厚生労働科学研究費補助金
エイズ対策研究事業

# HIV感染モデルマウスの樹立および HIV特異的細胞傷害性T細胞によるエイズ発症遅延機序の解析に関する研究

平成20年度　総括研究報告書

研究代表者　佐藤　義則

平成21(2009年)3月

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# I. 総括研究報告書

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総括研究報告書

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研究代表者: 佐藤 義則(熊本大学エイズ学研究センター COEリサーチ・アソシエイト)

研究要旨

長期 HIV 感染における免疫応答および病態の解析が困難である理由のひとつとして、小動物を用いた HIV 感染実験系が確立されていない点が挙げられる。 そこでヒト化マウスによる HIV 感染系の確立を目指し、新たな免疫不全マウス(NOD/SCID/Jak3 ノックアウトマウス(以下 NOK マウス))にヒト臍帯血由来幹細胞を移植して、ヒト免疫構築マウス(hu-NOK)を作製した。 Hu-NOK マウスでは、末梢血中にヒト CD19 陽性B細胞およびヒト CD3 陽性T細胞の発生が認められ 10 週以上継続的に定着することが明らかとなった。 発生したヒトT細胞は CD45RA+CCR7+CD27+CD28+のナイーブ表現型を示したヒトT細胞が多く含まれたマウス(Tn 群)と、CD45RA-CCR7-CD27+CD28+のエフェクターメモリー表現型を示したヒトT細胞が多く含まれたマウス(Tem 群)の2群に分かれることが判明した。 しかしながら、いずれのマウスにおいてもエフェクター表現型を示した細胞は認められなかった。 また、ウイルス感染細胞の破壊因子となるパーフォリンとグランザイムの各酵素群はパーフォリン $^{low}$、グランザイム$A^+$、グランザイム$B^-$の集団まで確認できた。 一方、PMA/Ionomycin 刺激によるヒト CD8T細胞のサイトカイン産生能を調べたところ、Tem 群のヒト CD8T細胞では各種サイトカイン(TNF-alfa, IFN-gamma, IL-2)産生能を示した。 これらの結果は、hu-NOKマウスで発生したヒトT細胞はエフェクターメモリーで留まっているものの、刺激に対しての反応が起こることから、エフェクターメモリー表現型までの正常な分化は起こっており、よりヒトの免疫応答に近いヒト化マウスによる HIV 感染系の確立ができる可能性を示した。

## A. 研究目的

長期にわたって HIV の増殖を抑えるためには HIV 特異的細胞傷害性 T 細胞によるウイルス感染細胞の排除が重要であることはよく知られている。 当研究室では、長期にわたってエイズを発症しない HIV 感染者から非常に強い HIV 増殖抑制能を示す細胞傷害性T細胞(CTL)の単離に成功した。 一方、長期 HIV 感染における免疫応答および病態の解析が困難である理由のひとつとして、小動物を用いた HIV 感染実験系が確立されていない点が挙げられる。 そこで我々は、新たに免疫不全マウス(NOD/SCID/Jak3 ノックアウトマウス(以下NOK マウス))を作製し、ヒト臍帯血由来幹細胞を移植したところ、マウス体内において

HIV-1 の標的細胞であるヒト CD4 T 細胞および細胞傷害性を担うヒト CD8 T 細胞の発生に成功した。そこで本研究では、1. NOK マウスで樹立したヒト免疫構築マウスにおける基礎知見を得るため、発生したヒトT細胞の分化・機能を解析し、免疫細胞のレパートリーについて解析する、2. 長期 HIV 感染における免疫応答と病態進行の機序を明らかにするため、ヒト免疫構築 NOK マウスにおける HIV 感染系の確立、および、HLA 発現ヒト免疫構築 NOK マウスを樹立し、HIV 感染マウスにおける HIV-1 特異的 CTL の動態を解析する、3. さらに我々の過去の報告に基づく、より効果的な HIV-1 特異的 CTL クローンの移入により、HIV 感染に対する免疫応答および免疫細胞療法の効果についても検討する。 本研究で樹立する HLA 発現ヒト免疫構築マウスでは、ヒトの造血・免疫系を確立した際に問題となる胸腺での発生・分化障害も解決でき、今までのモデルマウスより成熟したヒト免疫系の構築が可能である。 これは HLA ハプロタイプとエイズ進行の相関を動物モデルにおいて検討できる点が優れており、これらの研究を通じて、エイズ発症機序の詳細な解析と新規エイズ治療法の開発に大きく貢献することが本研究の目的である。

