クロファージ系やリンパ球系での検討が必要と思われた。

本研究では検討できなかったが、HIV-1 粒子の出芽部位が、MA 領域欠損により粗面小胞体へ（J Virol, 67: 4972-4980, 1993）、また MA 塩基性領域の変異によりエンドソームへ（J Virol, 83: 5375-5387, 2009）変化すること報告されていることから、これらの Gag 蛋白変異体をさらに改変する、すなわち、粒子として出芽せず分解経路に誘導されるようアセンブリードメインや出芽ドメインを削除する等も検討に値すると思われる。

E．結論

HeLa 細胞では、①HIV-1 Gag 抗原は主にプロテアソーム経路で分解された（半減期 8 時間）。②開始コドンアルギニンの Gag 蛋白はメチオニンの Gag 蛋白より効率良くプロテアソーム経路で分解された（N 末端法則）。③LAMP1 融合 Gag 蛋白はリソソーム経路で分解された。

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G．知的財産権の出願・登録状況（予定を含む。）

1　特許取得

なし。

2　実用新案登録

なし。

3　その他

なし

厚生労働科学研究費補助金（エイズ対策研究事業）
分担研究報告書

ヘルパーT 細胞反応に関する研究

研究分担者　寺原　和孝　国立感染症研究所　免疫部　研究員
研究協力者　光木　裕也　国立感染症研究所　免疫部 エイズ予防財団 RR

研究要旨

HIV 感染制御におけるヘルパーT リンパ球（HTL）の役割を明らかにするためには正確な機能評価を行うことが重要である。H23 年度は、SIV 感染サルの慢性持続感染期の HTL 反応に及ぼす Gag 発現センダイウイルスワクチンの影響について検討した。具体的には SIV 特異的 HTL における 5 種類のエフェクター因子（MIP-1β, IL-2, TNF-α, IFN-γ, CD107 a）の発現能を解析し、ウイルス制御との関連性について評価した。解析の結果、SIV 特異的 HTL の頻度はワクチン接種群と非接種群との間で明確な違いは認められなかった。しかしながら、ワクチン接種群においては IL-2 発現能を有する SIV 特異的 HTL の頻度が血中ウイルス量と有意な逆相関を示した。さらに、ウイルス制御個体（ワクチン接種群）においては、高レベル多機能性 HTL（4 種類以上のエフェクター因子を発現）の頻度もウイルス非制御個体（ワクチン接種群）ならびにワクチン非接種群と比較して有意に高いことを認めた。一方、多機能性指数（何種類のエフェクター因子を発現し得るかを示す指数）については 3 群間（ワクチン接種群／ウイルス制御個体、ワクチン接種群／ウイルス非制御個体、ワクチン非接種群）で有意差は認められず、血中ウイルス量との相関についてはワクチン非接種群においてのみ有意な逆相関が認められた。以上の結果から、ワクチン接種が SIV 特異的 HTL の機能発現に影響を及ぼすことが考えられ、IL-2 発現能とウイルス制御性との関連性が示唆された。

A．研究目的

CD8 陽性細胞傷害性 T リンパ球（CTL）は、慢性持続感染を成立させる HIV および SIV の体内複製制御において中心的役割を担う。一方、CD4 陽性ヘルパーT リンパ球（HTL）は、これら CTL の誘導・維持に重要であるとされつつも、HTL 自身が HIV の標的であることから、HIV 感染制御における役割についてはいまだ不明な点が多い。そこで本研究では、HIV 感染制御における HTL の免疫学的機能を明らかにすることを目的とした。このことは、CTL 誘導型エイズワクチンの有効性に対する科学的根拠を提示するうえでも重要な課題であると考えられる。

本年度は、SIV 感染サルの慢性持続感染期の HTL 反応に及ぼす Gag 発現センダイウイルスワクチン（SeV-Gag）の影響について検討した。

B．研究方法

ハプロタイプ B（90-120-Ib）、D（90-010-Id）、E（90-010-Ie）、J（90-088-Ij）共有群を含むビルマ産アカゲサルに対して SIVmac239 をチャレンジした。一部の個体についてはチャレンジ前に DNA プライム／SeV-Gag ブーストを施した。慢性持続感染期（感染後 20-34 週）の末梢血リンパ球を採取し、これと VSV-G シュードタイプ SIV を感染させた同一個体由来の B lymphoblastoid cell line（SIV-BLCL）を共培養後、CD4 陽性および CD8 陽性 T リンパ球における MIP-1β, IL-2, TNF-α, IFN-γ, CD107 a の発現をフローサイトメトリーにて解析した。

（倫理面への配慮）

遺伝子組換え生物等を用いる実験については、国立感染症研究所の承認あるいは文部科学大臣の確認を得て行った。

C．研究結果

1) SIV特異的HTL頻度

血中ウイルス量を検出限界以下まで抑制でき

た個体がワクチン接種群において認められたことから、ワクチン接種群についてはさらにウイルス制御群と非制御群に区分し、ワクチン非接種群を含め、計3群間の比較を行った。SIV特異的HTLの頻度（5種類のエフェクター因子のうち、いずれかを発現する細胞の頻度）は3群間で有意差は認められなかった。しかしながら、SIV特異的HTLの内、IL-2発現細胞の頻度は、ウイルス非制御群およびワクチン非接種群に対してウイルス制御群において有意に高かった。一方、他のエフェクター因子の発現細胞頻度については3群間で有意差は認められなかった（図1）。

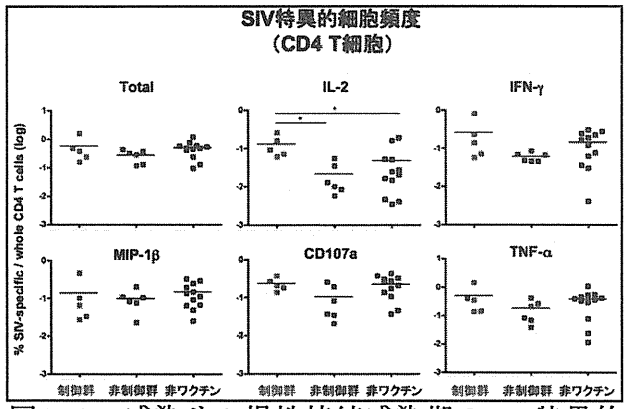

図1. SIV感染サル慢性持続感染期のSIV特異的HTL頻度

2) SIV特異的HTL頻度と血中ウイルス量の関連性

ワクチン接種群（ウイルス制御および非制御個体を含む）とワクチン非接種群における SIV 特異的 HTL 頻度（全頻度ならびに各エフェクター因子発現頻度）と血中ウイルス量との関連性を解析した結果、ワクチン接種群においては IL-2 発現 HTL 頻度、ワクチン非接種群においては IFN-γ発現 HTL 頻度のみが血中ウイルス量に対して有意な逆相関を示した（図 2）。

3) SIV 特異的 HTL の多機能性評価

高レベルの多機能性を保持したSIV特異的HTL（5 種類のエフェクター因子のうち 4 種類以上のエフェクター因子の発現が認められる細胞）の頻度について解析した結果、ウイルス制御群は非制御群と比較して有意に高い値を示した（図 3A）。さらに、SIV 特異的 HTL 集団として何種類のエフェクター因子を発現し得るかを指し示す多機能性指数についても、有意差は認められないもののウイルス制御群は非制御群と比較して高い傾向を示した（図 3B）。ワクチン非接種群においては高レベル多機能性 HTL 頻度、多機能性指数ともにワクチン接種／制御群・非制御群の両群に対して有意差を認めなかった（図 3）。

