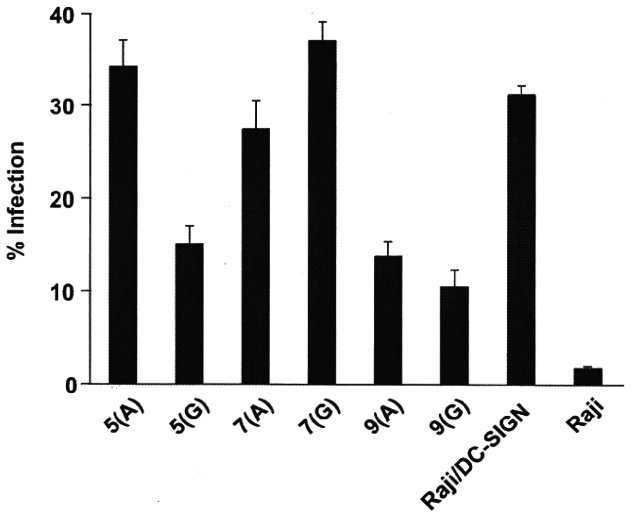

Fig. 3. HIV-1 trans-infection efficiency mediated by various DC-SIGNR molecules. Capture and transmission of HIV-$1_{NL\text{-}EGFP}$ were performed as described in Materials and methods. DC-SIGNR-expressing donor cells were incubated with HIV-$1_{NL\text{-}EGFP}$ for 3 h, then cells were washed and cocultivated with target MT4 cells. The percentage of GFP-expressing MT4 cells was determined by flow cytometry after 24 h. Raji/DC-SIGN cells and Raji cells were used as positive and negative donor cell controls, respectively. Each represents the mean value of three independent experiments.

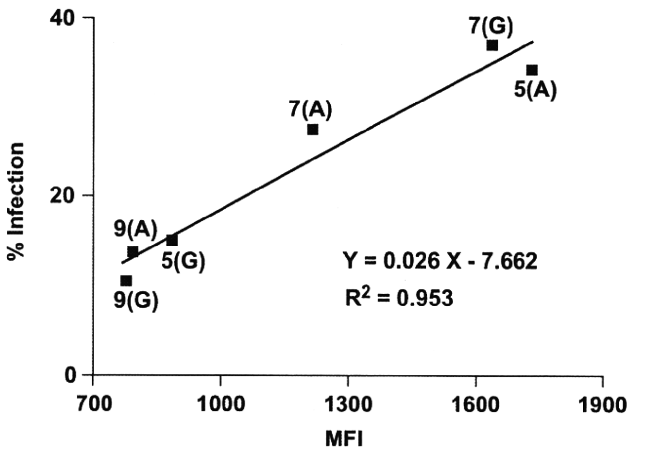

Fig. 4. The correlation of the HIV-1 trans-infection efficiency and the MFI of DC-SIGNR^{high} Raji cells. The abscissa represents the MFI of DC-SIGNR expression in various DC-SIGNR-expressing Raji cells shown in Fig. 2B. The ordinate shows the trans-infection efficiency shown in Fig. 3. The Pearson correlation coefficient (R) was used to show the extent of correlation between the trans-infection efficiency and the DC-SIGNR MFI.

Fig. 1 shows the structures of six kinds of cDNA encoding the DC-SIGNR alleles examined in this study. Raji cells were transfected with these plasmids by electroporation and DC-SIGNR-expressing cells (Raji/DC-SIGNR) were obtained. To obtain the stable DC-SIGNR-expressing cells, we selected G418-resistant cells for 2 weeks by culturing the transiently transfected Raji cells with G418. Raji cells with high levels of DC-SIGNR expression were collected by sorting the stable Raji/DC-SIGNR cells by BD FACSAria™ Cell Sorting System. After replicating these cells with G418 for 6 days, we checked the DC-SIGNR expression in these Raji cells (Fig. 2A and B). Though the mean fluorescent intensity (MFI) of DC-SIGNR observed from six kinds of Raji/DC-SIGNR varied from 777 to 1731, all the DC-SIGNR alleles were expressed on the cell surface efficiently and to a comparable degree. Only background levels of antibody-binding were observed in control Raji cells (data not shown).

Using these stable transformants, we determined the trans-infection efficiency that is expressed by the MFI value of the GFP expressed in MT4 cells. As shown in Fig. 3, Raji cells expressing DC-SIGNR were capable of trans-infecting MT4 cells to various extents. The assay is based on the fact that Raji cells are essentially non-permissive to HIV-1 infection due to the lack of CD4 molecules on the cell surface [4]. Therefore, the Raji cells expressing DC-SIGNR molecules on the cell surface serve as a platform that virions stop by transiently and as a station of delivery to target permissive MT4 cells [4]. GFP is expressed only when the viral cDNA integrates into the host chromosome, since HIV-1 used in our system carries a reporter gene, *GFP* [27]. DC-SIGNR-expressing Raji cells where HIV-1 particles bind did not express GFP thus showed no fluorescence (data not shown), whereas infected MT4 cells showed bright green fluorescence. Furthermore, we found a strong correlation between the HIV-1 trans-infection efficiency and the MFI of the DC-SIGNR expressed on Raji cells (Fig. 4, $R^2 = 0.95$).

Discussion

The report of differential HIV-1 susceptibility with respect to DC-SIGNR neck repeat polymorphism and the SNP (rs2277998) in Exon 5 [8] made us frame a working hypothesis that these polymorphic genotypes affect trans-infection efficiency. Contrary to our expectations, in our system, the polymorphic genotypes of the *DC-SIGNR* gene affected the amount of DC-SIGNR molecules on the cell surface and accordingly the trans-infection efficiency by DC-SIGNR molecules independently of the length of the neck region or the amino acid residue encoded by the SNP (Figs. 2B and 3). Differences in trans-infection efficiency could reflect differences in the amounts of DC-SIGNR on cell surface; that is, the more amounts of DC-SIGNR molecules are expressed on cell surface, the more efficiently trans-infection seems to take place. This idea is supported by the observed linear correlation between the expression level and trans-infection efficiency (Fig. 4). We concluded that the number of virions transferred from Raji/DC-SIGNR to MT4 cells is in proportion to the number of DC-SIGNR molecules on Raji/DC-SIGNR cells. This implies that the polymorphic genotypes do not affect the trans-infection efficiency per molecule but affect the amount of DC-SIGNR molecules on the cell surface.

Thus, our results do not provide molecular evidence supporting the clinical observation of a positive association in a Thai study [8], but instead suggest that other mechanisms may account for the association of the *DC-SIGNR* polymorphism with the reduced risk of HIV-1 infection. One possible mechanism is that the polymorphism cause in vivo relevant difference in the levels of expression on cell surface. In fact, the expression level of each isoform was consistently and reproducibly the same as shown in Fig. 2. The amount of the molecules on cell surface is likely to be due to stability of the molecule. The differences in stability of the DC-SIGNR molecule could reflect the differences in the oligomeric states of the DC-SIGNR isoforms. This idea is supported by some recent studies. Cell-surface DC-SIGNR as well as recombinant, soluble DC-SIGNR molecules forms tetramers [11,14–16]. The extended neck region of DC-SIGNR stabilizes tetramers of the molecules, as is reported in DC-SIGN oligomerization [11,15]. Therefore, it appears that increasing the number of neck region repeats promotes the stable state of DC-SIGNR molecules, and that this may affect their avidity for HIV-1 gp120. In fact, the number of tandem repeats in the soluble versions of DC-SIGNR proteins has been shown to influence binding affinity for HIV-1 gp120 [15]. Another possible mechanism is that the polymorphism may be associated with the functions of smaller DC-SIGNR isoforms in vagina. In addition to a regular splicing event, an alternative splicing event occurs in

DC-SIGNR, and generates a transcript that presumably encodes soluble molecules [21]. DC-SIGNR isoforms lacking the trans-membrane anchoring region may function as intracellular molecules or may be secreted [21,28]. Alternatively-spliced transcripts of *DC-SIGNR* have been identified in vaginal and rectal mucosal samples, and were much more abundant than the full-length *DC-SIGNR* transcript. In fact, no signal for DC-SIGNR on the vaginal cell surface was observed [29]. The soluble DC-SIGNR molecules may modulate efficiency of HIV-1 transmission and dissemination in the vagina. Thus, this possibility is plausible for explaining the differential HIV-1 susceptibility in males and females with respect to DC-SIGNR neck region repeat polymorphism in Thailand [8]. Albeit, we cannot exclude an indirect effect of *DC-SIGNR* polymorphism such as by modulating host immune responses to the HIV-1 infection. Finally, further studies are needed to determine whether the polymorphic genotypes affect the amount of DC-SIGNR isoforms in the virginal and rectal mucosa in vivo either expressed on the cell surface or secreted extracellular.

Acknowledgments

We thank Yoshio Koyanagi (Institute for Virus Research, Kyoto University) for pNL-EGFP. We thank Vineet KewalRamani (HIV Drug Resistance Program, National Cancer Institute) and Kunito Yoshiike (University of Tokyo) for discussion. This work was supported in part by the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases of the Ministry of Education, Culture, Sport, Science and Technology (MEXT); Global COE Program "Center of Education and Research for the Advanced Genome-Based Medicine – For personalized medicine and the control of worldwide infectious diseases" from MEXT; Grants for Research on HIV/AIDS and Research on Publicly Essential Drugs and Medical Devices from the Ministry of Health, Labour, and Welfare of Japan; Grant-in-Aid for Scientific Research (B) from the Japan Society for the Promotion of Science (JSPS).

References

[1] T.B. Geijtenbeek, D.S. Kwon, R. Torensma, S.J. van Vliet, G.C. van Duijnhoven, J. Middel, I.L. Cornelissen, H.S. Nottet, V.N. KewalRamani, D.R. Littman, C.G. Figdor, Y. van Kooyk, DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells, Cell 100 (2000) 587–597.

[2] R.M. Steinman, DC-SIGN: a guide to some mysteries of dendritic cells, Cell 100 (2000) 491–494.

[3] A.A. Bashirova, T.B. Geijtenbeek, G.C. van Duijnhoven, S.J. van Vliet, J.B. Eilering, M.P. Martin, L. Wu, T.D. Martin, N. Viebig, P.A. Knolle, V.N. KewalRamani, Y. van Kooyk, M. Carrington, A dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN)-related protein is highly expressed on human liver sinusoidal endothelial cells and promotes HIV-1 infection, J Exp Med 193 (2001) 671–678.

[4] S. Pohlmann, E.J. Soilleux, F. Baribaud, G.J. Leslie, L.S. Morris, J. Trowsdale, B. Lee, N. Coleman, R.W. Doms, DC-SIGNR, a DC-SIGN homologue expressed in endothelial cells, binds to human and simian immunodeficiency viruses and activates infection in trans, Proc. Natl. Acad. Sci. USA 98 (2001) 2670–2675.

[5] E.J. Soilleux, L.S. Morris, S. Rushbrook, B. Lee, N. Coleman, Expression of human immunodeficiency virus (HIV)-binding lectin DC-SIGNR: consequences for HIV infection and immunity, Hum. Pathol. 33 (2002) 652–659.

[6] D.A. Mitchell, A.J. Fadden, K. Drickamer, A novel mechanism of carbohydrate recognition by the C-type lectins DC-SIGN and DC-SIGNR. Subunit organization and binding to multivalent ligands, J. Biol. Chem. 276 (2001) 28939–28945.

[7] E.J. Soilleux, R. Barten, J. Trowsdale, DC-SIGN; a related gene, DC-SIGNR; and CD23 form a cluster on 19p13, J. Immunol. 165 (2000) 2937–2942.

[8] N. Wichukchinda, Y. Kitamura, A. Rojanawiwat, E.E. Nakayama, H. Song, P. Pathipvanich, W. Auwanit, P. Sawanpanyalert, A. Iwamoto, T. Shioda, K. Ariyoshi, The polymorphisms in DC-SIGNR affect susceptibility to HIV type 1 infection, AIDS Res. Hum. Retroviruses 23 (2007) 686–692.

[9] N. Kobayashi, H.T. Nakamura, M. Goto, T. Nakamura, K. Nakamura, W. Sugiura, A. Iwamoto, Y. Kitamura, Polymorphisms and haplotypes of the CD209L gene and their association with the clinical courses of HIV-positive Japanese patients, Jpn. J. Infect. Dis. 55 (2002) 131–133.

[10] K. Drickamer, Engineering galactose-binding activity into a C-type mannose-binding protein, Nature 360 (1992) 183–186.

[11] H. Feinberg, Y. Guo, D.A. Mitchell, K. Drickamer, W.I. Weis, Extended neck regions stabilize tetramers of the receptors DC-SIGN and DC-SIGNR, J. Biol. Chem. 280 (2005) 1327–1335.

[12] H. Feinberg, D.A. Mitchell, K. Drickamer, W.I. Weis, Structural basis for selective recognition of oligosaccharides by DC-SIGN and DC-SIGNR, Science 294 (2001) 2163–2166.

[13] S. Pohlmann, F. Baribaud, B. Lee, G.J. Leslie, M.D. Sanchez, K. Hiebenthal-Millow, J. Munch, F. Kirchhoff, R.W. Doms, DC-SIGN interactions with human immunodeficiency virus type 1 and 2 and simian immunodeficiency virus, J. Virol. 75 (2001) 4664–4672.

[14] G.A. Snyder, M. Colonna, P.D. Sun, The structure of DC-SIGNR with a portion of its repeat domain lends insights to modeling of the receptor tetramer, J. Mol. Biol. 347 (2005) 979–989.

[15] G.A. Snyder, J. Ford, P. Torabi-Parizi, J.A. Arthos, P. Schuck, M. Colonna, P.D. Sun, Characterization of DC-SIGN/R interaction with human immunodeficiency virus type 1 gp120 and ICAM molecules favors the receptor's role as an antigen-capturing rather than an adhesion receptor, J. Virol. 79 (2005) 4589–4598.