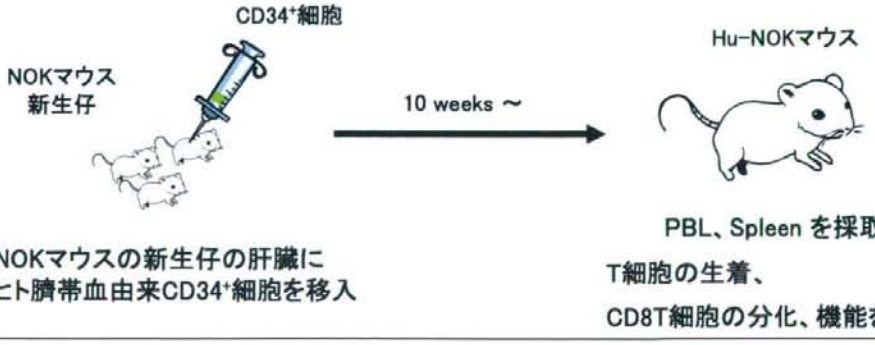
## B. 研究方法

ヒト免疫構築マウスを樹立するため、NOK マウスに幹細胞のマーカーである CD34 に対する特異的抗体がコートされた磁気ビーズをもちいて、臍帯血単核球より臍帯血幹細胞を分離し、NOK マウスの新生仔の肝臓へ移植した(図1)。 移植から 10 週間後、マウスの尾静脈より採血をし、T 細胞の生着を調べた。またT細胞の生着が確認できたマウスは、移植から 20 週間後、脾臓と血液を採取し、HIV-1 の標的細胞である CD4 T 細胞や細胞傷害性T細胞である CD8T 細胞を含む免疫細胞の発生・分化・成熟、および、それらの機能について、CD 抗原、リンパ球の分化マーカー、サイトカイン等に特異的な抗体をもちいてフローサイトメーターにて解析し、ヒト免疫構築マウスにおける免疫細胞のレパートリーについて解析した。

(倫理面への配慮)

本研究におけるヒト由来の検体の使用と遺伝子解析、および理化学研究所(理研)から購入した臍帯血単核球は提供者に対して既にインフォームド・コンセントを行い得たものであり、本研究によ

図1 方法



って新たに提供者に危険を及ぼすことは無く、当大学の倫理審査委員会にて承認済みである。また、動物実験についても既に当大学の動物実験委員会にて承認済みである。

## C. 研究結果

NOKマウス新生仔の肝臓にヒトCD34陽性臍帯血幹細胞を移植したマウスでは、図2で示すように、末梢血中にヒトB細胞と考えられた CD19 陽性細胞およびヒトT細胞と考えられた CD3 陽性細胞の発生が認められた。その発生頻度は移植後、T細胞では 10週以降から顕著に増加した。しかしながら、ヒトT細胞の発生頻度はヒトB細胞のそれに比べて約2分の1程度であることが明らかとなった。ヒトT細胞が発生したマウス群において、ヒトT細胞の分化・成熟の詳細な解析を行ったところ、CD8 T細胞およびCD4 T細胞の両者において、CD45RA+CCR7+CD27+CD28+のナイーブ表現型を示したT細胞が多く含まれたマウス(Tn 群)と(図3)、CD45RA-CCR7-CD27+CD28+のエフェクターメモリー表現型を示したT細胞が多く含まれたマウス(Tem 群)の2群に分かれることが判明した(データ示さず)。しかし、エフェクターT細胞を確認することは出来なかった。またヒトT細胞の機能解析を行った結果、ウイルス感染細胞の破壊因子となるパーフォリンとグランザイムの各酵素群の発現がパーフォリン$^{low}$、グランザイムA$^{+}$、グランザイムB$^{-}$の集団まで確認することができた(図4)。一方、PMA/Ionomycin 刺激による CD8 T細胞のサイトカイン産生能を調べたところ、Tem 群の CD8 T細胞では各種サイトカイン(TNF-alfa, IFN-gamma, IL-2)産生能を示し(図5)、Tn 群の CD8 T細胞ではそれらの産生能を示さなかった(データ示さず)。

