Japan). The $CD8^+$ T cells without peptide stimulation were used as a negative control. The number of spots for each peptide-specific T cell response was calculated by subtracting the number of negative-control spots (the number of spots in wells without peptides). Spots giving a mean of more than + 2 SD of the negative-control spots were defined as positive responses. In order to find cross-clade $CD8^+$ T cells in our cohort, we performed the ELISPOT assay by the same method with 11-mer single peptides that were the components of the cocktail peptides where 1) the frequency of responders was more than 20% or 2) the frequency was less than 20% but in which case at least 1 patient showed a high spot count (>750 spots).

2.5. Cells

721.221-CD4 cells expressing HLA-A*02:06, -A*33:03 or Cw*01:02 were generated by transfecting both human CD4 gene and one of these HLA-class I genes into 721.221 cells. These cells were maintained in RPMI medium containing 10% fetal calf serum (FCS) and 0.15 mg/mL hygromycin B. C1R cells expressing HLA-A*02:06 and those expressing HLA-A*33:03 were generated by transfecting C1R cells with HLA-A*02:06 and -A*33:03, respectively; and they were maintained in RPMI medium containing 10% FCS and 0.15 mg/mL hygromycin. C1R and 721.221 cells expressing other HLAs used in this study were previously generated and maintained in RPMI medium with 10% FCS and 0.15 mg/mL hygromycin B or 0.2 mg/mL neomycin [33–36].

2.6. Induction of peptide-specific CTLs from PBMCs

PBMCs from HIV-1-infected individuals who showed the responses to the cocktail peptides in the ELISPOT assay were stimulated with 11-mer single peptide or optimal peptide derived from consensus clade B HIV-1 (100 nM) and then cultured in culture medium (RPMI-1640 containing 10% FCS and 200 U/ml interleukin-2) for 2 weeks. These bulk cultured cells were used for intracellular IFN-γ staining assays.

2.7. HIV clones

The replication-competent molecular clones of p93JP-NH1 [37] and pNL-432 [38] reported previously were used in this study. Viral stocks were generated from plasmid DNA as described elsewhere [15,39].

2.8. HIV-1 infection of .221-CD4 cells expressing HLA molecules or not

.221-CD4 cells expressing HLA molecules or not were exposed to each virus for several days. These infected cells were used as stimulator cells for performing an intracellular cytokine staining assay (ICS) when approximately 30–60% of the cells had been infected, which infection was confirmed by intracellular staining for HIV-1 p24 antigen (KC-57-FITC; Beckman Coulter).

2.9. Intracellular cytokine staining assay (ICS)

After .221 cells or C1R cells had been incubated for 60 min with each peptide (0.01–100 nM), they were washed twice with RPMI-1640 containing 10% FCS. These peptide-pulsed or HIV-1–infected .221-CD4 cells (1×10^5 cells per well) and bulk cultured cells (2×10^4 cells per well) were added to wells of a 96-well round-bottomed plate, and then the cells were incubated for 2 h at 37 °C. Brefeldin A (10 μg/ml) was then added, after which the cells were incubated for a further 4 h. After having been stained with APC-labeled anti-CD8 mAb (DAKO, Glostrup, Denmark), the cells were fixed with 4% paraformaldehyde and then made permeable with the permeabilizing buffer (0.1% saponin and 5% FCS in PBS). Thereafter the cells were stained with FITC-labeled anti-IFN-γ mAb (BD Bioscience, CA). The percentage of IFN-γ^+CD8^+ cells was analyzed by flow cytometry.

3. Results

3.1. $CD8^+$ T cell responses to HIV-1 clade B-derived overlapping peptides by HIV-1 clade A/E-infected individuals

To clarify cross-clade responses of $CD8^+$ T cells between the clade B and A/E, we analyzed cross-clade responses of $CD8^+$ T cells from 26 clade A/E-infected Japanese individuals to 11-mer overlapping peptides derived from the consensus sequence of HIV-1 clade B Nef, Gag, and Pol regions. We measured the responses of $CD8^+$ T cells to cocktails including ten 11-mer overlapping peptides by performing the ELISPOT assay. The median of total magnitudes of the $CD8^+$ T cell responses to Nef, Gag, and Pol cocktails were 483, 1037, and 2538, respectively (Fig. 1A). There were no significant differences in total magnitude of the $CD8^+$ T cell responses between the clade A/E-infected and 401 clade B-infected Japanese individuals (the median of total magnitude against Nef, Gag and Pol in the clade B-infected individuals were 529,

Fig. 3. Identification of optimal epitopes Truncated peptides were designed based on HLA binding motif, and $CD8^+$ bulk T cells were induced from PBMCs of the following 6 responders. KI-648 for Nef 41, Gag 171, and Pol 141 peptides, KI-632 for Nef 67, Pol 297 and Pol 436 peptides, KI-388 for Gag 16, Gag 20, Gag 104, Gag 105, Gag 106, and Pol 383 peptides, KI-724 for Gag 138 peptide, KI-964 for Pol 212 and Pol 405 peptides, and KI-837 for Pol 455 peptide. IFN-g production by $CD8^+$ bulk T cells was measured by performing the ICS assay using the target C1R or .221 cells expressing HLA molecules prepulsed with truncated peptide or 11-mer peptide at a concentration of 100 nM. When the same level of response was seen at 100 nM, the ICS assay was performed again at concentrations from 0.1 to 100 nM. **A**. responses of HLA-A*02:06-restricted $CD8^+$ bulk T cells **B**. responses of HLA-B*40:02-restricted $CD8^+$ bulk T cells **C**. responses of HLA-A*33:03, A*24:02, Cw*01:02 or B*52:01-restricted $CD8^+$ bulk T cells.

1774, and 2300, respectively; H. Murakoshi et al. unpublished observation), although the identities of amino acid sequence in Nef, Gag, and Pol between clade the A/E and the clade B were 80.1, 84.3 and 92.3%, respectively. These results strongly suggest that cross-clade $CD8^+$ T cells were frequently elicited in the clade A/E-infected individuals.

3.2. Identification of cross-clade $CD8^+$ T cells elicited in HIV-1 clade A/E-infected individuals

To identify cross-clade $CD8^+$ T cells in the clade A/E-infected individuals, we focused on the $CD8^+$ T cell responses found to be strong or at a high frequency in these individuals (see Materials and methods). We selected the $CD8^+$ T cell responses to 13 cocktails including 2 Nef, 4 Gag, and 7 Pol cocktails (solid bars in Fig. 1B). First, to clarify which 11-mer peptides were recognized by the specific $CD8^+$ T cells, we selected the clade A/E-infected responders (KI-388, KI-632, KI-648, KI-659, KI-724, KI-837, and KI-964) and measured the $CD8^+$ T cell responses to ten 11-mer peptides in each cocktail by using the ELISPOT assay. We found positive responses to three 11-mer Nef peptides (Nef cocktail 5: Nef 41 and 42, Nef cocktail 7: Nef 67), to ten 11-mer Gag peptides (Gag cocktail 2: Gag 11, 16, 19, and 20, Gag cocktail 11: Gag 101, 104, 105, and 106, Gag cocktail 14: Gag 138, Gag cocktail 18: Gag 171), and to eleven 11-mer Pol peptides (Pol cocktail 15: Pol 141 and 142, Pol cocktail 22: Pol 211 and 212, Pol cocktail 30: Pol 297 and 298, Pol cocktail 39: Pol 383, Pol cocktail 41: Pol 405, Pol cocktail 44: Pol 436, Pol cocktail 46: Pol 455 and 456) (data not shown). We next sought to determine HLA restriction molecules in these responses. PBMCs from these responders were stimulated with the 11-mer peptides and then cultured for 14 days. In order to determine the HLA restriction molecules, responses of the cultured cells against the corresponding peptides were analyzed by performing the intracellular cytokine staining (ICS) assay using HLA class I gene-transfected C1R cells or 721.221 cells as stimulators. We found 10 HLA-A*02:06-restricted responses, 6 HLA-B*40:02-restricted responses, 3 A*33:03-restricted responses, 1 HLA-A*24:02-restricrted response, 1 HLA-B*52:01-restricted response, and 1 Cw*01:02-restricted response (Fig. 2).

We first analyzed the responses to the 10 HLA-A*02:06-restricted responses. Concerning the responses to overlapping peptides at 3 locations (Nef 41/42, Pol 141/142, and Pol211/212), we speculated that they would be the same epitope-specific $CD8^+$ T cell responses since the responses to these overlapping peptides were restricted by HLA-A*02:06. Therefore we focused on analyzing the response to Nef 41, Pol 141 or Pol 211, which showed higher responses than those to the other overlapping peptides (data not shown). We generated truncated peptides that were speculated based on HLA-A*02:06 binding motif (Ala, Thr or Gln at position 2) [40–43] and then analyzed these $CD8^+$ T cell responses to Nef 41, Pol 141, and Pol 212 by using them. As shown in Fig. 3A, we identified 3 optimal epitopes: Nef GL9 (GALDLSHFL), Pol YI9 (YTAFTIPSI), and Pol SV9 (SQIYAGIKV). $CD8^+$ T cell responses to Nef GL9 and Pol SV9 were detected among the responses to other overlapping peptides, Nef 42 and Pol 211, respectively (data not shown), indicating that the responses to Nef GL9 and Pol SV9 reflected those to Nef 42 and Pol 212, respectively. In contrast, Pol 142 did not contain Pol YI9. We analyzed the $CD8^+$ bulk T cells induced by Pol 142 by using the truncated peptides and identified Pol TI8 (TAFTIPSI) as an optimal epitope. However, the response to Pol TI8 in $CD8^+$ bulk T cells induced by Pol 142 (4.24% IFN-γ secretion at 100 nM peptide concentration) was much lower than that to Pol TI8 in $CD8^+$ bulk T cells induced by Pol 141 (12.07% at 100 nM; Fig. 3A), suggesting that Pol TI8 may have been a very weak epitope. Similarly, we analyzed the other 4 HLA-A*02:06-restricted responses (Gag16, Gag171, Pol383, and Pol405) by using truncated peptides and identified 4 optimal epitopes; Gag LL11 (LKHIVWASREL), Gag AA9 (ATLEEMMTA), Pol GI9 (GQVDCSPGI), and Pol GL9 (GQETAYFLL; Fig. 3A).

By using the same method identified the HLA-A*02:06-restricted epitopes, we attempted to identify other epitopes. We generated truncated peptides based on HLA binding motif [7,33,40,44–50] and then the responses to these truncated peptides were analyzed using the ICS assay. We finally identified 5 HLA-B*40:02-restricted epitopes (Gag RV8, Gag AP8, Gag AV9, Gag EG11, and Pol GI8; Fig. 3B), 2 HLA-A*33:03-restricted epitopes (Pol FR9 and Pol ER10; Fig. 3C), 1 HLA-A*24:02-restricted epitope (Nef RF10; Fig. 3C), 1 HLA-B*52:01-restricted epitope (Gag RI8; Fig. 3C) and 1 HLA-Cw*01:02-restricted epitope (Gag YI9; Fig. 3C).

