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G. 知的財産権の出願・登録状況

なし

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分担研究報告書

NK細胞によるHIVの認識と免疫逃避変異の認識に与える影響に関する研究

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研究要旨　ヒトナチュラルキラー(NK)細胞受容体 Killer cell Ig-like receptor (KIR)群は、細胞傷害性 T 細胞(CTL)にも発現し、主要組織適合性抗原(MHC)であるヒト白血球抗原(HLA)分子を認識することでウイルス感染に対する防御機構を制御している。抑制型 KIR 群はこれらの細胞の不活性化する機能を有する。そこで、本研究では、各種の HLA 分子に結合する HIV 由来ペプチドの同定を網羅的解析により行い、その KIR 群に対する親和性を検証する。これにより KIR 群を制御できる効果的な HIV ペプチド候補を開発することを目指している。本研究では、これまでに開発してきた簡易巻き戻し系と微量ゲルろ過法などを組み合せた HIV ペプチドの同定法を発展させ、HLA-Cw12 について系を確立し、スクリーニングを行った。その結果、HLA-Cw12 に提示される HIV 由来ペプチドを同定し、NK 細胞受容体 KIR2DL2、2DL3（抑制型）、2DS2（活性型）との表面プラズモン共鳴法による結合解析および実際に提示されているか質量分析法による確認を行った。また、実際に細胞内でペプチド断片化されて HLA-Cw12 に提示される HIV 由来ペプチドの同定を行うために、HEK293 細胞を用いたスクリーニング系の確立に向けた条件検討を重ね、スクリーニングを開始できる目途が立った。さらに、ペプチドワクチンの設計に向けて、HLA-Cw12 の構造情報を得るために、既知の HLA 由来ペプチドを結合した HLA-Cw12 の X 線結晶構造解析を行い、構造決定に成功した。

A. 研究目的

ヒトNK細胞や細胞傷害性T細胞(CTL)に発現する細胞表面受容体Killer cell Ig-like receptor (KIR)群はHLAを認識し、ウイルス感染を防御するNK細胞やCTLの制御に関与すると考えられている。これまでに、HIV-1由来ペプチドの変異が抑制型KIR群の活性を高め、NK細胞やCTLの活性を強く抑え、免疫不全を引き起こすと考えている。CTLだけではないHIVの新規の免疫逃避機構として提唱した (AIDS 2009)。本研究では、結合モチーフ情報を基にHIVゲノム配列から候補ペプチドを同定・合成を行い、HLA分子に提示されるかどうかを実験的に検証する。また、別の手法として、実際に細胞内消化過程を介してHLA-Cw12に提示されるHIV由来ペプチドの同定を行い、同定できたペプチドについては、ペプチドを提示したHLA分子を用いて抑制型および活性型のKIR群に対する親和性を網羅的に検証することにより、KIR群を適切に制御できる効果的なHIVペプチドワクチン候補分子を見いだすことを目的とする。

B. 研究方法

HIV ゲノム配列をもとに、HLA-Cw12 に提示

されうる候補配列を抽出したペプチドライブラリーを構築し、合成した。そのライブラリーを用いて、簡易巻き戻し系と微量ゲルろ過解析法を組み合せて、HLA-Cw12結合ペプチドを同定するためのスクリーニング条件を確立した。その結果、HLA-Cw12 に結合すると同定で来た新規ペプチドについて、NK細胞受容体 KIR2DL2、KIR2DL3（抑制型）と KIR2DS2(活性型)との結合解析を表面プラズモン共鳴法を用いて行うとともに、スケールを拡大して調製し、質量分析法により、実際に調製した HLA-Cw12 にペプチドが結合しているか確認した。また、細胞内でペプチド断片化されて HLA-Cw12 に提示される HIV 由来ペプチドの同定を行うために、HEK293細胞に HLA-Cw12、ヒトβ2m、HIV 由来遺伝子をそれぞれ導入することによって、HLA-Cw12 分子を分泌発現させ、質量分析法によって提示されたペプチドを同定するスクリーニング系の確立を目指した。さらに、ペプチドワクチン設計に向けて、原子レベルの構造情報を得るために、HLA-Cw12 の X 線結晶構造解析を行った。

(倫理面での配慮)
基礎的研究であり該当しない。

C. 研究結果

HLA-Cw12 結合モデルペプチドを用いて、微量巻き戻し法による網羅的スクリーニングを行う系の確立を目指して、巻き戻しおよびゲル濾過時の溶液条件を pH4.5～11 まで変化させて検討した。その結果、pH8 で巻き戻しを行った後に、pH6 でゲル濾過を行うことによって、HLA-Cw12/β2m/ペプチド複合体を明確に検出することができた。この条件で、結合モチーフ情報を基に合成した HIV 由来ペプチドライブラリー約140種についてスクリーニングを行ったところ、8 種のペプチドが HLA-Cw12 上に提示された。これらのペプチドについて、HLA-Cw12 と受容体 KIR2DL2、KIR2DL3（抑制型）、KIR2DS2(活性型)との相互作用解析を表面プラズモン共鳴法を用いて行った。各ペプチドを提示させた HLA-Cw12 を固定化したチップの上に、抑制型 KIR2DL1 および活性型 KIR2DS1 の可溶型分子を流すことにより、初期の結合解析を行ったところ、8 種のペプチドのうち、4 種はいずれの KIR にも結合せず、残る 4 種は抑制型の KIR2DL2 と最も強く結合することが分かった。8 種のペプチドについて、巻き戻しスケールを拡大し、精製後に質量分析法によって目的のペプチド結合の有無を確認したところ、実際にきちんと提示されていたのは 1 種類のみだった。