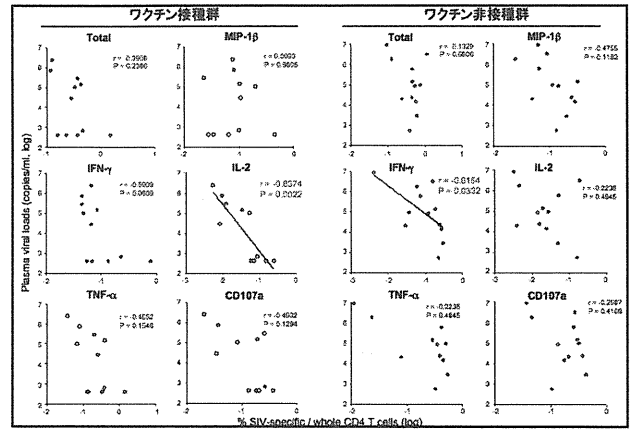

図2. SIV感染サル慢性持続感染期におけるSIV特異的 HTL 頻度と血中ウイルス量との関係

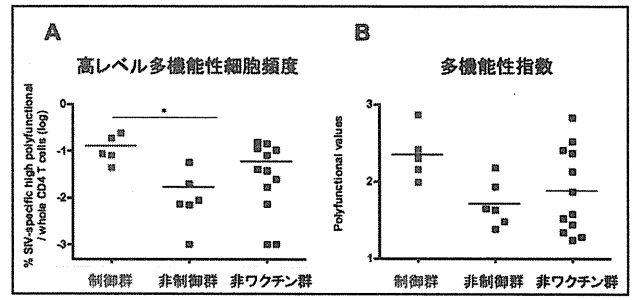

図 3. SIV 特異的 HTL の多機能性評価.（A）高レベル多機能性細胞頻度.（B）多機能性指数.

4) 多機能性指数と血中ウイルス量の関連性

ワクチン接種群と非接種群における SIV 特異的 HTL の多機能性指数と血中ウイルス量の相関を解析した結果、ワクチン接種群においては比較的高い関連性を示すものの有意性は認められず、非接種群においてのみ有意な逆相関が認められた（図 4）。

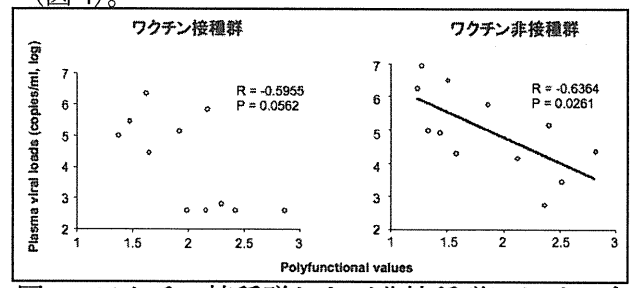

図 4. ワクチン接種群および非接種群における多機能性指数と血中ウイルス量の関係

D．考察

本年度の実験結果から、ワクチン接種／ウイルス制御群では IL-2 発現能を有する SIV 特異的 HTL の頻度がワクチン接種／非制御群やワクチ

ン非接種群と比較して有意に高いことが明らかとなった。また、多機能性指数については上記3群間で有意差は認められないものの、ワクチン接種／ウイルス制御群では4種類以上のエフェクター因子を同時に発現するSIV特異的HTLの頻度がワクチン接種／非制御群と比較して有意に高いことから、相対的に高い多機能性レベルを保持しているものと考えられる。

ウイルス制御との関連性について、具体的には、血中ウイルス量に対する関連性を解析した。その結果、血中ウイルス量に対して、ワクチン接種群ではIL-2発現能を有するSIV特異的HTLの頻度が有意な逆相関を示し、一方で、ワクチン非接種群ではIFN-γ発現能を有するSIV特異的HTLの頻度および多機能性指数が有意な逆相関を示した。この結果は、両群のSIV特異的HTL集団の機能的フェノタイプが異なることを示しており、ワクチン接種による免疫誘導がSIV特異的HTLの機能発現に対して慢性持続感染期に至るまで影響を及ぼしていると考えられる。

以上のことから、ウイルス制御に関連した抗原特異的HTLの機能を評価する際にはワクチン接種の有無について考慮する必要があると考えられる。つまり、ワクチン接種群においてはIL-2発現能、非接種群においては多機能性指数が最も適切な指標であると考えられる。

E．結論

CTL誘導を主たる目的としたSeV-Gagワクチンは、SIV特異的HTLの機能発現においても影響を及ぼし、IL-2発現能がウイルス制御と関連することが示された。

F．研究発表

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G．知的財産権の出願・登録状況（予定を含む。）

1　特許取得

なし。

2　実用新案登録

なし。

3　その他

なし。

厚生労働科学研究費補助金（エイズ対策研究事業）
分担研究報告書

中和抗体誘導に関する研究

研究分担者　横山　勝　　国立感染症研究所病原体ゲノム解析研究センター　主任研究官

研究要旨

HIV-1 gp120 が抗 V3 抗体中和を逃避するしくみを理解するため、V1/V2 ドメインを含む糖鎖付 HIV-1 gp120 三量体分子モデルの構築を行い、gp120 の抗 V3 抗体中和における V1/V2 ドメインの影響を検討した。HIV-1 gp120 三量体分子モデルより、V1/V2 ループは、ウイルス粒子の最外殻に配置され、まるで傘のように gp120 を覆う。さらに、糖鎖が gp120 外側ドメインおよび V1/V2 ドメインにあるため、gp120 はほぼ全体が糖鎖に覆われる。中和抵抗性株である gp120 三量体分子モデルは、同一の gp120 内の V1/V2 ドメインによる V3 エピトープのマスキングを示唆していた。したがって、V1/V2 ドメインと V3 の相対的配置が、中和逃避に重要な役割を果たしていると考えられる。

A．研究目的

HIV-1 gp120 の V3 は、感染受容体との相互作用に中心的役割を担う。そのため本来は、機能的制約が強く作用し、アミノ酸変異は抑制されるはずである。ところが V3 は高変異領域として知られる。これは、V3 は免疫原性が高く、持続感染には抗原変異を必要とするため、とされる。

V3 配列を糖鎖付加部位の有無と荷電量の違いで分類して多様性解析すると、V3 の荷電量の低下によって、V3 配列の多様性が低下することが示された。次に、V3 配列の荷電量の gp120 構造への影響を調べると、gp120 コアのアミノ酸配列が同じでも、V3 配列の荷電量の変化に伴い、V3 の配置や gp120 コアの構造を変えた。さらに、V3 荷電量の低下に伴い、抗 V3 結合抗体および CD4 結合部位を認識する中和抗体による中和感受性は低下した。ゆえに、HIV-1 gp120 V3 は荷電量に基づきウイルスの中和感受性と細胞指向性を司る機能領域と考えられる。

