TABLE 2. Virus-specific immune responses measured in circulating $CD8^+$ T cells

Monkey	Status	Wk p.i.	SIV Gag-specific response[a]
DB05	Controller	2.0	0.18
DB05	Controller	6.0	0.50
DB05	Controller	16.0	0.82
DA87	Controller	3.4	0.18
DA87	Controller	5.4	0.21
DA87	Controller	16.0	0.92
DA63	Controller	2.0	0.16
DA63	Controller	6.0	0.20
DA63	Controller	16.0	0.32
DA14	Noncontroller	4.1	1.07
DA14	Noncontroller	8.0	1.20
DA14	Noncontroller	16.0	2.70
DA21	Noncontroller	3.4	0.37
DA21	Noncontroller	6.4	0.11
DA21	Noncontroller	16.0	1.20
CL8P	Noncontroller	2.0	0.00
CL8P	Noncontroller	6.0	0.04
CL33	Untreated	3.0	0.97
CL33	Untreated	5.0	0.10
CL33	Untreated	14.0	0.27
CL2G	Untreated	2.0	0.16
CL2G	Untreated	6.0	0.20
CL2G	Untreated	16.0	0.32

[a] The frequency of $CD8^+$ $CD4^-$ memory T cells that produce TNF and/or Mip-1β after subtracting the frequency of memory $CD8^+$ T cells that spontaneously produce TNF and/or Mip-1β.

RNA copies/ml. The latter level was in the same general range as that observed for the two untreated SIV-infected control animals.

Not unexpectedly, the untreated macaques experienced depletions of memory $CD4^+$ T cells both in the blood and at an effector site (lung) during acute SIV infection (Fig. 2b and c). In contrast, this $CD4^+$ T-cell subset was preserved in all six of the infected animals during PMPA administration. However, two patterns of memory $CD4^+$ T-cell dynamics were observed in the drug-treated cohort after the discontinuation of ART that correlated with the two distinct levels of set point viremia. This is best seen with the $CD4^+$ T lymphocytes recovered by BAL (Fig. 2c). The three noncontroller monkeys experienced a nearly complete loss of $CD4^+$ T cells in BAL specimens by week 22, which was delayed relative to that observed in the untreated macaques. Two of the noncontrollers also sustained depletions of this T-lymphocyte subset in the blood (Fig. 2b). In contrast, two of the controller animals (DA63 and DB05) experienced no depletion of $CD4^+$ T cells in BAL samples during the first 6 months of their SIV infections, while the third controller (DA87) sustained a moderate loss of this lymphocyte subset. The loss of BAL fluid $CD4^+$ T cells in macaque DA87 may reflect its 10- to 20-fold-higher levels of peak viremia following the cessation of tenofovir therapy compared to that of the two other controller animals (DA63 and DB05) (Fig. 1a), which experienced no decline of this T-cell subset.

TABLE 3. Virus-specific immune responses measured in BAL $CD8^+$ T cells

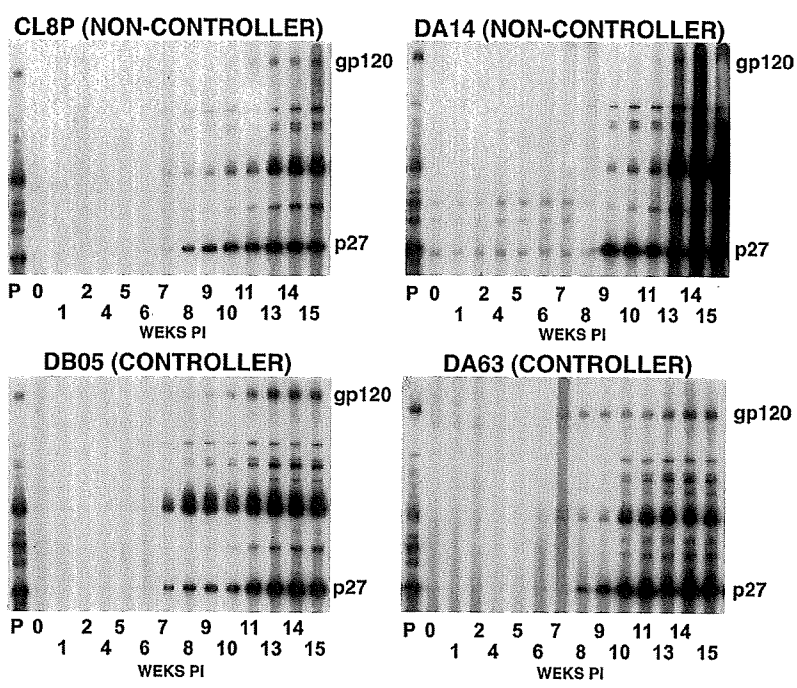
Monkey	Status	Wk p.i.	SIV Gag-specific response[a]
DA14	Noncontroller	2.0	1.90
DA14	Noncontroller	6.0	3.69
DA14	Noncontroller	10.0	1.18
CL8P	Noncontroller	3.0	3.32
CL8P	Noncontroller	5.0	0.36
CL8P	Noncontroller	7.0	0.71
CL8P	Noncontroller	13.0	0.38
DB05	Controller	2.0	0.84
DB05	Controller	6.0	0.77
DB05	Controller	10.0	1.56
DA63	Controller	3.0	1.31
DA63	Controller	5.0	1.18
DA63	Controller	7.0	1.64
DA63	Controller	13.0	1.36

[a] The frequency of $CD8^+$ T cells in BAL fluid that produce IFN-γ and CD69 after subtracting the frequency of $CD8^+$ T cells that spontaneously produce IFN-γ and CD69.

Virus-specific immune responses in tenofovir-treated animals. A major premise underlying the administration of PMPA at 48 h p.i. was that by potently suppressing SIV replication and preventing severe injury to the memory $CD4^+$ T-lymphocyte population during acute SIV infection, the immune system would be stimulated by low residual levels of virus production and generate a protective antiviral response when ART was stopped. We therefore initially examined whether T-cell-mediated immunity was induced in the treated macaques during the period in which they received ART. SIV Gag-specific $CD8^+$ T-cell responses were measured by intracellular staining for the production of either TNF or Mip-1β after stimulation with a peptide pool containing 125 15mer peptides (overlapping by 11 amino acids) spanning SIVmac239 Gag. As shown in Table 2, the frequency of virus-specific $CD8^+$ T cells remained at background levels during the period of tenofovir therapy, except for macaque DA14. As noted earlier, DA14 generated the highest levels of peak plasma viremia (1.3×10^7 RNA copies/ml) following drug treatment, possibly accounting for the elevated frequency of SIV-specific $CD8^+$ T cells measured in this animal at week 4.1. All five of the PMPA recipients, tested at week 16 p.i., generated increased frequencies of virus-specific $CD8^+$ T lymphocytes, presumably reflecting several months of antigen stimulation. In four of these animals, the responses at late times exceeded those measured for the untreated macaques.

SIV Gag-specific $CD8^+$ T-cell responses also were assessed in specimens recovered by BAL of four tenofovir-treated macaques by intracellular staining for IFN-γ following stimulation with the same SIVmac239 Gag peptide pool (Table 3). Increased frequencies of virus-specific $CD8^+$ T lymphocytes were detected both during and following the cessation of PMPA therapy. In general, the SIV Gag-specific responses were higher in the two noncontroller macaques (DA14 and CL8P), particularly during and immediately following drug administration.

a



b

ANIMAL ID	ANTI-SIV gp120 ELISA TITERS AT WEEK 10 PI
CL8P (NON-CONTROLLER)	2,500
DA14 (NON-CONTROLLER)	2,500
DA21 (NON-CONTROLLER)	2,500
DB05 (CONTROLLER)	2,500
DA63 (CONTROLLER)	12,500
DA87(CONTROLLER)	12,500

FIG. 3. Profiles of anti-SIV antibody responses of noncontroller and controller monkeys. (a) SIV proteins, prepared from detergent-treated pelleted particles, were transferred to PVDF membranes following PAGE and incubated with a 1:500 dilution of serially collected plasma samples from two noncontroller monkeys (upper) and two controller monkeys (bottom). The positions of SIV gp120 envelope glycoproteins and p27 CA are indicated. A plasma sample (diluted 1:500) collected from a chronically SIVmac239-infected monkey was used as a positive control in lane P. (b) Anti-SIV gp120 ELISA titers.

SIV-specific humoral responses initially were assessed by Western blot analyses using plasma samples from two controller and two noncontroller animals. As shown in Fig. 3a, antibody reacting with gp130 was delayed (weeks 11 to 13) in noncontrollers CL8P and DA14 compared to its earlier appearance (weeks 7 to 9) in controllers DA63 and DB05. Antiviral binding antibody, as measured by ELISA in all six treated macaques at week 10 p.i., revealed that two of the controller monkeys (DB05 and DB87) had generated the highest (1:12,500) anti-gp130 titers (Fig. 3b).

Virus neutralization assays were performed using TZM-bl cells and intact SIVmac239 virions in combination with plasma specimens recovered at weeks 10 and 22. No anti-SIVmac239 NAb activity was detected in any of the treated or untreated macaques at either time point with samples diluted 1:20 (data not shown).

