ニングの手順を繰り返し行った。

(2) gp120-capture ELISA による抗体産生の確認

クローニングにより増殖した抗体産生 B 細胞の抗 gp120 活性を有する抗体の産生の有無を“gp120-capture ELISA”をもちいて確認した。(2-a) gp120-captured plate の作成; 96well polypropyren plate (Falcon)に gp120-C5 sheep 抗体(D7324, Aalto Bioreagents, Dublin, Ireland) をコートし、blocking buffer でブロック後 1 μg/mlに希釈した単量体 gp120 (gp120-SF2;Austral Biologicals、San Ramon, California)を 50 μl ずつ加えた。2 時間室温に静置した後、ELISA wash buffer で 3 回洗浄し、gp120-captured plate とした。(2-b) 抗 gp120 抗体産生の確認;作製した gp120-captured plate に B 細胞培養上清を加えて室温で 2 時間反応させた後、ELISA wash buffer で 3 回洗浄し、抗ヒト IgG-ALP を加えて室温で 1 時間静置した。ELISA wash buffer で 3 回洗浄し、発色させ吸光度(405nm)を測定した。

(3)単クローン抗体の分類

gp120 に反応する抗体は反応エピトープによって次の 4 種類;CD4 結合部位(CD4binding site;CD4bs)、CD4 誘導エピトープ(CD4induced epitope;CD4i)、第3可変部位(V3loop;V3L)及びこれらに分類できないもの(other epitopes)に分類される。(3-a)抗 V3L 抗体の分類;V3L に結合する抗体かどうかは V3L のアミノ酸配列を持つ合成ペプチドを用いた ELISA(V3-peptide ELISA)で判定した。JR-FL 株の V3 配列(NNT20; NNTRISIHIGPGRAFVTIGK)をもつ合成ペプチド(NNT20)に反応し、コントロールとして用いた HIV-2 のペプチドに反応しないものを陽性と判断した。(3-b)抗 CD4bs、抗 CD4i 抗体の分類;抗 CD4bs 抗体と抗 CD4i 抗体は gp120-capture ELISA における可溶性 CD4{soluble CD4 (sCD4):R&D Systems, Inc. Minneapolis, MN}の影響が正反対である。CD4bs 抗体は sCD4 存在下に抗体の結合が抑制され、逆に CD4i 抗体の gp120 に対する反応性は sCD4 存在下に増強される。この性質を利用して、単クローン抗体の分類を行った。上述の gp120-capture ELISA の手順と同じ手順で gp120-captured plate を作成し、段階希釈した単クローン抗体を 0.5 μg/ml の sCD4 存在もしくは非存在下に 2 時間反応させ吸光度(405nm)を測定した。

(4) TZM-bl 細胞を用いた感染抑制の測定

TZM-bl 細胞を平底 96well プレートに 1 well 当り 2×10^4 となるように分注培養した。約 18 時間後に $200TCID_{50}$ の pseudo-typed virus と段階希釈をした抗体をインキュベーション後に TZM-bl 細胞に加え、37℃ 5%CO_2 下で培養した。48 時間後上清を除去し、Galact-starTM System を用い発色させ、TR717 Microplate luminometer (TROPIX™)にて測定した。中和活性は、{1−(t−c)/(n−c)}×100 (t; サンプルの発光強度、c; 細胞のみのバックグラウンド発光強度、n; 抗体無しサンプルの発光強度)で計算した。

(5) HIV-1 subtype B 臨床分離株の標準化パネル (HIV-1 subtype-B standard panel virus; SVPB) の調整;使用した SVPB エンベロープベクターは、NIH AIDS Research and Reference Reagent Program(ARRRP)より供給をうけた。12 名の HIV-1 subtype B 感染者の血漿 HIV-1 RNA および PBMC DNA から PCR で gp160 全長を増幅しクローニングされたもので、血漿 HIV-1 RNA 由来の産物は pcDNA3.1/His-TOPO (Invitrogen)に、PBMC DNA 由来の産物は pcDNA3.1/V5-His-TOPO (Invitrogen) に TA Cloning で組み込まれている。

(倫理面への配慮)

本研究を行うに当たり該当症例には研究の概要を説明し同意を得た上で採血し、解析した。なお本研究の倫理的・科学的妥当性は熊本大学病院の先進医療審査委員会で審査され、了承されている。

C. 研究結果

(1)単クローン抗体の作製と分類

広範なウイルス株に対して、中和活性を持つ一人の長期非進行症例より分離した末梢血 B 細胞にEBウイルスを感染させ、クローニングを繰り返し、抗gp120活性をもつ抗体産生細胞を分離した。分離された抗体産生細胞を培養し、gp120-capture ELISAにより、培養上清中に抗gp120活性を有する抗体産生細胞のスクリーニングを行い、20 種類のB細胞クローンを得た。クローンが産生する単クローン抗体について、V3 ペプチドに対する反応性を検討した。抗体の由来する症例のウイルスのV3配列に最も近いJR-FL株のV3配列(NNT20; NNTRISIHIGPGRAFVTIGK)を用いて、反応性を調べたところ、20 クローン中 6 クローン(0.5γ,5G2,2F8,3G8,3E4,1D9)で V3 ペプチド(NNT20)に対する結合活性が認められた。さらに、得られた単クローン抗体の sCD4 存在下、非存在下での gp120 に対する結合活性の変化を測定し、CD4bs 抗体及び CD4i 抗体の分類を行った。V3抗体はコントロールとして用いたKD-247を含めてsCD4 の影響を受けない。sCD4 存在下に結合活性が抑制されるクローン(CD4bs 抗体)が0.5・,42F9,49G2,4E3,7B5 の5種類あった。また、逆に sCD4 で反応が増強される抗体(CD4i 抗体)が、4C11,4E9B,5D6S 及び 7F11 の4種類あった(コントロールとして17bを用いた)。V3-peptide ELIAで反応が見られず、しかもsCD4の影響も見られないものが5種類あり、これらは epitope 特異性を確認できなかった(other epitopes)。

