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Original article

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

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Abstract

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

1. Introduction

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

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

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

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

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

2. Materials and methods

2.1. Patients

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

2.2. Cells

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

2.3. Synthetic peptides

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

2.4. Induction of peptide-specific T cells

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

2.5. Intracellular IFN-γ staining assay

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

2.6. CTL assay

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

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

3. Results

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

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

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

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

3.3. Identification of optimal epitope peptides

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

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

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

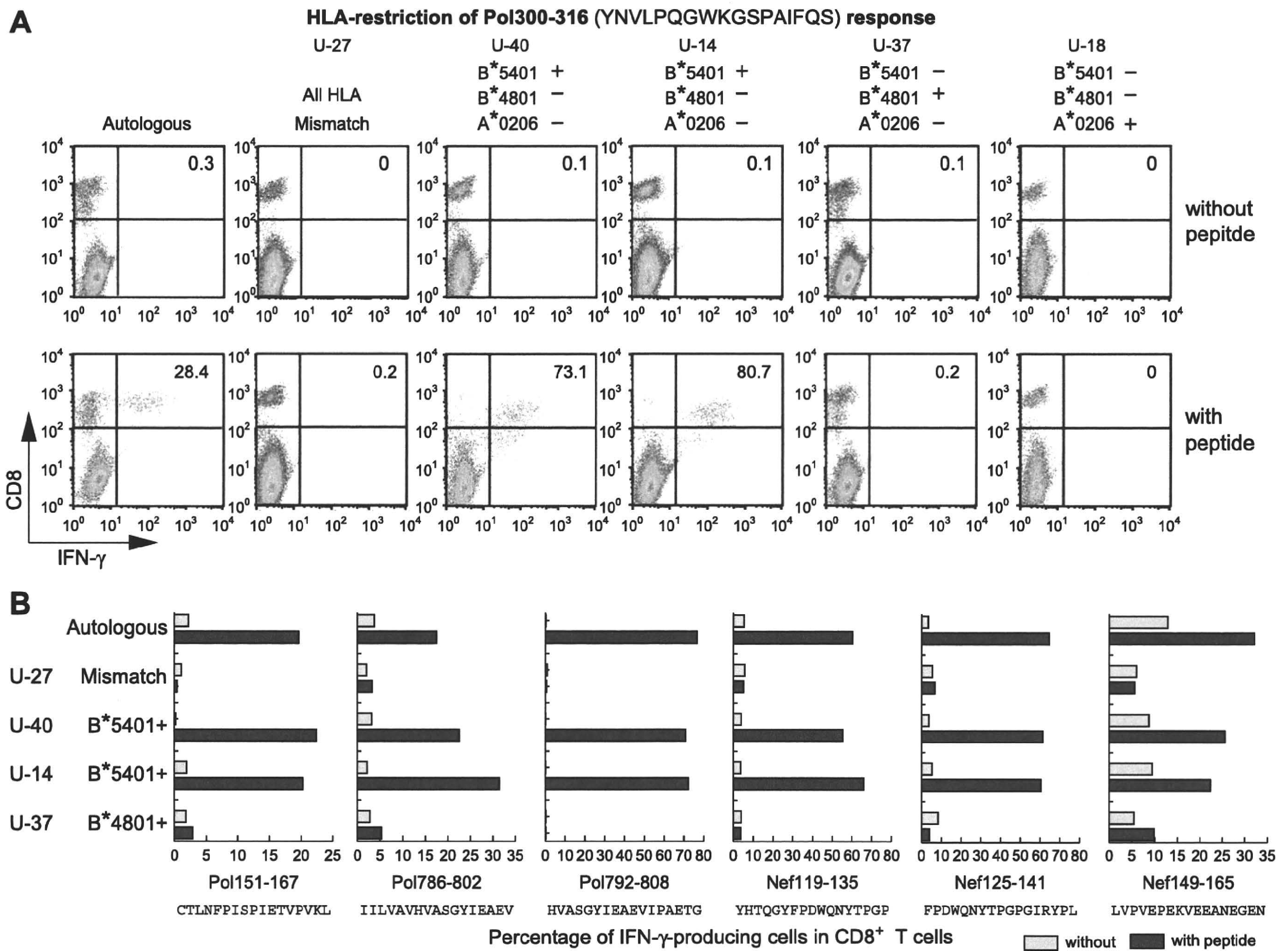

Fig. 1. Identification of HLA-B*5401-restricted HIV-1 CTL epitope candidates by using a panel of B-LCL pulsed with 17-mer peptides. A. PBMC from an HIV-1-seropositive individual KI-119 (A*0206/-, B*5401/B*4801) were stimulated with Pol300–316 peptide and then cultured for 2 weeks. The cultured cells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

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

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

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

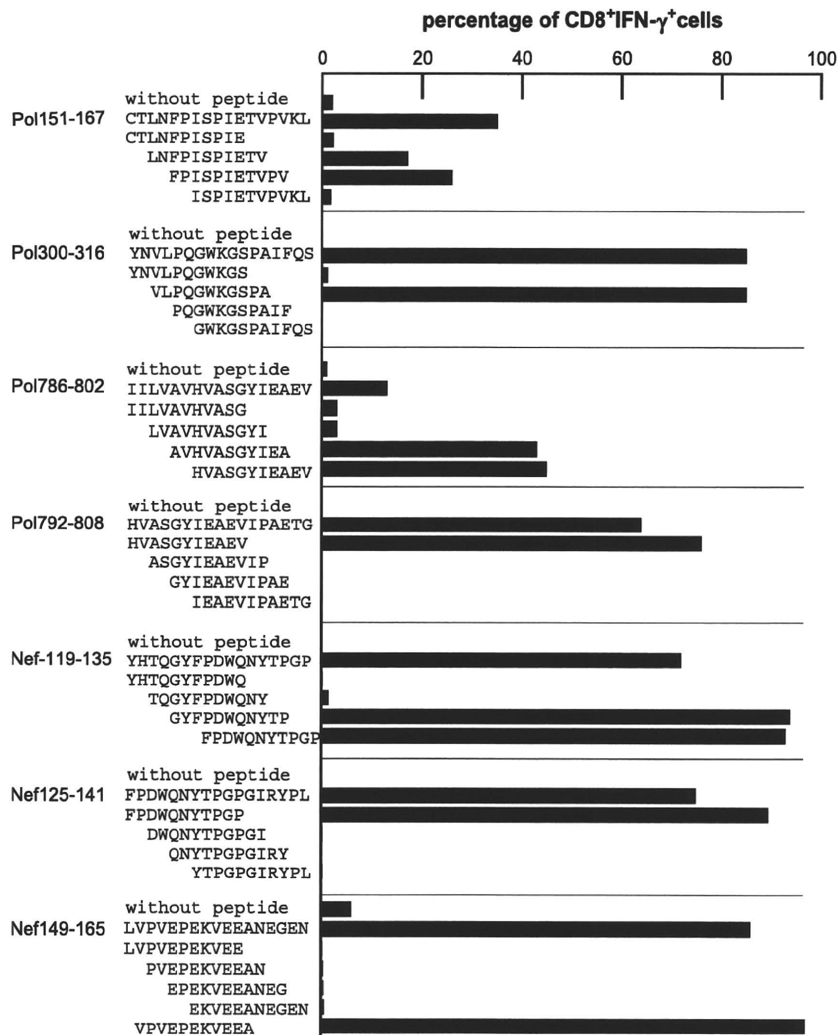

Fig. 2. Selection of 11-mer HIV-1 Pol and Nef peptides including HLA-B*5401-restricted epitopes. The 17-mer peptide-specific bulk CD8$^+$ T cells were stimulated with autologous B-LCL pre-pulsed with each overlapping 11-mer peptide included in the 17-mer peptides. The responsibility of the bulk CD8$^+$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

