Immunoprecipitation analysis using the anti-gp70 or a polyclonal rabbit anti-Lck antibody (Millipore, Tokyo, Japan) was performed as described previously [27].

2.4. Measurement of viral infectious titers

TELCeB6 cells plated at a density of 2×10^5 cells per well in 6-well plates were transfected with DNAs and two days later culture supernatants were harvested, filtered through a 0.45-μm-pore-size filter, and frozen until use. For measurement of viral infectious titers of these supernatants on NIH 3T3 cells, cells were seeded in 24-well plates (4×10^4 cells per well) and grown overnight. Infections were carried out by plating 0.5 ml of serially diluted viral supernatants in duplicate on NIH 3T3 cells in the presence of polybrene (8 μg/ml; Sigma–Aldrich, Tokyo, Japan), and after 4 h incubation, the virus-containing medium was replaced with fresh medium. Two days later, X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) staining was performed and the numbers of LacZ-positive colonies were counted to determine end-point viral titers. A representative result of two sets of experiments is shown in each figure.

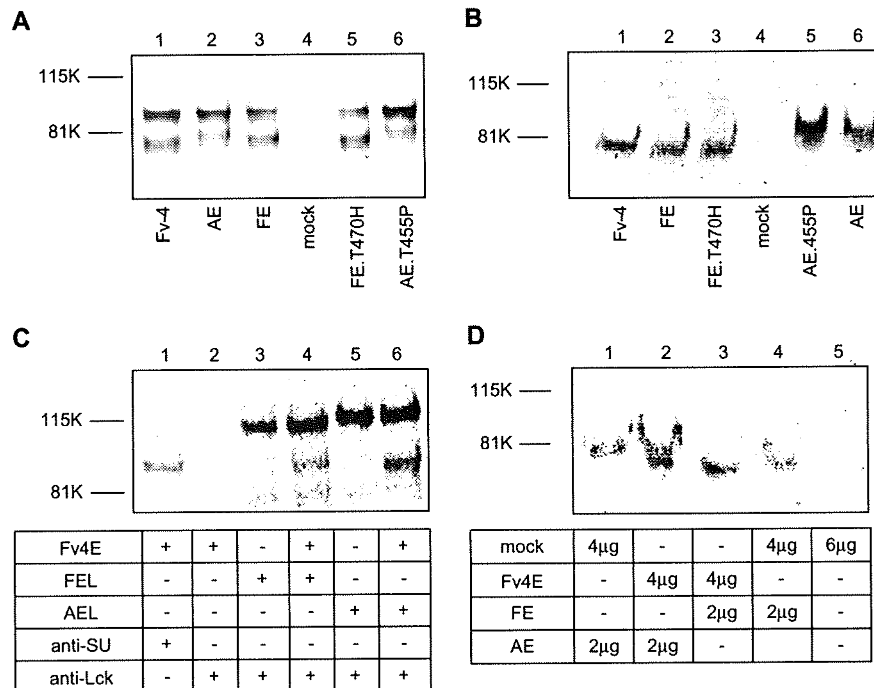
2.5. Syncytia assay on NIH 3T3 cells

NIH 3T3 cells were plated at a density of 8×10^4 cells per well in 24-well plates, incubated overnight, and transfected with DNAs. One day later, numbers of syncytia were counted.

3. Results

3.1. Fv-4 Env expression

In the present study, we used plasmids expressing the wild-type FMLV Env (FE), the wild-type amphotropic MLV Env (AE), a defective FMLV Env with a mutation in the receptor-binding domain of SU (FE.D86K), a defective FMLV Env with a mutation in the extracellular domain of TM (FE.T470H), a defective amphotropic MLV Env with a mutation in the extracellular domain of TM (AE.T455P), and Fv-4 Env, respectively (Fig. 1). These Env-expression plasmids were transfected into TELCeB6 cells, MoMLV *gag–pol* producer cells harboring MFGnlslacZ retroviral vector. Western blot analysis of cell lysates using an anti-MLV SU (anti-gp70) confirmed expression and processing of these Envs including Fv-4 Env (Fig. 2A). In



Fv4E	+	+	-	+	-	+
FEL	-	-	+	+	-	-
AEL	-	-	-	-	+	+
anti-SU	+	-	-	-	-	-
anti-Lck	-	+	+	+	+	+

mock	4μg	-	-	4μg	6μg
Fv4E	-	4μg	4μg	-	-
FE	-	-	2μg	2μg	-
AE	2μg	2μg	-		-

Fig. 2. Protein analyses. (A) Western blot analysis of cellular proteins. Lysates of TELCeB6 cells transfected with pCXN2Fv4E (lane 1), pCXN2AE (lane 2), pCXN2FE (lane 3), pCXN2 (mock) (lane 4), pCXN2FE.T470H (lane 5), and pCXN2AE.T455P (lane 6) were subjected to Western blotting using anti-gp70 antibody. (B) Western blot analysis of viral proteins. Lysates of viral pellets prepared from TELCeB6 cells transfected with pCXN2Fv4E (lane 1), pCXN2FE (lane 2), pCXN2FE.T470H (lane 3), pCXN2 (mock) (lane 4), pCXN2AE.T455P (lane 5), and pCXN2AE (lane 6) were subjected to Western blotting using anti-gp70 antibody. (C) Immunoprecipitation analysis. COS cells in a well of 6-well plates were transfected with 1 μg of pCXN2Fv4E (lanes 1 and 2), 1 μg of pCXN2FEL (lane 3), 1 μg of pCXN2FEL plus 1 μg of pCXN2Fv4E (lane 4), 1 μg of pCXN2AEL (lane 5), and 1 μg of pCXN2AEL plus 1 μg of pCXN2Fv4E (lane 6), respectively. Lysates of the labeled cells were subjected to immunoprecipitation analysis using anti-gp70 antibody (lane 1) or anti-Lck antibody (lanes 2–6). Cells were labeled for 30 min and only unprocessed Envs were detected. (D) Western blot analysis of coexpressed viral proteins. TELCeB6 cells in T25-flask were cotransfected with 2 μg of pCXN2AE plus 4 μg of pCXN2 (lane 1), 2 μg of pCXN2AE plus 4 μg of pCXN2Fv4 E (lane 2), 2 μg of pCXN2FE plus 4 μg of pCXN2Fv4 E (lane 3), 2 μg of pCXN2FE plus 4 μg of pCXN2 (lane 4), 6 μg of pCXN2 (lane 5), respectively, as shown in the lower panel. Lysates of viral pellets prepared from the supernatants were subjected to Western blotting using the anti-gp70 antibody.

the electrophoresis, the mobility of the processed Fv-4 Env, Fv-4-SU, was similar with that of FMLV Env SU, and slightly faster than that of amphotropic MLV Env SU. Western blot analysis of viral proteins obtained from supernatants of these transfected cells confirmed that all the Envs including Fv-4 Env were incorporated into the virion (Fig. 2B).

To examine interaction between Fv-4 and the wild-type MLV Envs, immunoprecipitation analysis using Lck-tagged FE and AE (FEL and AEL) was performed (Fig. 2C). FEL or AEL expression was not inhibited by Fv-4 Env coexpression. Fv-4 Env was coimmunoprecipitated with Lck-tagged FE by a monoclonal anti-Lck antibody. It was also coimmunoprecipitated with Lck-tagged AE by this antibody. These results indicate interaction of Fv-4 Env with amphotropic MLV Env as well as FMLV Env.

Then, we examined incorporation of coexpressed wild-type and Fv-4 Envs into the virion. In Fig. 2D, the amount of the incorporated SU detected in lane 3, the sum of FE-SU and Fv-4-SU, looked larger than that of the incorporated FE-SU detected in lane 4. Similarly, the sum amount of AE-SU and Fv-4-SU in lane 2 looked larger than the AE-SU amount in lane 1.

3.2. *Inhibitory effect of Fv-4 Env on infectious MLV production*

TELCeB6 cells expressing wild-type MLV Envs produce infectious pseudotyped MLVs bearing the Envs. We then examined the effect of Fv-4 Env coexpression on infectious pseudotyped MLV production from these cells. TELCeB6 cells were cotransfected with pCXN2FE and other expression plasmids at 1:2 molar ratio of the former to the latter, and infectious MLV titers in the supernatants were examined (Fig. 3A). While supernatant from TELCeB6 cells expressing FE.D86K, FE.T470H, AE.T455P, or Fv-4 Env alone did not show infectivity on NIH 3T3 cells (data not shown), the control supernatant from TELCeB6 cells transfected with pCXN2FE (plus mock plasmid) showed a titer of more than 1×10^4 IU (infectious units)/ml. Cotransfection of pCXN2FE.D86K had little effect on the titer, but pCXN2FE.T470H cotransfection resulted in approximately 10-fold decrease in the titer. Inhibitory effect of pCXN2Fv4E-cotransfection was more profound, and the titer of the supernatant from TELCeB6 coexpressing FE and Fv-4 Env was less than 1/100 of the control.

Next, we examined the effect of Fv-4 Env that can interact with AE as well as FE on infectious amphotropic MLV production by measuring infectious MLV titers in supernatants from TELCeB6 cells coexpressing AE and other Envs (Fig. 3B). The control supernatant from TELCeB6 cells transfected with pCXN2AE (plus mock plasmid) showed a titer of more than 1×10^4 IU/ml on NIH 3T3 cells. In contrast, the supernatant from TELCeB6 cells cotransfected with pCXN2AE plus pCXN2Fv4E showed much lower infectious titer, less than 1/50 of the control. These results indicate inhibitory effect of Fv-4 Env on infectious ecotropic and amphotropic MLV production.

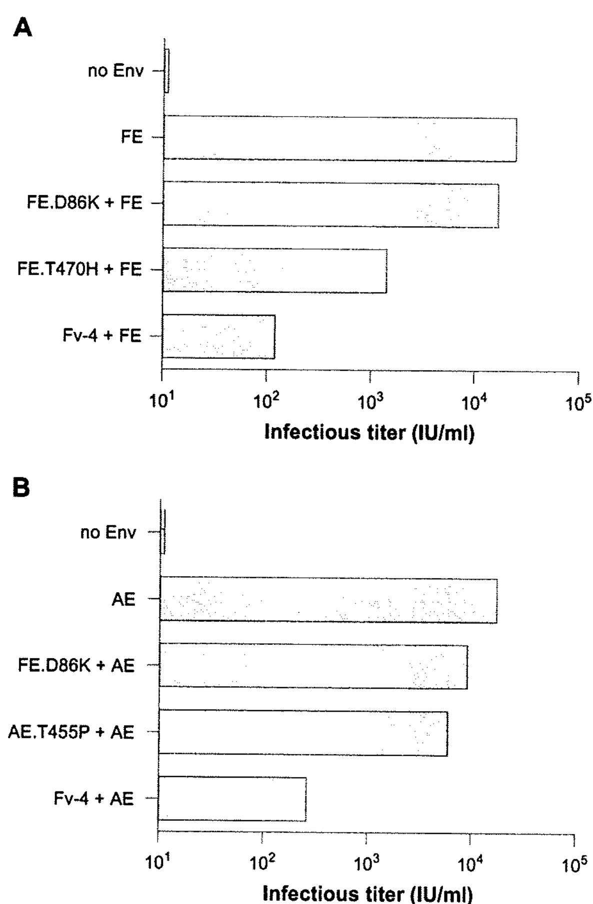


Fig. 3. Infectious viral titers on NIH 3T3 cells of supernatants from TELCeB6 cells coexpressing wild-type and mutant/Fv-4 Envs. (A) TELCeB6 cells were transfected with pCXN2 (no Env) or pCXN2FE (FE) or cotransfected with pCXN2FE and pCXN2FE.D86K (FE.D86K + FE), pCXN2FE.T470H (FE.T470H + FE), or pCXN2Fv4 (Fv-4 + FE) at 1:2 molar ratio of pCXN2FE to mutant/Fv-4 Env-expression plasmid. (B) TELCeB6 cells were transfected with pCXN2 (no Env) or pCXN2AE (AE) or cotransfected with pCXN2AE and pCXN2FE.D86K (FE.D86K + AE), pCXN2AE.T455P (AE.T455P + AE), or pCXN2Fv4 (Fv-4 + AE) at 1:2 molar ratio of pCXN2AE to mutant/Fv-4 Env-expression plasmid. Infectious titers of supernatants from these cells are shown.

We confirmed this inhibitory effect by cotransfection at different molar ratios of pCXN2FE or pCXN2AE to pCXN2Fv4E (Fig. 4). Lower ratio resulted in lower infectious MLV titer in supernatant. Remarkably, cotransfection with pCXN2FE and pCXN2Fv4E at the ratio of 1:3 resulted in decrease in the titer to be undetectable.