## D. 考察

小動物を用いた HIV 感染実験系の確立は、長期 HIV 感染における免疫応答および病態の解析を可能とするとともに、飼育に大掛かりな施設、設備、高額の飼育費を必要とするサルやチンパンジーにかわる代替動物として期待され、ヒト免疫構築マウスを用いた研究は既に国内外で始まっている。しかしながら、ヒト免疫構築マウスを用いた HIV 感染モデルの報告は数例あるものの、長期的 HIV 感染実験モデルや、HIV 感染マウスにおけるHIV-1特異的CTLの動態の解析についての報告は未だされていない。我々が今回用いている NOK マウスによるヒト免疫構築マウスでは、移植マウスの末梢血中にヒトB細胞およびヒトT細胞と考えられる免疫細胞の発生は認められたが、B細胞の発生頻度にくらべT細胞のそれは約2分の1程度であった。これはT細胞が分化する際に必要な胸腺における教育によってヒトT細胞の正の選択がされにくいこと(マウス MHC とヒト TCR の親和性不一致)が考えられる。また末梢 T 細胞は、CD8T細胞および CD4T細胞の両者で表現形が2群(Tn, Tem)に別れ、エフェクターT細胞の集団は確認することが出来なかった。さらに機能解析からパーフォリン $^{low}$、グランザイムA$^{+}$、グランザイムB$^{-}$の集団までが確認できたが、この集団はエフェクターメモリーが示す表現形であることからも、hu-NOK マウスで発生しているヒトT細胞はエフェクターメモリー表現形までの分化に留まっていることが明らかとなった。一方、Tem 群の

CD8 T細胞はPMA/Ionomycin刺激によって各種サイトカイン産生能を示したことから、T細胞の機能には問題がないと考えられる。 Hu-NOK マウスのヒトT細胞がエフェクターメモリー表現形で留まる理由として、マウスのSPF飼育環境に原因があると考えられる。 SPF飼育環境下はヒトの生活環境に比べクリーンな状態であることから、ヒトに比べ抗原刺激が少ないと思われる。 そのため、ヒト末梢血のT細胞ほど分化が進まないことが考えられる。 HIV 感染に対する免疫応答には HIV 特異的 CTL の機能がより重要となるため、ヒトT細胞の正常な分化は必要事項である。 そのため、今後は抗原刺激に対するT細胞の分化・機能を解析すること、さらにHLA発現ヒト免疫構築マウスでは胸腺におけるT細胞の教育が正常に行われ、今までのモデルマウスより成熟したヒト免疫系の構築が可能であると考え、現在HLA-B51発現NOKマウス(NOK/B51Tg マウス)を樹立中である。

## E. 結論

本研究では、長期 HIV 感染における免疫応答と病態進行の機序を明らかにするため、マウスによる HIV 感染系の確立、および、HLA 発現ヒト免疫構築マウスを樹立し、HIV 感染マウスにおける HIV-1 特異的 CTL の動態を解析し、最終的に HIV-1 特異的 CTL クローンの移入による免疫細胞治療法の効果についての検討を目指す。 ヒト免疫構築マウスをもちいた研究は既に国内外で始まっているが、本研究で樹立する HLA 発現ヒト免疫構築マウスでは、ヒトの造血・免疫系を確立した際に問題となる胸腺での分化障害も解決でき、今までのモデルマウスより成熟したヒト免疫系の構築が可能であり、また、HLA ハプロタイプとエイズ進行の相関を動物モデルにおいて検討できる点から、今後のエイズ治療研究に大きく貢献することが期待できる。

## F. 健康危険情報

なし。

## G. 研究発表

小林直樹（前研究代表者）

1）論文発表

1. Kitano M, Kobayashi N, Kawashima Y, Akahoshi T, Nokihara K, Oka S, Takighuchi M. Identification and characterization of HLA-B*5401-restricted HIV-1-Nef and Pol-specific CTL epitopes. *Microbes. Infect.* 10:764-772, 2008.