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

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

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

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

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

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

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

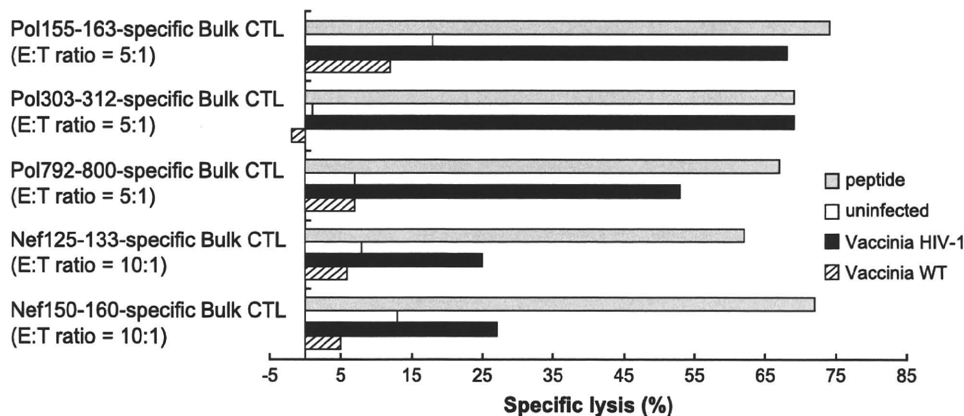

Fig. 4. Killing of r-HIV-1 vaccinia-infected or peptide-pulsed cells by the Pol- or Nef-specific CTLs. The activities of the five HIV-1-specific T cells toward B*5401$^+$B-LCL pre-pulsed with the corresponding peptide (1 μM), or those infected with recombinant vaccinia virus expressing the corresponding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

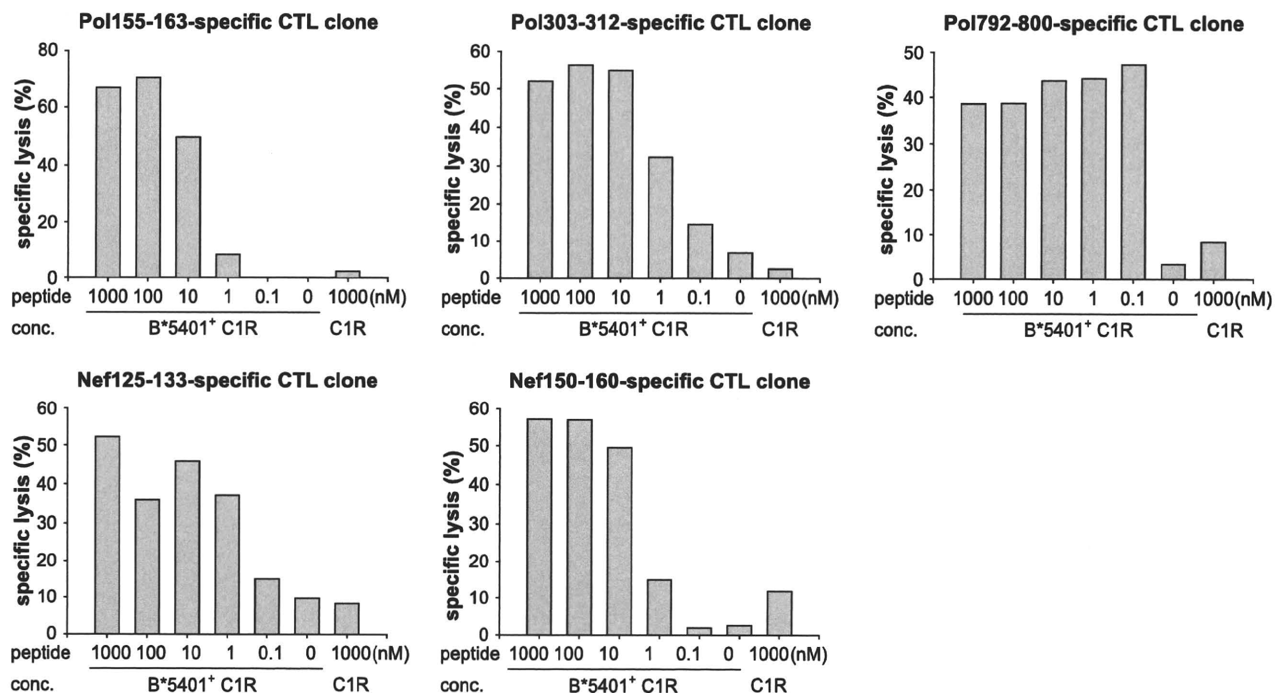

Fig. 5. Confirmation of HLA-B*5401-restricted recognition. Specific lysis of C1R-B*5401 cell lines by Pol155–163-specific, Pol303–312-specific, Pol792–800-specific, Nef125–133-specific, or Nef150–160-specific CTL clone was determined by performing the ^{51}Cr-release assay. The target cells were pulsed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

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

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

4. Discussion

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

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

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

[a] HIV-1-infected individuals with HLA-B*5401.
[b] Copies/ml.
[c] Cells/μl.

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

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

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

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

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

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Failure of Effector Function of Human CD8$^+$ T Cells in NOD/SCID/JAK3$^{-/-}$ Immunodeficient Mice Transplanted with Human CD34$^+$ Hematopoietic Stem Cells

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Abstract

Humanized mice, which are generated by transplanting human CD34$^+$ hematopoietic stem cells into immunodeficient mice, are expected to be useful for the research on human immune responses. It is reported that antigen-specific T cell responses occur in immunodeficient mice transplanted with both human fetal thymus/liver tissues and CD34$^+$ fetal cells, but it remains unclear whether antigen-specific T cell responses occur in those transplanted with only human CD34$^+$ hematopoietic stem cells (HSCs). Here we investigated the differentiation and function of human CD8$^+$ T cells reconstituted in NOD/SCID/Jak3$^{-/-}$ mice transplanted with human CD34$^+$ HSCs (hNOK mice). Multicolor flow cytometric analysis demonstrated that human CD8$^+$ T cells generated from the CD34$^+$ HSCs comprised only 3 subtypes, i.e., CD27^{high}CD28$^+$CD45RA$^+$CCR7$^+$, CD27$^+$CD28$^+$CD45RA$^-$CCR7$^+$, and CD27$^+$CD28$^+$CD45RA$^-$CCR7$^-$ and had 3 phenotypes for 3 lytic molecules, i.e., perforin(Per)$^-$granzymeA(GraA)$^-$granzymeB(GraB)$^-$, Per$^-$GraA$^+$GraB$^-$, and Per^{low}GraA$^+$GraB$^+$. These CD8$^+$ T cells failed to produce IFN-γ and to proliferate after stimulation with alloantigens. These results indicate that the antigen-specific T cell response cannot be elicited in mice transplanted with only human CD34$^+$ HSCs, because the T cells fail to develop normally in such mice.

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Introduction

Humanized mice are generated by transplanting human CD34$^+$ hematopoietic stem cells (HSCs) into immunodeficient mice and are expected to become a useful tool in studies on human T cell immune responses, infectious diseases, preclinical testing of vaccines, and new therapeutic strategies. Previous studies showed long-term human T cell and B cell reconstitution in NOD/SCID/γc^{null} immunodeficient mice transplanted with human CD34$^+$ HSCs (hNOG mice) [1–3]. Human IgM, IgG, and IgA were detectable in the serum of these mice; and class-switching of immunoglobulin in human cord blood (CB)-derived B cells properly occurred in the mice [4–7], indicating that the human B cells can develop from human CD34$^+$ HSCs in the hNOG mice and are functionally competent to produce immunoglobulins in them. Furthermore, previous studies demonstrated that human CD4/CD8 double-positive and human CD4/CD8 single positive T cells were observed in the thymus of the hNOG mice and that the latter were found in the spleen and peripheral blood of these animals [3–5,7,8]. These human T cells expressed predominantly αβ T cell receptors in the thymus and the spleen of the recipient, whereas CD45RA$^+$ naive T cells were identified in the spleen and the peripheral blood of the hNOG mice [4,9,10]. These results suggest the possibility that the human T cells respond to highly diverse molecules. Additionally, proliferation and IFN-γ expression of EBV-specific human CD8$^+$ T cells have been demonstrated in hNOG mice and in Rag2$^{-/-}$γc$^{-/-}$ mice transplanted with human CD34$^+$ HSCs after an EBV infection [8,11]. In contrast, high-dose injection of EBV caused a fatal lymphoproliferative disorder in the hNOG mice, whereas lower-dose injection induces an apparently asymptomatic persistent infection [11]. These findings suggest that the human T cell responses were not able to control the replication of EBV in the hNOG mice.