3.3. *Inhibitory effect of Fv-4 Env on syncytia formation*

Syncytia are not induced by wild-type MLV Env but by R-deleted Env in NIH 3T3 cells [18,19]. We then examined the effect of Fv-4 Env coexpression on syncytia formation by R-deleted FE (Fig. 5A). In NIH 3T3 cells, coexpression of FE with R-deleted FE induced larger number of syncytia than those by the R-deleted FE alone, whereas coexpression of FE.T470H resulted in decrease in the number. Fv-4 Env coexpression showed larger inhibitory effect on syncytia formation by R-deleted FE.

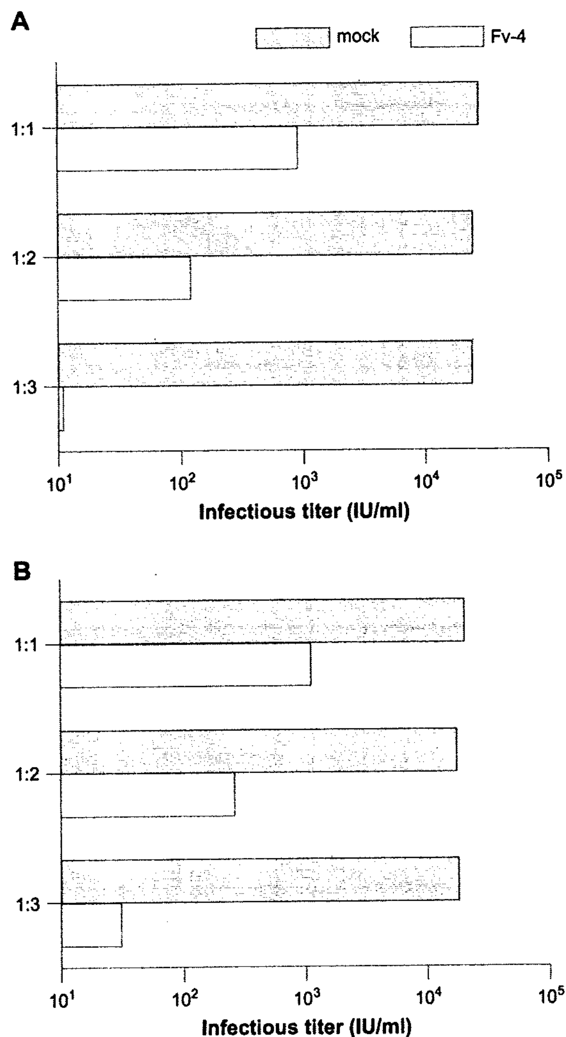


Fig. 4. Infectious viral titers on NIH 3T3 cells of supernatants from TELCeB6 cells coexpressing wild-type and Fv-4 Envs at different molar ratios. TELCeB6 cells were cotransfected with pCXN2FE (A) or pCXN2AE (B) and pCXN2 (mock) or pCXN2Fv4E (Fv-4) at different molar ratios (1:1, 1:2, and 1:3). Infectious titers of supernatants from these cells are shown.

We also examined the effect of Fv-4 Env coexpression on syncytia formation by R-deleted AE (Fig. 5B). Coexpression of AE with R-deleted AE resulted in decrease in syncytia number, and syncytia formation was impaired largely by coexpression of AE.T455P. Coexpression of FE induced larger number of syncytia while FE.T470H coexpression induced similar number compared with the control induced by R-deleted AE alone. Syncytia number induced by coexpression with Fv-4 Env and R-deleted AE was less than one-third of the control. Thus, Fv-4 Env coexpression impaired syncytia formation induced by R-deleted amphotropic as well as ecotropic MLV Envs.

4. Discussion

In the present study, Fv-4 Env coexpression resulted in reduction of infectious MLV titer in supernatant from TELCeB6 cells expressing the wild-type FMLV Env, indicating inhibitory effect of Fv-4 Env on infectious FMLV production from FMLV-infected cells. This may contribute to efficient resistance against ecotropic MLV infection in *Fv-4*-expressing mice. Interestingly, our results also indicate inhibitory effect of Fv-4 Env on infectious amphotropic MLV production, while this effect on amphotropic MLV replication may be limited [6].

Fv-4 Env was shown to be processed and incorporated into MLV virion as reported previously [30], and was indicated to interact with the wild-type Env to form hetero-oligomers. Thus, it can be speculated that TELCeB6 cells cotransfected with pCXN2FE and pCXN2Fv4E expressed FE homo-oligomers, Fv-4 Env homo-oligomers, and FE-Fv-4 Env hetero-oligomers and produced pseudotyped MLVs bearing these oligomers. FE homo-oligomers are functional, whereas Fv-4 Env oligomers are defective and unable to mediate viral entry. Large reduction in infectious titer of MLVs bearing FE and Fv-4 Env (Fig. 3A) suggests reduction in functional Env oligomers on the virion, implying that FE-Fv-4 Env hetero-oligomers are non-functional or unable to mediate viral entry efficiently. Our result in Fig. 2D indicates that TELCeB6 cells cotransfected with pCXN2FE and pCXN2Fv4E produced MLVs bearing both FE and Fv-4 Env, but this does not exclude a possibility of partial reduction in incorporation efficiency of FE-Fv-4 Env hetero-oligomers that may contribute to reduction in infectious MLV titer. Suppression of R(−)FE-mediated syncytia formation by Fv-4 Env coexpression suggests impairment of fusion function of these hetero-oligomers. Thus, Fv-4 Env can exert dominant negative effect on wild-type MLV function. The postbinding defective FE.T470H also suppressed infectious MLV production, but Fv-4 Env showed more profound inhibitory effect. This implies possible contribution of multiple determinants in Fv-4 *env* to this dominant negative effect, consistent with a previous report indicating contribution of Fv-4 SU as well as Fv-4 TM to the defect in Fv-4 Env function [10,30].

Binding of the defective Fv-4 Env to the ecotropic MLV receptor may contribute to the reduction in infectious titer of pseudotyped MLVs bearing FE and Fv-4 Env by interfering with FE binding to the receptor. Our results, however, revealed inhibitory effect of Fv-4 Env on infectious amphotropic MLV production and syncytia formation by R-deleted amphotropic MLV Env, confirming dominant negative effect of Fv-4 Env on wild-type MLV Env function.

In syncytia assay (Fig. 5), FE-coexpression with R(−)FE increased syncytia levels as compared with the mock control, reflecting larger numbers of fusion-competent oligomers (R[−]FE homo-oligomers and FE-R[−]FE hetero-oligomers) in the former. On the contrary, AE-coexpression with R(−)AE decreased syncytia levels as compared with the mock control. The discrepancy may be due to less fusion-activity of AE-R(−)AE hetero-oligomers.

Mice carrying the *Fv-4* gene show extremely efficient resistance to Friend virus infection [2,5]. While involvement of immune responses in the resistance have been indicated [8,9], the receptor interference by Fv-4 Env has been indicated to play a central role in the *Fv-4*-mediated resistance. However, the

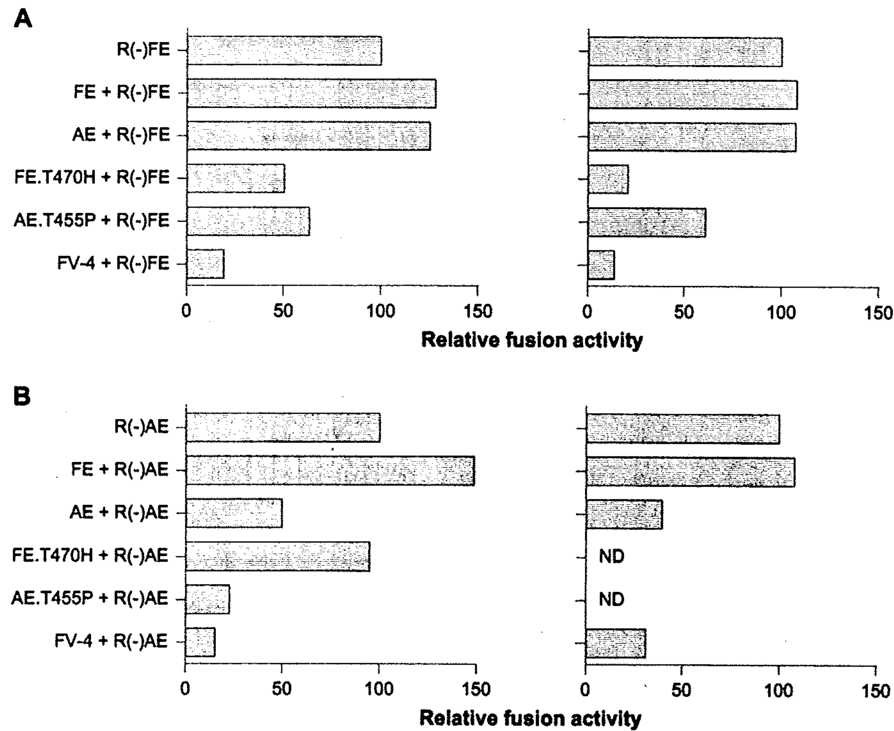


Fig. 5. Syncytia assay on NIH 3T3 cells. NIH 3T3 cells in a well of 24-well plates were cotransfected with 0.02 μg of pCXN2R(−)FE plus 0.04 μg of DNAs expressing the indicated Envs (A) or with 0.1 μg of pCXN2R(−)AE plus 0.2 μg of DNAs expressing the indicated Envs (B), respectively. Syncytia level is shown as a relative value compared to that obtained by mock-cotransfection (R[−]FE in [A] and R[−]AE in [B]) which is rated as 100. Two sets of experiments are shown. ND, not determined.

Fv-4 Env-mediated receptor interference does not result in complete block of FMLV entry into the cells in vitro [5,6]. The mechanism indicated in the present study, Fv-4 Env-mediated interference with the wild-type Env function would reinforce the resistance against Friend virus infection in mice carrying the *Fv-4* gene.

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References

[1] S. Suzuki, *Fv-4*; a new gene affecting the splenomegaly induction by Friend leukemia virus, Jpn. J. Exp. Med. 45 (1975) 473–478.
[2] H. Ikeda, T. Odaka, Cellular expression of murine leukemia virus gp70-related antigen on thymocytes of uninfected mice correlates with *Fv-4* gene-controlled resistance to Friend leukemia virus infection, Virology 128 (1983) 127–139.
[3] H. Ikeda, F. Laigret, M.A. Martin, R. Repaske, Characterization of a molecularly cloned retroviral sequence associated with *Fv-4* resistance, J. Virol. 55 (1985) 768–777.
[4] H. Ikeda, K. Kato, T. Suzuki, H. Kitani, Y. Matsubara, S. Takase-Yoden, R. Watanabe, M. Kitagawa, S. Aizawa, Properties of the naturally occurring soluble surface glycoprotein of ecotropic murine leukemia virus: binding specificity and possible conformational change after binding to receptor, J. Virol. 74 (2000) 1815–1826.
[5] H. Yoshikura, T. Odaka, Resistance of G mice to murine leukemia virus infection: apparent disparity in in vivo and in vitro resistances, J. Natl. Cancer Inst. 61 (1978) 461–463.
[6] H. Yoshikura, Y. Naito, K. Moriwaki, Unstable resistance of G mouse fibroblasts to ecotropic murine leukemia virus infection, J. Virol. 29 (1979) 1078–1086.
[7] M. Kitagawa, S. Aizawa, H. Kamisaku, H. Ikeda, K. Hirokawa, T. Sado, Cell-free transmission of *Fv-4* resistance gene product controlling Friend leukemia virus-induced leukemogenesis: a unique mechanism for interference with viral infection, Blood 86 (1995) 1557–1563.
[8] K. Higo, Y. Kubo, Y. Iwatani, T. Ono, M. Maeda, H. Hirai, T. Masuda, K. Kuribayashi, F. Zhang, T.Y. Lamin, A. Adachi, A. Ishimoto, Susceptibility of nude mice carrying the *Fv-4* gene to Friend murine leukemia virus infection, J. Virol. 71 (1997) 750–754.
[9] F. Zhang, L.T. Ya, Y. Iwatani, K. Higo, Y. Suzuki, M. Tanaka, T. Nakahara, T. Ono, H. Sakai, K. Kuribayashi, A. Ishimoto, Resistance to Friend murine leukemia virus infection conferred by the *Fv-4* gene is recessive but appears dominant from the effect of the immune system, J. Virol. 74 (2000) 6193–6197.
[10] G.M. Taylor, Y. Gao, D.A. Sanders, *Fv-4*: identification of the defect in Env and the mechanism of resistance to ecotropic murine leukemia virus, J. Virol. 75 (2001) 11244–11248.
[11] E. Hunter, Viral entry and receptors, in: J.M. Coffin, S.H. Hughes, H.E. Varmus (Eds.), Retroviruses, Cold Spring Harbor Laboratory Press, New York, 1997, pp. 71–120.