[16] O.K. Bernhard, J. Lai, J. Wilkinson, M.M. Sheil, A.L. Cunningham, Proteomic analysis of DC-SIGN on dendritic cells detects tetramers required for ligand binding but no association with CD4, J. Biol. Chem. 279 (2004) 51828–51835.

[17] Y. Guo, H. Feinberg, E. Conroy, D.A. Mitchell, R. Alvarez, O. Blixt, M.E. Taylor, W.I. Weis, K. Drickamer, Structural basis for distinct ligand-binding and targeting properties of the receptors DC-SIGN and DC-SIGNR, Nat. Struct. Mol. Biol. 11 (2004) 591–598.

[18] L.B. Barreiro, L. Quintana-Murci, DC-SIGNR neck-region polymorphisms and HIV-1 susceptibility: from population stratification to a possible advantage of the 7/5 heterozygous genotype, J. Infect. Dis. 194 (2006) 1184–1185 (author reply 1185–1187).

[19] H. Liu, M. Carrington, C. Wang, S. Holte, J. Lee, B. Greene, F. Hladik, D.M. Koelle, A. Wald, K. Kurosawa, C.R. Rinaldo, C. Celum, R. Detels, L. Corey, M.J. McElrath, T. Zhu, Repeat-region polymorphisms in the gene for the dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin-related molecule: effects on HIV-1 susceptibility, J. Infect. Dis. 193 (2006) 698–702.

[20] H. Liu, Y. Hwangbo, S. Holte, J. Lee, C. Wang, N. Kaupp, H. Zhu, C. Celum, L. Corey, M.J. McElrath, T. Zhu, Analysis of genetic polymorphisms in CCR5, CCR2, stromal cell-derived factor-1, RANTES, and dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin in seronegative individuals repeatedly exposed to HIV-1, J. Infect. Dis. 190 (2004) 1055–1058.

[21] S. Mummidi, G. Catano, L. Lam, A. Hoefle, V. Telles, K. Begum, F. Jimenez, S.S. Ahuja, S.K. Ahuja, Extensive repertoire of membrane-bound and soluble dendritic cell-specific ICAM-3-grabbing nonintegrin 1 (DC-SIGN1) and DC-SIGN2 isoforms. Inter-individual variation in expression of DC-SIGN transcripts, J. Biol. Chem. 276 (2001) 33196–33212.

[22] A. Rathore, A. Chatterjee, P. Sivarama, N. Yamamoto, T.N. Dhole, Role of homozygous DC-SIGNR 5/5 tandem repeat polymorphism in HIV-1 exposed seronegative North Indian individuals, J. Clin. Immunol. 28 (2008) 50–57.

[23] T. Gramberg, T. Zhu, C. Chaipan, A. Marzi, H. Liu, A. Wegele, T. Andrus, H. Hofmann, S. Pohlmann, Impact of polymorphisms in the DC-SIGNR neck domain on the interaction with pathogens, Virology 347 (2006) 354–363.

[24] M. Lichterfeld, H.D. Nischalke, J. van Lunzen, J. Sohne, N. Schmeisser, R. Woitas, T. Sauerbruch, J.K. Rockstroh, U. Spengler, The tandem-repeat polymorphism of the DC-SIGNR gene does not affect the susceptibility to HIV infection and the progression to AIDS, Clin. Immunol. 107 (2003) 55–59.

[25] L. Wu, T.D. Martin, M. Carrington, V.N. KewalRamani, Raji B cells, misidentified as THP-1 cells, stimulate DC-SIGN-mediated HIV transmission, Virology 318 (2004) 17–23.

[26] S. Harada, Y. Koyanagi, N. Yamamoto, Infection of HTLV-III/LAV in HTLV-I-carrying cells MT-2 and MT-4 and application in a plaque assay, Science 229 (1985) 563–566.

[27] Y. Miura, N. Misawa, Y. Kawano, H. Okada, Y. Inagaki, N. Yamamoto, M. Ito, H. Yagita, K. Okumura, H. Mizusawa, Y. Koyanagi, Tumor necrosis factor-related apoptosis-inducing ligand induces neuronal death in a murine model of HIV central nervous system infection, Proc. Natl. Acad. Sci. USA 100 (2003) 2777–2782.

[28] H. Liu, F. Hladik, T. Andrus, P. Sakchalathorn, G.M. Lentz, M.F. Fialkow, L. Corey, M.J. McElrath, T. Zhu, Most DC-SIGNR transcripts at mucosal HIV transmission sites are alternatively spliced isoforms, Eur. J. Hum. Genet. 13 (2005) 707–715.

[29] B. Jameson, F. Baribaud, S. Pohlmann, D. Ghavimi, F. Mortari, R.W. Doms, A. Iwasaki, Expression of DC-SIGN by dendritic cells of intestinal and genital mucosae in humans and rhesus macaques, J. Virol. 76 (2002) 1866–1875.

Microbiology and Immunology

Microbiol Immunol 2010; **54**: 196–205
doi:10.1111/j.1348-0421.2010.00206.x

ORIGINAL ARTICLE

Changes in impact of HLA class I allele expression on HIV-1 plasma virus loads at a population level over time

Michiko Koga[1], Ai Kawana-Tachikawa[1], David Heckerman[2], Takashi Odawara[1], Hitomi Nakamura[1], Tomohiko Koibuchi[3], Takeshi Fujii[3], Toshiyuki Miura[4] and Aikichi Iwamoto[1,5,6]

[1]Division of Infectious Disease, Advanced Clinical Research Center, [3]Department of Infectious Diseases and Applied Immunology, Research Hospital, [4]Department of Infectious Disease Control, International Research Center for Infectious Diseases, [5]Department of infectious Disease and Applied Immunology, and [6]Research Center for Asian Infectious Diseases, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo, 108-8639, Japan and [2]Microsoft Research, Redmond, Washington 98052

ABSTRACT

HLA class I allele types have differential impacts on the level of the pVL and outcome of HIV-1 infection. While accumulations of CTL escape mutations at population levels have been reported, their actual impact on the level of the pVL remains unknown. In this study HLA class I types from 141 untreated, chronically HIV-1 infected Japanese patients diagnosed from 1995–2007 were determined, and the associations between expression of individual HLA alleles and level of pVL analyzed. It was found that the Japanese population has an extremely narrow HLA distribution compared to other ethnic groups, which may facilitate accumulation of CTL escape mutations at the population level. Moreover while they uniquely lack the most protective HLA-B27/B57, they commonly express the alleles that are protective in Caucasians (A11:10.4%, A26:11.55%, B51:8.6% and Cw14:12.7%). Cross-sectional analyses revealed no significant associations between expression of individual alleles and the level of the pVL. The patients were then stratified by the date of HIV diagnosis and the analyses repeated. It was found that, before 2001, B51+ individuals displayed significantly lower pVL than the other patients (median: 5150 vs. 18 000 RNA copies/ml, $P = 0.048$); however thereafter this protective effect waned and disappeared, whereas no changes were observed for any other alleles over time. These results indicate that, at a population level, some HLA alleles have been losing their beneficial effects against HIV disease progression over time, thereby possibly posing a significant challenge for HIV vaccine development. However such detrimental effects may be limited to particular HLA class I alleles.

Key words CTL escape mutations, HIV-1, HLA class I, plasma virus loads.

HIV-1 is the causative agent for AIDS. Since the discovery of HIV-1 in 1983, although a myriad of studies focusing on the immunopathogenesis of HIV-1 infection have been conducted, a number of questions remained unanswered, hampering development of HIV/AIDS vaccines.

As the HIV-1 epidemic has continued, it has become evident that the rate of decline in CD4+ T cells varies considerably between infected people, and that untreated individuals with larger pVL during the asymptomatic phase of infection progress to AIDS more rapidly than those with

Correspondence
Aikichi Iwamoto, Division of Infectious Disease, Advanced Clinical Research Center, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo, 108-8639, Japan.
Tel: +81 3 5449 5359; fax: +81 3 6409 2008; email: aikichi@ims.u-tokyo.ac.jp

Received 28 October 2009; revised 23 December 2009; accepted 6 January 2010.

List of Abbreviations: CTL, cytotoxic T lymphocyte; HIV-1, human immunodeficiency virus type I; HLA, human leukocyte antigen; IQR, inter- quartile range; KIR, killer immunoglobulin-like receptors; MSM, men who have sex with men; PBMC, peripheral blood mononuclear cells; PCR-SSOP, polymerase chain reaction-sequence specific oligonucleotide probes; pVL, plasma virus loads.

lower pVL (1, 2). Host genetics, host innate and adaptive immune responses, and viral sequence variations have all been suggested as possible factors influencing the level of viremia and disease outcome (3–5). Amongst host genetic factors, HLA class I types are recognized to be the most influential with respect to disease progression (6–9), indicating that the effects of HLA class I molecules on HIV-1 specific CTL responses play a major role in controlling viremia. A number of studies have reported differential impacts of HLA class I allele expression on the level of the pVL and/or disease outcome: HLA-B27, B51 and B57 are associated with lower pVL and better clinical outcome (7, 10–12), whereas HLA-B*3502/3503 and B53 have a detrimental effect on these parameters (6, 8, 13, 14).

However, such studies have been performed either in Western countries, such as the United States (6, 7, 11), or in South Africa (12), where Caucasians and/or Africans dominate over other ethnic groups; accordingly information from Asian countries is largely lacking, although an estimated 5.0 million people were living with HIV/AIDS in Asia in 2007, accounting for 15% of the world total (15). Because people living in Asia have distinct patterns of HLA class I profiles, the known associations between HLA class I allele expression and HIV disease outcome may be applicable only to a limited geographical area on the globe. In order to design globally effective HIV vaccines that aim to induce CTL responses restricted by HLA class I molecules, it is crucial to identify the differential ability of HLA class I alleles to control viremia in different parts of the world.

Of importance, CTL escape mutations have been shown to accumulate in populations (16, 17), suggesting that we have been losing targeting epitopes. However, the actual impact on the level of pVL or clinical outcome has yet to be determined. There is an urgent need to investigate whether or not accumulations of CTL escape mutations at a population level increase the virulence of HIV-1 infection.

In the present study, we have examined the impact of HLA class I allele expression on the level of pVL and rate of CD4+ T cell decline in chronically HIV-1 infected Japanese patients who have distinct class I allele expression profiles compared to Caucasians or Africans, in that: (1) they express neither major protective alleles (HLA-B27/B57) nor detrimental alleles (HLA-B*3502/B*3503/B53); and (2) they have a much narrower HLA distribution as represented by around 70% of Japanese people expressing HLA-A24 (18), and thereby likely facilitate accumulation of CTL escape mutations at the population level. In a cross-sectional analysis, we found no significant associations between the level of pVL and individual HLA class I allele expression in this unique Asian population, including HLA-B51 which ranked as the third most protective allele in Caucasians (7). Further analysis revealed that HLA-B51 has been losing its ability to control viremia in this population as the epidemic matures. However this is not the case for the other alleles, suggesting that unfavorable consequences of the accumulation of CTL escape mutations might be limited to particular HLA class I alleles. Nonetheless, these differences still pose a significant challenge for those designing globally effective HIV vaccines.

MATERIALS AND METHODS

Study subjects

In the present study, a total of 141 Japanese subjects who had been diagnosed with HIV-1 infection from 1995 to 2007, and had remained untreated, were enrolled. In order to exclude individuals diagnosed during an acute/early phase of infection, only those who were fully Western blot positive were enrolled, while those with a history of being HIV seronegative within the year prior to their first visit to the clinics were excluded. Written informed consent was obtained from all participants, and the study was approved by the Institutional Review Boards of the Institute of Medical Science, the University of Tokyo (No. 11-2-0329). All the participants were Japanese and all had acquired HIV-1 through sexual intercourse; all but six were men, 96% of whom were MSM.

PVL and rate of decline in CD4+T cell counts

PVL were measured by the Roche HIV Amplicore (Roche Diagnostics, Indianapolis, IN, USA). PVL and CD4+ T cell counts at the first available time points were used for the analyses. The median pVL was 19 000 RNA copies/ml (IQR: 5000–49 000 RNA copies/ml). The median CD4+T cell count was 351/μl (IQR: 273–444/μl) at the corresponding time point for each individual. The rates of decline in CD4+ T cell count (cells/year) were calculated using the values at 6 and 18 months after the first visit to the hospital.

HLA class I allele typing

High resolution HLA genotyping (4–6 digits) was performed as follows: Genomic DNA was extracted from PBMC using the QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. The genotypes of HLA-A,-B, and -C, were determined by PCR-SSOP using the WAKFlow HLA typing kit (Wakunaga, Hiroshima, Japan) (19) and the Luminex Multi-Analyte Profiling system (xMAP, Luminex Corporation, Austin, TX, USA) (18, 19), according to the manufacturer's instructions. For most of the analyses, we used only 2-digit types.

Statistical analysis

Comparisons of level of pVL and CD4+ T cell decline between the two groups were performed by the Mann–Whitney U test, and a q-value approach was adopted for multiple comparisons (20). $q < 0.2$ were considered statistically significant.