On the other hand, antigen-specific T cell immune responses had definitely been shown in humanized NOD/SCID mice established by transplanting human fetal thymus/liver tissues and CD34$^+$ fetal liver cells into them (BLT mice) [12–16]. However, there are no studies providing reliable evidence as to whether or not antigen-specific T cell responses are induced in humanized mice established by transplanting only human CD34$^+$ HSCs into immunodeficient mice.

The phenotypic analysis of human T cells in humanized mice is useful to clarify their differentiation and effector function, because the phenotypic classification of human T cells reflects their differentiation and effector function. Previous studies demonstrated that human CD8$^+$ T cells change the expression levels of co-

stimulatory molecules (CD27, CD28, and CD45RA) [17–19] and chemokine receptor CCR7 on their surface according to their differentiation and maturation [20,21]. The phenotypic analysis of human CD8^{+} T cells showed that CD27^{high}CD28^{+}CD45-RA^{+}CCR7^{+}, CD27^{+}CD28^{+}CD45RA^{-}CCR7^{+}, CD27^{+}CD28^{+}-CD45RA^{-}CCR7^{-}, CD27^{low}CD28^{-}CD45RA$^{+/-}$CCR7^{-}, and CD27^{-}CD28^{-}CD45RA$^{+/-}$CCR7^{-} have characteristics of naive, central memory, early effector memory, late effector memory, and effector CD8^{+} T cells, respectively [22,23]. Moreover, human CD8^{+} T cells express 3 key cytolytic effector molecules, i.e., perforin (Per), granzyme A (GraA), and granzyme B (GraB) in response to their differentiation [24]. Five subpopulations of human CD8^{+} T cells defined by these cytolytic molecules exist and appear sequentially during CD8^{+} T cell differentiation: Per^{-}GraA^{-}GraB^{-}, Per^{-}GraA^{+}-GraB^{-}, Per^{low}GraA^{+}GraB^{-}, Per^{low}GraA^{+}GraB^{+}, and Per^{high}GraA^{+}-GraB^{+} [25]. Thus, the functional subsets of human CD8^{+} T cells can be identified by the phenotypic classification and the expression of these 3 cytolytic molecules [25]. By using these classifications of human CD8^{+} T cells, the differentiation and function of human CD8^{+} T cells reconstituted in humanized mice can be clarified in detail. A previous study showed that the CD45RA^{+}CCR7^{-} subset were detected in hNOG mice [10], suggesting the possibility that human CD8^{+} T cells can be differentiated in the mice. However, since this study did not demonstrated the existence of effector subset, CD27^{-}CD28^{-}CD45RA$^{+/-}$CCR7^{-}, it still remains unclear if human CD8^{+} T cells can be differentiated into effector cells in hNOG mice.

In the present study, to clarify antigen-specific human CD8^{+} T cell responses in immunodeficient mice transplanted with only human CD34^{+} HSCs, we established NOD/SCID/JAK3$^{-/-}$ (NOK) mice transplanted with human CD34^{+} HSCs (hNOK mice) and investigated the differentiation and function of human CD8^{+} T cells reconstituted in these mice. Especially, we focused on performing phenotypic classification based on the expression of the above-mentioned effector molecules and the alloreactivity of human CD8^{+} T cells reconstituted in the mice.

Results

Reconstitution of human T cells in hNOK mice

hNOK mice were established by transplanting human CD34^{+} HSCs isolated from human CB into the liver of newborn NOK mice. In order to investigate the reconstitution of the human immune system in these mice, we obtained PBMC from the mice at 10, 12 and 17 weeks after the transplantation and then analyzed them by using flow cytometry for detecting human T and B cells. As shown in Table 1, the cells expressing common human leukocyte antigen CD45 (hCD45^{+}) were found in all hNOK mice at 10 weeks after the transplantation (n = 31). Among the hCD45^{+} cells, human B cells, identified by the expression of CD19, were observed in all hNOK mice, whereas human T cells, identified by the expression of CD3, were observed in approximately 60% of the mice (Table 1). In the mice carrying human T cells, the proportion of human T cells gradually increased from 10 to 17 weeks after the transplantation and reached a level at 17 weeks similar to the proportion of CD8^{+} T cells in the human adult PBMC population (Figure 1A and 1B). The human T cells obtained during the period of 10-17 weeks post transplantation included both human CD4^{+} and CD8^{+} T cells (Figure 1C), though the ratio of human CD4/CD8 T cells gradually increased from the 10 to 17 weeks (Figure 1D). These results show that human CD8^{+} and CD4^{+} T cells were generated and maintained in the mice.

Phenotypic analysis of human T cells reconstituted in hNOK mice

Human peripheral CD8^{+} T cells are classified into the following 5 major populations based on their expression of 4 cell-surface

Table 1. Proportion of human immune cells in hNOK mice.

		% nucleated cells					% nucleated cells		
Cord blood			**hCD45 gated**		**Cord blood**			**hCD45 gated**	
Mouse No.		**hCD45^{+}**	**CD3^{+}**	**CD19^{+}**	**Mouse No.**		**hCD45^{+}**	**CD3^{+}**	**CD19^{+}**
Donor 1	1	12.8	0.0	54.6	Donor 4	1	62.1	46.7	48.3
	2	32.7	32.3	61.8		2	37.4	37.0	58.8
	3	55.1	55.7	40.3		3	57.4	23.3	67.8
	4	59.1	0.0	89.3		4	47.3	45.0	49.2
	5	5.1	0.5	94.9		5	21.4	0.0	91.0
	6	34.2	38.0	57.5		6	37.1	33.7	57.5
Donor 2	1	45.8	0.1	67.9	Donor 5	1	32.1	0.2	95.0
	2	35.1	13.1	57.9		2	10.3	0.0	92.9
	3	55.6	49.7	40.6		3	64.8	30.2	67.3
	4	19.3	0.0	65.5		4	44.2	0.0	96.5
	5	69.4	39.5	48.4		5	3.5	0.0	81.0
Donor 3	1	5.8	0.4	76.2	Donor 6	1	6.6	5.2	81.0
	2	18.9	47.0	43.4		2	7.7	81.3	13.2
	3	37.6	84.1	11.7	Donor 7	1	10.5	34.1	60.7
	4	10.7	0.2	79.7		2	50.3	42.2	54.0
	5	58.4	73.6	22.6					

For generation of hNOK mice, human CD34^{+} cells derived from 7 cord blood samples were transplanted into newborn NOK mice. PBMC of the hNOK mice were analyzed for the engraftment of human immune cells at 10 weeks after the transplantation (n = 31).