[12] J.M. Heard, O. Danos, An amino-terminal fragment of the Friend murine leukemia virus envelope glycoprotein binds the ecotropic receptor, J. Virol. 65 (1991) 4026–4032.
[13] R.A. Morgan, O. Nussbaum, D.D. Muenchau, L. Shu, L. Couture, W.F. Anderson, Analysis of the functional and host range-determining regions of the murine ecotropic and amphotropic retrovirus envelope proteins, J. Virol. 67 (1993) 4712–4721.
[14] J.L. Battini, O. Danos, J.M. Heard, Receptor-binding domain of murine leukemia virus envelope glycoproteins, J. Virol. 69 (1995) 713–719.
[15] D. Fass, R.A. Davey, C.A. Hamson, P.S. Kim, J.M. Cunningham, J.M. Berger, Structure of a retrovirus receptor-binding glycoprotein: murine leukemia virus SU domain at 2.0 angstrom resolution, Science 277 (1997) 1662–1666.
[16] E. Hunter, R. Swanstrom, Retrovirus envelope glycoproteins, Curr. Top. Microbiol. Immunol. 157 (1990) 187–253.
[17] N. Green, T.M. Shinnick, O. Witte, A. Ponticelli, J.G. Sutcliffe, R.A. Lerner, Sequence-specific antibodies show that maturation of Moloney leukemia virus envelope polyprotein involves removal of a COOH-terminal peptide, Proc. Natl. Acad. Sci. USA 78 (1981) 6023–6027.
[18] A. Rein, J. Mirro, J.G. Haynes, S.M. Ernst, K. Nagashima, Function of the cytoplasmic domain of a retroviral transmembrane protein: p15E-p2E cleavage activates the membrane fusion capability of the murine leukemia virus Env protein, J. Virol. 68 (1994) 1773–1781.
[19] J.A. Ragheb, W.F. Anderson, pH-independent murine leukemia virus ecotropic envelope-mediated cell fusion: implications for the role of the R peptide and p12E TM in viral entry, J. Virol. 68 (1994) 3220–3231.
[20] W. Koch, G. Hunsmann, R. Friedrich, Nucleotide sequence of the envelope gene of Friend murine leukemia virus, J. Virol. 45 (1983) 1–9.
[21] D.E. Ott, R. Friedrich, A. Rein, Sequence analysis of amphotropic and 10A1 murine leukemia viruses: close relationship to mink cell focus-inducing viruses, J. Virol. 64 (1990) 757–766.
[22] H. Niwa, K. Yamamura, J. Miyazaki, Efficient selection for high-expression transfectants with a novel eukaryotic vector, Gene 108 (1991) 193–199.
[23] H. Nakamura, A. Takeda, T. Matano, Postbinding fusion function contributed by a chimeric murine leukemia virus envelope protein, Arch. Virol. 146 (2001) 953–961.
[24] A.J. MacKrell, N.W. Soong, C.M. Curtis, W.F. Anderson, Identification of a subdomain in the Moloney murine leukemia virus envelope protein involved in receptor binding, J. Virol. 70 (1996) 1768–1774.
[25] Y. Zhao, S. Lee, W.F. Anderson, Functional interactions between monomers of the retroviral envelope protein complex, J. Virol. 71 (1997) 6967–6972.
[26] J.D. Marth, R. Peet, E.G. Krebs, R.M. Perlmutter, A lymphocyte-specific protein-tyrosine kinase gene is rearranged and overexpressed in the murine T cell lymphoma LSTRA, Cell 43 (1985) 393–404.
[27] T. Matano, T. Odawara, M. Ohshima, A. Iwamoto, H. Yoshikura, Interaction between the dominant negative mutant and the wild-type envelope proteins of Friend murine leukemia virus, J. Virol. 68 (1994) 6079–6082.
[28] F.-L. Cosset, Y. Takeuchi, J.L. Battini, R.A. Weiss, M.K.L. Collins, High-titer packaging cells producing recombinant retroviruses resistant to human serum, J. Virol. 69 (1995) 7430–7436.
[29] T. Matano, T. Odawara, M. Ohshima, H. Yoshikura, A. Iwamoto, Trans-dominant interference with virus infection at two different stages by a mutant envelope protein of Friend murine leukemia virus, J. Virol. 67 (1993) 2026–2033.
[30] M. Masuda, H. Yoshikura, Construction and characterization of the recombinant Moloney murine leukemia viruses bearing the mouse *Fv-4 env* gene, J. Virol. 64 (1990) 1033–1043.

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ORIGINAL PAPER

Copy number variations of *CCL3L1* and long-term prognosis of HIV-1 infection in asymptomatic HIV-infected Japanese with hemophilia

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Abstract We set up a cohort of HIV-infected, asymptomatic Japanese patients with hemophilia for follow-up study in 1995. All subjects who had been infected with HIV-1 for more than 10 years met the criteria for long-term nonprogressors (LTNPs) at the time of entry; however, some of them later developed lymphopenia and required antiretroviral treatment during five more years of observation. In this study, we investigated the impacts of the *CCL3L1* dose on the long-term prognosis in the subjects with chronic HIV-1 infection. We collected genomic DNA from 95 long-term survivors including 48 nonprogressors and 47 subjects receiving antiretroviral treatment. The distributions of *CCL3L1* copy number significantly differed between the 95 HIV-1-infected subjects with hemophilia and 205 controls. Average copy number of *CCL3L1* in the HIV-1-infected subjects was significantly lower than in control (5.00±0.22 vs 3.35±0.24, $p<0.001$). Moreover, the subjects possessing two or less copies of *CCL3L1* had significantly higher risk of acquiring HIV-1. However, *CCL3L1* copy number variations had no significant effect on the disease progression among the LTNP subjects who had been afflicted with chronic HIV-1 infection for more than 15 years, when compared between nonprogressors and patients under treatment (3.68±0.37 vs 3.02±0.29, ns). Furthermore, variations in the *CCL3L1* copy number had little effect on the levels of HIV-1 load among them. We conclude that variation in the *CCL3L1* copy number is apparently not a factor that determines the prognosis of chronic HIV-1 infection, even though it is linked to HIV-1 susceptibility.

Keywords HIV-1 infection · HIV-1 susceptibility · Long-term nonprogressors · *CCL3L1*

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Introduction

Worldwide, more than 50 million people have become infected with HIV-1 in the past 25 years, and a third of them have died (UNAIDS 2006). However, the clinical course of HIV-1 infection varies considerably from person to person. Some patients rapidly progress to acquired immunodeficiency syndrome (AIDS), while others spontaneously achieve viral control and relative immunologic stability. Much attention has been given to the small proportion of people, called "long-term nonprogressors" (LTNPs; Pantaleo et al. 1995; Hogan and Hammer 2001; Muñoz et al. 1995), who maintain sufficient CD4+T cell numbers not to develop symptoms for many years after infection. Understanding the mechanisms underlying the delayed disease progression has been one of the major areas of investigation for AIDS researchers over the last decade.

Several factors are linked to the delayed progression of HIV-1/AIDS. The main correlate with delayed progression is low viral burden (Henrard et al. 1995; Mellors et al. 1997), which probably reflects an effective immunologic response by HIV-1-specific CD4+T cells in the early course of infection. Thus, host genetic factors that determine the immune response to HIV-1 could account for the delayed disease progression. Some insights into the genetic determinants of HIV-1/AIDS susceptibility have come from studies of LTNPs. One of them involves genetic variations in chemokine receptor 5 (*CCR5*). Sequence variations in *CCR5*, which result in reduced or absent cell-surface expression of CCR5, decrease the susceptibility to HIV-1 infection (Dragic et al. 1996; McDermott et al. 1998; Ioannidis et al. 2001).

Recently, copy number variations in CC chemokine ligand 3-like 1 (*CCL3L1*) have also been reported to be linked to the susceptibility to HIV-1 infection (Gonzalez et al. 2005). CCL3L1, also known as macrophage-inflammatory protein 1α (MIP-1αP) or LD78β, is a natural ligand for the HIV-1 coreceptor CCR5 (Menten et al. 2002). *CCL3L1* exhibits copy number variations on chromosome 17q11.2 (Hirashima et al. 1992; Townson et al. 2002), and the possession of a lower copy number of *CCL3L1* is associated with markedly enhanced HIV/AIDS susceptibility. However, Shao et al. (2007) could not replicate the association in adolescents, and it remains controversial.

In an effort to understand the nature of protective immunity to HIV-1 infection, considerable attention has been given to the small proportion of people, LTNPs. Although called "LTNPs", many of these patients do eventually progress to disease, and it is important to identify the genetic factors linked to the prognosis and outcome under long-term chronic HIV-1 infection. In 1995, we set up a cohort of HIV-infected, asymptomatic patients with hemophilia for a follow-up study (Munkanta et al. 2005). All subjects who had been infected with HIV-1 for more than 10 years met the criteria for LTNPs at the time of entry; however, some of them later developed lymphopenia and required antiretroviral treatment during a further 5 years of observation. This cohort was deemed to be suitable to evaluate the genetic factors to determine the prognosis under chronic HIV-1 infection. In this study, we investigated the impact of *CCL3L1* dose on the long-term prognosis of chronic HIV-1 infection.

Materials and methods

Subjects

The protocol for the present study was approved by the Ethics Review Board of the Medical Research Institute of Tokyo Medical and Dental University as well as all of the hospitals in which the samples were taken. Upon the set-up of the cohort of HIV-1-infected Japanese patients with hemophilia in 1995, all patients had been infected for longer than 10 years but were nevertheless asymptomatic without any antiviral measures. Blood samples were collected from 95 well-characterized patients who were selected from the cohort after obtaining written informed consent. At the time of sample collection, 48 of them were still asymptomatic and maintained their CD4+T cells at a level of no less than 200/mm^3, but the rest of the 47 subjects were under antiretroviral treatment (HAART) because of a reduction of the CD4 count to beneath this level (less than 200/mm^3). Quantification of viral RNA in the plasma was evaluated by the Roche Amplicor version 1.5 assay (Roche Diagnostics, NJ). Controls were obtained from a random sampling of healthy volunteers (n=205). DNA was prepared from the blood sample by the use of the QuickGene DNA whole blood kit S (FUJIFILM, Japan).

Estimation of the copy number of *CCL3L1* in the genome

The copy number of *CCL3L1* in the genome was estimated according to the method of Gonzalez et al. (2005), with a few modifications. Briefly, real-time polymerase chain reaction (PCR) was performed by using a Bio-Rad iCycler iQ Real-Time PCR Detection Systems (Bio-Rad) detecting the emitted 6-carboxyfluorescein (FAM) fluorescence from the probe for *CCL3L1* and the probe for the *β-globin* gene (*HBB*) during amplification. The *CCL3L1* primer sequences were as follow: sense primer 5′-TGCCTATCTCCGTCTAGAGAGCTT-3′; antisense primer 5′-AGAAGGAGGCAGCAGGACACT-3′; probe 5′-FAM-TGACTCCAGGCAAGGG-MGB. The *HBB* primer sequences were: sense primer 5′-GGCAACCCTAAGGTGAAGGC-3′; antisense 5-GGTGAGCCAGGCCATCACTA-3′; probe 5′-FAM-CATGGCAAGAAAGTGCTCGGTGCCT-TAMRA (Applied Biosystems).

The cycle number at which the fluorescence reached a fixed threshold, termed the cycle threshold (CT), was determined. Eight serial 1:2 dilutions (100–0.78 ng/reaction) of genomic DNA having four copies of *CCL3L1* were used to generate standard curves of the CT value for *HBB* and for the *CCL3L1* gene. For each test sample, at least duplicate wells were set up for *CCL3L1* and *HBB*, the CT as determined, and converted into template quantity using the standard curves. The copy number is the ratio of the template quantity for *CCL3L1* to the template quantity for *HBB* multiplied by four.

Southern blotting

CCL3L1 copy numbers for the standard DNA sample and several additional DNA samples were evaluated by standard Southern blotting and a hybridization method (Hirashima et al. 1992) using AlkPhos Direct Labelling and Detection System (GE Healthcare, UK).

Statistical analysis

All statistical analyses in this study were performed using GraphPad InStat version 3.06 for Windows (GraphPad Software, CA).

Results

Copy number variations of CCL3L1 and HIV-1/AIDS susceptibility in HIV-1-infected Japanese with hemophilia

We evaluated the copy number of *CCL3L1* in 95 asymptomatic HIV-1-infected Japanese with hemophilia and 205 controls. All 95 asymptomatic subjects were classified into LTNPs at the entry. The copy number was estimated by the quantitative ratio of *CCL3L1* to *HBB* in the template genomic DNA. The quantities of *CCL3L1* and *HBB* in the template genomic DNA were evaluated by the quantitative TaqMan PCR method (Fig. 1).