(2)単クローン抗体の実験室株に対する中和活性

精製した単クローン抗体を用いて、4 種類のHIV-1株のenvelopeを用いたpseudo-typed virusに対する中和活性を抗体濃度10μg/ml及び2μg/mlで検討した。中和感受性が高い株として知られているSF162株に関してはテストした単クローン抗体のすべてに中和活性を認めた。一方、比較的中和抵抗性である89.6及びJR-FLに関してはV3 抗体{0.5γ(1C10)と5G2}には中和活性を認めたものの CD4bs 抗体、CD4i 抗体に関しては、中和活性を認めなかった。一方、V3-tip のアミノ酸配列が他のウイルスと異なる IIIB に関しては、V3 抗体は有効ではなかったが、CD4bs 抗体とCD4i抗体の一部に中和活性を認めた。

(3)自己由来の envelope を持つ pseudo-typed virus に対する中和感受性

感染者自己ウイルスに対する中和活性の評価をするにあたり、自己ウイルスの分離を試みたが、本症例は全経過を通じてウイルス量が測定感度前後であり、ウイルスの分離は困難であった。gp160全長のPCRによる増幅も困難であったため、env gp120のV1-V5部位をPCRにて増幅し、JRFL env gp160を発現するpCXN2-JRFL envベクターをKpn1とMfe1で処理し、断片を組み換えた。こうして、感染者自己ウイルスのエンベロープ(gp120)を発現するpseudo-typed virusを作製した。中和活性を持つと考えられる11クローンの抗体を用いて自己由来のenv 持つ pseudo-typed virus に対する中和活性を測定した。その結果 2 つの抗 V3 抗体(0.5γ,5G2)のみが自己ウイルスに対して 50%以上感染抑制を示すことがわかった。

(4)Subtype-B 臨床分離株標準化パネル (Subtype-B standard panel virus; SVPB)に対する感染抑制効果

Ming Li らは、HIV に対するワクチン候補が誘導する抗体の、臨床分離株に対する中和活性の評価を標準化するため、Subtype-B standard panel virus(SVPB)というpseudo-typed virusの標準化パネルを作成した(Li M. et.al,J.Virol.79,p10108-10125,2005)。LTNPより、今回樹立した単クローン抗体の、SVPB 標準化パネルに対する交差中和活性を、TZM-blを標的細胞とした中和アッセイにて検討した。コントロールとして、gp120のV3−tip部位の-IGPGRA-を認識するヒト型単クローン抗体・KD-247 を用いた。KD-247では、SVPB5,17,18において1.8μg/ml、17μg/ml、28μg/mlというIC_{50}レベルで中和活性が見られた。また、SVPB6,16に関しても50μg/ml

の濃度で、30～50%の有意な感染の抑制を観察した。これらをまとめると、KD-247 では 5/12 の SVPB に対して交差中和活性が検出可能であった。

HIV-1 gp120 V3-peptide に反応する抗 V3 単クローン抗体の中で実験室株に対して中和活性をもっていた 4 種類の抗体に関して SVPB に対する中和活性を解析した。これらのうち、2F8 と 3E4 には 50 μg/ml までの濃度で、パネルウイルスを 50%感染抑制できるものを認めなかったが、5G2 と 0.5γ では強力な交差中和活性を認めた。とくに、0.5γ は、SVPB5,6,11,14,16,17,18 に対し 0.23 ～38 μg/ml の IC_{50} レベルで中和活性が見られた。SVPB12,13,19 に関しても、80～110 μg/ml の IC_{50} レベルで中和活性を示した。このように、12 種類の SVPB のうち、計 10 に対して IC_{50} をこえる中和活性が得られた。また、中和抵抗性の SVPB8 に対しても 0.5γ は、150 μg/ml の濃度で有意な中和活性を示した。すなわち、V3-tip に、比較的まれな-GPGG-という配列をもつ SVPB15 以外は、必要な濃度に差があるものの、0.5γ は中和活性を持つことがわかった。

抗 CD4bs 抗体である、0.5δ (3D6)、42F9、49G2 についても SVPB に対する中和活性を検討した。IC_{50} が 50 μg/ml までの濃度で検出できたものは、0.5δ は 1/12、42F9 が 2/12、49G2 が 3/12 であった。50 μg/ml で抑制率 30%以上の有意な中和活性を認めたものは、0.5δ で 2/12、42F9 と 49G2 で 6/12 であった。一方、抗 CD4i 抗体の交差中和活性は見られなかった。注目すべきは、0.5γ が全く中和活性を示さない SVPB15 に対して、49G2 と 42F9 が交差中和を示すことである。このように、CD4bs 抗体と V3 抗体は相補的に中和活性を担っていると考えられる。