Eleven of the above 17 peptides were reported as epitopes in previous studies [7,33,40–43,45,47–50], whereas the other 6 peptides (HLA-A*02:06-restricted Gag LL11, Pol SV9, and Pol GI9, as well as HLA-B*40:02-restricted Gag RV8, Gag AP8, and Gag EG11) had not been previously reported to be epitopes. Therefore, we examined whether the $CD8^+$ bulk T cells specific for these 6 epitopes could recognize HIV-1 clade B virus-infected cells. We measured the IFN-γ production from the $CD8^+$ bulk T cells for target cells infected with HIV-1 clade B clone, NL4-3. These $CD8^+$ bulk T cells effectively produced IFN-γ (data not shown), indicating that these 6 peptides had been naturally processed and presented in cells infected with HIV-1.

3.3. Cross-recognition between the clade B and A/E

We sequenced each epitope region in 26 HIV-1 clade A/E-infected Japanese individuals and then compared these sequences to those from the clade A/E and B viruses reported in the database of the Los Alamos National Library. The results showed that the consensus amino acid sequences of these epitopes in our cohort were the same as those in the database of Los Alamos National Library. The clade B consensus sequences of 6 epitopes (Gag AA9, Pol YI9, Pol SV9, Pol GI9, Pol GL9, and Pol ER10) were identical to the clade A/E consensus ones, whereas other 11 epitopes showed different consensus sequences between the clade B and A/E viruses (Table 1).

Table 1
Frequency of amino-acid sequence for each epitope region of clade A/E viruses from Los Alamos National Library database and our cohort patients.

Epitope	HXB2 region	Sequence	Frequency of amino acid sequence of each clade virus		
			Clade B viruses for Los Alamos database	Clade A/E viruses for Los Alamos database	Clade A/E viruses for our cohort patients
Nef GL9	Nef(83–91)	GALDLSHFL[a]	**529/1494**	0/76	1/26
		—F——F-	12/1494	**47/76**	**16/26**
		Others	953/1494	29/76	9/26
Nef RF10	Nef(134–143)	RYPLTFGWCF[a]	**800/1494**	0/76	2/25
		———C——	94/1494	**56/76**	**10/25**
		others	600/1494	20/76	13/25
Gag LL11	p17(31–41)	LKHIVWASREL[a]	**1182/1644**	18/315	2/26
		M—L———	0/1644	**147/315**	**14/26**
		others	462/1644	150/315	10/26
Gag RV8	p17(39–46)	RELERFAV[a]	**1299/1644**	20/315	4/26
		————L	111/1644	**217/315**	**17/26**
		others	234/1644	78/315	5/26
Gag AP8	p24(78–85)	AEWDRLHP[a]	**1263/1644**	53/315	1/26
		———V-	128/1644	**185/315**	**10/26**
		others	253/1644	77/315	15/26
Gag AV9	p24(78–86)	AEWDRLHPV[a]	**1135/1644**	47/315	1/26
		———V-	121/1644	**178/315**	**8/26**
		others	388/1644	90/315	17/26
Gag EG11	p24(79–89)	EWDRLHPVHAG[a]	**921/1644**	48/315	1/26
		———V———	107/1644	**149/315**	**5/26**
		others	616/1644	118/315	20/26
Gag RI8	p24(143–150)	RMYSPTSI[a]	**1033/1644**	30/315	2/26
		———V-	351/1644	**212/315**	**15/26**
		others	260/1644	73/315	9/26
Gag YI9	p24(145–153)	YSPTSILDI[a]	**1032/1644**	30/315	2/26
		——V——	345/1644	**212/315**	**15/26**
		others	267/1644	73/315	9/26
Gag AA9	p24(209–217)	ATLEEMMTA[a]	**1468/1644**	**285/315**	**25/26**
		others	176/1644	30/315	1/26
Pol YI9	RT(127–135)	YTAFTIPSI[a]	**574/1003**	**44/59**	**16/26**
		others	429/1003	15/59	10/26
Pol SV9	RT(268–276)	SQIYAGIKV[a]	**438/1003**	**38/59**	**18/26**
		others	565/1003	21/59	8/26
Pol FR9	RT(440–448)	FYVDGAANR[a]	**838/1003**	9/59	9/26
		————S-	47/1003	**45/59**	**15/26**
		others	118/1003	5/59	2/26
Pol GI9	Integrase(50–60)	GQVDCSPGI[a]	**915/1003**	**55/59**	**26/26**
		others	88/1003	4/59	0/26
Pol GL9	Integrase(94–102)	GQETAYFLL[a]	**519/1003**	**52/59**	**19/26**
		others	484/1003	7/59	7/26
Pol ER10	Integrase(157–166)	ELKKIIGQVR[a]	**770/1003**	**40/59**	**21/26**
		others	233/1003	19/59	5/26
Pol GI8	Integrase(197–204)	GERIVDII[a]	**535/1003**	0/57	0/26
		——I—	378/1003	**56/59**	**23/26**
		others	90/1003	3/59	3/26

[a] Amino acid sequence in clade B consensus used in this study.

We investigated the cross-recognition of these 11 epitope peptides by $CD8^+$ T cells that had been induced by stimulating PBMCs from clade A/E virus-infected individuals with clade B-derived epitope peptides. The $CD8^+$ bulk T cells induced by Nef RF10, Gag RV8, Gag AP8, Gag AV9, or Gag YI9 peptides recognized both clades B and A/E peptides evenly (Fig. 4A). The $CD8^+$ bulk T cells induced by Gag EG11, Gag RI8, or Pol FR9 more strongly recognized the clade B-derived peptide than the clade A/E-derived one, whereas those induced by Nef GL9 or Pol GI8 more strongly recognized the clade A/E-derived peptide than the clade B-derived one (Fig. 4A). Interestingly, the $CD8^+$ bulk T cells induced by Gag 16 LL11 failed to recognized the clade A/E-derived Gag LL11-1M-4L peptide (Fig. 4A).

Next, we investigated whether $CD8^+$ T cells recognizing these 10 clade A/E peptides could recognize clade A/E-infected cells. We measured IFN-γ production from the $CD8^+$ bulk T cells for target cells infected with HIV-1 clade A/E clone or for those infected with HIV-1 clade B clone. The $CD8^+$ bulk T cells induced by Nef RF10, Gag RV8, Gag AP8, Gag AV9, Gag EG11, Gag YI9, Gag RI8, or Pol GI8 recognized not only the clade B virus-infected cells but also the clade A/E virus-infected ones (Fig. 4B), indicating that these cross-clade epitopes had been naturally processed

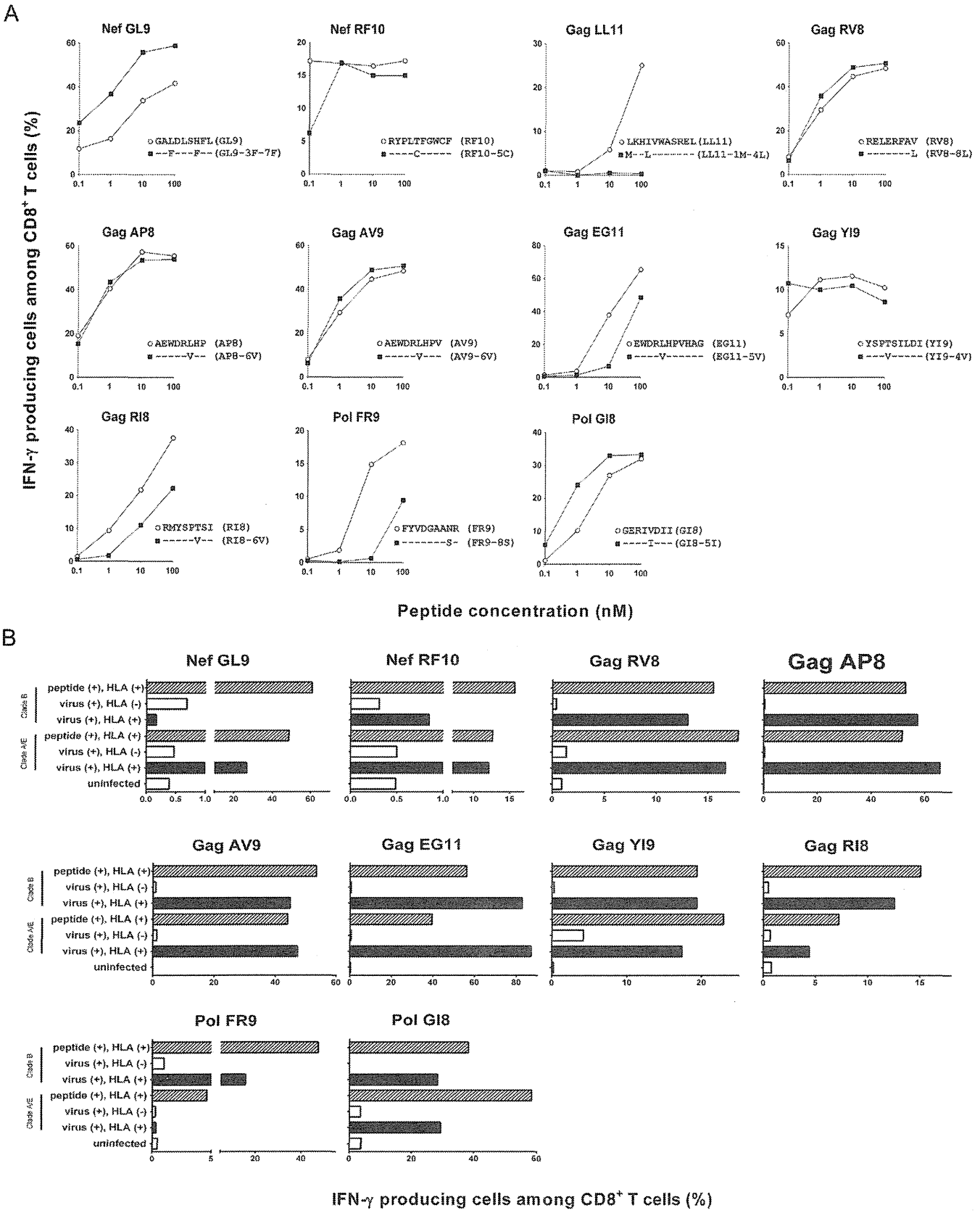

Fig. 4. Cross-recognition by $CD8^+$ T cells from HIV-1 clade A/E-infected individuals IFN-γ production by $CD8^+$ bulk T cells in response to stimulator cells with optimal epitope peptides and to cells infected with clade B or clade A/E viruses was measured by use of the ICS assay. Nef GL9 specific bulk T cells were induced from PBMCs of KI-648, Nef RF10 and Pol FR9 specific bulk T cells were induced from PBMCs of KI-632, Gag LL11, Gag RV8, Gag AP8, Gag AV9 and Gag EG11 specific bulk T cells were induced from PBMCs of KI-388, Gag RI8 and Gag YI9 specific bulk T cells were induced from KI-724, and Pol GI8 specific bulk