The distributions of the copy number of *CCL3L1* were different between the HIV-1-infected subjects with hemophilia and controls (Fig. 2). The subjects with a low copy number were more common among the HIV-1-infected subjects with hemophilia than in the control individuals. The average copy number of *CCL3L1* in the HIV-1-infected subjects with hemophilia was significantly lower than in the control (5.00±0.22 vs 3.35±0.24, $p<0.001$ by unpaired *t* test). The average copy number in the control was almost identical to that reported by Gonzalez et al. (2005). Moreover, the subjects possessing two or less copies of *CCL3L1*, who had a markedly enhanced risk of acquiring HIV-1 (Table 1), were more common among the HIV-1-infected subjects with hemophilia (Table 2).

Copy number variations of CCL3L1 and long-term prognosis of HIV-1 infection

Ninety five HIV-1-infected subjects with hemophilia were divided into two groups based on the clinical courses during further 5 years of follow-up; one with 48 subjects who maintained their CD4+T cell counts at a certain level (no less than 200/mm^3) without antiretroviral treatment (nonprogressor) and the other with 47 subjects who required antiretroviral treatment due to the depletion of CD4+T cells beneath this level (slow progressor). According to the AIDS surveillance case definitions (CDC 1992), individuals in the first group were categorized as A1 or A2 and the second group as A3. When compared, there was no significant difference in the distribution of the copy number of *CCL3L1* (Fig. 2). The average copy number of *CCL3L1* in nonprogressors was 3.68±0.37 and 3.02±0.29 in the patients undergoing antiretroviral treatment.

Copy number variations of CCL3L1 and HIV-1 load

The data for HIV-1 load after 7 to 8 years of observation period were available from 74 of the 95 subjects. There was no correlation between the *CCL3L1* copy number variations and HIV-1 load (data not shown). Moreover, the copy number of *CCL3L1* had little effect on the distribution of HIV-1 load in nonprogressors or slow progressors (Fig. 3). The levels of HIV-1 load from 28 subjects, 7 nonprogressors and 21 slow progressors, were under the detectable

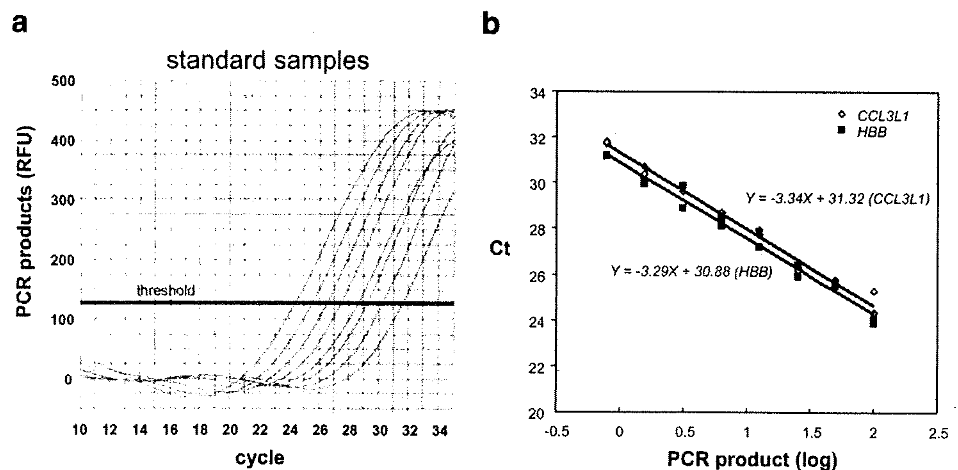

Fig. 1 **a** The amplification curves obtained using eight (1:2) serial dilutions of standard human genomic DNA with four copies of *CCL3L1* per diploid genome ranging from 100 to 0.56 ng. **b** The standard curves with the slope and the Pearson correlation coefficient (R^2) for CCL3L1 (*open diamonds*) and HBB(*closed squares*)

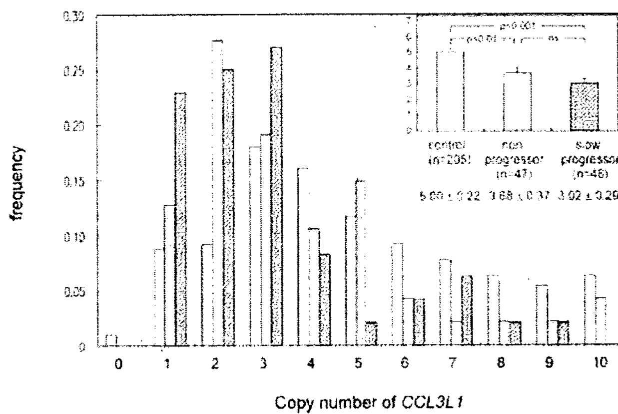

Fig. 2 The distribution of the copy number of *CCL3L1* in the HIV-1-infected asymptomatic subjects with no treatment (n=48), the HIV-1-infected asymptomatic subjects with antiretroviral treatment (n=47), and controls (n=205)

level; however, the rate of them in nonprogressors [0.133 (2/15) in CCL1L1≤2 vs 0.217(5/23) in CCL3L1>2, ns] and slow progressors [0.556(10/18) in CCL1L1≤2 vs 0.217 (11/18) in CCL3L1>2, ns] was not significantly different by the copy number of *CCL3L1*.

Discussion

Much attention has been paid to the small proportion of people, LTNPs, who maintain normal CD4+T cell numbers and remain symptom free for many years after infection. Many of these patients do eventually progress to disease. In our cohort, about half of the subjects who met the LTNP criteria at the time of entry also developed lymphopenia and required antiretroviral treatment during a further 5 years of

Table 1 Copy number of *CCL3L1* and risk of acquiring HIV-1 when compared between asymptomatic HIV-infected Japanese (n=95) with hemophilia and controls (n=205)

CCL3L1 copy number	Odds ratio	95% CI	Chi-square test with Yates correction	p-value
1	2.26	1.11–4.62	4.39	0.036*
2	3.50	1.81–6.74	13.74	0.0002**
3	1.37	0.76–2.48	0.77	0.379
4	0.55	0.25–1.19	1.85	0.174
5	0.69	0.30–1.61	0.43	0.511
6	0.43	0.14–1.30	1.69	0.194
7	0.52	0.17–1.60	0.83	0.362
8	0.32	0.07–1.44	1.64	0.200
9	0.38	0.08–1.75	0.97	0.324
10	0.32	0.07–1.44	1.64	0.200

*Significant association, p>0.05
**Significant association, p>0.001

Table 2 Copy number of *CCL3L1* among LTNPs (n=95) and controls (n=205)

	Control	LTNPs	
Mean of CCL3L1 copy number	5.00±0.22	3.35±0.25	p<0.001[a]
The rate of the subjects with CCL3L1≤2	0.190 (39/205)	0.389 (37/95)	p=0.0004 (χ^2=12.589)[b]

[a] Evaluated by unpaired t test
[b] Evaluated by chi-square test with Yates correction

observation. It is urgent to identify the genetic factors which determine the prognostic outcome of the patients afflicted with chronic HIV-1 infection, especially because of the recent dramatic increase in the number of such patients thanks to the remarkable progress of antiretroviral treatment. In this study, we investigated the impact of *CCL3L1* dose on this long-term prognosis.

Gonzalez et al. (2005) have reported the copy number variations of *CCL3L1* to be linked to the susceptibility to HIV-1 infection. Possession of a low *CCL3L1* copy number was found to be a determinant of enhanced HIV-1 susceptibility among the study population. Our study replicated these results in the subjects in our study with hemophilia. The subjects with a low *CCL3L1* copy number were more common among the HIV-1-infected subjects with hemophilia, and the subjects possessing two or fewer copies of *CCL3L1* had a higher risk of acquiring HIV-1. Independent studies have shown that an increased copy number of *CCL3L1* results in the enhanced secretion of CCL3L1 by activated leukocytes (Gonzalez et al. 2005;

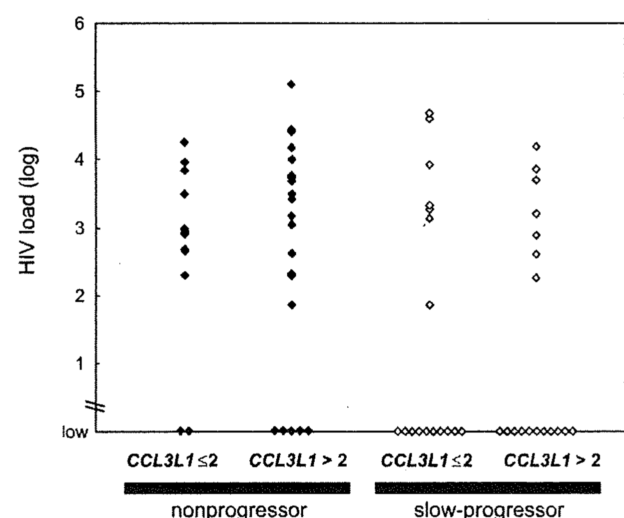

Fig. 3 Copy number of *CCL3L1* and HIV-1 load. *Low* means the plasma viral RNA levels under the detectable level by the Roche Amplicor version 1.5 assay (Roche Diagnostics, NJ)

Townson et al. 2002), so that altering the genetic expression of *CCL3L1* through copy number variations might contribute to the susceptibility to HIV-1 infection. It is reasonable that the reduced expression of *CCL3L1* might be associated with enhanced HIV-1 susceptibility because CCL3L1, which is the most potent known ligand for HIV-1 coreceptor CCR5, is a dominant HIV-1-suppressive chemokine (Menten et al. 2002).

In addition to influencing HIV-1 acquisition, Gonzalez et al. (2005) have reported the association of these variations with prognosis in HIV-1-infected patients. A lower *CCL3L1* dose was associated with an increased risk of progressing rapidly to AIDS or death. *CCL3L1* copy number was also associated with the viral set point and rate of change in CD4+T cell counts in a dose-dependent manner, which are well-established predictors of clinical outcome (Henrard et al. 1995; Mellors et al. 1997). Blocking of the interaction between HIV-1 glycoprotein 120 and CCR5 and/or the immune reaction against HIV through CCL3L1 itself might be related to the *CCL3L1* dose-dependent protective mechanism. However, we could not identify significant differences in the distribution of the *CCL3L1* copy number variations between the two groups with chronic HIV-1 infection, the one which maintained their CD4+T cells with no antiretroviral treatment and the other which received antiretroviral treatment due to the reduction of CD4+T cells. *CCL3L1* copy number variations had no significant effects on disease progression among subjects with chronic HIV-1 infection of more than 15 years. Furthermore, *CCL3L1* copy number variations had little effect on the levels of HIV-1 load in asymptomatic HIV-1-infected subjects with hemophilia. A possible explanation of this inconsistency is that the copy number of *CCL3L1* was associated with an effective immunologic response in the acute phase of HIV-1 infection but not chronic phase. Some patients with asymptomatic chronic HIV-1 infection nevertheless exhibit high rates of HIV-1 replication and destruction of CD4+T cells daily (Wei et al. 1995; Ho et al. 1995). Cell death and replacement are in near balance during the chronic phase of the illness in these patients. Thus, the maintenance of a steady state of cell counts and the viral load seems to be tightly linked to the long-term prognosis under chronic HIV-1 infection. It is unlikely that the *CCL3L1* dose would play a crucial role in maintaining this balance of HIV-1 replication with the destruction of CD4+T cells during the chronic phase. Further analyses are required to clarify the specific functional role of *CCL3L1* on the chronic phase of HIV-1 infection.

We conclude that the copy number variation of *CCL3L1* appears not to be a determining factor in the prognosis of chronic HIV-1 infection, even though it is linked to HIV-1 infection susceptibility. Our studies also suggest that the genetic factors which do determine the prognosis of HIV-1/AIDS may be different between the acute and chronic phases.

