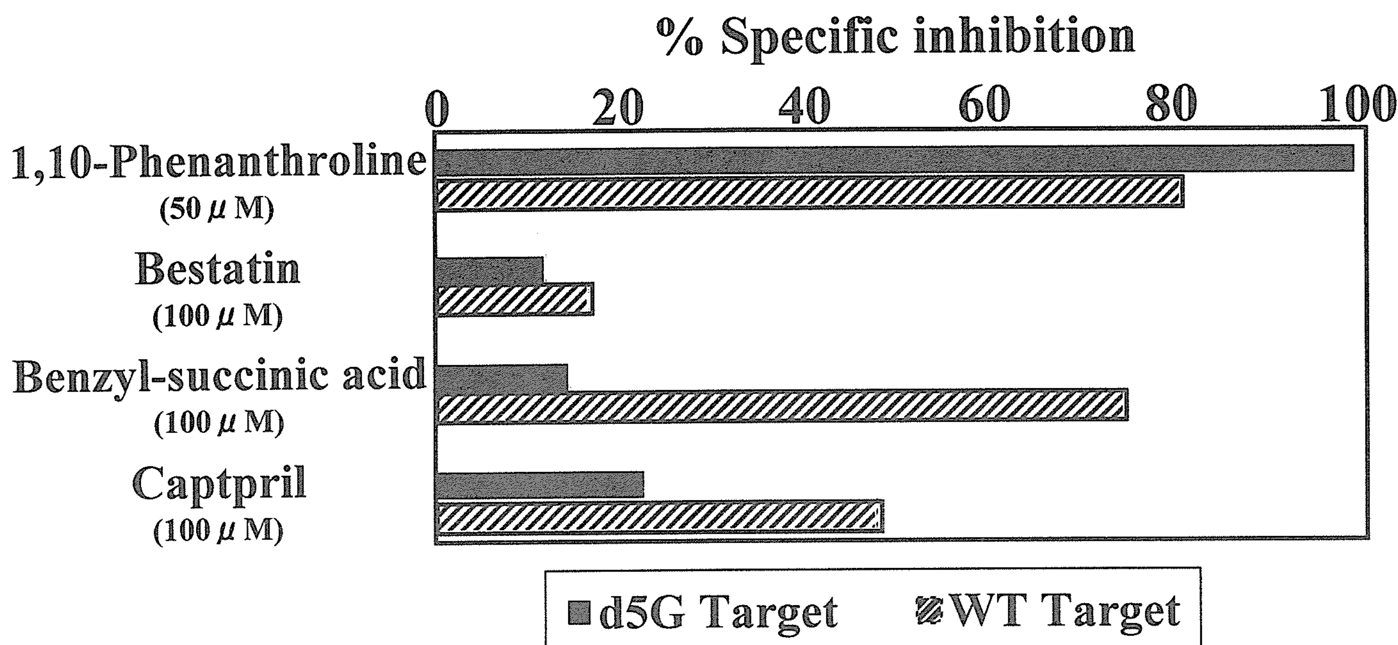
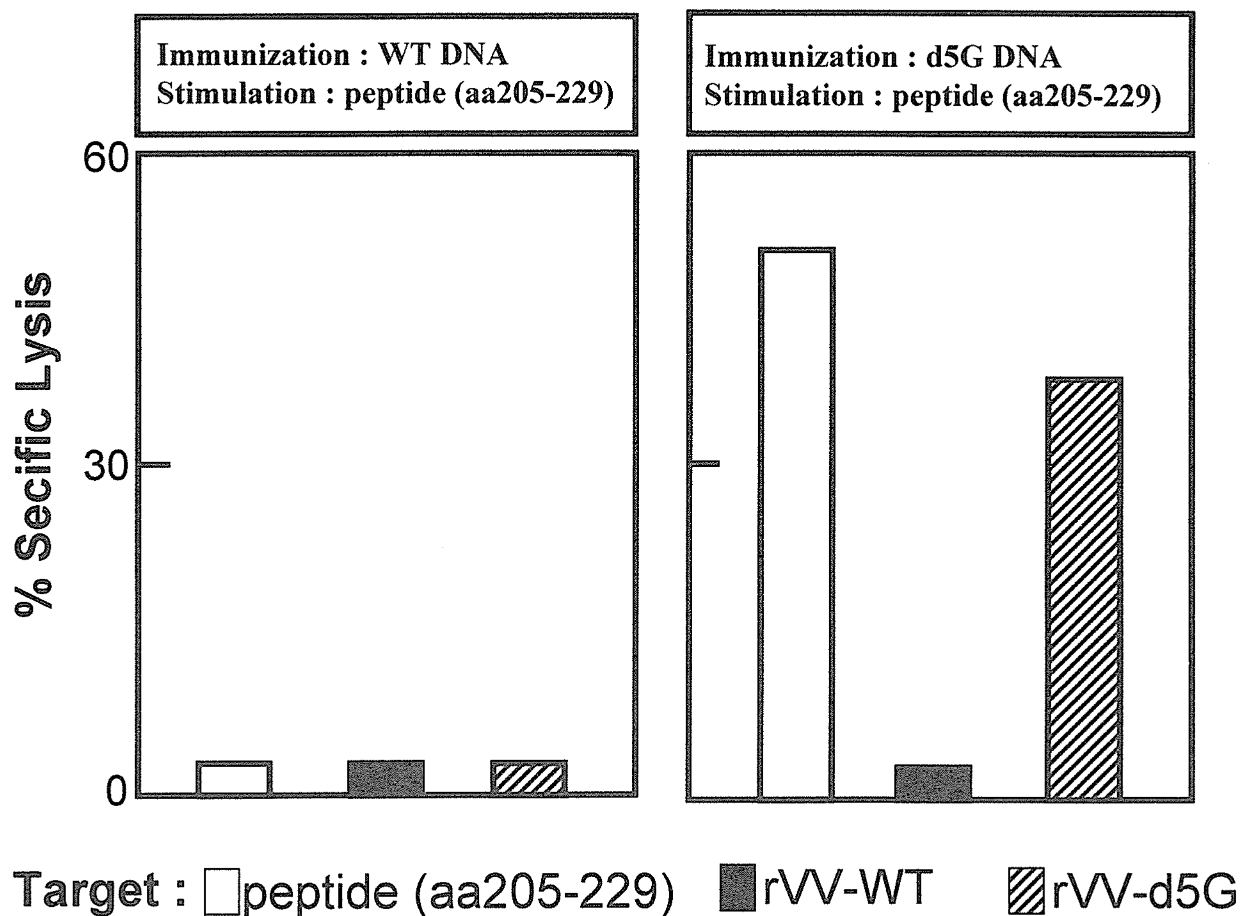


Inhibitor	Specificity
1,10-Phenanthroline	All metalloproteases and caspase-1
Bestatin	Metallo-aminopeptitases
Benzyl-succinic acid	Metallo-carboxypeptidases A and B
Captpril	ACE and ACE-like metalloprotease

Fig. 5. SIVenv WTおよびSIVd5G組み込みワクシニアウイルス感染標的細胞の酵素による処理
SIVenv WTおよびSIVd5G組み込みワクシニアウイルス感染標的細胞を各種酵素で処理し、DNAワクチンにより誘導されたCTLをエフェクター細胞とした時の細胞傷害活性。



<amino acid sequence>
aa.205-229 RCYMNHCNTSVIQESCDKHYWDAIR

Fig. 6 d5GとWTのエピトープ特異的CTLの誘導とCTLエピトープの発現
d5GおよびWT DNAワクチン免疫マウスの脾細胞をペプタイド(aa205-229)で刺激し、標的細胞をペプタイド、d5G組み込みワクシニアウイルス、WT組み込みワクシニアウイルスとしてCTL活性を比較した。

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分担研究報告書

SIV 複製及び宿主免疫回避に関わるウイルス側・宿主因子側の作用機構に関する解析

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研究要旨：本分担研究では、これまで HIV 及び SIV 由来 nef に共通する MHC-I 発現抑制機構を解明するための基盤として、HIV 由来 Nef 蛋白において同機能に寄与するアミノ酸残基の特定を試み、HIV/SIV 間で保存性の高い N 末端領域における Trp13, Val16 及び Met20 の 3 アミノ酸残基が MHC-I 発現制御に寄与していることを示した。そこで本年度は、このモチーフの意義を明らかにする目的で構造解析シミュレーションを行なった結果、本モチーフが輸送関連因子 AP-1 複合体の構成分子である μ1A サブユニットの結合ドメインであることを示唆する結果を得た。本研究成果は Nef による宿主免疫回避のメカニズムを解明するための重要な知見であると考えられる。

A．研究目的

アクセサリー遺伝子の一つである nef 遺伝子は宿主免疫の制御機能を司っていることが知られており、ウイルス感染細胞における MHC class-I 発現を抑制することで CTL による抗原認識を回避する。このような Nef 機能は HIV, SIV 共に認められ、機能的に補完性があることが知られている。そこで本研究では、HIV および SIV 由来 nef 遺伝子に共通する MHC class-I 発現抑制機構を明らかにするとともに、その機能発現に関わる宿主側因子の役割を解析することを最終目標とした。

昨年度まで我々は、Nef 蛋白において同機能に寄与するアミノ酸残基の特定を試みた。その結果、HIV/SIV 間で保存性の高い N 末端側 Val16 及び Met20 に加え、Trp13 が MHC-I 発現制御に寄与していることが明らかとなった。

今年度は、まず昨年度までに作成した各種 Nef 変異体を用いて、MHC class-I 発現抑制作用に寄与するアミノ酸残基の定量的機能解析を行なった。次に、本機能ドメイン近傍における NMR データおよび我々がこれまでに得たウイルス学的特性に関する解析結果を踏まえて、本機能領域に結合する宿主因子を推定し立体構造シミュレーションを行なった。

