そこで、本年は24種の霊長類についてTIM1配列を決定するとともにTIM1の3Dモデルを構築し、TIM1分子上で進化選択圧がかかったアミノ酸をマップした。その結果、進化選択圧は分子表面に位置する部分にかかったと考えられた（図7）。

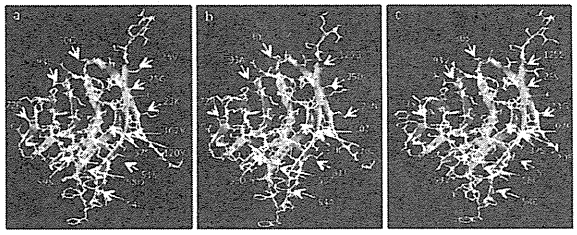

図7　TIM1分子の選択圧は表面にマップされる

一方、24種の霊長類で決定したTIM1遺伝子配列の系統樹を作製すると、霊長類の系統樹と一致したパターンが得られたが、極めて特徴的なことに、新世界ザルではLINE1配列の挿入あるいは点変異によってTIM1が偽遺伝子化していることが判明した（図8）。

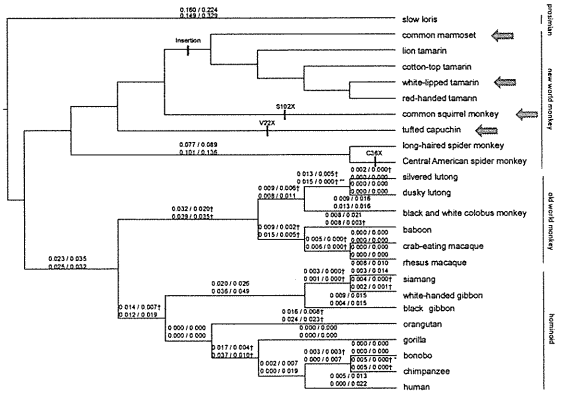

図8　霊長類におけるTIM1遺伝子系統樹

そこで、新世界ザル由来（図8で矢印で示した種由来）の4種類のT細胞株を用いて、RT-PCR法によってTIM1遺伝子の発現を検討した。その結果、いずれにおいてもTIM1発現が観察されなかった（図9）。

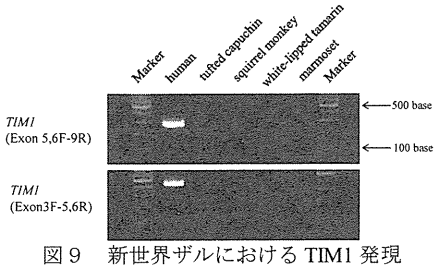

図9　新世界ザルにおけるTIM1発現

D．考察

SIVワクチン実験には主にアカゲザルが用いられているが、個々の実験個体の免疫遺伝学的背景は必ずしも明らかではない。我々はワクチン実験におけるCTL免疫応答の個体差を規定する最も重要な遺伝因子であるMHC、ことにMHCクラスIについて、多数の個体を解析し、実験個体の繁殖集団におけるMHCクラスIレパートリーの全貌をほぼ明らかにした。

また、CTL機能およびNK機能に関わるNKG2Dレセプターのリガンド多様性についての解析を進め、昨年までにMICおよびULBP4の多様性を明らかにし、本年はULBP2の多様性を検討した。ヒトと比較して、アカゲザルではMHCクラスI遺伝子座の遺伝子重複があり、さらに個々の遺伝子に多様性が大きいことが判明しているが、我々の研究によって、アカゲザルではULBP遺伝子座にも遺伝子重複があること、個々のULBP遺伝子の多様性がヒトより遥かに大きいことが明らかになった。ことに、ULBP4およびULBP2は、ヒトでは数種類のアリルしか見出されていないが、アカゲザル実験個体群にULBP4で少なくとも25種、ULBP2ではULBP2.1に少なくとも10種、ULBP2.2に少なくとも6種が確認された。ULBP多型の生物学的意義は不明であるが、ULBP4、ULBP2のいずれについても、その多型が分子表面に分布していないため、NKG2Dとの結合親和性が大きく変化することは考え難い。多数の実験個体について、ULBP多型とSIVワクチン後のCTL誘導性、中和抗体産生性を今後検討することで、その生物学的意義を推定できると考えられる。

本研究では、進化医科学的な手法でSIVワクチン反応性やHIV/AIDS感受性・抵抗性との関連を明らかにすることも視野に入れている。昨年度までにTIM1遺伝子の多様性がSIVワクチン接種アカゲザル個体における中和抗体産生性、タイ人およびインド人におけるHIV/AIDS感受性・抵抗性との関連を見出したが、本年はTIM1の進化において選択圧がかかったと考えられるアミノ酸部位がTIM1分子の表面に集中することを解明した。TIM1分子におけるこの領域（ドメイン）の機能は明らかではないが、他のタンパクとの結合に関わることが推定される。さらに、TIM1は新世界ザルにおいて、系統特異的に偽遺伝子化していることが判明した。ヒトではTIM1がA型肝炎ウイルス（HAV）のレセプターであることが知られているが、新世界ザルでもHAVに感染することから、TIM1以外のHAVレセプターの存在が示唆される。今後その意義をウイルス感染との関連で検討することが必要である。

E．結論

効率よいCTL誘導性SIVワクチンの開発において必須であるMHCクラスI遺伝子群の多様性分布を解明した。また、NKG2Dレセプターのリガンドである ULBP2 は、ヒトでは多型が乏しいが、アカゲザルでは大きな多様性を呈することが判明した。さらに、HIV/AIDS 関連遺伝子であるTIM1の特異な進化的特徴を明らかに証明した。

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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