RESULTS

The Japanese population has a narrow HLA class I allele distribution lacking the most protective, HLA-B27/B57, yet frequently expressing the third most protective, HLA-B51

In the present study, we aimed to identify HLA class I alleles that are associated with slow or rapid HIV disease progression in the Japanese population, and to investigate changes in the impact of individual HLA class I allele expression on disease progression at the population level over time. To this end, we initially sought to characterize HLA class I allele distribution in the Japanese population as compared to that in Western countries. We expected the Japanese to have a narrower spectrum of HLA class I types, since Japan is geographically isolated and had closed the door to other nations for a long time, as a result having very few immigrants. We reviewed the literature and compared HLA distributions in the general population between Japan and the USA (Fig. 1). We found that the total number of HLA class I alleles with over 1% of allelic frequency in the Japanese population was only 29 (A: 6, B: 15 and Cw: 8, $n = 1018$, Fig. 1a), which is considerably smaller than that found in European-Americans (total: 46, A: 14, B: 19, Cw: 13, $n = 265$, Fig. 1b), and in African-Americans (total: 50, A: 16, B: 21, Cw: 13, $n = 252$, Fig. 1c) (18, 21), confirming that the Japanese population is genetically much less diverse as compared to these other major ethnic groups. Furthermore, we noticed unique features in the Japanese population: (1) over 70% of people express HLA-A24; (2) the major protective alleles against HIV disease progression found in North America and in African countries are rarely seen (B27: 0.05% and B57: 0.0% of allelic- frequency) (18); (3) the major detrimental alleles (B*5802, B*3502/3503 and B53) are not observed at all (18); and (4) HLA-B51, which is widely known to be protective in Caucasians, is common in the Japanese population, almost 20% of people expressing this allele (Fig. 1a). These results indicate that HIV-1 circulating in this unique Asian population has been exposed to a distinct environment in terms of CTL selection pressures as compared to HIV-1 circulating in Caucasian or African populations.

Impact of individual HLA class I allele expression on pVL in the HIV-1 infected Japanese population

Given the distinctive HLA distribution in the Japanese population, we sought to find class I alleles associated with slow or rapid disease progression that have never been reported from the Western countries. We performed HLA class I genotyping on specimens from 141 untreated chronically HIV-1 infected Japanese (see Materials and Methods) and examined their impacts on level of pVL, which is known to be closely associated with the rate of HIV disease progression (1, 2). The distribution of alleles in HIV-1 infected Japanese was similar to that of the general Japanese population described above (data not shown). We then compared the level of pVL in terms of presence or absence of individual class I alleles (Table 1), and found that five alleles (HLA-A20, B07, B54, Cw01 and Cw15) were associated with lower or larger pVL, ($P < 0.05$ by Fisher's exact probability test). However, after determining q-values (20) none of the associations remained significant, indicating that there are no strongly protective or detrimental alleles in this unique Asian population. Notably, in this cross-sectional analysis, expression of HLA-B51, which is the third most beneficial allele after B57 and B27 in Caucasians (7, 22), proved to be not at all protective in Japan; likewise, HLA-A11, A26 and Cw14, which have also been reported to be protective in the USA in a study which controlled for ethnicity (7), did not show any protective effects in Japanese, either. Taken together, these results indicate that alleles which have protective effects in a given population do not necessarily behave similarly in other populations.

Level of pVL is not associated with expression of particular HLA class I supertypes or homozygotes for the Bw6 motif of HLA in the Japanese population

An HLA supertype is defined as a group of class I alleles sharing a similar peptide binding motif, thereby being able to present the same CTL epitopes (23). Some HLA class I supertypes have been reported to be associated with pVL in the USA: (B7s with larger pVL, and B27s/B58s with lower pVL) (24). We looked for such associations in the Japanese population by classifying alleles observed in our cohort into eight supertypes according to the literature (i.e., A1s, A2s, A3s, A24s, B7s, B27s, B44s, B62s) (23), and found that there were no significant associations between level of pVL and expression of particular class I supertypes in the Japanese population (data not shown). This finding may be due to the Japanese lacking HLA-B27/B57, which are major contributors to the protective supertypes in the USA (24).

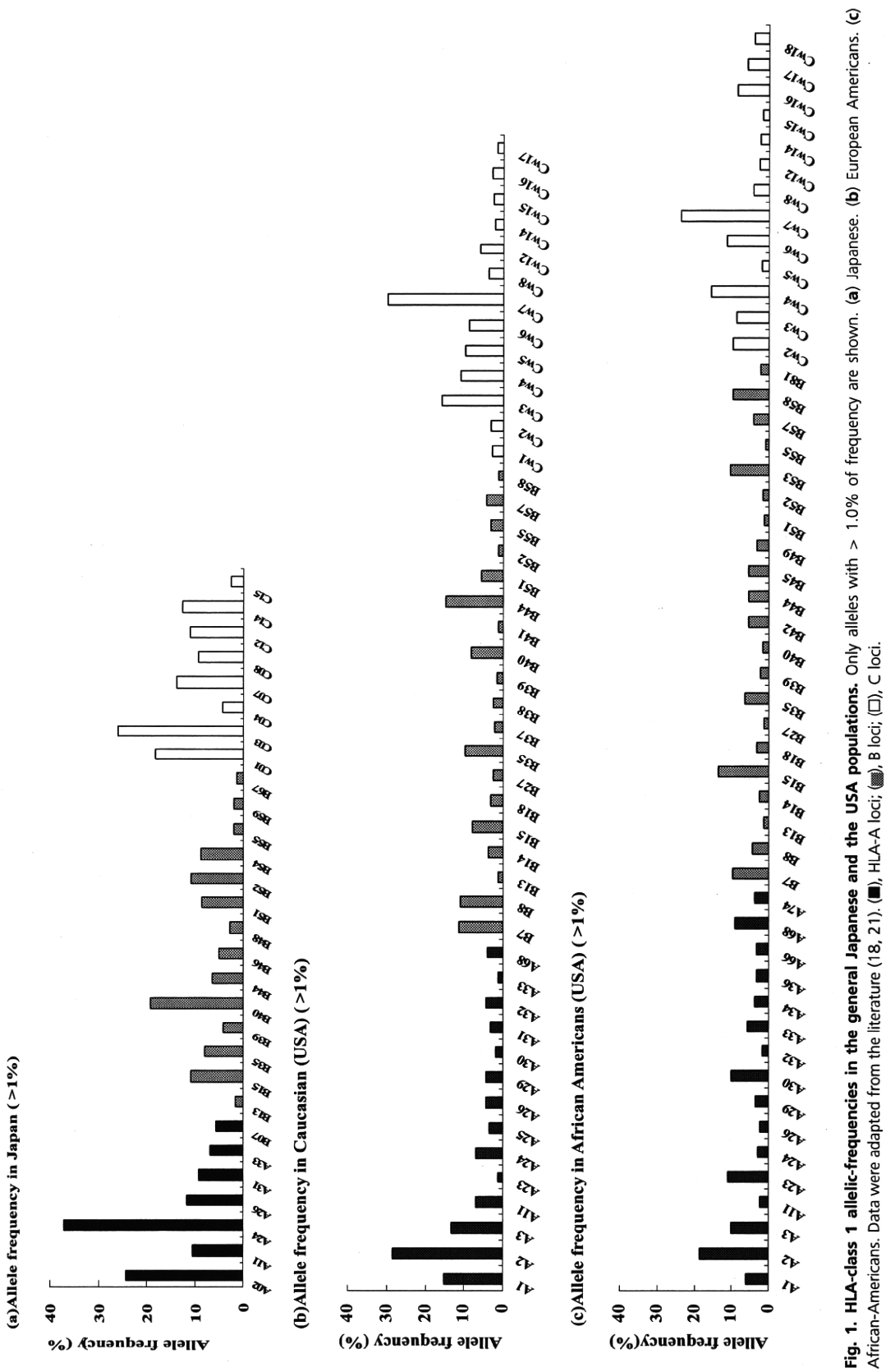

Fig. 1. HLA-class 1 allelic-frequencies in the general Japanese and the USA populations. Only alleles with > 1.0% of frequency are shown. (**a**) Japanese. (**b**) European Americans. (**c**) African-Americans. Data were adapted from the literature (18, 21). (■), HLA-A loci; (▩), B loci; (□), C loci.

Table 1. Association between the level of pVL and expression of individual HLA class I alleles ($n = 141$)

HLA allele	Absence of allele RNA copies/ml (log_{10})*	Presence of allele RNA copies/ml (log_{10})*	*P* value†	*q* value‡
A20	4.279	5.663	0.014	0.460
C01	4.230	4.491	0.027	0.460
C15	4.322	3.500	0.034	0.460
B07	4.342	3.703	0.040	0.460
B54	4.255	4.445	0.048	0.460
A01	4.267	4.699	0.088	0.639
B44	4.312	3.934	0.111	0.639
C05	4.290	3.041	0.178	0.639
A03	4.290	3.041	0.179	0.639
C14	4.301	4.216	0.201	0.639
C07	4.322	4.161	0.203	0.639
B48	4.312	3.462	0.215	0.639
A24	4.342	4.161	0.233	0.639
A33	4.312	4.079	0.249	0.639
B13	4.255	4.477	0.258	0.639
C03	4.243	4.322	0.274	0.639
A26	4.230	4.360	0.278	0.639
B55	4.279	4.405	0.280	0.639
B35	4.255	4.380	0.316	0.645
B51	4.312	4.176	0.341	0.645
B56	4.290	4.041	0.367	0.650
B27	4.279	4.771	0.375	0.650
B15	4.267	4.362	0.453	0.752
B67	4.301	4.073	0.497	0.771
A31	4.301	4.255	0.612	0.915
A02	4.255	4.332	0.621	0.915
B46	4.279	4.445	0.686	0.941
C12	4.279	4.300	0.730	0.947
B37	4.290	3.919	0.800	0.947
A30	4.279	4.477	0.803	0.947
B58	4.279	4.239	0.814	0.947
C04	4.279	4.322	0.855	0.947
A11	4.332	4.176	0.869	0.947
C08	4.300	4.204	0.874	0.947
B59	4.279	4.381	0.876	0.947
B52	4.255	4.332	0.883	0.947
C06	4.279	4.198	0.931	0.968
B40	4.322	4.204	0.964	0.974
B39	4.279	4.462	0.983	0.977

*median pVL are shown on a log_{10} scale.
†The Fisher exact test was performed and $P < 0.05$ was considered significant.
‡*q*-values (cut off < 0.2) for seeking strong specificity of the alleles are given.

We further assessed the impact on pVL of the Bw4/Bw6 motif of HLA class I molecules, which are known to act as ligands of KIR on natural killer cells and to modulate their activity (25, 26). Homozygosity for Bw6 motif has been reported to be associated with rapid disease progression, whereas the subtype of Bw4, which is carried by various alleles including HLA-B27/B57, is associated with slow disease progression (27, 28). However, there was no difference in the level of pVL between Bw4 and Bw6 homozygotes in the Japanese population (median: 26 000 vs. 20 500 RNA copies/ml, $P = 0.976$, Fig. 2), indicating that the findings reported from the USA cannot reliably be extended to other populations.

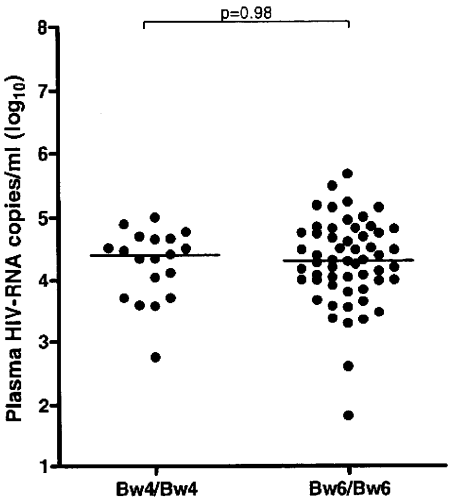

Fig. 2. Comparison of pVL according to expression of Bw motifs of HLA in the HIV-1 infected Japanese population. HLA class I B alleles from the 141 subjects were subcategorized as either Bw4 or Bw6 motifs according to the literature (28), and the level of pVL were compared between homozygotes for Bw4 and Bw6.

Changes in impact of expression of HLA class I alleles on HIV pVL at the population level over time

In the cross-sectional analyses, we did not find any associations between the level of pVL and expression of individual class I alleles, supertypes or Bw motifs in this unique Asian population. Notably, expression of HLA-B51, the third most protective allele in Caucasians, was not associated with lower pVL in this Japanese population. We have previously demonstrated that escape mutations from CTL restricted by HLA-A24, which is the most common allele in Japan (expressed in >70% of Japanese), has been accumulating amongst viral strains circulating in Japan, implying that individuals expressing HLA-A24 have been losing their targeting epitopes (16). Likewise, there is a report that the majority of recently-infected HLA-A02+ individuals in the USA cannot mount CTL responses to the epitopes that had been previously recognized in HLA-A02+ individuals, suggesting that escape mutations from this response have been accumulating in the USA population (29). Moreover, a recent study by Kawashima *et al.* has demonstrated accumulations of CTL escape mutations