B．研究方法

env 欠損型 HIV-1 分子クローンである pNL43Δenv を基に、Nef N 末端側においてサブタイプ間で保存性の高いアミノ酸残基に各種点変異を導入した。これらを VSV-G 発現ベクターと共に HeLa 細胞へ遺伝子導入し、48 時間後 Nef 変異体を含む VSV-G シュードタイプウイルス産生細胞を回収した。ウイルスを CEM-GFP 細胞に感染 2-3 日後、ウイルス感染細胞（GFP 陽性）の細胞表面上 MHC-I の発現量を flow cytometry にて解析した。同時に得られたウイルス産生細胞・ウイルス上清について、Western blotting 法により Nef 蛋白をはじめとするウイルスタンパク質の解析を行った。

Nef の N 末端領域の分子構造について、これまでに報告されている NMR データと上記の変異体解析の結果をあわせ、PYMOL ソフトウエアを用いた構造解析シミュレーションを行った。

C．研究結果

まず HIV/SIV 間で保存性の高い N 末端側におけるアミノ酸残基の点変異体について、定量的な MHC-I 発現抑制能を検討した。その結果、Trp13 は Met20 と同程度に機能発現に重要である一方、Val16 は両者と比較して中等度の機能への関与が示された。これらの変異体は、全て細胞内での定常的発現量及びウイルス粒子取り込み効率に差が見られなかった（図１）。すなわち MHC-I 発現抑制能における重要度は Met20=Trp13>Val16 と考えられる。

ところでこの保存性の高い N 末端側 Nef 領域は、αヘリックス構造を取ることが知られている。そこで PDB データを基にこの領域の構造シミュレーションを行なった（図２）。興味深いことに、保存性が高く、塩基性側鎖を有するアミノ酸残基（Arg17,19,21,22）はほぼ同方向側に局在する。この極性側鎖は、細胞脂質二重膜のリン脂質と相互作用することにより Nef 蛋白のラフト分画への局在に寄与すると考えられる。他方、疎水性側鎖を有するアミノ酸残基（Trp13, Val16, Met20）は前述の塩基性アミノ酸残基と対極に分布することが推定された。このことから、細胞膜に局在する Nef においてこれらの疎水性アミノ酸残基は細胞質側に向かい、MHC-I 発現抑制に関わる何らかの宿主因子への結合ドメインとなっていることが予想された。

これまで非常に多くの研究グループにより Nef 結合蛋白の探索が成されてきたにも関わらず、本疎水性ドメインへ結合する因子は同定されていない。ところで、近年 Collins らの研究グループは MHC-I 発現抑制を規定する宿主因子として、輸送関連因子 AP-1 複合体の構成分子であるμ1A サブユニットを提唱した（Roeth et al, J Cell Biol, 2005）。しかし Nef 蛋白におけるμ1A 蛋白結合モチーフ（Tyrosine motif; Yxxφ, di-leucine motif; D/ExxxLL）は C 末端側に存在し、かつこれらのモチーフは MHC-I 発現抑制に関与していないことが証明されていることから、Nef の MHC-I 発現抑制作用におけるμ1A サブユニットの意義は不明なままである。

ここで我々は、Trp13 を Tyr に置換してもほぼ正常な MHC-I 発現抑制機能を維持するというデータ（図１）に着目した。すなわち、Trp は Tyr と同様芳香族側鎖を有することから、Tyrosine motif として機能するのではないかと想定した。そこで、報告されているμ2（1BW8）の PDB ファイルを基に、SWISS-MODEL SERVER の THEORETICAL MODEL システムを使い、μ2 とのアラインメントその他のパラメータからμ1A のモデリングを行った後、PYMOL ソフトウエアにて Nef N 末端（９−２６アミノ酸残基）の結合をシミュレーションした。

その結果、μ1A との結合が報告されている EGFR におけるモデリングと同様、Nef N 末端の Trp13, Val16, Met20 が良くフィットすることが確認された。特に重要なことに、μ1A には存在するがμ2 には存在しないポケット構造が Met20 の疎水性側鎖を収納することで高い親和性とμ1A の特異性を示す事が示唆された。μ2 ではこの箇所がむしろ突出した形状を示しており、Met20 の側鎖が収納され無いことから、Nef N 末端との低親和性に繋がると類推される（図３）。

D．考察

Nef による MHC-I 発現抑制作用はエイズウイルスの生体内持続感染に重要な役割を持つことが知られている。本研究により、これまで明らかにされていなかった N 末端領域における本機能ドメイン WVM motif を同定した。さらに、このモチーフが AP-1 複合体の構成分子であるμ1A サブユニットと結合するモデルを構築した。

最近、Fc receptor における Trp-based motif が AP-2 μサブユニットとの結合ドメインとして機能していると報告されており（Wernick et al., J Biol Chem 280, 7309, 2005）、Nef の場合でも同様にμ1A と結合することが強く示唆される。特に MHC-I 発現抑制作用に特に重要な Met20 の binding pocket が、μ1A 特異的に存在していたことは、この考えを支持するものである。

これまでの Nef 結合蛋白の探索において、μ1A が同定されてこなかった理由として、多くの実験が Nef による MHC-I 発現抑制が機能しない細胞種、すなわち HeLa や 293T といった付着系細胞を使用

したことが挙げられる。また、N末端に存在するミリストイル基の存在しない大腸菌等を実験系に用いていたこともその一因と思われる。

現在、WVM motifとμ1Aの結合を直接的に証明するための co-immunoprecipitation 実験を進めているところである。また同時に、SIV由来Nef蛋白においても同様にMHC-I発現抑制に関わる機能ドメインの探索を進めている。

E．結論

本年度は、我々が新たに同定したNefにおけるMHC-I発現抑制作用を規定する疎水性アミノ酸モチーフ（Trp13, Val16, Met20）の意義を明らかにするため、詳細な構造解析シミュレーションを行なった。その結果、本モチーフが輸送関連因子AP-1複合体の構成分子であるμ1Aサブユニットの結合ドメインであることを示唆する結果を得た。本研究成果はNefによる宿主免疫回避のメカニズムを解明するための重要な知見であると考えられる。

F．健康危険情報

なし。

G．研究発表

1　論文発表

1) Hara M , Kikuchi T, Sata T, Nakajima N, Ami Y, Sato Y, Tanaka K, Narita T, Ono F, Akari H, Terao K, Mukai R. Detection of SRV/D shedding in body fluids of cynomolgus macaques and comparison of partial gp70 sequences in SRV/D-T isolates. Virus Genes, in press.

2) Ishii K, Iijima S, Kimura N, Lee Y-J, Ageyama N, Yagi S, Yamaguchi K, Maki N, Yoshizaki S, Machida S, Suzuki T, Iwata N, Sata T, Terao K, Miyamura T, Akari H: GBV-B as a pleiotropic virus: Distribution of GBV-B in extrahepatic tissues *in vivo. Microbes and Infection, in press.*

2　学会発表

(1) Iijima S, Lee Y-J, Arold S, Strebel K, Akari H. Tripartite hydrophobic residues as a potential myristoyl pocket are a determinant for MHC-I down-regulation by HIV-1 Nef. Cold Spring Harbor meeting on Retroviruses, New York, May 2006.