該当なし

2　実用新案登録

該当なし

3　その他

該当なし

厚生労働科学研究費補助金（創薬基盤推進研究事業）
分担研究報告書

SIV 各種抗原発現 SeV ベクター作製

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研究要旨

ワクチン抗原選択のための抗原提示解析用 F 遺伝子欠損型センダイウイルスベクター（SeV^{18+}FSUxxTM56/ΔF-GFP）について、ベクター調製を実施した。精製後、力価測定・配列確認・無菌試験などの QC 試験項目を行なった。

A．研究目的

センダイウイルス（SeV）ベクターは、一本鎖の非分節型マイナス鎖 RNA ベクターであり、その全生活環において DNA への変換がなく、転写ならびにゲノムの複製は細胞質内で、自前の RNA ポリメラーゼ（P および L 蛋白質）を利用して行われる。すなわち、治療用遺伝子を核内に挿入し染色体遺伝子に組み込むことなく、細胞質において直接発現することができる特徴があり、「細胞質型 RNA ベクター」と呼ばれている。このような特徴のあるベクターの開発にあたって、宿主細胞への侵入にかかわる膜融合蛋白質 F 遺伝子を欠失させることにより、二次感染性のない、非伝播型ベクターへ改良することに成功している（SeV/ΔF）。この F 遺伝子欠失については、in vivo および in vitro 解析用にクオリティーの高いベクター製造システムを構築している。このシステムを用いてベクターを調製し、SIV のワクチン抗原選択のための抗原提示解析用に、FSUxxTM56 を高発現し、更にモニタリング用に GFP を共発現するベクターを調製することを目的とした。

B．研究方法

(1)　ベクター調製

ベクターの調製には F 蛋白質を持続発現するパッケージング細胞株を利用し、再構成は公知の方法（国際公開第９７／１６５３８号、国際公開第９７／１６５３９号）をベースに一部改良して行った。

(2)　ベクターの品質検査

力価測定・配列確認・無菌試験などの QC 試験項目を設定し、実施した。

（倫理面への配慮）

SeV は実験室飼育下のネズミから単離されたパラインフルエンザウイルスであり、ヒトへの病原性は知られていない。野生型ウイルスでも文部科学省の指針ではバイオハザードレベル P2 であり、通常の実験室で使用でき、安全なウイルスと考えられている。さらに実験に使用するベクターは、ウイルスの感染融合に必須の F 蛋白質遺伝子をゲノムから欠損しているため、非伝播型に改良されており、理論的にも実験的にも伝播性が無いことが証明されている。この様に実験動物および環境等に与える影響は最小限にとどめる。なお当分担研究では動物等への投与実験は厳選して限定されたものとし、その際には動物愛護の基準に従うものとする。

C．研究結果

抗原提示解析用の FSUxxTM56 を高発現し、モニタリング用 GFP を共発現するベクター（SeV^{18+}FSUxxTM5/ΔF-GFP）について、パッケージング細胞株を利用してベクターを調製した。最終的には、遠心後 PBS 溶液に置換し、力価測定・配列確認・無菌試験などの QC 試験項目を実施し、解析用に十分使用可能なクオリティーでの調製に成功した。

D．考察

抗原提示解析用の FSUxxTM56 を高発現し、モニタリング用 GFP を共発現するベクター（SeV^{18+}FSUxxTM5/ΔF-GFP）について、問題なく再構成・製造が可能であり、試験に供することができた。

E．結論

「抗原提示解析用の FSUxxTM56 を高発現し、モニタリング用 GFP を共発現するベクター（SeV18+FSUxxTM5/ΔF-GFP）」の再構成・ベクター調製を実施した。精製後、力価測定・配列確認・無菌試験などの QC 試験項目を実施し、抗原提示解析用ベクターとして、クオリティーの高いベクターを供給した。

F．健康危険情報

なし。

G．研究発表

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2　学会発表

なし。

H．知的財産権の出願・登録状況

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	論文タイトル名	発表誌名	巻号	ページ	出版年
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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

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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

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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.