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References

CDC (1992) 1993 Revised classification system for HIV infection and expanded surveillance case definition for AIDS among adolescents and adults. MMWR 41(no. RR-17)

Dragic T, Litwin V, Allaway GP, Martin SR, Huang Y, Nagashima KA, Cayanan C, Maddon PJ, Koup RA, Moore JP, Paxton WA (1996) HIV-1 entry into CD4+cells is mediated by the chemokine receptor CC-CKR-5. Nature 381(6584):667–673

Gonzalez E, Kulkarni H, Bolivar H, Mangano A, Sanchez R, Catano G, Nibbs RJ, Freedman BI, Quinones MP, Bamshad MJ, Murthy KK, Rovin BH, Bradley W, Clark RA, Anderson SA, O, connell RJ, Agan BK, Ahuja SS, Bologna R, Sen L, Dolan MJ, Ahuja SK (2005) The influence of CCL3L1 gene-containing segmental duplications on HIV-1/AIDS susceptibility. Science 307 (5714):1434–1440

Henrard DR, Phillips JF, Muenz LR, Blattner WA, Wiesner D, Eyster ME, Goedert JJ (1995) Natural history of HIV-1 cell-free viremia. JAMA 274(7):554–558

Hirashima M, Ono T, Nakao M, Nishi H, Kimura A, Nomiyama H, Hamada F, Yoshida MC, Shimada K (1992) Nucleotide sequence of the third cytokine LD78 gene and mapping of all three LD78 gene loci to human chromosome 17. DNA Seq 3(4):203–212

Ho DD, Neumann AU, Perelson AS, Chen W, Leonard JM, Markowitz M (1995) Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature 373(6510):123–126

Hogan CM, Hammer SM (2001) Host determinants in HIV infection and disease. Part 2: genetic factors and implications for antiretroviral therapeutics. Ann Intern Med 134(10):978–996

Ioannidis JP, Rosenberg PS, Goedert JJ, Ashton LJ, Benfield TL, Buchbinder SP, Coutinho RA, Eugen-Olsen J, Gallart T, Katzenstein TL, Kostrikis LG, Kuipers H, Louie LG, Mallal SA, Margolick JB, Martinez OP, Meyer L, Michael NL, Operskalski E, Pantaleo G, Rizzardi GP, Schuitemaker H, Sheppard HW, Stewart GJ, Theodorou ID, Ullum H, Vicenzi E, Vlahov D, Wilkinson D, Workman C, Zagury JF, O'Brien TR (2001) Effects of CCR5-Delta32, CCR2-64I, and SDF-1 3'A alleles on HIV-1 disease progression: an international meta-analysis of individual-patient data. Ann Intern Med 135(9):782–795

McDermott DH, Zimmerman PA, Guignard F, Kleeberger CA, Leitman SF, Murphy PM (1998) CCR5 promoter polymorphism and HIV-1 disease progression. Multicenter AIDS Cohort Study (MACS). Lancet 352(9131):866–870

Mellors JW, Munoz A, Giorgi JV, Margolick JB, Tassoni CJ, Gupta P, Kingsley LA, Todd JA, Saah AJ, Detels R, Phair JP, Rinaldo CR

Jr (1997) Plasma viral load and CD4+lymphocytes as prognostic markers of HIV-1 infection. Ann Intern Med 126(12):946–954
Menten P, Wuyts A, Van Damme J (2002) Macrophage inflammatory protein-1. Cytokine Growth Factor Rev 13(6):455–481
Munkanta M, Terunuma H, Takahashi M, Hanabusa H, Miura T, Ikeda S, Sakai M, Fujii T, Takahashi Y, Oka S, Matsuda J, Ishikawa M, Taki M, Takashima Y, Mimaya J, Ito M, Kimura A, Yasunami M (2005) HLA-B polymorphism in Japanese HIV-1-infected long-term surviving hemophiliacs. Viral Immunol 18(3):500–505
Muñoz A, Kirby AJ, He YD, Margolick JB, Visscher BR, Rinaldo CR, Kaslow RA, Phair JP (1995) Long-term survivors with HIV-1 infection: Incubation period and longitudinal patterns of CD4+lymphocytes. J Acquir Immune Defic Syndr Human Retrovirol 8(5):496–505
Pantaleo G, Menzo S, Vaccarezza M, Graziosi C, Cohen OJ, Demarest JF, Montefiori D, Orenstein JM, Fox C, Schrager LK, Margolick JB, Buchbinder S, Giorgi JV, Fauci AS (1995) Studies in subjects with long-term nonprogressive human immunodeficiency virus infection. N Engl J Med 332(4):209–216
Shao W, Tang J, Song W, Wang C, Li Y, Wilson CM, Kaslow RA (2007) CCL3L1 and CCL4L1: variable gene copy number in adolescents with and without human immunodeficiency virus type 1 (HIV-1) infection. Genes Immun 8 (3):224–231
Townson JR, Barcellos LF, Nibbs RJ (2002) Gene copy number regulates the production of the human chemokine CCL3-L1. Eur J Immunol 32(10):3016–3026
UNAIDS (2006) 2006 Report on the global HIV/AIDS epidemic. Geneva: Joint United Nations Program on HIV/AIDS
Wei X, Ghosh SK, Taylor ME, Johnson VA, Emini EA, Deutsch P, Lifson JD, Bonhoeffer S, Nowak MA, Hahn BH, Saag MS, Shaw GM (1995) Viral dynamics in human immunodeficiency virus type 1 infection. Nature 373(6510):117–122

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Original article

Abrogation of AIDS vaccine-induced cytotoxic T-lymphocyte efficacy in vivo due to a change in viral epitope flanking sequences

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Abstract

A current promising AIDS vaccine strategy is to elicit $CD8^+$ cytotoxic T lymphocyte (CTL) responses that broadly recognize highly-diversified HIVs. In our previous vaccine trial eliciting simian immunodeficiency virus (SIV) mac239 Gag-specific CTL responses, a group of Burmese rhesus macaques possessing a major histocompatibility complex haplotype *90-120-Ia* have shown vaccine-based viral control against a homologous SIVmac239 challenge. Vaccine-induced $Gag_{206-216}$ epitope-specific CTL responses exerted strong selective pressure on the virus in this control. Here, we have evaluated in vivo efficacy of vaccine-induced $Gag_{206-216}$-specific CTL responses in two *90-120-Ia*-positive macaques against challenge with a heterologous SIVsmE543-3 that has the same $Gag_{206-216}$ epitope sequence with SIVmac239. Despite efficient $Gag_{206-216}$-specific CTL induction by vaccination, both vaccinees failed to control SIVsmE543-3 replication and neither of them showed mutations within the $Gag_{206-216}$ epitope. Further analysis indicated that $Gag_{206-216}$-specific CTLs failed to show responses against SIVsmE543-3 infection due to a change from aspartate to glutamate at Gag residue 205 immediately preceding the amino terminus of $Gag_{206-216}$ epitope. Our results suggest that even vaccine-induced CTL efficacy can be abrogated by a single amino acid change in viral epitope flanking region, underlining the influence of viral epitope flanking sequences on CTL-based AIDS vaccine efficacy.
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Keywords: AIDS vaccine; Simian immunodeficiency virus; Cytotoxic T lymphocyte; Escape

1. Introduction

Development of an effective AIDS vaccine is considered essential for controlling current AIDS pandemic. A current promising AIDS vaccine strategy is to elicit virus-specific $CD8^+$ cytotoxic T lymphocytes (CTLs) that broadly recognize highly-diversified HIVs [1–4]. However, it has remained unclear as to how broadly vaccine-induced CTLs can recognize heterologous viruses in vivo.

Vaccine efficacies have been evaluated in macaque AIDS models. Several vaccine trials eliciting virus-specific CTL responses have successfully shown viral control and prevention of acute AIDS progression after CXCR4-tropic simian-human immunodeficiency virus (SHIV) 89.6P challenge in rhesus macaques [5–9]. In the models of CCR5-tropic simian

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immunodeficiency virus (SIV) infections that induce acute depletion of $CCR5^+CD4^+$ effector memory T cells from mucosal effector sites and following chronic disease progression like HIV-1 infections in humans [10,11], DNA-prime/adenovirus vector-boost vaccine trials have recently shown transient, partial reduction in viral loads in Indian rhesus macaques, although most CTL-based vaccines have failed to show consistent viremia control after SIV challenge [12–15]. However, most of these trials have used SIVmac239 antigens for vaccination and homologous SIVmac239/251 for challenge [16]. There have been a few reports on heterologous challenge [17], but in vivo efficacy of vaccine-induced CTL responses has not yet been compared intensively between in the homologous and in the heterologous CCR5-tropic SIV challenge experiments.

We have developed an Env-independent DNA-prime/SIVmac239 Gag-expressing Sendai virus (SeV-Gag) vector-boost vaccine and shown its protective efficacy in macaque AIDS models [7,18]. A trial of a homologous SIVmac239 challenge has shown vaccine-based control of viral replication in a group of Burmese rhesus macaques possessing a major histocompatibility complex class I (MHC-I) haplotype *90-120-Ia* and suggested involvement of vaccine-induced $Gag_{206-216}$ (IINEEAADWDL) epitope-specific CTL responses in this control [18]. All the SIVmac239-infected macaques possessing MHC-I haplotype *90-120-Ia* selected a viral mutation that results in escape from this $Gag_{206-216}$-specific CTL recognition and loss of viral fitness, indicating strong suppressive pressure on SIVmac239 replication in vivo by this CTL.

In the present study, we have challenged MHC-I haplotype *90-120-Ia*-positive vaccinees with a heterologous SIVsmE543-3 that has the same $Gag_{206-216}$ epitope amino acid sequence with SIVmac239, and have evaluated in vivo efficacy of vaccine-induced $Gag_{206-216}$-specific CTL responses against this heterologous virus in those vaccinees. Remarkably, vaccine-induced $Gag_{206-216}$-specific CTLs failed to show responses against SIVsmE543-3 infection.

2. Materials and methods

2.1. Animal experiments

Burmese rhesus macaques (*Macaca mulatta*) possessing MHC-I haplotype *90-120-Ia* which were used in this study were maintained in accordance with the Guideline for Laboratory Animals of National Institute of Infectious Diseases and National Institute of Biomedical Innovation. Blood collection, vaccination, and virus challenge were performed under ketamine anesthesia.

2.2. Vaccination and challenge

Two MHC-I haplotype *90-120-Ia*-positive rhesus macaques received a DNA vaccine followed by a single boost with SeV-Gag before an intravenous SIVsmE543-3 challenge. The DNA, CMV-SHIVdEN, used for the priming was constructed from an *env*- and *nef*-deleted $SHIV_{MD14YE}$ [19] molecular clone DNA, SIVGP1 [7,18], and has the genes encoding SIVmac239 (GenBank accession no. M33262) Gag, Pol, Vif, and Vpx, SIVmac239-HIV-1_{DH12} chimeric Vpr, and HIV-1_{DH12} Tat and Rev as described previously [18]. At the DNA vaccination, animals received 5 mg of CMV-SHIVdEN DNA intramuscularly. Six weeks after the DNA priming, animals intranasally received a single boost with 1×10^8 cell infectious units (CIU) of replication-competent V-knocked-out SeV-Gag [20,21]. Approximately 3 months after the boost, animals were challenged intravenously with 100 $TCID_{50}$ (50% tissue culture infective dose) of SIVsmE543-3 [22]. An SIVsmE543-3 (GenBank accession number U72748) molecular clone DNA was provided by V. Hirsch, and the virus obtained from COS-1 cells transfected with the molecular clone DNA was propagated on rhesus macaque peripheral blood mononuclear cells (PBMCs) to prepare the SIVsmE543-3 challenge stock.

2.3. Vectors

The plasmid vectors, pEGFP-N1-$Gag_{202-216}$ and pEGFP-N1-$Gag_{202-216}$.205E, were constructed from pEGFP-N1 (Becton Dickinson, Tokyo, Japan) by adding epitope-coding regions into the 5′ end of EGFP cDNA to express $Gag_{202-216}$-EGFP and $Gag_{202-216}$.205E-EGFP fused proteins, respectively. The amino acid sequences added into the N-terminal portion of EGFP are MASRAAAIIRDIINEEAADWDLAAD PPVAT in $Gag_{202-216}$-EGFP and MASRAAAIIREIINEEA ADWDLAADPPVAT in $Gag_{202-216}$.205E-EGFP.

2.4. Quantitation of plasma viral loads

Plasma RNA was extracted using High Pure Viral RNA kit (Roche Diagnostics, Tokyo, Japan). For quantitation of SIVsmE543-3 RNA copies, serial five-fold dilutions of RNA samples were amplified in quadruplicate by reverse transcription (RT) and nested PCR using SIV *gag*-specific primers or SIVsmE543-3 *gag*-specific primers to determine the end point. The SIV *gag*-specific primers were TTGAAGCATGTAG TATGGGCAG and TGGGTAATTTCCTCCTCTGCC for the 1st RT–PCR and GATTAGCAGAAAGCCTGTTGG and TGTTCCTGTTTCCACCACTAG for the 2nd PCR (Sigma–Aldrich Japan, Ishikari, Japan). The SIVsmE543-3 *gag*-specific primers were AGAAACTCCGTCTTGTCAGG and CTAATAATTTGCATGGCTGC for the 1st RT–PCR and GATTAGCAGAAAGCCTGTTGG and TGCAGCCTTCTGA TAGCGC for the 2nd PCR. Plasma SIV RNA levels were calculated according to the Reed-Muench method as described previously [18]. The lower limit of detection is approximately 1×10^3 copies/ml. The plasma viral loads at several time points were confirmed by LightCycler real-time PCR system (Roche Diagnostics) using SIV *gag*-specific primers (GTAG TATGGGCAGCAAATGA and TGTTCCTGTTTCCACCA CTA) and probes (GCATTCACGCAGAAGAGAAAGTGAA ACA and ACTGAGGAAGCAAAACAAATAGTGCAGAGA) (Nihon Gene Research Laboratories Inc., Sendai, Japan).

