gag-Pol-HA or Gag-Pol-HA/ΔIN in 293T cells and subjected to immunoprecipitation assay (IP) using an anti-FLAG antibody. Gag-Pol and Huwe1 in immunoprecipitates were detected by western blotting analysis (WB) using anti-HA and anti-FLAG antibodies, respectively.

HIV-1 replication. Our results provide an intriguing insight into the role of an IN-binding protein in the production of HIV-1 virions.

So far, identification of IN-binding proteins have been conducted mostly by yeast two-hybrid screenings or co-immunoprecipitation assays [2]. In the present study, by employing a TAP procedure, tubulins and Huwe1 were identified as cellular interactors of MoMLV and HIV-1 IN (Fig. 1). α- and β-tubulins are the building blocks of microtubules and known to form heterodimers [36]. Microtubule is a key component of the cytoskeleton that regulates development and maintenance of cell shape, transport of components throughout cells, cell signaling, cell division, and mitosis [36]. The microtubule cytoskeletal network is also shown to be implicated in intracellular movement of retroviruses, and indeed several viral proteins are reported to interact with microtubule and its related factors [37]. Consistent with our data, previous studies have also reported that HIV-1 IN interacts with yeast microtubule-associated protein and human β-tubulin [2,38]. Although the physiological role of the binding of IN to microtubules is unclear, this interaction may function as one of the driving forces for intracellular trafficking of incoming viral core complexes such as PICs.

Huwe1 is a 482 kDa HECT-type E3 ubiquitin ligase and is also named Mule, ARF-BP-1, HectH9, E3$^{Histone}$, LASU1, and UREB1 [33]. This large protein was first identified as an E3 ligase that catalyzes ubiquitination of p53 and c-Myc [34,35]. Later on, histones, Cdc6, and N-myc were reported as substrates for Huwe1 [33]. Ubiquitination of these proteins by Huwe1 are implicated in proliferation, differentiation, apoptosis, gene expression, and DNA damage responses in cells [33]. However, there have been no reports regarding the interaction between Huwe1 and retroviral proteins.

In addition to the physical interaction with IN, we revealed that Huwe1 specifically binds to HIV-1 Gag-Pol protein in a C-terminal IN region dependent manner (Fig. 5). Furthermore, depletion of endogenous Huwe1 in T cells resulted in increasing infectivity of HIV-1 virions released from these cells (Fig. 4), suggesting that Huwe1 may regulate formation/production of virus particles in producer cells via an association with IN in the context of Gag-Pol. If this is the case, then how does Huwe1 modulate the HIV-1 infectivity? A number of studies have demonstrated that, during HIV-1 particle assembly, Gag and Env proteins are associated with lipid raft membrane subdomains, which is characterized by their insolubility against nonionic detergents such as NP-40 and Triton X-100 [39]. Likewise, it has been shown that Gag-Pol protein is also taken up by the detergent-resistant membrane (DRM) fraction through multimerization with Gag protein [40]. Since we could not find the incorporation of Huwe1 into HIV-1 virions (data not shown), one possibility would be that Huwe1 blocks the proper intracellular localization of the Gag-Pol precursor in virus producer cells: Huwe1 present in certain compartments of the cytoplasm could sequester a Gag-Pol precursor through its interaction with the IN region and, consequently, interfere with proper localization of Gag-Pol to specialized areas of the plasma membrane such as lipid rafts, where assembly of infectious virus particles take place with Gag. Supporting this hypothesis, our preliminary experiment showed that Huwe1 is indeed distributed in a NP-40-soluble fraction, whereas caveolin, a structural membrane protein of caveolae enriched in lipid rafts [41], remained in an insoluble fraction (data not shown). Therefore, Gag-Pol protein may accumulate in the DRM more efficiently by Huwe1 depletion, resulting in a significant increase in the virus infectivity (Fig. 4). Further biochemical analysis will be required to determine whether the distribution of Huwe1 disrupts proper association of Gag-Pol with lipid raft domains that serve as platforms for virion assembly.

An alternative and likely explanation for the negative modulation of HIV-1 infectivity by Huwe1 is that Huwe1 would mask the IN region of Gag-Pol precursor, thereby impeding the incorporation of another cellular factor(s) into virions, which is necessary for the next round of infection. Recent evidence indicates that INI1, a core subunit of the chromatin-remodeling complex SWI/SNF, is specifically packaged into HIV-1 virions [42]. It has been also shown that ectopic expression of a fragment of INI1 has a dominant negative effect on HIV-1 virion production via its interaction with IN within the context of Gag-Pol [16]. Another study revealed that IN and INI1 selectively recruits other cellular factors SAP18 and HDAC1, the components of a Sin3a-HDAC1 complex, into HIV-1 virions [43]. Interestingly, virion-associated HDAC1 activity appeared to enhance virus infectivity without any affect on particle production, and the enhancement of infectivity occurred at the early reverse transcription step in the next target cells [43]. In parallel with this observation, our results show that HIV-1 derived from Huwe1 knockdown cells exhibited increased activity in the synthesis of early RT products, albeit virion production was not affected (Fig. 4). Presently, our attempts to determine whether the recruitment of IN interacting proteins is competed with by the presence of Huwe1 will clarify the molecular mechanism underlying Huwe1-mediated modulation of HIV-1 infectivity.