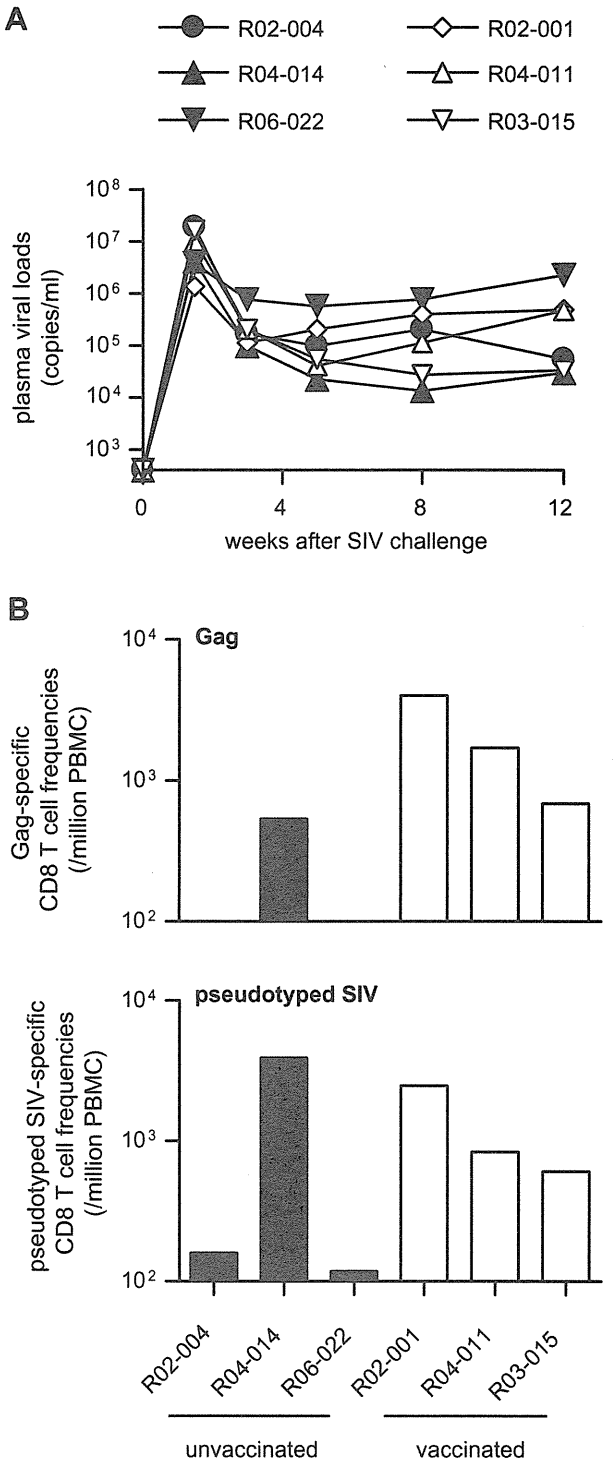

Fig. 1. CTL responses after SIVmac239 challenge in *90-088-Ij*-positive macaques. (A) Plasma viral loads after SIV challenge in unvaccinated (R02-004, R04-014, and R06-022) and DNA/SeV-Gag vaccinated animals (R02-001, R04-011, and R03-015). The viral loads (SIV gag RNA copies/ml) were determined as described 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

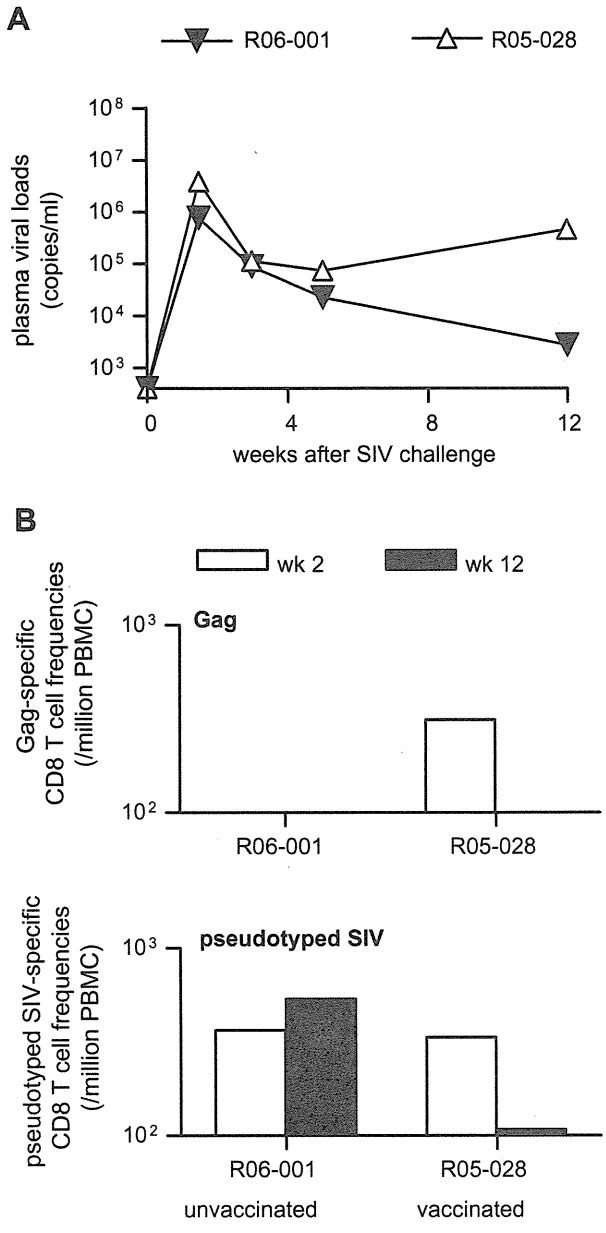

Fig. 2. CTL responses after SIVmac239 challenge in *90–120-Ib*-positive macaques. (A) Plasma viral loads after SIV challenge in unvaccinated R06–001 and SeV-Gag-vaccinated macaque R05-028. (B) Vaccine antigen Gag-specific (upper panel) and pseudotyped SIV-specific $CD8^+$ T cell frequencies (lower panel)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