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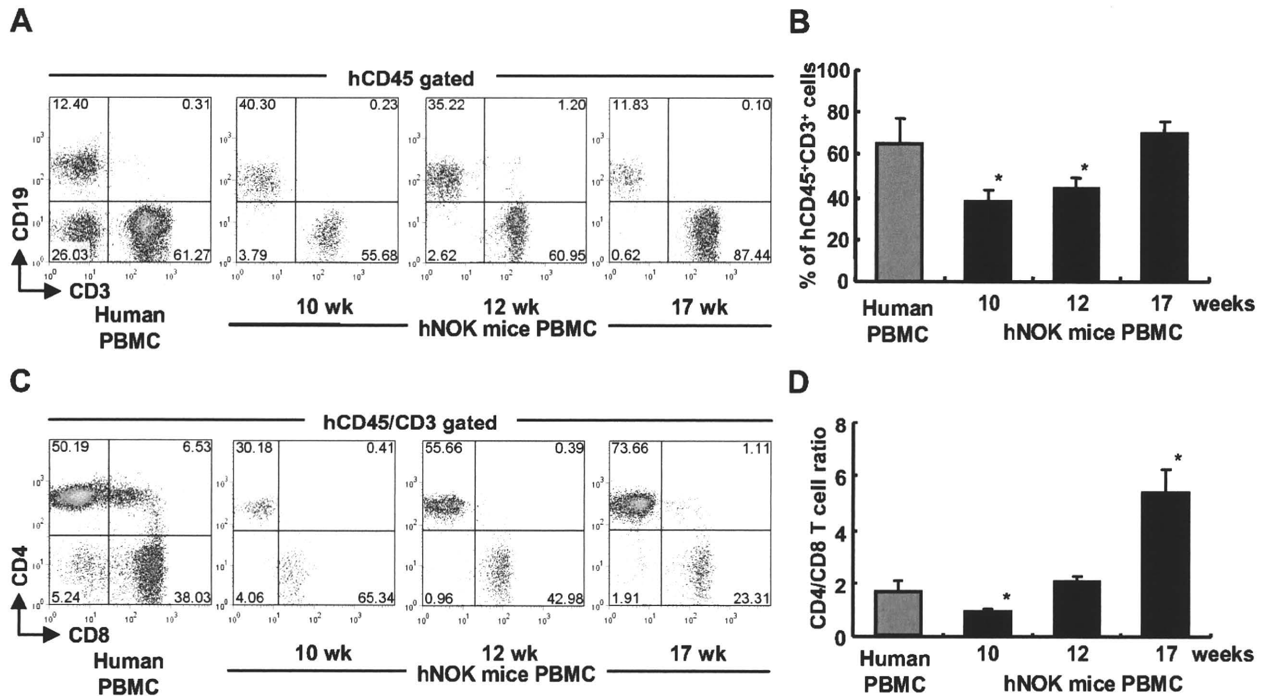

Figure 1. Flow cytometric analysis of human T cells reconstituted in hNOK mice. (A) A representative result for hCD45$^+$CD3$^+$ T cells and hCD45$^+$CD19$^+$ B cells among PBMC from hNOK mice at 10, 12, and 17 weeks after the transplantation. (B) Summarized results showing hCD45$^+$CD3$^+$ T cell proportion in PBMC from hNOK mice (n = 20) at 10, 12 and 17 weeks. (C) A representative result for human CD4$^+$ and CD8$^+$ T cell proportions in PBMC from hNOK at 10, 12 and 17 weeks after the transplantation. (D) Summarized results showing human CD4/CD8 T cell ratio for PBMC from hNOK mice at 10, 12, and 17 weeks. Human PBMC from healthy adult individuals (n = 4) are shown as a control. Bar graph data are shown as the mean ± SEM of 7 independent experiments. *, $p<0.05$, human PBMC vs. hNOK mouse PBMC.
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markers: CD27^{high}CD28$^+$CD45RA$^+$CCR7$^+$ (naive subset), CD27$^+$ CD28$^+$CD45RA$^-$CCR7$^+$ (central memory subset), CD27$^+$CD28$^+$ CD45RA$^-$CCR7$^-$ (early effector memory subset), CD27^{low}CD28$^-$ CD45RA$^{+/-}$CCR7$^-$ (late effector memory subset), and CD27$^-$ CD28$^-$CD45RA$^{+/-}$CCR7$^-$ (effector subset) [22,23]; whereas human peripheral CD4$^+$ T cells are also classified into 5 major populations by the same 4 cell-surface markers, i.e., CD27$^+$CD28$^+$ CD45RA$^+$CCR7$^+$ (naive subset), CD27$^+$CD28$^+$CD45RA$^-$CCR7$^+$ (central memory subset), CD27$^+$CD28$^+$CD45RA$^-$CCR7$^-$ (Th0 effector memory subset), CD27$^-$CD28$^+$CD45RA$^-$CCR7$^-$ (Th1/2 effector memory subset), and CD27$^-$CD28$^-$CD45RA$^-$CCR7$^-$ (effector subset) [26]. To examine the differentiation of human T cells reconstituted in the hNOK mice, we analyzed the phenotype of human T cells among PBMC from the mice at 17 weeks after the transplantation and that of T cells among PBMC from adult humans for comparison. Representative results for an individual hNOK mouse and an adult human are shown in Figure 2A; and a summary of the findings, in Figure 2B. The reconstituted human CD8$^+$ T cell population included naive (39.7±26.9%), central memory (18.9±9.7%), and early effector memory (18.4±15.9%) subsets. Late effector memory (0.9±1.6%) and effector (0.8±2.6%) subsets were hardly detected in the mice. These results suggest that the human CD8$^+$ T cells did not differentiate into late effector or effector human T cells in the mice.

The phenotypic analysis of the reconstituted human CD4$^+$ T cell population in the PBMC from the mice revealed that it included naive (27.5±23.0%), central memory (31.2±10.1%), Th0 effector memory (28.1±18.8%), and Th1/2 effector memory (9.0±11.4%) subsets. The effector (0.06±0.15%) subset was hardly detected, suggesting that the human CD4$^+$ T cells did not differentiate into effector T cells in these mice.

Expression of 3 cytolytic effector molecules in reconstituted human CD8$^+$ T cells

Human peripheral CD8$^+$ T cells are classified into the following 5 major populations based on their expression levels of 3 effector molecules: Per$^-$GraA$^-$GraB$^-$, Per$^-$GraA$^+$GraB$^-$, Per^{low}GraA$^+$ GraB$^-$, Per^{low}GraA$^+$GraB$^+$, and Per^{high}GraA$^+$GraB$^+$ [25]. To investigate the effector function of the human CD8$^+$ T cells reconstituted in the hNOK mice, we examined the expression of these effector molecules in human CD8$^+$ T cells among splenocytes from the mice. A representative result and summary of the analysis for 6 hNOK mice as well as for PBMC from 3 adult humans are shown in Figure 3A and 3B, respectively. The human CD8$^+$ T cells included Per$^-$GraA$^-$GraB$^-$, Per$^-$GraA$^+$GraB$^-$, and Per^{low}GraA$^+$GraB$^+$ cells. But they did not include Per^{low} GraA$^+$GraB$^-$ or Per^{high}GraA$^+$GraB$^+$ ones, though the latter were found among human adult PBMC. These results indicate that the reconstituted human CD8$^+$ T cells did not have effector function in the mice and that the human CD8$^+$ T cells did not differentiate into effector cells in the mice.

Ability of reconstituted human CD8$^+$ T cells to produce cytokines

Human CD8$^+$ T cells producing IL-2 but not IFN-γ are mainly found in naive and central memory CD8$^+$ T cell populations, whereas IFN-γ and TNF-α are mainly produced by effector memory and effector CD8$^+$ T cells among human PBMC [22,27].