(5)V3 抗体の 0.5γ と CD4bs 抗体の 0.5δ の JR-FL 株に対する相乗的増殖抑制効果

gp120capture ELISA を用いて検討すると 0.5γ の反応性は 0.5δ の存在下に著名に増強された。また、各濃度の 0.5δ と 0.5γ の組み合わせを用いて JR-FL 株に対する中和活性を MTT assay を用いて検討したところ、中和活性の相乗的増強が観察された。相乗効果の解析は Chow/Talaley らの分析法と 3-Dimensional 分析法を用いた。

D. 考察

HIV-1 の感染を阻止するワクチンの開発戦略の一つとして、広範な HIV-1 株に対して中和活性を示す抗体がどのようなものか同定することは、重要なことである。今回われわれは、一人の LTNP から多数の単クローン抗体を樹立した。その中でも、抗 V3 抗体は実験室分離株で比較的中和抵抗性である 89.6 及び JR-FL、さらに感染者自己由来ウイルスに関して、中和活性を示した。さらに、ワクチン開発の標準化のために作成された subtype B の国際標準化パネル(SVPB)に対する交差中和活性の検討から、広範囲の分離株に対して強い中和活性をもつ 0.5γ のような抗 V3 抗体が存在することが、明らかとなった。しかし、これら抗 V3 抗体は V3 に変異が入った実験室分離株である IIIB や、V3-tip が GPGG である SVPB15 に対して中和活性を示さなかった。一方、CD4bs 抗体は、中和能は強くないものの、実験室分離株および一部の SVPB に対して中和活性を示した。特に、0.5γ が中和しない IIIB や SVPB15 に対する中和活性が見られた。すなわち、同一 subtype 間においては、抗 CD4bs 抗体及び抗 V3 抗体が相補的に働き、広範な臨床分離株に対して交差中和を示すことが示唆された。また、V3 抗体と CD4bs 抗体は、相乗的に JR-FL 株を中和することを観察した。これらの in vitro の観察は、in vivo では V3 抗体と CD4bs 抗体が相互作用して、さらに強力な中和反応を起こしているのではないかと、推測させる。

E. 結論

一般の HIV-1 慢性感染症例では、交差中和活性は、ほとんど認められない。一方、長期非進行症例(LTNP)の中には、本症例のように、自己由

来のウイルスを含む広範囲の臨床分離株に対して中和抗体活性を有する症例が存在する。このことが病気の進行阻止と関連するかどうかは明らかではないが、十分強い中和抗体があれば、in vivo でもウイルスの増殖抑制効果が期待できると考えられる。今回の、我々の研究で、このような LTNP では複数の抗体が相補的、相乗的に働き、ウイルスの増殖を抑えている可能性が示唆された。

F. 健康危険情報:なし

G. 研究発表

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H. 知的所有権の出願・取得状況（予定含む）

特許出願;1 件

発明の名称 「抗 HIV モノクローナル抗体」、整理番号;A71717A, 平成 19 年 11 月 19 日

Ⅲ. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

主任研究者　滝口雅文

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Different immunodominance of HIV-1-specific CTL epitopes among three subtypes of HLA-A*26 associated with slow progression to AIDS

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Abstract

It is speculated that HLA-A*26-restricted HIV-1-specific CTLs can control HIV-1, since HLA-A*26 is associated with a slow progression to AIDS. In three major HLA-A*26 subtypes, HLA-A*2601-restricted, and HLA-A*2603-restricted HIV-1 epitopes have been identified, but HLA-A*2602-restricted ones have not. We here identified HLA-A*2602-restricted HIV-1 epitopes by using reverse immunogenetics and compared the immunodominance of the epitopes among the three subtypes. Out of 110 HIV-1 peptides carrying HLA-A*26 anchor residues, only the Gag169-177 peptide, which had been previously identified as an HLA-A*2601- and HLA-A*2603-restricted immunodominant epitope, induced Gag169-177-specific $CD8^+$ T cells from only two of six HLA-A*2602^+ HIV-1-infected individuals. No difference in affinity of this epitope peptide was found among these three HLA-A*26 subtypes, indicating that Gag169-177 was effectively presented by HLA-A*2602 but recognized as a subdominant epitope in HIV-1-infected HLA-A*2602^+ individuals. These findings indicate different immunodominance of Gag169-177 epitope among 3 HLA-A*26 subtypes.

Keywords: HLA-A*26; HLA-A*2602; HIV-1; Epitope; CTL

Human immunodeficiency virus type-1 (HIV-1)-specific cytotoxic T lymphocyte (CTL) responses play an important role in the control of HIV-1 infections [1–5]. However, it is thought that HIV-1 can escape from the host immune system, since it fails to completely eradiate HIV-1 from infected individuals. There are several proposed mechanisms that would allow HIV-1-infected cells to escape from being killed by HIV-1-specific $CD8^+$ T cells [6–11]. A mutation within the viral epitopes recognized by CTLs is one of these mechanisms [6,7]. Identification and characterization of HIV-1 CTL epitopes are therefore necessary for studies on the immunopathogenesis of AIDS. In addition, since HIV-1-specific CTLs are expected to suppress HIV-1 replication *in vivo*, characterization of these epitopes is also necessary for studies aimed at developing HIV-1 vaccines and immunotherapy to induce HIV-1-specific CTLs, either of which might be expected to prevent HIV-1 infection and the progression to AIDS.