The three controller monkeys express protective MHC class I alleles. The establishment of two distinct levels of viral set points in the six *Mamu-A*01*-negative, SIV-infected, tenofovir-treated recipients raised the possibility that this outcome might be genetically determined. It is now recognized that the expression of certain MHC class I alleles is strongly associated with reduced plasma viremia and slower disease progression in HIV- and SIV-infected humans and macaques, respectively (2, 15, 19, 20, 25, 34). In the rhesus macaque, MHC class I alleles associated with the control of SIV replication include Mamu-A*01, Mamu-A*02, Mamu-B*08, Mamu-B*17, and Mamu-B*29 (13, 15, 20, 25, 34). The MHC class I alleles expressed by the controller and noncontroller animals were determined following the RT-PCR amplification, cloning, and sequencing of full-length cDNAs for Mamu-A and Mamu-B alleles. Individual alleles were identified by comparing each cloned sequence to a database of published rhesus MHC class I sequences representing 56 Mamu-A and 157 Mamu-B alleles. This exhaustive approach was used to obtain a broad view of the MHC class I allele repertoire expressed in each macaque and to avoid limiting the analysis to only a few previously identified MHC alleles.

The repertoire of Mamu-A and Mamu-B alleles expressed by the six tenofovir-treated monkeys was very heterogeneous

TABLE 4. MHC class I alleles expressed by each SIV-infected tenofovir-treated rhesus macaque[a]

Animal	Mamu-A allele(s)			Mamu-B allele(s)			
Noncontroller							
RhDA21	A*08	A*1302	A*1303	B*12	B*38	B*71	B*30-like
				B*46-like	B*74-like	B*09-like	
RhDA14	A*1403			B*12	B*74-like	new B	new B
RhCL8P	A*0602			B*19	B*24	B*52	B*55-like
Controller							
RhDA87	A*06	A*07-like		B*01	**B*08**	new B	
RhDB05	**A*02**	A*04	A*1403	B*7301	B*31-like	B*74-like	
RhDA63	A*0602	A*28-like		**B*290101**	B*61	B*55-like	B*09-like

[a] The MHC alleles in bold have previously been reported to suppress SIV replication or are present at increased frequencies within elite controller cohorts (15, 34).

and included 10 Mamu-A and 17 Mamu-B alleles that had been identified previously (Table 4). No single MHC class I allele determined the segregation of our cohort into two distinct groups. Some MHC class I alleles (Mamu-A*0602 and Mamu-A*1403) that were present in both controller and noncontroller monkeys clearly were not involved in suppressing SIV replication in the former group. Two noncontroller macaques had a combination of Mamu-B alleles known to be present on the same haplotype (DA21, Mamu-B*12, Mamu-B*38, and Mamu-B*30; CL8P, Mamu-B*19 and Mamu-B*24) (26). Interestingly, two of the three controllers (DA87 and DA63) carried MHC alleles (Mamu-B*08 and Mamu-B*290101) previously reported to suppress SIV replication (15, 34). The third controller (DB05) expressed Mamu-A*02, an allele found at increased frequency within an elite controller cohort and associated with a partial reduction of plasma viral load in both the acute and the chronic phases of SIV infection (15). In contrast, none of the MHC class I alleles associated with reduced plasma viremia and slower disease progression were found in the three noncontroller monkeys.

Long-term effects of PMPA therapy. After week 20, the three noncontroller macaques continued to experience high set point plasma viremia levels (10^5 to 10^6 RNA copies/ml) and barely detectable $CD4^+$ T cells in BAL specimens (Fig. 2). These animals subsequently became symptomatic and were euthanized at weeks 34, 54, and 60, respectively, because of intractable diarrhea, anorexia, and marked weight loss. The control of plasma viremia varied greatly among the three controller monkeys. As shown in Fig. 2a, the plasma viral load in animal DA63 began to increase after week 27 p.i., reaching a set point of 2×10^5 RNA copies/ml by week 36 p.i. This increase in plasma viremia was accompanied by modest declines of memory $CD4^+$ T lymphocytes in blood and BAL samples in DA63 (Fig. 2b and c). Similarly, animal DB05 began to lose control of SIV replication after week 35 and experienced severe losses of memory $CD4^+$ T cells from both the blood and BAL specimens. Macaque DB05 had to be euthanized at week 80 p.i. because of its deteriorating clinical status. The third SIV controller (DA87) has maintained remarkably low levels of plasma viremia. Its plasma virus load at week 78 p.i. was 127 RNA copies/ml, and levels of memory $CD4^+$ T lymphocytes, in both the blood and BAL fluid, have been durably maintained.

Antiviral cytotoxic T-lymphocyte (CTL) responses in SIV-infected macaques are known to select for viral escape variants bearing mutations in, or close to, epitopes restricted by the selecting MHC class I alleles. We investigated if efficient immune responses restricted by protective MHC alleles led to viral evolution in controller macaques. The analysis was limited to virus present in RhDB05 and RhDA87, animals carrying Mamu-A*02 and Mamu-B*08, respectively, since several CTL epitopes have been characterized for these two MHC alleles but not for Mamu-B*290101 (14, 16, 32). Viral sequences encoding Gag, Env, Vif, or Nef were amplified from plasma samples. In viral sequences obtained from the Mamu-A*02 controller macaque RhDB05 at week 80 p.i., amino acid substitutions were identified in three Mamu-A*02-restricted epitopes (Gag GY9, Env RY8, and Nef YY9) (Fig. 4). Similarly, viral escape mutants in three Mamu-B*08-restricted epitopes (Vif RL9, Vif RL8, and Nef RL9) were detected in samples from the Mamu-B*08-positive macaque RhDA87 at week 78 p.i. Additionally, an alanine-to-proline substitution also was found one amino acid upstream from the Mamu-B*08 restricted epitope Nef RL10. This mutation, observed previously in SIV-infected Mamu-B*08-positive macaques, likely affects the proper generation of Mamu-B*08 binding peptide by the proteasome (14). Therefore, the presence of protective MHC alleles in two controller macaques was associated with the selection of CTL escape variants, suggesting that CTL activity restricted by protective MHC alleles played a role in controlling plasma viremia.

Thus, of the original six tenofovir-treated monkeys, only two

RhDB05 (A*02 epitopes)

Gag GY9	Env RY8	Nef YY9
GSENLKSLY	RTLLSRVY	YTSGPGIRY
********* (3)	*******F (4)	******P** (7)
D***** (3)	******A* (1)	
A********* (1)	*I**L*** (1)	

RhDA87 (B*08 epitopes)

Vif RL9	Vif RL8	Nef RL10	Nef RL9
RRAIRGEQL	RRDNRRGL	(A)RRHRILDIYL	RRLTARGLL
T********* (8)	*G****** (8)	(P)********** (6)	E**A***** (6)

FIG. 4. Amino acid changes associated with SIV escape variants in *Mamu-B*-08-* and *Mamu-A*02*-bearing rhesus macaques. The number of independent clones sequenced is indicated in parentheses. The Nef RL10 sequence contains an amino acid residue immediately N-terminal to the epitope, alanine (A), which has undergone mutation.

remained alive at week 80 p.i. Both carried protective MHC class I alleles. One (DA63), however, had high levels of plasma viremia and declining levels of $CD4^+$ T cells and was destined to develop immunodeficiency.

DISCUSSION

In these experiments, we have investigated whether a 28-day course of potent ART, initiated at a time (48 h p.i.) following SIV inoculation when the acquisition of a viral infection was virtually assured, would sufficiently sensitize the immune system to result in controlled virus replication when treatment was stopped. The administration of tenofovir 48 h after SIV inoculation did, in fact, delay virus replication in all of the treated rhesus macaques. However, the three animals deriving the greatest benefit (lowering the viral set point by nearly 3 logs for 6 months) were genetically privileged; they carried MHC class I alleles associated with the suppression of virus replication. The three PMPA-treated animals bearing nonprotective MHC alleles were not as fortunate; they generated levels of set point viremia comparable to that observed in control animals, and all three have died with symptoms of immunodeficiency.

This study is an extension of two previous reports describing ART initiated very early during acute SIV infection. When Lifson et al. initiated tenofovir treatment at 24 h p.i., three of four SIVsmE660-infected macaques experienced sterilizing protection both during and following drug administration (12). If PMPA was started at 72 h p.i., two of four animals became viremic during the 28-day course of therapy, and virus replication was measurable in all four monkeys following the cessation of ART. In a separate study using SIVmac239-inoculated animals also treated with PMPA beginning at 24 h p.i., the levels of viremia were low to undetectable during or following ART (11). However, in contrast to the SIVsmE660 macaque recipients of tenofovir, which generated SIV-specific proliferative responses in the absence of detectable virus replication during ART administration and resisted rechallenge, the treated SIVmac239 monkeys failed to produce measurable immune responses and readily became infected following rechallenge with homologous virus.

Therefore, 48 h after SIVmac239 infection seemed to be the appropriate time to begin tenofovir therapy. The establishment of an SIV infection would be ensured, and the possible development of effective immune responses might occur at a time when virus-induced damage to the immune system had been minimized. Our results show that PMPA was effective in suppressing SIV replication during the 4 weeks of ART. Cell-associated viral DNA was undetectable in all six treated monkeys, and plasma viremia was below detection limits in four of the six macaques. In addition, memory $CD4^+$ T cells in the blood and at an effector site (lungs) were maintained at pre-infection levels in all six animals during drug administration. Thus, the experimental regimen used had permitted infection acquisition, suppressed virus replication, and prevented injury to the immune system.

Unfortunately, however, all six of the PMPA-treated monkeys produced modest to high levels of plasma viremia within 1 to 2 weeks of ART cessation, suggesting that the potent suppression of virus replication mediated by 4 weeks of tenofovir treatment resulted in insufficient immune stimulation despite the preservation of the $CD4^+$ T-lymphocyte population. As had been reported previously by Lifson et al. for PMPA therapy initiated at 24 h p.i. in SIVmac239-inoculated monkeys (11), we also observed low to background levels of anti-SIVmac239 immune responses in circulating $CD8^+$ T cells during the 28-day course of treatment that was started at 48 h p.i. (Table 2). However, SIV Gag-specific responses were measurable in $CD8^+$ T cells present in BAL specimens obtained from most of the macaques during the period of PMPA therapy (Table 3), but this immune sensitization appeared to have minimal long-term effects.