一方、HIV-1 gp120 の V1/V2 ドメインは 50～90 アミノ酸残基からなり、抗体からの逃避に重要な役割を果たしていると考えられている。そのため、V1/V2 ドメインの構造解析は中和抗体からの逃避メカニズムを理解するために必須である。しかし、V1/V2 ドメインは何らかのフォールディングは取っていると思われるが、大きくゆらいでいるため、これまで V1/V2 ドメイン構造を決定できていなかった。最近、McLellan らにより、V1/V2 ドメイン構造が報告され（Nature 480, 336–343, 2011）、ほぼ全ての HIV-1 gp120 の構造が明らかになった。

本研究は、抗原部位構造を推定することで、ウイルスが抗 V3 抗体中和を逃避するしくみを解析し、抗 V3 抗体の中和能を人為的に高める方法を開発することを目的とする。成果をワクチン開発に還元することを目指す。

今年度は V1/V2 ドメインを含む糖鎖付 HIV-1 gp120 三量体分子モデルの構築を行い、gp120 の抗 V3 抗体中和における V1/V2 ドメインの影響を検討した。

B．研究方法

（1）HIV-1 gp120分子モデルの構築

これまでにHIV-1 gp120の構造はX線結晶構造解析やNMRを用いて、部分構造でのみ決定されている。X線結晶構造解析では構造を安定化するために、抗体などとの複合体としている。そのため、決定されている構造は抗体などの影響を受けていると考えられる。本研究では、抗体などの影響を軽減した全体構造を得るために、ホモロジーモデリング法と分子動力学計算を組み合わせることにより、V1/V2ドメインを含む糖鎖付HIV-1 gp120分子モデルを構築した。ホモロジーモデリングには、ターゲット配列として中和抵抗性株であるJR-FLのアミノ酸配列を用いた。全体構造のgp120分子モデルを構築するため、複数の構造を鋳型として用いた。使用した鋳型はgp120コア（PDB code: 3DWD）、V1/V2 stem（PDB code: 3IDX）、V1/V2（PDB code: 3U4E）、V3（PDB code:

2QAD)、V4（PDB code: 2B4C）である。gp120は20以上の糖鎖に覆われている。Glycoprotein Builder（http://glycam.ccrc.uga.edu/ccrc/gp/）を用いて、High mannose型である$Man_5GlcNAc_2$を付加した。得られたgp120分子モデルを初期構造に用いて、分子動力学計算により平衡構造を得た。分子動力学計算にはAmber10のpmemdモジュール、力場にはff99SB-ILDNおよびGlycam06を用いた。圧力は1atm、温度は310K、シミュレーション時間は7 nsとした。

（2）HIV-1 gp120三量体分子モデルの構築

HIV-1 gp120三量体分子モデルは、ホモロジーモデリング法と分子動力学計算を組み合わせることにより得た分子モデルを、クライオ電子電子顕微鏡法により得られた構造（PDB code: 3DNN）に重ね合わせることにより構築した。

（倫理面への配慮）

本研究では、特定の研究対象者は存在せず、倫理面への配慮は不要である。

C．研究結果

ホモロジーモデリング法と分子動力学計算を組み合わせることにより、V1/V2ドメインを含む糖鎖付HIV-1 gp120分子モデルを構築した。CD4結合ループを正面の方向から見ると、左上にgp120のN末端とC末端が配置され、右下にV3が配置される。Inner domain上部はβ-sandwich構造、下部は構造的に可塑性のレイヤー構造となっている。左から2つ目のレイヤー（レイヤー2）が下方に伸び、先端にV1/V2ドメインが配置される。V1/V2ドメインは他の部位に比べてgp120コアから最も離れて配置された。

HIV-1 gp120単量体分子モデルを、クライオ電子電子顕微鏡法により得られた既存の構造に重ね合わせることにより、HIV-1 gp120三量体分子モデルを構築した。gp120三量体において、V1/V2ドメインはウイルス粒子の最外殻に配置され、まるで傘のようにgp120を覆う。糖鎖がgp120外側ドメインおよびV1/V2ドメインにあるため、gp120はほぼ全体が糖鎖に覆われる。また、三量体分子モデルより、gp120単量体は他のgp120単量体との結合部位は無く、それぞれ離れて配置されていた。他のgp120単量体と最も接近して配置されている部位はV1/V2ドメインである。V1/V2ドメインは他のV1/V2ドメインと互い接近していた。

V1/V2ドメインはV3への抗V3中和抗体のアクセスを制限すると考えられる。三量体におけるV1/V2とV3の相対的な位置について検討した。V3の近傍には同一のgp120のV1/V2ドメインが配置され、V2のD164とV3のR310の間で水素結合やイオン結合を形成していた。これらの結合は、V2およびV3のゆらぎのために形成と消失を繰り返している。

D．考察

HIV-1 gp120 三量体分子モデルの妥当性について検討する。三量体分子モデルは、V2 と V3 が結合していることから、同一の gp120 内の V1/V2 ドメインによる V3 エピトープのマスキングを示唆している。本研究で構築した HIV-1 gp120 三量体分子モデルは中和抵抗性株である。ゆえに、この結果は L. Liu らの実験結果と一致する。(PNAS108(50):20148-53,2011.) したがって、本研究により構築した gp120 三量体分子モデルは妥当な構造であると考えられる。

V1/V2ドメインおよびV3が、HIV-1gp120の抗V3抗体中和に影響する要因ついて検討する。三量体分子モデルから、V1/V2ドメインは、中和抵抗性株においてV3近傍に配置され、V3エピトープのマスキングを示唆していた。一方、V3の荷電量の低下によってgp120 コアのアミノ酸配列が同じであっても、V3配列の荷電量によってV3の配置が変わり、中和感受性に影響を与える。これはV1/V2ドメインが同一であるとき、V3の配置がV3エピトープのマスキングの強さを決めることを意味している。したがって、V1/V2ドメインとV3の相対的配置が、抗V3抗体のV3へのアクセスのしやすさを決めると考えられる。

HIV-1 gp120 では抗 V3 中和抗体の結合能が三量体と単量体で異なることが知られている。三量体と単量体で結合能が異なる理由について検討する。gp120 三量体では、V1/V2 ドメインが他の gp120 単量体の V1/V2 ドメインと接近していた。そのため、gp120 三量体では、gp120 単量体よりも、V1/V2 ドメインのゆらぎが他の gp120 単量体の V1/V2 ドメインによって制限される。ゆえに、三量体では、V1/V2 ドメインが V3 とより接近している頻度が高くなる。したがって、三量体では、抗 V3 中和抗体による V3 への結合能が低下すると考えられる。

E．結論

V1/V2 ドメインを含む糖鎖付 HIV-1 gp120 三量体分子モデルの構築を行い、gp120 の抗 V3 抗体中和における V1/V2 ドメインの影響を検討した。中和抵抗性株である gp120 三量体分子モデルは、同一の gp120 内の V1/V2 ドメインによる V3 エピトープのマスキングを示唆していた。したがって、