H．知的財産権の出願・登録状況

なし。

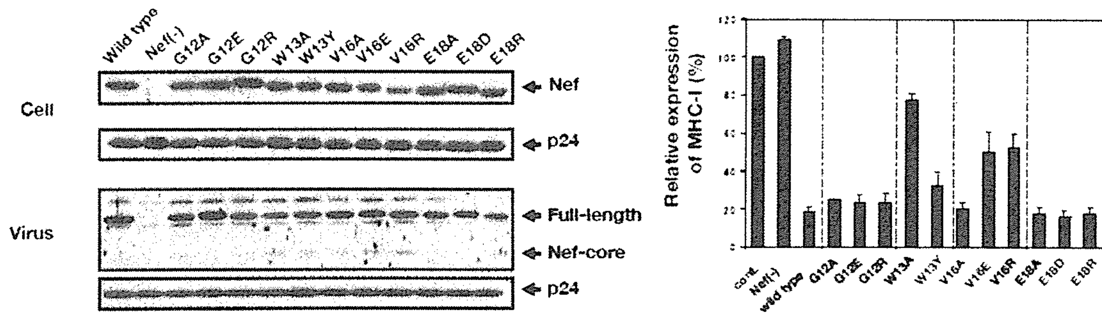

図１：Nef N 末端領域の各種点変異体における機能解析

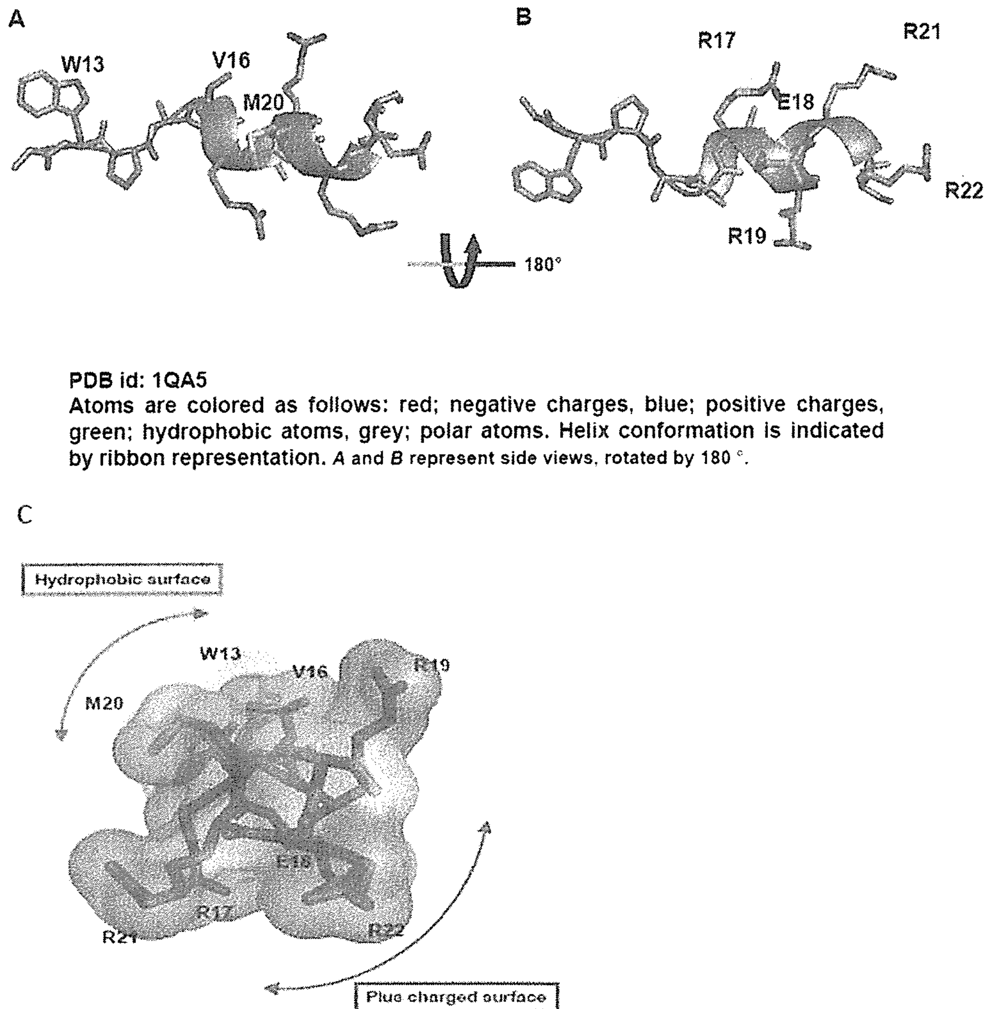

図２：Nef N 末端領域の構造シミュレーション解析

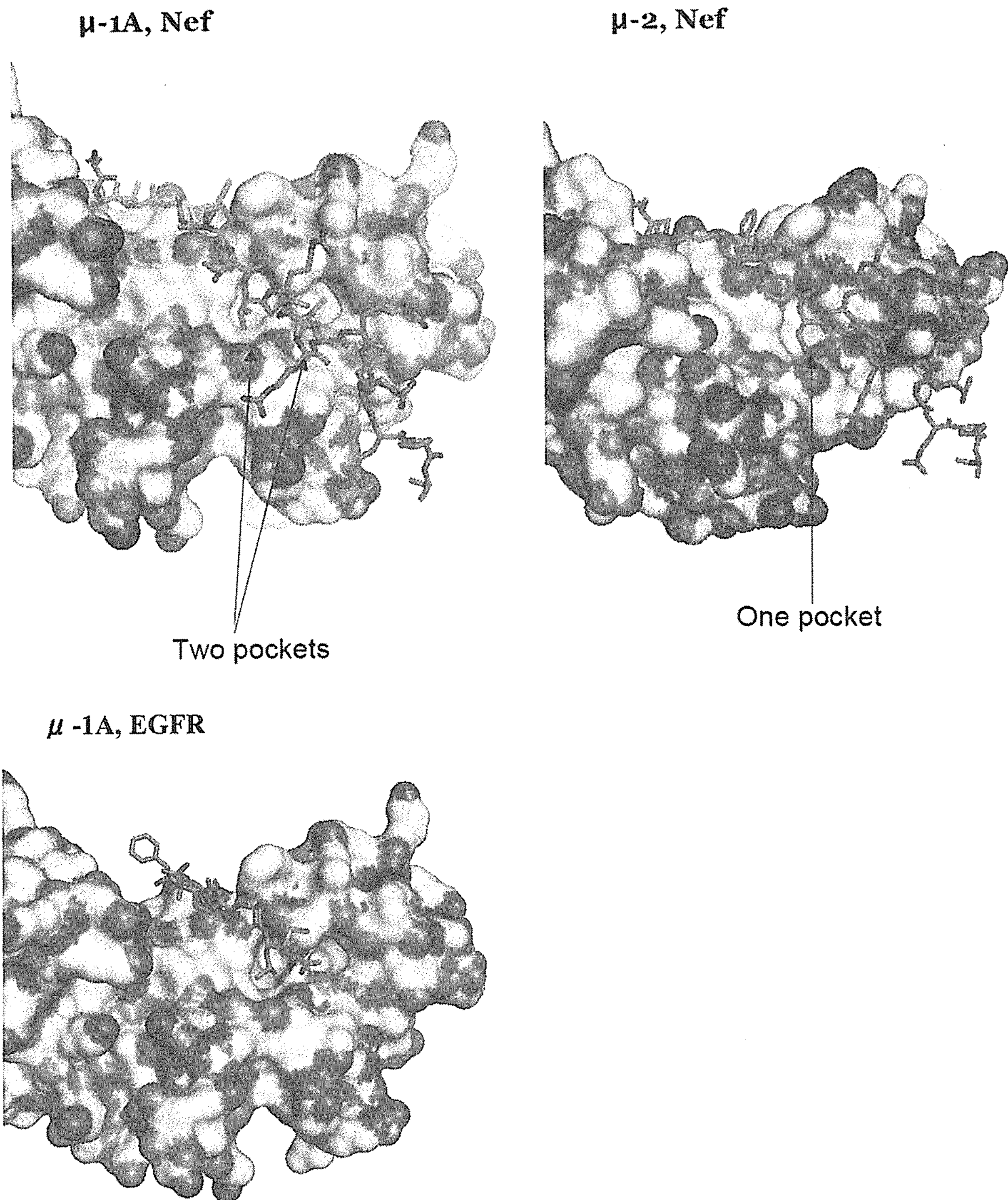

図３：PYMOL による Nef N 末端領域とアダプター蛋白複合体μサブユニットの結合シミュレーション解析

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

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
Yasutomi Y.	Chimeric recombinant hepatitis E virus–like particles presenting foreign epitopes as a novel vector of vaccine by oral administration.	Holland CR & Miyamura T	Structure-based viral replication.	World Scientific Publishing	USA	2007	in press

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Yamamoto H, Kawada M, Tsukamoto T, Takeda A, Igarashi H, Miyazawa M, Naruse T, Yasunami M, Kimura A, and Matano T.	Vaccine-based long-term stable control of simian-human immunodeficiency virus 89.6PD replication in rhesus macaques.	J Gen Virol	88	652-659	2007
Tanaka-Takahashi Y, Yasunami M, Naruse T, Hinohara K, Matano T, Mori K, Miyazawa M, Honda M, Yasutomi Y, Nagai Y, Kimura A.	Reference strand-mediated conformation analysis (RSCA) – based typing of multiple alleles in the rhesus macaques MHC class I Mamu-A and I Mamu-B loci.	Electrophoresis		in press	2007
Shibata H, Yasunami M, Obuchi N, Takahashi M, Kobayashi Y, Numano F, Kimura A.	Direct determination of SNP haplotype of NFKBIL1 promoter polymorphism by DNA conformation analysis and its application to association study of chronic inflammatory diseases.	Hum Immunol	67(4-5)	363-373	2006
Biasin M, Piacentini L, Lo Caputo S, Kanari Y, Magri G, Trabattoni D, Naddeo V, Lopalco L, Clivio A, Cesana E, Fasano F, Bergamaschi C, Mazzotta F, Miyazawa M, Clerici M.	APOBEC3G: A possible role in resistance of HIV-exposed seronegative individuals.	J Infec Dis		in press	2007
Kajikawa M, Baba T, Tomaru U, Watanabe Y, Koganei S, Tsuji-Kawahara S, Matsumoto N, Yamamoto K, Miyazawa M, Maenaka K, Ishizu A, Kasahara M.	MHC class I-like MILL molecules are β_2-microglobulin-associated, GPI-anchored glycoproteins that do not require TAP for cell surface expression.	J Immunol	177	3108-3115	2006
Kida Y, Tsuji-Kawahara S, Ostapenko V, Kinoshita S, Kajiwara E, Kawabata H, Yuasa T, Nishide I, Yukawa S, Ichinose M, Miyazawa M.	HLA-B polymorphism in Japanese HIV-1 infected long-term surviving hemophiliacs.	Cncer Immunol Immunother	55	1459-1469	2006

Hara M , Kikuchi T, Sata T, Nakajima N, Ami Y, Sato Y, Tanaka K, Narita T, Ono F, Akari H, Terao K, Mukai R.	Detection of SRV/D shedding in body fluids of cynomolgus macaques and comparison of partial gp70 sequences in SRV/D-T isolates.	Virus Genes		in press	2007
Ishii K, Iijima S, Kimura N, Lee Y-J, Ageyama N, Yagi S, Yamaguchi K, Maki N, Yoshizaki S, Machida S, Suzuki T, Iwata N, Sata T, Terao K, Miyamura T, Akari H.	GBV-B as a pleiotropic virus: Distribution of GBV-B in extrahepatic tissues *in vivo.*	Microbes and Infection		in press	2007

Ⅳ.　研究成果の刊行物・別刷

Vaccine-based, long-term, stable control of simian/human immunodeficiency virus 89.6PD replication in rhesus macaques

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The X4-tropic simian/human immunodeficiency virus (SHIV) 89.6P (or 89.6PD) causes rapid CD4$^+$ T-cell depletion leading to an acute crash of the host immune system, whereas pathogenic R5-tropic simian immunodeficiency virus (SIV) infection, like HIV-1 infection in humans, results in chronic disease progression in macaques. Recent pre-clinical vaccine trials inducing cytotoxic T lymphocyte (CTL) responses have succeeded in controlling replication of the former but shown difficulty in control of the latter. Analysis of the immune responses involved in consistent control of SHIV would contribute to elucidation of the mechanism for consistent control of SIV replication. This study followed up rhesus macaques that showed vaccine-based control of primary SHIV89.6PD replication and found that all of these controllers maintained viraemia control for more than 2 years. SHIV89.6PD control was observed in vaccinees of diverse major histocompatibility complex (MHC) haplotypes and was maintained without rapid selection of CTL escape mutations, a sign of particular CTL pressure. Despite the vaccine regimen not targeting Env, all of the SHIV controllers showed efficient elicitation of *de novo* neutralizing antibodies by 6 weeks post-challenge. These results contrast with our previous observation of particular MHC-associated control of SIV replication without involvement of neutralizing antibodies and suggest that vaccine-based control of SHIV89.6PD replication can be stably maintained in the presence of multiple functional immune effectors.