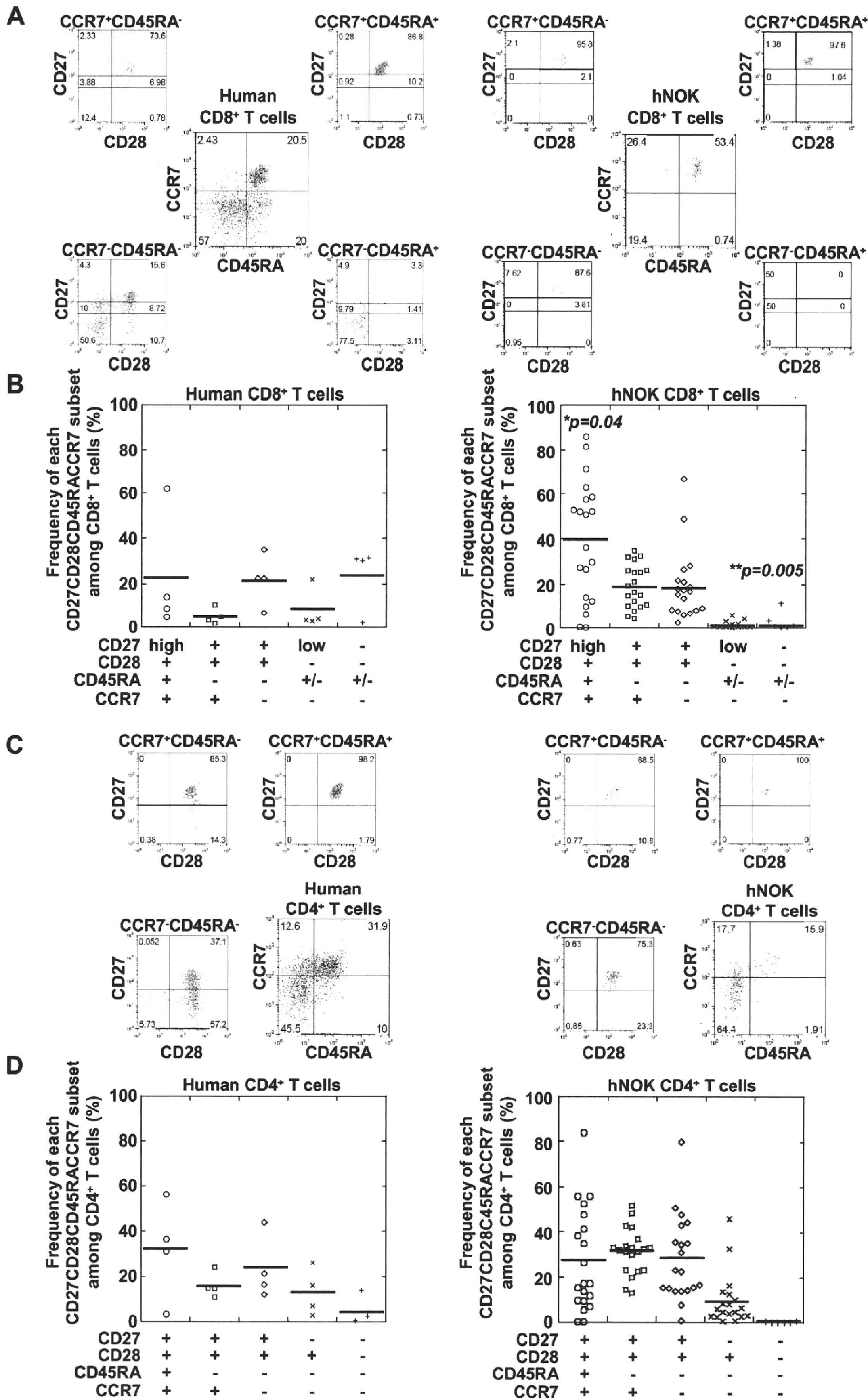
A
CCR7+CD45RA-
CCR7+CD45RA+
CCR7-CD45RA-
CCR7-CD45RA+
Human CD8+ T cells
hNOK CD8+ T cells
CD27
CD28
CCR7
CD45RA
B
Human CD8+ T cells
hNOK CD8+ T cells
Frequency of each CD27CD28CD45RACCR7 subset among CD8+ T cells (%)
*p=0.04
**p=0.005
CD27 high + + low -
CD28 + + + - -
CD45RA + - - +/- +/-
CCR7 + + - - -
C
Human CD4+ T cells
hNOK CD4+ T cells
D
Human CD4+ T cells
hNOK CD4+ T cells
Frequency of each CD27CD28CD45RACCR7 subset among CD4+ T cells (%)
CD27 + + + - -
CD28 + + + + -
CD45RA + - - - -
CCR7 + + - - -

Figure 2. Phenotypic classification of human CD8$^+$ T cells reconstituted in hNOK mice. Human T cells among PBMC from hNOK mice at 17 weeks after the transplantation were analyzed for the expression of the following cell-surface markers: CD4, CD8, CD27, CD28, CD45RA, and CCR7. (A) A representative result of 5-color flow cytometric analysis of the human CD8$^+$ T cell population among PBMC from a hNOK mouse (right data) and an adult human (left data). (B) Summarized results showing frequency of subsets among the human CD8$^+$ T cells isolated from the PBMC from hNOK mice (n = 20, right data) and human adult individuals (n = 4, left data). (C) A representative result of 5-color flow cytometric analysis of human CD4$^+$ T cell population in PBMC from hNOK mice (right data) and a human adult individual (left data). (D) Summarized result showing human CD4$^+$ T cell proportion in PBMC from hNOK mice (n = 20, right data) and human adult individuals (n = 4, left data). Each symbol represents 1 mouse; the mean value is shown as a horizontal solid line. *, *p<0.05*; **, *p<0.01*, human PBMC vs. hNOK mice PBMC.
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To investigate the ability of the human CD8$^+$ T cells reconstituted in the hNOK mice to produce these cytokines, we stimulated splenocytes isolated from these mice with PMA and ionomycin *in vitro* and measured the production of the above cytokines. A representative result and summary of the analysis for 12 hNOK mice are given in Figure 4A and 4B, respectively. The human CD8$^+$ T cells reconstituted in the mice produced IFN-γ, TNF-α, and IL-2 (Figure 4A). The frequency of IFN-γ-producing CD8$^+$ T cells positively correlated with that of the effector memory (CD27$^+$CD28$^+$CD45RA$^-$CCR7$^-$ plus CD27^{low}CD28$^-$CD45 RA$^{+/-}$CCR7$^-$) subset, whereas the frequency of IL-2$^+$IFN-γ$^-$TNF-α$^-$-producing CD8$^+$ T cells negatively correlated with that of effector memory subset (Figure 4B). These results indicate that the human CD8$^+$ T cells had the ability to produce these cytokines in the mice.

Alloreactivity of human CD8$^+$ T cells reconstituted in hNOK mice

It is well known that 0.1–10% of human T-cell repertoire react with alloantigens compared with a frequency of <1/100,000 for foreign antigens in humans [28,29]. We investigated alloreactivity of human CD8$^+$ T cells reconstituted in hNOK mice to clarify the responsiveness of antigen-specific human CD8$^+$ T cells. We immunized hNOK mice with irradiated human PBMC from a healthy donor with HLA-A*2402/A*2402, B*5201/B*5901, and DRB1*1502/DRB1*0405 (allo-PBMC) (n = 4) and analyzed the phenotype of human CD8$^+$ T cells reconstituted in the mice on 7 days or 31 days after the PBMC injection. Flow cytometric analysis demonstrated that CD27$^-$CD28$^-$CD45RA$^{+/-}$CCR7$^-$ effector CD8$^+$ T cell subsets and Per^{high}GraA$^+$GraB$^+$ cells were not induced in the mice on 7 days and 31 days after the PBMC injection (Figures S1A and S1B). We further analyzed the ability of the human CD8$^+$ T cells to produce IFN-γ after allo-PBMC stimulation. The splenocytes from the mice obtained at 31 days after injection of the irradiated PBMC into the mice were cultured with the irradiated PBMC *in vitro* for 7 days, and then the ability of the human CD8$^+$ T cells to produce IFN-γ was measured after stimulation with the irradiated PBMC. As shown in Figure 5A, the human CD8$^+$ T cells and the CD8$^-$ T cells (mostly CD4$^+$ T cells) reconstituted in the mice did not produce IFN-γ, suggesting that both CD8$^+$ T cells and CD4$^+$ T cells could not recognize alloantigen. In addition, we analyzed the ability of the human CD8$^+$ T cells to proliferate after stimulation with alloantigens. The splenocytes of the mice on 31 days were labeled with CFSE (carboxyfluorescein diacetate N-succinimidyl ester); and the cells were cultured for 3 days with irradiated 721.221 cells expressing HLA-A*2402 (.221-A*2402) or irradiated 721.221 expressing HLA-B*5201 (.221-B*5201) *in vitro*. As shown in Figure 5B, the human CD8$^+$ T cells and the CD8$^-$ T cells (mostly CD4$^+$ T cells) reconstituted in the mice did not proliferate in response to.221-A*2402 or. 221-B*5201 cells. In addition, they did not proliferate in response to splenocytes of C57BL/6(H-2^b) or Balb/c (H-2^d) mice (data not shown). These results together indicate that the human CD8$^+$ T cells could not recognize alloantigens in the mice.