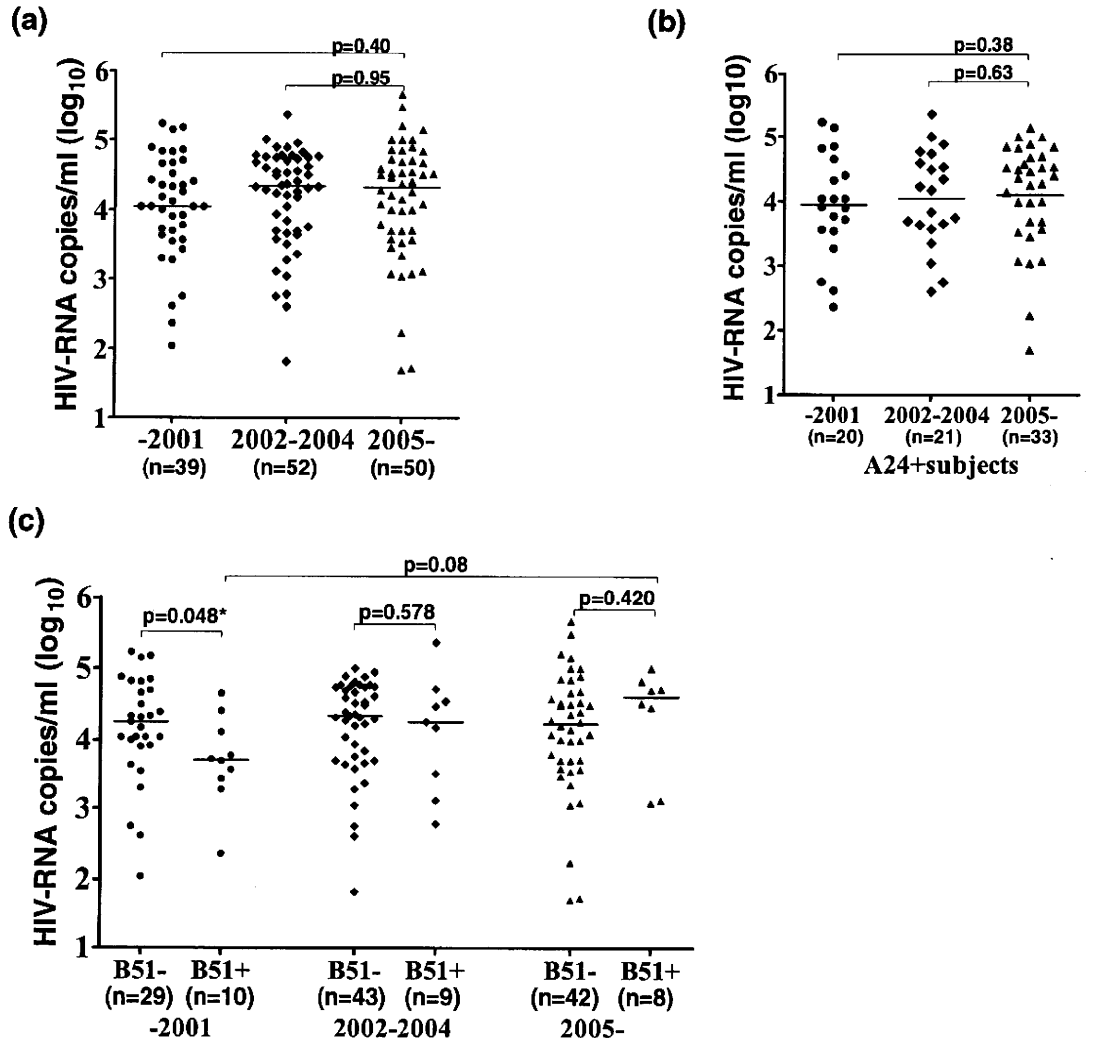

Fig. 3. Changes in the level of pVL at the population level over time. The level of pVL was compared between different time periods of HIV-1 diagnosis. (**a**) All 141 Japanese subjects (**b**) HLA-A24+ Japanese (**c**) HLA-B51+ and B51- Japanese subjects. *indicates $P < 0.05$. X-axis indicates the years of HIV diagnosis.

for various HLA class I alleles at population levels (17). However, it remains unknown how these accumulations of viral escape mutations in populations affect the course of the disease. We thought that the narrow HLA class I spectrum in the Japanese population might facilitate accumulation of CTL escape mutations, and thereby their influence on disease progression might be more evident in Japan than in other countries. We initially compared level of pVL between individuals diagnosed in the early days of the HIV epidemic and those diagnosed in later years by stratifying the subjects according to the year of HIV diagnosis, regardless of their HLA profiles, but found no difference in the level of pVL between the two phases of the epidemic (Fig. 3a). Next, we focused on HLA-A24, which is shared by over 70% of Japanese people and for which we have previously demonstrated accumulation of CTL escape mutations at the population level (16). However, no difference was observed between the A24+ Japanese diagnosed before 2001 and those diagnosed after 2005 (median: 9650 vs. 23 000 RNA copies/ml, $P = 0.379$, Fig. 3b). We then performed similar comparisons for the alleles considered protective in Caucasians and commonly expressed in the Japanese (A11: 10.4%, A26:11.6%, B51:8.6% and Cw14:12.7% of allelic-frequency) (7, 18), and observed a trend that individuals expressing HLA-B51 and diagnosed before 2001 had substantially lower pVL than those diagnosed after 2005 (median 5150 vs. 41 500 RNA copies/ml, $P = 0.08$, Fig. 3c). Moreover, while

HLA-B51+ persons displayed significantly lower pVL than B51 negative individuals before 2001 (median 5150 vs. 18 000 RNA copies/ml, $P = 0.048$), such differences were not observed between people diagnosed after 2005 (Fig. 3c). Given that Kawashima *et al.* have recently reported a similar trend for HLA-B51 (17), it appears evident that HLA-B51 has been losing its advantage over the other alleles. However, we did not see any changes in pVL over time related to expressions of the other alleles (data not shown), indicating that loss of protective superiority may be limited to particular class I alleles. Although level of pVL is closely associated with the rate of HIV disease progression, it does not measure disease progression directly. We therefore calculated the rate of decline in CD4+ T cell counts (see the Materials and Methods), and investigated their association with HLA allele expression as well, but failed to detect any alterations in the rate of decline as the HIV epidemic matured (data not shown). This may be due to the low statistical power of the present study, therefore larger scale studies are warranted in order to determine to what extent, and for which HLA alleles, such accumulations of CTL escape have been occurring, and how they have been affecting disease progression.

DISCUSSION

In the present study, we have demonstrated that: (1) there are no individual HLA class I alleles which are strongly associated with the level of pVL in the Japanese population at the current time; (2) the Japanese population has a narrow HLA distribution and lacks in the most protective HLA-B27/B57; (3) the proposed advantage of rare class I supertypes and the disadvantage of homozygotes for Bw6 motif cannot be applied to all ethnic groups across the globe; and (4) HLA-B51 has been losing its dominant effects at the population level over time, whereas this is not the case for the other alleles.

Despite substantial numbers of HIV-1 viremia controllers having been recognized in Japan, this population lacks the well-known protective alleles HLA-B27/B57. We therefore expected to discover novel associations between HIV disease progression and HLA class I alleles which are unique to Asian populations. However, in the cross-sectional analysis, we did not identify any significant associations between the level of pVL and expression of individual class I alleles, indicating that, regardless of the geographical part of the world, the protective effects of HLA alleles are greatly biased to a few of the prominent alleles like HLA-B27/B57. The discordant results for HLA supertypes and homozygosity of the Bw6 motif between Japan and the USA are likely also attributable to the lack of HLA-B27/B57 in the Japanese population. These two exceptional alleles are known to have targeting epitopes within Gag protein (10, 30–35). Likewise it has been suggested that expression of HLA alleles other than B27/57, but having targeting epitopes within Gag protein, are associated with lower pVL (8, 36–40). Therefore it is warranted to confirm that Gag specific CTL responses are associated with lower pVL in Japanese people who lack HLA-B27/57.

In the cross-sectional analysis, we did not identify significant associations between pVL and HLA-A11, 26, B51 or Cw14 expression, all of which have been shown to be protective in Caucasians (7), However, subsequent analysis revealed that HLA-B51, at least, was protective in the past, indicating that there has been loss of targeting epitopes in the viral strains circulating in this population. This result for HLA-B51 is supported by a recent report from Japan which demonstrated the accumulation of HLA-B51 escape mutation (17). Adaptation of HIV to HLA might be occurring at a greater speed in the Japanese population, which has a narrower HLA class I distribution as compared to other ethnic groups. In addition, the discordant rate of accumulation of CTL escape mutations between different populations will pose a significant challenge for designing globally effective HIV vaccines.

An increase in pVL over time was not observed for other alleles, including HLA-A24 for which the accumulation of CTL escape mutations amongst circulating viruses had been previously demonstrated (16). There are a number of feasible explanations for this unexpected observation: loss of viral replicative fitness due to CTL escape mutations may reduce viral burden *in vivo* (41–46); escape mutations may provide *de novo* CTL epitopes to the other HLA alleles; CTL restricted by these alleles can do nothing for viremia control from the start, and so on. In order to elucidate the mechanisms for these discordant results, detailed studies on viral sequences and specific CTL responses on an individual epitope basis are required.

We did not see any significant change in the rate of CD4+ T cell decline at the population level over time, though this might have been due to the low statistical power of the current study. Many health care providers have been claiming that recently diagnosed HIV infected individuals appear to progress more rapidly than did those diagnosed in previous years, and Crum-Cianflone *et al.* have reported significantly lower CD4+T cell counts at the first visit to clinics in individuals diagnosed in recent years (47), which may reflect adaptation of HIV to HLA. It is essential to elucidate whether the recent increase in HIV virulence has been caused by viral adaptation to HLA or to other host factors restricting proliferation of HIV.

There was a little concern that the improvement of the sensitivity of HIV-1 RNA quantification for non-B subtypes might have affected overall results; however, as described in the Materials and Methods section,

96% of studied Japanese were MSM; and in Japan virtually all MSM are considered to be infected with clade B. Therefore, inclusion of non-B infected subjects was extremely limited, and unlikely to affect the overall results.

The present study not only adds considerably to currently available knowledge but is also the first comprehensive study on associations between HLA alleles and HIV disease progression in Asia. However, there were a number of limitations: (1) the scale of the study was relatively small, which may have resulted in overlooking some true associations between HLA and the level of pVL; (2) the observation period might have not been long enough to see changes in pVL at the population level; (3) the incidence and prevalence of HIV infection in Japan might not be sufficiently high to see changes in pVL at the population level; and (4) we did not have viral sequence data, meaning that any accumulation of CTL escape mutations could not be demonstrated, though it is evident from the literature that such accumulation has occurred, at least for HLA-A24 and B51 (16, 17). In order to demonstrate that loss of protective effects of particular HLA alleles are attributable to accumulation of CTL escape mutations in the population, it is necessary to define CTL epitopes restricted by common HLA class I alleles in Japan systematically, and to identify escape mutations from those CTL responses. In spite of these limitations, the present study is valuable in consolidating the loss of predominance of some HLA class I alleles in a given population, and in raising concerns about both designing globally effective HIV vaccines and the future virulence of HIV-1.

DISCLOSURE

The authors declare no conflicts of interest related to this study.

ACKNOWLEDGMENTS

We thank the patients and clinical staff at the Research Hospital of the Institute of Medical Science, University of Tokyo, for their essential contributions to this research study. We also thank M. Motose for technical assistance. This work was supported in part by the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases of the Ministry of Education, Culture, Sports, Science and Technology (MEXT); Global COE Program (Center of Education and Research for Advanced Genome-Based Medicine) of MEXT; Grants for Research on HIV/AIDS and Research on Publicly Essential Drugs and Medical Devices from the Ministry of Health, Labor, and Welfare of Japan.

REFERENCES

1. Mellors J.W., Rinaldo C.R. Jr., Gupta P., White R.M., Todd J.A., Kingsley L.A. (1996) Prognosis in HIV-1 infection predicted by the quantity of virus in plasma. *Science (New York, NY)* **272:** 1167–70.
2. Egger M., May M., Chene G., Phillips A.N., Ledergerber B., Dabis F., Costagliola D., D'arminio Monforte A., De Wolf F., Reiss P., Lundgren J.D., Justice A.C., Staszewski S., Leport C., Hogg R.S., Sabin C.A., Gill M.J., Salzberger B., Sterne J.A. (2002) Prognosis of HIV-1-infected patients starting highly active antiretroviral therapy: a collaborative analysis of prospective studies. *Lancet* **360:** 119–29.
3. Telenti A., Goldstein D.B. (2006) Genomics meets HIV-1. *Nat Rev* **4:** 865–73.
4. Simon V., Ho D.D., Abdool Karim Q. (2006) HIV/AIDS epidemiology, pathogenesis, prevention, and treatment. *Lancet* **368:** 489–504.
5. Deeks S.G., Walker B.D. (2007) Human immunodeficiency virus controllers: mechanisms of durable virus control in the absence of antiretroviral therapy. *Immunity* **27:** 406–16.
6. Carrington M., O'brien S.J. (2003) The influence of HLA genotype on AIDS. *Annu Rev Med* **54:** 535–51.
7. O'brien S.J., Gao X., Carrington M. (2001) HLA and AIDS: a cautionary tale. *Trends Mol Med* **7:** 379–81.
8. Goulder P.J., Watkins D.I. (2008) Impact of MHC class I diversity on immune control of immunodeficiency virus replication. *Nat Rev Immunol* **8:** 619–30.
9. Stephens H.A. (2005) HIV-1 diversity versus HLA class I polymorphism. *Trends Immunol* **26:** 41–7.
10. Migueles S.A., Sabbaghian M.S., Shupert W.L., Bettinotti M.P., Marincola F.M., Martino L., Hallahan C.W., Selig S.M., Schwartz D., Sullivan J., Connors M. (2000) HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors. *Proc Natl Acad Sci U S A* **97:** 2709–14.
11. Kaslow R.A., Carrington M., Apple R., Park L., Munoz A., Saah A.J., Goedert J.J., Winkler C., O'brien S.J., Rinaldo C., Detels R., Blattner W., Phair J., Erlich H., Mann D.L. (1996) Influence of combinations of human major histocompatibility complex genes on the course of HIV-1 infection. *Nat Med* **2:** 405–11.
12. Kiepiela P., Leslie A.J., Honeyborne I., Ramduth D., Thobakgale C., Chetty S., Rathnavalu P., Moore C., Pfafferott K.J., Hilton L., Zimbwa P., Moore S., Allen T., Brander C., Addo M.M., Altfeld M., James I., Mallal S., Bunce M., Barber L.D., Szinger J., Day C., Klenerman P., Mullins J., Korber B., Coovadia H.M., Walker B.D., Goulder P.J. (2004) Dominant influence of HLA-B in mediating the potential co-evolution of HIV and HLA. *Nature* **432:** 769–75.
13. Carrington M., Nelson G.W., Martin M.P., Kissner T., Vlahov D., Goedert J.J., Kaslow R., Buchbinder S., Hoots K., O'brien S.J. (1999) HLA and HIV-1: heterozygote advantage and B*35-Cw*04 disadvantage. *Science (New York, NY)* **283:** 1748–52.
14. Gao X., Nelson G.W., Karacki P., Martin M.P., Phair J., Kaslow R., Goedert J.J., Buchbinder S., Hoots K., Vlahov D., O'brien S.J., Carrington M. (2001) Effect of a single amino acid change in MHC class I molecules on the rate of progression to AIDS. *N Engl J Med* **344:** 1668–75.
15. Unaids (2008) Status of the global HIV epidemic. 2008 Report on the global AIDS epidemic.
16. Furutsuki T., Hosoya N., Kawana-Tachikawa A., Tomizawa M., Odawara T., Goto M., Kitamura Y., Nakamura T., Kelleher A.D., Cooper D.A., Iwamoto A. (2004) Frequent transmission of cytotoxic-T-lymphocyte escape mutants of human immunodeficiency virus type 1 in the highly HLA-A24-positive Japanese population. *J Virol* **78:** 8437–45.

