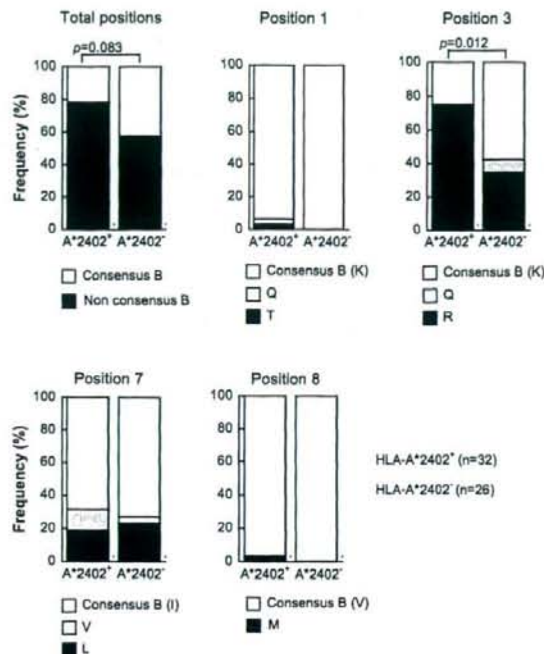



Fig. 2. Frequency of mutations in KW9 epitopes. The KW9 epitope sequence was analyzed in HLA-A*2402-positive and HLA-A*2402-negative individuals chronically infected with HIV-1. The consensus sequence of this epitope in clade B is KYKLKHIVW. The frequency of mutations in the total sequence of this epitope and those at a given position of the epitope are shown for both HLA-A*2402-positive and HLA-A*2402-negative donors. The $P$ values were determined by Fisher's exact test.

donors than in the HLA-A*2402$^-$ donors ($P = 0.012$, Fig. 2). In the flanking region, there was no significant difference between HLA-A*2402$^+$ and HLA-A*2402$^-$ individuals (data not shown). These results together suggest that only the 3R mutation was selected by the KW9-specific CTLs.

### *3.3. 3R is an escape mutant from KW9-specific CTLs*

To clarify that 3R is an escape mutant from KW9-specific CTLs, we investigated whether or not the KW9-specific CTLs could recognize the KW9-3R mutant epitope. We first tested the activity of KW9-specific CTL clones to kill target cells prepulsed with the KW9-3R mutant peptide. Two KW9-specific CTL clones and KW9-specific bulk CTLs effectively killed target cells prepulsed with the KW9 wild-type peptide but showed reduced ability to kill those prepulsed with the KW9-3R mutant peptide (Fig. 3A,C). Furthermore, to analyze whether or not KW9-specific CTLs could kill target cells infected with the KW9-3R mutant virus, we generated NL-432gag$^{SF2}$ virus in which the NL-432 gag gene was replaced with the SF2 gag gene as well as NL-432gag$^{SF2}$ carrying the KW9-3R epitope (NL-432gag$^{SF2}$ KW9-3R). The KW9-specific CTL clones and KW9-specific bulk CTLs effectively killed the target cells infected with NL-432gag$^{SF2}$, whereas they failed to kill those infected with NL-432gag$^{SF2}$ KW9-3R

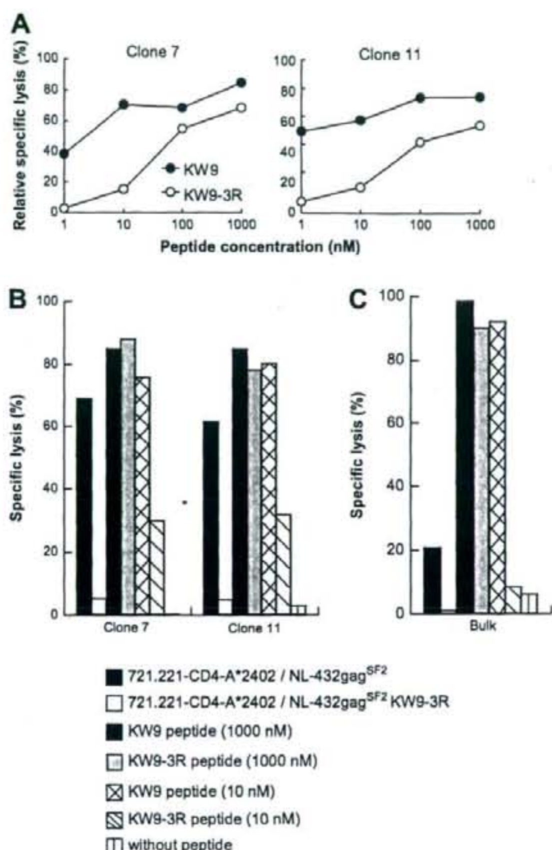



Fig. 3. Cytotoxic activity of KW9-specific CTLs against target cells pulsed with KW9-3R peptide and infected with NL-432gag$^{SF2}$ KW9-3R. (A) Cytolytic activities of KW9-specific CTL clones to kill C1R-A*2402 cells pulsed with KW9 or KW9-3R peptide. C1R-A*2402 cells were prepulsed with various concentrations of KW9 or KW9-3R peptide. Cytolytic activities of KW9-specific CTL clones were measured at an effector-to-target ratio of 2:1. (B) Cytotoxic activity of KW9-specific CTL clones against HIV-1-infected 721.221-CD4-A*2402. 721.221-CD4-A*2402 cells were infected with NL-432gag$^{SF2}$ or NL-432gag$^{SF2}$ KW9-3R. NL-432gag$^{SF2}$-infected (80.5% of total cells were p24 antigen-positive) or NL-432gag$^{SF2}$ KW9-3R-infected (81.9% of total cells were p24 antigen-positive) 721.221-CD4-A*2402 were used as target cells at an E:T ratio of 2:1. (C) Cytotoxic activity of KW9-specific bulk CTL cells against HIV-1-infected or epitope peptide-pulsed 721.221-CD4-A*2402. NL-432gag$^{SF2}$-infected (45.4% of total cells were p24 antigen-positive), NL-432gag$^{SF2}$ KW9-3R-infected (43.8% of total cells were p24 antigen-positive) 721.221-CD4-A*2402 or peptide-pulsed 721.221-CD4-A*2402 were used as target cells at an E:T ratio of 10:1.

(Fig. 3B,C), indicating that the Gag28-3R mutant is the escape one from KW9-specific CTLs.

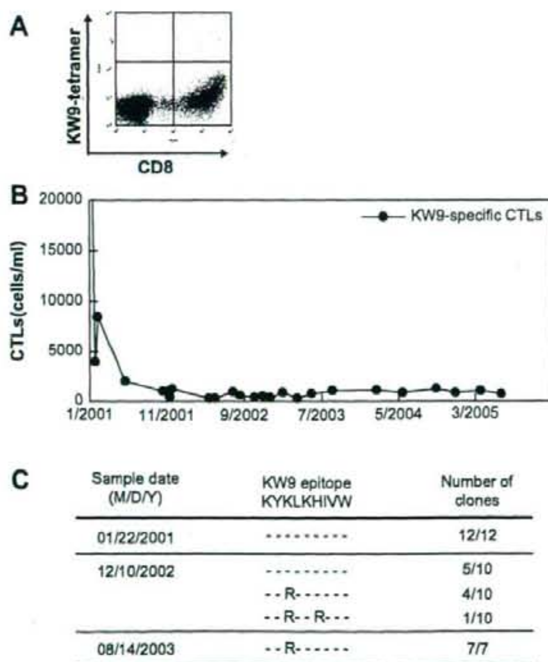
### *3.4. Longitudinal analysis of KW9 epitope and KW9-specific CD8$^+$ T cells in an HIV-1-infected HLA-A*2402$^+$ individual*

We furthermore performed longitudinal analysis of KW9-specific CTL responses and the KW9 epitope in an

HLA-A*2402$^+$ individual, KI-092, having an early HIV-1 infection. To investigate the number of Gag28-specific CTLs, we stained PBMCs from KI-092, who had been receiving STI therapy during the early phase, with the KW9-tetramer and anti-CD8 mAb. In this patient, KW9-specific CD8$^+$ T cells were effectively elicited during the early phase of the infection (Fig. 4A); however, the number of these cells became rapidly reduced within 10 months and remained low thereafter for approximately 4 years (Fig. 4B). We next analyzed the sequence of this epitope during the clinical course in this patient. As expected, the wild-type (WT) sequence of KW9 was found in the early phase. However, the patient showed the presence of both WT and 3R mutant viruses approximately 2 years later, and had only the 3R virus about 3 years later (Fig. 4C). These results strongly support the idea that KW9 is selected by KW9-specific T cells.

## 4. Discussion

Our previous studies identified 3 HLA-A*2402-restricted Gag epitopes and showed that KW9-specific cytolytic activities were detected in only 4 of 12 chronically HIV-1-infected HLA-A*2402$^+$ donors but that other Gag epitope-specific cytolytic activities were hardly detected [20,21]. The present study using the ICC assay demonstrated that KW9-specific CD8$^+$ T cells were detected in 8 of the 12 chronically HIV-1-infected HLA-A*2402$^+$ donors tested. The difference in frequency of KW9-specific T cells between the 2 studies may have been due to the difference in the assays used. The present study confirmed that KW9 is the immunodominant epitope and showed that KW9-specific T cells are elicited in chronically HIV-1-infected HLA-A*2402$^+$ donors more than was previously speculated.




| Sample date (M/D/Y) | KW9 epitope KYKLKHIVW | Number of clones |
|---|---|---|
| 01/22/2001 | --------- | 12/12 |
| 12/10/2002 | --------- | 5/10 |
| | --R------ | 4/10 |
| | --R--R--- | 1/10 |
| 08/14/2003 | --R------ | 7/7 |

Fig. 4. Longitudinal analysis of KW9-specific CD8$^+$ T cells and KW9 epitope in an HLA-A*2402$^+$ individual having an early HIV-1 infection. *Ex vivo* analysis of KW9-specific CTLs. KW9-specific CTLs in PBMC (2/1/2001) derived from an HLA-A*2402$^+$ individual, KI-092, who received STI therapy during the early phase of infection, were measured by using KW9-tetramer and anti-CD8 mAb (A). Results of *ex vivo* longitudinal analysis of KW9-specific CTLs (B) and the sequence of the KW9 epitope (C) derived from KI-092 are also shown.

A previous study showed that the KW9-3R mutant was detected in 2 of 3 HIV-1-infected HLA-A*2402-positive Japanese individuals and that HLA-A*2402-resricted KW9-specific CTLs failed to kill the target cells pulsed with KW9-3R peptide [22]. That study implied only the possibility that KW9-3R is a mutation for escape from the specific CTLs. In the present study, we showed that KW9-specific CTLs effectively killed target cells infected with the WT virus but failed to kill those infected with the KW9-3R mutant virus. In addition, the sequence analysis of this epitope in 58 chronically HIV-1-infected individuals showed that the frequency of the KW9-3R mutation was significantly higher in HLA-A*2402$^+$ Japanese individuals than in the HLA-A*2402$^-$ individuals, indicating that this mutation was selected in an HLA-A*2402-restricted fashion at the population level. This finding also supports the idea that KW9-3R is an escape mutant. Approximately 40% of the HLA-A*2402$^-$ Japanese individuals were infected with the HIV-KW9-3R mutant, suggesting that this mutant has accumulated in the Japanese population and implying that this mutant does not revert in HLA-A*2402$^-$ hosts.

Seven of 8 HLA-A*2402$^+$ donors who induced KW9-specific CTLs shown in Fig. 1B had the 3R mutation, while all 4 HLA-A*2402$^+$ donors who did not induce KW9-specific CTLs had this mutation (data not shown). This result indicates that HLA-A*2402$^+$ donors who have the 3R mutation can still maintain KW9-specific memory or effector memory T cells. However, it still remains unclear that the 3R mutation elicits the 3R-specific T cells.

Escape mutations occur at some site within the CTL epitope, where the substitution of an amino acid abrogates HLA binding, reduces the recognition of the TCR, and/or interferes with efficient antigen processing. HLA-A*2402-binding peptides have 2 anchor residues, Tyr at position 2 and Phe, Leu, Ile or Trp at the carboxyl terminus [26,27]. Since position 3 is not an anchor residue and the substitution from Lys to Arg is speculated not to affect the binding affinity, the KW9-3R peptide may bind to HLA-A*2402 molecules with similar affinity to the WT peptide. Therefore, the escape mechanism of the KW9-3R mutant virus may involve the failure of the recognition of the TCR and/or the disruption of cellular processing of the KW9-3R peptide.

Longitudinal analysis of the KW9 epitope in an HLA-A*2402$^+$ individual having an early HIV-1 infection revealed that half of the HIV-1 isolates were the 3R mutant approximately 2 years after the early phase of the infection when only the wild-type HIV-1 was detected. These results suggest that this escape mutant is slowly selected by the specific T cells,

and also support the idea that the 3R mutant is selected by KW9-specific CTLs. The analysis using the tetramers showed that the frequency of KW9-specific CTLs was reduced within 9 months. This finding implies that the 3R mutant had already appeared at this point in time.

The results obtained in the present study reveal that KW9-3R is selected as an escape mutant by KW9-specific CTLs. As this escape mutation has accumulated in the Japanese population, HLA-A*2402$^{+}$ new hosts may become infected with the 3R mutant virus; and the CTLs of such patients may fail to respond to this mutant virus. Further studies of this escape mutant will be necessary to clarify HIV-1-specific CTL responses in Japanese and other Asian populations.

## Acknowledgements

The authors thank Sachiko Sakai for her secretarial assistance. This research was supported by a grant-in-aid for scientific research from the Ministry of Health, Labor, and Welfare of the Government of Japan; a grant from the Japan Health Science Foundation; a grant-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) (No. 20390134) of Japan; and by the Global COE program "Global Education and Research Center Aiming at the Control of AIDS", MEXT, Japan.

## References

[1] P. Borrow, H. Lewicki, B.H. Hahn, G.M. Shaw, M.B. Oldstone, Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection, J. Virol 68 (1994) 6103–6110.

[2] R.A. Koup, J.T. Safrit, Y. Cao, C.A. Andrews, G. McLeod, W. Borkowsky, C. Farthing, D.D. Ho, Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome, J. Virol 68 (1994) 4650–4655.

[3] G.S. Ogg, X. Jin, S. Bonhoeffer, P.R. Dunbar, M.A. Nowak, S. Monard, J.P. Segal, Y. Cao, S.L. Rowland-Jones, Y. Cerundolo, A. Hurley, M. Markowitz, D.D. Ho, D.F. Nixon, A.J. McMichael, Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA, Science 279 (1998) 2103–2106.

[4] P.J. Goulder, R.E. Phillips, R.A. Colbert, S. McAdam, G. Ogg, M.A. Nowak, P. Giangrade, G. Luzzi, B. Morgan, A. Edwards, A.J. McMichael, S. Rowland-Jones, Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS, Nat. Med 3 (1997) 212–217.

[5] P. Borrow, H. Lewicki, X. Wei, M.S. Horwitz, N. Peffer, H. Meyers, J.A. Nelson, J.E. Gairin, B.H. Hahn, M.B. Oldstone, G.M. Shaw, Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus, Nat. Med 3 (1997) 205–211.