次に、HEK293 細胞を用いた実際に細胞内で消化され、HLA-Cw12 に提示されるペプチド同定スクリーニング法の確立に向けて、まず、HEK293 細胞での HLA-Cw12 分泌発現の系を確立した。その結果、HEK293 細胞に、HLA-Cw12 のみ遺伝子導入した場合には分泌発現せず、ヒトβ2m を一緒に導入した場合のみ HLA-Cw12 の分泌発現が認められた。分泌発現系が確立したため、次に、HIV 由来ペプチドを同定するために、HIV 各遺伝子断片（Env については膜貫通領域を除く）をクローニングし、HLA-Cw12、ヒトβ2m、HIV 遺伝子をすべて HEK293 細胞に導入した。分泌発現した HLA-Cw12 の一部はヒトβ2m、ペプチドと

会合していない重鎖のみで存在していたため、構造を認識する W6/32 モノクローナル抗体を用いてペプチドを提示している HLA-Cw12 のみを精製し、質量分析法によりペプチド同定を試みた。測定可能なタンパク質量を得るための大量培養条件検討を重ね、実際に、MALDI-TOF-MS によって目的の分子量（８～１０アミノ酸程度を想定）付近にシグナルが確認できたため、さらにペプチドを濃縮し、LC-MS/MS で配列を同定する条件を詰めている。

HLA-Cw12 の結晶構造解析については、分解能 2.5Å で構造決定することができた。

D. 考察

HLA-Cw12 に結合するペプチドの網羅的同定を行った結果、HIV ペプチドワクチン候補となる活性型受容体 KIR2DS2 に優位に結合するペプチドの同定には至らなかった。また、KIR と結合したものは４種類だったが、今回質量分析法により実際に結合を確認できたものは１種類のみだった。この１種類については、今後立体構造解析も行う予定である。残るペプチドについては、配列内にシステイン残基を含んでいるものもあり、重鎖とジスルフィド結合を形成している可能性も含め、さらに詳細な検討を重ねていく。今後、HLA-Cw12 に提示され、受容体 KIR との結合も示すことが明らかになった４種のペプチドを基に、変異を導入することによって、受容体との結合様式が変化するか検討する予定である。

実際に細胞内でペプチド断片化されて HLA-Cw12 に提示される HIV 由来ペプチドの同定に向けて、HEK293 細胞を用いた HLA-Cw12 の分泌発現・精製条件が確立し、結合ペプチドを同定できる目途が立った。随時、各 HIV 蛋白質をコードする遺伝子を Cw12 と強発現させ、実際にどのようなペプチドが提示されているか、その中に HIV 由来のペプチドが含まれているか検討しい、配列を同定していく予定である。また、HLA-Cw12 の立体構造を明らかにしたことで、HLA-Cw12 は比較的長いペプチド（11 アミノ酸残基）でも、中央部が外側に露出し、両端でペプチド溝に結合することによって、機能的に提示されることがわかった。結合ペプチドスクリーニングの際には、想定よりも分子量の大きいところまで解析する必要があることがわかった。

E. 結論

(1) HLA-Cw12 について、巻き戻し-ゲル濾過条件を検討し、ペプチドスクリーニング法を確立し、約140種のペプチドについてスクリーニングを行い、うち８種類のペプチドが結合することを明らかにした。

(2) HLA-Cw12 結合ペプチドを用いて、KIR2D の抑制型と活性化型との結合解析を行い、４種類のペプチドが実際に KIR2D と結合することを明らかにした。

(3) 細胞内消化により HLA-Cw12 に提示されるペプチド同定法の確立に向けて、HEK293 細胞での分泌発現-W6/32 抗体による精製-質量分析法によるペプチド同定のスクリーニング系を確立した。

(2)HLA-Cw12 の X 線結晶構造解析に成功し、ペプチドワクチン設計に向けて原子レベルの構造情報を得ることができた。

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H. 知的財産権の出願・登録状況

1. 特許取得
　無し
2. 実用新案登録
　無し
3. その他
　無し

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分担研究報告書

HIV-1 薬剤耐性発現機序の解明と薬剤耐性に対する強力な抗 HIV 阻害剤の研究開発

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（平成 24 年度：研究分担者、平成 25～26 年度：研究協力者）

研究要旨
HIV 感染症・AIDS 治療に対する多剤併用療法は一定の効果を上げているが、治療の長期化が薬剤耐性株の出現や薬剤の副作用の問題をより深刻なものとしており、新規薬剤の開発は常なる課題となっている。本研究は HIV が耐性化しにくい・耐性化しても他薬剤との交差耐性を有しない薬剤や、新規の作用機序で HIV の感染・複製を阻止する阻害剤の開発、及びウイルス学・細胞生物学・結晶解析学的な手法を駆使し開発の基礎となる前臨床的データの蓄積を目的として行った。その結果、oxatricyclic 構造を有する新規 HIV-1 プロテアーゼ阻害剤(PI), GRL-0519 を同定し、強力な抗 HIV 活性(IC_{50}: 0.5~0.7 nM)、耐性発現の遅延について報告した。更に cyclohexyl-*bis*-THF という新たな構造を有する有望な新規 PI, GRL-0739 を同定し、報告した。それ以外にも特徴的な polycycle 構造を有し複数の高度多剤耐性臨床分離株に対して抗ウイルス活性を高度に維持する強力な GRL-09510、また methoxy- chloro-benzene 構造を P2 部位に有し、強力な抗 HIV 活性及び HIV の同化合物に対する耐性獲得に著しい抵抗性を示す GRL-10413 といった新規化合物を同定、その詳細な検討を行った。また、新規の作用機序を有する薬剤としては、HIV Capsid 領域（CA）の特定の領域に結合する薬剤を docking simulation の手法により同定し、3 化合物が実際に HIV-1 CA の著明な自己崩壊を誘導する事を証明した（Amano, Mitsuya, 投稿準備中）。このような作用を有する化合物は現在までに報告が無く、全く新しい HIV-1 増殖阻害機序と考えられる。