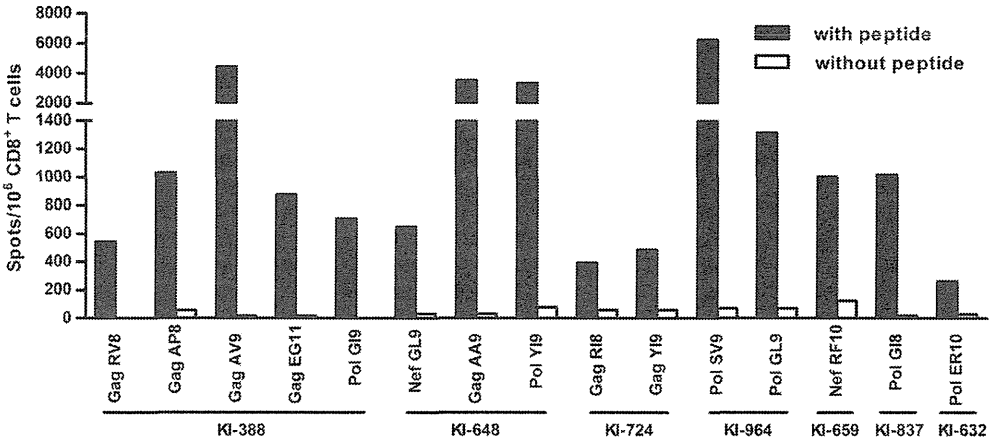

Fig. 5. $CD8^+$ T cell responses to clade B-drived epitope peptides in HIV-1 clade A/E-infected Japanese individuals $CD8^+$ T cell responses to 15 clade B-derived epitope peptides were analyzed by performing ELISPOT assay using $CD8^+$ T cells from seven clade A/E-infected individuals (KI-388, KI-632, KI-648, KI-659, KI-724, KI-837, and KI-964). >200 spots were evaluated as positive response.

and presented in cells infected with these viruses. On the other hand, $CD8^+$ bulk T cells induced by Pol FR9 recognized the clade B virus-infected cells but failed to recognize the clade A/E virus-infected cells (Fig. 4B). This finding is consistent with the low ability of these cells to recognize the clade A/E peptide (Fig. 4A). In contrast, $CD8^+$ T cells induced by Nef GL9 recognized the clade A/E virus-infected cells but failed to recognize the clade B virus-infected cells although these T cells could recognize GL9 peptide. This result may be explained by the fact that the amino acid sequence of the clade B consensus peptide is different than that of the clade B clone, NL4-3(Ala and Val at position 1 and 3, respectively, in Nef GL9 region). Thus, $CD8^+$ T cells induced by 8 out of 10 clade B-derived epitope peptides successfully recognized both the clade B virus-infected and clade A/E-infected cells.

3.4. Detection of cross-clade $CD8^+$ T cell responses in the clade A/E-infected Japanese individuals

To confirm $CD8^+$ T cell responses to the 15 epitopes including Nef GL9, we analyzed $CD8^+$ T cell responses to the clade B-derived epitope peptides in clade A/E-infected individuals who had HLA alleles restricting these epitopes. Positive $CD8^+$ T cell responses to these 15 clade B-derived epitope peptides were detected in PBMCs from chronically HIV-1 clade A/E-infected individuals (Fig. 5). These results indicate that these cross-clade CTLs are elicited in these individuals.

4. Discussion

Previous studies, which focused on known CTL epitopes for the clade B or C viruses, showed the existence of cross-clade CTLs in HIV-1-infected individuals by demonstrating that CTL clones established by using clade-matched peptides from the clade B-infected or the clade C-infected individuals recognize the cells infected with other clade viruses [13–17]. These studies also showed that conserved epitopes across the clades are more likely recognized by the T cell clones and suggested that conserved epitopes would be a more preferable target for a widely effective CTL vaccine than variable ones. In the present study, we for the first time performed a comprehensive analysis of cross-clade $CD8^+$ T cells by using 11-mer overlapping clade B-derived peptides to stimulate $CD8^+$ T cells from HIV-1 clade A/E-infected individuals. Interestingly, we found a similar level of $CD8^+$ T cell responses to clade B-derived Nef, Gag, and Pol peptides in the clade A/E virus-infected individuals as compared to those to the same peptides in clade B-infected individuals. These results strongly suggested the existence of a high number of cross-clade CTLs in the clade A/E virus-infected individuals. Indeed, we finally identified 15 cross-clade CTL epitopes from only 13 out of 85 overlapping peptide cocktails. These results strongly suggest that a large number of cross-clade CTLs were elicited in the clade A/E virus-infected individuals.

$CD8^+$ T cells induced by Pol FR9 recognized to a much lesser extent the clade A/E-derived peptide (FR9-8S) than the clade B-derived peptide and recognized cells infected with

T cells were induced from KI-837. **A**. Cross-recognition of HIV-1 clade B (open circle) and clade A/E (closed square) optimal epitope peptides of the consensus sequence. These analyses were performed at peptide concentrations from 0.1 to 100 nM. **B**. Cross-recognition of cells infected with clade B or clade A/E virus. IFN-γ production by $CD8^+$ bulk T cells in response to HLA-positive cells prepulsed with clade B or clade A/E consensus optimal peptide and that in response to HLA-negative cells infected with the virus and to uninfected HLA-positive cells were measured as positive and negative controls, respectively. All epitope sequences derived from the clade B or the clade A/E were identical to the sequences from clone virus (NL4-3 or 93JP-NH1) except for clade B Nef GL9 epitope (GALDLSHFL). NL4-3 has Ala and Val at positions 1 and 3 of this epitope, respectively.

clade B virus but not those infected with A/E viruses, suggesting that PolFR9-8S was not an epitope. Indeed, the HLA-A*33:03$^+$ individuals were infected with the clade A/E virus carrying Pol FR9 sequence but not Pol FR9-8S one (data not shown). The CD8$^+$ T cells induced by Nef GL9 recognized the clade A/E virus-infected cells, whereas they failed to recognize the clade B-infected ones. These T cells could recognize GL9 peptide, though they recognized more effectively Nef GL9-3F-7F peptides than the Nef GL9 one. However, CD8$^+$ T cells specific for both Nef GL9 and Nef GL9-3F-7F were detected in 3 of 7 HLA-A*02:06$^+$ individuals (data not shown). These results suggest that Nef GL9-3F-7F had been presented in the clade A/E-infected individuals. Therefore, the failure of the T cells to recognize cells infected with NL4-3 virus may have resulted from a different amino acid sequence of this epitope between the clade B consensus peptide and NL4-3 (Ala and Val at position 1 and 3, respectively, in Nef GL9 region). CD8$^+$ bulk T cells induced by 8 other diverse epitopes effectively recognized both the clade B-infected and the clade A/E-infected cells, suggesting that these diverse epitopes could be cross-recognized by the T cells.

We previously reported that Phe at position 2 of Nef RF10 is an escape mutation in the clade B virus [48]. This escape mutation was frequently found in the clade A/E virus, though the consensus sequence was RF10-5C (RYPLCFGWCF; Table 1). Since RF10 and RF10-5C were cross-recognized by the CD8$^+$ T cells induced by the RF10 peptide, these T cells would be expected to select 2F mutants in the clade A/E-infected individuals. These results indicate that RF10-5C was an HLA-A*24:02-restricted epitope in the clade A/E-infected individuals and that RF10-5C-specific CD8$^+$ T cells could cross-recognize the RF10 epitope.

Since these epitopes were restricted by Asian HLA alleles, vaccine targeting these epitopes can cover Asian countries including south-east Asia and China where clade A/E and clade B viruses are prevalent. An HLA-B*40:02-restricted Nef epitope was known to be presented by world-wise HLA allele HLA-B*40:01 [33]. In addition, a previous study showed that Pol GL9-specific CD8$^+$ T cells were elicited in a vaccinated individual carrying world-wise HLA allele, HLA-A*02:01 [42]. These studies together suggest that some of the HLA-B*40:02-resticted and HLA-A*02:06-restricted epitopes identified in this study may be CTL epitopes presented by these world-wise HLA alleles. Thus, vaccine targeting the cross-clade epitopes identified in this study may cover countries in Europe, and northern and southern Americas in addition to Asian countries.

In conclusion, we here performed the first comprehensive study of cross-clade T cell responses and demonstrated that CD8$^+$ T cell responses to clade B-derived Nef, Gag, and Pol peptides were successfully induced in the clade A/E virus-infected individuals. We finally identified the 15 cross-clade epitopes which include not only conserved epitopes but also polymorphic epitopes across the different clades. These epitopes can thus be candidate targets of CTL-based vaccines.

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HIV Subtype Influences HLA-B*07:02-Associated HIV Disease Outcome

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Abstract

Genetic polymorphisms within the MHC encoding region have the strongest impact on HIV disease progression of any in the human genome and provide important clues to the mechanisms of HIV immune control. Few analyses have been undertaken of HLA alleles associated with rapid disease progression. HLA-B*07:02 is an HLA class I molecule that is prevalent in most populations worldwide and that has previously been consistently linked to accelerated disease progression in B-clade infection. This study investigates the observation that HLA-B*07:02 is not associated with a high viral setpoint in C-clade infection. We examine the hypothesis that this clade-specific difference in association with disease outcome may be related to distinct targeting of $CD8^+$ T cell epitopes. We observed that C-clade-infected individuals with HLA-B*07:02 target a broader range of Gag epitopes, and to higher magnitudes, than do individuals infected with B-clade infection. In particular, a novel p17-Gag (Gag22-30, RPGGKKHYM) epitope is targeted in >50% of HLA-B*07:02-positive C-clade-infected individuals but clade-specific differences in this epitope result in nonimmunogenicity in B-clade infection. Only the C-clade p24-Gag "GL9" (Gag355-363, GPSHKARVL) epitope-specific $CD8^+$ T cell response out of 16 studied was associated with a low viral setpoint. Although this epitope was also targeted in B-clade infection, the escape mutant S357S is present at higher frequency in B-clade infection than in C-clade infection (70% versus 43% in HLA-B*07:02-negative subjects). These data support earlier studies suggesting that increased breadth of the Gag-specific $CD8^+$ T cell response may contribute to improved HIV immune control irrespective of the particular HLA molecules expressed.