[6] I. Couillin, F. Connan, B. Culmann-Penciolelli, E. Gomard, J.G. Guillet, J. Choppin, HLA-dependent variations in human immunodeficiency virus Nef protein alter peptide/HLA binding, Eur. J. Immunol 25 (1995) 728–732.

[7] A.D. Kelleher, C. Long, E.C. Holmes, R.L. Allen, J. Wilson, C. Conlon, C. Workman, S. Shaunak, K. Olson, P. Goulder, C. Brander, G. Ogg, J.S. Sullivan, W. Dyer, I. Jones, A.J. McMichael, S. Rowland-Jones, R.E. Phillips, Clustered mutations in HIV-1 gag are consistently required for escape from HLA-B27-restricted cytotoxic T lymphocyte responses, J. Exp. Med 193 (2001) 375–386.

[8] D.A. Price, P.J. Goulder, P. Klenerman, A.K. Sewell, P.J. Easterbrook, M. Troop, C.R. Bangham, R.E. Phillips, Positive selection of HIV-1 cytotoxic T lymphocyte escape variants during primary infection, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 1890–1895.

[9] R.E. Phillips, S. Rowland-Jones, D.F. Nixon, F.M. Gotch, J.P. Edwards, A.O. Ogunlesi, J.G. Elvin, J.A. Rothbard, C.R. Bangham, C.R. Rizza, A.J. McMichael, Human immunodeficiency virus genetic variation that can escape cytotoxic T cell recognition, Nature 354 (1991) 453–459.

[10] M. Fujiwara, J. Tanuma, H. Koizumi, Y. Kawashima, K. Honda, S. Mastuoka-Aizawa, S. Dohki, S. Oka, M. Takiguchi, Different abilities of escape mutant-specific cytotoxic T cells to suppress replication of escape mutant and wild-type human immunodeficiency virus type 1 in new hosts, J. Virol 82 (2008) 138–147.

[11] T.M. Allen, D.H. O'Connor, P. Jing, J.L. Dzuris, B.R. Mothe, T.U. Vogel, E. Dunphy, M.E. Libel, C. Emerson, N. Wilson, K.J. Kunsman, X. Wang, D.B. Allison, A.L. Hughes, R.C. Desrosiers, J.D. Altman, S.M. Wolinsky, A. Sette, D.I. Watkins, Tat-specific cytotoxic T lymphocytes select for SIV escape variants during resolution of primary viremia, Nature 407 (2000) 386–390.

[12] A.J. McMichael, S.L. Rowland-Jones, Cellular immune responses to HIV, Nature 410 (2001) 980–987.

[13] D.H. Barouch, J. Kunstman, M.J. Kuroda, J.E. Schmitz, S. Santra, F.W. Peyerl, G.R. Krivulka, K. Beaudry, M.A. Lifton, D.A. Gorgone, D.C. Montefiori, M.G. Lewis, S.M. Wolinsky, N.L. Letvin, Eventual AIDS vaccine failure in a rhesus monkey by viral escape from cytotoxic T lymphocytes, Nature 415 (2002) 335–339.

[14] D.H. Barouch, J. Kunstman, M.J. Kuroda, J.E. Schmitz, S. Santra, F.W. Peyerl, G.R. Krivulka, K. Beaudry, M.A. Lifton, D.A. Gorgone, D.C. Montefiori, M.G. Lewis, S.M. Wolinsky, N.L. Letvin, Viral escape from dominant simian immunodeficiency virus epitope-specific cytotoxic T lymphocytes in DNA-vaccinated rhesus monkeys, J. Virol 77 (2003) 7367–7375.

[15] B.D. Walker, B.T. Korber, Immune control of HIV: the obstacles of HLA and viral diversity, Nat. Immunol 2 (2001) 473–475.

[16] P. Kiepiela, K. Ngumbela, C. Thobakgale, D. Ramduth, I. Honeyborne, E. Moodley, S. Reddy, C. de Pierres, Z. Mncube, N. Mkhwanazi, K. Bishop, M. van der Stok, K. Nair, N. Khan, H. Crawford, R. Payne, A. Leslie, J. Prado, A. Prendergast, J. Frater, N. McCarthy, C. Brander, G.H. Learn, D. Nickle, C. Rousseau, H. Coovadia, J.I. Mullins, D. Heckerman, B.D. Walker, P. Goulder, CD8+ T-cell responses to different HIV proteins have discordant associations with viral load, Nat. Med 13 (2007) 46–53.

[17] M.E. Feeney, Y. Tang, K.A. Roosevelt, A.J. Leslie, K. McIntosh, N. Karthas, B.D. Walker, P.J. Goulder, Immune escape precedes breakthrough human immunodeficiency virus type 1 viremia and broadening of the cytotoxic T-lymphocyte response in an HLA-B27-positive long-term-nonprogressing child, J. Virol 78 (2004) 8927–8930.

[18] R. Draenert, S. Le Gall, K.J. Pfafferott, A.J. Leslie, P. Chetty, C. Brander, E.C. Holmes, S.C. Chang, M.E. Feeney, M.M. Addo, L. Ruiz, D. Ramduth, P. Jeena, M. Altfeld, S. Thomas, Y. Tang, C.L. Verrill, C. Dixon, J.G. Prado, P. Kiepiela, J. Martinez-Picado, B.D. Walker, P.J. Goulder, Immune selection for altered antigen processing leads to cytotoxic T lymphocyte escape in chronic HIV-1 infection, J. Exp. Med 199 (2004) 905–915.

[19] T. Imanishi, T. Akaza, A. Kimura, K. Tokunaga, T. Gojobori, in: K. Tsuji, M. Aizawa, T. Sasazuki (Eds.), Allele and Haplotype Frequencies for HLA and Complement Loci in Various Ethnic Groups, HLA 1991: Proceedings of the Eleventh International Histocompatibility Workshop and Conference. Oxford University Press, Oxford, 1992.

[20] Y. Ikeda-Moore, H. Tomiyama, M. Ibe, S. Oka, K. Miwa, Y. Kaneko, M. Takiguchi, Identification of a novel HLA-A24-restricted cytotoxic T-lymphocyte epitope derived from HIV-1 Gag protein, AIDS 12 (1998) 2073–2087.

[21] Y. Ikeda-Moore, H. Tomiyama, K. Miwa, S. Oka, A. Iwamoto, Y. Kaneko, M. Takiguchi, Identification and characterization of multiple HLA-A24-restricted HIV-1 CTL epitopes: strong epitopes are derived from V regions of HIV-1, J. Immunol 159 (1997) 6242–6252.

[22] Y. Yokomaku, H. Miura, H. Tomiyama, A. Kawana-Tachikawa, M. Takiguchi, A. Kojima, Y. Nagai, A. Iwamoto, Z. Matsuda, K. Ariyoshi, Impaired processing and presentation of cytotoxic-T-lymphocyte (CTL) epitopes are major escape mechanism from CTL immune pressure in human immunodeficiency virus type 1 infection, J. Virol 78 (2004) 1324–1332.
[23] S. Karaki, A. Kariyone, N. Kato, Y. Iwakura, M. Takiguchi, HLA-B51 transgenic mice as recipients for production of polymorphic anti-HLA-A, B-specific antibodies, Immunogenetics 37 (1993) 139–142.
[24] M. Okamoto, M. Makino, I. Kitajima, I. Maruyama, M. Baba, HIV-1-infected myelomonocytic cells are resistant to Fas-mediated apoptosis: effect of tumor necrosis factor-alpha on their Fas expression and apoptosis, Med. Microbiol. Immunol 186 (1997) 11–17.
[25] J.D. Altman, P.A.H. Moss, P.J.R. Goulder, D.H. Barouch, M.G. McHeyzer-Williams, J.I. Bell, A.J. McMichael, M.M. Davis, Phenotype analysis of antigen-specific T lymphocytes, Science 274 (1996) 94–96.
[26] R. Maier, K. Falk, O. Rotzschke, B. Maier, V. Gnau, S. Stevanovic, G. Jung, H.G. Rammensee, A. Meyerhans, Peptide motifs of HLA-A3, -A24, and -B7 molecules as determined by pool sequencing, Immunogenetics 44 (1994) 306–308.
[27] M. Ibe, Y. Ikeda-Moore, K. Miwa, Y. Kaneko, S. Yokota, M. Takiguchi, Role of strong anchor residues in the effective binding of 10-mer and 11-mer peptides to HLA-A*2402 molecules, Immunogenetics 44 (1996) 233–241.

Human Immunology 70 (2009) 170–174

Contents lists available at ScienceDirect

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

Brief communication

# Identification and characterization of 2 HIV-1 Gag immunodominant epitopes restricted by Asian HLA allele HLA-B*4801

Hayato Murakoshi [a], Mitsutaka Kitano [a], Tomohiro Akahoshi [a], Yuka Kawashima [a], Sachi Dohki [a], Shinichi Oka [b,c], Masafumi Takiguchi [a,*]

[a] Division of Viral Immunology, Center for AIDS Research, Kumamoto University, Kumamoto, Japan
[b] Division of Infectious Disease, Center for AIDS Research, Kumamoto University, Kumamoto, Japan
[c] AIDS Clinical Center, International Medical Center of Japan, Tokyo, Japan

ARTICLE INFO

*Article history:*
Received 22 October 2008
Accepted 18 December 2008
Available online 22 January 2009

*Keywords:*
HIV-1
Cytotoxic T lymphocytes
HLA-B*4801
Epitopes

ABSTRACT

HLA-B*4801 is frequently found in Asian populations but rarely in Caucasian or African populations. Although HLA-B*4801–restricted human immunodeficiency virus–1 (HIV-1) epitopes would be useful for acquired immune deficiency syndrome (AIDS) vaccine development in Asia, they have not been reported so far. In the present study, we sought to identify HLA-B*4801–restricted HIV-1 epitopes by using 17-mer overlapping peptides derived from HIV-1 Gag, Pol, and Nef as well as 8- to11-mer truncated peptides, and thereby identified two HLA-B*4801–restricted Gag epitopes. These epitope-specific CD8$^+$ T cells strongly responded to HIV-1–infected cells expressing HLA-B*4801, confirming that these Gag epitopes were endogenously presented by HLA-B*4801. These epitope-specific CD8$^+$ T cells were elicited in five of the seven tested chronically HIV-1–infected individuals with HLA-B*4801, suggesting them to be immunodominant epitopes. These epitopes will be useful for the studies of AIDS immunopathogenesis and the development of an HIV-1 vaccine in Asia.

© 2009 Society. Published by Elsevier Inc. All rights reserved.

## 1. Introduction

In human immunodeficiency virus type–1 (HIV-1) infection, cytotoxic T lymphocytes (CTLs) are elicited to control HIV-1 replication. HIV-1–specific CTLs strongly respond to HIV-1–infected cells in long-term non-progressors and slow progressors [1,2]. When monkeys whose CTLs are deleted *in vivo* are infected with simian immunodeficiency virus (SIV), they fail to control the virus [3,4]. Thus, HIV-1–specific or SIV-specific CTLs play an important role in the control of HIV-1 or SIV infection. On the other hand, HIV-1–specific CTLs cannot completely eradicate HIV-1 from infected individuals because HIV-1 escapes the host immune system. There are several proposed mechanisms that would allow HIV-1–infected cells to avoid being killed by HIV-1–specific CD8$^+$ T cells [5–10]. A mutation within CTL epitopes and the flanking region of them is one of the mechanisms for CTL escape [5,6]. Therefore, identification and characterization of HIV-1 CTL epitopes are very important for HIV-1 vaccine development.

To identify epitopes, we previously used the strategy of reverse immunogenetics based on the motif of HLA class I-binding peptides [11–13]. However, some epitopes may not be identified by this method in cases in which the epitopes are inconsistent with the motif of HLA class I–binding peptide [14,15]. The use of overlapping peptides is another useful method for identification of CTL epitopes [16–18]. This method has the advantage of identifying epitopes that are inconsistent with HLA class I–binding motifs. We recently identified HLA-B*5401–restricted HIV-1–specific CTL epitopes by using such overlapping peptides [19].

HLA-B48 is found in Asian populations but rarely in Caucasian or African populations [20–22]. The phenotypic frequencies of HLA-B*48 in Japanese, Chinese, Korean, Mogolian, and Thai populations are 6.4%, 3.8%, 8.0%, 9.4%, and 2.2%, respectively [20,23]. HLA-B*4801 is the only genotype of this allele in Japan [24]. Therefore, the identification of HLA-B*4801–restricted HIV-1 epitopes is important for studies of acquired immune deficiency syndrome (AIDS) immunopathogenesis and AIDS vaccine development in Japan and other Asian countries. So far, no HLA-B*4801–restricted HIV-1 epitopes have been reported.

In the present study, we used 17-mer overlapping peptides spanning Pol, Gag, and Nef to identify HLA-B*4801–restricted HIV-1 epitopes. We investigated the recognition of HIV-1–infected HLA-B*4801$^+$ cells by epitope-specific CTLs to clarify whether the identified epitopes were naturally occurring peptides. The induction of these epitope-specific CD8$^+$ T cells in chronically HIV-1–infected HLA-B*4801$^+$ individuals was further investigated to clarify the immunodominancy of these epitopes.

* Corresponding author.
E-mail address: masafumi@kumamoto-u.ac.jp (M. Takiguchi).

0198-8859/09/$32.00 - see front matter © 2009 Society. Published by Elsevier Inc. All rights reserved.
doi:10.1016/j.humimm.2008.12.011

## 2. Subjects and methods

### 2.1. Patients

Blood samples were obtained from HIV-1–seropositive Japanese individuals carrying HLA-B*4801. Informed consent was obtained from all subjects according to the Declaration of Helsinki.

### 2.2. Cells

Epstein-Barr virus (EBV)–transformed B-lymphoblastoid cell lines (B-LCL) were established by transforming B cells from peripheral blood mononuclear cells (PBMC) of laboratory volunteers and an HIV-1–seropositive individual as described previously [19]. C1R cells expressing HLA-B*4801 (C1R-B*4801) were generated by transfecting C1R cells with the HLA-B*4801 gene. For generation of .221 cells expressing CD4 (.221-CD4), .221 cells were transfected with the CD4 gene. Such cells expressing HLA-B*4801 (.221-CD4-B*4801) were subsequently generated by transfecting .221-CD4 cells with the HLA-B*4801 gene.

### 2.3. Synthetic peptides

We designed a panel of 281 overlapping peptides consisting of 17 amino acids in length and spanning Gag, Pol, and Nef of HIV-1 clade B sequences. Each 17-mer peptide was overlapped by at least 11 amino acids. The 281 peptides were synthesized by using an automated multiple peptide synthesizer. All peptides were purified by high-performance liquid chromatography (HPLC). The purity was examined by HPLC and mass spectrometry. Peptides with more than 90% purity were used in the present study.

### 2.4. Induction of peptide-specific T cells

The peptide-specific T cells were induced from PBMCs of HIV-1–seropositive individuals carrying HLA-B*4801. PBMCs were cultured with each peptide (1 $\mu$mol/l) in culture medium (RPMI-1640 containing 10% fetal calf serum (FCS) and 200 U/ml interleukin-2). Two weeks later, they were used in intracellular interferon (IFN)–$\gamma$ staining assays.