V1/V2 ドメインと V3 の相対的配置が、中和逃避に重要な役割を果たしていると考えられる。

F．研究発表

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G．知的財産権の出願・登録状況（予定を含む。）

1　特許取得

なし。

2　実用新案登録

なし。

3　その他

なし。

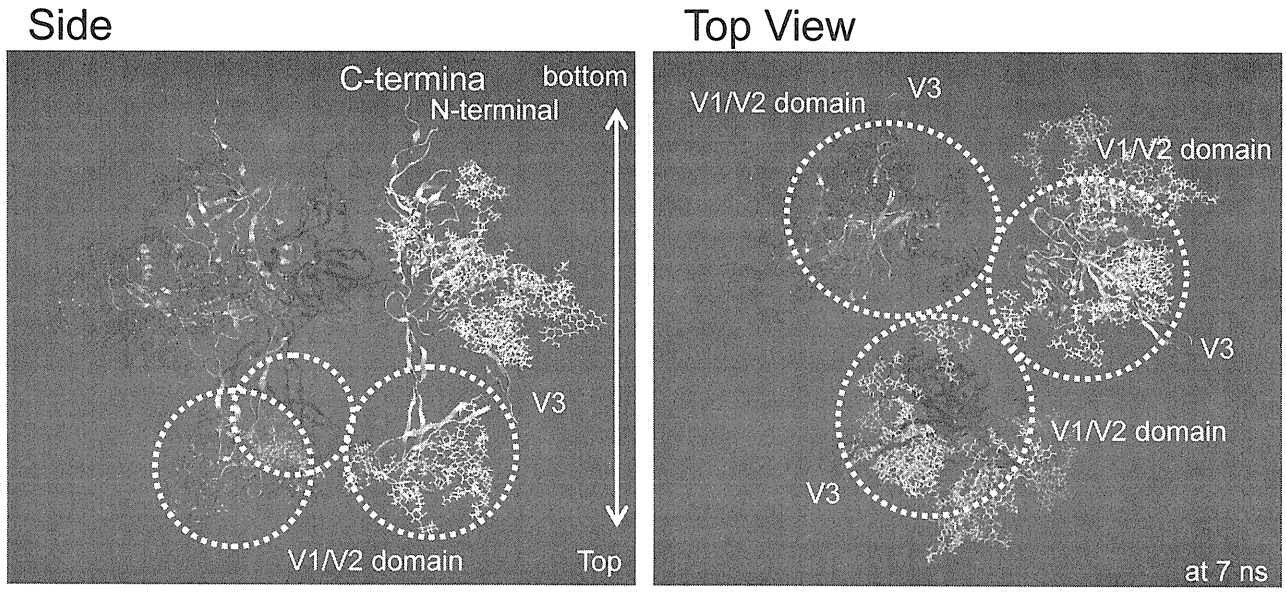

図１．V1/V2 ループを含む糖鎖付 HIV-1 gp120 三量体分子モデル。ホモロジーモデリング法および分子動力学計算により構築した HIV-1 gp120 分子モデルを、クライオ電子顕微鏡法により得られた構造に重ね合わせることにより構築した。

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

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Ⅳ.　研究成果の刊行物・別刷

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Dominant induction of vaccine antigen-specific cytotoxic T lymphocyte responses after simian immunodeficiency virus challenge

Yusuke Takahara [a,b], Saori Matsuoka [b], Tetsuya Kuwano [a], Tetsuo Tsukamoto [a], Hiroyuki Yamamoto [b], Hiroshi Ishii [a,b], Tadashi Nakasone [b], Akiko Takeda [b], Makoto Inoue [c], Akihiro Iida [c], Hiroto Hara [c], Tsugumine Shu [c], Mamoru Hasegawa [c], Hiromi Sakawaki [d], Mariko Horiike [d], Tomoyuki Miura [d], Tatsuhiko Igarashi [d], Taeko K. Naruse [e], Akinori Kimura [e], Tetsuro Matano [a,b,*]

[a] Division for AIDS Vaccine Development, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan
[b] AIDS Research Center, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan
[c] DNAVEC Corporation, 6 Ohkubo, Tsukuba, Ibaraki 300-2611, Japan
[d] Institute for Virus Research, Kyoto University, 53 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan
[e] Department of Molecular Pathogenesis, Medical Research Institute, Tokyo Medical and Dental University, 2-3-10 Kandasurugadai, Chiyoda-ku, Tokyo 101-0062, Japan

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ABSTRACT

Cytotoxic T lymphocyte (CTL) responses are crucial for the control of human and simian immunodeficiency virus (HIV and SIV) replication. A promising AIDS vaccine strategy is to induce CTL memory resulting in more effective CTL responses post-viral exposure compared to those in natural HIV infections. We previously developed a CTL-inducing vaccine and showed SIV control in some vaccinated rhesus macaques. These vaccine-based SIV controllers elicited vaccine antigen-specific CTL responses dominantly in the acute phase post-challenge. Here, we examined CTL responses post-challenge in those vaccinated animals that failed to control SIV replication. Unvaccinated rhesus macaques possessing the major histocompatibility complex class I haplotype *90-088-Ij* dominantly elicited SIV non-Gag antigen-specific CTL responses after SIV challenge, while those induced with Gag-specific CTL memory by prophylactic vaccination failed to control SIV replication with dominant Gag-specific CTL responses in the acute phase, indicating dominant induction of vaccine antigen-specific CTL responses post-challenge even in non-controllers. Further analysis suggested that prophylactic vaccination results in dominant induction of vaccine antigen-specific CTL responses post-viral exposure but delays SIV non-vaccine antigen-specific CTL responses. These results imply a significant influence of prophylactic vaccination on CTL immunodominance post-viral exposure, providing insights into antigen design in development of a CTL-inducing AIDS vaccine.

1. Introduction

In human and simian immunodeficiency virus (HIV and SIV) infections, cytotoxic T lymphocyte (CTL) responses exert strong suppressive pressure on viral replication but fail to control viremia leading to AIDS progression [1–5]. A promising AIDS vaccine strategy is to induce CTL memory resulting in more effective CTL responses post-viral exposure compared to those in natural HIV infections. It is important to determine how prophylactic CTL memory induction affects CTL responses in the acute phase post-viral exposure.

We previously developed a prophylactic AIDS vaccine (referred to as DNA/SeV-Gag vaccine) consisting of DNA priming followed by boosting with a recombinant Sendai virus (SeV) vector expressing SIVmac239 Gag [6]. Evaluation of this vaccine's efficacy against a SIVmac239 challenge in Burmese rhesus macaques showed that some vaccinees contained SIV replication [7]. In particular, vaccination consistently resulted in SIV control in those animals possessing the major histocompatibility complex class I (MHC-I) haplotype *90-120-Ia* [8]; $Gag_{206-216}$ (IINEEAADWDL) and $Gag_{241-249}$ (SSVDEQIQW) epitope-specific CTL responses were shown to be responsible for this vaccine-based SIV control [9]. Furthermore, in a SIVmac239 challenge experiment of *90-120-Ia*-positive macaques that received a prophylactic DNA/SeV vaccine expressing the $Gag_{241-249}$ epitope fused with enhanced green fluorescent protein (EGFP), all the vaccinees controlled SIV replication [10]. This single epitope vaccination resulted in dominant $Gag_{241-249}$-specific CTL responses with delayed $Gag_{206-216}$-specific CTL induction after SIV challenge, whereas $Gag_{206-216}$-specific and

* Corresponding author at: AIDS Research Center, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan. Fax: +81 3 5285 1165.
E-mail address: tmatano@nih.go.jp (T. Matano).

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$Gag_{241-249}$-specific CTL responses were detected equivalently in unvaccinated *90-120-Ia*-positive animals.