A. 研究目的
研究分担者は熊本大学満屋教授の下、AIDS 対する治療法の研究開発を続けている。本研究では米国研究グループと共同開発したユニークな構造を有する複数の新規 HIV-1 PI について、抗 HIV 活性や耐性発現について前臨床的なデータの確認を行った。同時に構造モデリングの手法を用い、これらの薬剤の作用発現機序について検討するなどの基礎研究も行った。また、新規の作用機序を有する薬剤として、研究協力者らが見出した HIV-1 の構造蛋白である Gag Capsid 領域（CA）の特定部位にアミノ酸挿入変異が入る事で、CA の異常な自己崩壊が生じ、変異ウイルスが増殖不能になるという現象（Amano, Mitsuya, 投稿準備中）を基に、そのような CA の特定部位近傍の蛋白表面に結合しうる低分子化合物を同定することで、HIV-1 の CA の自己崩壊を誘導する薬剤の開発を目標として研究を行った。

B. 研究方法
①野生株及び多剤耐性株に対する抗 HIV 活性は in vitro のアッセイシステム(MTT アッセイ、p24 アッセイ等)を用いて検討した。
②試験管内での薬剤耐性 HIV-1 株の誘導は、低濃度の薬剤存在下で野生型 HIV-1 を感染させた MT2 細胞を培養し、継代を重ねながら薬剤濃度を徐々に増加させ、最終的に高濃度の薬剤存在下でも増殖可能な HIV-1 株を誘導した。
③構造モデリングは Maestro version 9.3 (Schrödinger 社) を用い、HIV プロテアーゼと薬剤との相互作用について検討した。
④*in silico* flexible docking simulation の手法により HIV-1 CA の特定の cavity に結合する低分子化合物を同定し、それらの化合物について、抗 HIV 活性を調べた。

（倫理面への配慮）
当該研究は試験管内での細胞及び HIV-1 実験室株を用いた研究となっており、現時点では倫理面に問題はないと考えられる。Volunteers からの PBMC 採取のための採血については考えられる副作用の危険性について十分な説明を行い、承諾が得られた後に行った。

C. 研究結果

24-26 年度において研究分担者らは、oxatricyclic 構造を有する新規 HIV-1 PI, GRL-0519 を米国研究グループと共同開発、同化合物は野生株および高度多剤耐性株に対して非常に強力な抗 HIV 活性を発揮、試験管内耐性誘導では HIV-1 の同化合物に対する耐性獲得は著しく遅延することを明らかにした。また構造解析により、GRL-0519 の強力な抗 HIV 活性の機序として、2 つの THF 構造が HIV-1 プロテアーゼ（PR）活性中心部位のアミノ酸主鎖と強固に結合する事に加え、3 つ目の THF 環が PR の主要アミノ酸群と複数の相互作用を有する事を確認しこれを報告した（Amano, Mitsuya, *AAC.* 2013）。更に cyclohexyl-*bis*-THF という新たな構造を有する有望な新規 PI, GRL-0739 を同定開発し、最近報告を行った（Amano, Mitsuya, *AAC.* 2015)。また複数の高度多剤耐性臨床分離株に対して抗ウイルス活性が完全に維持される（臨床分離野生株に対する活性値と同等）、特徴的な polycycle 構造を有する強力な新規 PI である GRL-09510 を新たに同定し、同化合物への HIV の耐性誘導・結晶構造解析等を含めた評価検討を行った（Amano, Nakata, 投稿準備中）。また methoxy-chloro-benzene 構造を P2 部位に有し、強力な抗 HIV 活性及び HIV の同化合物に対する耐性獲得に著しい抵抗性を示す GRL-10413 を同定し、その詳細な検討を行った。

CA の自己崩壊を誘導する薬剤の開発については、CA の特定部位近傍の蛋白表面に低分子化合物が結合し得る充分な空間を有する疎水性 cavity を同定、8,555,483 個の化合物の構造データを用いて、*in silico* flexible docking simulation の手法により各化合物の CA 上の標的 cavity との結合スコアを算定、スコアの良い化合物に関しては実際に輸入購入し、試験管内での抗 HIV-1 活性の評価を行った。その結果、40 種類以上の抗 HIV-1 活性を有する新たな化合物群を同定しており、そのうち 3 化合物が実際に感染細胞内および細胞外に出芽した成熟ウイルス粒子内においても HIV-1 CA の著明な自己崩壊を誘導する事を証明した（Amano, Mitsuya, 投稿準備中)。

D.考察

本研究で同定された 4 種の新規 PIs はいずれも in vitro で強力な抗 HIV 活性と良好な耐性プロファイルを有しており、今後はヒト PBMC 移植マウスなどを用いた in vivo の系での活性を検討し、臨床試験につながる基礎データの蓄積を図る方針である。一方、CA の自己崩壊誘導低分子化合物は、これまでに報告のない全く新しい作用機序の薬剤であり、既存の薬剤と交叉耐性がなく、ウイルスの蛋白のみを標的とした副作用の少ない薬剤の開発が期待される。しかしながら、現時点では薬剤の抗 HIV 活性は既存の薬剤に比べ弱いため、今後はこれら化合物をリードとして合成展開を行うことで最適化を進めていく。また、本手法は他のウイルス蛋白と結合する低分子化合物の同定にも応用できるため、更なる新規作用機序の薬剤開発も期待される。