HLA-A*26 is one of the alleles associated with a slow progression to AIDS [12]. Therefore, identification and characterization of HIV-1-specific epitopes presented by this allele are necessary for studies on the immunopathogenesis of AIDS and vaccine development. Three HLA-A*26 subtypes, i.e., HLA-A*2601, HLA-A*2602, and HLA-A*2603, are found at a gene frequency of 7.7%, 2.3%, and 1.5%, respectively, in the Japanese population [13]. We previously identified four HLA-A*2601- and two HLA-A*2603-restricted HIV-1 epitopes by using reverse immunogenetics [14,15]. Both HLA-A*2601 and -A*2603 presented one immunodominant epitope, Gag169-177

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(EVIPMFSAL), which overlaps with the HLA-B*57-restricted immunodominant epitope KF11 (KAFSPEVIPMF) [16]. Gag169-177-specific CTLs have been speculated to control HIV-1 replication.

In the present study, we sought to identify HLA-A*2602-restricted HIV-1 epitopes by using reverse immunogenetics and to compare them with HLA-A*2601- and HLA-A*2603-restricted ones [14,15].

Materials and methods

Cells. C1R and TAP-defective cells of mouse cell line RMA-S were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). C1R cells expressing HLA-A*2601, -A*2602 or -A*2603 (C1R-A*2601, C1R-A*2602 or C1R-A*2603, respectively) were generated by transfecting the C1R cells with the HLA-A*2601, -A*2602 or -A*2603 gene, respectively [17]. RMA-S transfectants expressing HLA-A*2602 (RMA-S-A*2602) were previously generated [18]. C1R-A*2601, -A*2602 and -A*2603 were maintained in RPMI 1640 medium supplemented with 10% FCS and 0.2 mg/ml neomycin; and RMA-S-A* 2602, in RPMI 1640 medium supplemented with 10% FCS and 0.15 mg/ml hygromycin B.

Synthetic peptides. Sequences derived from four proteins of the human immunodeficiency virus type-1 SF2 strain (HIV-1: Env, Gag, Pol, and Nef) were screened for HLA-A*2602-binding motifs. Peptides were prepared by utilizing an automated multiple peptide synthesizer, with the Fmoc strategy followed by cleavage. The purity of the synthesized peptides was examined by mass spectrometry. Peptides with more than 90% of purity were used in the present study.

HLA-stabilization assay. Binding of HIV-1-derived peptides to HLA-A*2602 was measured as previously described by using RMA-S-A*2602 cells [18]. RMA-S-A*2602 cells were cultured for about 16 h. Then they were incubated with peptides at 26 °C for 1 h and subsequently at 37 °C for 3 h. Peptide-pulsed cells were stained with the HLA class I α_3 domain-specific mAb TP25.99 [19] and the FITC-conjugated IgG fraction of sheep anti-mouse Ig (Silenius Laboratories, Hawthorn Victoria, Australia). The mean fluorescence intensity (MFI) was measured by using a FACS Calibur (BD Bioscience, San Jose, CA, USA). HLA-A*2602-binding peptides were defined as those which at a concentration of 10^{-3} M caused a >25% increase in MFI compared with the MFI of control RMA-S-A*2602 cells cultured at 26 °C. The peptide concentration that yielded the half-maximal levels of the MFI was calculated and was reported as the BL50 value.

Patients. Blood samples were collected with informed consent from six HIV-1 clade B-infected patients with HLA-A*2602 (KO-003, KI-021, KI-030, KI-082, KI-382, and KI-478), 11 those with HLA-A*2601, and eight those with HLA-A*2603 at the AIDS Medical Center, National Hospital Organization, Osaka National Hospital or the AIDS Clinical Center, International Medical Center of Japan. Clinical stage of all patients tested was chronic one. Significant difference of CD4 count was not found among three subtype groups (HLA-A*2601:458±257, HLA-A*2602:564±299, HLA-A*2603:314±109). This study was approved by the ethical committees of Kumamoto University, International Medical Center of Japan, and Osaka National Hospital. Informed consent was obtained from all subjects, according to the Declaration of Helsinki.

Intracellular cytokine staining (ICC assay). After C1R-A*2601, C1R-A*2602 or C1R-A*2603 cells had been incubated for 60 min with each peptide (1 μM) or each peptide cocktail (1 μM concentration of each peptide), they were washed twice with RPMI-1640 containing 10% FCS. These C1R-A*26 cells and cultured PBMCs were incubated at 37 °C for 6 h at an effector-to-stimulator ratio of 1:4 after the addition of Brefeldin A (10 μg/ml). Next, the cells were stained with anti-CD8 mAb (DAKO Corporation, Flostrup, Denmark), fixed with 4% paraformaldehyde at 4 °C for 20 min, and then permeabilized at 4 °C for 10 min with PBS supplemented with 0.1% saponin containing 20% NCS (permeabilizing buffer). The cells were resuspended in the permeabilizing buffer and then stained with anti-IFN-γ mAb (BD Bioscience Pharmingen, San Diego, CA). The cells were finally resuspended in PBS containing 2% paraformaldehyde, and then the percentage of $CD8^+$ cells positive for intracellular IFN-γ was determined by using the FACSCalibur.

*ICC assay using C1R-A*2602 cells infected with recombinant HIV-1 vaccinia.* C1R-A*2602 cells were infected for 1 h at 37 °C with 10 plaque-forming units (per target cell) of recombinant vaccinia virus expressing HIV-1 SF2 Gag protein or of WT vaccinia virus and cultured for 16 h. These infected cells were washed twice with RPMI 1640 containing 10% FCS and then incubated with cultured effector cells at 37 °C for 6 h after the addition of Brefeldin A (10 μg/ml). The ability of the effector cells to produce IFN-γ was tested at an E:S ratio of 1:4. The cells were then stained with anti-CD8 mAb and anti-IFN-γ mAb.