### 2.5. Infection of .221-CD4-B*4801 cells with HIV-1

HIV-1 clones, NL-432, were produced as described previously [25,26]. The .221-CD4- B*4801 or .221-CD4 cells were incubated with HIV-1 clones for 4 days at 37°C. The cells were then harvested to determine the percentage of HIV-1–infected cells. They were fixed with 4% paraformaldehyde and then permeabilized with PBS containing 10% FCS and 0.1% saponin (permeabilizing buffer). Thereafter the cells were stained with fluorescein isothiocyanate (FITC)–conjugated anti–HIV-1 p24 MAb KC-57 (Beckman Coulter, Miami, FL). The percentage of cells positive for intracellular HIV-1 p24 was determined by flow cytometry.

### 2.6. Intracellular IFN-$\gamma$ staining assay

After B-LCL, C1R-B*4801, or C1R cells had been incubated for 60 minutes with each peptide (1 $\mu$mol/l), they were washed twice with RPMI-1640 containing 10% FCS. These peptide-pulsed, HIV-1–infected .221-CD4- B*4801, or .221-CD4 cells ($2 \times 10^5$ cells per well) and the cultured PBMCs ($1 \times 10^5$ cells per well) were added to a 96-well round-bottomed plate. Subsequently, Brefeldin A (10 $\mu$g/ml) was added, and these cells were incubated for 6 hours. After the cells had been stained with anti-CD8 mAb (DAKO, Glostrup, Denmark), they were fixed with 4% paraformaldehyde and then permeabilized with the permeabilizing buffer. Thereafter the cells were stained with anti–IFN-$\gamma$ mAb (BD Bioscience, CA). The percentage of CD8$^+$ cells positive for intracellular IFN-$\gamma$ was analyzed by flow cytometry.

## 3. Results

### 3.1. Identification of optimal epitope peptides

We previously showed that 8 Gag, 8 Pol, and 4 Nef 17-mer overlapping peptides could elicit the peptide-specific CD8$^+$ T cells among PBMCs from a chronically HIV-1–infected individual KI-119 carrying HLA-A*0206/A*0206 and HLA-B*5401/B*4801 [19]. To determine HLA restriction of these T-cell responses, we investigated the ability of the peptide-stimulated bulk cultured cells to produce IFN-$\gamma$ in response to a panel of B-LCLs sharing one HLA class I allele with KI-119. The CD8$^+$ T cells in the bulk cultures produced IFN-$\gamma$ only after stimulation with autologous or HLA-B*4801–positive B-LCLs (U-37) pre-pulsed with five (Gag37-53, Gag43-59, Gag313-329, Gag361-377, and Gag421-437) of the 20 17-mer peptides. The Gag44-53 peptide-specific CD8$^+$ T cells could recognize the peptide-pulsed B*4801$^+$ cells but not HIV-1–infected ones (data not shown), although both Gag37-53 and Gag43-59 17-mer peptides include this 10-mer peptide. The Gag361-377–specific CD8$^+$ T cell response was restricted by HLA-Cw*0801 (data not shown). Therefore, these results suggest that only Gag313-329 and Gag421-437 peptides include HLA-B*4801–restricted epitopes (Fig. 1A).

To identify the optimal epitope recognized by CD8$^+$ T cells specific for these two peptides, we first designed 11-mer peptides that overlapped 9 amino acids in the sequence of the 17-mer peptides and then investigated whether the 17-mer peptide-induced CD8$^+$ T cells could recognize these 11-mer peptides. The Gag313-329 (VKNWMTETLLVQNANPD)–induced CD8$^+$ T cells could not recognize any of these 11-mer overlapping peptides (Fig. 1B). Since HLA-B*4801-binding peptides have 2 anchor residues, Lys or Gln at position 2 and Leu at the C-terminus [27], 2K and 9L or 10L in the Gag313-329 peptide would be expected to serve as an anchor for HLA-B*4801. Therefore, to identify the optimal peptide, we generated 2 truncated peptides, Gag313-322 (VKNWMTETLL) and Gag313-321 (VKNWMTETL), and investigated whether Gag313-329–induced CD8$^+$ T cells could recognize these peptides. They recognized Gag313-321 but not Gag313-322 peptide (Fig. 1C), suggesting that L at the C-terminus of Gag313-322 affected the binding to HLA-B*4801 or the recognition by the T cells. To investigate whether L at the C-terminus of Gag313-321 was necessary for the specific CTL response, we generated truncated Gag313-320 (VKNWMTET). Gag313-329–induced CD8$^+$ T cells did not recognize the Gag313-320 peptide (data not shown). These results confirmed the Gag313-321 peptide to be an optimal epitope.

On the other hand, Gag421-437 (HQMKDCTERQANFLGKI)–induced CD8$^+$ T cells recognized Gag427-437 (TERQANFLGKI) peptide but not the three other 11-mers (Fig. 1D). Because Gln is also an anchor at position 2 (P2) for HLA-B*4801 [27], 4Q in this 11-mer peptide would be expected to be the P2 anchor for HLA-B*4801. We therefore generated three truncated peptides, Gag428-437 (ERQANFLGKI), Gag429-437 (RQANFLGKI), and Gag429-436 (RQANFLGK). Gag421-437–induced CD8$^+$ T cells recognized both Gag428-437 and Gag429-437 but not Gag429-436 (Fig. 1E). When we assayed the responsivenesses of the specific CTLs to Gag429-437 or Gag428-437 at various concentrations, they recognized lower concentrations of Gag429-437 peptides but only higher concentrations of Gag428-437 (Fig. 1F). These findings indicate that Gag429-437 is the optimal epitope.

### 3.2. Recognition of HIV-1–infected cells by specific CTLs

To confirm the restriction molecule of these two epitope-specific CTLs, we generated CTL clones specific for each peptide. We investigated the ability of the CTL clones to produce IFN-$\gamma$ after stimulation of them with HLA-B*4801–positive and –negative C1R cells pre-pulsed with each peptide. The two clones specific for each peptide induced the IFN-$\gamma$–producing cells after stimulation with

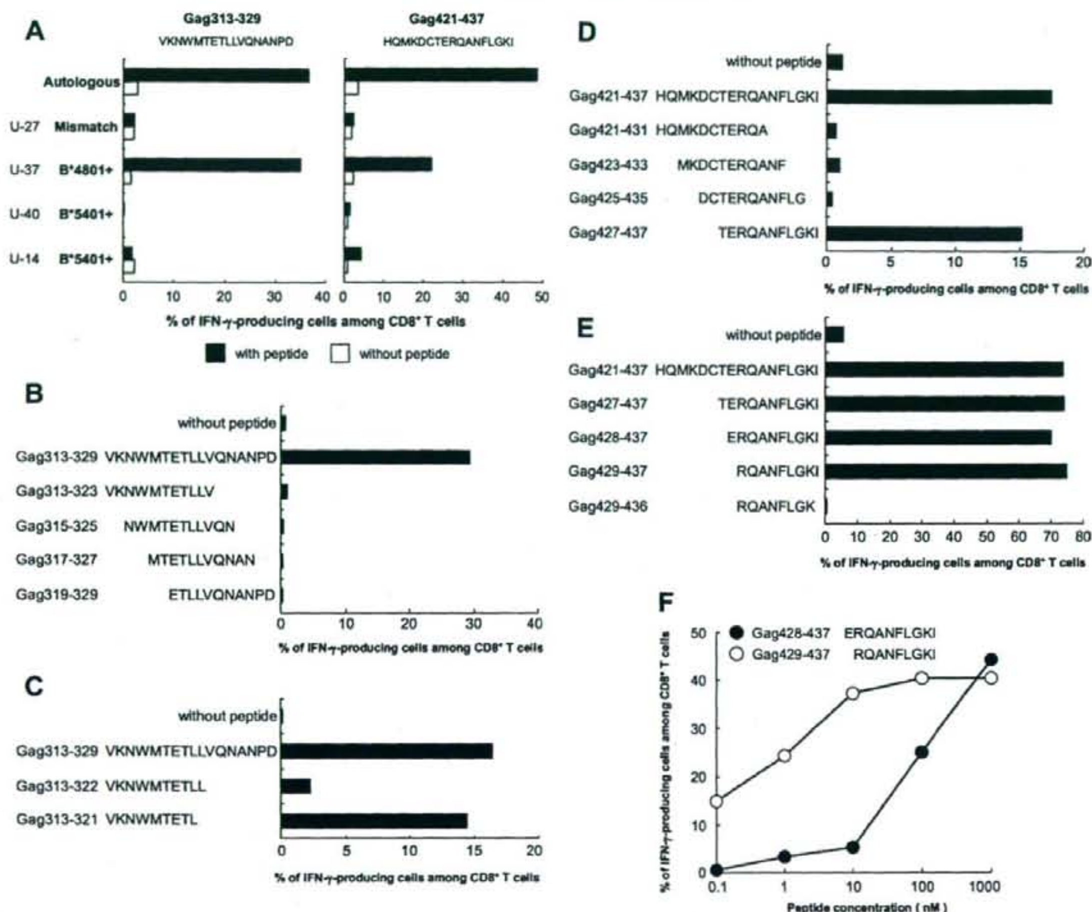



**Fig. 1.** Identification of optimal epitope peptides presented by HLA-B*4801. PBMCs from an HIV-1-seropositive individual KI-119 (A*0206/-, B*5401/B*4801) were stimulated with Gag313-329 or Gag421-437 peptide and then cultured for 2 weeks. The cultured cells were stimulated with Gag313-329 or Gag421-437-pulsed autologous B-LCL or B-LCLs sharing one HLA class I allele with KI-119 (A). Gag313-329-specific bulk CD8$^+$ T cells were stimulated with autologous B-LCL pre-pulsed with each overlapping 11-mer peptide (B) or 9- to 10-mer truncated peptide (C) at a concentration of 1000 nmol/l. Gag421-437-specific bulk CD8$^+$ T cells were stimulated with autologous B-LCL pre-pulsed with each overlapping 11-mer peptide (D) or 8- to 10-mer truncated peptide (E) at a concentration of 1000 nmol/l. The stimulator cells were pulsed with the 9- or 10-mer truncated peptide at concentrations from 0.1 to 1000 nmol/l (F). After the cultured cells were stimulated with each peptide-pulsed B-LCL for 6 hours, interferon (IFN)-γ-producing CD8$^+$ T cells specific for each peptide were measured by flow cytometry.

HLA-B*4801-positive C1R cells but not with HLA-B*4801-negative ones (Fig. 2A), confirming that the restriction molecule of Gag313-321-specific and Gag429-437-specific CTLs was indeed HLA-B*4801.

To clarify whether Gag313-321 and Gag429-437 epitopes are naturally occurring, we investigated the response of these peptide-specific CD8$^+$ T cells toward HLA-B*4801-expressing .221-CD4 cell lines infected with HIV-1 (NL-432) by using the intracellular IFN-γ staining assay. The .221 cells were infected with NL-432, and then incubated at 37°C for 4 days. The percentage of the HIV-1-infected cells was measured by using intracellular HIV-1 p24 staining (Fig. 2B). The Gag313-321-specific and Gag429-437-specific CTL clones responded to .221-CD4-B*4801 cells infected with HIV-1 but not to uninfected .221-CD4-B*4801 cells or to HLA-B*4801-negative.221-CD4 infected with HIV-1 (Fig. 2C). These results indicate that Gag313-321 and Gag429-437 peptides were naturally processed and presented by HLA-B*4801.

### *3.3. HLA-B*4801-restricted HIV-1-specific CD8$^+$ T cell responses in chronically HIV-1-infected individuals with HLA-B*4801*

To clarify whether these HLA-B*4801-restricted CD8$^+$ T cells were predominantly induced in chronically HIV-1-infected individuals bearing HLA-B*4801, we investigated the induction of the specific CD8$^+$ T cells in PBMCs from seven chronically HIV-1-infected HLA-B*4801-positive individuals by stimulating them with either of these two epitope peptides. The cells were cultured for 14 days, and the frequency of the specific CD8$^+$ T cells in the cultured cells was determined by using intracellular IFN-γ production assay. Both Gag313-321- and Gag429-437-specific CD8$^+$ T cells were found in five of the seven HIV-1-infected individuals (Table 1), indicating that these two Gag epitopes were immunodominant epitopes.

## 4. Discussion

A previous study analyzing the pool sequences of endogenously bound peptides showed that primary anchor residues for HLA-

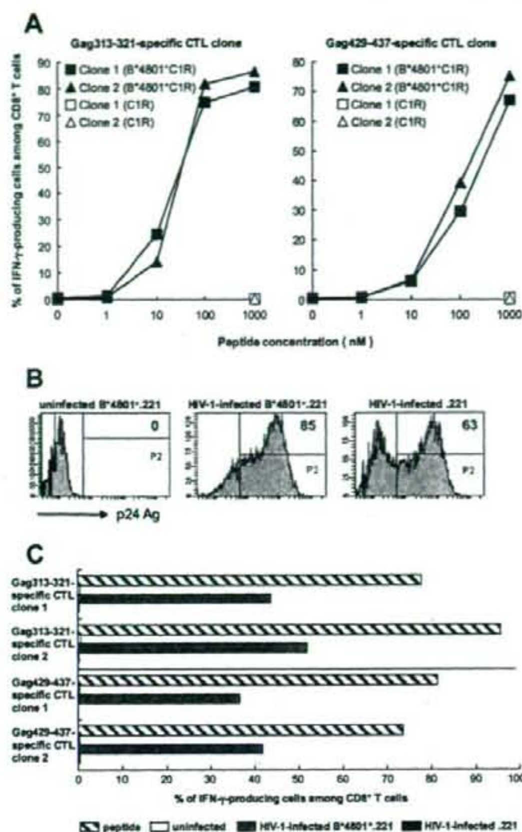



Fig. 2. HLA-B*4801-restricted recognition of HIV-1-infected cells by the Gag313-321 or Gag429-437-specific CTLs. (A) Confirmation of HLA-B*4801 restriction in Gag313-321- or Gag429-437-specific CTLs. The responsiveness of each epitope peptide-specific CTL clones to B*4801+C1R cell lines pre-pulsed with the corresponding peptide at concentrations from 1 to 1000 nmol/l was determined by performing the intracellular IFN-γ staining assay. (B) The 221-CD4 cell lines were infected with HIV-1 (NL-432) in incubation at 37°C for 4 days. The HIV-1-infected cells were determined by using intracellular p24 staining with anti-p24 MAb. The percentage of HIV-1-infected cells is shown in each figure. (C) The activities of each peptide-specific CTLs toward B*4801+.221-CD4 cell lines infected with HIV-1 or those pre-pulsed with the corresponding peptide (1 μmol/l) were measured by using the intracellular IFN-γ staining assay.

B*4801 were Lys or Gln at P2 and Leu at the C-terminus [27]. In the present study, we identified 2 HLA-B*4801-restricted HIV-1 Gag-specific CTL epitopes carrying Lys or Gln at P2 and Leu or Ile at the C-terminus. Thus these results confirm Lys and Gln as P2 anchor as well as Leu as the C-terminal anchor for HLA-B*4801 and identified an additional C-terminal anchor Ile. Leu, at the C-terminus of Gag313-322 (VKNWMTETLL), affected the recognition by specific T cells, suggesting that this additional Leu may reduce the affinity of the peptide for HLA-B*4801. However, it remains unknown why the 17-mer peptide Gag313-329 can be recognized by the T cells.