INTRODUCTION

The well-established importance of cytotoxic T lymphocyte (CTL) responses in the control of immunodeficiency virus replication has led the way to development of prophylactic AIDS vaccine regimens that augment virus-specific CTL responses (Borrow *et al.*, 1994; Koup *et al.*, 1994; Matano *et al.*, 1998; Ogg *et al.*, 1998; Jin *et al.*, 1999; Schmitz *et al.*, 1999; McMichael & Hanke, 2003; Goulder & Watkins, 2004). In a model of X4-tropic simian/human immunodeficiency virus (SHIV) 89.6P or 89.6PD infection (Reimann *et al.*, 1996; Lu *et al.*, 1998), which causes rapid CD4$^+$ T-cell depletion leading to an acute crash of the host immune system in macaques, several pre-clinical trials of prophylactic AIDS vaccines have successfully shown that efficient CTL induction results in control of virus replication and prevention of acute AIDS progression (Barouch *et al.*, 2000; Amara *et al.*, 2001; Matano *et al.*, 2001; Rose *et al.*, 2001; Shiver *et al.*, 2002; Willey *et al.*, 2003). In contrast, most trials of such CTL-based vaccines have failed to show viraemia control in models of R5-tropic simian immunodeficiency virus (SIV) infection, which result in chronic

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disease progression in macaques as in human immunodeficiency virus type 1 (HIV-1) infection in humans (Feinberg & Moore, 2002; Horton *et al.*, 2002; Casimiro *et al.*, 2005). Comparison of vaccine effects on virus replication in the acute AIDS model of X4-tropic SHIV infection with those in the chronic model of R5-tropic SIV infection could contribute to the development of an effective prophylactic AIDS vaccine for control of persistent HIV-1 replication.

We have developed a prophylactic AIDS vaccine using a DNA-prime/Gag-expressing Sendai virus (SeV-Gag) vector boost system and have shown its potential for efficient induction of Gag-specific CTL responses in Burmese rhesus macaques (Kano *et al.*, 2002; Matano *et al.*, 2004). In preclinical trials in an acute AIDS model, all of the macaques vaccinated with the DNA-prime/SeV-Gag vector boost system controlled SHIV89.6PD replication after challenge (Matano *et al.*, 2001; Takeda *et al.*, 2003). Furthermore, a trial of the prophylactic DNA-prime/SeV-Gag boost vaccine showed control of SIVmac239 replication leading to undetectable set-point plasma viraemia in five out of eight vaccinees (referred to as SIV controllers), despite failure of virus control in the other three vaccinees (referred to as SIV non-controllers) (Matano *et al.*, 2004). All of the SIV controllers showed rapid selection of viral CTL escape mutations, and analysis of the rhesus major histocompatibility complex (MHC) suggested that SIV control was associated with particular MHC haplotypes such as *90-120-Ia* and 'elite' CTL responses specific for the MHC-restricted epitopes (Matano *et al.*, 2004). Follow up of these SIV controllers revealed that some lost this control with accumulation of multiple viral CTL escape mutations (Kawada *et al.*, 2006).

In this study, we followed up, for more than 2 years, rhesus macaques that showed vaccine-based control of SHIV89.6PD replication (referred to as SHIV controllers). Our results showed durable and stable virus control in the SHIV controllers, contrasting with our previous observation in SIV controllers.

METHODS

Animal experiments. Ten vaccinated macaques used in our previous SHIV89.6PD challenge experiments (Matano *et al.*, 2001; Takeda *et al.*, 2003) were analysed in this study. The animal list is shown in Table 1. All were Burmese rhesus macaques (*Macaca mulatta*) and were maintained in accordance with the Guidelines for Laboratory Animals of the National Institute of Infectious Diseases and National Institute of Biomedical Innovation.

The immunization and challenge protocols have been described previously (Matano *et al.*, 2001; Takeda *et al.*, 2003). Of the ten macaques in the SHIV89.6PD challenge experiment, three (R00-013, R00-015 and R00-017) received a single intranasal immunization with replication-competent SeV expressing SIVmac239 Gag (SeV-Gag) (Kato *et al.*, 1996; Kano *et al.*, 2002) before challenge. Two (R99-007 and R99-011) received four immunizations with FMSIV DNA followed by a single SeV-Gag booster. The FMSIV plasmid DNA used in this DNA vaccination protocol (DNAv1) was constructed from an $SHIV_{MD14YE}$ molecular clone DNA (Shibata *et al.*, 1997a) by replacing SHIV *env* with ecotropic Friend murine leukemia virus (FMLV) *env*

Table 1. Summary of the vaccinees challenged with SHIV89.6PD

Animal	MHC I haplotype*	Vaccine protocol	Set-point virus load†	Virus load around year 2‡	Gag mutations around month 2§	Gag mutations after month 6ǁ
R00-013	ND	SeV-Gag	10^4–10^6	10^4–10^6 (at wk 52)	ND	ND
R00-015	*90-120-Ib*	SeV-Gag	<400	<400	None	None at wk 60
R00-017	*90-030-Ih*	SeV-Gag	<400	<400	None	None at wk 58
R99-007	ND	DNAv1/SeV-Gag	<400	<400 (at wk 28)	None	ND
R99-011	*90-010-Ie*	DNAv1/SeV-Gag	<400	<400	None	None at wk 51
R99-005	*90-010-Ie*	DNAv2/SeV-Gag	<400	<400	None	None at wk 49
R99-012	*90-030-Ih*	DNAv2/SeV-Gag	<400	<400	None	None at wk 51
R00-020	*90-122-Ie*	DNAv3/F^-SeV-Gag	<400	<400	None	None at wk 52
R00-023	ND	DNAv3/F^-SeV-Gag	<400	<400	None	None at wk 52
R00-024	*90-120-Ib*	DNAv3/F^-SeV-Gag	<400	<400	None	None at wk 52

*MHC I haplotype was determined by reference strand-mediated conformation analysis, as described previously (Arguello *et al.*, 1998; Matano *et al.*, 2004). MHC I haplotype *90-120-Ib* is derived from breeder R90-120, *90-010-Ie* from R90-010, *90-122-Ie* from R90-122 and *90-030-Ih* from R90-030. MHC I haplotypes *90-010-Ie* and *90-122-Ie* are identical.

†Plasma viral loads [RNA copies $(ml\ plasma)^{-1}$] around week 20.

‡Macaque R00-013 developed AIDS and was euthanized at week 53. Macaque R99-007 was euthanized at week 29 because of the limitation of available cage numbers.

§A *gag* gene fragment was amplified from plasma RNA at week 5 or from PBMC-derived DNA at week 7 or 8 and subjected to sequencing to determine predominant mutations leading to Gag amino acid changes. The results are shown in Table 2.

ǁA *gag* gene fragment was amplified from PBMC-derived DNA and subjected to sequencing to determine predominant mutations leading to Gag amino acid changes.

ND, Not determined.

(Matano *et al.*, 2000). Two macaques (R99-005 and R99-012) received four immunizations with both the FMSIV DNA and an FMLV receptor (mCAT1)-expression plasmid DNA (Albritton *et al.*, 1989) followed by a single SeV-Gag booster. This second DNA vaccination protocol (DNAv2) has been shown to elicit efficient CTL responses by confined mCAT1-dependent FMSIV replication (Matano *et al.*, 2000). Three macaques (R00-020, R00-023 and R00-024) received a single immunization with CMV-SHIVdEN DNA (DNAv3) followed by a single boost with an F-deleted replication-defective SeV-Gag (F^{-}SeV-Gag) (Li *et al.*, 2000; Takeda *et al.*, 2003). This CMV-SHIVdEN plasmid DNA was constructed from an *env*- and *nef*-deleted $SHIV_{MD14YE}$ molecular clone DNA and had the genes encoding SIVmac239 Gag, Pol, Vif and Vpx, SIVmac239/HIV-1_{DH12} chimeric Vpr and HIV-1_{DH12} Tat and Rev (Matano *et al.*, 2004). All ten animals were challenged intravenously with 10 $TCID_{50}$ SHIV89.6PD (Lu *et al.*, 1998) approximately 3 months after the last immunization. Four unvaccinated animals were also challenged with SHIV89.6PD and all failed to control virus replication.

Quantification of plasma viral loads. Plasma RNA was extracted using a High Pure Viral RNA kit (Roche Diagnostics). Serial fivefold dilutions of RNA samples were amplified in quadruplicate by nested RT-PCR using SIV *gag*-specific primers to determine the end point. Plasma SIV RNA levels were calculated according to the Reed–Muench method, as described previously (Matano *et al.*, 2004). The lower limit of detection was approximately 4×10^2 RNA copies ml^{-1}.

Sequencing of viral and proviral genomes. Plasma RNA was extracted as described above and genomic DNA was extracted from peripheral blood mononuclear cells (PBMCs) using a DNeasy kit (Qiagen). A fragment corresponding to nt 458–2185 (containing the entire *gag* region) in the SHIV89.6P genome (GenBank accession no. U89134) was amplified from plasma RNA by nested RT-PCR. Alternatively, fragments corresponding to nt 458–2185, 2019–3187, 3038–4197, 4056–5213, 5079–6250, 6065–7225, 7047–8176 and 7998–9172 in the SHIV89.6P genome were amplified from proviral DNA by nested PCR. The PCR products were sequenced using dye terminator chemistry and an automated DNA sequencer (Applied Biosystems). Alternatively, PCR products were subcloned into plasmids using a TOPO cloning system (Invitrogen) and sequenced.