E.結論

本研究では新規 PI 及び CA 崩壊誘導薬について、基礎的な生物学的活性を明らかにするとともに、その効果発現機序についても検討を行った。現在同定されている薬剤の in vivo での研究データの蓄積や、構造モデリングの手法を用いた最適化により、臨床試験への橋渡しとなる研究を進めて行く。

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2．天野将之、満屋裕明、HIV-1 Capsid 蛋白 (CA) の挿入変異がもたらす CA 自己崩壊の分子機構の解明およびCA 阻害活性を有する低分子化合物の検索・同定、2013年11月22日、第27回日本エイズ学会学術集会（熊本）
3．天野将之、Pedro Miguel Salcedo-Gomez、Arun K. Ghosh、満屋裕明、高い中枢神経系（CNS）移行性と強力な抗HIV-1活性を有する新規HIV関連神経認知障害（HAND）予防/治療薬の開発、2014年5月8日、第24回抗ウイルス療法研究会総会（山梨）

H. 知的財産権の出願・登録状況

該当なし

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

発表者氏名	論文タイトル	発表誌名	巻号	ページ	出版年
Chikata T, Carlson JM, Tamura Y, Borghan MA, Naruto T, Hashimoto M, Murakoshi H, Le AQ, Mallal S, John M, Gatanaga H, Oka S, Brumme ZL, Takiguchi M.	Host-specific adaptation of HIV-1 subtype B in the Japanese population.	Journal of Virology.	88(9)	4764-4775	2014
Rahman MA, Kuse N, Murakoshi H, Chikata T, Gatanaga H, Oka S, Takiguchi M.	Raltegravir and elvitegravir-resistance mutation E92Q affects HLA-B*40:02-restricted HIV-1-specific CTL recognition.	Microbes and Infection.	16(5)	434-438	2014
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Harada, S., Yoshimura, K., Yamaguchi, A., Yusa, K., Matsushita, S.	Impact of antiretroviral pressure on selection of primary HIV-1 envelope sequences in vitro.	J. Gen. Virol.	94	933-943	2013
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Ⅳ. 研究成果の刊行物・別刷

APs also correct for the confounding influences of viral phylogeny, HIV codon covariation, and linkage disequilibrium (LD) between HLA class I alleles (2, 16, 17, 21).

Associations between HLA class I alleles and HIV-1 amino acid polymorphisms in the Japanese and IHAC data sets were identified using a published phylogenetically corrected logistical-regression model that corrects for HLA LD, HIV phylogeny, and HIV codon covariation as potential confounders (17, 20). Briefly, maximum-likelihood phylogenetic trees were constructed using Gag, Pol, and Nef sequences (one tree per gene), and a model of conditional adaptation was inferred for each observed amino acid at each codon. Amino acids are assumed to evolve independently along the phylogeny to the tree tips (representing the present host). In each host, HLA-mediated selection and HIV amino acid covariation are directly modeled using weighted logistical regression, in which the individual's HLA repertoire and covarying HIV amino acids are used as binary predictors and the bias is determined by the possible transmitted sequences as inferred from the phylogeny (17). To identify which factors (HLA and/or HIV covariation) contribute to selection pressure, we employ a forward-selection procedure where the most significant association is iteratively added to the model, with *P* values computed using the likelihood ratio test. We performed *post hoc* filtering of the resulting HLA-associated-polymorphism list, restricting our output to instances in which at least 10 individuals carried the allele or polymorphism and at least 10 individuals did not carry the allele or polymorphism. Multiple tests were accounted for using *q* values, the *P* value analog of the false-discovery rate (FDR) (22). The FDR is the expected proportion of false positives among results deemed significant at a given threshold; for example, at a *q* value of <0.2, we expect 20% of identified associations to be false positives. In the analyses identifying HLA-APs, a significance threshold of a *q* value of <0.2 was employed.

Statistical analysis. Correlations between the total number of HLA-associated substitutions in each individual and clinical parameters (pVL and CD4 count) were performed using Spearman's correlation. To determine the total number of HLA-associated substitutions within a given HIV-1 sequence, we first identified all HIV-1 sites within that sequence known to be associated with any HLA allele. The specific residue at each site was counted as "HLA associated" if it matched any HLA-associated adapted form or any residue other than a nonadapted form identified at that position. The HLA alleles expressed by the individual were not considered (unless specifically stated); rather, our goal was to enumerate the HLA-APs associated with any HLA allele in each viral sequence. In analyses where host HLA alleles were not considered, HIV sites harboring residues that simultaneously represented a nonadapted and an adapted form associated with different HLA alleles were excluded from consideration.

Detection of differential escape between closely related HLA alleles and between cohorts. Two types of differential escape were investigated. First, we investigated differential escape between closely related HLA class I alleles, defined here as (four-digit) HLA subtype members belonging to the same (two-digit) allele group in the Japanese cohort. Specifically, seven HLA allele groups (A*02, A*26, B*15, B*40, C*03, C*08, and C*14) for which a minimum of two subtype members were represented in the Japanese cohort were investigated. For example, the HLA-A*02 allele group featured subtypes A*02:01, A*02:06, and A*02:07, while the A*26 allele group featured subtypes A*26:01 and A*26:03. For each allele group, we took the union of all HLA-APs identified for all subtype members of the group. Then, in a pairwise manner, we compared their strengths of selection between all HLA subtype members using a previously described phylogenetically corrected interaction test (17). In this analysis, thresholds of a *P* value of <0.05 and a *q* value of <0.2 were used to define significance.

