厚生労働科学研究費補助金

創薬基盤推進研究事業

（政策創薬総合研究事業）

ヒト抗原提示システムの包括的解析に基づく エイズワクチン戦略の再構築

平成20～22年度　総合研究報告書

研究代表者　上野 貴将

平成23（2011）年3月

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Ⅰ．総合研究報告書

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ヒト抗原提示システムの包括的解析に基づくエイズワクチン戦略の再構築

研究代表者	上野貴将	熊本大学エイズ学研究センター	准教授
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研究要旨

ヒトのHIV感染に伴って提示される細胞傷害性T細胞（CTL）抗原の包括的な解析を目指して、新たな蛋白化学的アプローチの基盤システム立ち上げと、これらを用いたCTL抗原ペプチドの蛋白化学解析を目指した。その結果、3年間の研究期間中に以下の成果を得た。（1）HIV特異的CTLの抗ウイルス機能は、抗原特異性によって大きく異なり、CTLに優れた抗ウイルス活性を与える抗原ペプチドは、HLAクラスI複合体として、熱力学的にきわめて安定な構造を形成していることを明らかにした。（2）T細胞レセプター（TCR）の相補性決定領域を抗体フレームワークに移植する新しい方法を確立して、抗原ペプチド・HLA複合体を特異的に認識する新規分子の創製に成功するとともに、IgG構造に組み込むことによって、機能を保持したまま、構造の安定化を達成した。（3）新たな高感度同定定量法としてのiTRAQ-MRM法を考案し、HIV感染細胞上の抗原ペプチドを10 att molレベルの高感度で定量的に解析する質量分析システムの立ち上げに成功した。ヒト検体を用いた帰納的解析と、熱力学、抗体工学、マススペクトロメトリーを用いた新しい試みにより、これまでに知られていなかったCTL抗原の性質を明らかにできた。将来のワクチン抗原の選別に、新たな科学的アプローチと情報を提供するものであると期待される。

A. 研究目的

ヒト感染免疫系に関する基盤情報は非常に限られており、エイズワクチン開発の障壁となっている。中でも抗原提示に関わる分子群は動物種間で大きく異なるため、ヒト検体での情報の充実化が望まれている。本研究では、プロテオームを主体とした新しい蛋白化学的アプローチを確立して、ヒトで提示される極微量のHIV抗原を包括的に明らかにすることにより、エイズワクチン開発と厚生労働行政に貢献することを目的とする。

B. 研究方法

（1）さまざまな病態にあるHIV感染者から提供していただいた血液検体（国立国際医療センター・岡先生の協力の下）を用いて、CTLの抗原特異性と抗ウイルス機能を解析した。さらにT細胞レセプター（TCR）遺伝子をクローニングして、TCRが欠損したT細胞に遺伝子導入し、抗原ペプチド、HLAクラスIおよびTCRの相互作用を詳細に解析した。

（2）T細胞レセプター(TCR)の相補性決定領域(CDR)を抗体可変領域断片のフレームワーク領域へ移植する構造分子モデリングを構築し、TCRグラフト抗体をデザインした。これをIgG発現ベクターにさらに組み込み、TCRグラフトIgG発現系を構築した。培養細胞に遺伝子導入して組換えIgGを発現させるとともに、精製して、フローサイトメトリーなどを用いた機能解析に供した。

（3）質量分析を用いた解析には、3台の高感度タンデム質量分析計、および付随するnanoレベルのクロマトグラフィー装置

（nanoLC），解析ソフト（AnalystQS, AnalystMRM, MRM pilot, MRM quant, scheduled MRM program, GPS, ProteinPilot, MASCOT 等）を用いた。高感度タンデム質量分析計 nanoLC-ESI-QqTOF (QStar Elite, Applied Biosystems)は網羅的なペプチドの同定用に、nanoLC-MALDI-TOF-TOF (MAALDI-TOF/TOF4700, 5800, Applied Biosystems)はペプチドの高感度検出用に、さらに nanoLC-ESI-ionTrapQQQ (QTRAP4000 Applied Biosystems)は高感度定量用に、それぞれ融合的に組み合わせて使用した。

(倫理面への配慮)
HIV 感染者から供与いただいた検体を用いた研究に関しては、関連する機関（熊本大学および国立国際医療センター）の倫理審査会の審議を受け、承認を得ている。また、HLA 遺伝子タイピングについては、ヒト遺伝子解析に関わる研究として、同じく関連機関の倫理審査委員会の審議を受け、承認されている。どちらの場合も、提供者の文書による承諾と個人情報の保護に万全を期すことを含め、承認を受けた研究計画に厳密にしたがって遂行した。