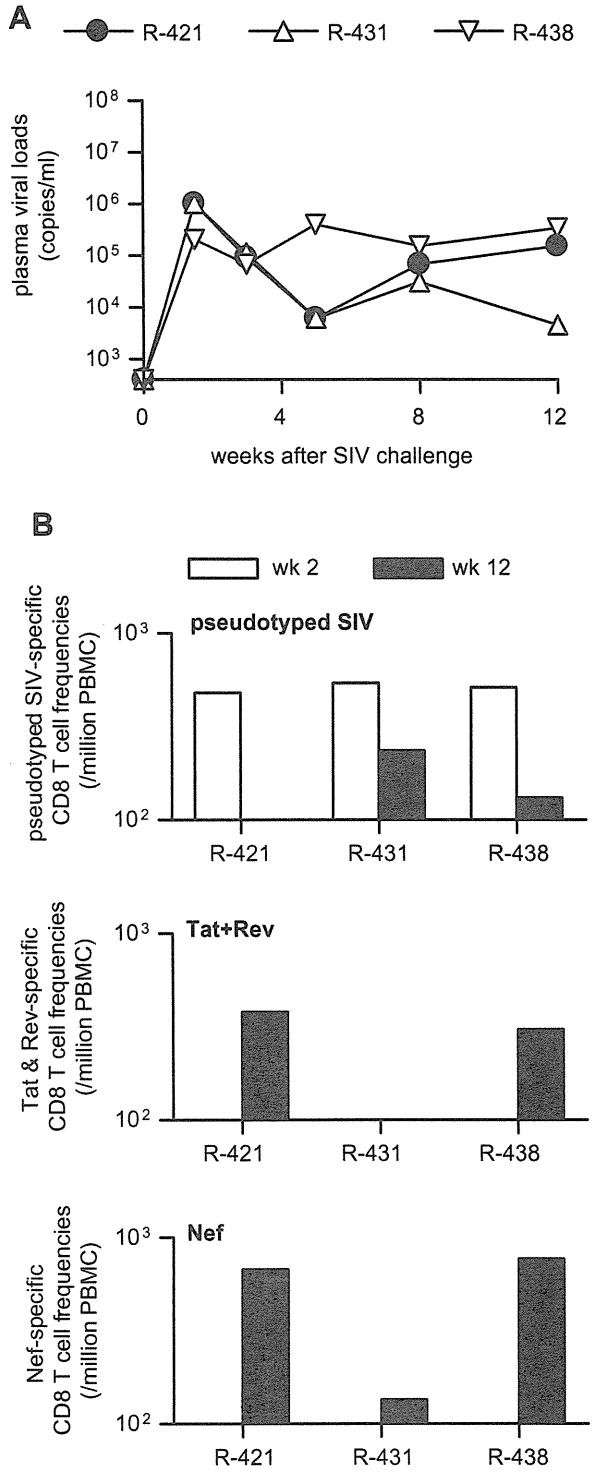

Fig. 3. CTL responses after SIVmac239 challenge in DNA-vaccinated macaques. The DNA used for the vaccination has the SIVmac239-derived region encoding Gag, Pol, Vif, and Vpx and is expected to induce pseudotyped SIV-specific CTL responses. (A) Plasma viral loads after SIV challenge in DNA vaccinated 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 ARTICLE

Major histocompatibility complex class I-restricted cytotoxic T lymphocyte responses during primary simian immunodeficiency virus infection in Burmese rhesus macaques

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ABSTRACT

Major histocompatibility complex class I (MHC-I)-restricted CD8$^+$ cytotoxic T lymphocyte (CTL) responses are crucial for the control of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) replication. In particular, Gag-specific CTL responses have been shown to exert strong suppressive pressure on HIV/SIV replication. Additionally, association of Vif-specific CTL frequencies with *in vitro* anti-SIV efficacy has been suggested recently. Host MHC-I genotypes could affect the immunodominance patterns of these potent CTL responses. Here, Gag- and Vif-specific CTL responses during primary SIVmac239 infection were examined in three groups of Burmese rhesus macaques, each group having a different MHC-I haplotype. The first group of four macaques, which possessed the MHC-I haplotype *90-010-Ie*, did not show Gag- or Vif-specific CTL responses. However, Nef-specific CTL responses were elicited, suggesting that primary SIV infection does not induce predominant CTL responses specific for Gag/Vif epitopes restricted by *90-010-Ie*-derived MHC-I molecules. In contrast, Gag- and Vif-specific CTL responses were induced in the second group of two *89-075-Iw*-positive animals and the third group of two *91-010-Is*-positive animals. Considering the potential of prophylactic vaccination to affect CTL immunodominance post-viral exposure, these groups of macaques would be useful for evaluation of vaccine antigen-specific CTL efficacy against SIV infection.

Key words cytotoxic T lymphocyte, human immunodeficiency virus, major histocompatibility complex, simian immunodeficiency virus.

Virus-specific CD8$^+$ CTL responses are crucial for the control of HIV and SIV replication (1–5). CTLs recognize specific epitopes which are presented on the target cell surface by binding to the MHC-I molecule. There have been many reports indicating association of MHC-I (HLA class I) genotypes with rapid or delayed AIDS progression in HIV-infected people (6–8). For instance, most of the HIV-infected individuals possessing *HLA-B*57* have a better prognosis and smaller viral loads, implicating HLA-B*57-restricted epitope-specific CTL responses in control

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List of Abbreviations: CTL, cytotoxic T lymphocyte; HIV, human immunodeficiency virus; HLA, human leukocyte antigen; IFN-γ, gamma interferon; MHC-1, major histocompatibility complex class I; PBMC, peripheral blood mononuclear cell; SIV, simian immunodeficiency virus.

of this virus (9, 10). Indian rhesus macaques possessing the MHC-I allele Mamu-B*17 tend to show smaller viral loads after SIVmac239 challenge (11). These findings imply possible HIV control by induction of particular effective CTL responses.

The potential of Gag-specific CTL responses to contribute to viral control was suggested by a cohort study indicating association of HIV control with the breadth of Gag-specific CTL responses (12). This was supported by an *in vitro* study indicating the ability of Gag-specific CTLs to respond rapidly to SIV infection (13). We previously developed a prophylactic AIDS vaccine using a Sendai virus vector expressing SIVmac239 Gag (14) and showed that Gag-specific CTL responses were responsible for vaccine-based SIV containment in a group of Burmese rhesus macaques possessing the MHC-I haplotype *90–120-Ia* (15, 16). Furthermore, our recent study analyzing the potential of $CD8^+$ cells to suppress SIV replication *in vitro* suggested association of *in vitro* anti-SIV efficacy with numbers of Vif-specific CTL frequencies (17). We also found weaker correlation between anti-SIV efficacy and numbers of Nef-specific CTL frequencies. These results imply the potency of Gag- and Vif-specific (and possibly Nef-specific) CTLs in suppressing HIV/SIV replication.

The immunodominance patterns of these potent CTL responses could be affected by host MHC-I genotypes (18, 19). Better understanding of these MHC-I-associated CTL immunodominance patterns during primary HIV/SIV infection would contribute to elucidation of the interaction between viral replication and host CTL responses. In the present study, we examined whether Gag- and Vif-specific CTL responses are efficiently induced during primary SIVmac239 infection in three groups of Burmese rhesus macaques possessing different MHC-I haplotypes. One group did not induce Gag- or Vif-specific CTL responses, whereas the other two groups elicited Gag- and Vif-specific CTL responses efficiently. These groups of macaques would be useful for analysis of the impact of Gag- and Vif-specific CTL responses on SIV replication *in vivo*.