Results and discussion

HLA-A*2602-binding peptides have two anchor residues, Val, Phe, Ile, Leu or Thr at position 2 and Tyr, Phe, Met or Leu at the C-terminus [17]. A previous study demonstrated that acidic amino acids (Asp and Glu) and a broad range of amino acids with the exception of positively charge amino acids function as an anchor at position 1 and the C-terminus, respectively [18]. Therefore, to identify HLA-A*2602-binding HIV-1 peptides, 8-mer to 11-mer sequences containing the anchor residues Asp or Glu at position 1, Val, Thr, Ile, Leu or Phe at position 2, and any amino acids except positively charged ones at the C-terminus were selected from the sequence of Gag, Pol, Nef, and Env proteins in the HIV-1 SF2 strain; and then 110 peptides matching these sequences were synthesized. The binding affinity of these synthesized peptides for the HLA-A*2602 molecule was tested by using the HLA-stabilization assay [18,20]. Representative results are shown in Fig. 1. Thirty-two out of these 110 peptides bound to HLA-A*2602 (Table 1). The frequency of HLA-A*2602-binding HIV-1 peptides was similar to that of HLA-A*2601- or HLA-A*2603-binding HIV-1 peptides previously identified by using the same 110 peptides [14,15].

PBMCs from three HLA-A^*2602^+ HIV-1-infected individuals (KO-003, KI-030 and KI-082) were stimulated

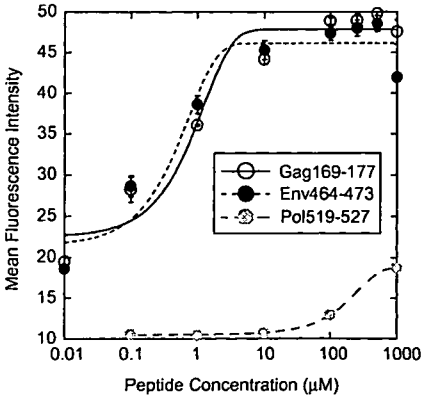

Fig. 1. Binding to HLA-A*2602 of HIV-1 peptides carrying HLA-A*2602 motif. Binding of the peptides carrying A*2602 anchors was measured by a stabilization assay using RMA-S-A*2602 cells. Representative results showing high-affinity peptides (Gag169-177 and Env464-473) and a very low-affinity peptide (Pol519-527) are given in this figure.

Table 1
HLA-A*2602-binding peptides

Sequence	Position	BL50[a]
EVFRPGGGDM	Env464-473	3.98×10^{-7}
EVIPMFSAL	Gag169-177	5.84×10^{-7}
ELKKIIGQV	Pol872-880	9.88×10^{-6}
EVNIVTDSQY	Pol647-656	1.21×10^{-5}
EVVIRSDNF	Env272-280	4.1×10^{-5}
ELNKRTQDF	Pol234-242	1.02×10^{-4}
EIKGEIKNCSF	Env147-157	1.62×10^{-4}
DIVIYQYMDDL	Pol332-342	1.94×10^{-4}
DTTNQKTEL	Pol626-634	2.78×10^{-4}
EIVASCDKCQL	Pol750-760	3.50×10^{-4}
ETVPVKLKPGM	Pol161-171	4.26×10^{-4}
ETWEAWWMEYW	Pol551-561	6.28×10^{-4}
ETWEAWWMEY	Pol551-560	$>1 \times 10^{-3}$
EICGHKAIGTV	Pol121-131	$>1 \times 10^{-3}$
EVVLGNVTENF	Env82-92	$>1 \times 10^{-3}$
DLRSLCLFSY	Env758-767	$>1 \times 10^{-3}$
DLWIYHTQGYF	Nef115-125	$>1 \times 10^{-3}$
EVIPLTEEA	Pol446-454	$>1 \times 10^{-3}$
ETPGIRYQY	Pol293-301	$>1 \times 10^{-3}$
EVYYDPSKDLV	Pol471-481	$>1 \times 10^{-3}$
EVYYDPSKDL	Pol471-480	$>1 \times 10^{-3}$
ETKLGKAGYV	Pol604-613	$>1 \times 10^{-3}$
EVHNVWATHAC	Env63-73	$>1 \times 10^{-3}$
EVQLGIPHPA	Pol244-253	$>1 \times 10^{-3}$
ELYPLTSLRS	Gag484-493	$>1 \times 10^{-3}$
DLNTMLNTV	Gag185-193	$>1 \times 10^{-3}$
DVKQLTEAV	Pol519-527	$>1 \times 10^{-3}$
ELYPLTSLRSL	Gag484-494	$>1 \times 10^{-3}$
DIQKLVGKL	Pol411-419	$>1 \times 10^{-3}$
DIAGTTSTL	Gag235-245	$>1 \times 10^{-3}$
ELRQHLLRW	Pol359-367	$>1 \times 10^{-3}$
DTKEALEKI	Gag96-104	$>1 \times 10^{-3}$

[a] The half maximal binding level was calculated as the peptide concentration yielding the half-maximal MFI.