2.5. Sequencing

A fragment corresponding to nucleotides 973–2690 (containing the entire *gag* region) in SIVsmE543-3 genome was amplified from plasma RNA by nested RT–PCR. For its amplification from plasma with low viral loads, plasma samples were concentrated five-fold by centrifugation at 25,000 × *g* for 2 h before RNA extraction. The PCR products were sequenced using dye terminator chemistry and an automated DNA sequencer (Applied Biosystems, Tokyo, Japan).

2.6. Measurement of virus-specific CD8$^+$ T-cell responses

We measured virus-specific CD8$^+$ T-cell levels by flow-cytometric analysis of interferon-γ (IFN-γ) induction after specific stimulation as described previously [18]. PBMCs were cocultured with autologous herpesvirus papio-immortalized B lymphoblastoid cell lines (B-LCLs) infected with vesicular stomatitis virus G (VSV-G)-pseudotyped SIVGP1 or VSV-G-pseudotyped SIVsmE543-3 for vaccine antigen-specific or SIVsmE543-3-specific stimulation. The pseudotyped viruses were obtained by cotransfection of COS-1 cells with a VSV-G-expression plasmid and SIVGP1 DNA or an SIVsmE543-3 molecular clone DNA. For peptide-specific stimulation, PBMCs were cocultured with B-LCLs pulsed with 1 μM or indicated concentrations of peptides (Sigma–Aldrich Japan). For stimulation with DNA-transfected cells, 10^6 B-LCLs were transfected with 10 μg of DNA by electroporation and 2 days later, one-fifth or half of them were cocultured with 10^6 PBMCs. Parts of the remaining DNA-transfected B-LCLs were subjected to flow-cytometric analysis for examining EGFP expression to confirm the transfection efficiency. Intracellular IFN-γ staining was performed using CytofixCytoperm kit (Becton Dickinson). Fluorescein isothiocyanate-conjugated anti-human CD4, Peridinin chlorophyll protein-conjugated anti-human CD8, allophycocyanin-conjugated anti-human CD3, and phycoerythrin-conjugated anti-human IFN-γ antibodies (Becton Dickinson) were used. Specific CD8$^+$ T-cell levels were calculated by subtracting non-specific IFN-γ$^+$ T-cell frequencies from those after antigen-specific stimulation. All the background IFN-γ$^+$CD8$^+$ T-cell frequencies in the present study (Figs. 2–4) were less than 100 cells/million PBMCs. Specific T-cell levels less than 100 cells/million PBMCs are considered negative.

3. Results

3.1. Failure in control of heterologous SIVsmE543-3 replication in 90-120-Ia-positive vaccinees

Two Burmese rhesus macaques (R00-018 and R01-006) possessing MHC-I haplotype *90-120-Ia* received a prophylactic DNA-prime/SeV-Gag-boost vaccination consisting of a single intramuscular priming with a DNA encoding SIVmac239 Gag, Pol, Vif, and Vpx followed by a single intranasal booster with an SeV expressing SIVmac239 Gag, and were challenged intravenously with SIVsmE543-3. After challenge, these two vaccinees failed to control SIVsmE543-3 replication with persistent high levels of plasma viremia (Fig. 1). Both of them finally exhibited AIDS-like symptoms and were euthanized around week 115.

We examined antigen-specific CD8$^+$ T-cell frequencies in PBMCs by flow-cytometric detection of IFN-γ induction after stimulation with VSV-G-pseudotyped virus-infected cells (Fig. 2). We used VSV-G-pseudotyped SIVGP1 and VSV-G-pseudotyped SIVsmE543-3 for measurement of frequencies of CD8$^+$ T cells responding to SIVGP1-transduced cells and those responding to SIVsmE543-3-transduced cells, respectively. We call the former vaccine antigen-specific CD8$^+$ T cells and the latter SIVsmE543-3-specific CD8$^+$ T cells, while some of the former cells are expected to respond to SIVsmE543-3-transduced cells and vice versa because of amino acid sequence homology between SIVmac239 and SIVsmE543-3 (e.g., approximately 90% in Gag). Vaccine antigen-specific CD8$^+$ T-cell responses were elicited efficiently after the vaccination but their expansion after SIVsmE543-3 challenge was not observed (in R00-018) or inefficient (in R01-006), suggesting that these vaccine-induced CD8$^+$ T cells did not efficiently respond to SIVsmE543-3 challenge. In

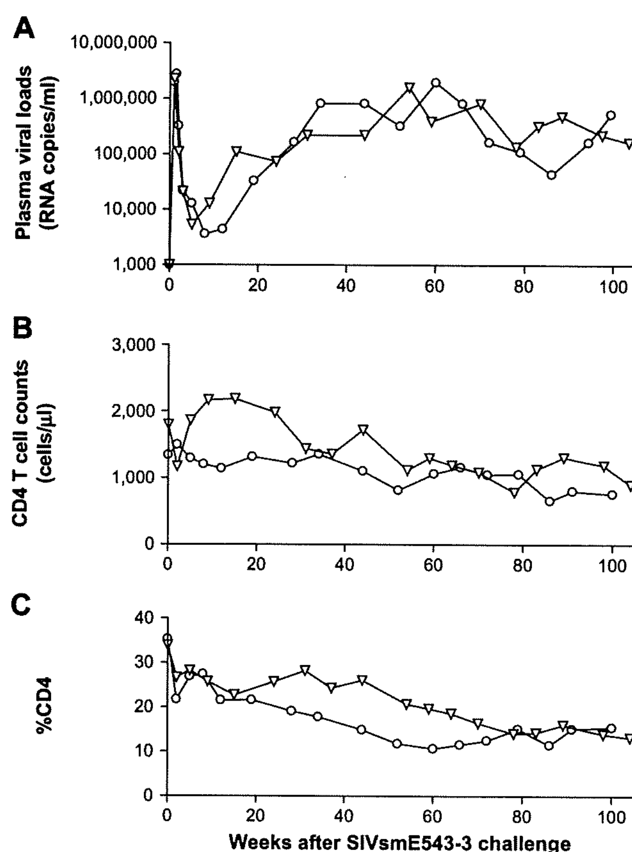

Fig. 1. Follow-up of the *90-120-Ia*-positive vaccinees (R00-018 indicated by circles and R01-006 indicated by triangles) after SIVsmE543-3 challenge. (A) Plasma viral loads (SIV RNA copies/ml plasma). (B) Peripheral CD4$^+$ T-cell counts (cells/μl). (C) Percentage of CD4$^+$ T cells in PBMCs.

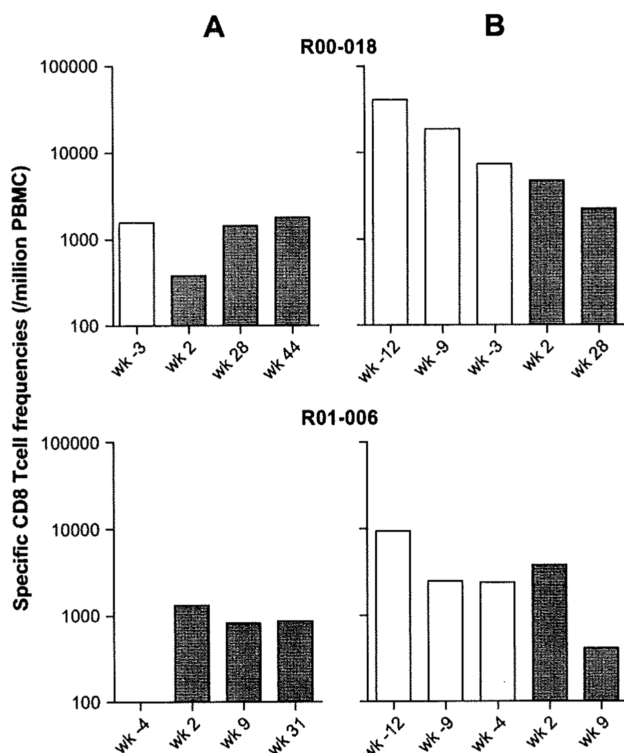

Fig. 2. Virus-specific $CD8^+$ T-cell frequencies in macaques R00-018 (upper panels) and R01-006 (lower panels). (A) SIVsmE543-3-specific $CD8^+$ T-cell frequencies in PBMCs. (B) Vaccine antigen-specific $CD8^+$ T-cell frequencies in PBMCs. The pre-challenge (indicated by minus week) and post-challenge frequencies are indicated by white and black bars, respectively.

R00-018, $CD8^+$ T cells responding to SIVsmE543-3-infected cells were detectable after vaccination but their levels did not increase at week 2 after challenge. In R01-006, these responses were undetectable just before challenge but induced after challenge.

3.2. No suppressive effect of vaccine-induced $Gag_{206-216}$-specific CTL responses on SIVsmE543-3 replication in vivo

Both macaques failed to control SIVsmE543-3 replication despite efficient elicitation of vaccine antigen-specific $CD8^+$ T-cell responses by the DNA-prime/SeV-Gag-boost vaccination. We then examined, in these two macaques, the levels of $Gag_{206-216}$-specific $CD8^+$ T-cell responses that have been indicated to exert suppressive pressure on SIVmac239 replication. Similarly to the previously-reported vaccinees possessing MHC-I haplotype *90-120-Ia* [18], both the vaccinees, R00-018 and R01-006, showed efficient induction of $Gag_{206-216}$-specific $CD8^+$ T-cell responses after vaccination (Fig. 3). Thus, these two macaques failed to control SIVsmE543-3 replication despite efficient induction of $Gag_{206-216}$-specific CTL responses by the prophylactic vaccination.

After SIVsmE543-3 challenge, the $Gag_{206-216}$-specific $CD8^+$ T-cell frequencies did not increase (Fig. 3), indicating no efficient expansion of these responses in these macaques. Sequencing of plasma viral genomes at several time points (Table 1) revealed no detectable mutations within the $Gag_{206-216}$ epitope-coding region even 1 year post-challenge, although all the previously-reported *90-120-Ia*-positive macaques infected with SIVmac239 have shown selection of a mutation within the $Gag_{206-216}$ epitope-coding region resulting in viral escape from recognition by $Gag_{206-216}$-specific CTLs [18]. These results indicate that the vaccine-induced $Gag_{206-216}$-specific CTLs did not respond to the heterologous challenge efficiently, exerting no suppressive pressure on SIVsmE543-3 replication, although the SIVsmE543-3 has the same $Gag_{206-216}$ epitope sequence, IINEEAADWDL, with SIVmac239.

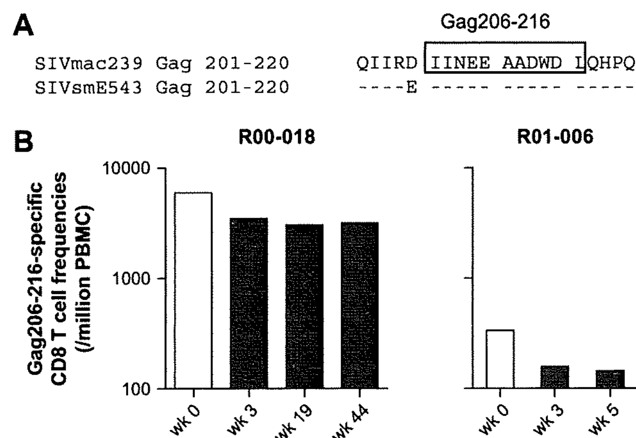

Fig. 3. $Gag_{206-216}$-specific $CD8^+$ T-cell frequencies. (A) Comparison of amino acid sequences around the $Gag_{206-216}$ epitope between SIVmac239 and SIVsmE543-3. (B) $Gag_{206-216}$ peptide-specific $CD8^+$ T-cell frequencies just before challenge (wk 0, white bars) and post-challenge (black bars) in macaques R00-018 (left panel) and R01-006 (right panel). PBMCs were stimulated by coculture with B-LCLs pulsed with $Gag_{206-216}$ peptide (IINEEAADWDL), and specific IFN-γ induction was measured.

Table 1
SIVsmE543-3 Gag amino acid changes in macaques[a]

R00-018					
Week 5	(no mutation)				
Week 12			V244A*		
Week 19			V244A		D465E
Week 28		T243S	V244A		
Week 60		T243S	V244A	V424I	
Week 100	P221L	T243S	V244A		
R01-006					
Week 9	(no mutation)				
Week 15			V244A		
Week 31		T243S			
Week 54		T243S			

[a] A *gag* fragment was amplified from plasma RNA by nested RT–PCR and subjected to sequencing. Dominant mutations resulting in amino acid changes are shown. Mutations within the $Gag_{206-216}$ epitope-coding region were undetectable. The V244A* at week 12 in R00-018 indicates that the wild-type and the mutant sequences were found equivalently.