Second, we investigated differential HLA-driven escape pathways between Japanese and IHAC cohorts. As outlined in the introduction, HLA-APs identified in human populations differ to some extent due to the presence (or enrichment) of certain HLA alleles in one population versus another. However, in this analysis, we were specifically interested in identifying cases where the same HLA allele drove significantly different escape pathways in the two cohorts. To do this, we took the union of all HLA-APs identified in the Japan and IHAC cohorts that were restricted by HLA subtypes observed a minimum of 10 times in both cohorts. We then compared the strength of selection of each HLA-AP in a pairwise manner between cohorts. The statistical methods used to investigate differential escape between the Japanese and IHAC cohorts are similar to those used to investigate differential escape between HLA subtype members (17), with some modifications, as follows. Briefly, a phylogenetically corrected logistical-regression model was constructed using a single HLA allele as a predictor. Using a likelihood ratio test, we then compared this model to a more expressive one that included an additional interaction term that was 1 if the individual expressed the HLA allele and was in the IHAC cohort or 0 otherwise. In this way, we could obtain a *P* value, testing the hypothesis that selection is the same in both cohorts (the null hypothesis) or whether selection differs across cohorts (alternative hypothesis). In contrast to the HLA-AP analyses described thus far, the present one does not feature corrections for HLA LD or HIV codon covariation and therefore yields odds ratios of association and *P* values that differ slightly from the original cohort-specific values. In the intercohort differential-escape analysis, significance was defined as a *P* value of <0.01 and a *q* value of <0.05.

Nucleotide sequence accession numbers. The accession numbers for the sequences determined in this study are AB873205 to AB873601 (Gag), AB873908 to AB874270 (Pol), and AB873602 to AB873907 (Nef).

RESULTS

Identification of HLA-associated polymorphisms in chronically HIV-1 clade B-infected Japanese individuals. The first objective of our study was to identify and characterize HLA-APs in Japan, a unique population in terms of its HLA class I distribution and predominantly HIV clade B epidemic. Toward this end, we analyzed linked HIV-HLA genotypes from 430 antiretroviral-therapy-naive Japanese individuals chronically infected with HIV-1 clade B. A total of 78 unique HLA class I alleles, defined at subtype level (four-digit) resolution, were observed in our cohort (see Fig. S1 in the supplemental material) at frequencies consistent with those in the published literature (23). Of these, 37 (including 9 HLA-A, 17 HLA-B, and 11 HLA-C alleles) were observed in at least 10 individuals and thus were included in the statistical analysis of HLA-APs (see Materials and Methods). Amplification and sequencing of HIV-1 Gag, Pol without the transframe (TF) protein, and Nef was successful for 397 (92.3%), 363 (84.4%), and 306 (71.2%) individuals, respectively. As described in Materials and Methods, HLA-APs within these three genes were identified using a phylogenetically corrected logistical-regression model that corrects for the confounding effects of viral phylogeny, HIV-1 codon covariation, and linkage disequilibrium between host HLA class I alleles (16, 17, 20). A false-discovery rate (*q* value) approach was employed to address multiple tests.

At a threshold of a *q* value of <0.2, a total of 284 HLA-APs, comprising 143 adapted and 141 nonadapted associations, were identified in Gag ($n = 94$ associations), Pol ($n = 86$ associations), and Nef ($n = 104$ associations) (Fig. 1; see Table S1 in the supplemental material). HLA-APs were more frequently detected in Nef (occurring at 45 of 206 codons [21.8%]) compared to Gag (51 of 500 codons [10.2%]) or Pol (51 of 947 codons [5.1%]). Although HLA class I allele frequencies in Japan are somewhat distinct globally, the distribution of HLA-APs across HIV-1 proteins was consistent with that reported in previous studies of other populations infected with clade B or C (1, 2, 6, 7, 16). Broken down by HLA locus, the numbers of HLA-A-, HLA-B-, and HLA-C-associated