HLA-B*1302 and HLA-B*3902 have the same peptide motif as HLA-B*4801 [28,29]. In fact, five HLA-B*1302-restricted HIV-1 epitopes were found to have the same motif [28], although no HLA-B*3902-restricted HIV-1 epitopes have been identified. Interestingly, the Gag429-437 epitope is presented by both HLA-B*1302 and HLA-B*4801 [28]. As a previous study showed that the viral load of HLA-B*13-positive subjects is significantly lower than that of B*13-negative subjects [28], we speculated that HLA-B*4801 is associated with successful immune control.

The two Gag epitope-specific CD8+ T cells were detected in five of seven chronically HIV-1-infected individuals with HLA-B*4801. These findings indicate that these epitopes are recognized as immunodominant ones in chronically HIV-1-infected individuals with HLA-B*4801. The sequences of Gag313-321 (VKNWMTETL) and Gag429-437 (RQANFLGKI) were found in 117 (93%) and 107 (85%) of 126 HIV-1 clade B isolates, respectively, and in 95 (17%) and 457 (83%) of 554 HIV-1 clade C ones, respectively, in reported HIV-1 sequences (Los Alamos National Laboratory HIV Molecular Immunology Database), indicating that both sequences are relatively conserved in clade B but only Gag429-437 in clade C. CTLs specific for these epitopes were frequently induced in HIV-1-infected individuals with HLA-B*4801. In addition, Gag313-321 and Gag429-437-specific CTL clones strongly responded to HIV-1-infected .221-CD4 expressing HLA-B*4801, suggesting that these specific CTLs may effectively kill HIV-1-infected cells. Thus, our findings suggest that these HLA-B*4801 epitopes may be useful in developing an HIV-1 vaccine to effectively induce specific CTLs.

A recent study reported that Gag-specific CTLs play a critical role in the control of HIV-1 replication [30]. These results suggest that HLA-B*4801-restricted Gag-specific CTLs may contribute to control of HIV-1 replication. A previous study reported that the affinity of binding of HLA-B*4801 to the CD8 co-receptor is weaker than that of other HLA class I molecules because of the mutation to threonine at position 245 in the α3 domain of HLA class I [27]. However we showed that the HIV-1 epitope-specific CTL clones strongly recognized HIV-1-infected .221-CD4 cells expressing HLA-B*4801, suggesting that these CTLs can recognize HIV-1-infected cells *in vivo*. Further studies are required to clarify the role of these CTLs *in vivo*.

HLA-B*4801 is found only in Asia. Therefore, this allele had not been analyzed for its effect on the progression to AIDS in Caucasian or African cohorts. In addition, there is no cohort study of HLA-B*4801 on an Asian cohort. Further analyses are required to clarify the effect of this allele in an Asian cohort.

We previously identified five HLA-B*5401-restricted CTL epitopes from Nef and Pol by using PBMCs from the same chronically HIV-1-infected individual, KI-119 [19]. In contrast, two HLA-B*4801-restricted CTL epitopes were identified from Gag by using PBMCs from the same individual. If Gag-specific CTLs are the most effective T cells to control HIV-1 replication, HLA-B*4801 and HLA-B*5401 may be associated with slow and rapid progression to AIDS, respectively. Both alleles are common ones in Asia, but the association of these alleles with progression to AIDS has not been analyzed.

In summary, we identified two novel HLA-B*4801-restricted HIV-1 Gag-specific CTL epitopes by using 17-mer overlapping peptides in this study. These epitopes were relatively conserved, and the specific T cells were predominantly induced in HIV-1-infected

**Table 1**
Induction of epitope-specific CD8+ T cells among PBMCs from HLA-B*4801+ HIV-1-infected individuals.

| Patients[a] | Viral load[b] | CD4[c] | CD8[c] | % of IFN-γ-producing cells in CD8+ T cells | |
|---|---|---|---|---|---|
| | | | | Gag313-321 | Gag429-437 |
| KI-119 | $3.0 \times 10^3$ | 536 | 1268 | 26.0 | 50.5 |
| KI-034 | $9.6 \times 10^4$ | 235 | 1239 | 56.1 | 65.8 |
| KI-092 | $2.2 \times 10^2$ | 971 | 1492 | 23.1 | 5.2 |
| KI-179 | $1.4 \times 10^3$ | 202 | 668 | 0.0 | 0.0 |
| KI-067 | $8.9 \times 10^4$ | 234 | 1198 | 4.9 | 50.2 |
| KI-134 | $1.1 \times 10^5$ | 335 | 1155 | 32.3 | 0.0 |
| KI-169 | $1.6 \times 10^5$ | 63 | 930 | 0.0 | 79.1 |

[a] HIV-1-infected individuals with HLA-B*4801.
[b] Copies/ml.
[c] Cells/μl.

individuals carrying HLA-B*4801. Thus these findings suggest that these epitopes are useful for the development of an HIV-1 vaccine and for analysis of the immunopathogenesis of AIDS.

## Acknowledgments

The authors thank Sachiko Sakai for secretarial assistance. This research was supported by a grant-in-aid for scientific research from the Ministry of Health, Labor, and Welfare of the government of Japan and by a grant from the Japan Health Science Foundation. H.M. is a JAIDS Foundation Research Fellow.

## References

[1] Barker E, Mackewicz CE, Reyes-Teran G, Sato A, Stranford SA, Fujimura SH, et al. Virological and immunological features of long-term human immunodeficiency virus-infected individuals who have remained asymptomatic compared with those who have progressed to acquired immunodeficiency syndrome. Blood 1998;92:3105–14.
[2] Klein MR, van Baalen CA, Holwerda AM, Kerkhof Garde SR, Bende RJ, Keet IP, et al. Kinetics of Gag-specific cytotoxic T lymphocyte responses during the clinical course of HIV-1 infection: A longitudinal analysis of rapid progressors and long-term asymptomatics. J Exp Med 1995;181:1365–72.
[3] McMichael AJ, Rowland-Jones SL. Cellular immune responses to HIV. Nature 2001;410:980–7.
[4] Schmitz JE, Kuroda MJ, Santra S, Sasseville VG, Simon MA, Lifton MA, et al. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. Science 1999;283:857–60.
[5] Borrow P, Lewicki H, Wei X, Horwitz MS, Peffer N, Meyers H, et al. Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. Nat Med 1997;3:205–11.
[6] Goulder PJ, Philips RE, Colbert RA, McAdam S, Ogg G, Nowak MA, et al. Late escape from an immunodominant cytotoxic T lymphocyte response associated with progression to AIDS. Nat Med 1997;3:212–7.
[7] Collins KL, Chen BK, Kalams SA, Walker BD, Blatimore D. HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes. Nature 1998;391:397–401.
[8] Xu XN, Laffert B, Screaton GR, Kraft M, Wolf D, Kolanus W, et al. Induction of Fas ligand expression by HIV involves the interaction of Nef with the T cell receptor zeta chain. J Exp Med 1999;189:1489–96.
[9] Mueller YM, De Rosa SC, Hutton JA, Witek J, Roederer M, Altman JD, et al. Increased CD95/Fas-induced apoptosis of HIV-specific CD8(+) T cells. Immunity 2001;15:871–82.
[10] Champagne P, Ogg GS, King AS, Knabenhans C, Ellefsen K, Nobile M, et al. Skewed maturation of memory HIV-specific CD8 T lymphocytes. Nature 2001; 410:106–11.
[11] Ikeda-Moore Y, Tomiyama H, Miwa K, Oka S, Iwamoto A, Kaneko Y, et al. Identification and characterization of multiple HLA-A24-restricted HIV-1 CTL epitopes: Strong epitopes are derived from V regions of HIV-1. J Immunol 1997;159:6242–52.
[12] Kawashima Y, Satoh M, Oka S, Takiguchi M. Identification and characterization of HIV-1 epitopes presented by HLA-A*2603: Comparison between HIV-1 epitopes presented by A*2601 and A*2603. Hum Immunol 2005;66:1155–66.
[13] Tomiyama H, Miwa K, Shiga H, Ikeda-Moore Y, Oka S, Iwamoto A, et al. Evidence of presentation of multiple HIV-1 cytotoxic T lymphocyte epitopes by HLA-B*3501 molecules that are associated with the accelerated progression of AIDS. J Immunol 1997;158:5026–34.
[14] Dong T, Boyd D, Rosenberg W, Alp N, Takiguchi M, McMichael AJ, et al. An HLA-B35-restricted epitope modified at an anchor residue results in an antagonist peptide. Eur J Immunol 1996;26:335–39.
[15] Goulder PJ, Reid SW, Price DA, O'Callaghan CA, McMichael AJ, Phillips RE, et al. Combined structural and immunological refinement of HIV-1 HLA-B8-restricted cytotoxic T lymphocyte epitopes. Eur J Immunol 1997;27:1515–21.
[16] Addo MM, Altfeld M, Rosenberg ES, Eldridge RL, Philips MN, Habeeb K, et al. The HIV-1 regulatory proteins Tat and Rev are frequently targeted by cytotoxic T lymphocytes derived from HIV-1-infected individuals. Proc Natl Acad Sci U S A 2001;98:1781–86.
[17] Goulder PJ, Addo MM, Altfeld MA, Rosenberg ES, Tang Y, Govender U, et al. Rapid definition of five novel HLA-A*3002-restricted human immunodeficiency virus-specific cytotoxic T-lymphocyte epitopes by ELISPOT and intracellular cytokine staining assays. J Virol 2001;75:1339–47.
[18] Draenert R, Altfeld M, Brander C, Basgoz N, Corcoran C, Wurcel AG, et al. Comparison of overlapping peptide sets for detection of antiviral CD8 and CD4 T cell responses. J Immunol Methods 2003;275:19–29.
[19] Kitano M, Kobayashi N, Kawashima Y, Akahoshi T, Nokihara K, Oka S, et al. Identification and characterization of HLA-B*5401-restricted HIV-1-Nef and Pol-specific CTL epitopes. Microbes Infect 2008;10:764–72.
[20] Imanishi T, Akaza T, Kimura A, Tokunaga K, Gojobori T. Allele and haplotype frequencies for HLA and complement loci in various ethnic groups. In: Tsuji K, Aizawa M, Sasazuki T, editors. HLA 1991: Proceedings of the Eleventh International Histocompatibility Workshop and Conference. Oxford, Oxford University Press, 1992:1065–220.
[21] Cao K, Hollenbach J, Shi X, Shi W, Chopek M, Fernandez-Vina MA. Analysis of the frequencies of HLA-A, -B, and C alleles and haplotypes in the five major ethnic groups of the United States reveals high levels of diversity in these loci and contrasting distribution patterns in these populations. Hum Immunol 2001;62:1009–30.
[22] Cao K, Hollenbach JA, Shi XJ, Shi WX, Chopek M, Fernandez-Vina MA. HLA-A, -B and -Cw allele frequencies in a Caucasian population from the USA. Hum Immunol 2004;65:1196–8.
[23] Shaw CK, Chen LL, Lee A, Lee TD. Distribution of HLA gene and haplotype frequencies in Taiwan: A comparative study among Min-nan, Hakka, Aborigines and Mainland Chinese. Tissue Antigens 1999;53:51–64.
[24] Itoh Y, Mizuki N, Shimada T, Azuma F, Itakura M, Kashiwase K, et al. High-throughput DNA typing of HLA-A, -B, -C, and -DRB1 loci by a PCR–SSOP–Luminex method in the Japanese population. Immunogenetics 2005; 57:717–29.
[25] Akari H, Arold S, Fukumori T, Okazaki T, Strebel K, Adachi A. Nef-induced major histocompatibility complex class I down-regulation is functionally dissociated from its virion incorporation, enhancement of viral infectivity, and CD4 down-regulation. J Virol 2000;74:2907–12.
[26] Tomiyama H, Akari H, Adachi A, Takiguchi M. Different effects of Nef-mediated HLA class I down-regulation on human immunodeficiency virus type 1-specific CD8+ T-cell cytolytic activity and cytokine production. J Virol 2002;76:7535–43.
[27] Martinez-Naves E, Barber LD, Madrigal JA, Vullo CM, Clayberger C, Lyu SC, et al. Interactions of HLA-B*4801 with peptide and CD8. Tissue Antigens 1997;50: 258–64.
[28] Honeyborne I, Prendergast A, Pereyra F, Leslie A, Crawford H, Payne R, et al. Control of human immunodeficiency virus type 1 is associated with HLA-B*13 and targeting of multiple Gag-specific CD8+ T-cell epitopes. J Virol 2007;81: 3667–72.
[29] Falk K, Rötzschke O, Takiguchi M, Gnau V, Stevanović S, Jung G, et al. Peptide motifs of HLA-B38 and B39 molecules. Immunogenetics 1995;41:162–4.
[30] Kiepiela P, Ngumbela K, Thobakgale C, Ramduth D, Honeyborne I, Moodley E, et al. CD8+ T-cell responses to different HIV proteins have discordant associations with viral load. Nat Med 2007;13:46–53.

ELSEVIER

INSTITUT PASTEUR

Microbes and Infection 10 (2008) 764–772

www.elsevier.com/locate/micinf

Original article

# Identification and characterization of HLA-B*5401-restricted HIV-1-Nef and Pol-specific CTL epitopes

Mitsutaka Kitano [a,1], Naoki Kobayashi [a,1], Yuka Kawashima [a], Tomohiro Akahoshi [a], Kiyoshi Nokihara [d], Shinichi Oka [b,c], Masafumi Takighuchi [a,*]

[a] *Division of Viral Immunology, Center for AIDS Research, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811, Japan*
[b] *Division of Infectious Disease, Center for AIDS Research, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811, Japan*
[c] *AIDS Clinical Center, International Medical Center of Japan, 1-21-1, Toyama, Shinjuku, Tokyo162-8655, Japan*
[d] *HiPep Laboratories, Nakatsukasacho 486-46, Kamigyo-ku, Kyoto 602-8158, Japan*

Received 14 March 2008; accepted 11 April 2008
Available online 22 April 2008

**Abstract**

The identification of HIV-1 cytotoxic T lymphocyte (CTL) epitopes presented by each HLA allele and the characterization of their CTL responses are important for the study of pathogenesis of AIDS and the development of a vaccine against it. In the present study, we focused on identification and characterization of HIV-1 epitopes presented by HLA-B*5401, which is frequently found in the Asian population, because these epitopes have not yet been reported. We identified these epitopes by using 17-mer overlapping peptides derived from HIV-1 Gag, Pol, and Nef. Seven of these 17-mer peptides induced HLA-B*5401-restricted $CD8^+$ T cell responses. Only five HLA-B*5401-restricted Pol- or Nef-specific $CD8^+$ T cell responses were detected in the analysis using 11-mer overlapping peptides. Three Pol and two Nef optimal peptides were identified by further analysis using truncated peptides. These epitope-specific CTLs effectively killed HLA-B*5401-expressing target cells infected with HIV-1 recombinant vaccinia virus, indicating that these peptides were naturally processed by HLA-B*5401 in HIV-1-infected cells. These epitope-specific $CD8^+$ T cells were elicited in more than 25% of chronically HIV-1-infected individuals carrying HLA-B*5401. Therefore, these epitopes should prove useful for studying the pathogenesis of AIDS in Asia and developing a vaccine against HIV-1.
© 2008 Elsevier Masson SAS. All rights reserved.