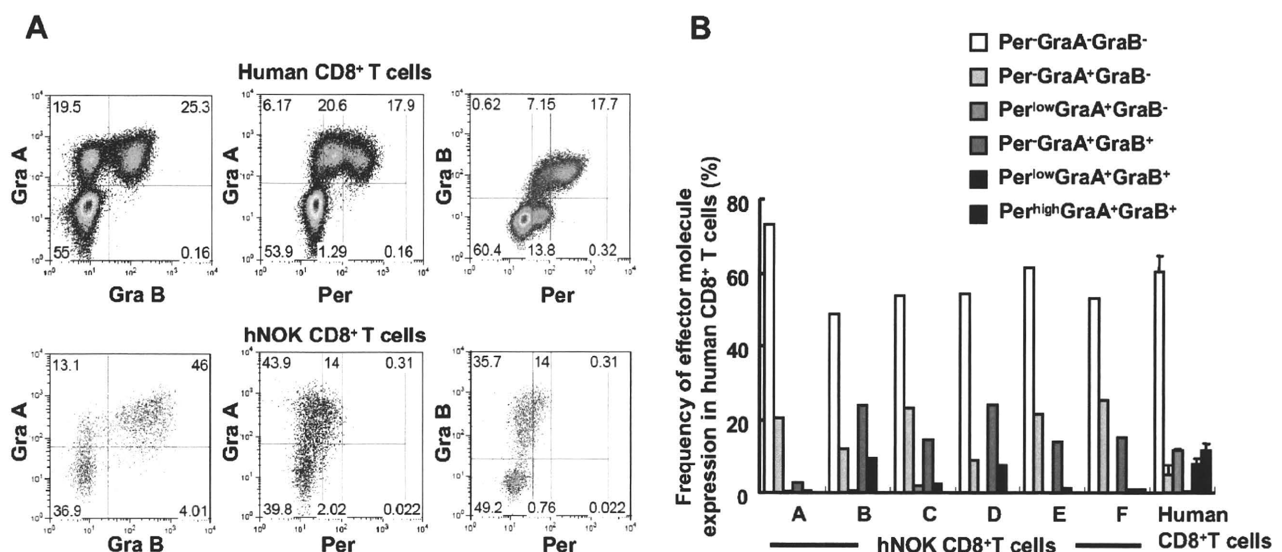

Figure 3. Expression of 3 cytolytic effector molecules of human CD8$^+$ T cells reconstituted in hNOK mice. Human CD8$^+$ T cells among splenocytes of hNOK mice were analyzed for the expression of 3 cytolytic effector molecules: Per, Gra A, and Gra B. (A) A representative result showing co-expression of 3 cytolytic effector molecules in human CD8$^+$ T cells from a hNOK mouse (lower data) and a human adult individual (upper data). (B) Summarized result for the expression of 3 the cytolytic effector molecules in human CD8$^+$ T cells from hNOK mice (n = 6) and human adult individuals (n = 3).
doi:10.1371/journal.pone.0013109.g003

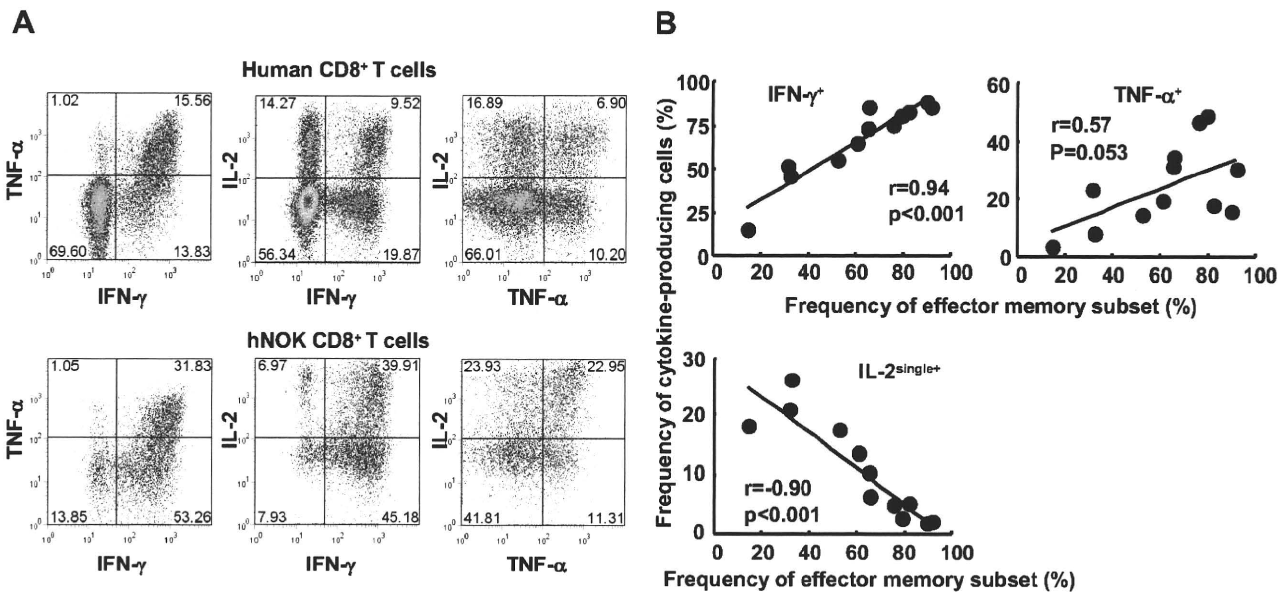

Figure 4. Cytokine production of human CD8$^+$ T cells reconstituted in hNOK mice. Splenocytes from hNOK mice were cultured with PMA and ionomycin for 6 hours and then the production of IFN-γ, TNF-α, and IL-2 cytokines by human CD8$^+$ T cells was measured by flow cytometry. (A) A representative result for cytokine production by human CD8$^+$ T cells from a hNOK mouse (lower data) and a human adult individual (upper data). (B) Correlation between frequency of IFN-γ-, TNF-α-, and IL-2$^+$IFN-γ$^-$TNF-α$^-$ (IL-2$^{single+}$)-producing CD8$^+$ T cells and that of effector memory (CD27$^+$CD28$^+$CCR7$^-$CD45RA$^-$ plus CD27^{low}CD28$^-$CD45RA$^{+/-}$CCR7$^-$) CD8$^+$ T cell subset (n = 12).