in vitro for 14 days with a cocktail of HLA-A*2602-binding peptides containing 5–7 peptides (cocktail 1: Env464-473, Gag169-177, Pol647-656, Pol872-880, and Pol551-560; cocktail 2: Env272-280, Pol121-131, Env82-92, Pol551-561, Env758-767, Pol234-242, and Nef115-125; cocktail 3: Pol446-454, Pol293-301, Pol471-481, Pol471-480, Pol604-613, Env147-157, and Env63-73; cocktail 4: Pol244-253, Gag484-493, Gag185-193, Pol626-634, Pol519-527, Gag484-494, and Pol411-419; and cocktail 5: Gag235-243, Pol359-367, Pol161-171, Gag96-104, Pol750-760, and Pol332-342). IFN-γ production by each bulk culture in response to C1R-A*2602 prepulsed with the corresponding peptide cocktail was assessed by intracellular IFN-γ staining. All five cocktails failed to induce specific $CD8^+$ T cells among the cells in bulk culture obtained from patients KI-030 and KI-082. On the other hand, only cocktail 1 induced specific $CD8^+$ T cells among the cells in bulk culture obtained from patient KO-003 (Fig. 2A). To determine which peptides in the cocktail induced the specific $CD8^+$ T cells, we re-stimulated the cells of this bulk culture with C1R-A*2602 cells prepulsed with each single peptide in this cocktail to detect the specific $CD8^+$ T cells. Only the Gag196-177 peptide induced $CD8^+$ T cells producing IFN-γ (Fig. 2B).

To clarify whether Gag169-177 was a naturally occurring peptide, we investigated the ability of these peptide-specific $CD8^+$ T cells to produce IFN-γ after having stimulated them with C1R-A*2602 cells infected with recombinant HIV-1 vaccinia virus (r-HIV vaccinia). IFN-γ-producing cells were induced in the Gag169-177-specific $CD8^+$ T cell culture after stimulation with r-HIV vaccinia-infected C1R-A*2602 cells, whereas they were not detected in that stimulated with WT vaccinia-infected C1R-A*2602 cells or r-HIV vaccinia-infected C1R cells (Fig. 2C). These results indicate that Gag169-177 is indeed a naturally occurring HIV-1 epitope peptide presented by HLA-A*2602.

Gag169-177-specific $CD8^+$ T cells were induced from only 1 of the 3 HLA-A*2602^+ HIV-1-infected individuals; whereas two HLA-A*2601 epitopes, Pol647-656 and Env464-473, which were also HLA-A*2602-binding peptides, failed to induce specific T cells in these individuals. To address lower frequency of these peptide-specific $CD8^+$ T cells, we investigated the induction of the specific $CD8^+$ T cells by stimulating PBMC from three additional donors carrying HLA-A*2602 with these three peptides. Only Gag169-177 peptide induced the specific $CD8^+$ T cells in one donor. Thus, Gag169-177-specific $CD8^+$ T cells were induced in 2 of 6 HLA-A*2602^+ HIV-1-infected individuals whereas they were induced in 8 of 11 HLA-A*2601^+ and 7 of 8 HLA-A*2603^+ HIV-1-infected ones (Fig. 2D). These results indicate that Gag169-177 is a subdominant epitope in the HLA-A*2602^+ donors.

Gag169-177-specific $CD8^+$ T cells were previously detected in five of seven HLA-A*2601^+ donors and in all four HLA-A*2603^+ ones [14,15], suggesting that Gag169-177 is a dominant epitope in HIV-1-infected individuals carrying either of these HLA-A*26 alleles. Additional experiments in the present study confirmed the immunodominance of this epitope in HLA-A*2601^+ and HLA-A*2603^+ donors (Fig. 2D). In contrast, they were detected in only two of six HLA-A*2602^+ donors, indicating Gag169-177 to be a subdominant epitope in HLA-A*2602^+ individuals. Interestingly, they were elicited in only a long-term non-progressor and a controller having low viral load. HLA-A*1101-restricted Gag349-, Nef73-, and/or Nef84-specific $CD8^+$ T cells were induces in two of four HLA-A*1101^+/A*2602^+ donors who did not have Gag169-177-specific $CD8^+$ T cells (data not shown), supporting that these HLA-A*2602^+ donors maintain HIV-1-specific cellular immunity. The affinity of Gag169-177 for HLA-A*2602 was similar to that for HLA-A*2601 and -A*2603 (Table 2), indicating that Gag169-177 was effectively presented by HLA-A*2602 but recognized as a subdominant epitope in HIV-1-infected individuals carrying HLA-A*2602. Pol604-612 and Env63-72 are HLA-A*2601 and HLA-A*2603 epitopes, respectively [14,15]. These epitope peptides failed to bind to HLA-A*2602 (data not shown). Since only one amino acid, at residue 116, differs between HLA-A*2602 and the other two subtypes (Asp