*Keywords:* Cytotoxic T lymphocytes; Epitopes; HIV-1

## 1. Introduction

Human immunodeficiency virus type 1 (HIV-1)-specific cytotoxic T lymphocytes (CTLs) play an important role in HIV-1 infections [1–4]. Previous studies demonstrated that HIV-1-specific CTL can inhibit viral replication in vitro [5,6] and that depletion of $CD8^+$ T cells by an anti-CD8 mAb results in failure of the clearance of the virus in rhesus macaques infected with chimeric simian/human immunodeficiency virus [7]. These studies suggest that the $CD8^+$ CTLs contribute to viral clearance and disease progression in HIV-1-infected individuals. Although high HIV-1-specific CTL activity is detected in the early phase of infection in HIV-1-infected individuals, CTL escape mutants are selected by these CTLs [8,9]. The patients in which these HIV-1 escape mutants appear may progress to AIDS. The CTL escape mutants are selected by strong immunological pressure via HIV-1-specific CTLs [10], and the disease progression to AIDS is associated with HLA class I alleles [11,12]. Therefore, the characterization of HIV-1 epitope-specific CTLs is important for understanding the pathogenesis of HIV and developing an AIDS vaccine. However, the number of identified HIV-1 CTL epitopes is limited and CTLs specific for a restricted number of epitopes have been investigated in detail.

*Abbreviations:* CTL, cytotoxic T lymphocytes; HLA, human leukocyte antigens.

* Corresponding author. Tel.: +81 96 373 6529; fax: +81 96 373 6532.
*E-mail address:* masafumi@kumamoto-u.ac.jp (M. Takighuchi).

[1] Equally contributed to this study.

1286-4579/$ - see front matter © 2008 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.micinf.2008.04.006

To identify HIV-1 epitopes, we previously used the strategy of reverse immunogenetics: (i) identification of the motif of HLA class I-binding peptides, (ii) selection of sequences matched to the motif of HLA class I-binding peptides from HIV proteins and synthesis of peptides, (iii) identification of HLA class I-binding HIV-1 peptides by a peptide-binding assay such as the HLA stabilization assay, (iv) induction of CTL by HLA class I-binding peptides in PBLs from HIV-1-infected individuals. We identified many HIV-1 CTL epitopes by using reverse immunogenetics and showed that it is a useful method to identify HLA-class-I-restricted HIV-1 epitopes [13–19]. However, some CTL epitopes may not be identified by this method, since some reported epitopes do not match the motif [20,21]. Identification of CTL epitopes by using overlapping peptides is another useful method [22–26]. This method is advantageous to identify epitopes that are inconsistent with HLA class I-binding motifs.

HLA-B54 is one of the serotypes in HLA-B22, which is a common allele in Asia. HLA-B*5401 is the only genotype of HLA-B54 in the Japanese population and is found in approximately 13% of the Japanese [27]. Therefore, the identification of HLA-B*5401-restricted HIV-1 epitopes is important in studies of immunopathogenesis and for vaccine development in Asia. So far, no HLA-B*5401 HIV-1 epitopes have been reported.

In the present study, we utilized 17-mer overlapping peptides to identify HLA-B*5401-restricted HIV-1 epitopes because those that are inconsistent with HLA-B*5401 motif can be identified by the method using overlapping peptide. Only Pol, Gag, and Nef were focused upon in the present study because these major proteins provide many CTL epitopes, and they are considered as vaccine targets. $CD8^+$ T cells specific for HLA-B*5401-restricted HIV-1 epitopes were further investigated in chronically HIV-1-infected individuals to clarify the immunodominancy of these epitopes.

## 2. Materials and methods

### 2.1. Patients

Blood samples were obtained from HIV-1-seropositive individuals carrying HLA-B*5401. The study was approved by the ethics committees of Kumamoto University and the International Medical Center of Japan. Informed consent was obtained from all subjects according to the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMCs) were separated from whole blood.

### 2.2. Cells

The EBV-transformed B-lymphoblastoid cell lines (B-LCL) were established by transforming B cells from PBMC of laboratory volunteers and an HIV-1-seropositive individual. The PBMC were plated at $3-4 \times 10^6$ cells per well in flat-bottomed 24-well plates in RPMI-1640 medium supplemented with 2 μg/ml cyclosporin A and the supernatant derived from B95-8 cultures. C1R cells expressing HLA-B*5401 (C1R-B*5401) were generated by transfecting C1R cells with the HLA-B*5401 gene. The C1R-B*5401 cells were maintained in the RPMI-1640 medium containing 10% FCS and 0.2 mg/ml of neomycin.

### 2.3. Synthetic peptides

We designed a panel of 281 overlapping peptides consisting of 17 amino acids in length and spanning Gag, Pol, and Nef of HIV-1 clade B sequences. Each 17-mer peptide was overlapped by at least 11 amino acids. The 281 peptides were synthesized by utilizing an automated multiple peptide synthesizer. Several peptides having difficult sequences were manually synthesized by monitoring of peptide-chain elongation. All peptides were purified by high-performance liquid chromatography (HPLC). The purity was examined by HPLC and mass spectrometry. Peptides with more than 90% purity were used in the present study.

### 2.4. Induction of peptide-specific T cells

The peptide-specific T cells were induced from PBMCs of HIV-1-seropositive individuals carrying HLA-B*5401. PBMCs were cultured with each peptide cocktail including eight kinds of 1 μM 17-mer peptides (totally 8 μM) or each 17-mer single peptide (1 μM) in culture medium (RPMI-1640 containing 10% FCS and 200 U/ml IL-2). Two weeks later, they were used in intracellular IFN-γ staining assays or CTL assays.

### 2.5. Intracellular IFN-γ staining assay

After B-LCL, C1R-B*5401 or C1R cells had been incubated for 60 min with each peptide cocktail containing eight kinds of 1 μM 17-mer peptide (totally 8 μM) or each 17-mer single peptide (1 μM), they were washed twice with RPMI-1640 containing 10% FCS. These peptide-pulsed autologous B-LCL ($2 \times 10^5$ cells per well) and peptide-stimulated PBMCs cells ($1 \times 10^5$ cells per well) were added to a 96-well round-bottomed plate, which was incubated for 2 h. Subsequently, Brefeldin A (10 μg/ml) was added, and incubation was continued for an additional 4 h. After the cells had been stained with anti-CD8 mAb (DAKO Corporation, Flostrup, Denmark), they were fixed with 4% paraformaldehyde at 4 °C for 20 min and then permeabilized with PBS containing 20% newborn calf serum (Summit Biotechnology, Greely, Co.) and 0.1% saponin (permeabilizing buffer) at 4 °C for 10 min. Thereafter, the cells were resuspended in the permeabilizing buffer and then stained with anti-IFN-γ mAb (BD Bioscience, CA, USA). The cells were finally resuspended in PBS containing 2% paraformaldehyde and then the percentage of $CD8^+$ cells positive for intracellular IFN-γ was analyzed by FACSCalibur (BD Bioscience).

### 2.6. CTL assay

The cytotoxicity of HIV-1-specific CTL was measured by the standard $^{51}Cr$ release assay. The HLA-B*5401$^+$ B-LCL

infected with recombinant vaccinia virus encoding *gag/pol*, or *nef* gene of HIV-1 SF2 or WT vaccinia virus were used as target cells. Target cells were incubated for 60 min with $Na_2^{51}$-$CrO_4$ (150 μCi) in saline, and washed three times with RPMI-1640 medium containing 10% NCS. The labeled target cells were added to each well of a 96-well round-bottomed plate with peptides and they were incubated for 1 h at 37 °C. Then, HIV-1-specific bulk CTL or clones as effector cells were added to the target cells and the mixtures were incubated for 4 h at 37 °C. The supernatants were collected and analyzed with a gamma counter. The spontaneous $^{51}Cr$ release (cpm spn) was determined by measuring the cpm in the supernatant in the wells containing only target cells. The maximum release (cpm max) was determined by measuring the release of $^{51}Cr$ from the target cells in the presence of 2.5% TritonX-100. Percent specific lysis was calculated as follows: percentage specific lysis = 100 × (cpm exp − cpm spn)/(cpm max − cpm spn), where cpm exp is the cpm in the supernatant from wells containing both target and effector cells. In another experiment, labeled C1R-B*5401 cells were pulsed with various concentrations ($0.1-10^3$ nM) of the corresponding peptide.

## 3. Results

### 3.1. Induction of HIV-1 peptide-specific CD8$^+$ T cells by using 17-mer overlapping peptide cocktails from PBMCs of chronically HIV-1-infected HLA-B*5401$^+$ individuals

PBMCs from KI-119, a chronically HIV-1-infected HLA-B*5401$^+$ individual, were stimulated in vitro for 12–14 days with Gag, Pol, and Nef peptide cocktails including eight 17-mer overlapping peptides. IFN-γ production by each bulk culture in response to autologous B-LCL pre-pulsed with the corresponding peptide cocktail was assessed by staining for intracellular IFN-γ. Bulk cultures from KI-119 responded to six Gag, seven Pol, and three Nef cocktails (data not shown). To determine which peptides in the each cocktail induced the specific CD8$^+$ T cell, we stimulated the bulk cultures with autologous B-LCL pre-pulsed with single 17-mer peptides found in the cocktails. Twelve Gag, nine Pol, and four Nef peptides induced CD8$^+$ T cells to produce IFN-γ (data not shown).

### 3.2. Candidates of HLA-B*5401-restricted 17-mer peptides

HLA restriction of the T cell response specific for these 17-mer peptides was subsequently determined by using the bulk cultured cells having a specific ability to recognize 17-mer peptide as well as a panel of B-LCLs sharing one HLA class allele with KI-119 carrying HLA-A*0206/A*0206 and HLA-B*5401/B*4801. Bulk cultured cells were incubated with either autologous B-LCL, HLA-A,-B-mismatched B-LCL or B-LCL sharing only one HLA class I allele with the donor. A representative result of flow cytometric analysis is shown in Fig. 1A. Pol300–316 peptide-pulsed autologous B-LCL or B-LCL expressing HLA-B*5401 induced IFN-γ production from CD8$^+$ T cells in the bulk culture cells having a specific ability for the Pol300–316 peptide. No significant response was found by stimulation with Pol300–316 peptide-pulsed HLA-B*5401-negative B-LCL. These results suggest that these peptide-specific CD8$^+$ T cells were restricted by HLA-B*5401. Similar results were obtained with bulk culture cells having a specific ability to recognize Pol151–167, Pol786–802, Pol792–808, Nef119–135, Nef125–141 or Nef149–165 peptide, suggesting that CD8$^+$ T cells specific for these peptides were also restricted by HLA-B*5401 (Fig. 1B). For some peptides, we could not test the entire panel at the same time due to sample limitation, while other 17-mer peptides were restricted by HLA-B*4801 or HLA-A*0206 (data not shown). Thus, these seven 17-mer peptides may include candidates of HLA-B*5401-restricted HIV-1 epitopes.

### 3.3. Identification of optimal epitope peptides

To identify the optimal epitope recognized by CD8$^+$ T cells specific for these peptides, we designed 11-mer peptides which were overlapping nine amino acids each in the sequence of the 17-mer peptide. IFN-γ production of each bulk culture in response to autologous B-LCL pre-pulsed with a 1 μM concentration of the corresponding 11-mer or 17-mer peptides was assessed by intracellular IFN-γ staining. The Pol151–167 (CTLNFPISPIETVPVKL)-induced CD8$^+$ T cells recognized LNFPISPIETV and FPISPIETVPV but not ISPIETVPVKL (Fig. 2). Since Pro is an anchor for HLA-B*5401 [28], 6P in this 17-mer is the anchor for HLA-B*5401 rather than 9P or 14P. Thus, we expected that the epitope would be included in FPISPIETVPV (Pol155–165). To identify the optimal peptide, we generated three truncated peptides (FP10: FPISPIETVP, FV9: FPISPIETV, and FT8: FPISPIET). Pol151–167 (CTLNFPISPIETVPVKL)-induced CD8$^+$ T cells recognized all of them (Fig. 3A), but at lower concentrations of the peptide they recognized FV9 and FP10 more than FT8 (Fig. 3B). The difference in T cell recognition between FV9 and FP10 is not significant though they seem to recognize FV9 more than FP10 at a lower concentration. These results suggest that a shorter peptide, FV9 (Pol 155–163), might be the optimal epitope rather than FP10, but it still remains possible that both peptides are presented and recognized by T cells.

Pol300–316 (YNVLPQGWKGSPAIFQS)-induced CD8$^+$ T cells recognized VLPQGWKGSPA but not the other three 11-mer peptides (Fig. 2), indicating that 5P in this 17-mer peptide is the anchor for HLA-B*5401 rather than 12P. We therefore generated three truncated peptides (LA10: LPQGWKGSPA, LP9: LPQGWKGSP, and LS8: LPQGWKGS) from Pol300–316. Pol300–316 (YNVLPQGWKGSPAIFQS)-induced CD8$^+$ T cells recognized both LA10 and LS8 (Fig. 3A), but they failed to recognize LS8 at lower concentrations of the peptide (Fig. 3C). These findings indicate that LA10 (Pol303–312) is the optimal epitope.