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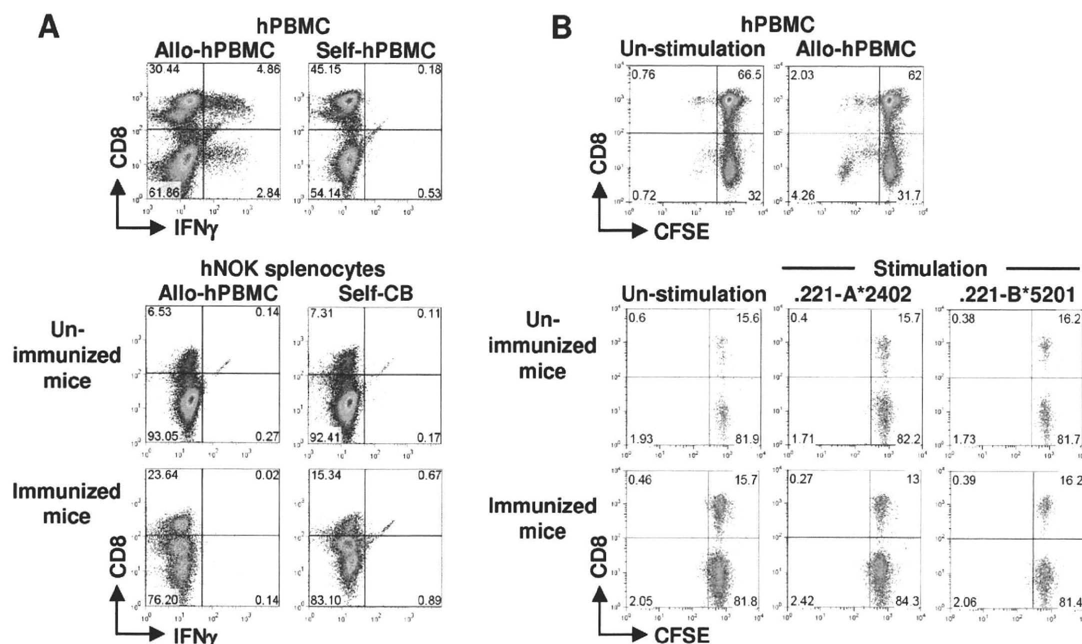

Figure 5. Alloreactivity of human CD8$^+$ T cells reconstituted in hNOK mice. The hNOK mice were immunized for 31 days with irradiated human PBMC (allo-hPBMC) from a healthy donor with HLA-A*2402/A*2402, HLA-B*5201/B*5901, and HLA-DRB1*1502/DRB1*0405 (immunized mice) or PBS (un-immunized mice). The splenocytes of each hNOK mice were harvested, and the cells were stained by using anti-human CD45, anti-human CD3, and anti-human CD8 mAbs. (A) Human adult PBMC from a healthy donor with HLA-A*2601/A*2403, HLA-B*3501/B*5101, and HLA-DRB1*0405/DRB1*0405 were cultured with the allo-hPBMC or the self-hPBMC for 7 days *in vitro*, and a representative result showing the IFN-γ production by the human CD8$^+$ T cells and the human CD8$^-$ T cells is shown as a control (upper data). The splenocytes from the immunized hNOK mice were cultured with the allo-hPBMC or self-CB for 7 days *in vitro*, and a representative result for IFN-γ production by the human CD8$^+$ T cells and the human CD8$^-$ T cells is shown (lower data). (B) Splenocytes from the immunized hNOK mice were cultured with irradiated 721.221 cells expressing HLA-A*2402 (.221-A*2402) or irradiated 721.221 expressing HLA-B*5201 (.221-B*5201) for 3 days *in vitro*, and a representative result for the proliferation of the human CD8$^+$ T cells or the human CD8$^-$ T cells is shown. One representative experiment from 3 experiments is shown.

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Discussion

It had remained unknown whether antigen-specific human T cell responses can be elicited in immunodeficient mice transplanted with only human $CD34^+$ HSCs. In the present study, we analyzed the differentiation of the reconstituted human T cells and the function of the human $CD8^+$ T cells stimulated by alloantigens in hNOK mice. Because human T cells express 4 cell-surface markers (CD27, CD28, CD45RA, and CCR7) and 3 cytolytic molecules (GraA, GraB, and Per) at different levels according to their stage of differentiation and maturation [22,23,25], the analysis of phenotype and effector molecule expression in the reconstituted human T cells is useful to clarify their differentiation status and effector function. We demonstrated that the $CD27^{low}CD28^-CD45RA^{+/-}CCR7^-$ (late effector memory) and the $CD27^-CD28^-CD45RA^{+/-}CCR7^-$ (effector) subsets did not exist among the human $CD8^+$ T cells from the hNOK mice. In addition, the reconstituted human $CD8^+$ T cells did not include the $Per^{high}GraA^+GraB^+$ (effector) cells. These results suggest that the reconstituted human $CD8^+$ T cells did not have their effector function *in vivo*. On the other hand, the reconstituted human $CD8^+$ T cells included the $CD27^+CD28^+CD45RA^-CCR7^-$ (early effector memory) subsets in the hNOK mice, suggesting that the human $CD8^+$ T cells could differentiate into memory T cells in the hNOK mice in response to some stimulation. Although early effector memory $CD8^+$ T cells among the human adult $CD8^+$ T cell population include $Per^{low}GraA^+GraB^-$ cells [25], the reconstituted human $CD8^+$ T cells did not include the Per^{low}-$GraA^+GraB^-$ ones. This finding suggests that some population of early effector memory $CD8^+$ T cells in the mice were defective and could not express Per in the course of their differentiation. These results further suggest that the human $CD8^+$ T cells did not have their effector function in the hNOK mice. On the other hand, the reconstituted human $CD8^+$ T cells showed the ability to produce cytokines similar to that of human adult $CD8^+$ T cells.

The linear differentiation model supports the idea that the naive T cells differentiate directly into effector cells during the acute phase of the response and that following contraction in the numbers of the effector cells at the end of the primary response, effector memory and central memory T cells become detectable [30]. However, the human $CD8^+$ T cells reconstituted in the hNOK mice showed the lack of Per expression and $Per^{low}GraA^+$-$GraB^-$ subset, implying that the cells could not differentiate into effector cells and subsequently differentiate into memory cells. The results of phenotypic analysis and effector molecule expression of the reconstituted human $CD8^+$ T cells in the hNOK mice support the idea that the human $CD8^+$ T cells asymmetrically divided into long-lived memory cells [30,31].

A previous study demonstrated the presence of alloantigen-specific cytotoxic human T cell clones in hNOG mice [5]. However, the data from that study do not exactly reflect T cell responses *in vivo*; because alloantigen-specific human $CD8^+$ T cell clones were established from the spleen cells of the mice cultured with allogeneic target cells *in vitro*. The frequency of the human alloreactive T cell repertoire was evaluated to reflect between 0.1% and 10% of the total T cell population, whereas that of T cells specific for foreign antigen is <1/100,000 [28,29]. Since the reconstituted human $CD8^+$ T cells did not respond to alloantigen in the hNOK mice, these reconstituted human $CD8^+$ T cells may not have a foreign antigen-specific function *in vivo*. This result implies that the reconstituted human $CD8^+$ T cells did not have the appropriate T cell receptor (TCR) repertoire against foreign antigens, because the human $CD8^+$ T cells were educated by mouse MHC-peptide complexes in the thymus. CD8 co-receptors bind with a nonpolymorphic region within the α3 domain of class I MHC proteins, and their binding has species specificity [32–36]. Considering that human CD8 molecules fail to bind to murine MHC, the human $CD8^+$ T cells in immunodeficient mice transplanted with only human $CD34^+$ HSCs are not educated in the murine thymus.