As mentioned above, Huwe1 is a HECT-type E3 ubiquitin ligase that targets cellular transcription factors, histones, and anti-apoptotic proteins for ubiquitination [33]. One question to ponder would be, does the ubiquitin ligase activity link to the modulatory function of Huwe1 in HIV-1 replication? HIV-1 IN is well known to undergo proteasome-mediated degradation via ubiquitination [15]. When we performed an *in vivo* ubiquitination assay in which Myc-tagged ubiquitin (Myc-Ub) was co-expressed with HIV-1 IN in 293T cells, and subsequently Myc-Ub-conjugated IN was isolated by immunoprecipitation, a comparable level of polyubiquitylated IN was detected from Huwe1 knockdown and control cells (data not shown). In addition, although we found that HIV-1 Gag-Pol is also polyubiquitylated in a similar *in vivo* ubiquitination assay [44], Huwe1 knockdown did not have an influence on the Gag-Pol ubiquitination (data not shown). Consistent with these results, neither the knockdown nor the overexpression of Huwe1 affected the stability of HIV-1 IN and Gag-Pol in 293T cells (data not shown), indicating that Huwe1 is not involved in ubiquitination and subsequent proteasomal-degradation of IN and Gag-Pol proteins. Given the result that Huwe1 associated with IN

through an extensive region independently of the C-terminal HECT domain, the catalytic domain responsible for the transfer of ubiquitin to substrate (Fig. 2C), one could speculate that Huwe1 acts as a scaffolding modulator, which interferes with proper localization and/or function of Gag-Pol precursor in HIV-1 producer cells.

In summary, the present study identified Huwe1 as a novel interactor of HIV-1 IN and Gag-Pol that has a negative impact on the formation of infectious virus. It should be noted that, by a recent report using a large-scale siRNA screening, Huwe1 has been found as a host factor implicated in HIV-1 infectivity [45]. However, in contrast to our study, knocking down of Huwe1 by siRNA in HeLa cells diminished virus infectivity to 37% compared to the virus derived from control cells [45]. Although we could not observe any effect of Huwe1 knockdown in HeLa cells on the virus infectivity, it will be intriguing to analyze cellular differences in the role of Huwe1 in HIV-1 replication. Additionally, further analysis of the mechanism by which Huwe1 modulates virus infectivity could be the basis of a future strategy for the treatment of HIV-1 infection.