These previous results in vaccine-based SIV controllers indicate dominant induction of vaccine antigen-specific CTL responses post-challenge, implying that prophylactic vaccination inducing vaccine antigen-specific CTL memory may delay CTL responses specific for viral antigens other than vaccine antigens (referred to as non-vaccine antigens) post-viral exposure. In these SIV controllers, the reduction of viral loads could be involved in delay of SIV non-vaccine antigen-specific CTL responses. Then, in the present study, we examined the influence of prophylactic vaccination on immunodominance post-challenge in those vaccinees that failed to control SIV replication. Our results showed dominant induction of vaccine antigen-specific CTL responses post-challenge even in these SIV non-controllers.

2. Materials and methods

2.1. Animal experiments

The first set of experiment used samples in our previous experiments of six Burmese rhesus macaques (Macaca mulatta) possessing the MHC-I haplotype *90-088-Ij* (macaques R02-004, R02-001, and R03-015, previously reported [7,11]; R04-014, R06-022, and R04-011, unpublished). Three of them, R02-001, R04-011, and R03-015, received a prophylactic DNA/SeV-Gag vaccine [7]. The DNA used for the vaccination, CMV-SHIVdEN, was constructed from *env*-deleted and *nef*-deleted simian–human immunodeficiency virus $SHIV_{MD14YE}$ [12] molecular clone DNA (SIVGP1) and has the genes encoding SIVmac239 Gag, Pol, Vif, and Vpx, SIVmac239-HIV chimeric Vpr, and HIV Tat and Rev. At the DNA vaccination, animals received 5 mg of CMV-SHIVdEN DNA intramuscularly. Six weeks after the DNA prime, animals received a single boost intranasally with 6×10^9 cell infectious units (CIUs) of F-deleted replication-defective SeV-Gag [13,14]. All six *90-088-Ij*-positive animals including three unvaccinated and three vaccinated were challenged intravenously with 1000 50% tissue culture infective doses (TCID50) of SIVmac239 [15] approximately 3 months after the boost. At week 1 after SIV challenge, macaque R03-015 was inoculated with nonspecific immunoglobulin G as previously described [11].

In the second set of experiment, unvaccinated (R06-001) and vaccinated (R05-028) rhesus macaques possessing the MHC-I haplotype *90-120-Ib* were challenged intravenously with 1000 TCID50 of SIVmac239. The latter R05-028 were immunized intranasally with F-deleted SeV-Gag approximately 3 months before the challenge.

In the third, three rhesus macaques received FMSIV plus mCAT1-expressing DNA vaccination three times with intervals of 4 weeks. The FMSIV DNA was constructed by replacing *nef*-deleted $SHIV_{MD14YE}$ with Friend murine leukemia virus (FMLV) *env*, carrying the same SIVmac239-derived antigen-coding regions with SIVGP1, as described before [16]. Vaccination of macaques with FMSIV and a DNA expressing the FMLV receptor (mCAT1) [17] three times with intervals of a week was previously shown to induce mCAT1-dependent confined FMSIV replication resulting in efficient CTL induction while vaccination three times with intervals of 4 weeks in the present study resulted in marginal levels of responses (data not shown). These three DNA-vaccinated animals were challenged intravenously with 1000 TCID50 of SIVmac239 approximately 2 months after the last vaccination.

Some animal experiments were conducted in the Tsukuba Primate Research Center, National Institute of Biomedical Innovation, with the help of the Corporation for Production and Research of Laboratory Primates, in accordance with the guidelines for animal experiments at the National Institute of Infectious Diseases, and others were in Institute for Virus Research, Kyoto University in accordance with the institutional regulations.

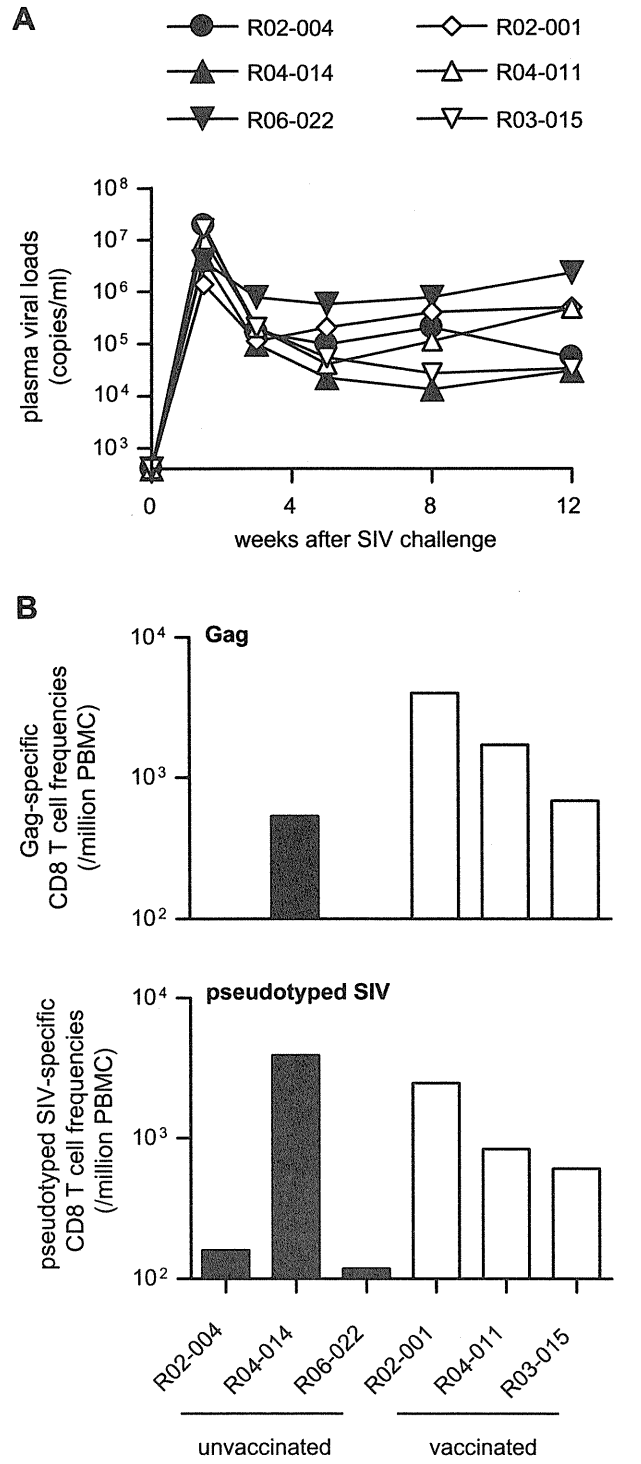

Fig. 1. CTL responses after SIVmac239 challenge in *90-088-Ij*-positive macaques. (A) Plasma viral loads after SIV challenge in unvaccinated (R02-004, R04-014, and R06-022) and DNA/SeV-Gag vaccinated animals (R02-001, R04-011, and R03-015). The viral loads (SIV gag RNA copies/ml) were determined as described previously [7]. (B) Vaccine antigen Gag-specific (upper panel) and pseudotyped SIV-specific $CD8^+$ T cell frequencies (lower panel) at week 2 after SIV challenge.