Introduction

THREE LARGE GENOME-WIDE association studies (GWAS) show that genetic variation within the MHC encoding region has the greatest impact on HIV disease progression[1–3] consistent with previous studies showing that HLA allele expression has a major impact on HIV viral load setpoint, both in B-clade[4] and C-clade infection.[5–8] In particular, the expression of alleles at the HLA-B locus has the greatest contribution to viral load setpoint.[1,5] The exact mechanisms by which HLA alleles are consistently linked with differential disease outcomes remain unresolved. However, several explanations have been proposed.[9]

First, the Gag specificity of the $CD8^+$ T cell response has been linked to immune control[7,10,11] exemplified by dominant Gag-specific responses restricted by protective HLA-B alleles, such as HLA-B*27 and HLA-B*57.[12–15] Escape mutations from these responses that occur within the structurally

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conserved Gag protein typically result in reduced viral replicative capacity.[15–19]

Second, the HLA alleles associated with low viral load setpoints mostly carry the HLA-Bw4 motif,[20] which may also reduce viral load through interaction with KIR3DS1 and KIR3DL1 on natural killer cells.[21,22]

Third, restrictive peptide-binding motifs, such as those found for the protective HLA-B*27 and HLA-B*57 alleles,[4,12,15,23] may reduce the number of self-reactive peptides bound during thymic selection, leaving a broader T cell receptor (TCR) repertoire available in the periphery to accommodate HIV viral sequence diversity. In contrast, alleles traditionally associated with "disease susceptibility," including HLA-B*07 and HLA-B*35, bind a broader repertoire of self-peptides leaving a more narrow TCR repertoire in the periphery for recognition of viral sequence diversity.[24]

Additional factors that may influence control of HIV via HLA expression include the interaction of HLA class I with leukocyte immunoglobulin-like receptors (LILRs) expressed on dendritic cells. This mechanism, leading to impaired dendritic cell function, has been proposed to contribute to the more rapid progression to HIV disease in people expressing certain HLA-B*35 subtypes.[25]

We recently showed that HLA-B*35:01, an allele consistently associated with disease progression in B-clade infection,[3,26] is somewhat protective in C-clade infection. This outcome hinges on a specific $CD8^+$ T cell response to a single Gag epitope only available in C-clade infection.[27] Similarly, we observed that HLA alleles belonging to the B*07 superfamily, including HLA-B*07:02, were associated with disease progression in B-clade infection, but not in C-clade infection. Consistent with these findings, recent studies of 3,622 B-clade-infected study subjects found that HLA-B*07:02 was associated with disease progression,[3,28] but this was not the case in a large cohort of 1,210 C-clade-infected individuals from Durban, South Africa.[6]

We here test the hypothesis that, like HLA-B*35:01, subjects with HLA-B*07:02 impose a broader and more dominant Gag-specific $CD8^+$ T cell response in C-clade infection leading to improved virologic outcomes, compared to a more narrow Gag-specific response in B-clade infection that is associated with disease progression.

Materials and Methods

Ethics statement

Ethics approval was given by University of KwaZulu-Natal Review Board and the Massachusetts General Hospital Review Board (Durban cohort), the Office of Human Research Administration, Harvard School of Public Health, and the Oxford Research Ethics Committee (Thames Valley and other cohorts). Study subjects from all cohorts gave written informed consent for their participation.

Study cohorts

We studied a total of 2,718 adults with chronic, antiretroviral therapy (ART)-naive HIV-1 infection, recruited from four cohorts as follows: (1) Durban, South Africa (C-clade, $n=1,218$), as previously described,[5,7,10,15] (2) Thames Valley cohort, UK (mixed clades, $n=237$), as previously described,[5,29] and (3) B-clade-infected cohorts from Kumamoto, Japan (B-clade, $n=242$) and (4) the United States ($n=1,021$), as previously described.[30] Viral loads were performed using the Roche Amplicor version 1.5 assay.

HLA typing and classification

HLA typing was undertaken from genomic DNA by sequence-based typing as previously described.[5] Locus-specific polymerase chain reactions (PCR) of exons 2 and 3 were amplified and sequenced.

*Definition of HLA-B*07:02-restricted epitopes*

To define a comprehensive list of HLA-B*07:02-restricted epitopes, we identified previously characterized epitopes from studies of predominantly B-clade-infected subjects (Los Alamos "A list"; www.lanl.gov),[31] and also sought and identified novel HLA-B*07:02-restricted epitopes by testing recognition of 410 overlapping 18-mer peptides in a cohort of C-clade-infected and B-clade-infected subjects. From this approach, 16 HLA-B*07:02-restricted epitopes were identified for further analysis (Table 1).

Interferon (IFN)-γ ELISpot assays

IFN-γ ELISpot assays were undertaken using fresh or cryopreserved peripheral blood mononuclear cells (PBMCs). We screened for HIV-1-specific responses statistically associated ($q<0.05$) with the expression of HLA-B*07:02 by testing a total of 1,010 C-clade and 401 B-clade chronically infected subjects against a panel of 410 overlapping peptides (OLPs) spanning the entire HIV proteome, as previously described.[10,14] Significant associations were determined using Fisher's exact test and corrected for multiple comparisons using a q-value (FDR, false detection rate) approach as previously described.[7,14,32]

We used B-clade and C-clade-specific optimal peptides to test for IFN-γ responses in HLA-B*07:02-positive B-clade-infected individuals recruited from the UK and Japan ($n=58$) and C-clade-infected individuals recruited from the UK ($n=11$).

Virus from all study subjects in the Japan cohort was sequenced to confirm clade of infection, and only those who were B-clade infected were included in the study. A response of 100 spot-forming cells (SFCs)/10^6 PBMCs was defined as significantly above the background response in control wells, which in most cases were zero.

Epitope fine mapping and HLA class I tetramer assay

We confirmed RM9-p17 (RPGGKKHYM), (Gag 22–30) as an optimal HLA-B*07:02-restricted epitope by stimulating PBMCs with truncated peptides in p-17-RM9 responder cells from ID: R045 and used peptide pulsed BCL lines partially HLA matching the responder p-17-RM9-specific 10 day expanded CTLs in an ICS assay. The corresponding p17-RM9 CTL line responses were validated using HLA-B*07:02 tetramers and controlled by a mismatched HLA tetramer. A pretitrated concentration of PE-conjugated tetramers[33] was used to stain p17-RM9-specific CTLs, incubated for 30 min and stained with pretitrated extracellular antibodies CD8-Pacific Blue (BD Pharmingen) and CD3-PacificOrange (Invitrogen). Dead cells were excluded by using Vivid Live/dead marker

TABLE 1. HLA-B*07:02-RESTRICTED EPITOPES WITH PEPTIDE-BINDING AFFINITITES AND PEPTIDE-BINDING HALF-LIFE (STABILITY)

Protein	*OLP*	*Clade*	*OLP sequence*	*Optimal epitope*	*Epitope name*	*Binding K_d [nM]*	*Stability [h]*
p17 Gag	3	B	EKIRLRPGGKKKYKLKHI	RPGGKKKYK[a]	RK9 p17	>20,000	0.0
		C	EKIRLRPGGKKHYMLKHL	------H-M[a]	RM9 p17	14	4.2
p24 Gag	20	B	QMVHQAISPRTLNAWVKV	SPRTLNAWV	SV9 p24	3	2.4
		C	QMVHQAISPRTLNAWVKV	---------			
	25	B	GATPQDLNTMLNTVGGH	TPQDLNTML	TL9 p24	1,162	0.8
		C	GATPQDLNTMLNTVGGH	---------			
	29	B	AAEWDRLHPVHAGPIA	HPVHAGPIA[a]	HA9 p24	32	3.6
		C	AAEWDRLHPVHAGPIA	---------[a]			
	48	B	ACQGVGGPGHKARVLAEA	GPGHKARVL	GL9 p24	342	1.1
		C	ACQGVGGPSHKARVLAEA	--S------		9	3.4
Nef	76	B	EVGFPVRPQVPLRPMTYK	RPQVPLRPM[a]	RM9 Nef	13	8.7
		C	EVGFPVRPQVPLRPMTFK	---------[a]			
	76	B	EVGFPVRPQVPLRPMTYK	FPVRPQVPL	FL9 Nef	21	4.5
		C	EVGFPVRPQVPLRPMTFK	---------			
	77	B	QVPLRPMTYKAAVDLSHF	RPMTYKAAV	RV9 Nef	2	nd
		C	QVPLRPMTFKGAFDLSFF	--------F	RF9 Nef	7	4.4
	84	B	NYTPGPGIRYPLTFGWCF	TPGPGIRYPL	TL10 Nef	6	6.6
		C	NYTPGPGVRYPLTFGWCF	-----V----	TL10 Nef	27	6.6
Rev	102	B	ILSTYLGRPAEPVPLQL	RPAEPVPLQL	RL10 Rev	65	2.9
		C	ILSTCLGRPAEPVPLQL	----------			
Pol	187	B	QGWKGSPAIFQSSMTKIL	SPAIFQSSM	SM9 RT	37	9.6
		C	QGWKGSPAIFQSSMTKIL	---------			
	244	B	MASDFNLPPVVAKEIVA	LPPVVAKEI[a]	LI9 Int	12,353	0.1
		C	MASEFNLPPIVAKEIVA	---I-----[a]		2,128	0.2
Vpr	281	B	ELKNEAVRHFPRIWLHSL	FPRIWLHSL	FL9 Vpr	3	2.8
		C	ELKQEAVRHFPRPWLHGL	---P---G-		6	4.1
Env	328	B	NCTRPNNNTRKSIHI	RPNNNTRKSI	RI10 Env	3	3.7
		C	VCTRPNNNTRKSIRI	----------			
	401	B	HIPRRIRQGLERALL	IPRRIRQGL	IL9 Env	95	4.2
		C	NIPRRIRQGFEAALQ	--------F	IF9 Env	33	2.7
Vif	407	B	RHHYESTHPRISSEVHI	HPRISSEVHI	HI10 Vif	4	4.3
		C	RHHYESRHPKVSSEVHI	--K-------		178	2.3

[a]Not listed in the Los Alamos "A" list database.
OLP, overlapping peptide.

(Invitrogen). FACS data were analyzed using FlowJo version 8.8.6 (Treestar, USA).

Peptide-MHC-binding studies

HLA-peptide-binding studies were undertaken using a luminescent oxygen channeling immunoassay (LOCI) as previously described.[34] We tested binding for 16 HLA-B*07:02 epitopes as shown. Binding assays were performed in quadruplicate; the reported result is the mean of the four values obtained. Stability of binding (binding half-life) was performed as described previously.[35] Briefly, biotinylated HLA-I heavy chain, ^{125}I-labeled β_2-microglobulin (B2m), and peptide were allowed to fold into peptide-HLA-I complexes in streptavidin-coated scintillation microplates (Flashplate PLUS, Perkin Elmer, Boston, MA) for 24 h at 18°C. Excess of unlabeled B2m was added and dissociation was initiated by placing the microplate in a scintillation reader (TopCount NXT, Perkin Elmer, Boston, MA) operating at 37°C. The scintillation signal was monitored by continuous reading of the microplate for 24 h. Half-lives were calculated from dissociation curves using the exponential decay equation in Prism v.5.0a (GraphPad, San Diego, CA). Assays were performed in duplicate; the mean value of two experiments is reported.

Statistical analysis

Statistical analysis was undertaken using GraphPad Prism v.5.0a (GraphPad, San Diego, CA). Overlapping peptide responses and HLA expression were determined using Fisher's exact test and corrected for multiple comparisons using a q-value (false detection rate), as previously described.[7,32] Comparing responders and nonresponder subjects for peptide recognition and viral sequences was determined by Fisher's exact test. The Mann–Whitney U test was used to compare viral load setpoints for GL9-Gag in B-clade-infected and C-clade-infected individuals. Correlation between percent optimal peptide recognition and peptide binding IC_{50} values and peptide-binding half-lives (hours) was determined by Spearman rank correlations. Data presentation and statistical analysis were undertaken by GraphPad Prism v.6.0c.

Results

*Dominant Gag-specific HLA-B*07:02-restricted CD8⁺ T cell responses in C-clade infection compared to B-clade infection*

Previously we had shown no impact of HLA-B*07:02 on viral setpoint in a highly-powered study of >2,000 HIV-infected subjects in Southern Africa.[6,36–38] In contrast, several studies have consistently shown a strong independent effect of HLA-B*07:02 on disease progression in B-clade infection,[3,26,28] but not in C-clade infection,[6,27] and therefore suggest a consistent impact of HIV clade on the association of HLA-B*07:02 with rapid HIV disease progression.