Bulk cultured cells stimulated with Pol786–802 or Pol792–808 responded to the same 11-mer peptide, HVASGYIEAEV (Fig. 2), suggesting that both bulk cultured

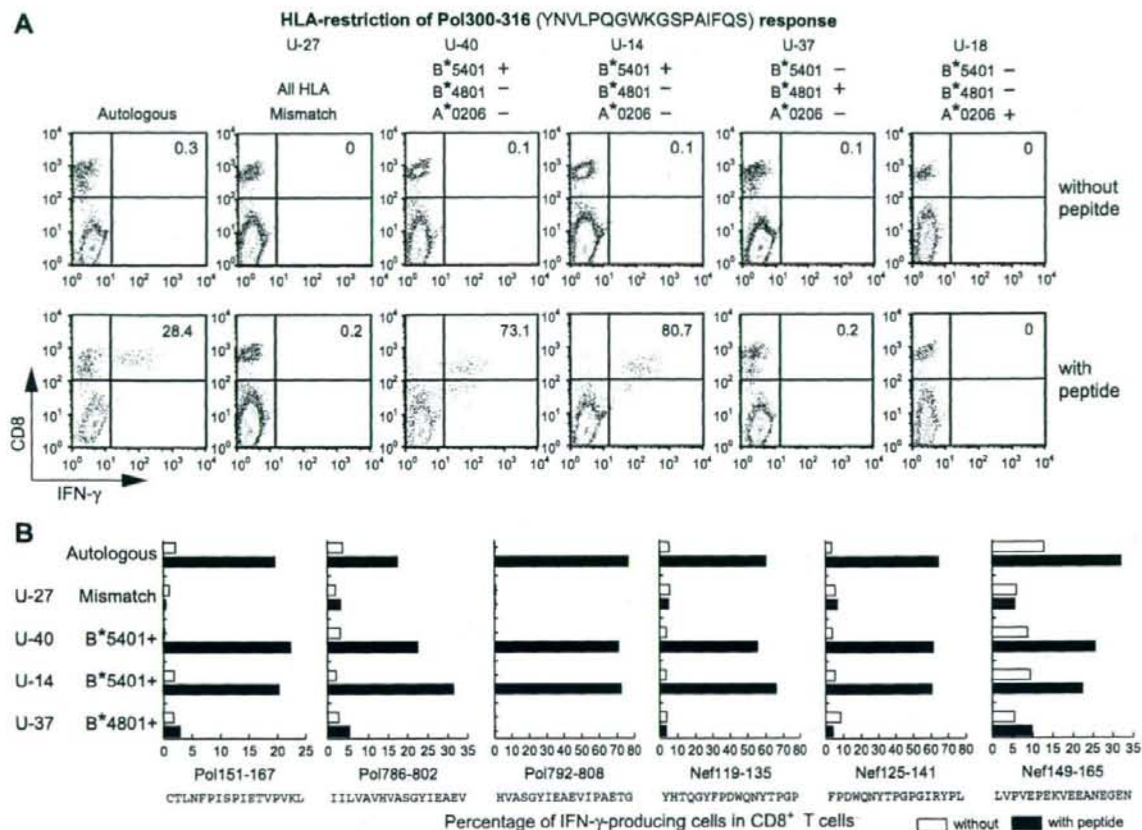



Fig. 1. Identification of HLA-B*5401-restricted HIV-1 CTL epitope candidates by using a panel of B-LCL pulsed with 17-mer peptides. A. PBMC from an HIV-1-seropositive individual KI-119 (A*0206/-, B*5401/B*4801) were stimulated with Pol300–316 peptide and then cultured for 2 weeks. The cultured cells were stimulated with Pol300–316 peptide-pulsed autologous B-LCL or allo B-LCL sharing only one HLA class I allele with the donor. Pol 300–316-specific $CD8^+$ T cells were detected by using the intracellular IFN-γ staining assay. The percentage of IFN-γ-producing cells among $CD8^+$ T cells are shown in each plot. B. The same assays shown in "A" were performed by using other 17-mer HIV-1 Pol and Nef peptides (Pol151–167, Pol786–802, Pol792–808, Nef119–135, Nef125–141 and Nef149–165). The percentage of IFN-γ-producing cells among $CD8^+$ T cells are shown in each figure.

$CD8^+$ T cells recognize this peptide (Pol790–800). Pol790–800 did not include the B*5401 anchor residue Pro. Since Ala is an amino acid with characteristics similar to those of Pro, we synthesized three peptides carrying A at position 2 (VV10: VASGYIEAEV, VE9: VASGYIEAE, and VA8: VASGYIEA). Pol790–800-specific bulk $CD8^+$T cells failed to recognize these three peptides (Fig. 3A). We therefore synthesized three truncated peptides (HA9: HVASGYIEA, AE10: AVHVASGYIE, and VA10: VHVASGYIEA) and tested whether Pol790–800-specific bulk $CD8^+$ T cells could recognize them. The result showed that they recognized VA10 and HA9 but not AE10 (Fig. 3A). However, they failed to recognize lower concentrations of VA10 peptide (Fig. 3D). These results indicate that HA9 (Pol 792–800) is the optimal epitope.

Similarly FPDWQNYTPGP was recognized by bulk cultured $CD8^+$ T cells stimulated with either Nef119–135 or Nef125–141. Bulk cultured $CD8^+$ T cells stimulated with Nef119–135 recognized both FPDWQNYTPGP and GYFPDWQNYTP, whereas Nef125–141-induced $CD8^+$ T cells recognized FPDWQNYTPGP but not the other 11-mers (Fig. 2). According to peptide-binding motif of HLA-B*5401, which has Pro at position 2, we speculated that FPDWQNYTP (overlapped between GYFPDWQNYTP and FPDWQNYTPGP) would be the optimal epitope peptide, and so we synthesized three truncated peptides (GT10: GYFPDWQNYT, FP9: FPDWQNYTP, and PP8: PDWQNYTP) from Nef123–133. The result showed that Nef125–141-induced $CD8^+$ T cells recognized FP9 but not GT10 and PP8 (Fig. 3A), thus indicating FP9 (Nef125–133) to be the optimal peptide.

In the case of Nef149–165, we found that the bulk culture cells stimulated with the Nef149–165 peptide failed to produce IFN-γ by stimulation with B-LCL pre-pulsed with four 11-mer peptides in Nef149–165 (Fig. 2). Nef149–165 has two Pro residues, but the bulk cells failed to respond to EPEKVEEANEG, suggesting that Pro at position 2 of

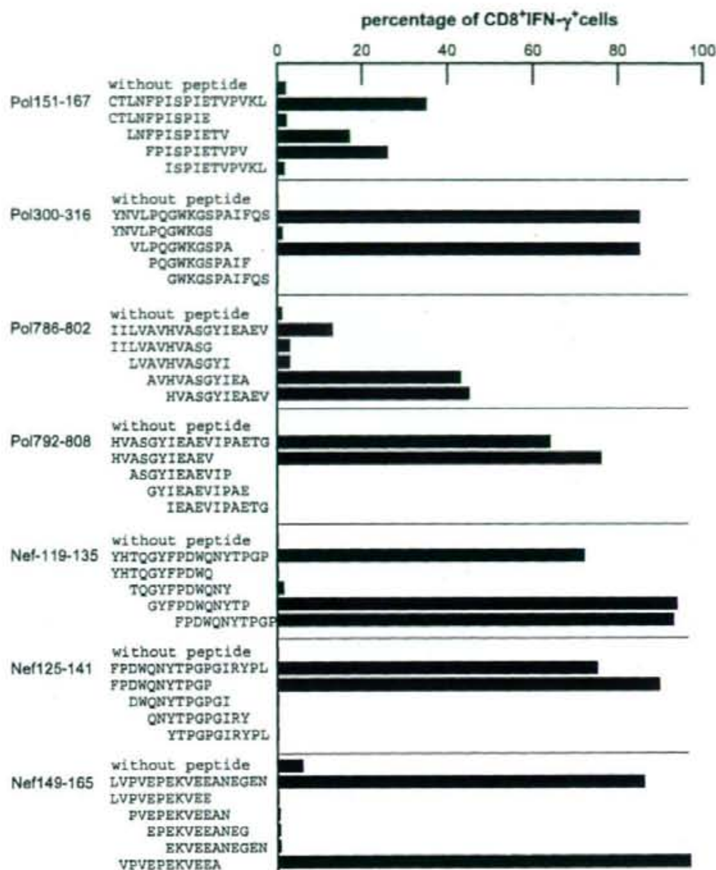



Fig. 2. Selection of 11-mer HIV-1 Pol and Nef peptides including HLA-B*5401-restricted epitopes. The 17-mer peptide-specific bulk CD8$^+$ T cells were stimulated with autologous B-LCL pre-pulsed with each overlapping 11-mer peptide included in the 17-mer peptides. The responsibility of the bulk CD8$^+$ T cells toward each 11-mer peptide was measured by using the intracellular IFN-γ staining assay. The percentages of IFN-γ-producing cells among CD8$^+$ T cells are shown in the figure.

VPVEPEKVEEA (Nef150–160) is the anchor residue of the epitope. Therefore, we generated the Nef150–160 peptide and investigated whether the bulk cultured cells would respond to the stimulator cells pre-pulsed with this 11-mer peptide. The results showed that they produced IFN-γ production in response to Nef150–160 (Fig. 2). The finding that the bulk cells did not respond to LVPVEPEKVEE (Nef149–159) excluded the possibility that one of the three shorter peptides (VPVEPEKV, VPVEPEKVE or VPVEPEKVEE) was the epitope. These results strongly suggest that Nef150–160 is the optimal epitope peptide.

### 3.4. Killing of HIV-1-recombinant vaccinia-infected cells by specific CTLs

To clarify whether Pol155–163, Pol303–312, Pol792–800, Nef125–133, and Nef150–160 epitopes are naturally occurring peptides, we investigated the ability of these peptide-specific CD8$^+$ T cells to kill HLA-B*5401 expressing B-LCL infected with recombinant HIV-1 (r-HIV-1) vaccinia virus. They effectively killed HLA-B*5401 expressing B-LCL infected with r-HIV-1 vaccinia virus but not the cells infected with the wild-type vaccinia virus (Fig. 4). There was a difference in killing activity toward r-HIV vaccinia-infected cells between Pol- and Nef-specific bulk CTLs. A previous study showed that HLA class I is downregulated in cells infected with HIV-1 nef recombinant vaccinia [28]. The difference might be explained by Nef-mediated HLA-A and -B down-regulation. These results confirm these peptides to be naturally occurring ones presented by HLA-B*5401.

### 3.5. Confirmation of HLA-B*5401-restriction in five HIV-1-epitope-specific CTLs

To confirm the restriction molecule of these five HIV-1 epitopes, we generated CTL clones specific for these

Fig. 3. Recognition of the 8- to 10-mer truncated peptides by HIV-1 Pol- or Nef-specific CD8+ T cells. A. The 17-mer peptide-specific bulk CD8$^+$ T cells were stimulated with autologous B-LCL pre-pulsed with each 8- to 10-mer truncated peptide. For determination of the optimal epitopes in Pol155–165 (B), Pol302–312 (C), and Pol790–800 (D), bulk CTL were co-cultured with autologous B-LCL pre-pulsed with each truncated peptide at concentrations from 0.1 to 1000 nM. The responsiveness of the bulk CD8$^+$ T cells toward each truncated peptide was measured by conducting the intracellular IFN-γ staining assay. The percentages of IFN-γ-producing cells among CD8$^+$ T cells are shown in the figure.

epitopes as well as HLA-B*5401-transfected C1R cells (C1R-B*5401 cells). We used both C1R-B*5401 cells and C1R cells as target cells for the CTL clones specific for these epitopes. These CTL clones killed C1R-B*5401 cells pre-pulsed with the corresponding peptide but failed to kill the C1R cells that were similarly treated (Fig. 5). These results confirm that these CTLs recognized HLA-B*5401-restricted epitopes.

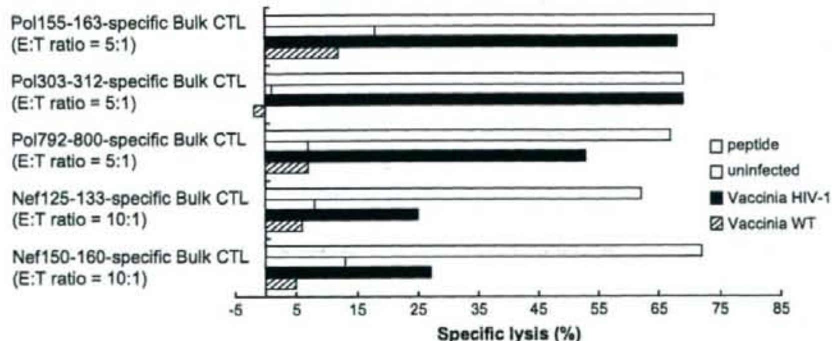



Fig. 4. Killing of r-HIV-1 vaccinia-infected or peptide-pulsed cells by the Pol- or Nef-specific CTLs. The activities of the five HIV-1-specific T cells toward B*5401$^+$B-LCL pre-pulsed with the corresponding peptide (1 μM), or those infected with recombinant vaccinia virus expressing the corresponding proteins Pol and Nef (vaccinia-HIV-1) or wild-type vaccinia virus (vaccinia-WT) were measured at an effector-to-target (E:T) ratio of 5:1 or 10:1.

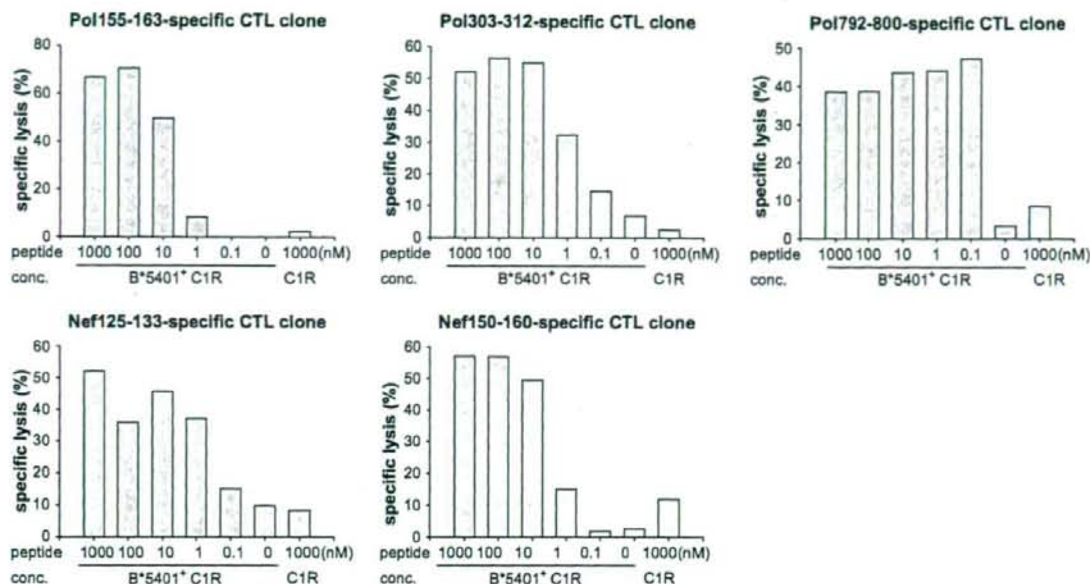



Fig. 5. Confirmation of HLA-B*5401-restricted recognition. Specific lysis of C1R-B*5401 cell lines by Pol155–163-specific, Pol303–312-specific, Pol792–800-specific, Nef125–133-specific, or Nef150–160-specific CTL clone was determined by performing the $^{51}$Cr-release assay. The target cells were pulsed with each peptide at concentrations from 0.1 to 1000 nM, and the assays were performed at a 2:1 ratio of effector cells to target cells. The percentage of specific lysis is shown in each graph.

### *3.6. Frequency of HLA-B*5401-restricted HIV-1-specific CD8$^+$ T cells in HIV-1-infected individuals with HLA-B*5401*

To clarify whether CD8$^+$ T cells specific for these epitopes were predominantly induced in chronically HIV-1-infected individuals bearing HLA-B*5401, we investigated the induction of the specific CD8$^+$ T cells in PBMCs from eight chronically HIV-1-infected HLA-B*5401-positive individuals by stimulating them with these epitope peptides. Pol155–163-specific CD8$^+$ T cells were found in four of the eight HIV-1-infected individuals. Pol792–800-, Nef125–133-, and Nef150–160-specific CD8$^+$ T cells were found in three individuals; and Pol303–312-specific CD8$^+$ T cells, in two of them (Table 1).