17. Kawashima Y., Pfafferott K., Frater J., Matthews P., Payne R., Addo M., Gatanaga H., Fujiwara M., Hachiya A., Koizumi H., Kuse N., Oka S., Duda A., Prendergast A., Crawford H., Leslie A., Brumme Z., Brumme C., Allen T., Brander C., Kaslow R., Tang J., Hunter E., Allen S., Mulenga J., Branch S., Roach T., John M., Mallal S., Ogwu A., Shapiro R., Prado J.G., Fidler S., Weber J., Pybus O.G., Klenerman P., Ndung'u T., Phillips R., Heckerman D., Harrigan P.R., Walker B.D., Takiguchi M., Goulder P. (2009) Adaptation of HIV-1 to human leukocyte antigen class I. *Nature.*
18. Itoh Y., Mizuki N., Shimada T., Azuma F., Itakura M., Kashiwase K., Kikkawa E., Kulski J.K., Satake M., Inoko H. (2005) High-throughput DNA typing of HLA-A, -B, -C, and -DRB1 loci by a PCR-SSOP-Luminex method in the Japanese population. *Immunogenetics* **57:** 717–29.
19. Ogata S., Shi L., Matsushita M., Yu L., Huang X.Q., Shi L., Sun H., Ohashi J., Muramatsu M., Tokunaga K., Chu J.Y. (2007) Polymorphisms of human leucocyte antigen genes in Maonan people in China. *Tissue Antigens* **69:** 154–60.
20. Storey J.D., Tibshirani R. (2003) Statistical significance for genome wide studies. *Proc Natl Acad Sci U S A* **100:** 9440–5.
21. Cao K., Hollenbach J., Shi X., Shi W., Chopek M., Fernandez-Vina M.A. (2001) 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* **62:** 1009–30.
22. Pereyra F., Addo M.M., Kaufmann D.E., Liu Y., Miura T., Rathod A., Baker B., Trocha A., Rosenberg R., Mackey E., Ueda P., Lu Z., Cohen D., Wrin T., Petropoulos C.J., Rosenberg E.S., Walker B.D. (2008) Genetic and immunologic heterogeneity among persons who control HIV infection in the absence of therapy. *J Infect Diseases* **197:** 563–71.
23. Sette A., Sidney J. (1999) Nine major HLA class I supertypes account for the vast preponderance of HLA-A and -B polymorphism. *Immunogenetics* **50:** 201–12.
24. Trachtenberg E., Korber B., Sollars C., Kepler T.B., Hraber P.T., Hayes E., Funkhouser R., Fugate M., Theiler J., Hsu Y.S., Kunstman K., Wu S., Phair J., Erlich H., Wolinsky S. (2003) Advantage of rare HLA supertype in HIV disease progression. *Nat Med* **9:** 928–35.
25. Carrington M., Martin M.P., Van Bergen J. (2008) KIR-HLA intercourse in HIV disease. *Trends Microbiol* **16:** 620–7.
26. Alter G., Altfeld M. (2009) NK cells in HIV-1 infection: evidence for their role in the control of HIV-1 infection. *J Intern Med* **265:** 29–42.
27. Flores-Villanueva P.O., Yunis E.J., Delgado J.C., Vittinghoff E., Buchbinder S., Leung J.Y., Uglialoro A.M., Clavijo O.P., Rosenberg E.S., Kalams S.A., Braun J.D., Boswell S.L., Walker B.D., Goldfeld A.E. (2001) Control of HIV-1 viremia and protection from AIDS are associated with HLA-Bw4 homozygosity. *Proc Natl Acad Sci U S A* **98:** 5140–5.
28. Martin M.P., Gao X., Lee J.H., Nelson G.W., Detels R., Goedert J.J., Buchbinder S., Hoots K., Vlahov D., Trowsdale J., Wilson M., O'brien S.J., Carrington M. (2002) Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. *Nat Genet* **31:** 429–34.
29. Altfeld M., Allen T.M., Kalife E.T., Frahm N., Addo M.M., Mothe B.R., Rathod A., Reyor L.L., Harlow J., Yu X.G., Perkins B., Robinson L.K., Sidney J., Alter G., Lichterfeld M., Sette A., Rosenberg E.S., Goulder P.J., Brander C., Walker B.D. (2005) The majority of currently circulating human immunodeficiency virus type 1 clade B viruses fail to prime cytotoxic T-lymphocyte responses against an otherwise immunodominant HLA-A2-restricted epitope: implications for vaccine design. *J Virol* **79:** 5000–5.
30. Goulder P.J., Brander C., Tang Y., Tremblay C., Colbert R.A., Addo M.M., Rosenberg E.S., Nguyen T., Allen R., Trocha A., Altfeld M., He S., Bunce M., Funkhouser R., Pelton S.I., Burchett S.K., Mcintosh K., Korber B.T., Walker B.D. (2001) Evolution and transmission of stable CTL escape mutations in HIV infection. *Nature* **412:** 334–8.
31. Goulder P.J., Phillips R.E., Colbert R.A., Mcadam S., Ogg G., Nowak M.A., Giangrande P., Luzzi G., Morgan B., Edwards A., Mcmichael A.J., Rowland-Jones S. (1997) Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. *Nat Med* **3:** 212–7.
32. Streeck H., Lichterfeld M., Alter G., Meier A., Teigen N., Yassine-Diab B., Sidhu H.K., Little S., Kelleher A., Routy J.P., Rosenberg E.S., Sekaly R.P., Walker B.D., Altfeld M. (2007) Recognition of a defined region within p24 Gag by CD8+ T cells during primary human immunodeficiency virus type 1 infection in individuals expressing protective HLA class I alleles. *J Virol* **81:** 7725–31.
33. Altfeld M., Kalife E.T., Qi Y., Streeck H., Lichterfeld M., Johnston M.N., Burgett N., Swartz M.E., Yang A., Alter G., Yu X.G., Meier A., Rockstroh J.K., Allen T.M., Jessen H., Rosenberg E.S., Carrington M., Walker B.D. (2006) HLA alleles associated with delayed progression to AIDS contribute strongly to the initial CD8(+) T Cell response against HIV-1. *PLoS Med* **3:** e403.
34. Altfeld M., Addo M.M., Rosenberg E.S., Hecht F.M., Lee P.K., Vogel M., Yu X.G., Draenert R., Johnston M.N., Strick D., Allen T.M., Feeney M.E., Kahn J.O., Sekaly R.P., Levy J.A., Rockstroh J.K., Goulder P.J., Walker B.D. (2003) Influence of HLA-B57 on clinical presentation and viral control during acute HIV-1 infection. *AIDS (London, England)* **17:** 2581–91.
35. Leslie A.J., Pfafferott K.J., Chetty P., Draenert R., Addo M.M., Feeney M., Tang Y., Holmes E.C., Allen T., Prado J.G., Altfeld M., Brander C., Dixon C., Ramduth D., Jeena P., Thomas S.A., St John A., Roach T.A., Kupfer B., Luzzi G., Edwards A., Taylor G., Lyall H., Tudor-Williams G., Novelli V., Martinez-Picado J., Kiepiela P., Walker B.D., Goulder P.J. (2004) HIV evolution: CTL escape mutation and reversion after transmission. *Nat Med* **10:** 282–9.
36. Kiepiela P., Ngumbela K., Thobakgale C., Ramduth D., Honeyborne I., Moodley E., Reddy S., De Pierres C., Mncube Z., Mkhwanazi N., Bishop K., Van Der Stok M., Nair K., Khan N., Crawford H., Payne R., Leslie A., Prado J., Prendergast A., Frater J., Mccarthy N., Brander C., Learn G.H., Nickle D., Rousseau C., Coovadia H., Mullins J.I., Heckerman D., Walker B.D., Goulder P. (2007) CD8 +T-cell responses to different HIV proteins have discordant associations with viral load. *Nat Med* **13:** 46–53.
37. Borghans J.A., Molgaard A., De Boer R.J., Kesmir C. (2007) HLA alleles associated with slow progression to AIDS truly prefer to present HIV-1 p24. *PLoS ONE* **2:** e920.
38. Brumme Z.L., Tao I., Szeto S., Brumme C.J., Carlson J.M., Chan D., Kadie C., Frahm N., Brander C., Walker B., Heckerman D., Harrigan P.R. (2008) Human leukocyte antigen-specific polymorphisms in HIV-1 Gag and their association with viral load in chronic untreated infection. *AIDS (London, England)* **22:** 1277–86.
39. Geldmacher C., Currier J.R., Herrmann E., Haule A., Kuta E., Mccutchan F., Njovu L., Geis S., Hoffmann O., Maboko L., Williamson C., Birx D., Meyerhans A., Cox J., Hoelscher M. (2007) CD8 T-cell recognition of multiple epitopes within specific Gag regions is associated with maintenance of a low steady-state viremia in human immunodeficiency virus type 1-seropositive patients. *J Virol* **81:** 2440–8.
40. Honeyborne I., Prendergast A., Pereyra F., Leslie A., Crawford H., Payne R., Reddy S., Bishop K., Moodley E., Nair K., Van Der Stok

M., Mccarthy N., Rousseau C.M., Addo M., Mullins J.I., Brander C., Kiepiela P., Walker B.D., Goulder P.J. (2007) 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* **81:** 3667–72.

41. Miura T., Brockman M.A., Schneidewind A., Lobritz M., Pereyra F., Rathod A., Block B.L., Brumme Z.L., Brumme C.J., Baker B., Rothchild A.C., Li B., Trocha A., Cutrell E., Frahm N., Brander C., Toth I., Arts E.J., Allen T.M., Walker B.D. (2009) HLA-B57/B*5801 human immunodeficiency virus type 1 elite controllers select for rare gag variants associated with reduced viral replication capacity and strong cytotoxic T-lymphocyte recognition. *J Virol* **83:** 2743–55.
42. Goepfert P.A., Lumm W., Farmer P., Matthews P., Prendergast A., Carlson J.M., Derdeyn C.A., Tang J., Kaslow R.A., Bansal A., Yusim K., Heckerman D., Mulenga J., Allen S., Goulder P.J., Hunter E. (2008) Transmission of HIV-1 Gag immune escape mutations is associated with reduced viral load in linked recipients. *J Exp Med* **205:** 1009–17.
43. Chopera D.R., Woodman Z., Mlisana K., Mlotshwa M., Martin D.P., Seoighe C., Treurnicht F., De Rosa D.A., Hide W., Karim S.A., Gray C.M., Williamson C. (2008) Transmission of HIV-1 CTL escape variants provides HLA-mismatched recipients with a survival advantage. *PLoS Pathog* **4:** e1000033.
44. Martinez-Picado J., Prado J.G., Fry E.E., Pfafferott K., Leslie A., Chetty S., Thobakgale C., Honeyborne I., Crawford H., Matthews P., Pillay T., Rousseau C., Mullins J.I., Brander C., Walker B.D., Stuart D.I., Kiepiela P., Goulder P. (2006) Fitness cost of escape mutations in p24 Gag in association with control of human immunodeficiency virus type 1. *J Virol* **80:** 3617–23.
45. Schneidewind A., Brockman M.A., Yang R., Adam R.I., Li B., Le Gall S., Rinaldo C.R., Craggs S.L., Allgaier R.L., Power K.A., Kuntzen T., Tung C.S., Labute M.X., Mueller S.M., Harrer T., Mcmichael A.J., Goulder P.J., Aiken C., Brander C., Kelleher A.D., Allen T.M. (2007) Escape from the dominant HLA-B27-restricted cytotoxic T-lymphocyte response in Gag is associated with a dramatic reduction in human immunodeficiency virus type 1 replication. *J Virol* **81:** 12382–93.
46. Brockman M.A., Schneidewind A., Lahaie M., Schmidt A., Miura T., Desouza I., Ryvkin F., Derdeyn C.A., Allen S., Hunter E., Mulenga J., Goepfert P.A., Walker B.D., Allen T.M. (2007) Escape and compensation from early HLA-B57-mediated cytotoxic T-lymphocyte pressure on human immunodeficiency virus type 1 Gag alter capsid interactions with cyclophilin A. *J. Virol* **81:** 12608–18.
47. Crum-Cianflone N., Eberly L., Zhang Y., Ganesan A., Weintrob A., Marconi V., Barthel R.V., Fraser S., Agan B.K., Wegner S. (2009) Is HIV becoming more virulent? Initial CD4 cell counts among HIV seroconverters during the course of the HIV epidemic: 1985–2007. *Clin Infect Dis.*

Journal of General Virology (2010), 91, 919–930 DOI 10.1099/vir.0.016766-0

Identification and structural definition of H5-specific CTL epitopes restricted by HLA-A*0201 derived from the H5N1 subtype of influenza A viruses