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## References

[1] M.K. Lewinski, F.D. Bushman, Retroviral DNA integration—mechanism and consequences, Adv. Genet. 55 (2005) 147–181.
[2] F. Turlure, E. Devroe, P.A. Silver, A. Engelman, Human cell proteins and human immunodeficiency virus DNA integration, Front. Biosci. 9 (2004) 3187–3208.
[3] R. Swanstrom, J.W. Wills, Synthesis, Assembly, and Processing of Viral Proteins. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1997.
[4] X. Wu, H. Liu, H. Xiao, J.A. Conway, E. Hunter, J.C. Kappes, Functional RT and IN incorporated into HIV-1 particles independently of the Gag/Pol precursor protein, EMBO J. 16 (1997) 5113–5122.
[5] C.G. Shin, B. Taddeo, W.A. Haseltine, C.M. Farnet, Genetic analysis of the human immunodeficiency virus type 1 integrase protein, J. Virol. 68 (1994) 1633–1642.
[6] A. Engelman, G. Englund, J.M. Orenstein, M.A. Martin, R. Craigie, Multiple effects of mutations in human immunodeficiency virus type 1 integrase on viral replication, J. Virol. 69 (1995) 2729–2736.
[7] T. Masuda, V. Planelles, P. Krogstad, I.S. Chen, Genetic analysis of human immunodeficiency virus type 1 integrase and the U3 att site: unusual phenotype of mutants in the zinc finger-like domain, J. Virol. 69 (1995) 6687–6696.
[8] A.D. Leavitt, G. Robles, N. Alesandro, H.E. Varmus, Human immunodeficiency virus type 1 integrase mutants retain in vitro integrase activity yet fail to integrate viral DNA efficiently during infection, J. Virol. 70 (1996) 721–728.
[9] A. Bukovsky, H. Gottlinger, Lack of integrase can markedly affect human immunodeficiency virus type 1 particle production in the presence of an active viral protease, J. Virol. 70 (1996) 6820–6825.
[10] N. Tsurutani, M. Kubo, Y. Maeda, T. Ohashi, N. Yamamoto, M. Kannagi, T. Masuda, Identification of critical amino acid residues in human immunodeficiency virus type 1 IN required for efficient proviral DNA formation at steps prior to integration in dividing and nondividing cells, J. Virol. 74 (2000) 4795–4806.
[11] M.S. Briones, C.W. Dobard, S.A. Chow, Role of human immunodeficiency virus type 1 integrase in uncoating of the viral core, J. Virol. 84 (2010) 5181–5190.
[12] L.Q. Al-Mawsawi, N. Neamati, Blocking interactions between HIV-1 integrase and cellular cofactors: an emerging anti-retroviral strategy, Trends Pharmacol. Sci. 28 (2007) 526–535.
[13] B. Studamire, S.P. Goff, Host proteins interacting with the Moloney murine leukemia virus integrase: multiple transcriptional regulators and chromatin binding factors, Retrovirology 5 (2008) 48.
[14] S. Hamamoto, H. Nishitsuji, T. Amagasa, M. Kannagi, T. Masuda, Identification of a novel human immunodeficiency virus type 1 integrase interactor, Gemin2, that facilitates efficient viral cDNA synthesis in vivo, J. Virol. 80 (2006) 5670–5677.
[15] A. Mousnier, N. Kubat, A. Massias-Simon, E. Segeral, J.C. Rain, R. Benarous, S. Emiliani, C. Dargemont, von Hippel Lindau binding protein 1-mediated degradation of integrase affects HIV-1 gene expression at a postintegration step, Proc. Natl. Acad. Sci. U.S.A 104 (2007) 13615–13620.
[16] E. Yung, M. Sorin, A. Pal, E. Craig, A. Morozov, O. Delattre, J. Kappes, D. Ott, G.V. Kalpana, Inhibition of HIV-1 virion production by a transdominant mutant of integrase interactor 1, Nat. Med. 7 (2001) 920–926.
[17] F. Christ, W. Thys, J. De Rijck, R. Gijsbers, A. Albanese, D. Arosio, S. Emiliani, J.C. Rain, R. Benarous, A. Cereseto, Z. Debyser, Transportin-SR2 imports HIV into the nucleus, Curr. Biol. 18 (2008) 1192–1202.
[18] C.L. Woodward, S. Prakobwanakit, S. Mosessian, S.A. Chow, Integrase interacts with nucleoporin NUP153 to mediate the nuclear import of human immunodeficiency virus type 1, J. Virol. 83 (2009) 6522–6533.
[19] M. Sorin, E. Yung, X. Wu, G.V. Kalpana, HIV-1 replication in cell lines harboring INI1/hSNF5 mutations, Retrovirology 3 (2006) 56.
[20] X. Wei, J.M. Decker, H. Liu, Z. Zhang, R.B. Arani, J.M. Kilby, M.S. Saag, X. Wu, G.M. Shaw, J.C. Kappes, Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy, Antimicrob. Agents Chemother. 46 (2002) 1896–1905.
[21] J. Colicelli, S.P. Goff, Sequence and spacing requirements of a retrovirus integration site, J. Mol. Biol. 199 (1988) 47–59.
[22] T. Burckstummer, K.L. Bennett, A. Preradovic, G. Schutze, O. Hantschel, G. Superti-Furga, A. Bauch, An efficient tandem affinity purification procedure for interaction proteomics in mammalian cells, Nat. Methods 3 (2006) 1013–1019.
[23] H. Nishitsuji, T. Hayashi, T. Takahashi, M. Miyano, M. Kannagi, T. Masuda, Augmentation of reverse transcription by integrase through an interaction with host factor, SIP1/Gemin2 Is critical for HIV-1 infection, PLoS One 4 (2009) e7825.
[24] O.N. Jensen, A. Podtelejnikov, M. Mann, Delayed extraction improves specificity in database searches by matrix-assisted laser desorption/ionization peptide maps, Rapid Commun. Mass Spectrom. 10 (1996) 1371–1378.
[25] Y. Shinoda, K. Hieda, Y. Koyanagi, Y. Suzuki, Efficient transduction of cytotoxic and anti-HIV-1 genes by a gene-regulatable lentiviral vector, Virus Genes 39 (2009) 165–175.
[26] K. Niwano, M. Arai, N. Koitabashi, A. Watanabe, Y. Ikeda, H. Miyoshi, M. Kurabayashi, Lentiviral vector-mediated SERCA2 gene transfer protects against heart failure and left ventricular remodeling after myocardial infarction in rats, Mol. Ther. 16 (2008) 1026–1032.
[27] K. Sato, J. Aoki, N. Misawa, E. Daikoku, K. Sano, Y. Tanaka, Y. Koyanagi, Modulation of human immunodeficiency virus type 1 infectivity through incorporation of tetraspanin proteins, J. Virol. 82 (2008) 1021–1033.