Measurement of virus-specific T-cell levels by intracellular cytokine staining. We measured virus-specific T-cell levels by flow cytometric analysis of gamma interferon (IFN-γ) induction after specific stimulation, as described previously (Matano *et al.*, 2001, 2004). In brief, PBMCs were co-cultured with autologous herpesvirus papio-immortalized B lymphoblastoid cell lines (B-LCLs) infected with a vesicular stomatitis virus G (VSV-G)-pseudotyped SIVGP1 for SHIV-specific stimulation. The pseudotyped virus was obtained by co-transfection of COS-1 cells with a VSV-G-expression plasmid and the SIVGP1 DNA, an *env*- and *nef*-deleted SHIV molecular clone DNA, constructed by removing the whole FMLV *env* region from the FMSIV DNA. Alternatively, PBMCs were co-cultured with B-LCLs pulsed with peptide mixture (final concentration of each peptide, 0.5–2 μM) for peptide-specific stimulation. A panel of 117 overlapping peptides (15–17 aa in length and overlapping by 10–12 aa) spanning the entire SIVmac239 Gag sequence (Sigma-Aldrich) were divided into ten pools (1–10) each consisting of 11 or 12 peptides. Intracellular IFN-γ staining was performed using a Cytofix/Cytoperm kit (Becton Dickinson) according to the manufacturer's instructions. 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 antibodies (Becton Dickinson) were used. Specific T-cell levels were calculated by subtracting non-specific IFN-γ^{+} T-cell frequencies from those after SHIV-specific or peptide-specific stimulation. Specific T-cell levels of <100 cells per 10^6 PBMCs were considered negative.

Measurement of virus-specific neutralizing titres. We performed a neutralizing assay for the measurement of virus-specific neutralizing titres in plasma, as described previously (Shibata *et al.*, 1997b). Serial twofold dilutions of heat-inactivated plasma were prepared in duplicate and mixed with 10 $TCID_{50}$ SHIV89.6PD. In each mixture, 5 μl diluted plasma was incubated with 5 μl virus. After a 45 min incubation at room temperature, each 10 μl mixture was added to 5×10^4 MT4 cells in a well of a 96-well plate. After 12 days of culture, supernatants were harvested. Progeny virus production in the supernatants was examined by ELISA for detection of SIV p27 core antigen (Beckman Coulter) to determine the 100 % neutralizing end point. The lower limit of detection was a titre of 1 : 2.

RESULTS

MHC haplotypes of the SHIV controllers

In our previous SHIV89.6PD challenge experiment (Matano *et al.*, 2001; Takeda *et al.*, 2003), three animals received a single SeV-Gag vaccination alone, whilst the remaining seven animals were immunized with a DNA-prime/SeV-Gag boost vaccine before challenge (Table 1). The seven animals vaccinated with the prime–boost vaccine (R99-007, R99-011, R99-005, R99-012, R00-020, R00-023 and R00-024) were able to control virus replication, with undetectable set-point plasma viraemia. Two (R00-015 and R00-017) of the three animals vaccinated with SeV-Gag alone were also able to control viraemia, but the remaining one (R00-013) failed to control virus replication and showed acute $CD4^{+}$ T-cell depletion. This animal R00-013 developed AIDS and was euthanized at week 53.

In the present study, we determined the MHC class I (MHC I) haplotypes of the SHIV controllers and their viral genome sequences at around 1 or 2 months after challenge to examine whether SHIV controllers showed rapid selection of CTL escape mutations as observed in our previous analysis, in particular MHC-associated control of SIV replication. Importantly, control of SHIV89.6PD replication was observed in vaccinees with diverse MHC haplotypes (Table 1). Analysis of the proviral *gag* region in PBMCs at around week 8 showed a predominance of the wild-type sequence in all nine SHIV controllers (Table 2). Sequencing of the plasma viral *gag* region at week 5 in three of them confirmed the lack of dominant mutations (Table 2). Thus, the SHIV controllers controlled virus replication without rapid selection of CTL escape mutations.

Follow-up of the SHIV controllers

We followed up eight of the nine SHIV controllers except for one animal, R99-007, which was euthanized at week 29 because of a limitation on available cage numbers (Table 1). All eight SHIV controllers maintained control of virus replication for more than 2 years (Fig. 1). Viraemia was undetectable and peripheral $CD4^{+}$ T-cell counts were maintained during the observation period. Analysis of the *gag* region in PBMC-derived proviral DNA revealed that the wild-type sequence was still dominant around 1 year after challenge in all eight (Table 1). Additionally, we succeeded

Table 2. Mutations in SHIV *gag* at 1 or 2 months post-challenge

A *gag* gene fragment was amplified by nested PCR from PBMC-derived DNA at week 7 (in R00-017) or week 8 (in others) or by nested RT-PCR from plasma RNA at week 5. The viral *gag* fragment was amplified from plasma RNA in only three of the nine SHIV controllers (R00-017, R00-023 and R00-024); this was due to lower viral loads at week 5 in the remaining SHIV controllers.

Animal	Frequency*	Position of Gag changes (aa)†
Proviral DNA at week 7 or 8		
−R00-015	8/9	None
	1/9	373
−R00-017	9/10	None
	1/10	384
−R99-007	8/9	None
	1/9	485
−R99-011	9/10	None
	1/10	141
−R99-005	8/9	None
	1/9	495
−R99-012	8/10	None
	1/10	210
	1/10	372, 456
−R00-020	9/9	None
−R00-023	7/10	None
	3/10	385
−R00-024	7/7	None
Plasma RNA at week 5		
−R00-017	2/9	None
	1/9	49
	1/9	208
	1/9	443
	1/9	49, 103
	1/9	270, 448
	1/9	59, 232, 293
	1/9	391, 481, 499
−R00-023	2/11	None
	2/11	218
	1/11	27
	1/11	434
	1/11	444, 493
	1/11	76, 182, 379
	1/11	118, 272, 380
	1/11	5, 140, 312, 434
	1/11	6, 17, 112, 205
−R00-024	2/9	None
	1/9	227
	1/9	42, 301
	1/9	272, 434
	1/9	9, 48, 367
	1/9	50, 176, 247
	1/9	103, 364, 386
	1/9	108, 137, 364, 386, 411

*Number of clones with change(s)/total number of clones.

†Amplified *gag* fragments were subcloned into plasmids for sequencing and the positions of amino acid changes in SHIV Gag in each clone are shown.

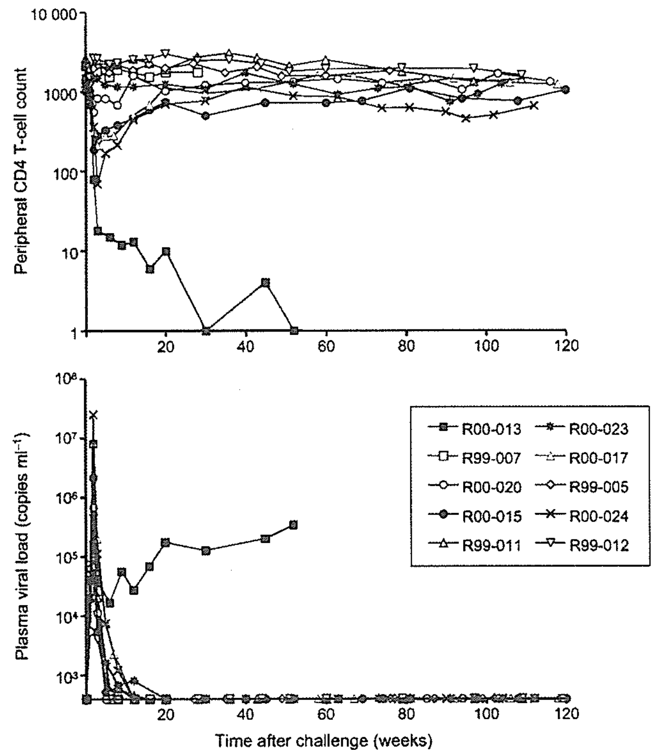

Fig. 1. Follow-up of vaccinated macaques after SHIV89.6PD challenge. Macaque R00-013 was a non-controller that failed to control virus replication, with acute $CD4^+$ T-cell depletion, whereas the other nine animals were SHIV controllers. (a) Peripheral $CD4^+$ T-cell counts μl^{-1}. (b) Plasma viral loads [viral RNA copies (ml plasma)$^{-1}$].

in amplifying almost the entire coding region of the proviral genomes from three (R00-015, R00-017 and R00-023) of the eight controllers at around 1 year for sequencing and found no dominant non-synonymous mutations except for one leading to a change in aa 401 in Env in macaque R00-015, suggesting inefficient virus replication during the period of SHIV control.

Virus-specific T-cell responses

We next examined changes in virus-specific T-cell frequencies during the period of SHIV89.6PD control. The SHIV controllers did not rapidly lose SHIV-specific T cells but most showed a gradual decrease in SHIV-specific T-cell levels, except for macaque R99-011, which maintained constant SHIV-specific $CD8^+$ T-cell levels (Fig. 2). Thus, none of the SHIV controllers showed a significant increase in SHIV-specific T-cell levels, suggesting stable virus control without any sign of a virus replication burst in the chronic phase.