There are several reports that antigen-specific responses are detectable in BLT mice infected with EBV or HIV [12,14,16]. Furthermore, the BLT mice produce high levels of human IgM and IgG antibodies and mediate strong immune responses *in vivo*, as demonstrated by skin xenograft rejection [13]. These results suggest that the reconstituted human T cells educated in the human thymus can have appropriate antigen-specific function in mice. A recent study demonstrated that the human $CD8^+$ T cells reconstituted in HLA transgenic NOG mice transplanted with only human $CD34^+$ HSCs expressed IFN-γ production and EBV-specific cytolytic activity in response to an EBV infection [37,38], suggesting that the reconstituted human T cells educated by human HLA class I molecules on mouse thymus cells have appropriate effector function in the mice.

In the present study, we demonstrated impaired differentiation of human $CD8^+$ T cells in the hNOK mice established with only human $CD34^+$ T cells and lack of ability to induce the effector function. Previous studies of hNOG mice showed very weak immune responses to viral antigens [8,9,11]. Thus both hNOK and hNOG mice seems to have no or weak ability in HLA-restricted T cell responses although it is unclear whether some differences exist in human T cell function between 2 humanized mice. HLA transgenic immunodeficient mice are expected to be useful for the generation of a good mouse model having human immunity.

Materials and Methods

Establishment of humanized mice

The NOK mouse strain was established by backcrossing $JAK3^{-/-}$ mice with the NOD.Cg-Prkdcscid strain for 10 generations. The NOK mice were maintained under specific pathogen-free conditions in the Center for Animal Resources and Development. Animal experiments were conducted according to the Regulation for Animal Experiments in Kumamoto University (Approval ID, C22-168: Analysis of immune responses against viral diseases by using humanized and HLA transgenic mice). Human CB was purchased from Riken Cell Bank (Tsukuba, Japan). Human $CD34^+$ cells were isolated from human CB by using a Direct CD34 Progenitor Cell Isolation Kit and an MS Column (Miltenyi Biotec, Gladbach, Germany). The usual purity of the human $CD34^+$ cells was approximately 95%. The hNOK mice were generated by injecting the isolated human $CD34^+$ cells (5×10^4 cells/mouse) into the liver of newborn NOK mice.

Blood samples

Human blood samples were taken from healthy adult individuals. Peripheral blood mononuclear cells (PBMC) were isolated from the blood by using Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden).

Cell lines

721.221 cells are human B cell lines lacking HLA class I but not HLA class II [39]. 721.221 cells expressing HLA-A*2402 (.221-A*2402) and those expressing HLA-B*5201 (.221-B*5201) were generated by transfecting of HLA-A*2402 and HLA-B*5201 genes into 721.221 cells, respectively.

Flow cytometric analysis

Human leukocytes reconstituted in hNOK mice were stained with various combinations of monoclonal antibodies (mAbs): FITC-labeled anti-mouse CD45 (mCD45), PECy7-labeled human CD45 (hCD45), allophycocyanin-labeled anti-CD4, allophycocyanin, Cy7-labeled anti-CD4, AmCyan-labeled anti-CD8, Pacific blue-labeled anti-CD8, PE-labeled anti-CD19, FITC-labeled anti-Per, PE-labeled anti-GraA, Alexa647-labeled anti-GraB, PE-Cy7-labeled anti-CCR7, FITC-labeled anti-CD45RA, PE-labeled anti-CD28, allophycocyanin-Cy7-labeled anti-CD27, FITC-labeled IFN-γ and PE-Cy7-labeled TNF-α mAbs, all were purchased from BD Biosciences (San Diego, CA). ECD-labeled anti-CD3 mAb was obtained from Beckman Coulter (Fullerton, CA); and allophycocyanin-labeled IL-2 mAb, from e-bioscience (San Diego, CA). To analyze the phenotype of human T cells reconstituted in the hNOK mice, we first stained the human T cells with anti-CCR7 mAb for 30 min at room temperature, and subsequently with specific antibody against surface markers at 4°C for 30 min. The cells were washed twice with PBS containing 10% fetal calf serum (FCS; Sigma-Aldrich, St. Louis, MO). To analyze the intracellular expression of IFN-γ, Per, GraA, and GraB, we fixed cells with 4% paraformaldehyde PBS at 4°C for 20 min, and then made them permeable by incubating them at 4°C for 10 min in PBS containing 0.1% saponin (Sigma-Aldrich) and 20% FCS (permeabilizing buffer). The cells were then stained with anti-IFN-γ, anti-Per, anti-GraA, and anti-GraB mAbs at 4°C for 20 min. Finally, the stained cells were washed 3 times in the permeabilizing buffer at 4°C. The stained cells were analyzed by using a FACSCant II flow cytometer (BD Biosciences, San Jose, CA). All flow cytometric data were analyzed by using FlowJo software (Tree Star, Inc, Ashland, OR).

Cytokine production of human $CD8^+$ T cells stimulated with PMA and ionomycin

The splenocytes of hNOK mice were cultured at a density of 1×10^6 cells in 96- well plates with phorbol 12-myristate 13-acetate (PMA) (10 ng/ml) and ionomycin (1 ng/ml). Two hours later, brefeldin A (10 ng/ml) was added to each well. After a further 4-hour incubation, the splenocytes were stained with Allophycocyanin-labeled anti-IL-2, FITC-labeled anti-IFN-γ, and PE-Cy7-labeled anti-TNF mAbs, and the stained cells were analyzed on the FACSCant II flow cytometer.

Assay for alloreactivity of human $CD8^+$ T cells

PBMC (5×10^6/mouse) from a healthy donor with HLA types HLA-A*2402/A*2402, B*5201/B*5901, and DRB1*1502/DRB1*0405 were irradiated and then intraperitoneally injected into hNOK mice. At 4 weeks after the injection, PBMC and splenocytes of the hNOK mice were harvested and analyzed for their phenotype by using the FACSCant II. To investigate IFN-γ expression of human T cells against alloantigen, we cultured the splenocytes for 6 hours at a density of 1×10^6 cells in 96-well plates with the irradiated PBMC or irradiated self-CB. Then the cultured cells were stained with anti-CD3 mAb, anti-CD8 mAb, and anti-IFN-γ mAb. For proliferation assays, carboxyfluorescein diacetate N-succinimidyl ester (CFSE, Molecular Probes, Willow Creek Road Eugene, OR) for a final concentration of 0.5 μM was added to the cell suspension. After a 15-min incubation, the excess CFSE was removed by washing with 5% FCS/PBS; and then the cells were resuspended in RPMI1640 containing 10% FCS. The CFSE-labeled cells were cultured for 3 days with 721.221 cells expressing HLA-A*2402 or HLA-B*5201 and then were analyzed on the FACSCant II.

Statistical analysis

Results shown as bar graphs were expressed as the means ± S.E.M. One-way ANOVA followed by Dunnet's test was used for multiple comparisons. Differences were considered to be statistically significant when the p-value was less than 0.05.

Supporting Information

Figure S1 Phenotypic analysis of reconstituted human $CD8^+$ T cells stimulated with alloantigen in hNOK mice hNOK mice were immunized for 31 days with irradiated human PBMC from a healthy donor with HLA-A*2402/A*2402, HLA-B*5201/B*5901, and HLA-DRB1*1502/DRB1*0405. Then the phenotype of human $CD8^+$ T cells among PBMC from the hNOK mice was analyzed on 0, 7, and 31 days after the immunization. Splenocytes from the same mice were examined at day 31. (A) Representative results of 5-color flow cytometric analysis of CCR7CD45RACD27CD28 subsets in human $CD8^+$ T cell population of PBMC and splenocytes are shown. (B) Representative results for Per, GraA, and GraB expression by the human $CD8^+$ T cells among splenocytes from the hNOK mice are shown.
Found at: doi:10.1371/journal.pone.0013109.s001 (1.97 MB TIF)

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Author Contributions

Conceived and designed the experiments: MT. Performed the experiments: YS HT NK SN. Analyzed the data: YS HT NK TU. Contributed reagents/materials/analysis tools: NN. Wrote the paper: MT.

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