2）学会発表

1. Kobayashi N, Takata H, Takiguchi M. The immature differentiation of human T cells in humanaized NOD/SCID/Jak3$^{-/-}$ mice. 9th Kumamoto AIDS Seminar, September 18-19, 2008. Aso Resort Grand Vrio Hotel, Kumamoto, Japan.

## H. 知的財産権の出願・登録状況（予定を含む。）

なし。

図2. NOKマウスにおけるヒトT細胞の再構築

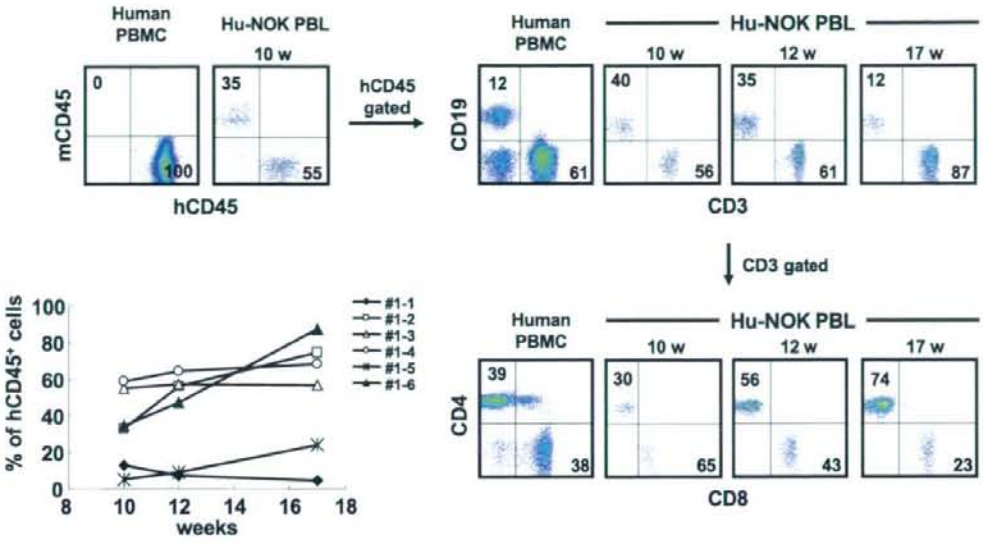



図3. Hu-NOKマウスにおけるヒトCD8T細胞分化の解析

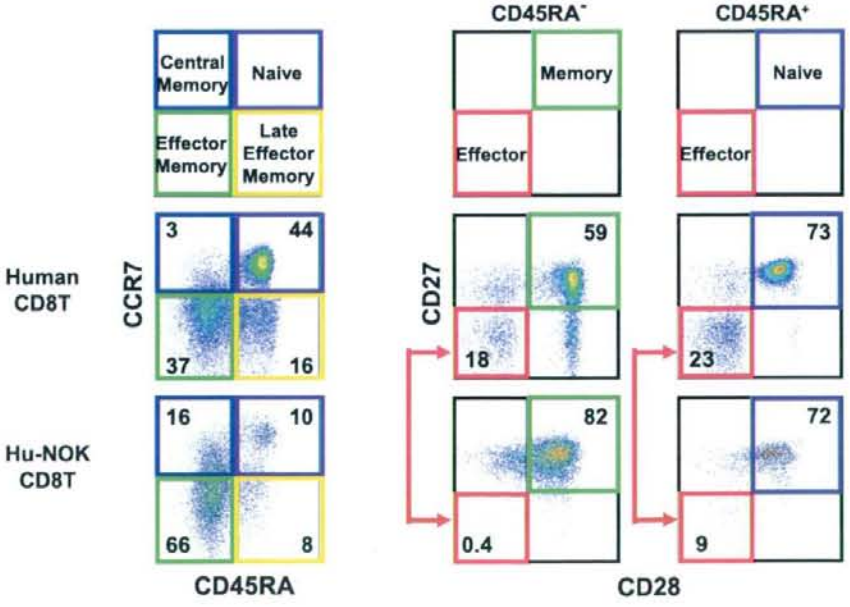


図4. Hu-NOKマウスにおけるヒトCD8T細胞の機能解析

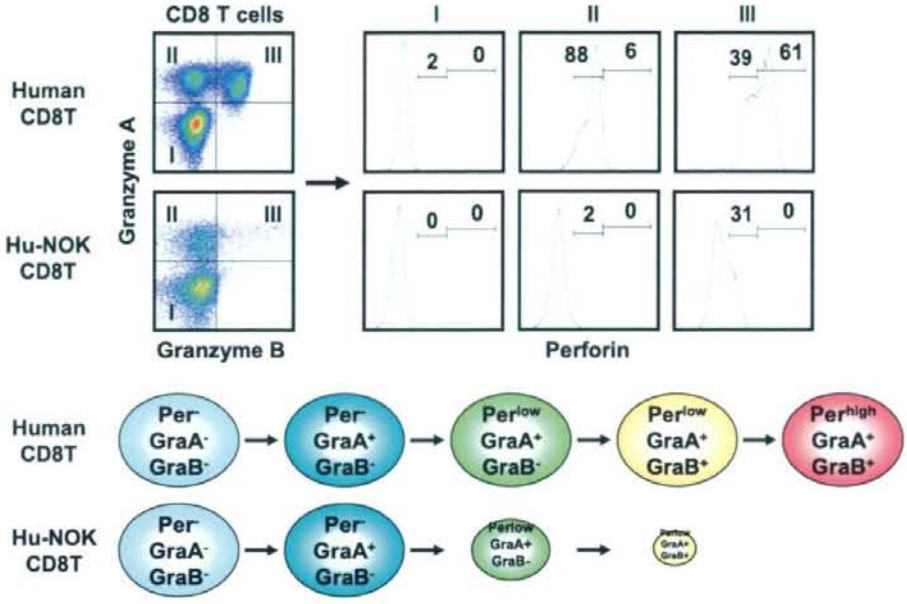



図5. Hu-NOKマウスにおけるヒトCD8T細胞のサイトカイン産生能の解析

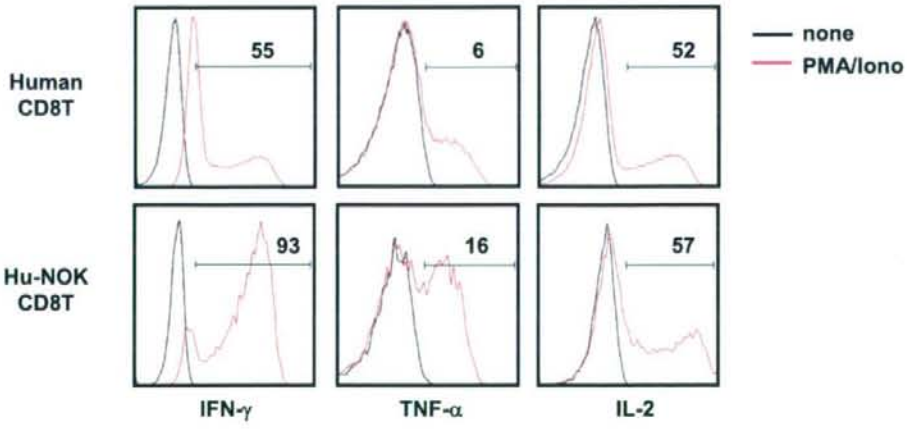
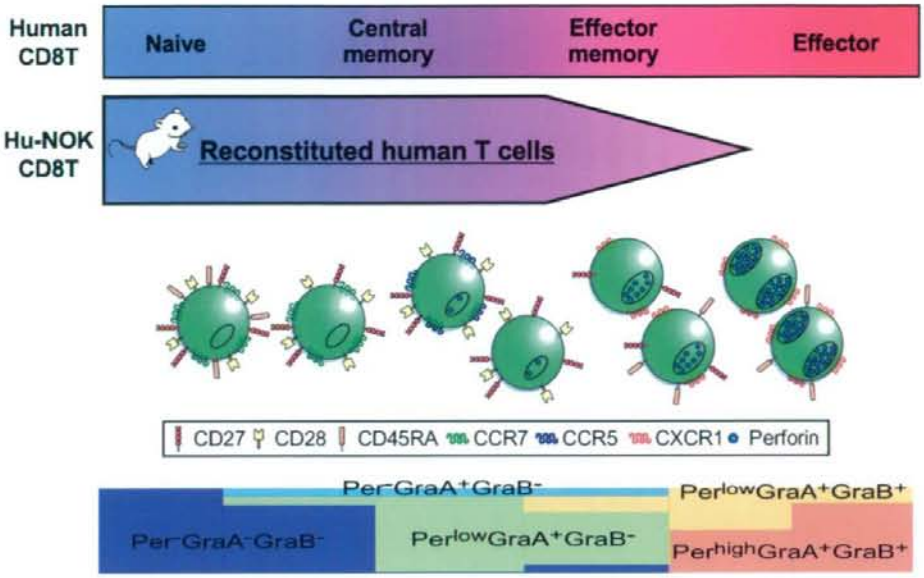