Given that *Mamu-A*01*-negative and otherwise randomly selected macaques were used in this study, initially we were intrigued by the resolution of viral set points at two discrete levels, differing by 300-fold, in controller and noncontroller animals at week 15 p.i. In the three controller animals, set points were maintained at a range of 1×10^3 RNA copies/ml plasma for the initial 6 months of their viral infections. This degree of control rarely is observed in SIVmac239-infected Indian-origin rhesus macaques. To determine whether any virologic or immunologic parameter could predict controller or noncontroller status, data collected during and immediately following tenofovir treatment was evaluated retrospectively. Clearly, peak viremia following ART was not a predictor of the subsequent clinical course. For example, and not unexpectedly, the mean level of peak plasma viremia immediately following the cessation of PMPA treatment was 20-fold lower in controller than in noncontroller animals (1×10^5 and 2.1×10^6 RNA copies/ml, respectively) (Fig. 1b). Nevertheless, one controller macaque (DB05), with a relatively low peak virus load (4.0×10^4 RNA copies/ml), eventually died from immunodeficiency at week 80 p.i., whereas another controller monkey (DA87), with the highest peak viremia (8.3×10^5 RNA copies/ml at week 6 p.i.), was asymptomatic, with a plasma load of only 127 RNA copies/ml at week 78 p.i.

Similarly, the frequencies of circulating virus-specific $CD8^+$ T lymphocytes present during or immediately after PMPA treatment did not predict which animals were destined to become controllers or noncontrollers. The levels of these SIV-specific $CD8^+$ T cells were low to undetectable during the 4-week course of tenofovir therapy and increased in both groups following the cessation of ART. As noted earlier, SIV Gag-specific $CD8^+$ T-cell responses in BAL samples were higher in two noncontroller animals during and immediately following drug administration, most likely reflecting somewhat higher levels of antigen production in these macaques rather than foretelling clinical outcomes.

ELISA and immunoblotting experiments both revealed that the controller macaques generated earlier and higher-titer anti-SIV gp130 antibody responses compared to those of the noncontrollers. However, because virus-specific NAbs were undetectable at weeks 10 and 22 in both controller and noncontroller macaques, the significance of the binding antibody results presently is unclear.

As noted earlier, the establishment of viral set points at either high or low levels in the treated monkeys correlated with the presence of MHC class I alleles that are reported to control SIV replication. Only controller animals carried protective MHC alleles, and every one suppressed its set point viremia at or below the level of 10^3 viral RNA copies/ml for at least 6

months. The emergence of CTL escape variants in two of the controller macaques suggested that CTL activity restricted by protective MHC alleles played a role in controlling plasma viremia when ART was discontinued. One could ask whether the suppression of virus replication observed in the controller group of treated animals simply reflected the presence of protective MHC alleles rather than a consequence of early postinoculation PMPA therapy. At present, we have no direct experimental data supporting the latter suggestion, although some previously published reports pertaining to SIV elite controllers are relevant to this question. The *Mamu-B*08* allele previously has been reported to be overrepresented in a significant fraction (38%) of SIV elite controllers (15). Although SIV-infected *Mamu-B*08*$^+$ rhesus macaques do not experience significant reductions in peak viremia during acute infections, they are able to reduce set point virus loads by 7.3-fold compared to the loads of other elite controllers. In this regard, the suppression of plasma viremia to 127 RNA copies/ml at week 78 p.i. in monkey DA87 (carrying the *Mamu-B*08* allele) would appear to be quite unusual. In addition, although *Mamu-A*02*$^+$ and *Mamu-B*29*$^+$ macaques also are overrepresented in elite controller cohorts, their capacity to suppress set point viremia is quite limited compared to that of the more potent control reported for Mamu-B*08 and Mamu-B*17 (15). It was surprising, therefore, that controller macaques DB05 and DA63, carrying the *Mamu-A*02* and *Mamu-B*29* alleles, respectively, were able to effectively control plasma viremia to the range of 10^3 viral RNA copies/ml for 6 to 9 months. In the context of previously published studies, the potent suppression of virus replication in these two animals may not be due solely to the modest protective effects of the *Mamu-A*02* and *Mamu-B*29* alleles.

Clearly, the prevention of immediate virus-induced damage to the immune system mediated by p.i. ART by itself did not lead to augmented immunologic responses capable of controlling SIV in monkeys not bearing protective MHC alleles. These results emphasize the incredibly narrow window of opportunity for controlling de novo SIV and, by extension, HIV infections in individuals not bearing protective MHC alleles. Starting PMPA treatment at 24 h did confer the durable control of SIV replication in most, but not all, inoculated animals. ART commencing at 48 h p.i. clearly was too late; the suppression of virus replication at 48 h p.i. did not sensitize the immune system for subsequent control SIV replication but merely delayed the emergence of detectable virus. In this context, we have reported previously that the transfer of high titers of antiviral NAbs at 6 h p.i., but not at 24 h p.i., potently controlled virus replication in the macaque model (24), compressing even further the time interval between lentivirus exposure and successful antiviral intervention.

ACKNOWLEDGMENTS

We are indebted to Boris Skopets and Rahel Petros for their diligence and assistance in the maintenance of our animals; Ronald Willey for performing virus neutralization assays; Ranjini Iyengar and Robin Kruthers for determining plasma viral RNA levels; Martha Vazquez and Norbert Bischofberger, Gilead Science, Inc., for providing tenofovir; and John Loffredo for providing information about protective MHC class I alleles.

This work was supported by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

REFERENCES

1. **Brenchley, J. M., T. W. Schacker, L. E. Ruff, D. A. Price, J. H. Taylor, G. J. Beilman, P. L. Nguyen, A. Khoruts, M. Larson, A. T. Haase, and D. C. Douek.** 2004. CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. J. Exp. Med. **200**:749–759.
2. **Carrington, M., and S. J. O'Brien.** 2003. The influence of HLA genotype on AIDS. Annu. Rev. Med. **54**:535–551.
3. **Committee on the Care and Use of Laboratory Animals.** 1985. Guide for the care and use of laboratory animals. Department of Health and Human Services publication no. NIH 85-23. National Institutes of Health, Bethesda, MD.
4. **Endo, Y., T. Igarashi, Y. Nishimura, C. Buckler, A. Buckler-White, R. Plishka, D. S. Dimitrov, and M. A. Martin.** 2000. Short- and long-term clinical outcomes in rhesus monkeys inoculated with a highly pathogenic chimeric simian/human immunodeficiency virus. J. Virol. **74**:6935–6945.
5. **Guadalupe, M., E. Reay, S. Sankaran, T. Prindiville, J. Flamm, A. McNeil, and S. Dandekar.** 2003. Severe CD4$^+$ T-cell depletion in gut lymphoid tissue during primary human immunodeficiency virus type 1 infection and substantial delay in restoration following highly active antiretroviral therapy. J. Virol. **77**:11708–11717.
6. **Hill, C. M., H. Deng, D. Unutmaz, V. N. Kewalramani, L. Bastiani, M. K. Gorny, S. Zolla-Pazner, and D. R. Littman.** 1997. Envelope glycoproteins from human immunodeficiency virus types 1 and 2 and simian immunodeficiency virus can use human CCR5 as a coreceptor for viral entry and make direct CD4-dependent interactions with this chemokine receptor. J. Virol. **71**:6296–6304.
7. **Igarashi, T., O. K. Donau, H. Imamichi, M. J. Dumaurier, R. Sadjadpour, R. J. Plishka, A. Buckler-White, C. Buckler, A. F. Suffredini, H. C. Lane, J. P. Moore, and M. A. Martin.** 2003. Macrophage-tropic simian/human immunodeficiency virus chimeras use CXCR4, not CCR5, for infections of rhesus macaque peripheral blood mononuclear cells and alveolar macrophages. J. Virol. **77**:13042–13052.
8. **Jayaraman, P., T. Zhu, L. Misher, D. Mohan, L. Kuller, P. Polacino, B. A. Richardson, H. Bielefeldt-Ohmann, D. Anderson, S. L. Hu, and N. L. Haigwood.** 2007. Evidence for persistent, occult infection in neonatal macaques following perinatal transmission of simian-human immunodeficiency virus SF162P3. J. Virol. **81**:822–834.
9. **Lafont, B. A., C. M. McGraw, S. A. Stukes, A. Buckler-White, R. J. Plishka, R. A. Byrum, V. M. Hirsch, and M. A. Martin.** 2007. The locus encoding an oligomorphic family of MHC-A alleles (Mane-A*06/Mamu-A*05) is present at high frequency in several macaque species. Immunogenetics **59**:211–223.
10. **Li, Q., L. Duan, J. D. Estes, Z. M. Ma, T. Rourke, Y. Wang, C. Reilly, J. Carlis, C. J. Miller, and A. T. Haase.** 2005. Peak SIV replication in resting memory CD4+ T cells depletes gut lamina propria CD4+ T cells. Nature **434**:1148–1152.
11. **Lifson, J. D., M. Piatak, Jr., A. N. Cline, J. L. Rossio, J. Purcell, I. Pandrea, N. Bischofberger, J. Blanchard, and R. S. Veazey.** 2003. Transient early postinoculation anti-retroviral treatment facilitates controlled infection with sparing of CD4+ T cells in gut-associated lymphoid tissues in SIVmac239-infected rhesus macaques, but not resistance to rechallenge. J. Med. Primatol. **32**:201–210.
12. **Lifson, J. D., J. L. Rossio, R. Arnaout, L. Li, T. L. Parks, D. K. Schneider, R. F. Kiser, V. J. Coalter, G. Walsh, R. J. Imming, B. Fisher, B. M. Flynn, N. Bischofberger, M. Piatak, Jr., V. M. Hirsch, M. A. Nowak, and D. Wodarz.** 2000. Containment of simian immunodeficiency virus infection: cellular immune responses and protection from rechallenge following transient postinoculation antiretroviral treatment. J. Virol. **74**:2584–2593.
13. **Loffredo, J. T., A. T. Bean, D. R. Beal, E. J. Leon, G. E. May, S. M. Piaskowski, J. R. Furlott, J. Reed, S. K. Musani, E. G. Rakasz, T. C. Friedrich, N. A. Wilson, D. B. Allison, and D. I. Watkins.** 2008. Patterns of CD8$^+$ immunodominance may influence the ability of Mamu-B*08-positive macaques to naturally control simian immunodeficiency virus SIVmac239 replication. J. Virol. **82**:1723–1738.
14. **Loffredo, J. T., T. C. Friedrich, E. J. Leon, J. J. Stephany, D. S. Rodrigues, S. P. Spencer, A. T. Bean, D. R. Beal, B. J. Burwitz, R. A. Rudersdorf, L. T. Wallace, S. M. Piaskowski, G. E. May, J. Sidney, E. Gostick, N. A. Wilson, D. A. Price, E. G. Kallas, H. Piontkivska, A. L. Hughes, A. Sette, and D. I. Watkins.** 2007. CD8+ T cells from SIV elite controller macaques recognize Mamu-B*08-bound epitopes and select for widespread viral variation. PLoS ONE **2**:e1152.
15. **Loffredo, J. T., J. Maxwell, Y. Qi, C. E. Glidden, G. J. Borchardt, T. Soma, A. T. Bean, D. R. Beal, N. A. Wilson, W. M. Rehrauer, J. D. Lifson, M. Carrington, and D. I. Watkins.** 2007. Mamu-B*08-positive macaques control simian immunodeficiency virus replication. J. Virol. **81**:8827–8832.
16. **Loffredo, J. T., J. Sidney, C. Wojewoda, E. Dodds, M. R. Reynolds, G. Napoe, B. R. Mothe, D. H. O'Connor, N. A. Wilson, D. I. Watkins, and A. Sette.** 2004. Identification of seventeen new simian immunodeficiency virus-derived CD8+ T cell epitopes restricted by the high frequency molecule, Mamu-