In addition to virus-specific T-cell levels, we examined epitopes that were recognized by CTLs. We focused on two SHIV controllers, R00-015 and R00-017, that were vaccinated with SeV-Gag alone and examined CTL responses

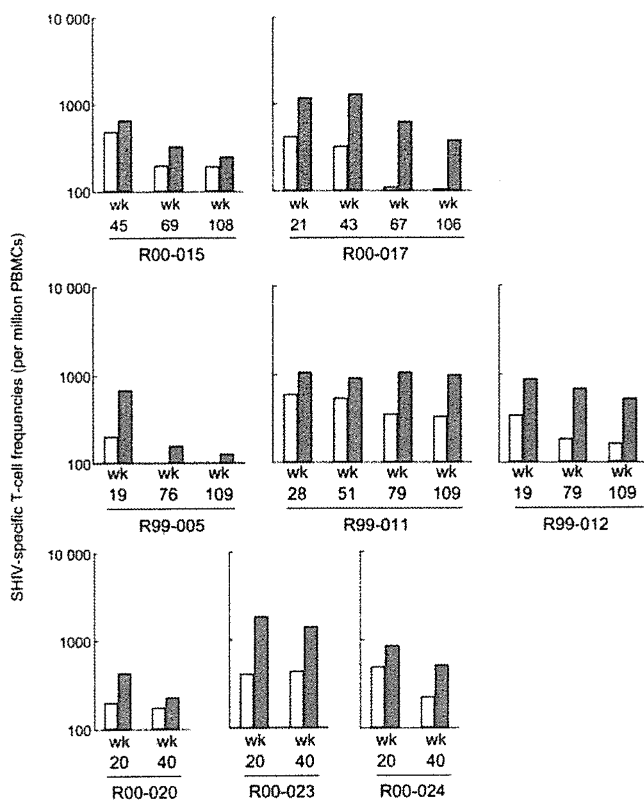

Fig. 2. SHIV-specific T-cell levels in SHIV controllers at various time points after challenge. The frequencies of SHIV-specific CD4$^+$ T cells (open bars) and CD8$^+$ T cells (shaded bars) in PBMCs are shown.

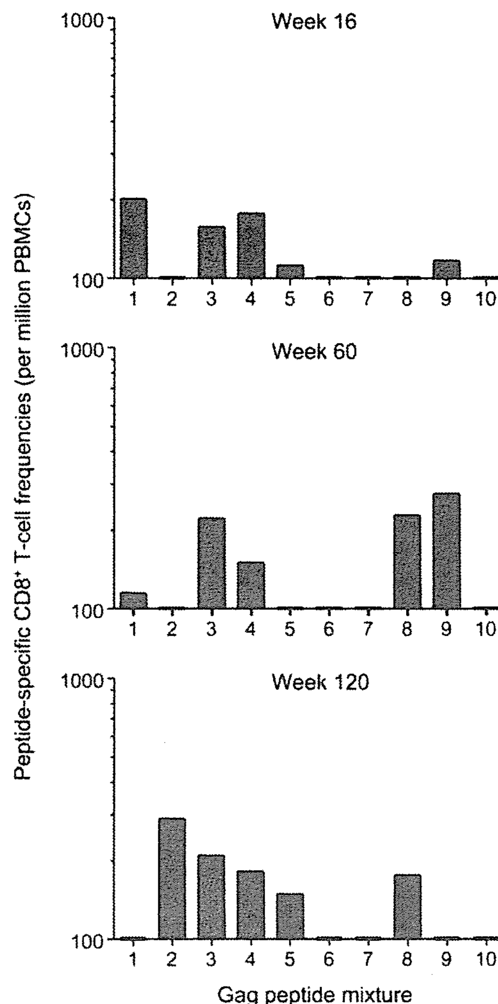

Fig. 3. Changes in frequencies of CD8$^+$ T cells specific for pools of Gag peptides in PBMCs of macaque R00-015 during the period of virus control. The frequencies at week 16 (top panel), week 60 (middle panel) and week 120 (bottom panel) after SHIV89.6PD challenge are shown. A panel of overlapping peptides spanning the entire SIV Gag sequence was divided into ten pools: 1 (aa 1–65), 2 (aa 55–114), 3 (aa 104–165), 4 (aa 155–213), 5 (aa 202–265), 6 (aa 255–316), 7 (aa 306–364), 8 (aa 354–416), 9 (aa 406–464) and 10 (aa 453–510). Each pool was used for stimulation to detect peptide-pool-specific CD8$^+$ T cells.

specific for ten pools of Gag-overlapping peptides. In macaque R00-015, significant Gag peptide pool 1-specific CD8$^+$ T-cell responses were detected at week 16 but became undetectable by week 120, whereas pool 8- and 2-specific CD8$^+$ T-cell responses that were undetectable at week 16 appeared at week 60 or 120, respectively, and pool 3- and 4-specific CD8$^+$ T-cell responses were detectable throughout the observation period (Fig. 3). A similar pattern of disappearance (pool 10-specific), appearance (pool 3- and 9-specific) and maintenance (pool 6- and 8-specific) of CD8$^+$ T-cell responses during the period of SHIV control was also observed in macaque R00-017 (Fig. 4). These results suggested that SHIV89.6PD replication was not completely contained in these macaques.

Virus-specific neutralizing antibody responses

We next examined virus-specific neutralizing antibody responses by determining the end-point plasma titres required to neutralize the replication of 10 $TCID_{50}$ of virus on MT4 cells. Our vaccine regimens did not utilize Env as an immunogen and no neutralizing antibody responses were induced before challenge in any of the vaccinees, as expected. Remarkably, however, SHIV89.6PD-specific neutralizing antibodies appeared rapidly between weeks 3 and 6 post-challenge and were maintained during the observation period in all of the SHIV controllers (Fig. 5). In contrast to such efficient induction of neutralizing antibodies in SHIV controllers, macaque R00-013, which failed to control SHIV replication, showed no neutralizing antibody induction after challenge.

DISCUSSION

Long-term control of X4-tropic pathogenic SHIV has been reported in follow-up studies of several pre-clinical AIDS

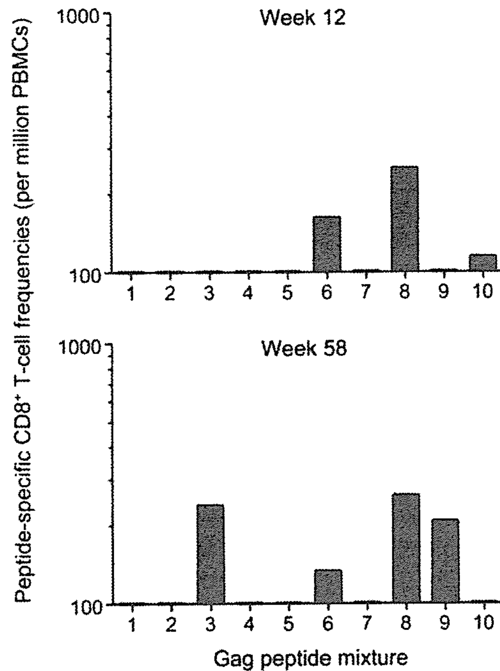

Fig. 4. Changes in frequency of $CD8^+$ T cells specific for pools of Gag peptides in PBMCs of macaque R00-017 during the period of virus control. The frequencies at week 12 (top panel) and week 58 (bottom panel) after SHIV89.6PD challenge are shown. Ten pools of Gag peptides were used for stimulation to detect peptide pool-specific $CD8^+$ T cells, as described in the legend to Fig. 3.

vaccine trials (Willey *et al.*, 2003; Sadagopal *et al.*, 2005). Whilst these vaccine regimens utilized Env as an immunogen (Amara *et al.*, 2002), we have developed vaccine regimens not targeting Env and demonstrated their efficacies leading to control of SHIV89.6PD replication in rhesus macaques (Matano *et al.*, 2001; Takeda *et al.*, 2003). In the present study, we followed up these SHIV controllers for more than 2 years after challenge. All maintained this control with undetectable plasma viraemia, indicating that efficient CTL induction by a prophylactic AIDS vaccine not targeting Env can result in sustained control of virus replication and protection from AIDS progression in a model of X4-tropic SHIV infection.

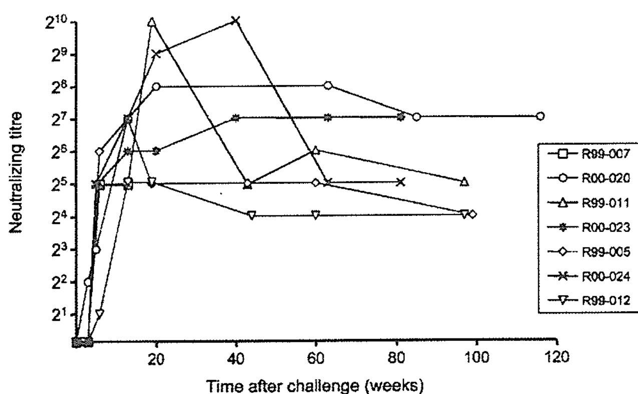

Fig. 5. SHIV89.6PD-specific neutralizing antibody levels in plasma of SHIV controllers. Plasma titres that neutralized replication of 10 $TCID_{50}$ SHIV89.6PD in seven out of nine SHIV controllers are shown. In the remaining SHIV controllers, R00-015 and R00-017, we confirmed induction of neutralizing antibodies at weeks 5, 12 and 20, but their titres were not determined.

X4-tropic SHIV and R5-tropic SIV target different $CD4^+$ T-cell subsets in rhesus macaques and this difference has been indicated as resulting in their divergent clinical courses (Nishimura *et al.*, 2004). Indeed, it has been shown that X4-tropic SHIV targets $CXCR4^+$ naive $CD4^+$ T cells for depletion, whereas R5-tropic SIV, like HIV-1 infection in humans, eliminates $CCR5^+$ effector memory $CD4^+$ T cells in rhesus macaques during the acute phase of infection (Picker *et al.*, 2004; Li *et al.*, 2005; Mattapallil *et al.*, 2005; Nishimura *et al.*, 2005; Picker & Watkins, 2005). In the latter chronic AIDS model, several CTL vaccine trials have recently shown partial reductions in viral loads with amelioration of acute memory $CD4^+$ T-cell loss, but this partial control was transient and unstable (Letvin *et al.*, 2006; Mattapallil *et al.*, 2006; Wilson *et al.*, 2006). In our previous study (Matano *et al.*, 2004), SIV control was observed consistently in the three vaccinees possessing MHC I haplotype *90-120-Ia*, but this control was not stable and two of them lost viraemia control around week 60 after challenge. In the present study showing long-term, stable SHIV control, we found several differences between X4-tropic SHIV controllers and R5-tropic SIV controllers.