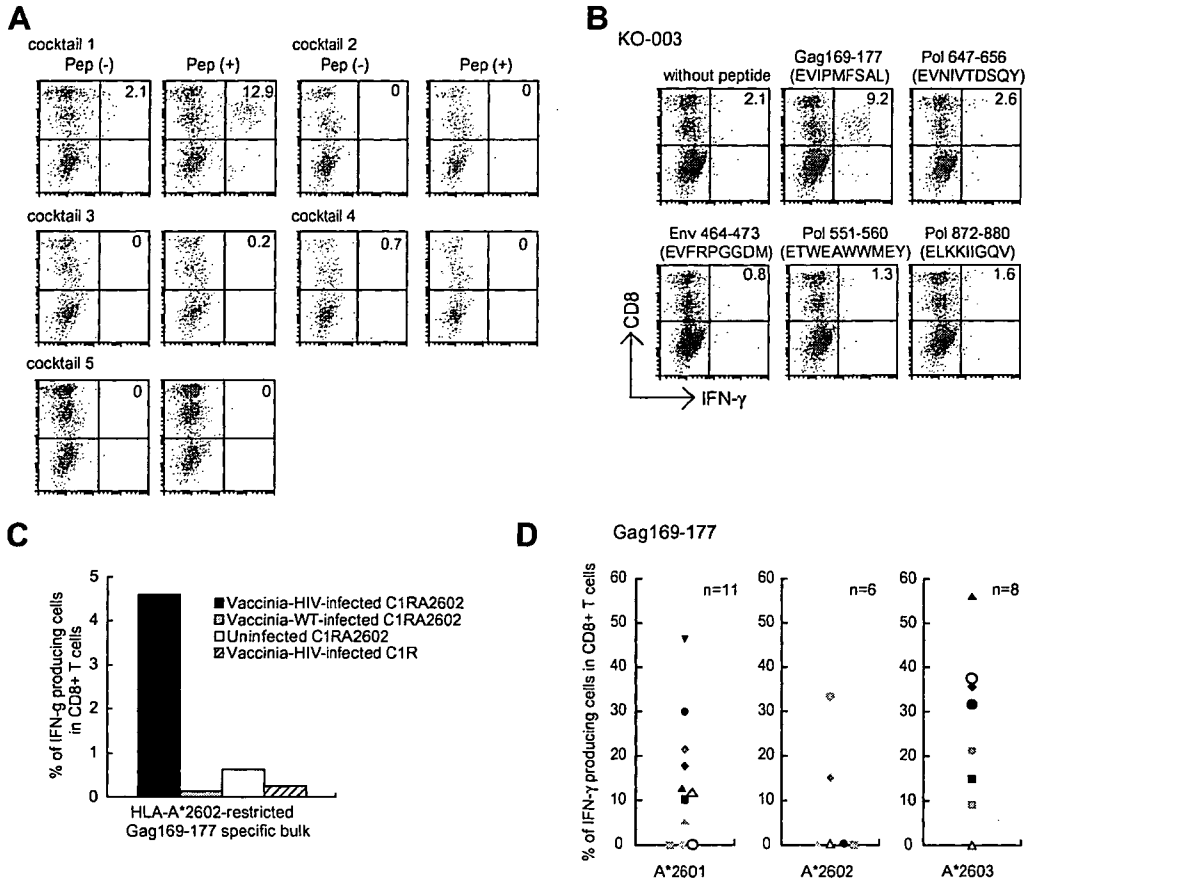

Fig. 2. Identification and recognition of Gag169-177-specific $CD8^+$ T cells. (A) PBMCs from HIV-1-infected individuals with HLA-A*2602 (KO-003) were cultured for 10–14 days after they had been stimulated with the indicated cocktails of HLA-A*2602-binding peptides. The cultured cells were then tested for IFN-γ production by $CD8^+$ T cells after stimulation with C1R-A*2602 cells prepulsed with the peptide cocktails. (B) Induction of Gag169-177-specific $CD8^+$ T cells. PBMCs from KO-003 were stimulated with peptide cocktail 1 and cultured for 10–14 days. The cultured cells were stimulated with C1R-A*2602 cells prepulsed with each single peptide included in cocktail 1. The population of IFN-γ-producing $CD8^+$ T cells was determined by using flow cytometry. The percentage of IFN-γ-producing $CD8^+$ T cells is presented at the right of the upper right-hand quadrant. (C) Presentation of Gag169-177 by HLA-A*2602 on r-HIV-1 vaccinia-infected cells. Bulk cultures containing Gag169-177-specific $CD8^+$ T cells were examined for IFN-γ production after they had been stimulated with C1R-A*2602 cells infected with wild-type vaccinia (Vaccinia-WT) or with C1R-A*2602 cells or with C1R cells infected with r-HIV-1Gag-vaccinia (Vaccinia-HIV-1), or uninfected C1R-A*2602 cells (Uninfected). The percentage of IFN-γ-producing $CD8^+$ T cells was measured by using flow cytometry. (D) Percentage of Gag169-177-specific $CD8^+$ T cells in HIV-1-infected individuals having three HLA-A*26 subtypes. The percentage of IFN-γ-producing cells among $CD8^+$ T cells from each individual was plotted in the graph. The percentage of IFN-γ-producing cells in the cultures was measured by using flow cytometry after they had been stimulated with the corresponding C1R-A*26 cells prepulsed with Gag169-177 peptide.

Table 2
Comparison of binding affinity of HLA-A*26 epitope peptides among three HLA-A*26 subtypes and induction of the peptide-specific $CD8^+$ T cells

	Binding affinity (BL50)			Comparison of binding affinity		Frequency[a]		
	A*2601	A*2602	A*2603	A*2602/A*2601	A*2602/A*2603	A*2601	A*2602	A*2603
Gag169-177	7.5×10^{-7}	5.8×10^{-7}	2.1×10^{-6}	0.77	0.28	8/11	2/6	7/8
Env63-72	1.1×10^{-4}	No binding	7.6×10^{-7}	—	—	0/11	NT	3/8
Pol604-612	6.5×10^{-5}	No binding	No binding	—	—	10/11	NT	NT
Pol647-656	6.3×10^{-5}	1.2×10^{-5}	6.6×10^{-4}	0.19	0.02	1/11	0/6	0/8
Env464-473	1.5×10^{-6}	4.0×10^{-7}	3.7×10^{-5}	0.27	0.01	1/11	0/6	0/8

NT, not tested.
[a] The number of individuals in whom peptide specific $CD8^+$ T cells were induced/the number of tested individuals.

for HLA-A*2601 and -A*2603, but Asn for HLA-A*2602), this substitution in the floor of the peptide binding groove is thought to affect the binding of these peptides.