A*02, and potential escape from CTL recognition. J. Immunol. **173**:5064–5076.
17. **Mattapallil, J. J., D. C. Douek, B. Hill, Y. Nishimura, M. Martin, and M. Roederer.** 2005. Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection. Nature **434**:1093–1097.
18. **Mehandru, S., M. A. Poles, K. Tenner-Racz, A. Horowitz, A. Hurley, C. Hogan, D. Boden, P. Racz, and M. Markowitz.** 2004. Primary HIV-1 infection is associated with preferential depletion of CD4+ T lymphocytes from effector sites in the gastrointestinal tract. J. Exp. Med. **200**:761–770.
19. **Migueles, S. A., M. S. Sabbaghian, W. L. Shupert, M. P. Bettinotti, F. M. Marincola, L. Martino, C. W. Hallahan, S. M. Selig, D. Schwartz, J. Sullivan, and M. Connors.** 2000. HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors. Proc. Natl. Acad. Sci. USA **97**:2709–2714.
20. **Muhl, T., M. Krawczak, P. Ten Haaft, G. Hunsmann, and U. Sauermann.** 2002. MHC class I alleles influence set point viral load and survival time in simian immunodeficiency virus-infected rhesus monkeys. J. Immunol. **169**: 3438–3446.
21. **Naidu, Y. M., H. W. Kestler III, Y. Li, C. V. Butler, D. P. Silva, D. K. Schmidt, C. D. Troup, P. K. Sehgal, P. Sonigo, M. D. Daniel, et al.** 1988. Characterization of infectious molecular clones of simian immunodeficiency virus (SIVmac) and human immunodeficiency virus type 2: persistent infection of rhesus monkeys with molecularly cloned SIVmac. J. Virol. **62**:4691–4696.
22. **Nishimura, Y., C. R. Brown, J. J. Mattapallil, T. Igarashi, A. Buckler-White, B. A. Lafont, V. M. Hirsch, M. Roederer, and M. A. Martin.** 2005. Resting naive CD4+ T cells are massively infected and eliminated by X4-tropic simian-human immunodeficiency viruses in macaques. Proc. Natl. Acad. Sci. USA **102**:8000–8005.
23. **Nishimura, Y., T. Igarashi, A. Buckler-White, C. Buckler, H. Imamichi, R. M. Goeken, W. R. Lee, B. A. Lafont, R. Byrum, H. C. Lane, V. M. Hirsch, and M. A. Martin.** 2007. Loss of naive cells accompanies memory $CD4^+$ T-cell depletion during long-term progression to AIDS in simian immunodeficiency virus-infected macaques. J. Virol. **81**:893–902.
24. **Nishimura, Y., T. Igarashi, N. L. Haigwood, R. Sadjadpour, O. K. Donau, C. Buckler, R. J. Plishka, A. Buckler-White, and M. A. Martin.** 2003. Transfer of neutralizing IgG to macaques 6 h but not 24 h after SHIV infection confers sterilizing protection: implications for HIV-1 vaccine development. Proc. Natl. Acad. Sci. USA **100**:15131–15136.
25. **O'Connor, D. H., B. R. Mothe, J. T. Weinfurter, S. Fuenger, W. M. Rehrauer, P. Jing, R. R. Rudersdorf, M. E. Liebl, K. Krebs, J. Vasquez, E. Dodds, J. Loffredo, S. Martin, A. B. McDermott, T. M. Allen, C. Wang, G. G. Doxiadis, D. C. Montefiori, A. Hughes, D. R. Burton, D. B. Allison, S. M. Wolinsky, R. Bontrop, L. J. Picker, and D. I. Watkins.** 2003. Major histocompatibility complex class I alleles associated with slow simian immunodeficiency virus disease progression bind epitopes recognized by dominant acute-phase cytotoxic-T-lymphocyte responses. J. Virol. **77**:9029–9040.
26. **Otting, N., C. M. Heijmans, R. C. Noort, N. G. de Groot, G. G. Doxiadis, J. J. van Rood, D. I. Watkins, and R. E. Bontrop.** 2005. Unparalleled complexity of the MHC class I region in rhesus macaques. Proc. Natl. Acad. Sci. USA **102**:1626–1631.
27. **Pitcher, C. J., S. I. Hagen, J. M. Walker, R. Lum, B. L. Mitchell, V. C. Maino, M. K. Axthelm, and L. J. Picker.** 2002. Development and homeostasis of T cell memory in rhesus macaque. J. Immunol. **168**:29–43.
28. **Pitcher, C. J., C. Quittner, D. M. Peterson, M. Connors, R. A. Koup, V. C. Maino, and L. J. Picker.** 1999. HIV-1-specific CD4+ T cells are detectable in most individuals with active HIV-1 infection, but decline with prolonged viral suppression. Nat. Med. **5**:518–525.
29. **Shibata, R., F. Maldarelli, C. Siemon, T. Matano, M. Parta, G. Miller, T. Fredrickson, and M. A. Martin.** 1997. Infection and pathogenicity of chimeric simian-human immunodeficiency viruses in macaques: determinants of high virus loads and CD4 cell killing. J. Infect. Dis. **176**:362–373.
30. **Van Rompay, K. K., J. M. Cherrington, M. L. Marthas, C. J. Berardi, A. S. Mulato, A. Spinner, R. P. Tarara, D. R. Canfield, S. Telm, N. Bischofberger, and N. C. Pedersen.** 1996. 9-[2-(Phosphonomethoxy)propyl]adenine therapy of established simian immunodeficiency virus infection in infant rhesus macaques. Antimicrob. Agents Chemother. **40**:2586–2591.
31. **Veazey, R. S., M. DeMaria, L. V. Chalifoux, D. E. Shvetz, D. R. Pauley, H. L. Knight, M. Rosenzweig, R. P. Johnson, R. C. Desrosiers, and A. A. Lackner.** 1998. Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection. Science **280**:427–431.
32. **Vogel, T. U., T. C. Friedrich, D. H. O'Connor, W. Rehrauer, E. J. Dodds, H. Hickman, W. Hildebrand, J. Sidney, A. Sette, A. Hughes, H. Horton, K. Vielhuber, R. Rudersdorf, I. P. De Souza, M. R. Reynolds, T. M. Allen, N. Wilson, and D. I. Watkins.** 2002. Escape in one of two cytotoxic T-lymphocyte epitopes bound by a high-frequency major histocompatibility complex class I molecule, Mamu-A*02: a paradigm for virus evolution and persistence? J. Virol. **76**:11623–11636.
33. **Wei, X., J. M. Decker, S. Wang, H. Hui, J. C. Kappes, X. Wu, J. F. Salazar-Gonzalez, M. G. Salazar, J. M. Kilby, M. S. Saag, N. L. Komarova, M. A. Nowak, B. H. Hahn, P. D. Kwong, and G. M. Shaw.** 2003. Antibody neutralization and escape by HIV-1. Nature **422**:307–312.
34. **Yant, L. J., T. C. Friedrich, R. C. Johnson, G. E. May, N. J. Maness, A. M. Enz, J. D. Lifson, D. H. O'Connor, M. Carrington, and D. I. Watkins.** 2006. The high-frequency major histocompatibility complex class I allele Mamu-B*17 is associated with control of simian immunodeficiency virus SIVmac239 replication. J. Virol. **80**:5074–5077.