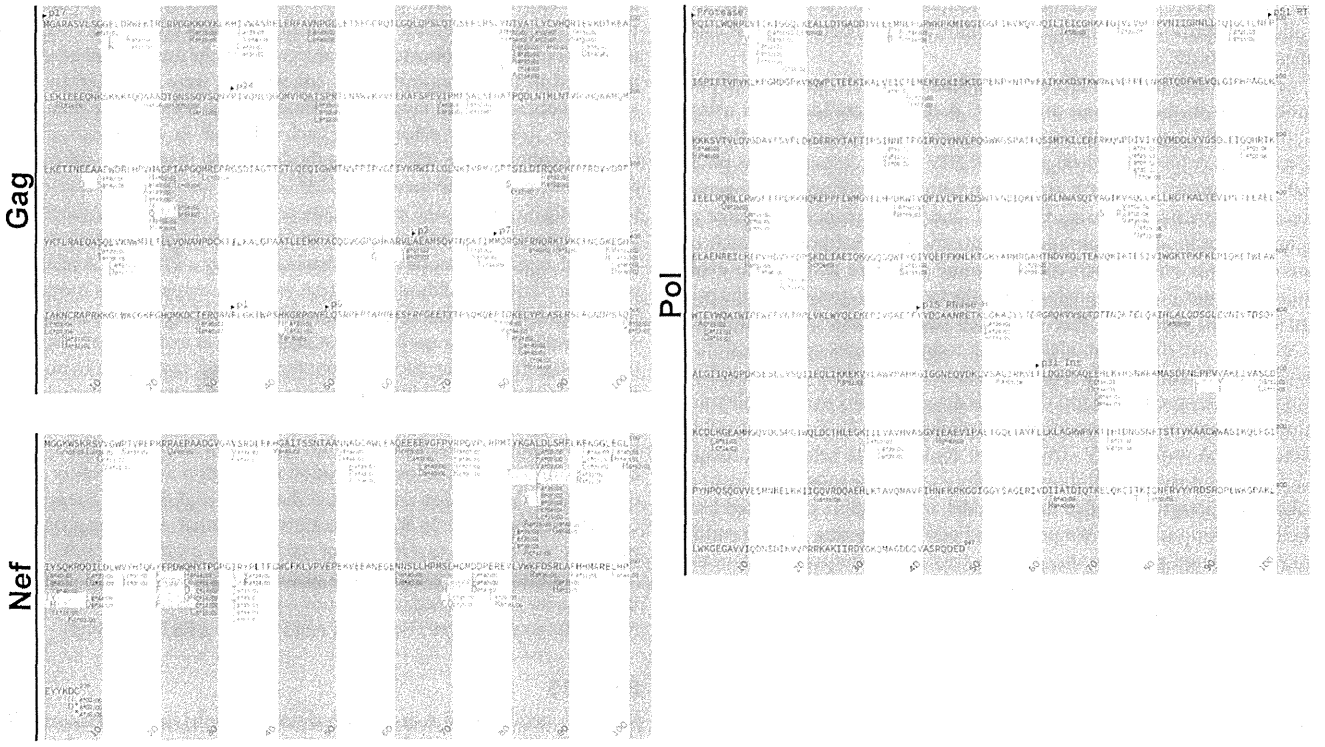

FIG 1 Escape map of HLA-APs for Gag, Pol, and Nef in the Japanese cohort. The escape maps indicate the locations, specific residues, and HLA restrictions of HLA-APs (all $q < 0.2$). The global HIV-1 clade B consensus amino acid sequence is used as a reference. The shaded vertical bars separate blocks of 10 amino acids. Adapted amino acids (those significantly overrepresented in the presence of a given HLA allele) are red. Nonadapted amino acids (those significantly underrepresented in the presence of a given HLA allele) are blue. Polymorphisms associated with the same HLA allele that occur in proximity to one another are grouped together in yellow boxes. A list of all HLA-APs is provided in Table S1 in the supplemental material.

polymorphisms were 78, 140, and 66, respectively, numbers that were also consistent with previous reports from Caucasian and African cohorts that HLA-B alleles restrict more associations than HLA-A or HLA-C alleles (1, 6, 18).

Correlation between the total number of HLA-associated substitutions and clinical parameters in Japanese individuals. We next wished to investigate the relationship between the presence of HLA-associated substitutions in each gene and the patient HIV-1 pVL and CD4 count in the Japanese cohort. As described in Materials and Methods, substitutions within a given HIV-1 sequence were counted as HLA associated if they had been identified as being associated with any HLA class I allele in our study, regardless of the HLA alleles expressed by the patient. For example, Gag-9S is an HLA-B*15:01-associated nonadapted polymorphism (Fig. 1; see Table S1 in the supplemental material); as such, any amino acid other than S at codon 9 was counted as an HLA-associated substitution. Similarly, Gag-123G is an HLA-C*01:02-associated adapted polymorphism (but no specific nonadapted forms, restricted by C*01:02 or others, were identified at this position); as such, any sequence harboring G at codon 123 was counted as having an HLA-associated substitution at this site.

A weak yet statistically significant inverse correlation was observed between pVL and the total number of HLA-associated substitutions in Pol (Spearman's $R = -0.11$; $P = 0.04$) (Fig. 2A). However, no such correlations were observed for Gag (Spearman's $R = -0.056$; $P = 0.3$) or Nef (Spearman's $R = -0.029$; $P = 0.6$) (Fig. 2A). Moreover, no significant correlations were observed between the total number of HLA-associated substitutions in any HIV protein and the CD4 count (Fig. 2A). Though the overall association is weak, the results raise the intriguing hypothesis that selection of certain HLA-driven substitutions in Pol could modulate the pVL in the Japanese population.

We next wondered whether the observed correlation between Pol polymorphisms and lower pVL could be attributed to polymorphisms restricted by HLA alleles that are protective in Japanese populations. HLA-B*67:01 and the HLA-B*52:01-HLA-C*12:02 haplotype are examples of such protective alleles (24). As such, we investigated whether they could play a role in the observed pVL correlation. No HLA-B*67:01-associated substitution was identified in Pol, whereas four HLA-B*52:01-associated and one HLA-C*12:02-associated substitutions were detected in the protein (see Table S1 in the supplemental material). Exclusion of the single HLA-C*12:02-associated substitution from analysis did not affect the relationship between the number of HLA-associated substitutions in Pol and pVL (data not shown). In contrast, exclusion of the four HLA-B*52:01-associated Pol substitutions substantially weakened the overall relationship between the number of HLA-associated Pol substitutions and pVL (Spearman's $R = -0.057$; $P = 0.3$) (Fig. 2B). Similarly, specific consideration of only HLA-B*52:01-associated Pol substitutions revealed a highly significant inverse correlation with pVL (Spearman's $R = -0.18$; $P = 0.0007$) (Fig. 2C) that represented the strongest such relationship detected in Pol for common HLA alleles observed in our cohort (see Fig. S2 in the supplemental material). We therefore