3.3. Failure in $Gag_{206-216}$ epitope presentation due to a single amino acid change, Gag D205E, in the epitope flanking region

Comparison of amino acid sequences around the $Gag_{206-216}$ epitope between SIVmac239 and SIVsmE543-3 revealed a single amino acid change at the 205th amino acid in Gag, from aspartate (D) in SIVmac239 to glutamate (E) in SIVsmE543-3 (Fig. 3A). We then examined the effect of this single amino acid difference in the epitope flanking region on recognition of the epitope by $Gag_{206-216}$-specific CTL.

We first prepared amino (N) terminal-extended 15-mer peptides, SIVmac239 $Gag_{202-216}$ (IIRDIINEEAADWDL) and $Gag_{202-216}$.205E (SIVsmE543-3 $Gag_{202-216}$, IIREIINEEAADWDL), and examined frequencies of $CD8^+$ T cells that recognize these peptide-pulsed cells in PBMCs derived from vaccinated *90-120-Ia*-positive macaques (Fig. 4). No significant difference was observed between $Gag_{202-216}$ peptide-specific $CD8^+$ T-cell and $Gag_{202-216}$.205E peptide-specific $CD8^+$ T-cell frequencies, indicating that $Gag_{206-216}$-specific CTLs were able to recognize $Gag_{202-216}$ peptide-pulsed cells and $Gag_{202-216}$.205E peptide-pulsed cells equivalently.

Next, we constructed plasmid vectors, pEGFP-N1-$Gag_{202-216}$ and pEGFP-N1-$Gag_{202-216}$.205E expressing $Gag_{202-216}$-EGFP and $Gag_{202-216}$.205E-EGFP fused proteins, respectively (Fig. 5A). Efficient IFN-γ induction was observed after stimulation with pEGFP-N1-$Gag_{202-216}$-transfected cells but not with pEGFP-N1-$Gag_{202-216}$.205E-transfected cells (Fig. 5B). Thus, $Gag_{206-216}$-specific CTLs were able to recognize the cells expressing $Gag_{202-216}$-EGFP fusion proteins but not those expressing $Gag_{202-216}$.205E-EGFP fused proteins efficiently. These results suggest failure in $Gag_{206-216}$ epitope presentation due to the single amino acid change, Gag205D to Gag205E, in the epitope flanking region of SIVsmE543-3.

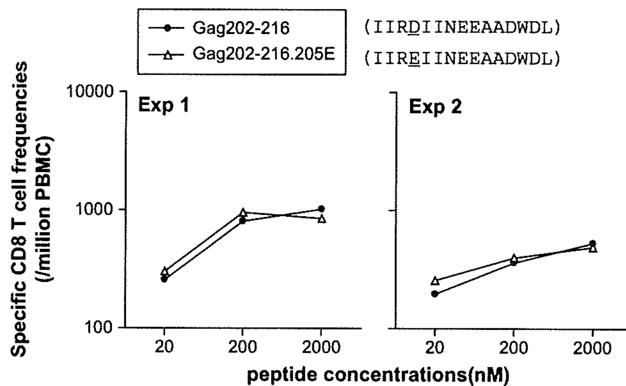

Fig. 4. Recognition of $Gag_{202-216}$ peptide-pulsed cells by $Gag_{206-216}$-specific $CD8^+$ T cells. Results with PBMCs obtained at one week after SeV-Gag boost from a DNA-prime/SeV-Gag-vaccinated macaque (#1) used in other experiment are shown in the left panel (Expt. 1), and those with PBMCs at 4 weeks after boost from another (#2) are in the right (Expt. 2). PBMCs were stimulated by coculture with B-LCLs pulsed with indicated concentrations of the SIVmac239 $Gag_{202-216}$ peptide or the $Gag_{202-216}$.205E peptide (purity: approximately 80%), and specific IFN-γ induction was measured.

4. Discussion

The previous study has shown control of SIVmac239 replication in the group of MHC-I haplotype *90-120-Ia*-positive macaques vaccinated with DNA-prime/SeV-Gag-boost [18]. These controllers showed high levels of $Gag_{206-216}$-specific CTL responses and rapid selection of a mutant escaping from this CTL recognition. The mutation leading to a substitution from leucine to serine at the 216th amino acid in Gag resulted in loss of viral fitness, indicating strong selective pressure of $Gag_{206-216}$-specific CTL responses on homologous SIVmac239 [18,23]. In the present study, we immunized macaques possessing MHC-I haplotype *90-120-Ia* with the vaccine expressing SIVmac239-derived antigens and challenged them with SIVsmE543-3. We then examined the efficacy of vaccine-induced $Gag_{206-216}$-specific CTLs against this heterologous virus that has the same $Gag_{206-216}$ epitope sequence. The vaccinees possibly able to control homologous SIVmac239 replication failed to contain the heterologous SIVsmE543-3 challenge despite efficient $Gag_{206-216}$-specific CTL induction by vaccination.

The vaccinees did not show detectable secondary $Gag_{206-216}$-specific CTL responses after the heterologous SIVsmE543-3 challenge nor exhibited mutations within the $Gag_{206-216}$ epitope-coding region even in the chronic phase. These results indicate that $Gag_{206-216}$-specific CTLs did not efficiently respond to SIVsmE543-3 infection or exert suppressive pressure on SIVsmE543-3 replication in vivo. Involvement of $Gag_{241-249}$ (SSVDEQIQW)-specific CTL responses in vaccine-based SIVmac239 control has also been suggested in the group of MHC-I haplotype *90-120-Ia*-positive macaques [24], but those $Gag_{241-249}$-specific CTLs did not show detectable responses against SIVsmE543-3 that has a different amino acid sequence (STVEEQIQW) within this epitope region (data not shown). Thus, neither vaccine-induced $Gag_{206-216}$-specific CTL nor $Gag_{241-249}$-specific CTL responses were effective against SIVsmE543-3 replication. However, SIVsmE543-3 challenge into unvaccinated *90-120-Ia*-positive control animals showed inefficient viral replication even in the absence of these CTL responses (data not shown). Its mechanism remains unclear, but vaccine-induced dominant CTL responses ineffective against the heterologous SIV may possibly exert worse effect on viral control.

Both SIVmac239-derived $Gag_{202-216}$ peptide-pulsed cells and SIVsmE543-3-derived $Gag_{202-216}$.205E peptide-pulsed cells were recognized equivalently (Fig. 4) whereas $Gag_{202-216}$-EGFP-expressing cells but not $Gag_{202-216}$.205E-EGFP-expressing cells were recognized by $Gag_{206-216}$-specific CTLs (Fig. 5), suggesting failure in recognition of SIVsmE543-3-infected cells by $Gag_{206-216}$-specific CTLs due to a single amino acid change from D in SIVmac239 to E in SIVsmE543-3 at Gag residue 205 immediately preceding the N terminus of $Gag_{206-216}$ epitope. Our results suggest failure in the epitope presentation on SIVsmE543-3-infected cells due to this amino acid change. It may be speculated that the $Gag_{206-216}$ epitope is processed at its carboxy terminus by proteasomes and at its N-terminus by aminopeptidases for

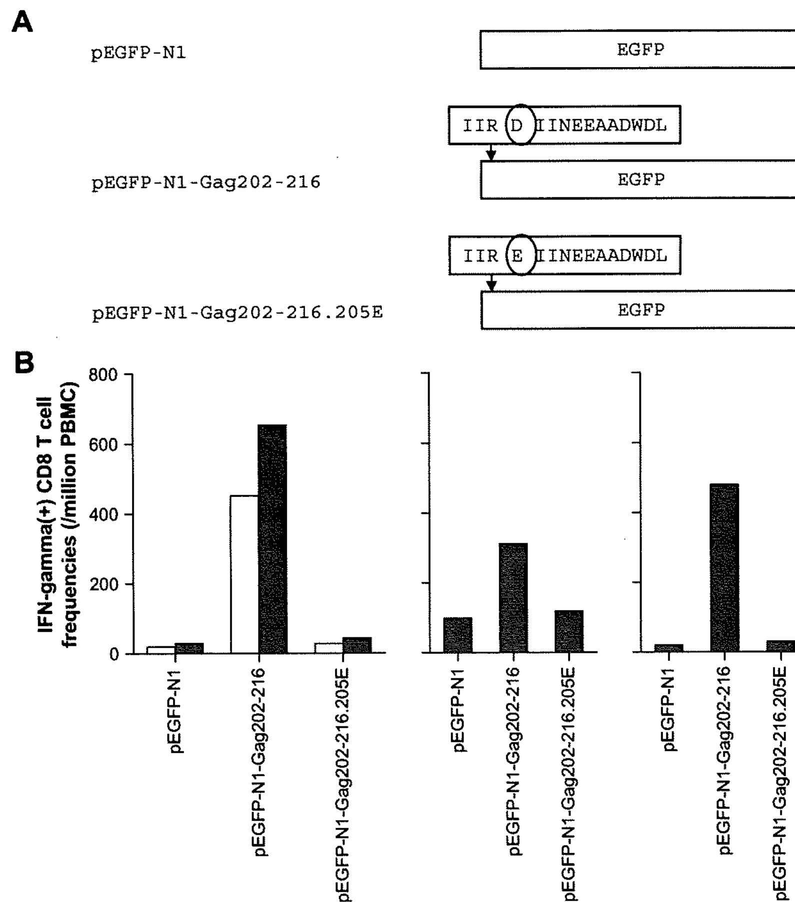

Fig. 5. Recognition of $Gag_{202-216}$-expressing cells by $Gag_{206-216}$-specific $CD8^+$ T cells. (A) Schema of DNA constructs. (B) IFN-γ induction in $CD8^+$ T cells by stimulation with $Gag_{202-216}$-expressing cells but not by $Gag_{202-216}$.205E-expressing cells. Three sets of experiments using PBMCs obtained at 2 weeks after SeV-Gag boost from DNA-prime/SeV-Gag-vaccinated macaques (#1 in Expt. 1, #2 in Expt. 2, and #3 in Expt. 3) are shown. Million PBMCs were stimulated by coculture with one-fifth (white bars in Expt. 1) or half (black bars in Expt. 1, Expt. 2, and Expt. 3) of million B-LCLs transfected with pEGFP-N1, pEGFP-N1-$Gag_{202-216}$, or pEGFP-N1-$Gag_{202-216}$.205E by electroporation and IFN-γ-positive $CD8^+$ T-cell frequencies were measured. The transfection efficiencies determined by EGFP expression in Expt. 1 were 6.1%, 6.2%, and 7.1%, respectively; 3.3%, 3.6%, and 4.1% in Expt. 2; not determined in Expt. 3.

its presentation [25,26] and that the D-to-E change in its N-terminal flanking region results in impairment of its N-terminal processing. However, amino acid changes in epitope flanking region do not always result in impairment of epitope processing [27]. Indeed, there has been no report showing aminopeptidases which do not recognize E but D for processing and the exact mechanism of impairment of the $Gag_{206-216}$ epitope presentation by the D-to-E change in its N-terminal flanking region remains unclear.

This study presents the first case, in macaques, of SIV escape from CTL recognition by changes in viral epitope flanking sequences. HIV-1 escape from CTL recognition by changes in epitope flanking sequences has been shown in HIV-1-infected individuals [28]. The escape observed in patients naturally infected with HIV-1 was from CTLs induced after infection and these post-infection-induced CTLs may not be fully functional because of possible immune impairment after HIV-1 infection [29,30]. Because it is difficult to know whether viral escape from CTL recognition in vivo is complete or not, it is important to clarify how much extent viral escape mutations can abrogate efficacy of functional CTLs in vivo. Indeed, it has remained unclear if changes in epitope flanking sequences can abrogate vaccine-induced CTL efficacy. In this study, the vaccine-induced $Gag_{206-216}$-specific CTLs effective against SIVmac239 did not efficiently respond to or show efficacy against SIVsmE543-3 infection in macaques, indicating a possibility of abrogation of vaccine-induced CTL efficacy in vivo by a single amino acid change in viral epitope flanking region. These results underline the influence of viral epitope flanking sequences on CTL-based AIDS vaccine efficacy, suggesting an important implication for development of an effective vaccine against highly-diversified HIVs.

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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] 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.

[3] 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.

[4] A.J. McMichael, T. Hanke, HIV vaccines 1983–2003, Nat. Med 9 (2003) 874–880.

[5] K.A. Reimann, J.T. Li, R. Veazey, M. Halloran, I.W. Park, G.B. Karlsson, J. Sodroski, N.L. Letvin, A chimeric simian/human immunodeficiency virus expressing a primary patient human immunodeficiency virus type 1 isolate env causes an AIDS-like disease after in vivo passage in rhesus monkeys, J. Virol 70 (1996) 6922–6928.

[6] R.R. Amara, F. Villinger, J.D. Altman, S.L. Lydy, S.P. O'Neil, S.I. Staprans, D.C. Montefiori, Y. Xu, J.G. Herndon, L.S. Wyatt, M.A. Candido, N.L. Kozyr, P.L. Earl, J.M. Smith, H.L. Ma, B.D. Grimm, M.L. Hulsey, J. Miller, H.M. McClure, J.M. McNicholl, B. Moss, H.L. Robinson, Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine, Science 292 (2001) 69–74.