Biochemical and Biophysical Research Communications 380 (2009) 838–843

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Inhibition of human immunodeficiency virus type 1 (HIV-1) nuclear import via Vpr–Importin α interactions as a novel HIV-1 therapy

Tatsunori Suzuki [a], Norio Yamamoto [a,b], Mizuho Nonaka [a], Yoshie Hashimoto [a], Go Matsuda [a], Shin-nosuke Takeshima [a], Megumi Matsuyama [c], Tatsuhiko Igarashi [c], Tomoyuki Miura [c], Rie Tanaka [d], Shingo Kato [d], Yoko Aida [a,*]

[a] Viral Infectious Diseases Unit, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan
[b] Department of General Medicine, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan
[c] Institute for Virus Research, Kyoto University, Kyoto 606-8507, Japan
[d] Department of Microbiology and Immunology, Keio University School of Medicine, 35 Shinanomachi, Shinjyuku-ku, Tokyo 160-8582, Japan

ARTICLE INFO

Article history:
Received 18 January 2009
Available online 4 February 2009

Keywords:
HIV-1 inhibitor
Vpr
Nuclear import inhibition
Importin α
Small molecule
Macrophage

ABSTRACT

The development of multidrug-resistant viruses compromises the efficacy of anti-human immunodeficiency virus (HIV) therapy and limits treatment options. Therefore, new targets that can be used to develop novel antiviral agents need to be identified. One such target is the interaction between Vpr, one of the accessory gene products of HIV-1 and Importin α, which is crucial, not only for the nuclear import of Vpr, but also for HIV-1 replication in macrophages. We have identified a potential parent compound, hematoxylin, which suppresses Vpr–Importin α interaction, thereby inhibiting HIV-1 replication in a Vpr-dependent manner. Analysis by real-time PCR demonstrated that hematoxylin specifically inhibited nuclear import step of pre-integration complex. Thus, hematoxylin is a new anti-HIV-1 inhibitor that targets the nuclear import of HIV-1 via the Vpr–Importin α interaction, suggesting that a specific inhibitor of the interaction between viral protein and the cellular factor may provide a new strategy for HIV-1 therapy.

Macrophages are a major target of human immunodeficiency virus type 1 (HIV-1) and serve as a viral reservoir for the release of small amounts of viral particles in symptomatic carriers [1]. HIV-1 in latently infected macrophages in some lymphoreticular tissues cannot be eradicated by highly active anti-retroviral therapy (HAART), and these cells may produce viral particles that can spread throughout the body [2]. A striking feature of HIV-1 is its ability to replicate in non-dividing cells, in particular, macrophages. Replication in non-dividing cells depends on the active nuclear import of the viral pre-integration complex (PIC) [3]. The HIV-1 PIC exhibits biophysical properties typical of a large nucleo–protein complex and contains the viral proteins reverse transcriptase, integrase (IN), nucleocapsid, Vpr, and small amounts of matrix (MA), in addition to the viral nucleic acids [4–7]. Three PIC-associated proteins, MA, IN, and Vpr, have been proposed as karyophilic agents that act probably via their interactions with Importin (Imp) α, Imp β, and/or Imp 7 [8]. In addition, a recent study indicates that transportin-SR2, which shuttles the essential splicing factor, mediates PIC nuclear import, thereby facilitating HIV infection [9]. Moreover, a novel partner, tRNA, has been shown to facilitate PIC nuclear import [10]. However, the molecular mechanisms of PIC nuclear import and its role in viral replication in macrophages are still not completely understood.

Several studies have shown that Vpr is essential for the nuclear import of PIC in macrophages [11,12], while others do not support such observations [13]. However, our studies have clearly shown that Vpr is targeted to the nuclear envelope and then transported into the nucleus by Imp α alone, in an Imp β-independent manner [12,14]. Furthermore, the interaction between Imp α and the N-terminal α-helical domain (αH1) of Vpr, amino acid residues 17–34, is indispensable, not only for nuclear import of Vpr, but also for HIV-1 replication in macrophages [12]. Thus, it appears that Vpr–Imp α binding precedes a novel nuclear import process, which is a potential target for therapeutic intervention in macrophages, which is crucial for subsequent viral spread to lymphoid organs and T-helper lymphocytes [15]. In addition to nuclear transport, Vpr also has other important functions, including the induction of cell cycle arrest at the G_2 phase [16], the regulation of apoptosis [16,17] and splicing [18,19], carried out through interactions with a variety of cellular partners. These observations suggest that drug targeting of Vpr may lead to pleiotropic effects on the HIV life cycle.

As a promising target for blocking HIV-1 replication in macrophages, by screening a large collection of chemical compounds,

* Corresponding author. Fax: +81 48 462 4399.
E-mail address: aida@riken.jp (Y. Aida).

0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.01.180

we here discovered several compounds that selectively inhibit the nuclear import of Vpr in an Imp α-dependent manner. Importantly, hematoxylin blocks HIV-1 replication in a Vpr-dependent manner in macrophages by blocking the nuclear import of PIC, but does not block Vpr-induced G_2 cell cycle arrest, the virion incorporation function of Vpr and nuclear import of karyophilins, which possess the classical nuclear localization signal (cNLS).

Materials and methods

Enzyme linked immuno-sorbent assay (ELISA)-based binding assay. The wells of 96-well microplates (NUNC) were coated with an anti-glutathione *S*-transferase (GST)-specific monoclonal antibodies (MAb) (Sigma) in 50 mM $NaHCO_3$ (pH 9.8) for 6 h at 4 °C. After washing the wells, GST or GST-N17C74 (0.5 μg/well) in phosphate-buffered saline (PBS) containing 5% bovine serum albumin (BSA) were added to the wells and incubated for 2 h at 4 °C. Imp α-histidine tag_6 (His_6) was added to the wells together with Imp β or test compounds and incubated for 2 h at 4 °C. The horseradish peroxidase (HRP)-conjugated anti-His tag MAb (Sigma) was added. Following incubation at 22 °C for 1 h, the microplates were washed three times and tetramethylbenzidine (TMB) (Pierce) was added. After incubation at 37 °C for 30 min, the amount of surface-bound Imp α was estimated by monitoring the optical density of the wells at 450 nm using an ELISA plate reader (Wallac ARVO™ SX 1420; Perkin-Elmer).

Viral infection assay. Primary macrophages in 24-well plates were inoculated with vesicular stomatitis virus G (VSV-G) pseudotyped reporter virus [NL-Luc-E^-R^+ (VSV-G) or NL-Luc-E^-R^- (VSV-G); 4 ng of p24 antigen], cultured in the absence or presence of hematoxylin for 2 days, harvested, and lysed in luciferase assay substrate (Promega). Luciferase activity was measured using a Wallac ARVO™ SX 1420 luminometer (Perkin-Elmer). Moreover, primary macrophages were exposed to diluted virus stocks (containing 2 or 20 ng of p24 antigen), the JR-CSF strain of the HIV-1 macrophage-tropic virus for 3 h at 37 °C. The cells were then washed three times and seeded in a 24-well tissue-culture plate (NUNC) for primary macrophages. Cells were maintained in RPMI-1640 that contained 10% fetal calf serum. Culture supernatants were harvested at 4-, 8-, 12-, 16-day intervals for primary macrophages and viral production was monitored by sequential quantitation of p24 antigen in cell-free supernatants using an HIV-1 $p24^{gag}$ ELISA kit (LUMIPULSE; Fuji REBIO).

Quantitative real-time PCR. Real-time PCR for quantification of total viral DNA, 2-long terminal repeat (LTR) circular DNA, was performed as follows. Differentiated primary macrophages were infected with VSV-G pseudotyped reporter viruses [NL-Luc-E^-R^+ (VSV-G) or NL-Luc-E^-R^- (VSV-G)] containing 4 ng of p24 antigen and genomic DNA was isolated at 24 h. Total DNA, 2-LTR DNA, and β-globin were quantified with specific primers (2-LTR-forward, ccctcagacccttttagtcagtg; 2-LTR-reverse, tggtgtgtagttctgccaatca; U5gag forward, gtagtgtgtgcccgtctgttg; U5gag reverse, caagccgagtcctgcgt; β-globin primers were those used by Yamamoto et al. [20]).

Other assays. Other assays were described in Supplementary materials.

Results

To identify compounds inhibiting HIV-1 replication, we focused on the nuclear entry of HIV-1 via the Vpr–Imp α interaction as a target for therapeutic strategies and performed ELISA-based binding assays using natural product libraries derived from microbial and fungal metabolites. For these experiments, we used the Vpr N17C74 fragment (containing residues 17–74), because this is a functionally transportable region [12,14]. Among 49 compounds that specifically inhibited the interaction between N17C74 and Imp α, as measured by the ELISA-based binding assay (Fig. 1B), one compound, hematoxylin, inhibited specific binding of Vpr to Imp α in a dose-dependent manner, as assessed by *in vitro* pull-down assays (Fig. 1A and C). Next, we tested the effect of this compound in an *in vitro* nuclear import assay using digitonin-permeabilized HeLa cells. Interestingly, Imp α-mediated nuclear import of Vpr was dose-dependently inhibited by the addition of hematoxylin with a mean 50% inhibitory concentration (IC_{50}) of 5 μM, suggesting that hematoxylin is a potent small-molecule inhibitor of Vpr nuclear entry. Furthermore, we demonstrated the effect of hematoxylin on Vpr-mediated G_2 phase cell cycle arrest, which is one of the major roles of Vpr in HIV-1 replication (Fig. 2B). Flow-cytometry analysis showed that hematoxylin failed to inhibit the Vpr-induced G_2 phase cell cycle arrest. The αH1 which is essential for the nuclear import of Vpr appeared to be critical for the expression, stability and incorporation of Vpr into the viral particle [14,21]. Therefore, we analyzed whether hematoxylin has an effect on the incorporation of Vpr into viral particle using the virion incorporation assay. Hematoxylin had no effect on the virion incor-