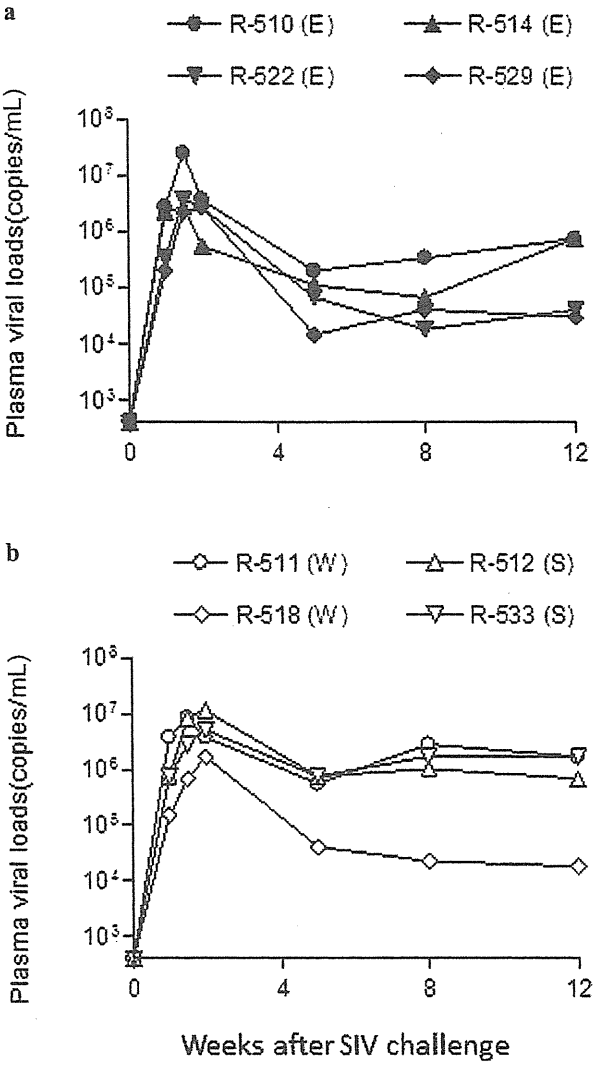

Fig. 1. Plasma viral loads after SIV challenge. (a) The first group of Burmese rhesus macaques, which possessed MHC-I haplotype *90-010-Ie* (R-510, R-514, R-522, and R-529) and (b) the second group, which possessed *89-075-Iw* (R-511 and R-518) and the third group, which possessed *91-010-Is* (R-512 and R-533) were challenged with SIVmac239. The viral loads (SIV gag RNA copies/mL) were determined as described previously (15).

MATERIALS AND METHODS

Animal experiments

Animal experiments using Burmese rhesus macaques (*Macaca mulatta*) possessing either the MHC-I haplotypes *90-010-Ie*, *89-075-Iw* or *91-010-Is* were performed in the Institute for Virus Research, Kyoto University, in accordance with the institutional regulations approved by the Committee for Experimental Use of Non-human Primates. The MHC-I haplotypes of macaques were determined as described previously (20, 21). These animals were challenged intravenously with 1000 50% tissue culture infective doses ($TCID_{50}$) of SIVmac239 (22).

Analysis of virus-specific cytotoxic T lymphocyte responses

Virus-specific $CD8^+$ T-cell frequencies were measured by flow cytometric analysis of IFN-γ induction after specific stimulation as described previously (17). PBMCs were cocultured with autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines pulsed with peptide pools using panels of overlapping peptides

spanning the entire SIVmac239 Gag, Pol, Vif, Vpx, Vpr, Tat, Rev, Nef, and Env amino acid sequences. Intracellular IFN-γ staining was performed with a Cytofix-Cytoperm kit (Becton Dickinson, Tokyo, Japan) and fluorescein isothiocyanate-conjugated anti-human CD4 (Becton Dickinson), peridinin chlorophyll protein-conjugated anti-human CD8 (Becton Dickinson), allophycocyanin-conjugated anti-human CD3 (Becton Dickinson), and phycoerythrin-conjugated anti-human IFN-γ monoclonal antibodies (BioLegend, Tokyo, Japan). Specific $CD8^+$ T-cell frequencies were calculated by subtracting nonspecific IFN-γ^+ $CD8^+$ T-cell frequencies from those after peptide-specific stimulation. Specific $CD8^+$ T-cells counts of less than 100 per million PBMCs were considered negative.

RESULTS

In the present study, we used eight Burmese rhesus macaques consisting of four animals possessing MHC-I haplotype *90-010-Ie*, two possessing *89-075-Iw*, and two possessing *91-010-Is*. After a SIVmac239 challenge, all these animals failed to control viral replication and had high set-point plasma viral loads (geometric mean: 3×10^5 copies/mL) (Fig. 1).

We examined SIV-specific $CD8^+$ T cell responses at week 2 and week 6 or 12 after SIV challenge in these animals by detection of specific IFN-γ induction after stimulation using peptide mixtures (Figs. 2 and 3). At week 6 or 12, we examined $CD8^+$ T cell responses specific for the N-terminal half of Gag (Gag-N), the C-terminal half of Gag (Gag-C), Vif, Nef, the N-terminal half of Pol (Pol-N), the C-terminal half of Pol (Pol-C), Vpx, Vpr, the N-terminal half of Env (Env-N), the C-terminal half of Env (Env-C), Tat, and Rev. At week 2, however, we examined only Gag-N-, Gag-C-, Vif- and Nef-specific $CD8^+$ T cell responses because of limited availability of PBMCs.