Yeping Sun,[1,2] Jun Liu,[1,2] Meng Yang,[1] Feng Gao,[1,3] Jianfang Zhou,[4] Yoshihiro Kitamura,[1,5,6] Bin Gao,[1,2,6] Po Tien,[1] Yuelong Shu,[4] Aikichi Iwamoto,[5,6] Zhu Chen[7] and George F. Gao[1,2,6,8]

Correspondence
George F. Gao
gaof@im.ac.cn

[1]CAS Key Laboratory of Pathogenic Microbiology and Immunology, Institute of Microbiology, Chinese Academy of Sciences (CAS), Beijing, PR China

[2]Graduate University, CAS, Beijing, PR China

[3]Institute of Biophysics, CAS, Beijing, PR China

[4]Chinese National Influenza Center, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing, PR China

[5]Division of Infectious Diseases, Advanced Clinical Research Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan

[6]China–Japan Joint Laboratory of Molecular Immunology and Molecular Microbiology, Institute of Microbiology, CAS, Beijing, PR China

[7]Institute of Hematology, Rui Jin Hospital affiliated to Shanghai Second Medical University, Shanghai, PR China

[8]Beijing Institutes of Life Science, CAS, Beijing, PR China

Received 17 September 2009
Accepted 1 December 2009

The haemagglutinin (HA) glycoprotein of influenza A virus is a major antigen that initiates humoral immunity against infection; however, the cellular immune response against HA is poorly understood. Furthermore, HA-derived cytotoxic T-lymphocyte (CTL) epitopes are relatively rare in comparison to other internal gene products. Here, CTL epitopes of the HA serotype H5 protein were screened. By using *in silico* prediction, *in vitro* refolding and a T2 cell-binding assay, followed by immunization of HLA-A2.1/K^b transgenic mice, an HLA-A*0201-restricted decameric epitope, RI-10 (H5 HA205–214, RLYQNPTTYI), was shown to elicit a robust CTL epitope-specific response. In addition, RI-10 and its variant, KI-10 (KLYQNPTTYI), were also demonstrated to be able to induce a higher CTL epitope-specific response than the influenza A virus dominant CTL epitope GL-9 (GILGFVFTL) in peripheral blood mononuclear cells of HLA-A*0201-positive patients who had recovered from H5N1 virus infection. Furthermore, the crystal structures of RI-10–HLA-A*0201 and KI-10–HLA-A*0201 complexes were determined at 2.3 and 2.2 Å resolution, respectively, showing typical HLA-A*0201-restricted epitopes. The conformations of RI-10 and KI-10 in the antigen-presenting grooves in crystal structures of the two complexes show significant differences, despite their nearly identical sequences. These results provide implications for the discovery of diagnostic markers and the design of novel influenza vaccines.

INTRODUCTION

Influenza A virus has always been a major cause of morbidity and mortality throughout history. Both the ability of the virus genome to mutate at a very high rate and its special gene segment-reassortment mechanism often lead to the emergence of highly virulent strains that may potentially become the cause of a pandemic, or at least some panic worldwide, for example the recent outbreak of swine-origin influenza A (H1N1).

The highly pathogenic avian influenza virus (HPAIV) H5N1 has aroused global attention since 1997, when it was transmitted directly from chicken to humans in Hong Kong and resulted in a severe respiratory disease in 18 humans, of whom six died (Claas *et al.*, 1998). From late

2003, H5N1 re-emerged and reached epizootic levels in domestic fowl and even spread to wild birds. In April 2005, a large-scale outbreak of H5N1 infection occurred in migratory waterfowl in Qinghai Lake Nature Reserve of western China, killing more than 6000 wild birds (Chen *et al.*, 2005, 2006; Liu *et al.*, 2005; Wang *et al.*, 2008; Zhou *et al.*, 2006a). Since then, migratory birds have been recognized as a significant source of international spread of HPAIV (Kilpatrick *et al.*, 2006; Normile, 2006; Olsen *et al.*, 2006; Wang *et al.*, 2008). Between December 2003 and 23 April 2009, 421 human H5N1 infection cases and 257 deaths were detected in 15 countries (WHO, 2009), although so far no sustained human-to-human transmission has been found. However, the possibility of a human influenza pandemic resulting from either mutations or avian-origin virus reassortments with human strains caused great international concern and pandemic preparedness has been accelerated (Salomon & Webster, 2009).

In addition to the antibody-induced humoral immune response, cellular immunity plays a variety of roles in defending against influenza virus infection and these have been increasingly documented (Bender & Small, 1992; Rimmelzwaan *et al.*, 2007; Thomas *et al.*, 2006; Yap *et al.*, 1978). In many observations, influenza virus-specific cytotoxic T lymphocytes (CTLs) target mainly viral internal proteins (Gotch *et al.*, 1987; Kees & Krammer, 1984; Townsend & Skehel, 1984; Wahl *et al.*, 2009). As a result, many studies have focused on the cross-reactive CTL response against internal proteins conserved between the H5N1 influenza subtype and seasonal influenza viruses, assuming that a cross-reactive CTL response against the conserved regions of the viral proteome may provide partial protection against H5 influenza virus and dampen the impact of the next pandemic (Ahmed *et al.*, 2007; Doherty & Kelso, 2008; Heiny *et al.*, 2007; Kreijtz *et al.*, 2008; Lee *et al.*, 2008).

Although there are lines of evidence that the viral surface haemagglutinin (HA) elicits a CTL response (Bennink *et al.*, 1984; Townsend *et al.*, 1986; Wabuke-Bunoti *et al.*, 1984), HA-specific CTL epitopes are rare compared with those specific for the viral internal proteins. HA glycoproteins are responsible for virus binding to host receptors, enabling entry into the host cell through endocytosis and subsequent membrane fusion (Wilson & Cox, 1990). HA is recognized as the primary target of neutralizing antibodies and is an important target for both drug and vaccine development (Stevens *et al.*, 2006). However, the role of HA in CTL-based cellular immunity and the major histocompatibility complex (MHC or HLA in humans)-restricted HA epitopes are poorly understood. In the present study, we have successfully identified CTL epitopes in H5 HA restricted by HLA-A*0201 and have shown an immune response in patients who have recovered from H5N1 infection. HLA-A*0201 is an allele expressed by nearly half of the world's population and the peptide–HLA (pHLA) complexes defined here are clearly seen by X-ray crystallography, confirming the authenticity of the epitope presentation.

RESULTS

Selection of potential HLA-A*0201-binding peptides within the H5 protein

Based on BIMAS software analysis, 15 candidate nonameric and decameric peptides with the highest estimated half-life of dissociation were synthesized (Table 1). To evaluate the binding ability of these peptides to the HLA-A*0201 molecule, we employed a peptide-induced stabilization assay (complex refolding) of HLA-A*0201 heavy chain and β2 microglobulin (β2m) *in vitro*. In this experiment, the correctly refolded complex is detected if the right peptide is presented by the HLA heavy chain and β2m. Among the 15 synthesized peptides, nine could be refolded with HLA-A*0201 heavy chain and β2m molecules (Fig. 1). To further test the peptide presentation of HLA-A*0201, a T2 cell-binding assay was employed, which measures the increase of HLA-A*0201 molecules induced on the surface of T2 cells following exposure to an exogenous HLA-A*0201-binding peptide. As they are a TAP-defective HLA-A2-expressing cell line, T2 cells cannot present endogenous peptides. However, when T2 cells are exposed to an exogenous HLA-A2-binding peptide, the expression of HLA-A2 molecules on the cell surface will increase, because peptide binding stabilizes the HLA-A2 molecules on the cell surface. Among nine candidate peptides that can refold with HLA-A*0201, seven could be presented on the cell surface (Table 1).

CTL epitope-induced gamma interferon (IFN-γ) production in HLA-A2.1/K^b transgenic (Tg) mice by vaccinia–H5 or pcDNA3.0–H5 immunization

In order to examine whether the peptides that can bind to the HLA-A*0201 molecule are able to induce a CTL epitope-specific response, we immunized HLA-A2.1/K^b Tg mice using vaccinia virus expressing the H5 HA of H5N1 virus strain A/bar-headed goose/Qinghai/1/05 (vaccinia–H5) three times at an interval of 4 weeks. The splenocytes were isolated 10 days after the last immunization and a peptide-specific putative CTL response was detected by ELISPOT assay. (As cells were stimulated with CTL epitopes, we presumed that the IFN-γ was produced by CD8$^+$ cells and referred to it as a putative CTL response, although we did not do a CTL killing assay. IFN-γ-producing CD4$^+$ or $\gamma\delta$ T cells cannot be ruled out completely.) A significantly higher number of spot-forming cells (SFCs) were produced in the splenocytes from vaccinia-immunized HLA-A2.1/K^b Tg mice compared with those from PBS-immunized HLA-A2.1/K^b Tg mice ($P<0.05$, t test), suggesting that vaccinia–H5 immunization induced a high IFN-γ-producing response. Noticeably, in the splenocytes of mice immunized with vaccinia–H5,

Table 1. Predicted HLA-A*0201-restricted peptides for H5 HA epitopes and presentation by and binding to HLA-A*0201

The prediction is based on the HA sequence of AIV strain A/bar-headed goose/Qinghai/1/05 (H5N1).

No.	Start position*	Name†	Sequence	Score‡	Refolding§	T2 binding‖
P1	6	LV-9	LLLAIVSLV	1006.209	+	–
P2	86	FI-10	FLNVPEWSYI	448.715	+	+
P3	5	VLL-9	VLLLAIVSL	309.050	–	–
P4	463	NL-10	NLYDKVRLQL	280.275	–	–
P5	314	TV-9	TIGECPKYV	215.655	+	+
P6	205	RI-10	RLYQNPTTYI	183.617	+	+
P7	404	KV-9	KMNTQFEAV	163.681	+	+
P8	34	TV-10	TIMEKNVTV	145.077	–	–
P9	446	VL-9	VLMENERTL	110.183	+	+
P10	429	KMV-9	KMEDGFLDV	87.653	–	–
P11	531	QV-9	QILSIYSTV	53.077	+	+
P12	207	YV-10	YQNPTTYISV	48.657	+	+
P13	304	SL-10	SMPFHNIHPL	35.485	–	–
P14	547	IM-10	IMVAGLSLWM	33.548	–	–
P15¶	205	KI-10	KLYQNPTTYI	642.660	+	+
P16#	58	GL-9	GILGFVFTL	550.927	+	+
P17**	181	CM-9	CTPYDINQM	0.159	–	–

*The position of the first amino acid of the signal peptide in the sequence is defined as 1.

†The peptides are denominated with their first and last residue code and the number of their residues. If two peptides have the same name, then one of them is denominated by the first two residue codes, the last residue code and the number of their residues (e.g. VLL-9).

‡Estimated half-time of dissociation ($T_{1/2}$) of HLA-A*0201 peptide complexes, calculated by using BIMAS (http://bimas.dcrt.nih.gov/molbio/hla_bind/index.html).

§In the refolding assay, if an elution peak representing the complex of the peptide and HLA-A*0201 appears in gel filtration, the peptide is thought to be able to bind to HLA-A*0201 and is defined as +; otherwise it is defined as –.

‖In the T2-cell binding assay, the affinity of the peptide to HLA-A*0201 is measured by the fluorescence index (FI; see Methods). If the FI of a given peptide is $\geqslant 1$, it is defined as +; otherwise it is defined as –.

¶P15 (KI-10) is a variant of P6 (RI-10), found in the HA sequence of some human H5N1 virus isolates.

#P16 (GL-9), the HLA-A*0201-restricted influenza virus M1 (58–66) peptide, was used as a positive control for HLA-A*0201-binding ability.

**P17 (CM-9), the Mamu-A*01 restricted (SIV)-derived peptide gag (aa 181–189), was used as negative control.

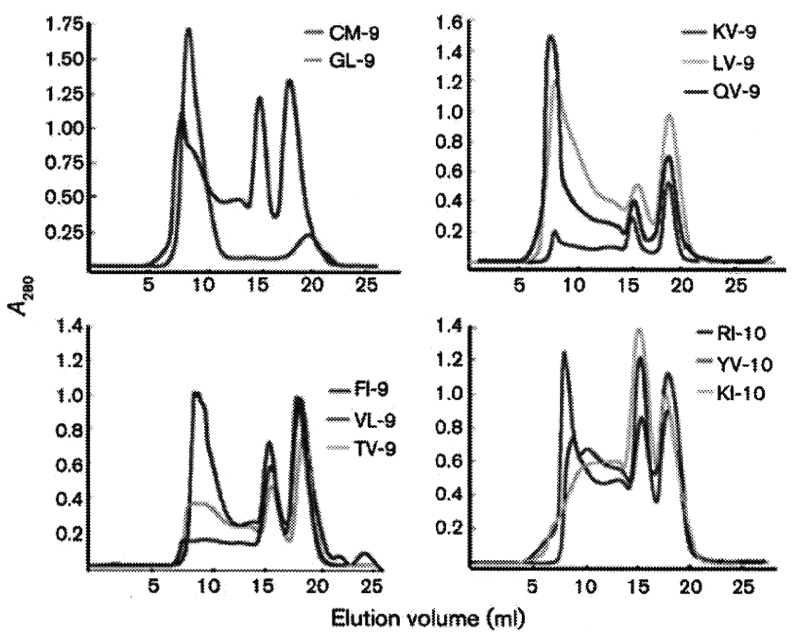

Fig. 1. Peptide-induced stabilization assay of HLA-A*0201 molecules by refolding. In the refolding assay, the refolded complexes, eluted with the expected molecular mass of 45 kDa, were analysed by FPLC Superdex G200 gel-filtration chromatography. The positive control GL-9 peptide is HLA-A*0201-restricted influenza virus M1 aa 58–66 (GILGFVFTL) and the negative control CM-9 peptide is a non-HLA-A*0201-restricted unrelated peptide derived from SIV. Peptides KV-9, LV-9, QV-9, VL-9, TV-9, FI-10, RI-10, YV-10 and KI-10, which could refold with HLA-A*0201 heavy chain and β2m, showed peaks with an elution volume of 15 ml in comparison with other peptides.

the numbers of SFCs increased significantly in the presence of two overlapping decameric peptides, RI-10 (RLYQNPTTYI) and YV-10 (YQNPTTYISV), compared with that in the absence of peptide stimulation (NS) ($P<0.01$ and $P<0.05$, respectively; Student's t test). In contrast, with the stimulation of other candidate peptides, the numbers of SFCs did not increase significantly (Fig. 2a).