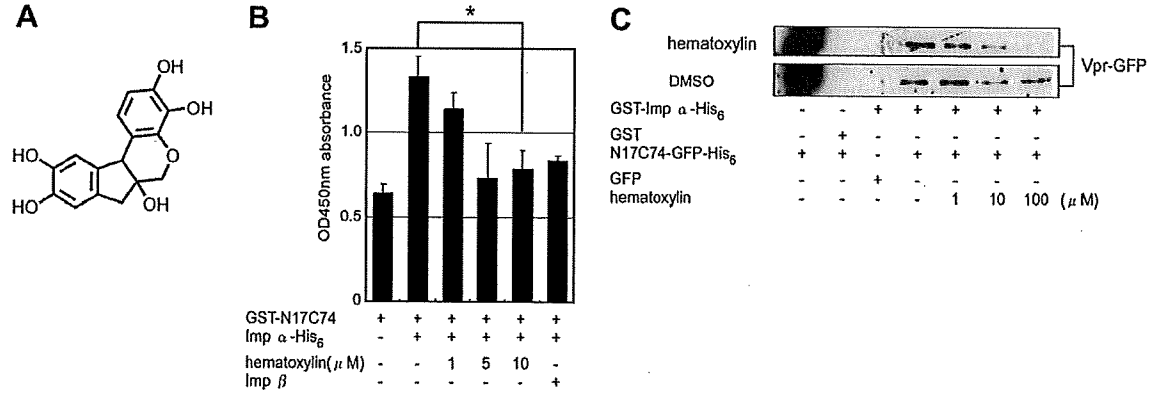


Fig. 1. Identification of a small-molecule that inhibits the interaction between Vpr and Imp α. (A) Chemical structure of hematoxylin. (B) Binding of GST-N17C74 to Imp α-His_6 was quantified using an ELISA-based binding assay. The bound GST-N17C74 was incubated with Imp α-His_6 in the absence (–) or presence (+) of Imp β or hematoxylin. Bound Imp α-His_6 was detected using a HRP-conjugated anti-His MAb. The data are presented as means ± standard deviation of three independent experiments. The asterisk (*) indicates a statistically significant difference ($p < 0.05$). (C) Glutathione-Sepharose beads coupled with GST-Imp α-His_6 or GST were incubated with N17C74-green fluorescence protein (GFP)-His_6 or GFP in the absence (–) or presence of 1, 10 or 100 μM hematoxylin, or 1, 10 or 100 μl dimethyl sulfoxyde (DMSO). The bound fractions were analyzed by Western blotting with anti-GFP MAb.

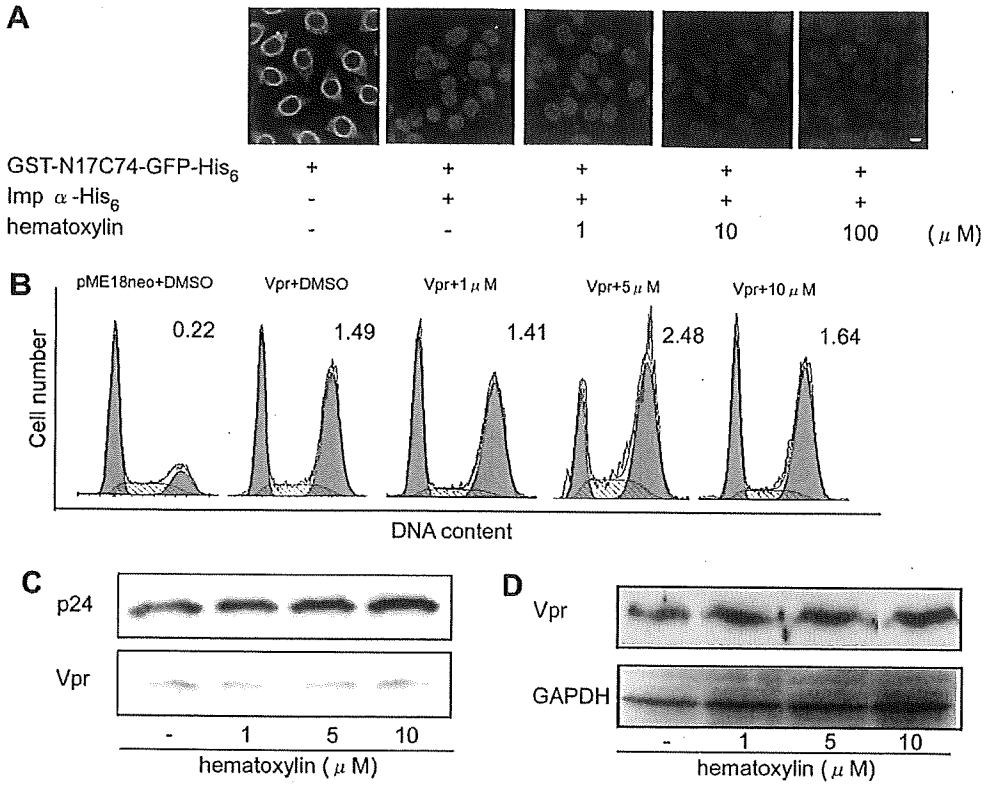


Fig. 2. Hematoxylin inhibits the nuclear import of Vpr by Imp α. (A) Nuclear import of Vpr by Imp α. Digitonin-permeabilized HeLa cells were incubated with 1 μM of GST-N17C74-GFP-His_6 in the absence (−) or presence (+) of 2 μM Imp α, or the indicated concentration of hematoxylin. After fixation, cells were analyzed by confocal laser-scanning microscopy. Bar = 10 μm. (B) Vpr-mediated G_2 arrest. HeLa cells were transfected with pME18Neo encoding Flag-tagged Vpr, or the pME18Neo-Flag, together with pEGFP-N1. At 42 h after addition of 1, 5 or 10 μm hematoxylin, cells were fixed and stained with propidium iodide (PI) for analysis of DNA content and GFP-positive cells were analyzed by flow-cytometry. The proportion of cells in the G_2 phase is indicated at the upper right in each panel. (C) Incorporation of Vpr into viral particles. At 6 h post transfection with pNL4-3, 293T cells were cultured in the absence or presence of hematoxylin, and viral particles were analyzed by Western blotting with anti-Vpr antibody or anti-HIV-1 p24 MAb. (D) Stability of Vpr in HeLa cells. At 6 h post transfection with pME18Neo encoding Flag-tagged Vpr, HeLa cells were cultured in the absence or presence of hematoxylin cells were harvested, lysed and analyzed by Western blotting with anti-Flag M2 MAb and anti-GAPDH MAb.

poration function of Vpr (Fig. 2C). As regards the stability of Vpr, Western blotting analysis showed that the expression level of Vpr in HeLa cells was not affected by hematoxylin 48 h post transfection (Fig. 2D). These results clearly indicate that although hematoxylin has a specific effect on the nuclear import of Vpr, it does not affect the ability of the Vpr to induce G_2 cell cycle arrest or the virion incorporation function of Vpr.

Given the key role played by Vpr in the nuclear import of PIC in non-dividing cells, treatment of infected macrophages with hematoxylin might block HIV-1 replication. Initial experiments were designed to determine a concentration of hematoxylin that could be used with minimal effects on cell viability and cell cycle progression. We investigated the survival of hematoxylin treated human primary macrophages. Macrophages were incubated with hematoxylin at various concentrations in complete culture medium for 15 days and cell viability was evaluated using a 3-(4, 5-dimethylthylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Fig. 3A). For primary macrophages, hematoxylin was toxic with a 50% cytotoxity concentration (CC_{50}) of >40 μM, respectively. Hematoxylin had no effect on the host cell cycle progression, as indicated by flow-cytometry analysis (Fig. 3B). Given the results here, hematoxylin had no adverse effect on the cell growth and viability of the cells at the IC_{50} concentrations for the binding and nuclear import assays, determined in Figs. 1 and 2. Furthermore, we tested whether nuclear import of the cNLS, which requires the formation of a ternary complex of Imp β and Imp α, was inhibited by hematoxylin. The cNLS-Imp α interaction and classical transport were not inhibited by hematoxylin (Fig. 3C and D), implying that hematoxylin has a specific action on the Imp α-mediated nuclear import of Vpr.

Vpr is essential for the nuclear import of PIC in macrophages. Therefore, specific inhibition of nuclear import by hematoxylin via the Vpr–Imp α interaction led us to investigate whether hematoxylin blocks HIV-1 replication in macrophages. Primary macrophages were infected with the macrophage-tropic JR-CSF HIV-1 strain at low viral input (2 ng of p24 antigen) and cultured for 8 days in the presence or absence of hematoxylin at indicated concentrations (Fig. 4A, left panel). Virus replication was monitored at 4 or 8 days post infection by p24 ELISA. Hematoxylin blocked virus replication efficiently and in a dose-dependent manner, reaching inhibition levels up to about 50% and 70% in 4 and 8 days, respectively, at 20 μM (IC_{50} = 1.64 μM). Similarly, results of infection at high viral input (20 ng of p24 antigen) showed same tendency (Fig. 4A, right panel). Furthermore, to confirm the effect of hematoxylin on HIV-1 replication in macrophages, macrophages were infected with a VSV-G-pseudotype HIV-1 strain that encoded either the wild-type Vpr or a truncated Vpr which can only support a single round of HIV-1 replication, and cultured for 2 days in the absence or presence of 2.5, 5, 10 or 20 μM hematoxylin (Fig. 4B). When hematoxylin was added at the time of infection, the replication of Vpr^+ virus was suppressed by hematoxylin in a dose-dependent manner with a mean of IC_{50} of 5 μM (Fig. 4B). Importantly, levels of luciferase activity of the Vpr^- virus were not effective in each concentration of hematoxylin, indicating that inhibition of viral replication by hematoxylin is a Vpr-dependent manner.