## 4. Discussion

In the present study, we could identify 5 HLA-B*5401-restricted epitopes in HIV-1 Pol and Nef by using 17-mer overlapping peptides. A previous study had shown that Pro at position 2 is the primary anchor residue and that Phe, Met, Arg, Tyr or Asp at position 3, and Ala at position 9 is the secondary anchor residue for HLA-B*5401 [29]. However, CTL epitopes are not always consistent with the peptide-binding motif of HLA [20,21]. In fact, out of the five HLA-B*5401-restricted HIV-1 epitopes, one does not have Pro at position 2. Pol792–800 epitope (HVASGYIEA) has Ala residue at position 3. This Ala is a candidate of the anchor at position 2 because Ala has similar characteristics as Pro, but the specific

Table 1
Induction of epitope-specific CD8$^+$ T cells among PBMCs from HLA-B*5401$^+$ HIV-1-infected individuals

| Patients[a] | Viral load[b] | CD4[c] | CD8[c] | Percentage of IFN-γ-producing cells in CD8$^+$ T cells | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Pol155–163 | Pol303–312 | Pol792–800 | Nef125–133 | Nef150–160 |
| KI-119 | $3.0 \times 10^3$ | 536 | 1268 | 16.1 | 58.0 | 39.8 | 26.3 | 21.1 |
| KI-160 | $3.5 \times 10^4$ | 360 | 831 | 3.9 | 0.0 | 0.5 | 0.0 | 0.0 |
| KI-172 | $1.8 \times 10^4$ | 512 | 558 | 0.0 | 0.0 | 0.0 | 8.1 | 0.0 |
| KI-115 | $5.3 \times 10^3$ | 264 | 721 | 19.9 | 7.0 | 0.0 | 0.1 | 2.1 |
| KI-150 | $2.4 \times 10^4$ | 307 | 1411 | 0.0 | 0.0 | 3.5 | 0.0 | 0.0 |
| KI-167 | $4.2 \times 10^4$ | 281 | 1055 | 0.0 | 0.0 | 0.0 | 48.5 | 0.0 |
| KI-141 | $1.7 \times 10^5$ | 578 | 1414 | 0.8 | 0.0 | 0.0 | 0.4 | 0.0 |
| KI-201 | <50 | 518 | 374 | 7.2 | 0.0 | 12.8 | 0.0 | 3.5 |

[a] HIV-1-infected individuals with HLA-B*5401.

[b] Copies/ml.

[c] Cells/μl.

$CD8^+$ T cells failed to recognize three truncated peptides carrying Ala at position 2. Thus, this epitope is not consistent with the HLA-B*5401 peptide motif. We note that the Pol792–800 epitope cannot be identified by reverse immunogenetics. Interestingly, the Pol792–800-specific CTL clone showed high cytotoxicity toward B*5401-transfected C1R cells pulsed with peptides at low concentrations (Fig. 5), thus suggesting that the Pol792–800 peptide may be a high-affinity HLA-B*5401-binding peptide.

In the present study, we used 17-mer overlapping peptides to identify HIV-1-specific CTLs, because the cost of making shorter peptides is much cheaper than that for the longer ones. The optimal length of epitope peptides presented by HLA class I molecules is thought to be 8–11 amino acid residues [30]. Therefore, the affinity of 17-mer peptides toward HLA class I molecules is thought to be low. This suggests that some epitopes are not identified by this approach using 17-mer overlapping peptides.

Interestingly, Gag-specific epitopes were not identified in the present study, although Pol- and Nef-specific CTL ones were. We used PBMCs from only HIV-1-infected individual KI-119. Therefore, we speculate that this individual does not have any ability to elicit CTL specific for Gag. However, KI-119 showed strong HLA-A*0206-restricted or HLA-B*4801-restricted $CD8^+$ T cells responses to Gag (data not shown), suggesting that HLA-B*5401-restricted Gag-specific T cell responses are hardly induced. This suggests the possibility that Gag does not include a high-affinity peptide carrying HLA-B*5401 motif. Recent studies reported that Gag-specific CTLs play a critical role in the control of viral replication, because their frequency was correlated with viral loads in HIV-1-infected individuals [31]. If this is also the case in Japanese and other Asian populations, HLA-B*5401 may be associated with rapid progression to AIDS. The role of these HLA-B*5401-restricted CTLs still remains unknown. Further analysis of these CTLs will be required to clarify the role of HLA-B*5401-restricted CTLs in Asian populations.

When we examined the frequency of these five epitope-specific CTL in eight chronically HIV-1-infected individuals, these CTLs were detected in two to four of eight chronically HIV-1-infected individuals with HLA-B*5401 (Table 1), indicating that these five epitopes were relatively recognized ones in chronically HIV-1-infected individuals. These epitopes except Nef150–160 are relatively conserved in clade B (approximately more than 80% of clade B has consensus sequences: Los Alamos National Laboratory HIV Molecular Immunology Database, http://www.hiv.lanl.gov/content/immunology/maps/ctl/ctl.pdf). In contrast, many substitutions are found in Nef150–160. They include D at position 4, D at position 6, Q/E/R at position 8, I at position 8, and K at position 10. These results imply that these CTLs play an important role in the control of HIV-1. Further analysis of these epitopes such as escape mutants is now under investigation.

In summary, we identified five novel HLA-B*5401-restricted HIV-1 epitopes in HIV-1-infected individuals by using 17-mer overlapping peptides derived from HIV-1 Gag, Pol, and Nef. In addition, one of them, Pol792–800, did not have an amino acid sequence matching the HLA-B*5401 peptide motif. These epitopes identified by using 17-mer overlapping peptides will be useful to clarify immune response toward HIV-1 and to develop a population-based AIDS vaccine.

## Acknowledgements

The authors thank Sachiko Sakai for her secretarial assistance. This research was supported by a grant-in aid for scientific research from the Ministry of Health, Labor, and Welfare of the government of Japan and by a grant from the Japan Health Science Foundation.

## References

[1] R.A. Koup, J.T. Safrit, Y. Cao, C.A. Andrews, G. McLeod, W. Borkowsky, C. Farthing, D.D. Ho, Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome, J. Virol. 68 (1994) 4650–4655.

[2] G. Pantaleo, J.F. Demarest, H. Soudeyns, C. Graziosi, F. Denis, J.W. Adelsberger, P. Borrow, M.S. Saag, G.M. Shaw, P.S. Sekaly, A.S. Fauci, Major expansion of CD8+ T cells with a predominant Vβ usage during the primary immune response to HIV, Nature 370 (1994) 463–467.

[3] P.A. Moss, S.L. Rowland-Jones, P.M. Frodsham, S. McAdam, P. Giangrande, A.J. McMichael, J. Bell, Persistent high frequency of human immunodeficiency virus-specific cytotoxic T cells in peripheral blood of infected donors, Proc. Natl. Acad. Sci. U. S. A. 192 (1995) 5773–5777.

[4] S. Rowland-Jones, J. Sutton, K. Ariyoshi, T. Dong, F. Gotch, S. McAdam, D. Whitby, S. Sabally, A. Gallimore, T. Corrah, M. Takiguchi, T. Schultz, A. McMichael, H. Whittle, HIV-1 specific cytotoxic T cells in HIV-exposed but uninfected Gambian women, Nat. Med. 1 (1995) 59–64.

[5] C.M. Walker, D.J. Moody, D.P. Stites, J.A. Levy, CD8+ lymphocytes can control HIV infection in vitro by suppressing virus replication, Science 234 (1986) 1563–1566.

[6] O.O. Yang, B.D. Walker, CD8+ cells in human immunodeficiency virus type I pathogenesis: cytolytic and noncytolytic inhibition of viral replication, Adv. Immunol. 66 (1997) 273–311.

[7] T. Matano, R. Shibata, C. Siemon, M. Connors, H.C. Lane, M.A. Martin, Administration of an anti-CD8 monoclonal antibody interferes with the clearance of chimeric simian/human immunodeficiency virus during primary infections of rhesus macaques, J. Virol. 72 (1998) 164–169.

[8] P. Borrow, H. Lewicki, X. Wei, M.S. Horwitz, N. Peffer, H. Meyers, J.A. Nelson, J.E. Gairin, B.H. Hahn, M.B. Oldstone, G.M. Shaw, Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus, Nat. Med. 3 (1997) 205–211.

[9] P.J. Goulder, R.E. Philips, R.A. Colbert, S. McAdam, G. Ogg, M.A. Nowak, P. Giangrande, G. Luzzi, B. Morgan, A. Edwards, A.J. McMichael, S. Rowland-Jones, Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS, Nat. Med. 3 (1997) 212–217.

[10] A.J. Frater, H. Brown, A. Oxenius, H.F. Günthard, B. Hirschel, N. Robinson, A.J. Leslie, R. Payne, H. Crawford, A. Prendergast, C. Brander, P. Kiepiela, B.D. Walker, P.J. Goulder, A. McLean, R.E. Phillips, Effective T-cell responses select human immunodeficiency virus mutants and slow disease progression, J. Virol. 81 (2007) 6742–6751.

[11] S.J. O'Brien, X. Gao, M. Carrington, HLA and AIDS: a cautionary tale, Trends Mol. Med. 7 (2001) 379–381.

[12] M. Altfeld, E.T. Kalife, Y. Qi, H. Streeck, M. Lichterfeld, M.N. Johnston, N. Burgett, M.E. Swartz, A. Yang, G. Alter, X.G. Yu, A. Meier, J.K. Rockstroh, T.M. Allen, H. Jessen, E.S. Rosenberg, M. Carrington, B.D. Walker, HLA alleles associated with delayed progression to

AIDS contribute strongly to the initial CD8+ T cell response against HIV-1, PLoS Med. 3 (2006) e403.
[13] Y. Ikeda-Moore, H. Tomiyama, K. Miwa, S. Oka, A. Iwamoto, Y. Kaneko, M. Takiguchi, Identification and characterization of multiple HLA-A24-restricted HIV-1 CTL epitopes: strong epitopes are derived from V regions of HIV-1, J. Immunol. 159 (1997) 6242–6252.
[14] K. Fukada, H. Tomiyama, Y. Chujoh, K. Miwa, Y. Kaneko, S. Oka, M. Takiguchi, HLA-A*1101-restricted cytotoxic T lymphocyte recognition for a novel epitope derived from the HIV-1 Env protein, AIDS 13 (1999) 2597–2599.
[15] H. Tomiyama, T. Sakaguchi, K. Miwa, S. Oka, A. Iwamoto, Y. Kaneko, M. Takiguchi, Identification of multiple HIV-1 CTL epitopes presented by HLA-B*5101 molecules, Hum. Immunol. 60 (1999) 177–186.
[16] M.S. Hossain, H. Tomiyama, T. Inagawa, S. Ida, S. Oka, M. Takiguchi, Identification and characterization of HLA-A*3303-restricted, HIV type 1 Pol- and Gag-derived cytotoxic T cell epitopes, AIDS Res. Hum. Retroviruses 19 (2003) 503–510.
[17] M. Satoh, Y. Takamiya, S. Oka, K. Tokunaga, M. Takiguchi, Identification and characterization of HIV-1-specific CD8+ T cell epitopes presented by HLA-A*2601, Vaccine 23 (2005) 3783–3790.
[18] Y. Kawashima, M. Satoh, S. Oka, M. Takiguchi, Identification and characterization of HIV-1 epitopes presented by HLA-A*2603: comparison between HIV-1 epitopes presented by A*2601 and A*2603, Hum. Immunol. 66 (2005) 1155–1166.
[19] M.A. Borghan, S. Oka, M. Takiguchi, Identification of HLA-A*3101-restricted cytotoxic T-lymphocyte response to human immunodeficiency virus type 1 (HIV-1) in patients with chronic HIV-1 infection, Tissue Antigens 66 (2005) 305–313.
[20] T. Dong, D. Boyd, W. Rosenberg, N. Alp, M. Takiguchi, A. McMichael, S. Rowland-Jones, An HLA-B35-restricted epitope modified at an anchor residue results in an antagonist peptide, Eur. J. Immunol. 26 (1996) 335–339.
[21] P.J. Goulder, S.W. Reid, D.A. Price, C.A. O'Callaghan, A.J. McMichael, R.E. Phillips, E.Y. Jones, Combined structural and immunological refinement of HIV-1 HLA-B8-restricted cytotoxic T lymphocyte epitopes, Eur. J. Immunol. 27 (1997) 1515–1521.
[22] M. Altfeld, M.M. Addo, R.L. Eldridge, X.G. Yu, S. Thomas, A. Khatri, D. Strick, M.N. Phillips, G.B. Cohen, S.A. Islam, S.A. Kalams, C. Brander, P.J. Goulder, E.S. Rosenberg, B.D. WalkerHIV Study Collaboration, Vpr is preferentially targeted by CTL during HIV-1 infection, J. Immunol. 167 (2001) 2743–2752.
[23] M.M. Addo, M. Altfeld, E.S. Rosenberg, R.L. Eldridge, M.N. Philips, K. Habeeb, A. Khatri, C. Brander, G.K. Robbins, G.P. Mazzara, P.J. Goulder, B.D. WalkerHIV Controller Study Collaboration, The HIV-1 regulatory proteins Tat and Rev are frequently targeted by cytotoxic T lymphocytes derived from HIV-1-infected individuals, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 1781–1786.
[24] P.J. Goulder, M.M. Addo, M.A. Altfeld, E.S. Rosenberg, Y. Tang, U. Govender, N. Mngqundaniso, K. Annamalai, T.U. Vogel, M. Hammond, M. Bunce, H.M. Coovadia, B.D. Walker, Rapid definition of five novel HLA-A*3002-restricted human immunodeficiency virus-specific cytotoxic T-lymphocyte epitopes by elispot and intracellular cytokine staining assays, J. Virol 75 (2001) 1339–1347.
[25] R. Draenert, M. Altfeld, C. Brander, N. Basgoz, C. Corcoran, A.G. Wurcel, D.R. Stone, S.A. Kalams, A. Trocha, M.M. Addo, P.J. Goulder, B.D. Walker, Comparison of overlapping peptide sets for detection of antiviral CD8 and CD4 T cell responses, J. Immunol. Methods 275 (2003) 19–29.
[26] H.T. Maecker, H.S. Dunn, M.A. Suni, E. Khatamzas, C.J. Pitcher, T. Bunde, N. Persaud, W. Trigona, T.M. Fu, E. Sinclair, B.M. Bredt, J.M. McCune, V.C. Maino, F. Kern, L.J. Picker, Use of overlapping peptide mixtures as antigens for cytokine flow cytometry, J. Immunol. Methods 255 (2001) 27–40.
[27] T. Imanishi, T. Akaza, A. Kimura, K. Tokunaga, T. Gojobori, in: K. Tsuji, M. Aizava, T. Sasazuki (Eds.), Allele and Haplotype Frequencies for HLA and Complement Loci in Various Ethnic Groups, HLA 1991: Proceedings of the Eleventh International Histocompatibility Workshop and Conference, Oxford University Press, Oxford, 1992.
[28] A.D. Blagoveshchenskaya, L. Thomas, S.F. Feliciangeli, C.-H. Hung, G. Thomas, HIV-1 Nef downregulates MHC-1 by a PACS-1- and PI3K-regulated ARF6 endocytic pathway, Cell 111 (2002) 853–866.
[29] L.D. Barber, B. Gillece-Castro, L. Percival, X. Li, C. Clayberger, P. Parham, Overlap in the repertoires of peptides bound in vivo by a group of related class I HLA-B allotypes, Curr. Biol. 1 (1995) 179–190.
[30] J.W. Yewdell, J.R. Bennink, Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses, Annu. Rev. Immunol. 17 (1999) 51–88.
[31] P. Kiepiela, K. Ngumbela, C. Thobakgale, D. Ramduth, I. Honeyborne, E. Moodley, S. Reddy, C. de Pierres, Z. Mncube, N. Mkhwanazi, K. Bishop, M. van der Stok, K. Nair, N. Khan, H. Crawford, R. Payne, A. Leslie, J. Prado, A. Prendergast, J. Frater, N. McCarthy, C. Brander, G.H. Learn, D. Nickle, C. Rousseau, H. Coovadia, J.I. Mullins, D. Heckerman, B.D. Walker, P. Goulder, CD8+ T-cell responses to different HIV proteins have discordant associations with viral load, Nat. Med. 13 (2007) 46–53.