First, patterns of *de novo* neutralizing antibody induction were completely different between the two. Although the vaccine regimens did not target Env, SHIV-specific neutralizing antibodies appeared rapidly and became detectable by week 6 post-challenge in the SHIV controllers, whereas no neutralizing antibody induction was observed in the SHIV non-controllers. Thus, SHIV-specific neutralizing antibodies can be rapidly induced if animals are protected by CTLs from complete $CD4^+$ T-cell depletion in the acute phase and may be involved in viraemia control at the set point and after (Rasmussen *et al.*, 2002). In contrast, SIV-specific neutralizing antibody induction in the SIV controllers was poor and less efficient than the SIV non-controllers (data not shown), indicating that neutralizing antibody responses are not involved in SIV control.

Secondly, all of the SIV controllers showed rapid selection of viral CTL escape mutations, whereas this sign of particular CTL pressure (Borrow *et al.*, 1997; Goulder *et al.*, 1997; Price *et al.*, 1997; Goulder & Watkins, 2004; Matano *et al.*, 2004) was not observed in any of the SHIV controllers. Additionally, SIV control was associated with some MHC haplotypes such as *90-120-Ia*, but SHIV control was observed in vaccinees with diverse MHC haplotypes. Indeed, none of the SHIV controllers had the MHC

haplotype *90-120-Ia* associated with SIV control. Although the involvement of functional virus-specific $CD4^+$ T-cell responses remains unclear, these results support the notion that multiple target-specific CTL effectors are involved in SHIV control, whereas relatively limited regions of viral antigens are targeted by effectors responsible for SIV control.

All of the SHIV controllers maintained virus control for more than 2 years. Sequencing of viral genomes revealed a predominance of the wild-type sequence around 1 year after SHIV89.6PD challenge, and analysis of SHIV-specific T-cell levels showed no signs of a burst of virus replication during the chronic phase. These results indicated stable virus control in the chronic phase in the SHIV controllers. Interestingly, however, analysis of Gag peptide-specific $CD8^+$ T-cell responses in some of the SHIV controllers showed a shift of targeting epitopes during the period of virus control, suggesting that virus replication was inefficient but not completely contained, even in the SHIV controllers.

In summary, the present study revealed several differences in vaccine-based virus control in a model of X4-tropic SHIV compared with R5-tropic SIV infections. Our results suggest that, compared with virus control with limited effectors in SIV controllers, the control of X4-tropic SHIV89.6PD replication may be maintained more stably in the presence of multiple functional immune effectors.

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REFERENCES

Albritton, L. M., Tseng, L., Scadden, D. & Cunningham, J. M. (1989). A putative murine ecotropic retrovirus receptor gene encodes a multiple membrane-spanning protein and confers susceptibility to virus infection. *Cell* 57, 659–666.

Amara, R. R., Villinger, F., Altman, J. D., Lydy, S. L., O'Neil, S. P., Staprans, S. I., Montefiori, D. C., Xu, Y., Herndon, J. G. & other authors (2001). Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine. *Science* 292, 69–74.

Amara, R. R., Smith, J. M., Staprans, S. I., Montefiori, D. C., Villinger, F., Altman, J. D., O'Neil, S. P., Kozyr, N. L., Xu, Y. & other authors (2002). Critical role for Env as well as Gag-Pol in control of a simian-human immunodeficiency virus 89.6P challenge by a DNA prime/recombinant modified vaccinia virus Ankara vaccine. *J Virol* 76, 6138–6146.

Arguello, J. R., Little, A. M., Pay, A. L., Gallardo, D., Rojas, I., Marsh, S. G., Goldman, J. M. & Madrigal, J. A. (1998). Mutation detection and typing of polymorphic loci through double-strand conformation analysis. *Nat Genet* 18, 192–194.

Barouch, D. H., Santra, S., Schmitz, J. E., Kuroda, M. J., Fu, T. M., Wagner, W., Bilska, M., Craiu, A., Zheng, X. X. & other authors (2000). Control of viremia and prevention of clinical AIDS in rhesus monkeys by cytokine-augmented DNA vaccination. *Science* 290, 486–492.

Borrow, P., Lewicki, H., Hahn, B. H., Shaw, G. M. & Oldstone, M. B. (1994). Virus-specific $CD8^+$ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *J Virol* 68, 6103–6110.

Borrow, P., Lewicki, H., Wei, X., Horwitz, M. S., Peffer, N., Meyers, H., Nelson, J. A., Gairin, J. E., Hahn, B. H. & other authors (1997). Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTL) during primary infection demonstrated by rapid selection of CTL escape virus. *Nat Med* 3, 205–211.

Casimiro, D. R., Wang, F., Schleif, W. A., Liang, X., Zhang, Z. Q., Tobery, T. W., Davies, M. E., McDermott, A. B., O'Connor, D. H. & other authors (2005). Attenuation of simian immunodeficiency virus SIVmac239 infection by prophylactic immunization with DNA and recombinant adenoviral vaccine vectors expressing Gag. *J Virol* 79, 15547–15555.

Feinberg, M. B. & Moore, J. P. (2002). AIDS vaccine models: challenging challenge viruses. *Nat Med* 8, 207–210.

Goulder, P. J. & Watkins, D. I. (2004). HIV and SIV CTL escape: implications for vaccine design. *Nat Rev Immunol* 4, 630–640.

Goulder, P. J., Phillips, R. E., Colbert, R. A., McAdam, S., Ogg, G., Nowak, M. A., Giangrande, P., Luzzi, G., Morgan, B. & other authors (1997). Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. *Nat Med* 3, 212–217.

Horton, H., Vogel, T. U., Carter, D. K., Vielhuber, K., Fuller, D. H., Shipley, T., Fuller, J. T., Kunstman, K. J., Sutter, G. & other authors (2002). Immunization of rhesus macaques with a DNA prime/modified vaccinia virus Ankara boost regimen induces broad simian immunodeficiency virus (SIV)-specific T-cell responses and reduces initial viral replication but does not prevent disease progression following challenge with pathogenic SIVmac239. *J Virol* 76, 7187–7202.

Jin, X., Bauer, D. E., Tuttleton, S. E., Lewin, S., Gettie, A., Blanchard, J., Irwin, C. E., Safrit, J. T., Mittler, J. & other authors (1999). Dramatic rise in plasma viremia after $CD8^+$ T cell depletion in simian immunodeficiency virus-infected macaques. *J Exp Med* 189, 991–998.

Kano, M., Matano, T., Kato, A., Nakamura, H., Takeda, A., Suzaki, Y., Ami, Y., Terao, K. & Nagai, Y. (2002). Primary replication of a recombinant Sendai virus vector in macaques. *J Gen Virol* 83, 1377–1386.

Kato, A., Sakai, Y., Shioda, T., Kondo, T., Nakanishi, M. & Nagai, Y. (1996). Initiation of Sendai virus multiplication from transfected cDNA or RNA with negative or positive sense. *Genes Cells* 1, 569–579.

Kawada, M., Igarashi, H., Takeda, A., Tsukamoto, T., Yamamoto, H., Dohki, S., Takiguchi, M. & Matano, T. (2006). Involvement of multiple epitope-specific cytotoxic T-lymphocyte responses in vaccine-based control of simian immunodeficiency virus replication in rhesus macaques. *J Virol* 80, 1949–1958.

Koup, R. A., Safrit, J. T., Cao, Y., Andrews, C. A., McLeod, G., Borkowsky, W., Farthing, C. & Ho, D. D. (1994). Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J Virol* 68, 4650–4655.

Letvin, N. L., Mascola, J. R., Sun, Y., Gorgone, D. A., Buzby, A. P., Xu, L., Yang, Z. Y., Chakrabarti, B., Rao, S. S. & other authors (2006). Preserved $CD4^+$ central memory T cells and survival in vaccinated SIV-challenged monkeys. *Science* 312, 1530–1533.

Li, H. O., Zhu, Y. F., Asakawa, M., Kuma, H., Hirata, T., Ueda, Y., Lee, Y. S., Fukumura, M., Iida, A. & other authors (2000). A cytoplasmic RNA vector derived from nontransmissible Sendai virus with efficient gene transfer and expression. *J Virol* **74**, 6564–6569.

Li, Q., Duan, L., Estes, J. D., Ma, Z. M., Rourke, T., Wang, Y., Reilly, C., Carlis, J., Miller, C. J. & Haase, A. T. (2005). Peak SIV replication in resting memory CD4$^+$ T cells depletes gut lamina propria CD4$^+$ T cells. *Nature* **434**, 1148–1152.

Lu, Y., Pauza, C. D., Lu, X., Montefiori, D. C. & Miller, C. J. (1998). Rhesus macaques that become systemically infected with pathogenic SHIV 89.6-PD after intravenous, rectal, or vaginal inoculation and fail to make an antiviral antibody response rapidly develop AIDS. *J Acquir Immune Defic Syndr Hum Retrovirol* **19**, 6–18.

Matano, T., Shibata, R., Siemon, C., Connors, M., Lane, H. C. & Martin, M. A. (1998). Administration of an anti-CD8 monoclonal antibody interferes with the clearance of chimeric simian/human immunodeficiency virus during primary infections of rhesus macaques. *J Virol* **72**, 164–169.

Matano, T., Kano, M., Odawara, T., Nakamura, H., Takeda, A., Mori, K., Sato, T. & Nagai, Y. (2000). Induction of protective immunity against pathogenic simian immunodeficiency virus by a foreign receptor-dependent replication of an engineered avirulent virus. *Vaccine* **18**, 3310–3318.

Matano, T., Kano, M., Nakamura, H., Takeda, A. & Nagai, Y. (2001). Rapid appearance of secondary immune responses and protection from acute CD4 depletion after a highly pathogenic immunodeficiency virus challenge in macaques vaccinated with a DNA prime/ Sendai virus vector boost regimen. *J Virol* **75**, 11891–11896.