In the first group of macaques, which possessed *90-010-Ie*, neither Gag- nor Vif-specific $CD8^+$ T cell responses were induced efficiently at week 2 (Fig. 2). Even at week 12, these responses were undetectable in most of the animals. In contrast, Nef-specific $CD8^+$ T cell responses were detected at week 2, 6, or 12 in all four animals. Env-specific $CD8^+$ T cell responses were detectable at week 12 in three of them. These results indicate that, during primary SIV infection in *90-010-Ie*-positive macaques, Gag- or Vif-specific $CD8^+$ T cell responses are not induced, however Nef-specific $CD8^+$ T cell responses are.

In the second group of macaques, which possessed *89-075-Iw*, Gag- and Vif-specific $CD8^+$ T cell responses were elicited efficiently (Fig. 3a). In the third group of macaques, which possessed *91-010-Is*, Gag-, Vif- and Nef-specific $CD8^+$ T cell responses were elicited efficiently (Fig. 3b). Other SIV antigen-specific $CD8^+$ T cell responses were not efficiently induced in these two groups except for Tat-specific $CD8^+$ T cell responses in macaque

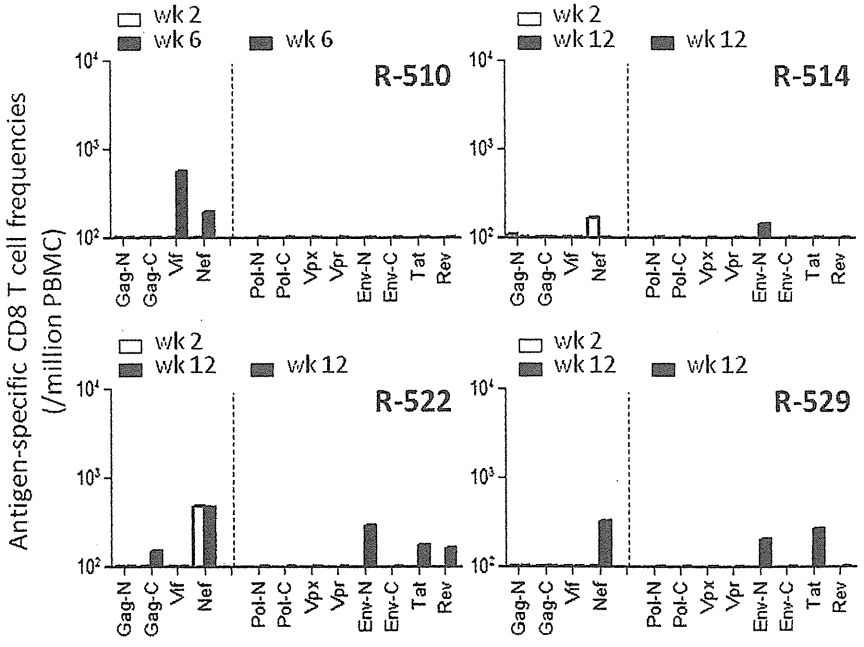

Fig. 2. SIV antigen-specific $CD8^+$ T cell frequencies in the first group of macaques, which possessed *90-010-Ie*. Gag-, Vif- and Nef-specific $CD8^+$ T cell frequencies at week 2 and Gag-, Vif-, Nef-, Pol-, Vpx-, Vpr-, Env-, Tat- and Rev-specific $CD8^+$ T cell frequencies at weeks 6 or 12 are shown.

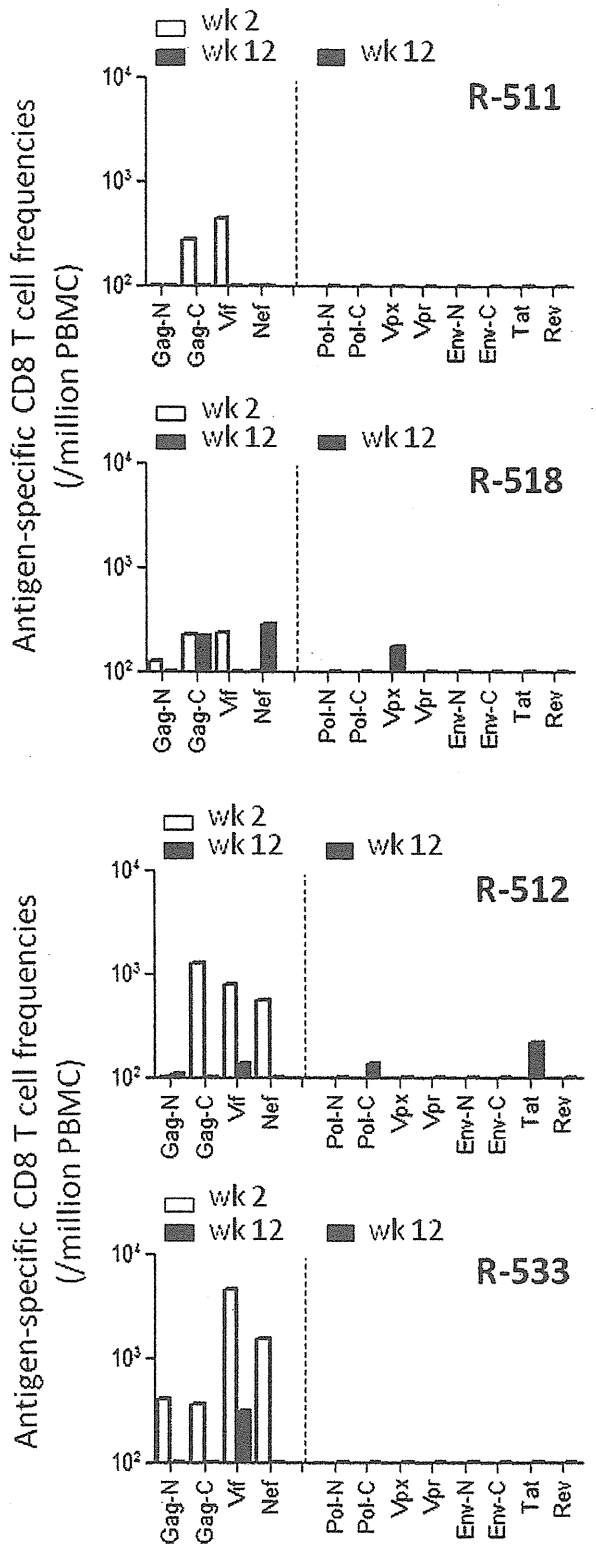

Fig. 3. SIV antigen-specific CD8$^+$ T cell frequencies in (a) the second group of macaques, which possessed *89-075-Iw* and (b) the third, which possessed *91-010-Is*.