図6. Hu-NOK(ヒト化マウス)におけるヒトCD8T細胞の分化、機能解析のまとめ




Hu-NOKマウスのヒトCD8T細胞は、表現型および機能解析からエフェクターメモリー表現型まで分化していることが明らかとなった。

# II. 研究成果の刊行に関する一覧表

小林直樹（前主任研究者）

| 発表者氏名 | 論文タイトル名 | 発表誌名 | 巻号 | ページ | 出版年 |
|---|---|---|---|---|---|
| Kitano M, Kobayashi N, Kawashima Y, Akahoshi T, Nokihara K, Oka S, Takighuchi M. | Identification and characterization of HLA-B*5401-restricted HIV-1-Nef and Pol-specific CTL epitopes. | Microbes. Infect. | 10 | 764-772 | 2008 |

# III. 研究成果の刊行物・別刷

ELSEVIER

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Microbes and Infection 10 (2008) 764–772

www.elsevier.com/locate/micinf

Original article

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

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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.


*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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[1] Equally contributed to this study.

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

### 2.6. CTL assay

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

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

## 3. Results

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

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

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

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

### *3.3. Identification of optimal epitope peptides*

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

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

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

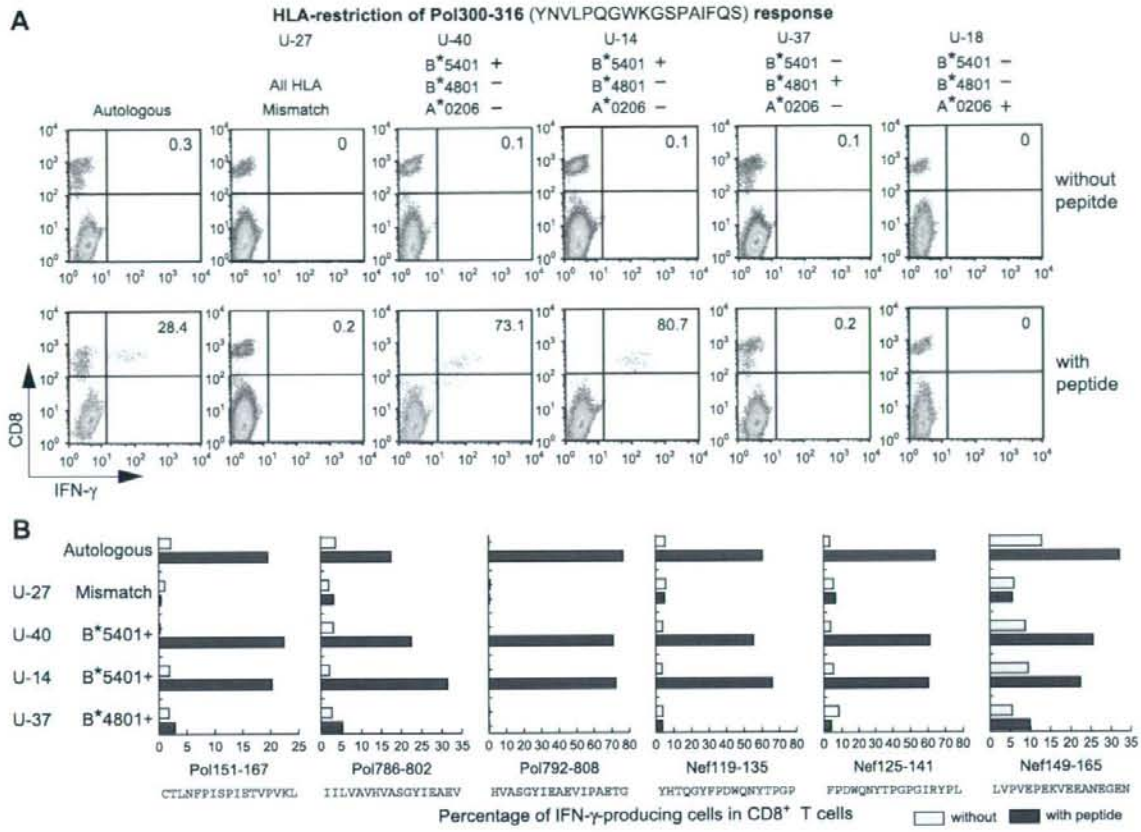


Fig. 1. Identification of HLA-B*5401-restricted HIV-1 CTL epitope candidates by using a panel of B-LCL pulsed with 17-mer peptides. A. PBMC from an HIV-1-seropositive individual KI-119 (A*0206/-, B*5401/B*4801) were stimulated with Pol300–316 peptide and then cultured for 2 weeks. The cultured cells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

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

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

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

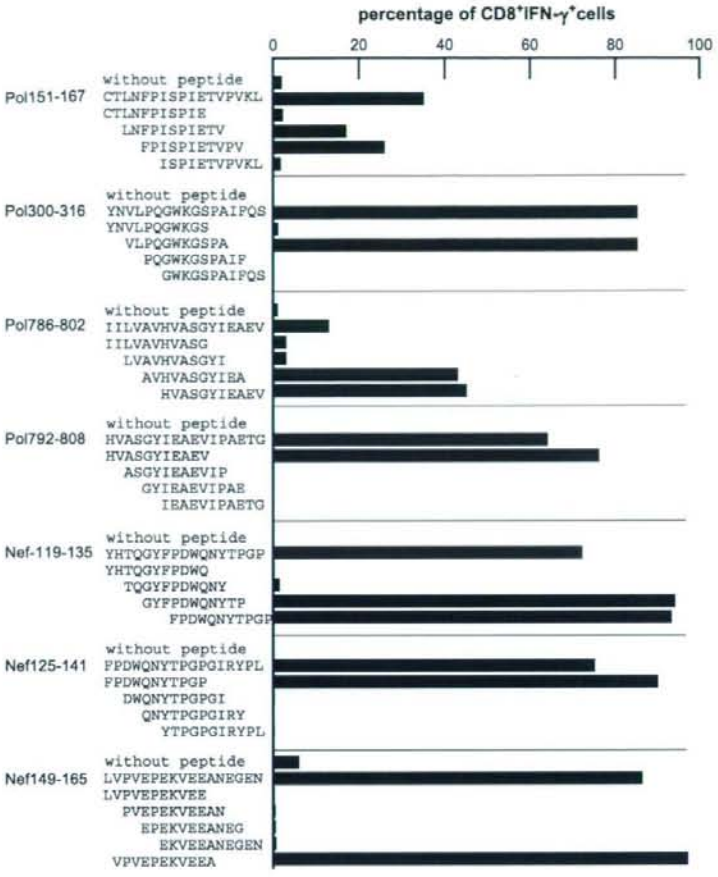


Fig. 2. Selection of 11-mer HIV-1 Pol and Nef peptides including HLA-B*5401-restricted epitopes. The 17-mer peptide-specific bulk $CD8^+$ T cells were stimulated with autologous B-LCL pre-pulsed with each overlapping 11-mer peptide included in the 17-mer peptides. The responsibility of the bulk $CD8^+$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