Because there were many IFN-γ-producing cells in the absence of peptide stimulation in the splenocytes of vaccinia–H5-immunized Tg mice, the SFCs increased by only about two times with the stimulation of RI-10 and YV-10, although the statistical difference is significant. However, when we stimulated the splenocytes of the Tg mice immunized with pcDNA3.0–H5 with the eight peptides refolded with HLA-A*0201 molecules, RI-10 and YV-10 elicited a vigorous response ($P<0.01$ compared with NS; Student's t test). These results suggested that, among the eight peptides that could refold with HLA-A*0201, only RI-10 and YV-10 were naturally processed CTL epitopes with potent antigenicity (Fig. 2b).

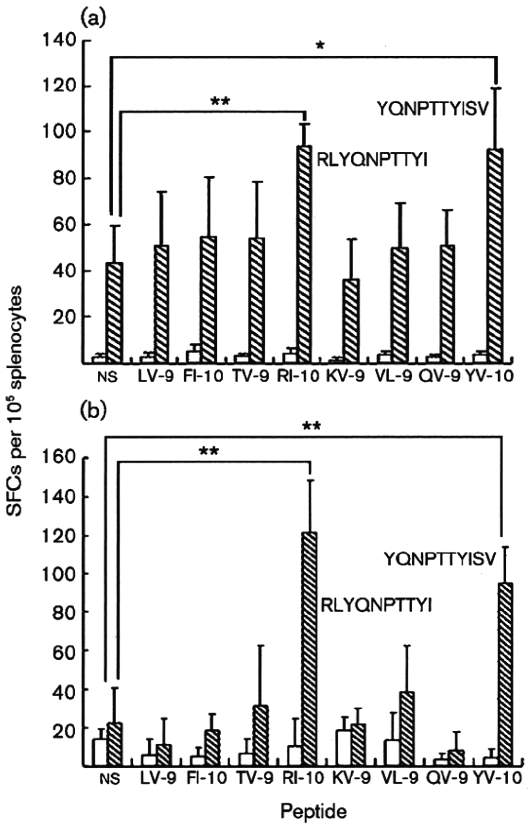

Fig. 2. Detection of CTL-epitope-specific IFN-γ-producing cells in vaccinia–H5- (a) or pcDNA3.0–H5- (b) immunized HLA-A2.1/K^b Tg mice (hatched bars) by ELISPOT assay. Empty bars represent negative controls [PBS (a); pcDNA3.0 (b)]. Female HLA-A2.1/K^b Tg mice, 6–8 weeks old, were immunized with 3–10^6 p.f.u. vaccinia–H5 in 20 µl PBS or PBS alone at weeks 0, 4 and 8. Ten days after the final boosting, splenocytes were isolated and CTL epitope-specific IFN-γ-producing cells were evaluated by ELISPOT assay. In the ELISPOT assay, splenocytes were stimulated *in vitro* in the presence of 20 U interleukin-2 (IL-2) ml^{-1} and 20 µM of the designated peptide for 24 h. NS represents cells that were cultured in the absence of any peptide. * ($P<0.05$) and ** ($P<0.01$) indicate significant differences between peptides and NS, using Student's t test.

Induction of CTL epitope-specific IFN-γ-producing T cells in HLA-A2.1/K^b Tg mice by peptide immunization

In order to further examine the antigenicity of RI-10 and YV-10, we immunized HLA-A2.1/K^b Tg mice with RI-10, YV-10 or GL-9 peptide. This was done following our previous regime by using incomplete Freund's adjuvant and murine GP96 N-terminal fragment N333 as adjuvant, which has been confirmed to be very effective (Li *et al.*, 2005). The peptide-specific IFN-γ-producing T-cell-response was detected by ELISPOT assay and tetramer staining. After immunization with the peptides three times at intervals of 2 weeks *in vivo* and stimulation with the peptides for 2 weeks *in vitro*, peptide-specific T cells were detected. The ELISPOT assay showed that RI-10 induced a significant IFN-γ response that was even higher than that with the influenza dominant epitope GL-9 ($P<0.01$, Student's t test). YV-10 also induced a certain degree of IFN-γ response, but it was much weaker than that induced by RI-10 ($P<0.01$, Student's t test) and was not significantly different from the control group ($P>0.05$, Student's t test; Fig. 3a). Similarly, tetramer staining also showed that RI-10 elicited a robust CTL epitope-specific CD8$^+$ T-cell response and that RI-10-specific CD8$^+$ cells accounted for 20.6% of CD8$^+$ T cells, whereas the proportion of GL-9-specific CD8$^+$ cells was 10.1% (Fig. 3b). However, YV-10 induced a much weaker specific CD8$^+$ T-cell response and the proportion of YV-10-specific CD8$^+$ T cells was only 2.3%. These results suggest that RI-10 is a CTL epitope with potent antigenicity, but that the antigenicity of YV-10 is much weaker in this scenario.

The reason that YV-10 elicited a comparable response with RI-10 in vaccinia–H5-immunized HLA-A*0201/K^b Tg mice, but induced a much weaker CD8$^+$ T-cell response in peptide-immunized Tg mice, is unknown. However, YV-10 may not bind well to HLA-A2 because it does not harbour a typical anchor motif for HLA-A2. Indeed, it showed a much lower binding affinity for the HLA-A*0201 molecule on the cell surface in the T2 cell-binding assay compared with RI-10 (data not shown), in spite of its ability to bind to HLA-A2 in the refolding assay.

We did not test KI-10 in the Tg mice, although it showed obvious binding to the HLA-A*0201 molecule in the refolding/T2 assays, because it is different from RI-10 only in the first residue. The first residue of both peptides is a basic amino acid. We speculated that KI-10 would share similar antigenicity with RI-10. This was confirmed by

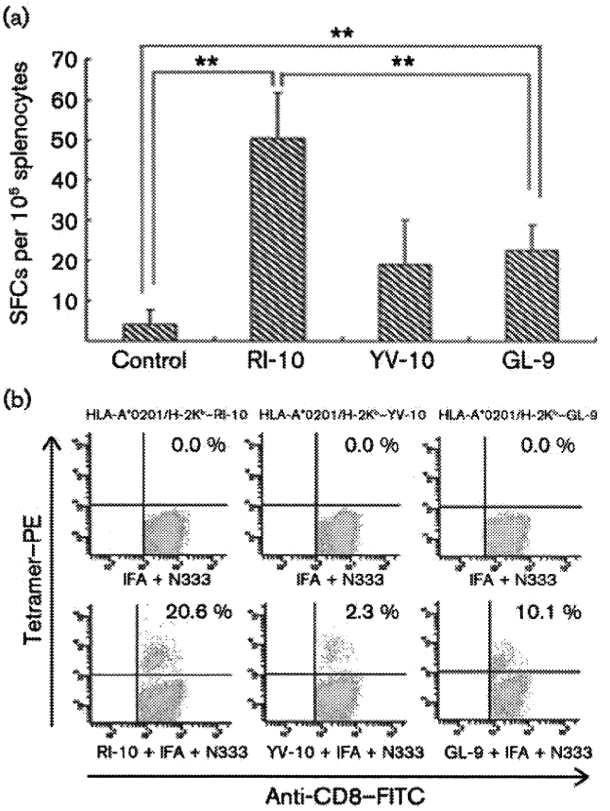

Fig. 3. Detection of peptide-specific IFN-γ-producing cells in peptide-immunized HLA-A2.1/K^b Tg mice by ELISPOT assay and tetramer staining. Female Tg mice, 6–8 weeks old, were immunized with a mixture of peptides, incomplete Freund's adjuvant (IFA) and the N-terminal fragment N333 (aa 22–355) of murine gp96 three times with intervals of 2 weeks. Control mice were immunized with only IFA and N333. Splenocytes were harvested 10 days after the last immunization and stimulated in the presence of 20 U IL-2 ml^{-1} and 10 μM peptide for 2 weeks *in vitro*. Then, the cells were harvested for ELISPOT assay (a) and tetramer staining (b) as described in Methods. ** indicates a significant difference ($P<0.01$, Student's *t* test).

detection of a specific $CD8^+$ T-cell response in the peripheral blood mononuclear cells (PBMCs) of patients who had recovered from H5N1 infection in the following experiments.

Detection of CTL epitope-specific $CD8^+$ T cells in PBMCs of patients who had recovered from avian influenza virus (AIV) infection

In order to confirm whether the results obtained in HLA-A2 Tg mice could also be applied to humans, we examined whether RI-10 and its variant KI-10 could induce a CTL epitope-specific $CD8^+$ T-cell response in PBMCs from human samples. We do not exclude the possibility that other candidate peptides that can bind to the HLA-A2 molecule, but do not induce a CTL response in HLA-A*0201/K^b Tg mice, may induce a CTL epitope-specific response in humans. All HLA-A2-binding peptides will be tested in human samples when more blood samples of HLA-A2-positive patients who have recovered from H5N1 virus infection are available to us. Here, we focus on the most promising candidates, RI-10 and its variant KI-10, due to sample limitation.

PBMC samples were collected from an HLA-A2-positive healthy donor and three survivors of AIV H5N1 infection. Two of the survivors are HLA-A*0201-positive (named LXC and HJR) and one is HLA-A2-negative. The samples of the three patients were collected about 15–18 months after the onset of disease. The PBMCs were stimulated for 7 days in the presence of 10 μM RI-10, KI-10 or GL-9 peptide and 20 U recombinant human IL-2 (rhIL-2) ml^{-1}. They were then stained with RI-10–HLA-A*0201, KI-10–HLA-A*0201 or GL-9–HLA-A*0201 tetramer. The proportion of peptide-specific and cross-reactive $CD8^+$ T cells was detected by flow cytometry. As shown in Fig. 4, when stained with RI-10 or KI-10-tetramer, CTL epitope-specific $CD8^+$ T cells were detected in the PBMCs of patient LXC at 1.5 and 0.5 % in $CD8^+$ T cells, respectively. The proportions of CTL epitope-specific $CD8^+$ T cells for RI-10 and KI-10 in the PBMCs of patient HJR were 1.8 and 2.7 %, respectively. When staining the PBMCs with GL-9 (a common HLA-A*0201-restricted CTL epitope) tetramer, specific CTLs were detected at 1.2 and 0.2 % in $CD8^+$ T cells in the two PBMC samples, which are both slightly lower than the values of the specific RI-10 or KI-10 tetramer staining. In contrast, the CTL epitope-specific $CD8^+$ T cells could not be detected with RI-10, KI-10 or GL-9 tetramer staining in PMBCs of the HLA-A2-negative AIV-recovered patient. Specific CTLs were not detectable with RI-10 or KI-10 tetramer staining in the PBMCs of an HLA-A2-positive healthy control, but the proportion of GL-9-specific CTLs was 1.1 % in his $CD8^+$ T cells.

Crystal structures of the HLA-A*0201 complexes with RI-10 or KI-10 peptide

The RI-10–HLA-A*0201 and KI-10–HLA-A*0201 complexes were both crystallized in the P1 space group. The crystals diffracted X-rays to 2.3 and 2.2 Å resolution for the RI-10 and KI-10 complexes, respectively (Table 2). The structures were determined by molecular replacement, using a previously determined HLA-A2 structure as the search probe (PDB accession no. 1JF1). The RI-10–HLA-A2 complex structure has been refined to R_{work} and R_{free} of 20.2 and 24.7 %, respectively. The KI-10–HLA-A2 complex structure has been refined to R_{work} and R_{free} of 19.2 and 23.7 %, respectively. The crystallographic asymmetrical unit contains four complex molecules with the peptides adopting the same conformation and there were no direct lattice contacts involving the peptides. The electron densities of RI-10 and KI-10 peptides unambiguously identify their anchor residues Leu2 and Ile10, which point