To determine whether the observed differential HLA-B*07:02-associated HIV disease outcomes in B-clade and C-clade-infected cohorts are related to clade-specific differences in $CD8^+$ T cell responses, we tested a set of 16 clade-specific HLA-B*07:02-restricted optimal epitopes (Table 1). This panel comprised previously defined optimal epitopes that are detailed within the Los Alamos "A-list"[31] and an additional four epitopes identified by testing 1,010 C-clade-infected[10] and 401 B-clade-infected individuals[39] for recognition of 410 overlapping peptides spanning the C-clade and B-clade proteomes, respectively. Based on these screenings we suggest an additional four epitopes restricted by HLA-B*07:02 and not listed in the Los Alamos "A-list" (Table 1). The novel epitope, RPGGKKHYM (Gag 22-30) (p17-RM9), was optimized and unequivocally defined as restricted by HLA-B*07:02 (Fig. 1).

Reactivity to this comprehensive panel of clade-specific optimal epitopes was tested in HLA-B*07:02-positive subjects with B-clade infection ($n=58$) and in subjects with C-clade infection ($n=11$) using IFN-γ ELISpot assays (Fig. 2). We observed significantly more responses directed toward Gag for C-clade compared to B-clade-infected individuals ($p=0.02$). At the individual epitope level, the statistically significant clade-specific difference was the response to two Gag epitopes, p17-RM9 and p24-TL9, targeted more by C-clade-infected individuals compared to B-clade-infected individuals ($p=4\times10^{-5}$ and 1×10^{-3}, respectively) (Fig. 2B), whereas Vif-HI10 was targeted only by B-clade-infected individuals, $p=0.05$ (Fig. 2D). Of note, no significant difference in cross-recognition of the p24-GL9 "3S" (C-clade) and "3G" (B-clade) version was observed for 6/11=55% and 17/58=29% in C-clade and B-clade-infected individuals, respectively ($p=0.16$) and confirmed by peptide titration in three C-clade

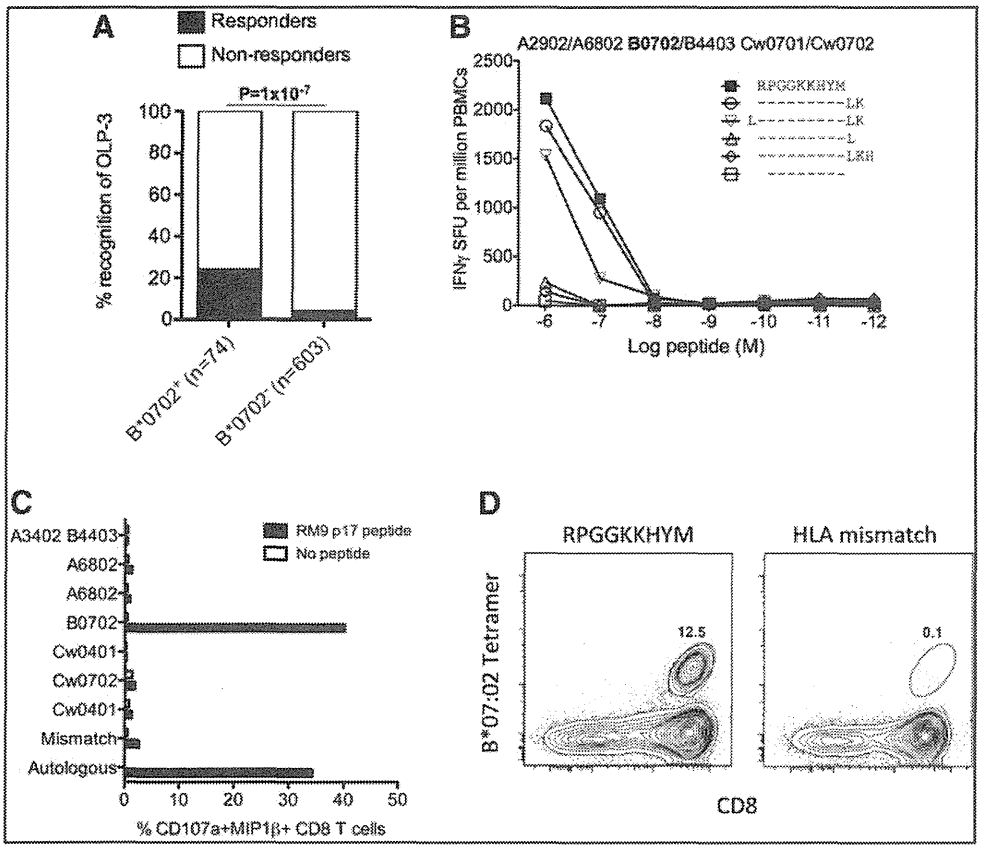

FIG. 1. Identification and characterization of the p17-RM9 Gag epitope. **(A)** Association of overlapping peptide (OLP)-3 (EKIRLRPGGKKHYMLKHL) response to HLA-B*07:02 expression after removal of individuals expressing HLA-A*03:01, B*08:01, B*42:01, and B*42:02, known to restrict other epitopes within OLP-3 (left panel). **(B)** Peptide truncations of RPGGKKYHYM used in interferon (IFN)-γ ELISpot assay with peripheral blood mononuclear cells (PBMCs) from individual H034 (HLA-A*29:02/68:02, B*07:02/44:03, Cw*04:01/07:02). **(C)** HLA-B*07:02 restriction using a 10 day $CD8^+$ T cell line grown from PBMCs from individual R045 (HLA-A*29:02/68:02, HLA-B*07:02/44:03, HLA-Cw*04:01/07:02) and tested using ICS against a complete set of B cell lines partially HLA matching the effector CTLs. **(D)** The epitope is unequivocally confirmed by HLA-B*07:02 tetramer staining using HLA-B*44:03 as mismatched control gated on the live $CD3^+$ lymphocyte population.

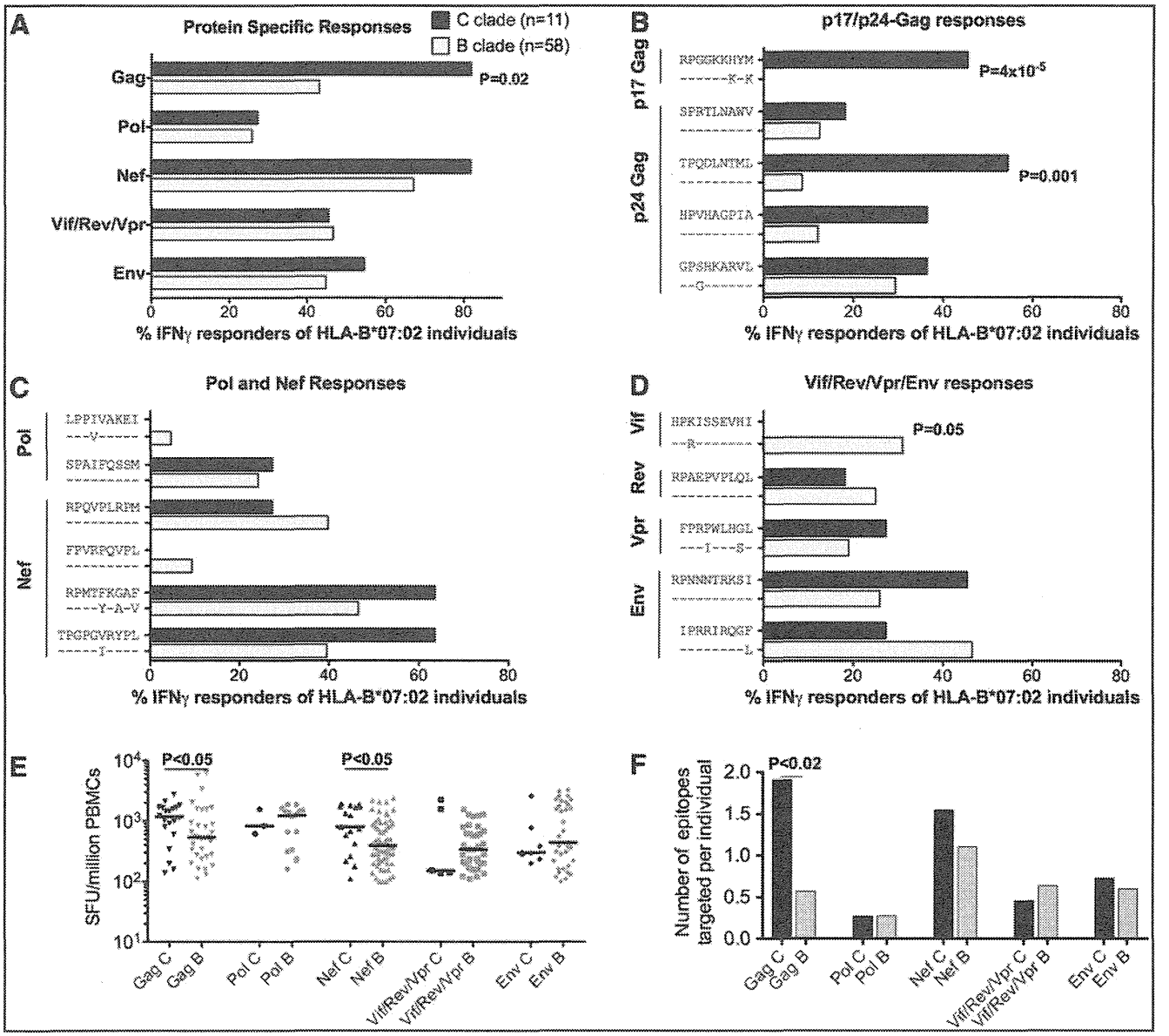

FIG. 2. Percentage of HLA-B*07:02 subjects making IFN-γ ELISpot responses to 16 B-clade and C-clade-specific HLA-B*07:02-restricted optimal epitopes in B-clade and C-clade-infected subjects. **(A)** Protein-specific and **(B–D)** optimal epitope-specific IFN-(ELISpot responses tested in B-clade and C-clade-infected HLA-B*07:02 individuals using a cut-off of 100 spot-forming units per million PBMCs. **(E)** Comparing magnitudes of protein-specific responses expressed as SFU/million input PBMCs with horizontal lines representing median values. **(F)** Showing the mean number of epitopes targeted per individual for C-clade and B-clade-infected individuals. Significant differences ($p < 0.05$) by Fisher's exact test **(A–C)**, Mann–Whitney *U* test **(E)**, and unpaired *t*-test **(F)**. Individuals coexpressing HLA-B*39:10, B*42:01, B*81:01, and Cw*08:02 were excluded for TL9-p24 analysis as these HLA alleles also restrict TL9-p24.

and two B-clade-infected subjects (data not shown). Increased magnitudes of Gag and Nef-specific responses were detected for C-clade-infected compared to B-clade-infected individuals ($p < 0.05$) (Fig. 2E) with increased breadth of Gag-specific epitope targeting for C-clade compared to B-clade-infected individuals ($p < 0.02$) (Fig. 2F).