C. 研究結果

（1）ヒト T 細胞による HIV 抗原認識の解析(上野)

日本人 HIV 感染者の検体を用いて、ヒト CTL が応答する HIV 抗原とその階層性を解析し、CTL が認識する抗原は病態進行とともに経時的に変化することを明らかとした。次に、こうした CTL 抗原の経時的変化が、CTL の抗ウイルス活性に与える影響を解析し、急性期に見られる CTL の方が、慢性期に見られる CTL よりも、抗ウイルス活性に優れていることを見いだした。さらに、抗原ペプチドと HLA 複合体に対して、示差熱解析(DSC)を用いることにより、CTL に強い抗ウイルス活性を示す抗原ペプチドの熱力学的特性を明らかにした。

（2）抗体工学を用いた HIV 抗原検出プローブの開発と応用（熊谷）

ヒト細胞上の HIV 抗原の追跡に必要な抗体作製を、T 細胞受容体(TCR)と抗体の機能・構造が類似している点に着目し、TCR の相補性決定領域(CDR)を抗体フレームワークに移植することで、HIV 抗原ペプチド-主要組織適合性抗原複合体(pHLA)認識能を賦与した TCR グラフト抗体断片の作製を行った。
初めに試みた TCR グラフト Fv, scFv 断片には、抗原特異性は認められたものの、蛋白質としての構造安定性に難があった。そこで、さらに、抗体断片を IgG 化して、抗原特異性を維持したまま、安定性を向上させることに挑んだ。その結果、得られた TCR グラフト IgG 抗体は、培養時間の増加に伴い一部分解してしまう傾向がみられたが、動物細胞を用いた一過性発現系によって調製することに成功し、フローサイトメトリー、およびビアコアによってリガンドに対する結合能を確認した。

（3）プロテオミクスによる HIV 抗原の網羅的解析（荒木）

nanoLC-ESI-QqTOF(四重極飛行時間型ハイブリッド型質量分析計）および nano-LC-ESI-trapQQQ(四重極型タンデム質量分析計）を用いて、iTRAQ (isobaric Tagging for Relative and Absolute Quantitation) 法および MRM (Multiple Reaction Monitoring)法を確立し、少なくともスタンダードペプチドの定量解析において、1-10 att mol の感度で検出同定可能であることが判明した。さらに、実際のクルードな細胞画分からのペプチドの検出法を検討した。その結果、10-20 attmol レベルで、細胞表面から回収したペプチド分画から、Immuno Epitope Database(IEDB),T cell Epitope prediction MHC-1 binding prediction を活用することで、HLA class1 に結合するペプチドの同定と大まかな定量が可能となった。

D. 考察

（1）HIV 感染急性期と慢性期で、CTL の抗ウイルス応答に機能的な差が生じる原因は、CTL の抗原特異性の経時的な推移と相関していること、CTL の抗ウイルス機能は、抗原特異性によって大きく異なること、CTL

の優れた抗ウイルス活性発現には、ターゲットとする抗原ペプチドがHLAクラスI複合体と安定な複合体を形成し、細胞表面上に長時間にわたって提示されることが重要であることを明らかとした。さらに熱力学的な解析を行うことにより、強いCTL活性を与える抗原ペプチドの性質を明らかにした。

（2）TCRの特異性を抗体断片に移植する新しい技術の作成に成功した。さらにこうした抗体断片をIgG化するとともに、培養細胞で分泌生産する系の立ち上げに成功した。さらに、TCRグラフト抗体の機能・特異性を維持したまま、IgG化による安定性の向上も達成した。

（3）MRMは同位体を内部標準とすることで、タンパク質の絶対定量、相対定量が可能であるが、iTRAQ法を用いることにより高感度に多検体のサンプル間の相対的な定量が可能であることが明らかとなった。HLAクラスIに、自己由来の多種類のペプチドが載っているコントロール細胞と、対象となるHIV抗原を結合している細胞から、各々の提示ペプチドを回収し、各々から目的ペプチドを高感度に定量的に同定できる可能性が高い。HIV抗原ペプチドのレパートリーは最大で300通りと考えられているため、これら全ての一度に定量的に解析することが可能と考えられる。