# CTL-Mediated Selective Pressure Influences Dynamic Evolution and Pathogenic Functions of HIV-1 Nef[1]

**Takamasa Ueno,[2]* Chihiro Motozono,* Sachi Dohki,* Philip Mwimanzi,* Susanne Rauch,‡ Oliver T. Fackler,‡ Shinichi Oka,† and Masafumi Takiguchi***

**HIV-1 Nef plays multiple roles in modulating immune responses, even though it is a dominant CTL target itself. How Nef accomplishes the balance between such conflicting selective pressures remains elusive. By genetic and functional studies, we found that $Arg^{75}$Thr and $Tyr^{85}$Phe mutations, located in a well-conserved proline-rich region in Nef, were differently associated with escape from CTL responses specific for two overlapping HLA-B35-restricted epitopes. CTLs specific for an epitope, that selected $Tyr^{85}$Phe, were elicited earlier and had more potent functional avidities than did those that selected $Arg^{75}$Thr. Although the double mutant could escape from both CTLs, the mutations are rarely observed in combination naturally. Introduction of both mutations reduced Nef's HLA class I down-regulation activity and increased the susceptibility of virus-infected cells to recognition by CTLs targeting other epitopes. Moreover, the mutant Nef was impaired in the association with activated cellular kinases and in the enhancement of viral replication. These results highlight CTL immunosurveillance as important modulators of Nef's biological activity in the infected host.** ***The Journal of Immunology,*** **2008, 180: 1107–1116.**

The accessory gene product Nef is a critical determinant for the pathogenesis of the primate lentiviruses, HIV-1, HIV-2, and SIV. The importance of Nef in viral pathogenesis was first shown in rhesus macaques, where a large deletion of the *nef* gene severely reduced SIV pathogenicity (1). This finding was supported by the fact that a cohort consisting of one blood donor and eight transfusion recipients infected with Nef-defective HIV-1 demonstrated dramatically decreased rates of disease progression (2, 3). The impact on the outcome of HIV/SIV infection likely results from the synergy of multiple functions exerted by Nef that may be differentially regulated over time (4). Nef enhances viral replication and virion infectivity (5–7) and affects cells in many ways, including altering T cell activation and maturation (6, 8–11), subverting the apoptotic machinery, and down-regulating a number of cell surface receptors including CD4 and HLA class I (7, 12, 13). The down-regulation of MHC class I (MHC-I)[3] by SIV Nef in rhesus macaques limits CD8 T cell-mediated killing and contributes to the pathogenic effect of Nef in vivo, highlighting the importance of Nef-mediated immunoevasion to facilitate disease progression (14).

The initial peak of viral replication after primary HIV infection begins to decline simultaneously with the appearance of HIV-specific CD8 T lymphocytes (15, 16) that can eliminate HIV-infected cells directly by MHC-I-restricted cytolysis or indirectly through the production of soluble factors such as cytokines and chemokines (17, 18). The biological relevance of HIV-specific CTLs in HIV infection is also supported by the results of in vivo studies demonstrating a dramatic rise of viremia and an accelerated clinical disease progression in SIV-infected macaques after the artificial depletion of $CD8^+$ cells (19, 20). Among HIV proteins targeted by HIV-specific CTLs, HIV Nef protein is expressed at high levels early in an HIV infection (21) and elicits a strong CTL response in a number of subjects (22, 23). Most antigenic determinants are located within a multirestricted, immunodominant central region spanning residues 73–94 and 113–147 (22, 24), including a highly conserved proline-rich region containing an Src homology 3 (SH3)-binding motif, PxxP ($Nef_{73-82}$: PVRPQVPLRP) critical for several but not all Nef functions (6, 7, 25–27). In particular, HIV-infected subjects expressing the HLA-B*3501 molecule, which prefers a proline residue on the second position of its antigenic peptides, show vigorous HLA-B35-restricted CTL responses toward the proline-rich region of Nef (22, 28, 29).

In the present study, we focused on HLA-B35-restricted CD8 T cell responses toward the functionally important PxxP region of HIV-1 Nef to ask whether CTL responses can impose constraints on Nef activity. Remarkably, sequence analysis of autologous viruses revealed the association of two different mutations with patients carrying HLA-B*35, one of which was earlier shown to be a naturally occurring variation that can modulate Nef functions (25). Further detailed analyses of CTL responses and Nef functions demonstrated that Nef balances between the conflicting selective pressures during the course of an HIV-1 infection. These findings suggest an important role of HIV-1 Nef-specific CTL responses in the control of Nef activity during the progression of an HIV-1 infection.

*Division of Viral Immunology, Center for AIDS Research, Kumamoto University, Kumamoto, Japan; †Division of Infectious Diseases, Center for AIDS Research, Kumamoto University, Kumamoto, Japan and AIDS Clinical Center, International Medical Center of Japan, Tokyo, Japan; and ‡Department of Virology, University Hospital of Heidelberg, Heidelberg, Germany

Received for publication September 28, 2007. Accepted for publication November 8, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan (to T.U.), by a Grant-in-Aid for AIDS Research from the Ministry of Health, Labor, and Welfare of Japan (to T.U., S.O., and M.T.), and by Deutsche Forschungsgemeinschaft Grant SFB 638 (Project A11, to O.T.F.).

[2] Address correspondence and reprint requests to Dr. Takamasa Ueno, Division of Viral Immunology, Center for AIDS Research, Kumamoto University, 2-2-1 Honjo, Kumamoto, 860-0811, Japan. E-mail address: uenotaka@kumamoto-u.ac.jp

[3] Abbreviations used in this paper: MHC-I, MHC class I; SH3, Src homology 3; 7-AAD, 7-aminoactinomycin D; wt, wild type; IVKA, in vitro kinase assay.

Copyright © 2008 by The American Association of Immunologists, Inc. 0022-1767/08/$2.00

Table I. *Summary of HLA-B35⁺ subjects used in this study*[a]

| Pt. | HLA Class I Allele | Months since Seroconversion | Viral Load ($\log_{10}$/ml) | CD4 ($mm^{-3}$) | Antiretoviral Therapy | Nef Sequence | PBMC Availability |
|---|---|---|---|---|---|---|---|
| 001 | *A2402/A2603, B3501/B4002* | 132 | ND | 227 | + | RPQVPLRPMT**F** | − |
| | | 192 | 3.9 | 223 | + | **T**PQVPLRPMTY | + |
| 003 | *A2402/A2601, B3501/B5101* | 72 | ND | 480 | − | RPQVPLRPMT**F** | − |
| | | 144 | ND | 252 | + | **T**PQVPLRPMTY | + |
| 006 | *A24/A26, B35/B52* | 48 | ND | 102 | + | RPQVPLRPMT**F** | − |
| 015 | *A11/A24, B35/B54* | 147 | BD | 383 | + | **T**PQVPLRPMTY | + |
| 016 | *A26/A33, B35/B44* | 7 | ND | 43 | − | RPQVPLRPMT**F** | − |
| 017 | *A2/A24, B35/B48* | 192 | BD | 254 | + | **T**PQVPLRPMTY | − |
| 019 | *A2402/−, B3501/B5201* | 18 | 4.7 | 524 | − | RPQVPLRPMT**F** | − |
| | | 80 | BD | 1574 | + | **T**PQVPLRPMTY | + |
| 025 | *A24/A31, B35* | 26 | ND | 50 | + | **T**PQVPLRPMTY | − |
| 027 | *A24/A26, B35/B44* | 4 | ND | 84 | + | RPQVPLRPMT**F** | − |
| 033 | *A0207/A3101, B3501/B4601* | 72 | 5.3 | 326 | − | **T**PQVPLRPMTY | + |
| 034 | *A2402/A2601, B3501/B4801* | 48 | 4.4 | 201 | − | **T**PQVPLRPMTY | + |
| 042 | *A24/A31, B35/B60* | 59 | 3.8 | 311 | − | **T**PQVPLRPMTY | + |
| 046 | *A2, B35/B61* | 48 | BD | 263 | + | **T**PQVPLRPMTY | + |
| 099 | *A2402/−, B3501/B61* | 12 | 3.9 | 984 | − | RPQVPLRPMT**F** | + |
| 100 | *A2601/−, B3501/B4001* | 16 | 5.0 | 614 | − | RPQVPLRPMT**F** | + |
| 102 | *A2402/A0206, B3501/B0702* | 17 | 2.8 | 482 | − | RPQVPLRPMT**F** | + |
| 131 | *A2402/A0207, B3501/B4601* | 10 | 1.9 | 563 | + | RPQVPLRPMT**F** | + |
| 136 | *A2402/A2601, B3501/B5201* | 15 | 4.4 | 308 | − | RPQVPLRPMT**F** | + |
| 141 | *A0201/A3101, B3501/B5401* | 10 | 5.3 | 382 | − | RPQVPLRPMTY | + |
| | | 20 | 5.1 | 360 | + | RPQVPLRPMT**F** | + |
| 145 | *A0207/A2601, B3501/B5101* | 6 | BD | 645 | − | RPQVPLRPMTY | − |
| | | 18 | 4.6 | 685 | − | RPQVPLRPMT**F** | + |
| 161 | *A2402/A2601, B3501/B5401* | 13 | 2.3 | 955 | − | RPQVPLRPMT**F** | + |
| 168 | *A2601/−, B3501/−* | 5 | 2.3 | 408 | + | RPQVPLRPMTY | + |
| 178 | *A2601/A3101, B3501/B4601* | 8 | 2.7 | 568 | + | RPQVPLRPMTY | + |

[a] ND, Not determined; BD, below detection limit. Bold, underlined letters in the sequences represent mutations.

## Materials and Methods

*Subjects*

A total of 23 individuals (HLA-B35⁺) with HIV infection followed at the AIDS Clinical Center (International Medical Center of Japan) were enrolled for functional analysis of HIV-specific CD8 T cells and autologous HIV-1 sequence analysis in this study. Subjects were selected based on the availability of plasma and PBMC samples as well as HLA-B*35 expression. Clinical data of all subjects are listed in Table I. Patients 01, 03, and 17 are hemophiliacs who had been infected with HIV-1 through contaminated blood products. Because the time of HIV-1 infection or the time of seroconversion was not known for these subjects, we suspect that their infection occurred in 1983 based on a survey done on Japanese hemophiliacs. In addition, 41 individuals (negative for HLA-B*35) with HIV infections were enrolled for autologous HIV-1 sequence analysis. The study was conducted in accordance with the human experimentation guidelines of the International Medical Center of Japan and Kumamoto University.

*Sequence analysis of autologous HIV-1*

HIV-1 particles were precipitated by ultracentrifugation (50,000 rpm, 30 min) of patients' plasma, after which the viral RNA was extracted from them. DNA fragments encoding Nef proteins were amplified by a nested PCR, gel purified, and sequenced directly as described (29). The fragments were cloned into a plasmid and then sequenced for phylogenetic tree analysis.

For phylogenetic tree analysis of intrapatient evolution of the *nef* gene (HXB2 coordinate, 8932–9555), nucleotide sequences were initially aligned by using Clustal W and then manually adjusted to maximize alignment of codon triplet as needed. Regions that could not be unambiguously aligned were removed from subsequent phylogenetic analysis. The MEGA3 package of sequence analysis programs was used for detailed phylogenetic analysis (30). Pairwise evolutionary distances were calculated by using the Kimura 2-parameter model for estimation of distances, and phylogenetic trees were constructed by the neighbor-joining method.

*Generation of T cell clones*

CTL clones or lines were established by stimulation of PBMC with a synthetic peptide, as previously described (31). Briefly, a bulk CTL culture was seeded at a density of 0.8 or 5 cells/well with a cloning mixture (irradiated allogeneic PBMC and C1R-B3501 cells pulsed with 1 μM peptide in RPMI 1640 with 10% FCS and 100 U/ml rIL-2). Two weeks later, cells showing substantial Ag-specific cytolytic activity were maintained in the medium with peptide stimulation weekly.

*Preparation of HIV-1 variants*

The full-length HIV-1 pNL43 derivatives in which the *nef* gene was completely removed (pNL43ΔNef) or replaced with SF2 *nef* (pNL43SF2Nef) were created earlier (32). The $Arg^{75}$ to Thr and $Tyr^{85}$ to Phe mutations were achieved by site-directed mutagenesis based on SF2 *nef*. 293T cells were transfected with each of the constructs, and the infectious HIV-1 virions released into the medium were collected 48 h later. The p24 Ag concentrations of virus stocks were determined by p24 Ag ELISA.

*Flow cytometric analysis*

***HLA stabilization assay.*** Peptide-binding activity for HLA-B*3501 was assessed by an HLA stabilization assay using RMA-S cells expressing HLA-B*3501 as described earlier (31).

***HLA tetramer analysis.*** The HLA-B3501 tetramers in complex with the VY8 and RY11 peptides were prepared as previously described (31). Cryopreserved PBMC of HIV-positive ($2 \times 10^6$) or -negative donors ($3 \times 10^6$) were stained with the PE- and allophycocyanin-labeled tetramers at 37°C for 15 min followed by anti-CD8-PerCP (BD Biosciences/BD Pharmingen) and anti-CD3-FITC (DakoCytomation) at 4°C for 15 min. The $CD3^+CD8^+$ cells were gated and then analyzed for binding with the tetramers by flow cytometry (FACSCalibur; BD Biosciences).

***Intracellular cytokine staining assay.*** Intracellular cytokine staining of Ag-specific CTL clones was done as previously described (33). Briefly, CTL clones ($4 \times 10^4$ cells) were incubated with C1R-B3501 cells ($4 \times 10^4$ cells) alone or pulsed with various concentrations of peptides for 6 h at 37 °C in the presence of brefeldin A (10 μg/ml). The cells were stained first with anti-CD8 mAb and 7-aminoactinomycin D (7-AAD), permeabilized in a detergent buffer, and then stained with mAb specific for IFN-γ or TNF-α (BD Biosciences/BD Pharmingen).

*Cytotoxic assays*

***Toward peptide-loaded cells.*** The cytotoxic activity of the CTL clones was determined by a standard $^{51}Cr$-release assay as described previously (31).