Clade-specific viral sequence differences determine epitope immunogenicity

The single HLA-B*07:02-restricted epitope not targeted in B-clade infection is the novel Gag epitope p17-RM9, which differs from the C-clade consensus sequence at two positions, H7K and M9K. The change M → K at the carboxy-terminal position (PC) does not fit the preferred peptide-binding motif of isoleucine/leucine at the C terminal residue in the F pocket of HLA-B*07:02.[23] Peptide-binding half-life (stability) studies were undertaken, revealing that only the C-clade version was stable in complex with HLA-B*07:02 (Fig. 3A), which was then confirmed by the determination of peptide-binding affinities to HLA-B*07:02 (RPGGKKHYM vs. RPGGKKKYK, $K_d = 14$ nM and >20,000 nM, respectively, Table 1). The non-immunogenicity of p17-RM9 in B-clade infection was further confirmed by lack of cross-recognition from C-clade

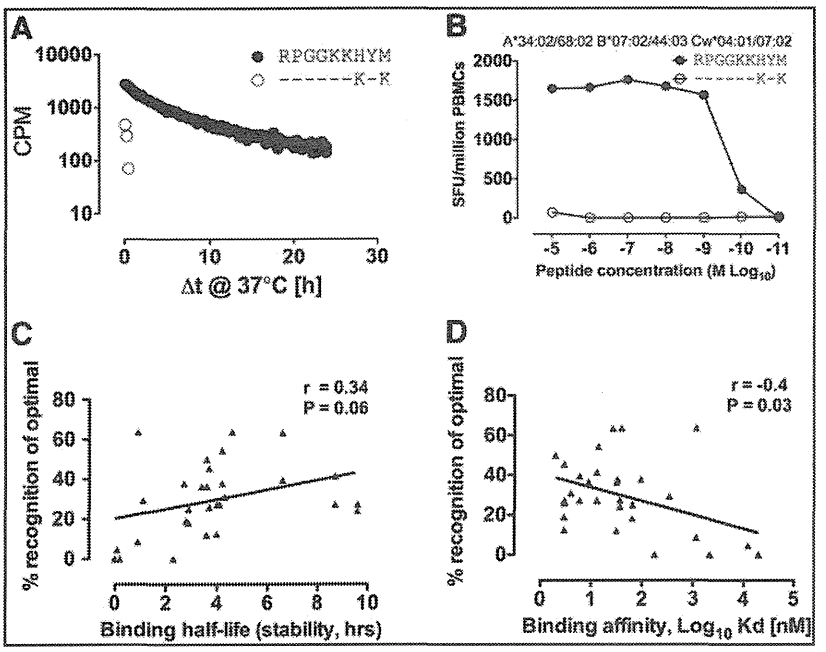

FIG. 3. Clade-specific differences influence HLA-B*07:02 peptide binding and dictate epitope immunogenicity. **(A)** p17-RM9 B-clade and C-clade epitope binding half-life (stability); **(B)** lack of cross-reactivity in PBMCs from subject H034 (HLA-A*34:02/A*68:02, B*07:02/B*44:03, Cw04:01/Cw07:02) using IFN-γ ELISpot to peptide RM9 and variant RK9; **(C)** correlation between optimal epitope binding half-life and **(D)** affinity with recognition by IFN-γ ELISpot in HLA-B*07:02 individuals pooled for B-clade and C-clade infection.

p17-RM9-specific CD8$^+$ T cells against the nonbinding B-clade version of this epitope (Fig. 3B). A subset of clade B-infected subjects recognized the C-clade version of the p17-RM9 epitope, although still significantly less frequent than C-clade-infected individuals (4/58=7%, $p=0.004$) (data not shown). In addition, the single epitope, Vif-HI10, targeted significantly more in B-clade infection and nonimmunogenic in C-clade-infected individuals, had a 40-fold stronger binding affinity in the B-clade ($K_d=4$ nM and 178 nM, in B-clade and C-clade, respectively) and was also less stable in complex with HLA-B*07:02 (half-life 4.3 h versus 2.3 h, respectively) (Table 1). Overall, we observed a weak correlation between peptide-binding affinity and binding half-life (stability) and the frequency of epitope recognition ($r=0.34$, $p=0.06$ and $r=-0.4$, $p=0.03$, respectively) (Fig. 3C and D) as previously observed for other HLA-B-restricted responses.[14]

p24 Gag-GL9 response in C-clade infection is associated with lower viral setpoint

To assess the antiviral efficacy of each of the 16 HLA-B*07:02-restricted responses in C-clade and B-clade infection (Table 1), we compared the viral load setpoint of responding and nonresponding HLA-B*07:02-positive individuals and found that the single response associated with a significantly lower viral load setpoint was the C-clade version of the

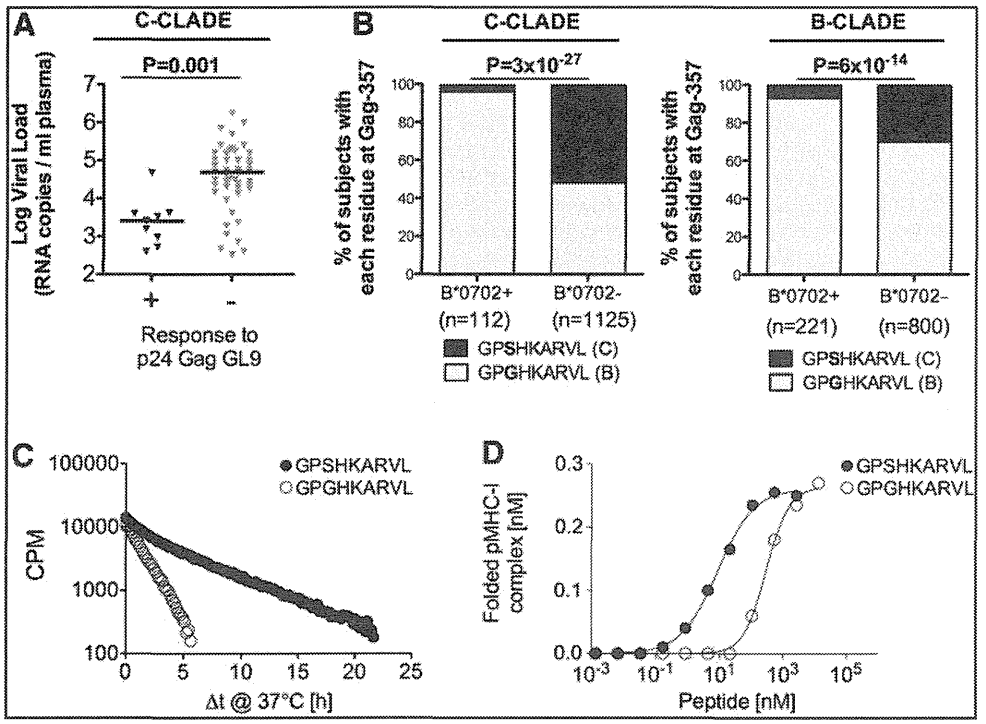

FIG. 4. Peptide binding and stability influence antiviral activity and HIV selection pressure. **(A)** Viral load setpoints for HLA-B*07:02-positive individuals with C-clade infection making IFN-γ ELISpot response to Gag OLP-48 (ACQGVGGPSHKARVLAEA), which contains only p24-GL9, compared to individuals not making this response; **(B)** selection of Gag-357G HIV sequence polymorphism in two large B-clade and C-clade cohorts; **(C)** p24 Gag-GL9-3S and GL9-3G peptides tested for binding half-life and **(D)** affinity to the HLA-B*07:02 molecule.

p24-GL9 (Gag 355–363, GPSHKARVL) specific response ($p=0.001$, Fig. 4A). In contrast, this response was not associated with any change in viral load setpoint in B-clade-infected individuals when tested against both the B-clade and C-clade versions of this peptide (data not shown).

Approximately one-third (36%) of HLA-B*07:02-positive individuals show detectable responses to p24-GL9 in chronic C-clade infection, but this response drives strong selection pressure in acute infection[30] on the virus for the selection of the S357G escape mutant: 96% of HLA-B*07:02-positive subjects carry the GL9-357G mutation compared to 43% of HLA-B*07:02-negative subjects ($p=3\times10^{-27}$) (Fig. 4B). The GL9-357G mutation is also selected in HLA-B*07:02-positive subjects in B-clade infection, but 70% of B-clade sequences carry GL9-357G. In addition, the S357G mutation has a significant impact on the peptide-binding half-life and binding affinity (3.4 h vs. 1.1 h and K_d= = 9 nM vs. 342 nM, respectively) (Fig. 4C and D) (Table 1).

Taken together, these data show that clade-specific differences influence epitope immunogenicity and antiviral CD8$^+$ T cell response efficacy. Alteration of the peptide-HLA-B*07:02 interaction ultimately results in improved Gag-specific epitope targeting in C-clade infection over B-clade infection at the population level.

Discussion

We consistently show here that HLA-B*07:02 is not inherently linked to HIV disease progression, but that individuals infected with a C-clade virus have improved immune control compared to individuals infected with a B-clade virus. Overall, individuals infected with a C-clade virus had a greater breadth and magnitude of HLA-B*07:02-restricted responses targeting Gag epitopes. At the individual epitope level, C-clade-infected individuals targeted two Gag epitopes significantly more often, one in p17-RM9 Gag and one in p24-TL9 Gag, whereas B-clade-infected individuals targeted the Vif-HI10 epitope significantly more frequently. Clade-specific differences in the p17-RM9 Gag epitope resulted in nonbinding and therefore nonimmunogenicity in B-clade infection.

A second observation was that, for the p24-GL9 Gag epitope, targeting frequencies did not differ significantly between B-clade and C-clade-infected subjects, but a response to this epitope was associated with lower viral load setpoints only in C-clade infection. This may be related to the fact that the consensus sequence in B-clade is predominantly the relatively poor binding variant and therefore elicits a different qualitative CD8$^+$ T cell response compared to the S357 C-clade version of the epitope. In addition, the strong selection of the G357S mutation by HLA-B*07:02 expressing individuals in both C-clade and B-clade infection, but the higher frequency of consensus B-clade version of the p24-GL9 epitope (3G) in the HLA-B*07:02-negative B-clade-infected individuals compared to HLA-B*07:02-negative C-clade-infected individuals, may be a consequence of the higher frequency of HLA-B*07:02 expression in the B-clade (22%) than in the C-clade (9%)-infected cohorts studied here.[30] It seems that the S357G mutation is selected very early in acute infection and tends not to revert posttransmission, so this may be an example of a mutant that would accumulate over the course of the epidemic, especially rapidly in populations where HLA-B*07:02 is highly prevalent.[30]

These data support the recent study on clade-specific differences in HLA-B*35:01 in control of HIV infection, which indicated that the protein specificity of the CD8$^+$ T cell response makes an important contribution to viral setpoints and therefore to HLA associations with HIV disease outcome.[27] These HLA molecules are both HLA-Bw6 and therefore do not contribute to the HLA-B-KIR interactions that previous studies have shown make important contributions to immune control of HIV.[21,22] It is also striking that HLA-B*07:02 and HLA-B*35:01, which are the two HLA class I molecules that have been proposed to precipitate rapid HIV progression as a result of a more narrow TCR repertoire,[24] both are linked to a better immune control in C-clade infection along with other HLA-B alleles belonging to the B*07 superfamily.[6,27] These studies therefore are consistent with the hypothesis that increased targeting of Gag epitopes is associated with immune control.