E. 結論

それぞれの分担課題について、下記の3点について発展的な成果を得ることができた。（1）CTLの抗原特異性とCTLの抗ウイルス活性が互いに関連することを明らかとし、CTLに強い抗ウイルス機能を与える抗原ペプチドの熱力学的な性質を明らかにした。（2）TCRグラフティングという新たな分子の創製に成功するとともに、抗体断片をIgG化することにより、抗原特異性を維持したまま、蛋白質としての安定性を向上させることに成功した。（3）プロテオミクスの高感度かつhigh throughputな新技術によるアプローチによって、ヒトで提示されるHIV抗原を網羅的、経時的、定量的に解析するシステムを構築し、新たな高感度同定定量法のプロトコール化、生体サンプルへの応用へ向けての最適化に成功した。HIV抗原の高感度定量的同定法として有用な方法を提示することができた。

F. 研究発表

詳細は別紙参照。

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熊谷泉

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G．知的財産権の出願・登録状況

特願 2008-135007

発明者：熊谷泉、梅津光央、渡邊秀樹、上野貴将、本園千尋

発明の名称：T 細胞受容体を模倣する抗体断片及びその製造方法

Ⅱ．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

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
荒木令江	融合プロテオミクスによる病態メカニズムの解析	小田吉哉・長野光司編集	創薬のためのタンパク質・プロテオミクス解析	羊土社	東京	2010年	p182-190

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Ⅲ. 研究成果の刊行物・別刷

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.

Table I. *Summary of HLA-B35$^+$ subjects used in this study*[a]

Pt.	HLA Class I Allele	Months since Seroconversion	Viral Load (log_{10}/ml)	CD4 (mm^{-3})	Antiretoviral Therapy	Nef Sequence	PBMC Availability
001	*A2402/A2603, B3501/B4002*	132	ND	227	+	RPQVPLRPMT**F**	−
		192	3.9	223	+	**T**PQVPLRPMTY	+
003	*A2402/A2601, B3501/B5101*	72	ND	480	−	RPQVPLRPMT**F**	−
		144	ND	252	+	**T**PQVPLRPMTY	+
006	*A24/A26, B35/B52*	48	ND	102	+	RPQVPLRPMT**F**	−
015	*A11/A24, B35/B54*	147	BD	383	+	**T**PQVPLRPMTY	+
016	*A26/A33, B35/B44*	7	ND	43	−	RPQVPLRPMT**F**	−
017	*A2/A24, B35/B48*	192	BD	254	+	**T**PQVPLRPMTY	−
019	*A2402/−, B3501/B5201*	18	4.7	524	−	RPQVPLRPMT**F**	−
		80	BD	1574	+	**T**PQVPLRPMTY	+
025	*A24/A31, B35*	26	ND	50	+	**T**PQVPLRPMTY	−
027	*A24/A26, B35/B44*	4	ND	84	+	RPQVPLRPMT**F**	−
033	*A0207/A3101, B3501/B4601*	72	5.3	326	−	**T**PQVPLRPMTY	+
034	*A2402/A2601, B3501/B4801*	48	4.4	201	−	**T**PQVPLRPMTY	+
042	*A24/A31, B35/B60*	59	3.8	311	−	**T**PQVPLRPMTY	+
046	*A2, B35/B61*	48	BD	263	+	**T**PQVPLRPMTY	+
099	*A2402/−, B3501/B61*	12	3.9	984	−	RPQVPLRPMT**F**	+
100	*A2601/−, B3501/B4001*	16	5.0	614	−	RPQVPLRPMT**F**	+
102	*A2402/A0206, B3501/B0702*	17	2.8	482	−	RPQVPLRPMT**F**	+
131	*A2402/A0207, B3501/B4601*	10	1.9	563	+	RPQVPLRPMT**F**	+
136	*A2402/A2601, B3501/B5201*	15	4.4	308	−	RPQVPLRPMT**F**	+
141	*A0201/A3101, B3501/B5401*	10	5.3	382	−	RPQVPLRPMTY	+
		20	5.1	360	+	RPQVPLRPMT**F**	+
145	*A0207/A2601, B3501/B5101*	6	BD	645	−	RPQVPLRPMTY	−
		18	4.6	685	−	RPQVPLRPMT**F**	+
161	*A2402/A2601, B3501/B5401*	13	2.3	955	−	RPQVPLRPMT**F**	+
168	*A2601/−, B3501/−*	5	2.3	408	+	RPQVPLRPMTY	+
178	*A2601/A3101, B3501/B4601*	8	2.7	568	+	RPQVPLRPMTY	+

[a] ND, Not determined; BD, below detection limit. Bold, underlined letters in the sequences represent mutations.