In summary, Gag169-177 was not an HIV-1 immunodominant epitope in HIV-1-infected individuals carrying HLA-A*2602, whereas it was one in those carrying HLA-

A*2601 or -A*2603. These findings imply the possibility that HLA-A*2602 is not an allele associated with a slow progression to AIDS. However, it still remains unknown that Gag169-177-specific CTLs can control HIV-1. A further study using a cohort of a large number of subjects will clarify the association of these HLA-A*26 subtypes or Gag169-177-specific CTLs with the progression of AIDS.

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CTL-Mediated Selective Pressure Influences Dynamic Evolution and Pathogenic Functions of HIV-1 Nef[1]

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HIV-1 Nef plays multiple roles in modulating immune responses, even though it is a dominant CTL target itself. How Nef accomplishes the balance between such conflicting selective pressures remains elusive. By genetic and functional studies, we found that Arg^{75}Thr and Tyr^{85}Phe mutations, located in a well-conserved proline-rich region in Nef, were differently associated with escape from CTL responses specific for two overlapping HLA-B35-restricted epitopes. CTLs specific for an epitope, that selected Tyr^{85}Phe, were elicited earlier and had more potent functional avidities than did those that selected Arg^{75}Thr. Although the double mutant could escape from both CTLs, the mutations are rarely observed in combination naturally. Introduction of both mutations reduced Nef's HLA class I down-regulation activity and increased the susceptibility of virus-infected cells to recognition by CTLs targeting other epitopes. Moreover, the mutant Nef was impaired in the association with activated cellular kinases and in the enhancement of viral replication. These results highlight CTL immunosurveillance as important modulators of Nef's biological activity in the infected host. *The Journal of Immunology,* **2008, 180: 1107–1116.**

The accessory gene product Nef is a critical determinant for the pathogenesis of the primate lentiviruses, HIV-1, HIV-2, and SIV. The importance of Nef in viral pathogenesis was first shown in rhesus macaques, where a large deletion of the *nef* gene severely reduced SIV pathogenicity (1). This finding was supported by the fact that a cohort consisting of one blood donor and eight transfusion recipients infected with Nef-defective HIV-1 demonstrated dramatically decreased rates of disease progression (2, 3). The impact on the outcome of HIV/SIV infection likely results from the synergy of multiple functions exerted by Nef that may be differentially regulated over time (4). Nef enhances viral replication and virion infectivity (5–7) and affects cells in many ways, including altering T cell activation and maturation (6, 8–11), subverting the apoptotic machinery, and down-regulating a number of cell surface receptors including CD4 and HLA class I (7, 12, 13). The down-regulation of MHC class I (MHC-I)[3] by SIV Nef in rhesus macaques limits CD8 T cell-mediated killing and contributes to the pathogenic effect of Nef in vivo, highlighting the importance of Nef-mediated immunoevasion to facilitate disease progression (14).

The initial peak of viral replication after primary HIV infection begins to decline simultaneously with the appearance of HIV-specific CD8 T lymphocytes (15, 16) that can eliminate HIV-infected cells directly by MHC-I-restricted cytolysis or indirectly through the production of soluble factors such as cytokines and chemokines (17, 18). The biological relevance of HIV-specific CTLs in HIV infection is also supported by the results of in vivo studies demonstrating a dramatic rise of viremia and an accelerated clinical disease progression in SIV-infected macaques after the artificial depletion of CD8$^+$ cells (19, 20). Among HIV proteins targeted by HIV-specific CTLs, HIV Nef protein is expressed at high levels early in an HIV infection (21) and elicits a strong CTL response in a number of subjects (22, 23). Most antigenic determinants are located within a multirestricted, immunodominant central region spanning residues 73–94 and 113–147 (22, 24), including a highly conserved proline-rich region containing an Src homology 3 (SH3)-binding motif, PxxP (Nef$_{73-82}$: PVRPQVPLRP) critical for several but not all Nef functions (6, 7, 25–27). In particular, HIV-infected subjects expressing the HLA-B*3501 molecule, which prefers a proline residue on the second position of its antigenic peptides, show vigorous HLA-B35-restricted CTL responses toward the proline-rich region of Nef (22, 28, 29).

In the present study, we focused on HLA-B35-restricted CD8 T cell responses toward the functionally important PxxP region of HIV-1 Nef to ask whether CTL responses can impose constraints on Nef activity. Remarkably, sequence analysis of autologous viruses revealed the association of two different mutations with patients carrying HLA-B*35, one of which was earlier shown to be a naturally occurring variation that can modulate Nef functions (25). Further detailed analyses of CTL responses and Nef functions demonstrated that Nef balances between the conflicting selective pressures during the course of an HIV-1 infection. These findings suggest an important role of HIV-1 Nef-specific CTL responses in the control of Nef activity during the progression of an HIV-1 infection.

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[3] Abbreviations used in this paper: MHC-I, MHC class I; SH3, Src homology 3; 7-AAD, 7-aminoactinomycin D; wt, wild type; IVKA, in vitro kinase assay.