2.2. Analysis of virus-specific CTL responses

We measured virus-specific $CD8^+$ T-cell levels by flow cytometric analysis of gamma interferon (IFN-γ) induction after specific

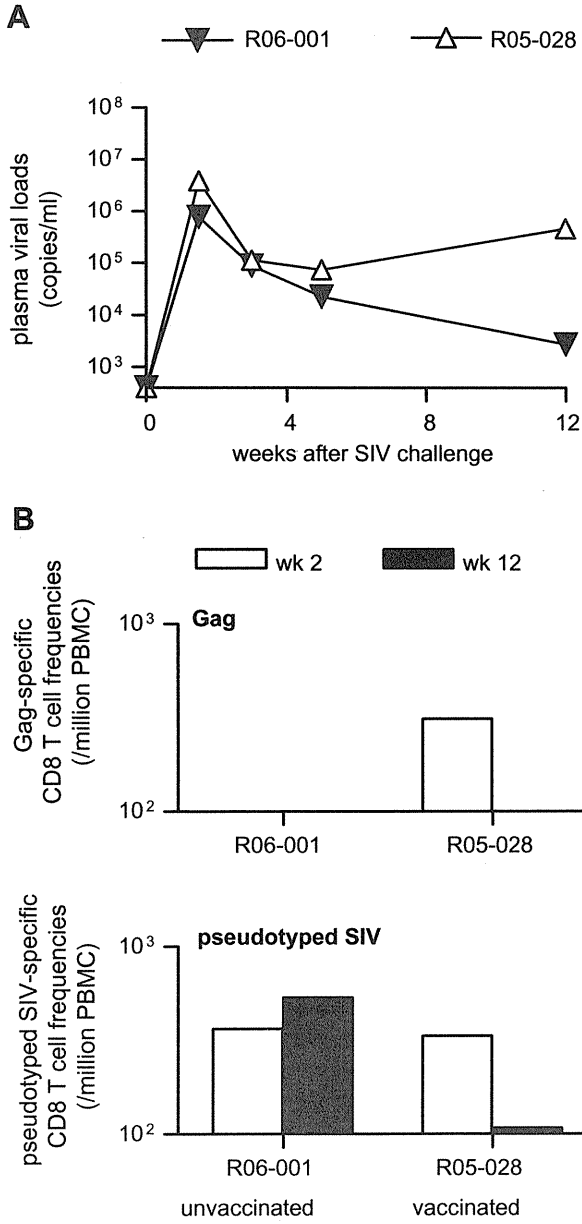

Fig. 2. CTL responses after SIVmac239 challenge in *90–120-Ib*-positive macaques. (A) Plasma viral loads after SIV challenge in unvaccinated R06–001 and SeV-Gag-vaccinated macaque R05-028. (B) Vaccine antigen Gag-specific (upper panel) and pseudotyped SIV-specific $CD8^+$ T cell frequencies (lower panel) at weeks 2 (white bars) and 12 (black bars) after SIV challenge.

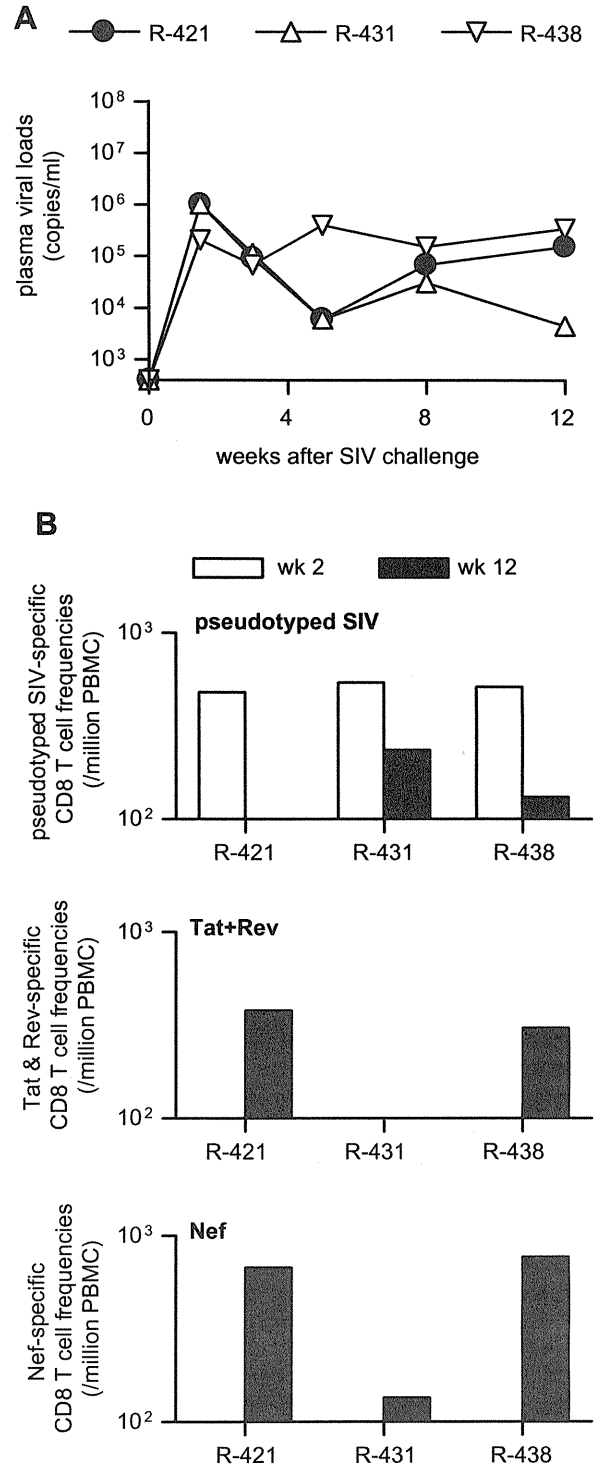

Fig. 3. CTL responses after SIVmac239 challenge in DNA-vaccinated macaques. The DNA used for the vaccination has the SIVmac239-derived region encoding Gag, Pol, Vif, and Vpx and is expected to induce pseudotyped SIV-specific CTL responses. (A) Plasma viral loads after SIV challenge in DNA vaccinated macaques R-421, R-431, and R-438. (B) Vaccine antigen (pseudotyped SIV)-specific (top panel), Tat-plus-Rev-specific (middle panel), and Nef-specific $CD8^+$ T cell frequencies (bottom panel) at weeks 2 (white bars) and 12 (black bars) after SIV challenge. In macaque R-438, CTL responses at week 5 instead of week 12 are shown.

stimulation as described previously [18,19]. Peripheral blood mononuclear cells (PBMCs) were cocultured with autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines (B-LCLs) infected with a vaccinia virus vector expressing SIVmac239 Gag for Gag-specific stimulation or a vesicular stomatitis virus G protein (VSV-G)-pseudotyped SIV for pseudotyped SIV-specific stimulation. The pseudotyped SIV was obtained by cotransfection of COS-1 cells with a VSV-G-expression plasmid and SIVGP1 DNA. Alternatively, PBMCs were cocultured with B-LCLs pulsed with peptide pools using panels of overlapping peptides spanning the entire SIVmac239 Tat, Rev, and Nef amino acid sequences. Intracellular IFN-γ staining was performed with a CytofixCytoperm kit (Becton Dickinson, Tokyo, Japan) and fluorescein isothiocyanate-conjugated anti-human CD4, peridinin chlorophyll protein-conjugated anti-human CD8, allophycocyanin-conjugated anti-human CD3, and phycoerythrin-conjugated anti-human IFN-γ monoclonal antibodies (Becton Dickinson). Specific $CD8^+$ T-cell levels were calculated by subtracting nonspecific IFN-γ^+ $CD8^+$ T-cell frequencies from those after Gag-specific, pseudotyped

	vaccine antigen					non-vaccine antigen										
	Gag				Vif	Vpr	Tat					Rev		Nef		
	165	333	375	376	143	73	23	115	120	122	125	45	50	63	100	124
wk 5																
R-421					++											
R-431					+											
R-438	++		+							++						
wk 12																
R-421		++			++				+		+	+	+			++
R-431					+		+			++						
R-438	++			++		+		++						++	++	