Materials and Methods

Subjects

A total of 23 individuals (HLA-B35$^+$) with HIV infection followed at the AIDS Clinical Center (International Medical Center of Japan) were enrolled for functional analysis of HIV-specific CD8 T cells and autologous HIV-1 sequence analysis in this study. Subjects were selected based on the availability of plasma and PBMC samples as well as HLA-B*35 expression. Clinical data of all subjects are listed in Table I. Patients 01, 03, and 17 are hemophiliacs who had been infected with HIV-1 through contaminated blood products. Because the time of HIV-1 infection or the time of seroconversion was not known for these subjects, we suspect that their infection occurred in 1983 based on a survey done on Japanese hemophiliacs. In addition, 41 individuals (negative for HLA-B*35) with HIV infections were enrolled for autologous HIV-1 sequence analysis. The study was conducted in accordance with the human experimentation guidelines of the International Medical Center of Japan and Kumamoto University.

Sequence analysis of autologous HIV-1

HIV-1 particles were precipitated by ultracentrifugation (50,000 rpm, 30 min) of patients' plasma, after which the viral RNA was extracted from them. DNA fragments encoding Nef proteins were amplified by a nested PCR, gel purified, and sequenced directly as described (29). The fragments were cloned into a plasmid and then sequenced for phylogenetic tree analysis.

For phylogenetic tree analysis of intrapatient evolution of the *nef* gene (HXB2 coordinate, 8932–9555), nucleotide sequences were initially aligned by using Clustal W and then manually adjusted to maximize alignment of codon triplet as needed. Regions that could not be unambiguously aligned were removed from subsequent phylogenetic analysis. The MEGA3 package of sequence analysis programs was used for detailed phylogenetic analysis (30). Pairwise evolutionary distances were calculated by using the Kimura 2-parameter model for estimation of distances, and phylogenetic trees were constructed by the neighbor-joining method.

Generation of T cell clones

CTL clones or lines were established by stimulation of PBMC with a synthetic peptide, as previously described (31). Briefly, a bulk CTL culture was seeded at a density of 0.8 or 5 cells/well with a cloning mixture (irradiated allogeneic PBMC and C1R-B3501 cells pulsed with 1 μM peptide in RPMI 1640 with 10% FCS and 100 U/ml rIL-2). Two weeks later, cells showing substantial Ag-specific cytolytic activity were maintained in the medium with peptide stimulation weekly.

Preparation of HIV-1 variants

The full-length HIV-1 pNL43 derivatives in which the *nef* gene was completely removed (pNL43ΔNef) or replaced with SF2 *nef* (pNL43SF2Nef) were created earlier (32). The Arg^{75} to Thr and Tyr^{85} to Phe mutations were achieved by site-directed mutagenesis based on SF2 *nef*. 293T cells were transfected with each of the constructs, and the infectious HIV-1 virions released into the medium were collected 48 h later. The p24 Ag concentrations of virus stocks were determined by p24 Ag ELISA.

Flow cytometric analysis

HLA stabilization assay. Peptide-binding activity for HLA-B*3501 was assessed by an HLA stabilization assay using RMA-S cells expressing HLA-B*3501 as described earlier (31).

HLA tetramer analysis. The HLA-B3501 tetramers in complex with the VY8 and RY11 peptides were prepared as previously described (31). Cryopreserved PBMC of HIV-positive (2×10^6) or -negative donors (3×10^6) were stained with the PE- and allophycocyanin-labeled tetramers at 37°C for 15 min followed by anti-CD8-PerCP (BD Biosciences/BD Pharmingen) and anti-CD3-FITC (DakoCytomation) at 4°C for 15 min. The CD3$^+$CD8$^+$ cells were gated and then analyzed for binding with the tetramers by flow cytometry (FACSCalibur; BD Biosciences).

Intracellular cytokine staining assay. Intracellular cytokine staining of Ag-specific CTL clones was done as previously described (33). Briefly, CTL clones (4×10^4 cells) were incubated with C1R-B3501 cells (4×10^4 cells) alone or pulsed with various concentrations of peptides for 6 h at 37 °C in the presence of brefeldin A (10 μg/ml). The cells were stained first with anti-CD8 mAb and 7-aminoactinomycin D (7-AAD), permeabilized in a detergent buffer, and then stained with mAb specific for IFN-γ or TNF-α (BD Biosciences/BD Pharmingen).

Cytotoxic assays

Toward peptide-loaded cells. The cytotoxic activity of the CTL clones was determined by a standard ^{51}Cr-release assay as described previously (31).