In summary, this study examines a clade-specific difference in mechanisms of immune control mediated by HLA-B*07:02, in which B-clade is associated with rapid progression, while C-clade is not. The data presented indicate that clade-specific differences can alter the CD8$^+$ T cell response, and suggest that these have an important impact on sustained control of HIV.[40] This suggests that immune control of viral replication can be achieved, irrespective of the restricting HLA molecule.[10,41] Ultimately, this provides hope that a vaccine focused on inducing responses targeting conserved regions of the proteome such as Gag will be effective, regardless of the HLA expression of the infected individual.

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H.K. performed the study and wrote the paper. E.A. and M.K. performed experiments. A.S. generated tetramers. M.H. performed peptide HLA binding assays. P.M. analyzed data. C.B., B.W., T.N., and M.T. established and oversaw the HIV cohorts. S.B. supported and established tetramer and peptide binding data. P.G. provided supervision and financial support.

Author Disclosure Statement

No competing financial interests exist.

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Sensitive detection of measles virus infection in the blood and tissues of humanized mouse by one-step quantitative RT-PCR

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Live attenuated measles virus (MV) has long been recognized as a safe and effective vaccine, and it has served as the basis for development of various MV-based vaccines. However, because MV is a human-tropic virus, the evaluation of MV-based vaccines has been hampered by the lack of a small-animal model. The humanized mouse, a recently developed system in which an immunodeficient mouse is transplanted with human fetal tissues or hematopoietic stem cells, may represent a suitable model. Here, we developed a sensitive one-step quantitative reverse transcription (qRT)-PCR that simultaneously measures nucleocapsid (N) and human RNase P mRNA levels. The results can be used to monitor MV infection in a humanized mouse model. Using this method, we elucidated the replication kinetics of MV expressing enhanced green fluorescent protein both *in vitro* and in humanized mice in parallel with flow-cytometric analysis. Because our qRT-PCR system was sensitive enough to detect MV expression using RNA extracted from a small number of cells, it can be used to monitor MV infection in humanized mice by sequential blood sampling.

Keywords: measles virus infection, humanized mouse, quantitative RT-PCR, EGFP expression, flow cytometry

INTRODUCTION

Measles, a highly contagious childhood disease caused by the measles virus (MV), affects more than 20 million people each year. MV infection is characterized by a high fever with typical Koplik's spots followed by the appearance of a generalized maculopapular rash, and is often associated with respiratory and neuronal complications (Griffin, 2007). Since the implementation of vaccination programs using an effective live attenuated MV vaccine, global measles deaths have decreased dramatically. Nevertheless, measles is still one of the leading causes of death among young children under the age of 5 years, especially in countries with weak health infrastructures, and approximately 158,000 measles death occurred in 2011 (http://www.who.int/mediacentre/factsheets/fs286/en/). The ongoing global vaccination strategy aims to protect small children at high risk.

The MV vaccine is safe, effective, and inexpensive. Based on its long and successful vaccination history, several groups have taken advantage of reverse-genetics technology to utilize the live attenuated MV vaccine strain as a viral vector to elicit immune responses against foreign antigens from various pathogens, such as Env or Gag of human immunodeficiency virus (HIV; Lorin et al., 2004; Stebbings et al., 2012), hepatitis B surface (S) antigen (Singh et al., 1999; Reyes-del Valle et al., 2009), fusion protein of respiratory syncytial virus (Sawada et al., 2011), and envelope glycoprotein of West Nile virus (Despres et al., 2005; Brandler et al., 2012). MV is a human-tropic virus that uses CD46, signaling of lymphocyte activation molecule (SLAM, CD150), and the recently identified epithelial-cell receptor nectin-4 (PVRL4, see review in Kato et al., 2012) as receptors. To test the immune response against MV-based recombinant vaccines, both MV receptor-transgenic mice (Singh et al., 1999; Lorin et al., 2004; Despres et al., 2005) and non-human primates have been used as animal models (Reyes-del Valle et al., 2009; Brandler et al., 2012; Stebbings et al., 2012).

Although non-human primates are susceptible to MV, and they develop pathologies similar to those that occur in humans, the expense of using monkeys in research limits the number of animals that can be used for studies. To overcome such practical problems, various types of human MV receptor-transgenic mice expressing CD46 or CD150 have been developed (review in

Sellin and Horvat, 2009). Unfortunately, MV infection of all of these human MV receptor-expressing mouse models is severely restricted by the presence of murine type I IFN; to establish MV infection, it is necessary to introduce the IFNα receptor knockout into the MV receptor-transgenic mice, even in strains expressing CD150 driven by a native human promoter (Ohno et al., 2007). The IFNα receptor knockout/CD150 knock-in mouse is highly susceptible to MV infection and reproduces some aspects of MV infection in humans, including immunosuppression (Koga et al., 2010). This makes it a useful mouse model for study purposes. However, one problem is the lack of an initial innate immune response, which may modify the outcome of MV infection. Thus, the model may not truly reflect the outcome in humans.

In the early 2000s, a series of immunodeficient mice were developed that allow efficient transplantation of human cells or tissues; these systems are collectively termed "humanized mice." A large number of studies have described the development of human hematopoietic cells and their immunological functions in humanized mice, and technical modifications have been made for the study of various human diseases (Ito et al., 2012). Currently, humanized mouse systems are widely used as alternatives to non-human primate models, especially for the study of human-tropic infectious diseases such as HIV, human T cell leukemia virus (HTLV), dengue virus, HCV, and EB virus (Akkina, 2013). Of the different humanized mice models, the BM/Liver/Thymus transplanted (BLT) mouse, which is transplanted with human fetal liver and thymus tissue in addition to hematopoietic stem cells (HSCs), is recognized as the model that most closely mimics the human immune response (Wege et al., 2008). However, the use of this model is limited, mainly because of the ethical issues surrounding human fetal organs/tissues.

We have recently established an HIV infection model in NOD/SCID/Jak3null (NOJ) mouse transplanted with human cord blood HSCs (Terahara et al., 2013). To study MV infection in humanized NOJ (hNOJ), we infected an MV vaccine strain (AIK-C) expressing enhanced green fluorescent protein (EGFP) into hNOJ and analyzed the MV-infected cells by flow cytometry. The hNOJ mouse is highly susceptible to MV infection; in that study, we observed that GFP^+ cells were present in systemic lymphoid tissues and bone marrow (BM). Because it is important to assess MV infection kinetics in an animal without sacrificing the infected mouse, we developed a highly sensitive one-step quantitative reverse transcription-PCR (qRT-PCR) system to monitor MV infection in human peripheral blood mononuclear cells (PBMCs) circulating in the blood of humanized mice. In this study, we describe how this monitoring system works and demonstrate that the results obtained reflect the actual frequency of MV-infected cells, as determined by flow cytometry.

MATERIALS AND METHODS

CELL FRACTIONATION OF PBMCs

Peripheral blood mononuclear cells were obtained from human blood samples of healthy volunteers. Samples were collected after obtaining the approval of the institutional ethical committee of the National Institute of Infectious Diseases (NIID; No. 350) and written informed consent from each subject. PBMCs were separated by Ficoll–Hypaque density-gradient centrifugation (Lymphosepal; IBL, Gunma, Japan).

To obtain monocyte-derived dendritic cells (MDDCs), monocytes were enriched from PBMCs using CD14 microbeads (Miltenyi Biotec) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and antibiotics in the presence of interleukin-4 (IL-4) and granulocyte–macrophage colony-stimulating factor (GM-CSF; both 10 ng/ml, from PeproTech Inc., London, UK) for 1 week. T cells were isolated from CD14-negative PBMCs using the Total T Cell Enrichment Kit (STEMCELL technologies, Vancouver, BC, Canada).

PREPARATION OF RNA

Total RNA was extracted from mouse blood, BM, and spleen of humanized mice, human PBMCs, and Jurkat cells expressing human SLAM (Jurkat/hSLAM) using the RNeasy Mini Kit (QIAGEN, Valencia, CA, USA) or the Total RNA Isolation Mini Kit (Agilent Technologies, Santa Clara, CA, USA).

To prepare a standard of MV RNA, the cDNA encoding measles virus nucleocapsid (N) (MV-N: AB052821) was subcloned into the pBluescript II vector, and then MV-N RNA was produced by *in vitro* RNA transcription using the T7 RiboMAX^{TM} Express Large Scale RNA Production System (Promega, Madison, WI, USA). The RNA product was purified by DNase treatment, followed by phenol–chloroform extraction and ethanol precipitation, according to the protocol supplied by the manufacturer. The final concentration of RNA was measured using an ND-1000 spectrophotometer (Thermo, Waltham, MA, USA).

PREPARATION OF STANDARD TEMPLATE DNA

To prepare a standard template DNA, cDNAs of human CD45 (hCD45: NG_007730) and RNase P (NM_006413) were synthesized from total RNA of CEM cells by reverse transcription (RT)-PCR using SuperScript III RT/Platinum Taq Mix (Invitrogen, Carlsbad, CA, USA). The products were further amplified by PCR using TaKaRa Ex Taq Hot Start Version (TAKARA, Otsu, Shiga, Japan) for hCD45, or AmpliTaq Gold 360 (Applied Biosystems, Carlsbad, CA, USA) for RNase P. These PCR products of hCD45 and RNase P were subcloned into plasmids using the pGeneBLAzer TOPO TA Expression kit (Invitrogen) and pGEM-T (Easy) Vector Systems (Promega), respectively.

REAL-TIME RT-PCR ASSAY

To perform real-time qRT-PCR, SuperScript III Platinum One-Step Quantitative RT-PCR system (Invitrogen) was used according to the manufacturer's instructions. Briefly, each reaction contained 1× reaction mix, ROX reference dye, SuperScript III RT/Platinum TaqMix, 0.2 μM specific primers, and 0.1 μM TaqMan probe. Reactions were performed on an Mx3000P qPCR system (Agilent Technologies). Thermocycling parameters included a RT step at 50°C for 20 min, followed by a DNA polymerase activation step at 95°C for 2 min and 50 PCR cycles (95°C for 20 s, 60°C for 30 s). Threshold cycle (C_t) values were calculated for each reaction; C_t represents the cycle at which a statistically significant increase in the emission intensity of the reporter relative to the passive reference dye is first detected.

For detection of hCD45 mRNA, the following sequences were used: forward primer, 5′-GGA AGT GCT GCA ATG TGT CAT T-3′; reverse primer; 5′-CTT GAC ATG CAT ACT ATT ATC TGA TGT CA-3′; TaqMan probe; 5′-FAM-ACA ACT AAA AGT GCT CCT CCA AGC CAG GTC T-BHQ1-3′ (Hamaia et al., 2001). For detection of RNase P mRNA: forward primer, 5′-AGA TTT GGA CCT GCG AGC G-3′; reverse primer, 5′-GAG CGG CTG TCT CCA CAA GT-3′; TaqMan probe, 5′-FAM-TTC TGA CCT GAA GGC TCT GCG CG-BHQ1-3′ (Kimberly et al., 2005). For detection of MV-N RNA: forward primer, 5′-CGA TGA CCC TGA CGT TAG CA-3′; reverse primer, 5′-GCG AAG GTA AGG CCA GAT TG-3′; TaqMan probe, 5′-FAM-AGG CTG TTA GAG GTT GTC CAG AGT GAC CAG-BHQ1-3′ (Hummel et al., 2006).