Matano, T., Kobayashi, M., Igarashi, H., Takeda, A., Nakamura, H., Kano, M., Sugimoto, C., Mori, K., Iida, A. & other authors (2004). Cytotoxic T lymphocyte-based control of simian immunodeficiency virus replication in a preclinical AIDS vaccine trial. *J Exp Med* **199**, 1709–1718.

Mattapallil, J. J., Douek, D. C., Hill, B., Nishimura, Y., Martin, M. A. & Roederer, M. (2005). Massive infection and loss of memory CD4$^+$ T cells in multiple tissues during acute SIV infection. *Nature* **434**, 1093–1097.

Mattapallil, J. J., Douek, D. C., Buckler-White, A., Montefiori, D. C., Letvin, N. L., Nabel, G. J. & Roederer, M. (2006). Vaccination preserves CD4 memory T cells during acute simian immunodeficiency virus challenge. *J Exp Med* **203**, 1533–1541.

McMichael, A. J. & Hanke, T. (2003). HIV vaccines 1983–2003. *Nat Med* **9**, 874–880.

Nishimura, Y., Igarashi, T., Donau, O. K., Buckler-White, A., Buckler, C., Lafont, B. A., Goeken, R. M., Goldstein, S., Hirsch, V. M. & Martin, M. A. (2004). Highly pathogenic SHIVs and SIVs target different CD4$^+$ T cell subsets in rhesus monkeys, explaining their divergent clinical courses. *Proc Natl Acad Sci U S A* **101**, 12324–12329.

Nishimura, Y., Brown, C. R., Mattapallil, J. J., Igarashi, T., Buckler-White, A., Lafont, B. A., Hirsch, V. M., Roederer, M. & Martin, M. A. (2005). Resting naive CD4$^+$ T cells are massively infected and eliminated by X4-tropic simian-human immunodeficiency viruses in macaques. *Proc Natl Acad Sci U S A* **102**, 8000–8005.

Ogg, G. S., Jin, X., Bonhoeffer, S., Dunbar, P. R., Nowak, M. A., Monard, S., Segal, J. P., Cao, Y., Rowland-Jones, S. L. & other authors (1998). Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. *Science* **279**, 2103–2106.

Picker, L. J. & Watkins, D. I. (2005). HIV pathogenesis: the first cut is the deepest. *Nat Immunol* **6**, 430–432.

Picker, L. J., Hagen, S. I., Lum, R., Reed-Inderbitzin, E. F., Daly, L. M., Sylwester, A. W., Walker, J. M., Siess, D. C., Piatak, M., Jr & other authors (2004). Insufficient production and tissue delivery of CD4$^+$ memory T cells in rapidly progressive simian immunodeficiency virus infection. *J Exp Med* **200**, 1299–1314.

Price, D. A., Goulder, P. J., Klenerman, P., Sewell, A. K., Easterbrook, P. J., Troop, M., Bangham, C. R. & Phillips, R. E. (1997). Positive selection of HIV-1 cytotoxic T lymphocyte escape variants during primary infection. *Proc Natl Acad Sci U S A* **94**, 1890–1895.

Rasmussen, R. A., Hofmann-Lehmann, R., Li, P. L., Vlasak, J., Schmitz, J. E., Reimann, K. A., Kuroda, M. J., Letvin, N. L., Montefiori, D. C. & other authors (2002). Neutralizing antibodies as a potential secondary protective mechanism during chronic SHIV infection in CD8$^+$ T-cell-depleted macaques. *AIDS* **16**, 829–838.

Reimann, K. A., Li, J. T., Veazey, R., Halloran, M., Park, I. W., Karlsson, G. B., Sodroski, J. & Letvin, N. L. (1996). A chimeric simian/human immunodeficiency virus expressing a primary patient human immunodeficiency virus type 1 isolate env causes an AIDS-like disease after in vivo passage in rhesus monkeys. *J Virol* **70**, 6922–6928.

Rose, N. F., Marx, P. A., Luckay, A., Nixon, D. F., Moretto, W. J., Donahoe, S. M., Montefiori, D., Roberts, A., Buonocore, L. & Rose, J. K. (2001). An effective AIDS vaccine based on live attenuated vesicular stomatitis virus recombinants. *Cell* **106**, 539–549.

Sadagopal, S., Amara, R. R., Montefiori, D. C., Wyatt, L. S., Staprans, S. I., Kozyr, N. L., McClure, H. M., Moss, B. & Robinson, H. L. (2005). Signature for long-term vaccine-mediated control of a simian and human immunodeficiency virus 89.6P challenge: stable low-breadth and low-frequency T-cell response capable of coproducing gamma interferon and interleukin-2. *J Virol* **79**, 3243–3253.

Schmitz, J. E., Kuroda, M. J., Santra, S., Sasseville, V. G., Simon, M. A., Lifton, M. A., Racz, P., Tenner-Racz, K., Dalesandro, M. & other authors (1999). Control of viremia in simian immunodeficiency virus infection by CD8$^+$ lymphocytes. *Science* **283**, 857–860.

Shibata, R., Maldarelli, F., Siemon, C., Matano, T., Parta, M., Miller, G., Fredrickson, T. & Martin, M. A. (1997a). Infection and pathogenicity of chimeric simian-human immunodeficiency viruses in macaques: determinants of high virus loads and CD4 cell killing. *J Infect Dis* **176**, 362–373.

Shibata, R., Siemon, C., Czajak, S. C., Desrosiers, R. C. & Martin, M. A. (1997b). Live, attenuated simian immunodeficiency virus vaccines elicit potent resistance against a challenge with a human immunodeficiency virus type 1 chimeric virus. *J Virol* **71**, 8141–8148.

Shiver, J. W., Fu, T. M., Chen, L., Casimiro, D. R., Davies, M. E., Evans, R. K., Zhang, Z. Q., Simon, A. J., Trigona, W. L. & other authors (2002). Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. *Nature* **415**, 331–335.

Takeda, A., Igarashi, H., Nakamura, H., Kano, M., Iida, A., Hirata, T., Hasegawa, M., Nagai, Y. & Matano, T. (2003). Protective efficacy of an AIDS vaccine, a single DNA-priming followed by a single booster with a recombinant replication-defective Sendai virus vector, in a macaque AIDS model. *J Virol* **77**, 9710–9715.

Willey, R. L., Byrum, R., Piatak, M., Kim, Y. B., Cho, M. W., Rossio, J. L., Jr, Bess, J., Jr, Igarashi, T., Endo, Y. & other authors (2003). Control of viremia and prevention of simian-human immunodeficiency virus-induced disease in rhesus macaques immunized with recombinant vaccinia viruses plus inactivated simian immunodeficiency virus and human immunodeficiency virus type 1 particles. *J Virol* **77**, 1163–1174.

Wilson, N. A., Reed, J., Napoe, G. S., Piaskowski, S., Szymanski, A., Furlott, J., Gonzalez, E. J., Yant, L. J., Maness, N. J. & other authors (2006). Vaccine-induced cellular immune responses reduce plasma viral concentrations after repeated low-dose challenge with pathogenic simian immunodeficiency virus SIVmac239. *J Virol* **80**, 5875–5885.

ELSEVIER

Direct Determination of Single Nucleotide Polymorphism Haplotype of *NFKBIL1* Promoter Polymorphism by DNA Conformation Analysis and Its Application to Association Study of Chronic Inflammatory Diseases

Hiroki Shibata, Michio Yasunami, Nobuhisa Obuchi, Megumi Takahashi, Yasushi Kobayashi, Fujio Numano[1], and Akinori Kimura

ABSTRACT: We previously revealed that one of the human leukocyte antigen-linked susceptibility genes for Takayasu's arteritis (TA) was mapped between *TNFA* and *MICB* loci and that −63T allele of *NFKBIL1*, which is between *TNFA* and *MICB* loci, was associated with rheumatoid arthritis (RA) in the Japanese population. We have developed a novel typing method based on reference strand-mediated conformation analysis for the upstream sequence of the *NFKBIL1* gene, where −422 $(T)_8/(T)_9$, −325 C/G, −263 A/G, and −63 T/A polymorphisms were found. Upon the analysis of the patients with TA (n = 84), those with RA (n = 120), and healthy control subjects (n = 217), five common haplotypes named IKBLp*01 through IKBLp*05 were found in the Japanese population. The frequency of IKBLp*03 was significantly increased in the patient with TA (57.1% vs 35.0%, giving an odds ratio of 2.47). In addition, the frequency of IKBLp*01, but not that of other −63T-bearing alleles, was increased in the patients with RA (73.3% vs 58.1%, giving an odds ratio of 1.99), suggesting that the susceptibility to RA was conferred not by −63T alone but by combination of single nucleotide polymorphisms in the *NFKBIL1* promoter. A higher promoter activity associated with IKBLp*03 and a lower activity associated with IKBLp*01 may contribute to the susceptibility to TA and RA, respectively. *Human Immunology 67, 363–373 (2006).* © American Society for Histocompatibility and Immunogenetics, 2006. Published by Elsevier Inc.

KEYWORDS: HLA class III region; SNP haplotype; DNA conformation analysis; association study

ABBREVIATIONS

TA	Takayasu's arteritis
RA	rheumatoid arthritis
LD	linkage disequilibrium
RSCA	reference strand-mediated conformation analysis
SNP	single nucleotide polymorphism
HLA	human leukocyte antigen
SSCP	single strand conformation polymorphism

INTRODUCTION

The HLA class III region of the short arm of human chromosome 6 contains multiple genes involved in the host defense mechanism against pathogens and inflammatory processes. Polymorphisms in the genes in this region were associated with several infectious diseases and autoimmunity. In addition to the association with HLA-B alleles, B*5201 and B*3902 [1], we previously reported that an allele of microsatellite polymorphic locus *C1_2_A* and certain alleles of other markers in the *TNFA-MICB* interval of the HLA class III region [2] were associated with Takayasu's arteritis (TA), also known as aortitis syndrome or pulseless disease, which is a chronic inflammatory disease mainly affecting the aorta

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