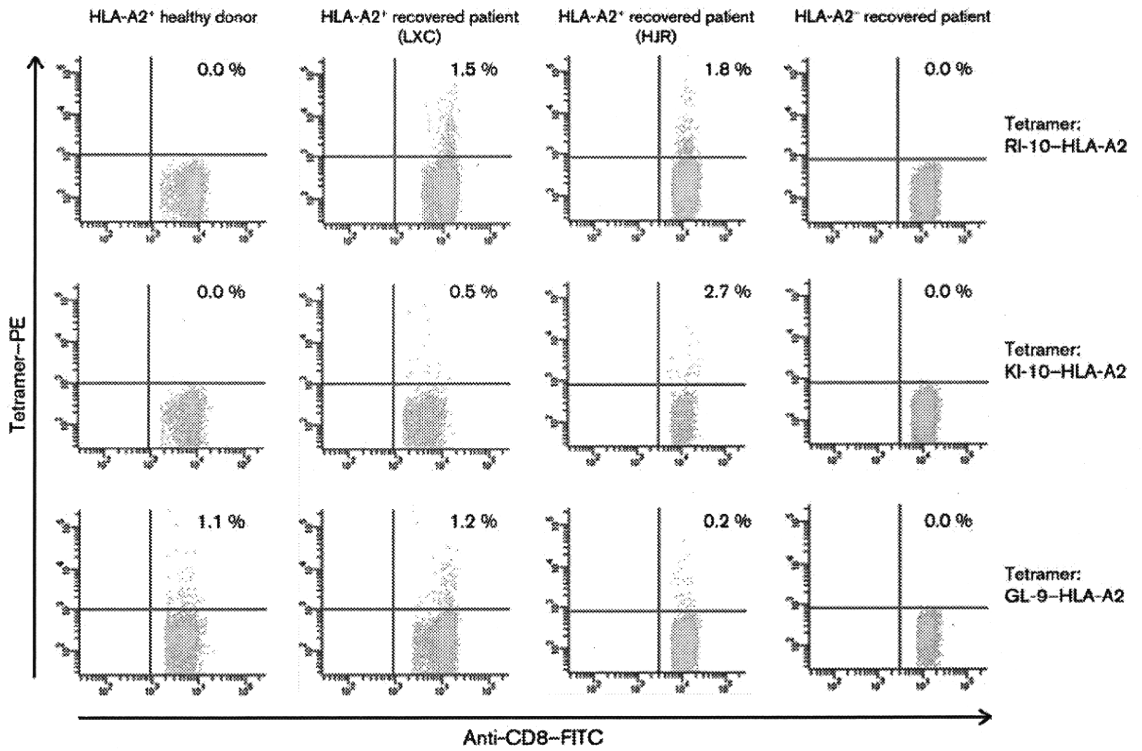

Fig. 4. Measurement of RI-10-, KI-10- and GL-9-specific $CD8^+$ T cells in the PBMCs of AIV-infection-recovered donors and a healthy donor by tetramer staining. PBMCs of an HLA-$A2^+$ healthy donor and three patients recovered from AIV infection (two HLA-$A2^+$ AIV patients, named LXC and HJR, and an HLA-$A2^-$ AIV patient) were stimulated with 10 μM RI-10, KI-10 or GL-9 in the presence of 20 U IL-2 ml^{-1} for 1 week and the cells were harvested and stained using phycoerythrin (PE)-conjugated HLA-A*0201–RI-10, HLA-A*0201–KI-10 or HLA-A*0201–GL-9 tetramer, along with FITC-conjugated anti-CD8 monoclonal antibodies (mAbs) for cell staining. The numbers shown represent the percentage of tetramer$^+$$CD8^+$ cells within $CD8^+$ T lymphocytes. The results are the representative of three independent experiments.

downwards and tether the peptides in the antigen-binding groove (Fig. 5a, b).

Except for the side chain of the first amino acid (Arg for RI-10 and Lys for KI-10), the three N-terminal and the two C-terminal residues of RI-10 and KI-10 superimpose well. However, the conformations of the main chains and the side chains of the central residues of RI-10 and KI-10, from Gln4 to Thr8, show significant differences, in spite of their similar sequences (Fig. 5c, d). The root mean square (RMS) deviation is 1.367–1.606 between any RI-10 and KI-10 peptide chain of the four complex molecules in their respective asymmetrical unit. The peptides are in the same conformation in the four molecules in one asymmetrical unit (RMS deviation $\leqslant$0.280 between any two peptide chains of the four complex molecules in the same asymmetrical unit). The conformational difference between RI-10 and KI-10 is not caused by lattice contact. A previous study suggested that a single amino acid substitution could alter the entire conformation of the bound peptide (Madden *et al.*, 1993). Subsequent studies have demonstrated that even subtle changes in anchor residues can change the peptide–MHC (pMHC) surface. For example, as in the HLA-B8 human immunodeficiency virus 1 (HIV-1) gag p17 structure, substitution of arginine for lysine at the anchor of position 5 (Reid *et al.*, 1996). More importantly, evidence has shown that N-terminal residues are energetically important and that chemical modification or removal of the N-terminal residue of the bound peptide lowers the stability of complexes of HLA-A2 and peptide. In these studies, chemical modification or removal of the N-terminal residue does not alter the conformation of the peptides significantly unless they are involved in direct lattice contact (Bouvier *et al.*, 1998; Khan *et al.*, 2000). Therefore, it is a novel finding that a subtle change of the N-terminal residue of the bound peptide may profoundly impact the whole conformation of the central residues.

For both RI-10 and KI-10, the main chains of the centrally located residues formed arches centred at Pro6 and tended to protrude out of the antigen-binding groove at this site. Gln4, Pro6 and Tyr9, whose side chains point upwards, are the most exposed residues as measured by the solvent-accessible surface and these are the most probable recognizing sites for the T-cell receptors (TCRs).

Table 2. Data collection and refinement statistics

	RI-10	KI-10
Non-hydrogen atoms		
Protein	13679	12680
Water	975	1087
Space group	P1	P1
Unit cell dimension	a=63.21, b=79.32, c=87.15 ; α=90.00, β=90.00, γ=90.02	a=63.172, b=79.304, c=86.739; α=90.02, β=89.99, γ=89.96
Molecules in AU	4	4
Resolution (Å)	33.6–2.30	25.5–2.20
Measured reflections	282436	165362
Unique reflections	75342	76982
Data completeness (%)	96.2	95.8
I/σ (I)	15.1	23.0
R_{work}	0.2022	0.1920
R_{free}	0.2470	0.2367
RMS* deviations from ideality		
Bond lengths (°)	0.002	0.004
Bond angles (°)	0.575	0.817
Dihedrals (°)	15.309	16.316
Ramachandran plot		
Most favoured	91.8	91.5
Allowed region	7.9	8.2
Generously allowed region	0.3	0.3
B factors (Å^2)		
Average main chain	32.09	31.34
Average side chain	33.50	32.12
Average water molecule	36.73	37.14

*RMS, Root mean square.

DISCUSSION

Immune responses against influenza A virus have been characterized intensively for several decades and over 100 CTL epitopes within numerous subtypes and strains have been identified. Nevertheless, little work has been done on the highly pathogenic H5N1 subtype (Bui *et al.*, 2007). In this study, we successfully identified a strongly immunogenic CTL epitope in the H5 HA protein by using a strategy that was applied in screening and identification of CTL epitopes of severe acute respiratory syndrome coronavirus in our laboratory, i.e. starting from a computer motif

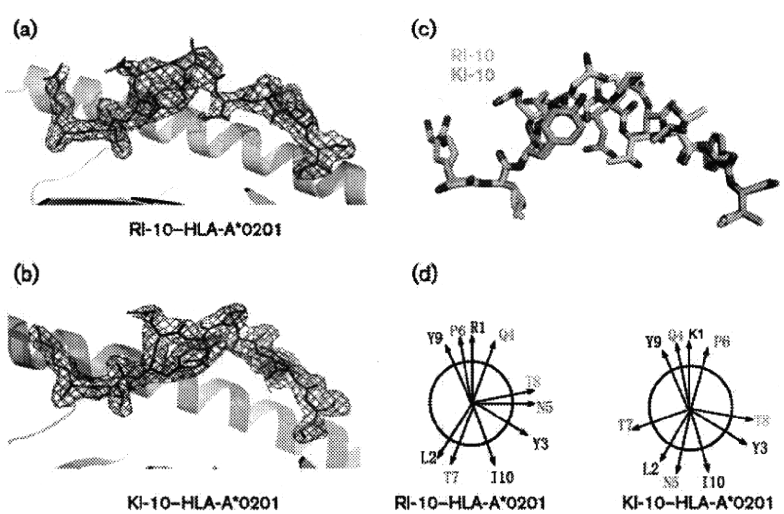

Fig. 5. Crystal structures of RI-10/KI-10 in complex with HLA-A*0201. (a, b) 2Fo–Fc electron density maps contoured at 1.0 σ for RI-10 (a) and KI-10 (b). (c) Superimposition of RI-10 and KI-10. (d) Orientation of the peptide side chains in the HLA-A*0201-bound RI-10 and KI-10, viewing along the peptide from the N-terminal to the C-terminal end. Magenta (Gln4), red (Asn5), blue (Pro6), purple (Thr7) and green (Thr8) lettering indicates the central residues that show significant positional variation between RI-10 and KI-10.

prediction, followed by *in vitro* complex refolding and then coming back to a standard T2-binding assay (Zhou *et al.*, 2006b). We found that nine of 15 H5 HA-derived peptides could bind to HLA*0201 in the refolding assay and seven of them could bind to HLA*0201 on the cell surface in the T2-binding assay. Among the nine peptides that could refold, two overlapping peptides, RI-10 (H5 HA205-214, RLYQNPTTYI) and YV-10 (H5 HA207-216, YQNPTTYISV), induced a vigorous CTL epitope-specific response in HLA-A*0201/K^b Tg mice immunized with vaccinia–H5 or pcDNA3.0–H5. An intriguing coincidence is that two overlapping K^d-restricted CTL epitopes, H2 HA204–212 (LYQNVGTYV) and H2 HA210–219 (TYVSVGTSTL), have been defined in the same region of the HA molecule of the influenza virus A/JAP/305/57 (H2N2) (Braciale *et al.*, 1989; Cao *et al.*, 1996). The former epitope H2 HA204–212 is immunodominant, eliciting vigorous CTL responses in BALB/c mice immunized with A/Japan/57 virus, whereas H2 HA210–219 is sub-immunodominant (Braciale *et al.*, 1989; Cao *et al.*, 1996). The RI-10 and KI-10 epitopes identified in this study share certain sequence similarity with H2 HA204–212. This may imply that this region is a 'hot spot' for a putative CTL response against influenza A virus. Although YV-10 elicits a comparable CTL epitope-specific response with RI-10 in Tg mice immunized with vaccinia–H5 or pcDNA3.0–H5, it induces a much weaker CTL epitope-specific response in the peptide-immunized Tg mice. Therefore, we focused on RI-10 and its variant KI-10 in this study.

Here, we show that RI-10/KI-10 induced a significant CTL epitope-specific response in patients who had recovered from H5N1 infection and carried the HLA-$A2^+$ haplotype. It is noteworthy that virus sequence from patient HJR has a KI-10 epitope, but that patient LXC's virus sequence is not available for this region. Our results are somewhat unexpected, because RI-10 and KI-10 induced a higher CTL epitope-specific response than the GL-9 epitope. The GL-9 epitope is suggested as a dominant CTL epitope in influenza A virus infection (Stewart-Jones *et al.*, 2003). As the HA of influenza A virus does not have more copies and is not expressed earlier than the matrix protein, the dominance of RI-10 and KI-10 observed here is worthy of future investigation. Furthermore, the sequences of RI-10 and KI-10 not only harbour typical anchor residues for HLA-A2, but also match the requirement of TAP transporter binding perfectly. Namely, they each possess a basic amino acid residue (Arg or Lys) stabilized at position 1, and contain favoured hydrophobic and aromatic residues at positions 2 and 3 (Uebel & Tampe, 1999; Uebel *et al.*, 1997). Our results suggest that the hierarchy of CTL response against highly pathogenic H5N1 virus may be different from those of other human influenza A virus subtypes, such as H1N1 and H3N2. It is necessary in the future study to re-evaluate the relative roles played by these newly identified CTL epitopes in comparison with those in other influenza A virus subtypes.

We present the crystal structures of RI-10 and KI-10 in a complex with HLA-A*0201 and β2m. To the best of our knowledge, these are the second crystal structures to be released of influenza virus-derived CTL epitopes restricted by HLA-A*0201 in addition to the dominant epitope GL-9. The most interesting finding is that the two peptides, RI-10 and KI-10, adopt different conformations in their central residues, from Gln4 to Thr8, despite their sequence similarity. It is unknown whether RI-10 and KI-10 can be recognized by a single TCR or whether their responding TCR repertoires are actually not cross-reactive. Despite the conformational difference between the two peptides, they may still be recognized by a single TCR, because conformational adjustment can be adopted at the binding interface when pMHC engages TCR. For example, Lee *et al.* (2004) demonstrated that the immunodominant HLA-A2-restricted HIV gag epitope (SLFNTVATL) and its variant (SLYNTVATL) show marked differences in structure when bound to HLA-A2. However, their on-rate kinetics of TCR binding were identical, implying that conformational changes at the TCR–peptide–MHC binding interface occur after an initial permissive antigen contact (Lee *et al.*, 2004). In our observations, RI-10 and KI-10 are cross-reactive in tetramer staining in the PBMCs of patients who have recovered from H5N1 virus infection. The characterization of their responding TCR repertoires should be demonstrated in the near future.

HA sequence alignment shows that the sequences of RI-10/KI-10 are unique compared with relevant 10-mer peptide sequences from seasonal 'flu (H1 or H3) and other subtypes of influenza virus. However, the sequences relevant to RI-10/KI-10 in the HA of serotypes H1 and H3 (and most other serotypes) also have a leucine as the second residue and most of these sequences have a valine or isoleucine as the last residue (Fig. 6). In other words, most of these sequences have typical anchor residues for HLA-A2. Therefore, it is highly possible that these relevant peptides can also bind to HLA-A2. However, whether they can also elicit a significant $CD8^+$ T-cell response should be demonstrated in a future study. Even if they can induce a certain $CD8^+$ T-cell response, it is less likely that the response is cross-reactive to RI-10/KI-10, because the central part of RI-10/KI-10 is largely different from that of the HA proteins of other serotypes (Fig. 6). At least in our experiment, we did not observe any cross-reactivity between RI-10/KI-10 and seasonal 'flu (H1 or H3). As shown in Fig. 4, GL-9-specific $CD8^+$ T cells could be detected in the PBMCs of the HLA-$A2^+$ healthy donor, suggesting that the donor used to be infected by seasonal 'flu. However, no RI-10/KI-10-specific $CD8^+$ T cells were detected in the healthy donor's PBMCs, so our data do not support a cross-reaction between RI-10/KI-10 and their relevant putative epitopes in seasonal 'flu.

The strong immunogenicity of RI-10 and KI-10 that we observed suggests that they are potential targets for the design of new vaccines. However, whether they can induce any protection against H5N1 virus or whether they actually may have a pathogenic effect during H5N1 infection remains to be elucidated. Nevertheless, RI-10 and KI-10