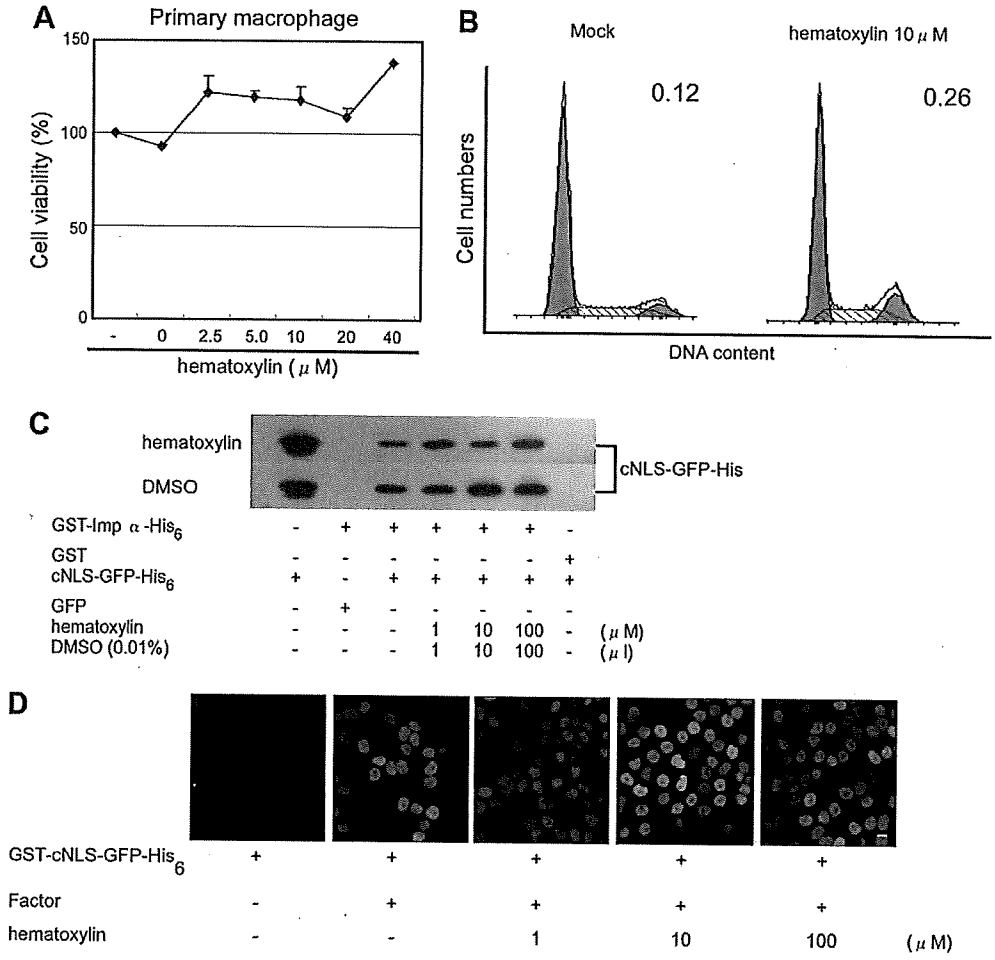


Fig. 3. Analysis of the effect of hematoxylin on normal cell functions. (A) Differentiated primary macrophages were treated with the indicated concentrations of hematoxylin for 15 days. Cell viability was determined using an MTT assay. (B) HeLa cells were treated with 10 μM hematoxylin for 48 h and cell cycle profiles were analyzed by flow cytometry. (C) Glutathione-Sepharose beads coupled with GST–Imp α-His_6 or GST were incubated with cNLS-GFP-His_6 or GFP as a control, in the absence (–) or presence of hematoxylin or DMSO at indicated concentrations. The bound fractions were analyzed by Western blotting with a GFP-specific MAb. (D) Digitonin-permeabilized HeLa cells were incubated with GST-cNLS-GFP-His_6 in the absence (–) or presence (+) of hematoxylin and soluble factors as described previously [12], and then analyzed by confocal laser-scanning microscopy. Bar = 10 μm.

To determine the target of hematoxylin in HIV-1 life cycle, we quantified the viral nucleic acid species present in macrophages after infection. We analyzed the effect of hematoxylin on the formation of late reverse transcripts, as an indicator of total viral DNA (U5 gag as shown in the text), 2-LTR circular DNA, as a marker for successful nuclear import of viral genomic DNA [22]. Treatment with hematoxylin dramatically reduced the amount of 2-LTR circular forms without affecting the copy numbers of U5 gag total DNA in a Vpr-dependent manner (Fig. 4C). These results strongly indicate that the anti-retroviral activity of hematoxylin is likely to be mediated by inhibition of HIV-1 nuclear transport, in macrophages, rather than other steps, such as virus entry and virus maturation.

Discussion

In this study, we explored the anti-HIV activity of hematoxylin, a compound screened by ELISA-binding assays to target the interaction between Vpr and Imp α. By targeting the Vpr–Imp α interaction, we successfully identified a compound inhibiting HIV-1 replication. This result indicates that a specific inhibitor of an interaction between a viral protein and a host cellular factor may provide a new therapeutic strategy for blocking HIV-1 replication. Our results further demonstrate that hematoxylin can efficiently block the nuclear import of Vpr and PIC, and potently blocked HIV-1 replication with Vpr-dependent manner in macrophages. By contrast, hematoxylin, did not inhibit HIV-1 replication in peripheral blood mononuclear cells (PBMCs), which limits the effectiveness of nuclear import inhibitors (unpublished data). This result strongly suggests that Vpr–Imp α interaction is a valuable new drug target which can be exploited for the development of HIV-1 therapies targeting macrophages which cannot be eradicated by HAART.

In present study, by targeting the interaction between Imp α and αH1, we identified a compound inhibiting HIV-1 replication. However, whether hematoxylin directly interacts with Imp α or Vpr remains to be clarified. Recently, using a new hematoxylin derivative and photo-cross-linked small-molecule affinity matrix assay, we obtained evidence to suggest that hematoxylin derivative directly interacts with the αH1 domain of Vpr, but does not interact with Imp α (unpublished data). We postulate that hematoxylin directly bind αH1 and that specifically inhibit Vpr–Imp α interaction without disrupting the global structure of Vpr, because hematoxylin did not inhibit the incorporation of Vpr into viral particle, which is mediated by the αH1 domain. Moreover, the modified Wu-Kabat variability index clearly showed that the αH1 of Vpr is highly conserved among various HIV-1 strains, compared to the