R-512. Thus, in the four animals possessing *89-075-Iw* or *91-010-Is*, Gag- or Vif-specific CD8$^+$ T cell responses were induced more efficiently than Nef-specific ones at week 2. These responses in PBMCs were mostly diminished at week 12; possibly reflecting the considerable CTL consumption in the effector sites in animals with high viral loads.

DISCUSSION

Previous studies have indicated the potential of Gag-specific CTL responses to suppress HIV/SIV replication *in vivo* (12, 13, 16). Further, our recent study suggested the potency of Vif-specific CTL responses (17). Then, in the present study, we examined Gag- and Vif-specific CTL responses during primary SIV infection in three groups of animals, each group having a different MHC-I haplotype. Although the numbers of CTL frequencies differed between groups, the CTL responses tended have similar patterns.

Our previous study showed vaccine efficacy in a group of macaques with the MHC-I haplotype *90-120-Ia* (15, 16). Unvaccinated *90-120-Ia*-positive macaques predominantly induce Gag-specific CTL responses but fail to control viremia, while vaccinated ones show enhanced Gag-specific CTL responses and control SIV replication. $Gag_{206–216}$ epitope-specific and $Gag_{241–249}$ epitope-specific CTL responses were shown to be responsible for this vaccine-based viral control (16). However, some Gag-specific CTLs may be effective while others are not. Further analysis of this type of vaccine efficacy would contribute to understanding the requisites for vaccine-based viral control. Possibly, the *89-075-Iw*-positive or *91-010-Is*-positive animals presented in this study may be a candidate model for such analysis.

In primary SIVmac239 infection, it is speculated that some MHC-I haplotypes (referred to as type 1) are associated with Gag/Vif-specific CTL responses while others (referred to as type 2) are not. The MHC-I haplotype *90-120-Ia* described above belongs to type 1. In the present study, the second group, which possess MHC-I haplotype *89-075-Iw*, and the third, which possess *91-010-Is*, both showed efficient Gag- and Vif-specific CTL responses in primary SIV infection, although it remains undetermined whether these MHC-I haplotypes belong to type 1. In contrast, the first group of macaques, which possess MHC-I haplotype *90-010-Ie* did not show efficient Gag- or Vif-specific CTL responses in primary SIV infection. Instead, Nef-specific CTL responses were induced in all four animals. This suggests that the MHC-I haplotype *90-010-Ie* belongs to type 2; that is, primary SIV infection induces no predominant CTL responses specific for Gag/Vif epitopes

restricted by *90-010-Ie*-derived MHC-I molecules. Our results imply that CTLs exerted selective pressure on SIV *gag* and *vif* in the second/third groups but not in the first group. Larger number of animals would enable us to compare those with type 1 and 2 MHC-I haplotypes, which would contribute to our understanding of the efficacy of Gag- and Vif-specific CTL responses against SIV infection.

In developing a prophylactic CTL-inducing AIDS vaccine, it would be important to induce CTL memory resulting in potent CTL responses post-HIV exposure, while prophylactic vaccination can affect the immunodominance patterns of CTL responses post-viral exposure (23, 24). Gag- and Vif-specific CTL memory induction may be a promising vaccine strategy, but the influence of prophylactic vaccination on the patterns of CTL responses post-viral exposure would be affected by MHC-I genotypes. In the hosts in which Gag- and Vif-specific CTL responses are induced during the natural course of SIV infection, Gag- and Vif-specific CTL memory induction by prophylactic vaccination would predominantly enhance these CTL responses. In contrast, in those in whom no Gag- or Vif-specific CTL responses occurred during the natural course of SIV infection, prophylactic vaccination inducing Gag- and Vif-specific CTL responses would result in broader CTL responses. Macaques in which both MHC-I haplotypes belong to type 2 may be ideal for evaluation of this type of vaccine efficacy, but it is very difficult to accumulate those animals. It would be reasonable to use groups of macaques possessing type 2 haplotypes such as the group 1 (*90-010-Ie*-positive macaques) presented in this study for such evaluation.

In summary, by focusing on Gag- and Vif-specific CTL responses, we found two types of rhesus macaques that showed different patterns of CTL responses during primary SIV infection; one elicited Gag- and Vif-specific CTL responses but the other did not. Accumulated analyses in both types of animals would contribute to understanding the impact of these potent CTL responses on primary SIV infection.