GENERATION OF HUMANIZED MICE

Humanized NOD/SCID/JAK3null mice were established as described previously (Terahara et al., 2013). In brief, NOJ mice were transplanted with human HSCs (0.5–1 × 10^5 cells) enriched from human umbilical cord blood cells into the livers of irradiated (1 Gy) newborn mice within 2 days after birth. All mice were maintained under specific pathogen-free conditions in the animal facility at NIID and were treated in accordance with the guidelines issued by the Institutional Animal Care and Committee of NIID.

Human umbilical cord blood was donated by the Tokyo Cord Blood Bank (Tokyo, Japan) after obtaining informed consent. The use of human umbilical cord blood cells was approved by the Institutional Ethical Committees of NIID and the Tokyo Cord Blood Bank. Human HSCs were isolated using the CD133 MicroBeads Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity was approximately 90% as assessed by flow cytometry.

PREPARATION AND INFECTION OF MV

Recombinant wild-type MV (IC323: AB016162) expressing EGFP (IC323-EGFP; Hashimoto et al., 2002) and a recombinant vaccine strain of MV (AIK-C: S58435) expressing EGFP (AIK-C-EGFP; Fujino et al., 2007) were grown in Vero/hSLAM cells. Virus titers were determined by plaque assay using Vero/hSLAM cells.

Jurkat/hSLAM cells were infected with various doses of MV [multiplicity of infection (MOI) = 0.25, 0.05, and 0.01] by incubation at 37°C for 1 h, washed twice with phosphate buffered saline (PBS), and seeded on 24-well plates. Cells were harvested immediately after washing (time 0) or 6, 12, 18, or 24 h later. The harvested cells were either lysed for RNA extraction or analyzed by flow cytometry.

Humanized NOD/SCID/JAK3null mice were challenged intravenously (i.v.) with different doses [200, 2,000, 10,000, or 20,000 plaque-forming units (pfu)] of AIK-C-EGFP. Peripheral blood was obtained from MV-infected hNOJ mice at 3, 5, 7, 10, 14, and 21 days post-infection (p.i.). In some experiments, MV-infected hNOJ mice were sacrificed at day 7 p.i. At the time of sacrifice, peripheral blood, BM, spleen, and mesenteric lymph nodes (MLNs) were harvested, and red blood cells were lysed in ACK buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM EDTA-2Na; pH 7.2–7.4).

FLOW-CYTOMETRIC ANALYSIS OF MV-INFECTED CELLS

PE-conjugated anti-human CD150 (A12) and Pacific Blue-conjugated anti-hCD45 (HI30) monoclonal antibodies (mAbs) were purchased from BioLegend Inc. (San Diego, CA, USA). Cells were stained with these mAbs, fixed with 2% formalin/PBS for 15 min at room temperature, washed, and kept at 4°C prior to flow-cytometric analysis. Dead cells were stained with a LIVE/DEAD Fixable Dead Cell Stain Kit (L34957; Invitrogen). Data were collected using a FACScanto (BD Biosciences, San Jose, CA, USA) and analyzed using the FACSDiva (BD Biosciences) or FlowJo (Tree Star, San Carlos, CA, USA) software.

STATISTICAL ANALYSIS

Non-parametric one-way ANOVA was performed to compare cell type-specific differences in hCD45 and RNase P mRNA expression. Spearman's rank correlation coefficient test was also performed to compare the level of MV-N expression and frequency of MV-infected cells. Prism ver.5 software (GraphPad Software, San Diego, CA, USA) was used for all analyses. $P < 0.05$ was considered statistically significant.

RESULTS

HUMAN-SPECIFIC qRT-PCR SYSTEM FOR THE DETECTION OF MV INFECTION

For the detection of MV infection in clinical specimens, Hummel et al. (2006) established a sensitive qRT-PCR system that used primer and probe sets targeting the MV-N gene. In our humanized mouse model, it is necessary to analyze endogenous mRNA expression in human PBMCs to determine the level of human cell-associated MV infection in mouse blood. We initially assumed that hCD45 expression would be suitable to discriminate human hematopoietic cells from co-existing mouse hematopoietic cells *in vivo*. On that basis, we designed human-specific primer and TaqMan probe sets for hCD45 and compared their usefulness with a primer/probe set for a widely used housekeeping gene, RNase P. RNA was extracted from humanized (hu-mouse) or non-humanized (non-hu-mouse) murine splenocytes, and the level of mRNA was measured by one-step qRT-PCR. Both hCD45 and RNase P primer/probe sets detected mRNA expression of target genes from human PBMCs present in hu-mouse spleen, at similar sensitivities, but neither set detected expression in non-hu-mouse (**Figure 1A**). Thus, both primer/probe sets are human-specific. Next, we enriched $CD14^+$ monocytes and T cells from PBMCs by positive and negative magnetic-bead selection, respectively, and then determined the copy numbers of hCD45 and RNase P in these cell fractions from each of five donors. In **Figure 1B**, the expression levels of hCD45 (left panel) and RNase P (right panel) in monocytes and T cells are depicted relative to the level in each donor's PBMCs. Because RNase P expression was less affected by cell type than CD45 expression ($^*P < 0.05$), in subsequent experiments we exclusively used RNase P primer/probe sets as an endogenous control for mRNA expression.

PARALLEL INCREASE IN THE TIME COURSE OF MV-INFECTED CELL FREQUENCY AND MV-N RNA LEVEL *IN VITRO*

Because wild-type MV mainly utilizes SLAM as the receptor for entry into lymphoid cells (Tatsuo et al., 2000), the kinetics of MV

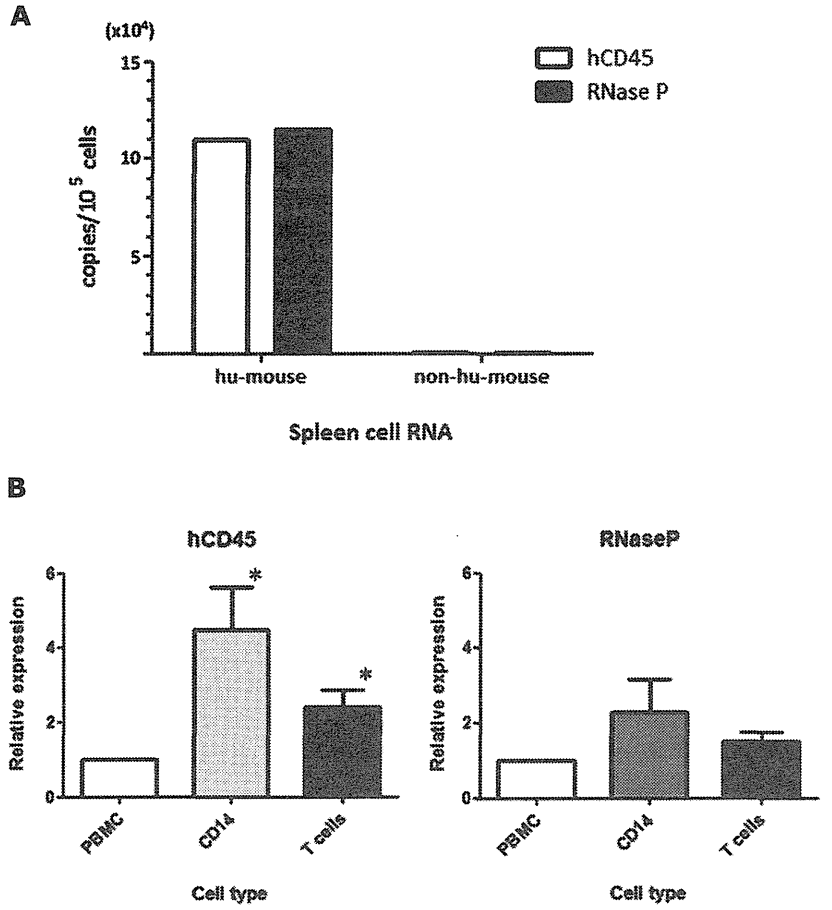

FIGURE 1 | Selection of an endogenous control for the analysis of MV-infected human PBMCs. (A) RNA was extracted from spleen cells of hNOJ and non-humanized NOJ, and one-step qRT-PCR was performed using primer and probe sets designed against the human-specific hCD45 and RNaseP mRNAs. To calculate copy numbers of these genes, the PCR products of human CD45 and RNase P were subcloned into plasmids and used as standard DNAs. **(B)** Human PBMCs from five donors were fractionated into CD14$^+$ monocytes and T cells. RNA from these cell populations was extracted, and the expression levels of hCD45 and RNase P were analyzed by qRT-PCR. The graph depicts the expression levels in these fractionated cells relative to the levels in PBMCs (defined as 1). Statistical differences in hCD45 and RNase P expression among these cell populations were evaluated by non-parametric one-way ANOVA test (*$P<0.05$).

infection in Jurkat/hSLAM cells can be clearly visualized by flow cytometry. We infected Jurkat/hSLAM cells with a wild-type MV encoding EGFP (IC323-EGFP) at MOI of 0.01, 0.05, and 0.25. Cells were washed and harvested at 6, 12, 18, or 24 h after MV infection. A subset of the cells in each sample was analyzed by flow cytometry, and the remainder of the sample was used for RNA extraction. The mRNA levels of MV-N and RNase P were determined by qRT-PCR, and the level of MV-N mRNA relative to RNase P RNA was calculated. Representative results of three experiments are shown in **Figure 2A** (flow cytometry) and **Figure 2B** (qRT-PCR). Because of the rapid and strong cytopathic effect by MV at the highest MOI (0.25), we omitted the flow cytometry data corresponding to that condition. At MOI 0.01, a similar frequency of GFP$^+$ cells was detectable at 12 and 18 h p.i., whereas at MOI 0.05, the GFP$^+$ cell frequency was already high at 12 h p.i. Note that the level of hSLAM was not down-modulated by MV infection. Over the time course, relative MV-N expression level at all three MOIs increased in parallel over two orders of magnitude, indicating that these two methods yield comparable results (as shown in **Figure 2C**) and are useful for monitoring the replication kinetics of MV infection *in vitro*.

PARALLEL INCREASE OF MV-INFECTED CELL FREQUENCY AND MV-N RNA LEVELS *IN VIVO*

We then applied these detection systems *in vivo* in MV-infected hNOJ mice. hNOJ mice were infected with an MV vaccine strain expressing EGFP (AIK-C-EGFP) at 2000 pfu, and the animals were sacrificed 7 days later. Blood PBMCs and BM cells were washed with PBS, and a subset of the cells in each sample were stained with anti-hCD45 mAb. Representative results of flow-cytometric analysis of BM cells from three mice are shown in **Figure 3A**. The percentages of GFP$^+$ cells in mice 127-1, 127-4, and 127-5 mice were low (0.002%), high (0.35%), and intermediate (0.028%), respectively. The number of human PBMCs obtained from mouse blood was not sufficient to determine GFP$^+$