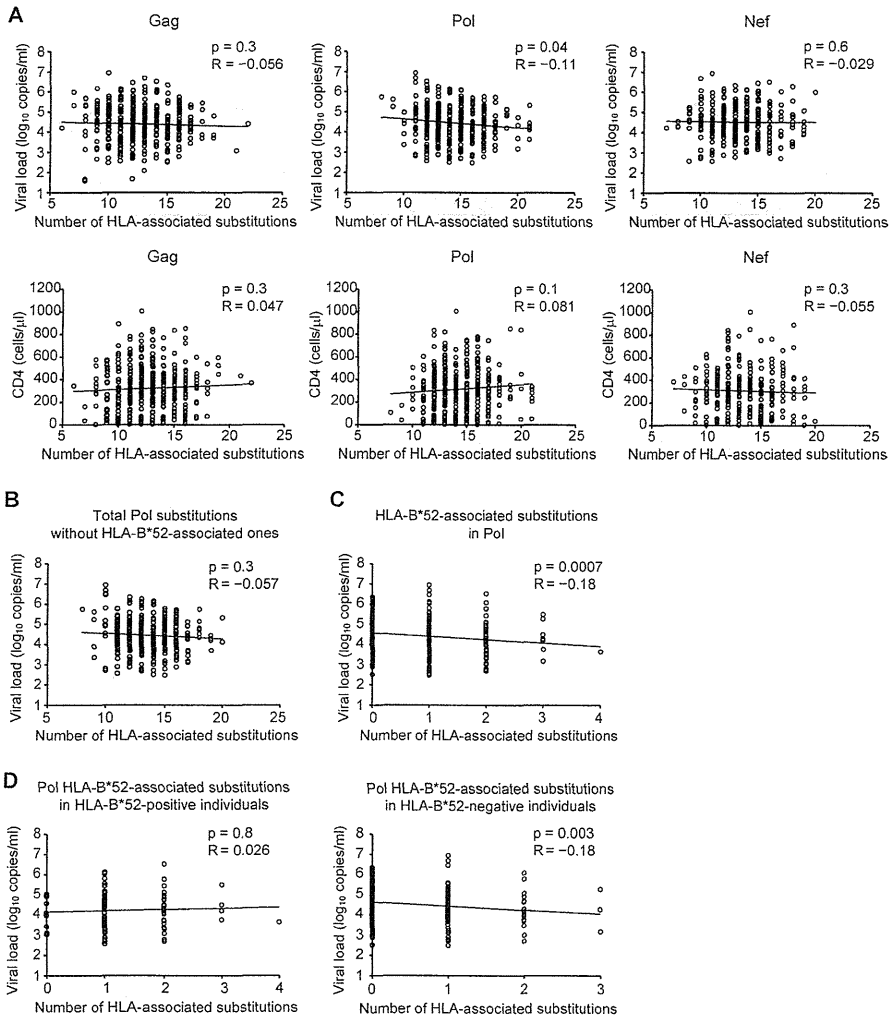

FIG 2 Correlations between HLA-associated substitutions in Gag, Pol, and Nef and viral load or CD4 count. The total number of HLA-associated substitutions in each subject's Gag, Pol, and Nef sequence was determined (see Materials and Methods). (A) Correlation between the number of HLA-associated substitutions in Gag, Pol, or Nef and pVL or CD4 count. (B) Correlation between pVL and the number of HLA-associated substitutions in Pol, with HLA-B*52:01-associated substitutions excluded. (C) Correlation between pVL and the number of HLA-B*52:01-associated substitutions in Pol (all patients). (D) Correlation between the number of HLA-B*52:01-associated substitutions in Pol in HLA-B*52:01-positive individuals (left) and HLA-B*52:01-negative individuals (right). Analyses were performed using Spearman's correlation. Linear regression lines are included in the plots.

reasoned that B*52:01-restricted substitutions were likely to be critical mediators of the observed pVL effect.

Finally, stratification of B*52:01-associated Pol substitutions by host B*52:01 expression revealed that the inverse correlation with pVL remained strongly detectable in HLA-B*52:01$^-$ individuals (Spearman's $R = -0.18$; $P = 0.003$), but not in HLA-B*52:01$^+$ individuals (Spearman's $R = 0.026$; $P = 0.8$) (Fig. 2D). We interpret our observations as suggesting that HLA-B*52:01-restricted Pol substitutions possess fitness costs that manifest themselves in terms of lower pVL upon transmission to, and persistence in, HLA-B*52:01$^-$ individuals. In contrast, no such pVL effects are detectable in B*52:01$^+$ individuals, likely because the fitness costs of these substitutions are outweighed by the advantages conferred by immune escape.

Differential escape between HLA subtypes in Japanese individuals. Our final goal in characterizing HLA-APs in Japan was to investigate the extent of differential escape between closely related HLA subtypes. In particular, we hypothesized that HLA subtype members differing with respect to the amino acids located within in the peptide-binding groove of the HLA molecule may differ with respect to the nature (or binding affinity) of the specific HIV epitopes presented (25–28), and therefore, that they may exhibit differential escape pathways. In contrast, we hypothesized that HLA subtype members that differ with respect to amino acids located outside the peptide-binding groove may be more likely to present the same epitopes (29–31) and therefore will generally exhibit less evidence for differential escape between them. Of the 284 HLA-APs identified in our cohort, 128 were restricted by HLA allele groups (A*02, A*26, B*15, B*40, C*03, C*08, and C*14) containing two or more subtype members (see Table S1 in the supplemental material). For five of these allele groups (A*02, A*26, B*15, B*40, and C*08), subtype members differed by substitutions within the peptide-binding groove (see Fig. S3 in the supplemental material), supporting their potential as candidates