Fig. 4. Viral mutations in DNA-vaccinated macaques. Plasma viral genome sequencing was performed as described previously [18] to determine mutations resulting in amino acid substitutions in SIV Gag, Pol, Vif, Vpx, Vpr, Tat, Rev, and Nef antigens (except for Env) at weeks 5 and 12 in DNA-vaccinated macaques. The amino acid positions showing mutant sequences dominantly (++) or equivalently with wild type (+) are shown. While we found a mutation leading to a lysine-to-arginine alteration at the 40th amino acid in Rev in all animals, this mutation is not shown because the wild-type sequence at this position in the SIVmac239 molecular clone is considered to be a suboptimal nucleotide that frequently reverts to an alternative sequence in vivo [18,23].

SIV-specific, or peptide-specific stimulation. Specific $CD8^+$ T-cell levels lower than 100 per million PBMCs were considered negative.

3. Results and discussion

In our previous SIVmac239 challenge experiments, the prophylactic DNA/SeV-Gag vaccination did not result in viral control in rhesus macaques possessing the MHC-I haplotype *90-088-Ij*. These vaccinated animals showed similar levels of plasma viral loads as those in unvaccinated *90-088-Ij*-positive animals after SIV challenge (Fig. 1A). Analysis of virus-specific $CD8^+$ T-cell responses using PBMCs at week 2 after challenge showed equivalent Gag-specific and pseudotyped SIV-specific (Gag-, Pol-, Vif-, and Vpx-specific) CTL responses in all three vaccinees (Fig. 1B). Pseudotyped SIV-specific CTL responses were also detected in all three unvaccinated animals, but Gag-specific CTL responses were undetectable in two out of the three; even the Gag-specific CTL responses detected in macaque R04-014 were much lower than pseudotyped SIV-specific CTL responses, indicating dominant induction of CTL responses specific for SIV antigens other than Gag (Fig. 1B). Thus, in the acute phase of SIV infection, SIV non-Gag antigen-specific CTL responses were dominantly induced in unvaccinated *90-088-Ij*-positive macaques, whereas vaccine antigen (Gag)-specific CTL responses were dominant in *90-088-Ij*-positive vaccinees.

We then analyzed another vaccinees that failed to control a SIVmac239 challenge; these macaques were vaccinated with SeV-Gag alone or DNA alone. First, we compared post-challenge CTL responses in unvaccinated and SeV-Gag-vaccinated macaques possessing the MHC-I haplotype *90-120-Ib*. Both macaques failed to control SIV replication after challenge (Fig. 2A). In the unvaccinated animal R06-001, Gag-specific CTL responses were undetectable but pseudotyped SIV-specific CTL responses were induced efficiently at weeks 2 and 12 (Fig. 2B). In contrast, Gag-specific CTL responses were induced efficiently at week 2 in the SeV-Gag-vaccinated animal R05-028 (Fig. 2B). At week 12, Gag-specific CTL responses became undetectable while pseudotyped SIV-specific CTL responses were still detectable in this animal. These results indicate that, in the acute phase after SIVmac239 challenge, the unvaccinated *90-120-Ib*-positive macaque dominantly elicited SIV non-Gag antigen-specific CTL responses whereas the SeV-Gag-vaccinated *90-120-Ib*-positive macaque dominantly induced vaccine antigen (Gag)-specific CTL responses.

Next, we analyzed post-challenge CTL responses in three DNA-vaccinated macaques. These animals failed to control SIVmac239 replication after challenge (Fig. 3A). The DNA used for the vaccination and the pseudotyped SIV genome both have the same SIVmac239-derived region encoding Gag, Pol, Vif, and Vpx, thus expected to induce pseudotyped SIV-specific CTL responses. Pseudotyped SIV-specific CTL responses, namely vaccine antigen-specific CTL responses, were induced efficiently at week 2 but diminished after that in all three animals (Fig. 3B). In contrast, Tat/Rev- and Nef-specific CTL responses were undetectable at week 2 but induced later (Fig. 3B). Again, vaccine antigen-specific CTL responses were dominantly induced in the acute phase after SIV challenge and non-vaccine antigen-specific CTL responses were elicited later.

All three animals showed viral genome mutations leading to amino acid substitutions in Gag or Vif at week 5 (Fig. 4). Further analysis indicated that viral mutations in vaccine antigen-coding regions appeared earlier than those in other regions. These results may reflect selective pressure on SIV by vaccine antigen-specific CTL responses dominantly induced in the acute phase, although it remains undetermined whether these mutations are CTL escape ones. Disappearance of vaccine antigen-specific CTL responses at week 12 may be explained by rapid selection of CTL escape mutations in vaccine antigen-coding regions. However, analysis using peptides found Gag-specific CTL responses in macaques R-421 and R-431 that had no gag mutations at week 5 (data not shown), suggesting involvement of immunodominance [20] in the disappearance of vaccine antigen-specific CTL responses at week 12.

In summary, the present study indicates that vaccine antigen-specific CTL responses are induced dominantly in the acute phase after viral exposure, with delayed induction of CTL responses specific for SIV non-vaccine antigens (SIV antigens other than vaccine antigens). While this delay previously-observed in vaccine-based SIV controllers [10] can be explained not only by immunodominance but also by reduction in viral loads, the delay in vaccinated non-controllers in the present study might reflect the immunodominance in CTL responses. Thus, in development of a prophylactic, CTL-inducing AIDS vaccine, it is important to select vaccine antigens leading to effective CTL responses post-viral

exposure [21,22]. These results imply a significant influence of prophylactic vaccination on the immunodominance pattern of CTL responses post-viral exposure, providing insights into antigen design in development of a CTL-inducing AIDS vaccine.

Acknowledgments

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ORIGINAL PAPER

ULBP4/RAET1E is highly polymorphic in the Old World monkey

Taeko K. Naruse · Yukiko Okuda · Kazuyasu Mori · Hirofumi Akari · Tetsuro Matano · Akinori Kimura

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Abstract Natural-killer group 2 member D (NKG2D) is an activating receptor that plays an important role in the immune response mediated by NK cells, $\gamma\delta^+$ T cells, and $CD8^+$ T cells. In humans, MHC class I chain-related genes and UL-16 binding protein (ULBP)/retinoic acid early transcript 1 (REAT1) gene family encode ligands for NKG2D. The rhesus and crab-eating macaques, which belong to the Old World monkeys, are widely used as non-human primate models in medical researches on the immunological process. In the present study, we investigated the polymorphisms of *ULBP4/RAET1E*, a member of the *ULBP/RAET1* family, and found 25 and 14 alleles from the rhesus and crab-eating macaques, respectively, of which diversities were far more extended than in humans. A phylogenetic study suggested that the allelic diversification of ULBP4/RAET1E predated the divergence of rhesus and crab-eating macaques.