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

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

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

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

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

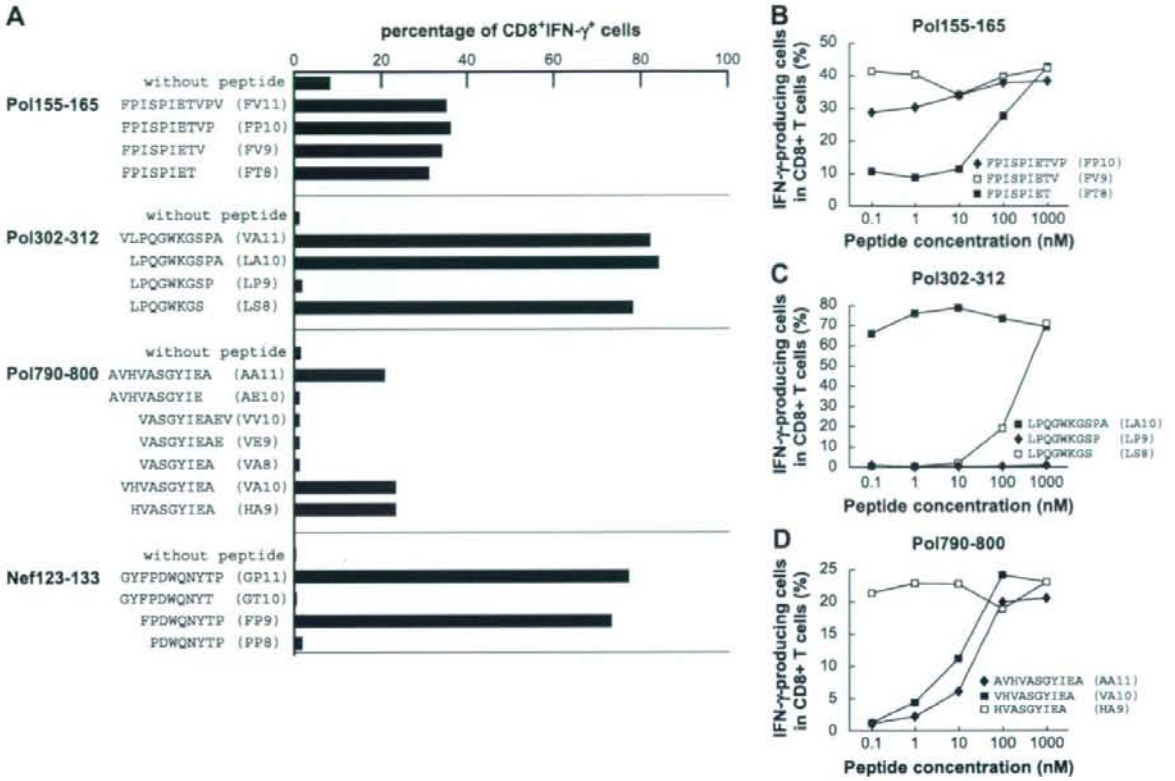


Fig. 3. Recognition of the 8- to 10-mer truncated peptides by HIV-1 Pol- or Nef-specific CD8+ T cells. A. The 17-mer peptide-specific bulk $CD8^+$ T cells were stimulated with autologous B-LCL pre-pulsed with each 8- to 10-mer truncated peptide. For determination of the optimal epitopes in Pol155–165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

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

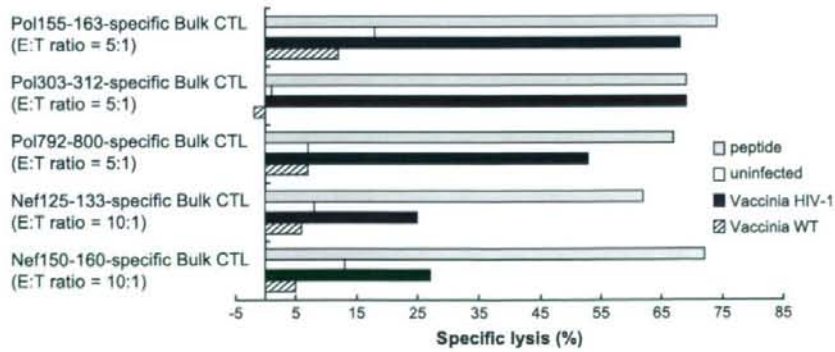


Fig. 4. Killing of r-HIV-1 vaccinia-infected or peptide-pulsed cells by the Pol- or Nef-specific CTLs. The activities of the five HIV-1-specific T cells toward $B*5401^+$B-LCL pre-pulsed with the corresponding peptide (1 μM), or those infected with recombinant vaccinia virus expressing the corresponding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