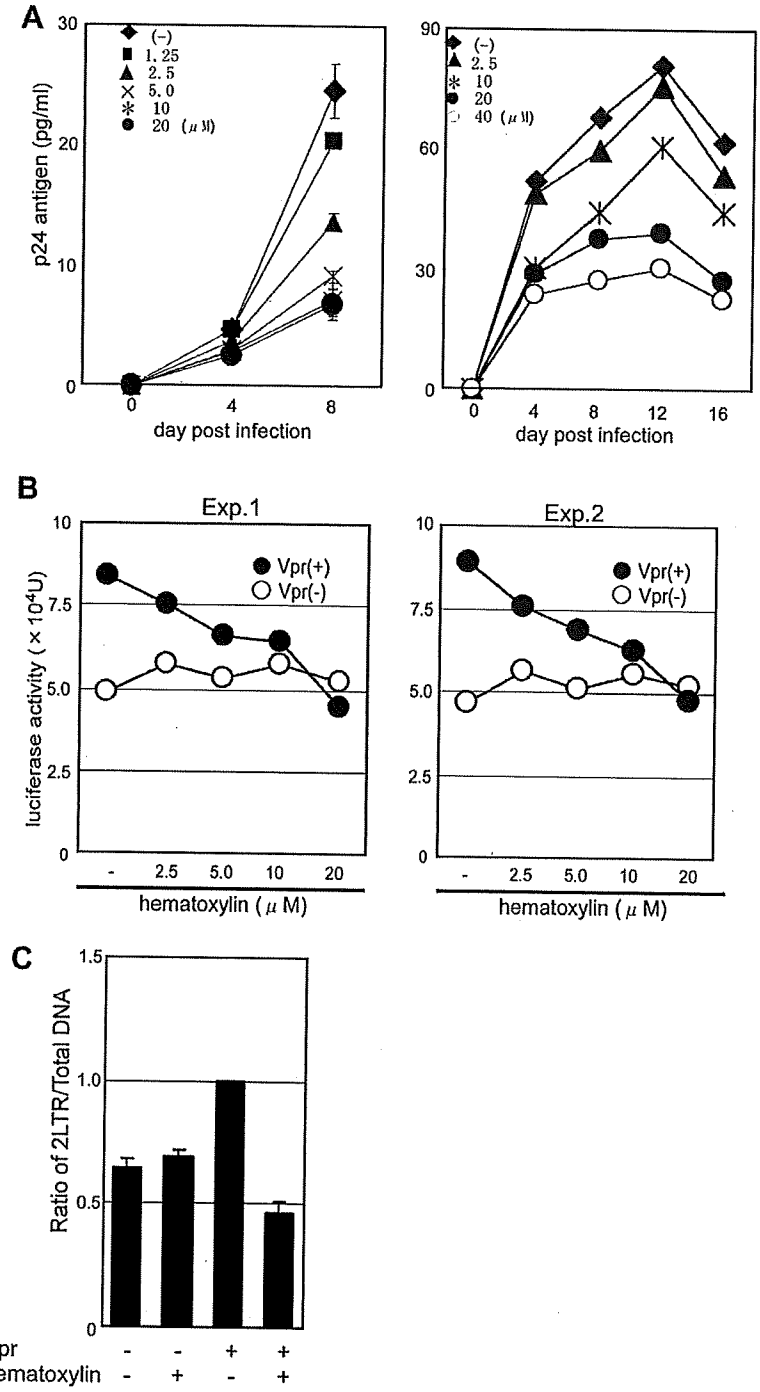


Fig. 4. Hematoxylin inhibition of HIV-1 replication depends on the Vpr in macrophages. (A) Macrophages were infected with HIV-1 (JR-CSF strain) at 2 ng (left panel) or 20 ng (right panel) of p24 antigen in the absence or presence of hematoxylin. Cells were maintained for 4, 8, 12 and 16 days, and the levels of virus production in the culture supernatants were measured by p24 antigen ELISA. (B) Macrophages from two healthy donors were infected with VSV-G-pseudotype virus encoded wild-type Vpr (closed circle) or truncated Vpr (open circle) at 4.0 ng of p24 antigen, and cultured in the absence or presence of hematoxylin. Proviral gene expression was analyzed by luciferase assays 2 days after the infection. (C) Macrophages were infected with the VSV-G-pseudotype virus encoding wild-type Vpr or truncated Vpr at 4 ng of p24 antigen and cultured in the absence or presence of 10 μM hematoxylin. Total HIV-1 DNA and 2-LTR DNA were determined using real-time PCR at 24 h post infection. All samples were tested in duplicate or triplicate, and the data are presented the mean levels of p24 antigen or luciferase activity.

α-helix 2 and α-helix 3 domains, suggesting that the αH1 domain may play crucial roles in HIV-1 survival (unpublished data). Although these data are preliminary, the fact that hematoxylin directly binds to αH1 predicts that HIV-1 resistance to hematoxylin would occur far less frequently than resistance to other conventional drugs. More importantly, understanding of the detailed mechanism of the interaction between Vpr and hematoxylin is essential.

Three HIV-1 proteins, Vpr, MA, and IN, have been proposed as karyophilic agents that recruit the cellular nuclear import machin-

ery to the PIC [8]. Other machineries in addition to the Vpr likely contribute to HIV-1 nuclear import; nevertheless, potent inhibition of HIV-1 replication was achieved by hematoxylin inhibition of Vpr–Imp α interaction. As shown in Fig. 4A, although hematoxylin blocked viral replication efficiently, resulting in 70% on 8 or 16 day, it did not block viral replication completely. This may indicate that there are PIC nuclear import pathways that mediate PIC nuclear import independently of the Vpr; for example, Imp β, Imp 7, Imp β/Imp 7 heterodimer and transportin-SR2 are involved in the nuclear import of PIC into macrophages. In addition, IN, MA, and Vpr either work sequentially or synergistically to regulate PIC nuclear import.

Our results demonstrate that the Vpr–Imp α interaction, a virus protein–cellular protein interaction, is a potential target for an antiviral agent that inhibits nuclear entry. To clarify the mechanism of action of hematoxylin, we are conducting ongoing studies to analyze the crystal structures of the hematoxylin–αH1 domain complex and the αH1 domain–Imp α complex. Detailed descriptions of these interactions will provide new therapeutic strategies for rational drug design. Nuclear import-blocking drugs may be useful not only against HIV, but also against other viruses that require nuclear import for replication, such as the influenza virus, hepatitis B virus, or herpes viruses. The development of such compounds is likely to provide a valuable enrichment of our arsenal of antiviral drugs.

Acknowledgments

We thank Drs. K. Tokunaga, A. Koito, and K. Strebel for kindly providing VSV-G pseudotyped reporter viruses and anti-Vpr antibody. This work was supported in part by a Health Sciences Research Grant from the Ministry of Health, Labor and Welfare of Japan (Research on HIV/AIDS), by the program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO) of Japan, and by the Chemical Biology Research Project (RIKEN).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.01.180.

References

[1] J.M. Orenstein, C. Fox, S.M. Wahl, Macrophages as a source of HIV during opportunistic infections, Science 276 (1997) 1857–1861.
[2] J.M. Orenstein, M. Feinberg, C. Yoder, L. Schrager, J.M. Mican, D.J. Schwartzentruber, R.T. Davey Jr., R.E. Walker, J. Falloon, J.A. Kovacs, K.D. Miller, C. Fox, J.A. Metcalf, H. Masur, M.A. Polis, Lymph node architecture preceding and following 6 months of potent antiviral therapy: follicular hyperplasia persists in parallel with p24 antigen restoration after involution and CD4 cell depletion in an AIDS patient, AIDS 13 (1999) 2219–2229.
[3] M.I. Bukrinsky, N. Sharova, M.P. Dempsey, T.L. Stanwick, A.G. Bukrinskaya, S. Haggerty, M. Stevenson, Active nuclear import of human immunodeficiency virus type 1 preintegration complexes, Proc. Natl. Acad. Sci. USA 89 (1992) 6580–6584.
[4] M.I. Bukrinsky, S. Haggerty, M.P. Dempsey, N. Sharova, A. Adzhubei, L. Spitz, P. Lewis, D. Goldfarb, M. Emerman, M. Stevenson, A nuclear localization signal within HIV-1 matrix protein that governs infection of non-dividing cells, Nature 365 (1993) 666–669.
[5] C.M. Farnet, W.A. Haseltine, Determination of viral proteins present in the human immunodeficiency virus type 1 preintegration complex, J. Virol. 65 (1991) 1910–1915.
[6] A. Fassati, S.P. Goff, Characterization of intracellular reverse transcription complexes of human immunodeficiency virus type 1, J. Virol. 75 (2001) 3626–3635.
[7] M.D. Miller, C.M. Farnet, F.D. Bushman, Human immunodeficiency virus type 1 preintegration complexes: studies of organization and composition, J. Virol. 71 (1997) 5382–5390.
[8] J. De Rijck, L. Vandekerckhove, F. Christ, Z. Debyser, Lentiviral nuclear import: a complex interplay between virus and host, Bioessays 29 (2007) 441–451.
[9] 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.
[10] L. Zaitseva, R. Myers, A. Fassati, TRNAs promote nuclear import of HIV-1 intracellular reverse transcription complexes, PLoS Biol. 4 (2006) e332.
[11] M. Bukrinsky, A. Adzhubei, Viral protein R of HIV-1, Rev. Med. Virol. 9 (1999) 39–49.
[12] Y. Nitahara-Kasahara, M. Kamata, T. Yamamoto, X. Zhang, Y. Miyamoto, K. Muneta, S. Iijima, Y. Yoneda, Y. Tsunetsugu-Yokota, Y. Aida, Novel nuclear import of Vpr promoted by Importin α is crucial for human immunodeficiency virus type 1 replication in macrophages, J. Virol. 81 (2007) 5284–5293.
[13] M. Bouyac-Bertoia, J.D. Dvorin, R.A. Fouchier, Y. Jenkins, B.E. Meyer, L.I. Wu, M. Emerman, M.H. Malim, HIV-1 infection requires a functional integrase NLS, Mol. Cell 7 (2001) 1025–1035.
[14] M. Kamata, Y. Nitahara-Kasahara, Y. Miyamoto, Y. Yoneda, Y. Aida, Importin-α promotes passage through the nuclear pore complex of human immunodeficiency virus type 1 Vpr, J. Virol. 79 (2005) 3557–3564.
[15] O.J. Cohen, A.S. Fauci, Current strategies in the treatment of HIV infection, Adv. Intern. Med. 46 (2001) 207–246.
[16] X. Wen, K.M. Duus, T.D. Friedrich, C.M. de Noronha, The HIV1 protein Vpr acts to promote G2 cell cycle arrest by engaging a DDB1 and Cullin4A-containing ubiquitin ligase complex using VprBP/DCAF1 as an adaptor, J. Biol. Chem. 282 (2007) 27046–27057.
[17] A. Azuma, A. Matsuo, T. Suzuki, T. Kurosawa, X. Zhang, Y. Aida, Human immunodeficiency virus type 1 Vpr induces cell cycle arrest at the G(1) phase and apoptosis via disruption of mitochondrial function in rodent cells, Microbes Infect. 8 (2006) 670–679.
[18] M. Kuramitsu, C. Hashizume, N. Yamamoto, A. Azuma, M. Kamata, Y. Tanaka, Y. Aida, A novel role for Vpr of human immunodeficiency virus type 1 as a regulator of the splicing of cellular pre-mRNA, Microbes Infect. 7 (2005) 1150–1160.
[19] C. Hashizume, M. Kuramitsu, X. Zhang, T. Kurosawa, M. Kamata, Y. Aida, Human immunodeficiency virus type 1 Vpr interacts with spliceosomal protein SAP145 to mediate cellular pre-mRNA splicing inhibition, Microbes Infect. 9 (2007) 490–497.
[20] N. Yamamoto, C. Tanaka, Y. Wu, M.O. Chang, Y. Inagaki, Y. Saito, T. Naito, H. Ogasawara, I. Sekigawa, Y. Hayashida, Analysis of human immunodeficiency virus type 1 integration by using a specific, sensitive and quantitative assay based on real-time polymerase chain reaction, Virus Genes 32 (2006) 105–113.
[21] S. Mahalingam, V. Ayyavoo, M. Patel, T. Kieber-Emmons, D.B. Weiner, Nuclear import, virion incorporation, and cell cycle arrest/differentiation are mediated by distinct functional domains of human immunodeficiency virus type 1 Vpr, J. Virol. 71 (1997) 6339–6347.
[22] B. Bowerman, P.O. Brown, J.M. Bishop, H.E. Varmus, A nucleo–protein complex mediates the integration of retroviral DNA, Genes Dev. 3 (1989) 469–478.