T. K. Naruse · A. Kimura (✉)
Department of Molecular Pathogenesis, Medical Research Institute, Tokyo Medical and Dental University,
1-5-45 Yushima, Bunkyo-ku,
Tokyo 113–8510, Japan
e-mail: akitis@mri.tmd.ac.jp

Y. Okuda · A. Kimura
Laboratory of Genome Diversity, Graduate School of Biomedical Science, Tokyo Medical and Dental University,
Tokyo, Japan

K. Mori · T. Matano
AIDS Research Center, National Institute of Infectious Diseases,
Tokyo, Japan

H. Akari
Primate Research Institute, Kyoto University,
Inuyama, Japan

T. Matano
International Research Center for Infectious Diseases,
The Institute of Medical Science, The University of Tokyo,
Tokyo, Japan

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Introduction

Non-human primates, such as rhesus and crab-eating macaques, are important animal models for the study of infectious diseases, autoimmune diseases, and organ transplantation. These macaques are members of the Old World monkeys, and it has been reported that the genetic diversity in the rhesus macaque is quite unique, that is, more than 60% of the rhesus macaque-specific expansions are found in the protein coding sequences (Gibbs et al. 2007). To evaluate the results of immunological experiments in the macaque models, it is essential to characterize the genetic diversity of immune-related molecules which may control the individual differences in the immune response against foreign antigens and/or pathogens. It has been reported that the gene copy number in the major histocompatibility complex (MHC) loci in the rhesus and crab-eating macaques is higher than that in humans (Kulski et al. 2004; Gibbs et al. 2007; Otting et al. 2007). In addition, the extent of genetic diversity differed, in part, depending on the geographic areas, and we have reported that the diversity of MHC class I genes in the rhesus macaque is considerably different depending on habitat (Naruse et al. 2010).

Because the innate immune system is involved in the response to environmental pathogens, it is necessary to consider the function of natural killer (NK) cells in the experimental animal models. Natural-killer group 2 member D (NKG2D), a C-type lectin molecule, is an activating receptor expressed on the cell surface of NK, $\gamma\delta^+$, and $CD8^+$ T cells, which plays an important role in the immune response (Wu et al. 1999; Raulet 2003). In humans, MHC class I chain-related genes (MIC) and UL-16 binding protein (ULBP)/retinoic acid early transcript 1 (REAT1)

gene family are known to encode ligands for NKG2D (Bauer et al. 1999; Cosman et al. 2001; Chalupny et al. 2003; Bacon et al. 2004). These ligand molecules are usually stress-inducible, and their recognition by NKG2D can lead to the activation of NK cells, consequently killing virus-infected and tumor cells (Pende et al. 2002; Eagle et al. 2006; Pappworth et al. 2007; Ward et al. 2007).

The human *ULBP/RAET1* gene family is located on chromosome 6q24.2, which is composed of ten members including six functional genes, *ULBP1*, *2*, *3*, *4*, *5*, and *6*, corresponding to *RAET1I*, *H*, *N*, *E*, *G*, and *L*, respectively (Radosavljevic et al. 2001; Chalupny et al. 2003; Eagle et al. 2009a, b). In addition, several sequence polymorphisms in each *ULBP* gene have been identified (Romphruk et al. 2009; Antoun et al. 2010). Although it is evident that the cell surface expression of the ligand molecules on target cells is differentially regulated (Eagle et al. 2006), genetic polymorphisms in the coding regions might have a functional impact. We have previously investigated the genetic polymorphisms of *ULBP/RAET1* genes and have found that the *ULBP4/RAET1E* gene is the most polymorphic, with the allelic distribution differing among ethnic groups (Romphruk et al. 2009).

On the other hand, rhesus macaque *ULBP4/RAET1E* (GenBank: NW_001116520) is mapped on the long arm of chromosome 4 (i.e., positions from 31, 164, 822 to 31, 175, 032 of chromosome 4 in the rhesus genome; data obtained from the UCSC Genome Browser at http://genome.ucsc.edu/cgi-bin/hgGateway; Gibbs et al. 2007). However, its genetic polymorphisms are poorly characterized, although the MIC gene polymorphisms are well studied in the rhesus macaque (Seo et al. 1999, 2001; Doxiadis et al. 2007; Averdam et al. 2007). In the present study, we investigated the polymorphisms of *ULBP4/RAET1E* in rhesus and crab-eating macaques. This is the first report demonstrating the extreme diversity of the NKG2D ligand in the Old World monkey.

Materials and methods

Animals

A total of 38 rhesus macaques from seven lineages previously analyzed for the MHC polymorphisms (Naruse et al. 2010) and 24 crab-eating macaques from five lineages were the subjects. They were maintained in the breeding colonies in Japan. The founders of the rhesus macaque colonies were captured in Myanmar and Laos, whereas the founders of crab-eating macaque colonies were captured in Indonesia, Malaysia, and the Philippines. All care, including blood sampling of animals, were in accordance with the guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication 85–23, revised 1985) and were subjected to prior approval by the local animal protection authority.

DNA extraction and sequencing analysis

Genomic DNAs from B lymphoblastoid cell lines of the rhesus macaque (Naruse et al. 2010) and from whole blood sample of the crab-eating macaque were extracted by using the QuickGene DNA kit (Fujifilm, Tokyo, Japan) according to the manufacturer's instructions. The genomic gene for *ULBP4/RAET1E* of rhesus and crab-eating macaques was amplified by polymerase chain reaction (PCR) with a primer pair designed for the region spanning from introns 1 to 3 of the rhesus gene (NC007861), ULBP4F (5′-TGGGCCTCTTCCCCTGTCC) and ULBP4R (5′-GTGGGAGGTGGGATGGG), using FastStart Taq DNA polymerase (Roche, Mannheim, Germany). The PCR condition was composed of the following steps: denaturation at 95°C for 4 min; 30 cycles of 95°C for 30 s, 63°C for 30 s, and 72°C for 45 s; and additional extension at 72°C for 7 min. The PCR products of about 1,200 bp in length were cloned into pSTBlue-1 AccepTer vector (Novagen, WI, USA) according to the manufacturer's instructions and were transformed to Nova Blue Single™ competent cells (Merck4Biosciences Japan, Tokyo, Japan). Ten to 20 independent transformant colonies were picked up for each sample and subjected to sequencing on both strands by using a BigDye Terminator cycling system and an ABI 3730 automated sequence analyzer (Applied Biosystems, CA, USA).

Data analyses

Nucleotide sequences of *ULBP4/RAET1E* from cloned DNAs were aligned using the Genetyx software package (version 8.0, Genetyx Corp., Japan). If at least three clones from independent PCR or from different individuals showed identical sequences, the sequences were submitted to the DNA Data Bank of Japan (DDBJ). Neighbor-joining trees were constructed with Kimura's 2-parameter method for a phylogenetic analysis of *ULBP4/RAET1E* sequences spanning from exons 2 to 3 including intron 2 by using the Genetyx software. Bootstrap values were based on 5,000 replications. The *ULBP4/RAET1E* sequences from humans (GenBank accession number AY252119), chimpanzees (AY032638), and rhesus (NC007861) were included in the analysis as references.

Structure model analysis

A three-dimensional (3-D) structure model of rhesus ULBP4/RAET1E, with amino acid positions from 1 to 178, was created by a molecular visualization software RasTop2.2 (http://sourceforge.net/projects/rastop/), and the