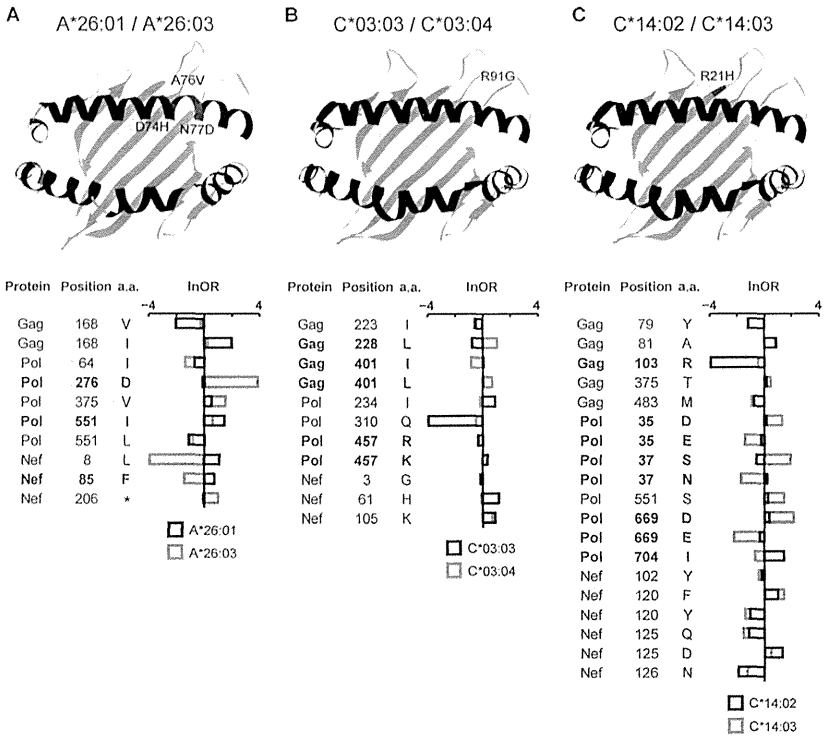

FIG 3 Polymorphic positions in HLA class I molecules and differential escape between pairs of HLA subtypes. In each ribbon diagram depicting the HLA-peptide-binding groove, the locations of residues differing among subtype members of the HLA-A*26 (A), HLA-C*03 (B), and HLA-C*14 (C) allele groups are highlighted in red and labeled with their locations and amino acids. HLA-AP comparisons between subtype members are shown in the corresponding plot below. The horizontal bars represent the lnORs, with colors indicating the restricting allele. Infinite lnORs are set to values of ±4. Boldface type indicates HLA-APs whose strengths of selection are statistically significantly different between the two subtype members ($P < 0.05$; $q < 0.2$). a.a., amino acid.

for differential HLA-AP selection. In contrast, members of the C*03 and C*14 subtypes differed by substitutions outside the peptide-binding groove (see Fig. S3 in the supplemental material), suggesting that their epitope repertoires (and thus escape pathways) would be more similar to one another.

We began by simply comparing HLA-APs identified in the context of the different HLA subtypes. As expected, viral polymorphisms associated with HLA subtype members differing within their peptide-binding grooves appeared to be quite specific to each HLA subtype (see Fig. S3A to D and F in the supplemental material). Surprisingly, however, viral polymorphisms associated with HLA subtype members differing only with respect to amino acids located outside their peptide-binding grooves also appeared to be quite specific to each HLA subtype (see Fig. S3E and G in the supplemental material). For example, HLA-C*03:03 and C*03:04, which differ only by substitutions at position 91 that have no contact with the groove (29–31), were associated with a total of 11 HLA-APs, none of which appeared to be shared (see Fig. S2E in the supplemental material). Similarly, HLA-C*14:02 and C*14:03, which differ only by a substitution at position 21 located outside the floor of the peptide-binding groove (see Fig. S2G in the supplemental material), shared only 10 of the 24 HLA-APs identified between them.

However, qualitative comparisons of HLA-APs meeting a specific significance threshold, such as those described above, are not statistically robust (since individual associations may fail to meet the threshold and thus not be detected, or variations in allele frequency may limit the power to detect associations). Thus, to explicitly investigate whether the above-mentioned examples represent statistically significant instances of differential escape between subtype members, we applied a phylogenetically corrected interaction test to compare their strengths of selection between subtypes (17). For each HLA allele group, we took the union of all HLA-APs identified for all subtype members and compared their strengths of selection between all subtype members in a pairwise manner. Representative examples of our results are shown in Fig. 3. For example, HLA-A*26:01 and -A*26:03 differ with respect to substitutions at amino acids 74, 76, and 77, located within the peptide-binding groove of the HLA molecule (see Fig. S3B in the supplemental material). A total of 10 HLA-APs, located at 8 HIV codons, were originally identified as associated with either HLA-A*26:01 or -A*26:03 (see Fig. S3B in the supplemental material). Although qualitatively, all 10 HLA-APs appear to be differentially selected by HLA-A*26:01 or -A*26:03 (see Fig. S3B in the supplemental material), the phylogenetically corrected interaction test revealed only 3 of them (located at Pol residues 276 and 551 and Nef residue 85) to be significantly differentially selected in terms of their natural logarithms of the odds ratios (lnORs) of association ($P < 0.05$; $q < 0.2$) (Fig. 3A). Surprisingly, significant differential escape was also observed between subtype members that differed only with respect to substitutions outside their peptide-binding grooves: 3 of 9 (33.3%) sites restricted by HLA-C*03 allele group members and 5 of 14 (35.7%) sites restricted by C*14 allele group members similarly exhibited statistically significant evidence of differential selection (Fig. 3B and C).

To determine whether the extent of differential escape between