[7] T. Matano, M. Kano, H. Nakamura, A. Takeda, Y. Nagai, Rapid appearance of secondary immune responses and protection from acute CD4 depletion after a highly pathogenic immunodeficiency virus challenge in macaques vaccinated with a DNA prime/Sendai virus vector boost regimen, J. Virol 75 (2001) 11891–11896.

[8] J.W. Shiver, T.M. Fu, L. Chen, D.R. Casimiro, M.E. Davies, R.K. Evans, Z.Q. Zhang, A.J. Simon, W.L. Trigona, S.A. Dubey, L. Huang, V.A. Harris, R.S. Long, X. Liang, L. Handt, W.A. Schleif, L. Zhu, D.C. Freed, N.V. Persaud, L. Guan, K.S. Punt, A. Tang, M. Chen, K.A. Wilson, K.B. Collins, G.J. Heidecker, V.R. Fernandez, H.C. Perry, J.G. Joyce, K.M. Grimm, J.C. Cook, P.M. Keller, D.S. Kresock, H. Mach, R.D. Troutman, L.A. Isopi, D.M. Williams, Z. Xu, K.E. Bohannon, D.B. Volkin, D.C. Montefiori, A. Miura, G.R. Krivulka, M.A. Lifton, M.J. Kuroda, J.E. Schmitz, N.L. Letvin, M.J. Caulfield, A.J. Bett, R. Youil, D.C. Kaslow, E.A. Emini, Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity, Nature 415 (2002) 331–335.

[9] Y. Nishimura, T. Igarashi, O.K. Donau, A. Buckler-White, C. Buckler, B.A. Lafont, R.M. Goeken, S. Goldstein, V.M. Hirsch, M.A. Martin, Highly pathogenic SHIVs and SIVs target different $CD4^+$ T cell subsets in rhesus monkeys, explaining their divergent clinical courses, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 12324–12329.

[10] Q. Li, L. Duan, J.D. Estes, Z.M. Ma, T. Rourke, Y. Wang, C. Reilly, J. Carlis, C.J. Miller, A.T. Haase, Peak SIV replication in resting memory $CD4^+$ T cells depletes gut lamina propria $CD4^+$ T cells, Nature 434 (2005) 1148–1152.

[11] J.J. Mattapallil, D.C. Douek, B. Hill, Y. Nishimura, M. Martin, M. Roederer, Massive infection and loss of memory $CD4^+$ T cells in multiple tissues during acute SIV infection, Nature 434 (2005) 1093–1097.

[12] M.B. Feinberg, J.P. Moore, AIDS vaccine models: challenging challenge viruses, Nat. Med. 8 (2002) 207–210.

[13] J.J. Mattapallil, D.C. Douek, A. Buckler-White, D. Montefiori, N.L. Letvin, G.J. Nabel, M. Roederer, Vaccination preserves CD4 memory T cells during acute simian immunodeficiency virus challenge, J. Exp. Med 203 (2006) 1533–1541.

[14] N.L. Letvin, J.R. Mascola, Y. Sun, D.A. Gorgone, A.P. Buzby, L. Xu, Z.Y. Yang, B. Chakrabarti, S.S. Rao, J.E. Schmitz, D.C. Montefiori, B.R. Barker, F.L. Bookstein, G.J. Nabel, Preserved CD4+ central memory T cells and survival in vaccinated SIV-challenged monkeys, Science 312 (2006) 1530–1533.

[15] N.A. Wilson, J. Reed, G.S. Napoe, S. Piaskowski, A. Szymanski, J. Furlott, E.J. Gonzalez, L.J. Yant, N.J. Maness, G.E. May, T. Soma, M.R. Reynolds, E. Rakasz, R. Rudersdorf, A.B. McDermott, D.H. O'connor, T.C. Friedrich, D.B. Allison, A. Patki, L.J. Picker, D.R. Burton, J. Lin, L. Huang, D. Patel, G. Heindecker, J. Fan, M. Citron, M. Horton, F. Wang, X. Liang, J.W. Shiver, D.R. Casimiro, D.I. Watkins, Vaccine-induced cellular immune responses reduce plasma viral concentrations after repeated low-dose challenge with pathogenic simian immunodeficiency virus SIVmac239, J. Virol. 80 (2006) 5875–5885.

[16] H.W. Kestler 3rd, D.J. Ringler 3rd, K. Mori, D.L. Panicali, P.K. Sehgal, M.D. Daniel, R.C. Desrosiers, Importance of the nef gene for maintenance of high virus loads and for development of AIDS, Cell 65 (1991) 651–662.

[17] D.H. Barouch, J. Kunstman, J. Glowczwskie, K.J. Kunstman, M.A. Egan, F.W. Peyerl, S. Santra, M.J. Kuroda, J.E. Schmitz, K. Beaudry, G.R. Krivulka, M.A. Lifton, D.A. Gorgone, 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.

[18] T. Matano, M. Kobayashi, H. Igarashi, A. Takeda, H. Nakamura, M. Kano, C. Sugimoto, K. Mori, A. Iida, T. Hirata, M. Hasegawa, T. Yuasa, M. Miyazawa, Y. Takahashi, M. Yasunami, A. Kimura, D.H. O'Connor, D.I. Watkins, Y. Nagai, Cytotoxic T lymphocyte-based control of simian immunodeficiency virus replication in a preclinical AIDS vaccine trial, J. Exp. Med 199 (2004) 1709–1718.

[19] R. Shibata, F. Maldarelli, C. Siemon, T. Matano, M. Parta, G. Miller, T. Fredrickson, M.A. Martin, Infection and pathogenicity of chimeric simian-human immunodeficiency viruses in macaques: determinants of high virus loads and CD4 cell killing, J. Infect. Dis 176 (1997) 362–373.

[20] A. Kato, Y. Sakai, T. Shioda, T. Kondo, M. Nakanishi, Y. Nagai, Initiation of Sendai virus multiplication from transfected cDNA or RNA with negative or positive sense, Genes. Cells 1 (1996) 569–579.

[21] M. Kano, T. Matano, A. Kato, H. Nakamura, A. Takeda, Y. Suzaki, Y. Ami, K. Terao, Y. Nagai, Primary replication of a recombinant Sendai virus vector in macaques, J. Gen. Virol 83 (2002) 1377–1386.

[22] V. Hirsch, D. Adger-Johnson, B. Campbell, S. Goldstein, C. Brown, W.R. Elkins, D.C. Montefiori, A molecularly cloned, pathogenic, neutralization-resistant simian immunodeficiency virus, J. Virol 71 (1997) 1608–1620 SIVsmE543-3.

[23] P.J. Goulder, D.I. Watkins, HIV and SIV CTL escape: implications for vaccine design, Nat. Rev. Immunol 4 (2004) 630–640.

[24] M. Kawada, H. Igarashi, A. Takeda, T. Tsukamoto, H. Yamamoto, S. Dohki, M. Takiguchi, T. Matano, Involvement of multiple epitope-specific cytotoxic T-lymphocyte responses in vaccine-based control of simian immunodeficiency virus replication in rhesus macaques, J. Virol 80 (2006) 1949–1958.

[25] P.M. Kloetzel, Generation of major histocompatibility complex class I antigens: functional interplay between proteasomes and TPPII, Nat. Immunol 5 (2004) 661–669.

[26] K.L. Rock, I.A. York, A.L. Goldberg, Post-proteasomal antigen processing for major histocompatibility complex class I presentation, Nat. Immunol 5 (2004) 670–677.

[27] C. Brander, O.O. Yang, N.G. Jones, Y. Lee, P. Goulder, R.P. Johnson, A. Trocha, D. Colbert, C. Hay, S. Buchbinder, C.C. Bergmann, H.J. Zweerink, S. Wolinsky, W.A. Blattner, S.A. Kalams, B.D. Walker, Efficient processing of the immunodominant, HLA-A*0201-restricted human immunodeficiency virus type 1 cytotoxic T-lymphocyte epitope despite multiple variations in the epitope flanking sequences, J. Virol 73 (1999) 10191–10198.

[28] 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.

[29] V. Appay, D.F. Nixon, S.M. Donahoe, G.M. Gillespie, T. Dong, A. King, G.S. Ogg, H.M. Spiegel, C. Conlon, C.A. Spina, D.V. Havlir, D.D. Richman, A. Waters, P. Easterbrook, A.J. McMichael, S.L. Rowland-Jones, HIV-specific CD8(+) T cells produce antiviral cytokines but are impaired in cytolytic function, J. Exp. Med 192 (2000) 63–75.

[30] P. Champagne, G.S. Ogg, A.S. King, C. Knabenhans, K. Ellefsen, M. Nobile, V. Appay, G.P. Rizzardi, S. Fleury, M. Lipp, R. Forster, S. Rowland-Jones, R.P. Sekaly, A.J. McMichael, G. Pantaleo, Skewed maturation of memory HIV-specific CD8 T lymphocytes, Nature 410 (2001) 106–111.

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HLA-DPβ chain may confer the susceptibility to hepatitis C virus-associated hypertrophic cardiomyopathy

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Summary

Hypertrophic cardiomyopathy (HCM) is a heart muscle disease characterized by hypertrophy and diastolic dysfunction of cardiac ventricles. It is suggested that one possible aetiology of HCM is the hepatitis C virus (HCV) infection, but molecular mechanisms underlying development of HCV-associated HCM (HCV-HCM) remains unknown. Because the human leucocyte antigen (HLA) molecule is involved in the control of progression/suppression of viral infection, extensive *HLA* allelic diversity may modulate the post-infectious course of HCV and pathogenesis of HCV-HCM. Here we undertook a case-control study with 38 patients with HCV-HCM and 132 unrelated healthy controls to reveal the potential impact of polymorphisms in seven classical and two non-classical *HLA* genes on the pathogenesis of HCV-HCM. It was found that *DPB1*0401* and *DPB1*0901* were significantly associated with increased risk to HCV-HCM in dominant model ($P < 0.028$, OR = 3.94, 95% confidence interval (CI) = 1.19, 13.02) and in recessive model ($P < 0.007$, OR = 9.85, 95% CI = 1.83, 53.04), respectively. The disparity in the gene–dose effect by two susceptible DPB1 alleles may be attributable to the difference between the susceptible (36 A and 55 A) and resistant (8L, 9F, 11G, 57E and 76M) residue-combination consisting of DPβ anchor pocket for antigenic peptide-binding. These results implied that the HLA-DP molecules with specificity pocket appropriate for HCV antigen(s) might confer the progressive process of HCM among the HCV-infected individuals.

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Introduction

Primary cardiomyopathy is a heart muscle disease that often leads to severe heart failure and sudden death. There are two different types of cardiomyopathy; one is hypertrophic cardiomyopathy (HCM) characterized by cardiac hypertrophy and diastolic ventricular dysfunction, and the other is dilated cardiomyopathy (DCM) characterized by chamber dilation and contractile dysfunction (Seidman & Seidman, 2001). It is well known that more than half of patients with HCM and about 20–30% of patients with DCM have apparent family history of the disease (familial cardiomyopathy) mainly consistent with the autosomal dominant genetic trait, and mutations in the genes for sarcomere or Z-disc components could be found in the patients with familial cardiomyopathy (Seidman & Seidman, 2001; Richard *et al.*, 2006). On the other hand, there are many patients who have no family history of the disease (30–50% of HCM patients and 70–80% of DCM patients), that is, patients with sporadic cardiomyopathy. Molecular mechanisms underlying the pathogenesis of sporadic HCM or sporadic DCM have not been fully understood, because gene mutations are usually not found in the patients with sporadic cardiomyopathy albeit that a part of sporadic HCM patients and sporadic DCM patients carried antibodies to hepatitis C virus (HCV). These findings have suggested a possible involvement of HCV in the pathogenesis of sporadic HCM and/or sporadic DCM (Matsumori *et al.*, 1995, 1996, 2006; Teragaki *et al.*, 2003; Matsumori, 2005).

HCV causes not only liver disease but also a broad clinical spectrum of extrahepatic manifestation (Mayo, 2003). Immunohistological studies demonstrated the presence of HCV in extrahepatic organs including heart (Takeda *et al.*, 1999), and it was reported that the prevalence of patients with anti-HCV antibodies (seropositive patients) was about 9.5% and 6.7% in Japanese HCM and DCM, respectively (Matsumori *et al.*, 2002). On the other hand, transgenic mice with high expression of HCV-core in the heart exhibited morphological and functional changes similar in part to both HCM and DCM without apparent inflammatory cell infiltration (Omura *et al.*, 2005), demonstrating the role of HCV infection in the development of cardiomyopathy. However, not all of the HCV infected-patients exhibited abnormal findings in the electrocardiogram and echocardiography such as arrhythmia and

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