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Intranasal Sendai viral vector vaccination is more immunogenic than intramuscular under pre-existing anti-vector antibodies

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ABSTRACT

Viral vectors are promising vaccine tools for eliciting potent cellular immune responses. Pre-existing anti-vector antibodies, however, can be an obstacle to their clinical use in humans. We previously developed a Sendai virus (SeV) vector vaccine and showed the potential of this vector for efficient CD8+ T-cell induction in macaques. Here, we investigated the immunogenicity of SeV vector vaccination in the presence of anti-SeV antibodies. We compared antigen-specific CD8+ T-cell responses after intranasal or intramuscular immunization with a lower dose (one-tenth of that in our previous studies) of SeV vector expressing simian immunodeficiency virus Gag antigen (SeV-Gag) between naive and pre-SeV-infected cynomolgus macaques. Intranasal SeV-Gag immunization efficiently elicited Gag-specific CD8+ T-cell responses not only in naive but also in pre-SeV-infected animals. In contrast, intramuscular SeV-Gag immunization induced Gag-specific CD8+ T-cell responses efficiently in naive but not in pre-SeV-infected animals. These results indicate that both intranasal and intramuscular SeV administrations are equivalently immunogenic in the absence of anti-SeV antibodies, whereas intranasal SeV vaccination is more immunogenic than intramuscular in the presence of anti-SeV antibodies. It is inferred from a recent report investigating the prevalence of anti-SeV antibodies in humans that SeV-specific neutralizing titers in more than 70% of people are no more than those at the SeV-Gag vaccination in pre-SeV-infected macaques in the present study. Taken together, this study implies the potential of intranasal SeV vector vaccination to induce CD8+ T-cell responses even in humans, suggesting a rationale for proceeding to a vaccine clinical trial using this vector.

1. Introduction

Virus-specific CD8+ T-cell responses are crucial for the control of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) replication [1–6]. Efficient induction of virus-specific CD8+ T-cell responses is an important strategy for AIDS vaccine development, and recombinant viral vectors are promising vaccine tools for CD8+ T-cell induction [7,8]. Recent studies have indicated the potential of prophylactic viral vector immunization to induce virus-specific CD8+ T-cell responses and reduce postchallenge viral loads in macaque AIDS models [9–13]. Most of the parental or related viruses of these vectors can induce natural infection in humans. Thus, pre-existing antibodies against the vector virus itself could be an obstacle to viral vector-based CD8+ T-cell induction in humans. Indeed, a clinical trial of a vaccine using adenovirus serotype 5 (AdV5) vectors has shown reduction in efficiency of vaccine-based CD8+ T-cell induction in people with pre-existing anti-AdV5 antibodies [14–17].

We previously developed an AIDS vaccine using a recombinant Sendai virus (SeV) vector and showed that intranasal SeV vector immunization results in efficient induction of antigen-specific CD8+ T-cell responses in macaques [9,18,19]. SeV, murine parainfluenza virus type 1 (PIV-1), is an enveloped virus with a negative-sense RNA genome. SeV replication is localized in the airway because it requires a protease localized in the airway epithelium for envelope protein processing [20]. Thus, replication-competent SeV vectors [21] have been administered intranasally, while replication-defective SeV vectors [22] may be administered intramuscularly as well as intranasally. However, we have not

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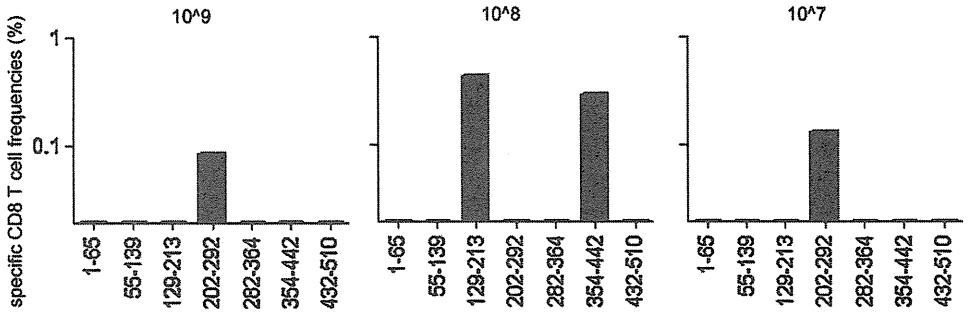

Fig. 1. Gag-specific CD8⁺ T-cell responses after intranasal boost with lower doses of F(−)SeV-Gag. Cynomolgus macaques received a DNA vaccination, and six weeks later, were intranasally boosted with 6×10^9 (10^9), 6×10^8 (10^8), or 6×10^7 (10^7) CIU of F(−)SeV-Gag, respectively. Gag peptide pool-specific CD8⁺ T-cell frequencies (percent in CD8⁺ T lymphocytes) two weeks after the boost are shown. A panel of overlapping peptides spanning the entire SIV Gag amino acid (aa) sequence was divided into 7 pools, 1–65 (corresponding to the 1st–65th aa in SIV Gag), 55–139 (55th–139th aa), 129–213 (129th–213th aa), 202–292 (202nd–292nd aa), 282–364 (282nd–364th aa), 354–442 (354th–442nd aa), and 432–510 (432nd–510th aa), and used for the stimulation to detect peptide pool-specific CD8⁺ T cells, respectively.

yet examined the immunogenicity of intramuscular SeV vector vaccination.

The natural host of SeV is mice and its natural infection has not been observed in primates including humans [20]. Antibodies against human PIV-1 (hPIV-1), whose natural infection frequently occurs in humans, are known to cross-react with SeV [23,24]. Our recent analyses in macaques showed efficient Gag-specific CD8⁺ T-cell induction by an intranasal immunization with 6×10^9 CIU of F(−)SeV-Gag more than one year after an initial SeV vector inoculation, suggesting a possibility of antigen-specific CD8⁺ T-cell induction by SeV vector administration in the presence of SeV-specific neutralizing antibody (NAb) responses [25,26]. However, it remains unclear to what extent SeV-specific NAbs could have adverse effect on CD8⁺ T-cell induction by SeV vector vaccination.

In the present study, we investigated antigen-specific CD8⁺ T-cell responses after intranasal or intramuscular immunization with a lower dose of SeV vector in macaques pre-infected with SeV to sensitively examine the effect of pre-SeV-infection on SeV-based CD8⁺ T-cell induction. Our results revealed that intranasal SeV administration is more immunogenic than intramuscular in the presence of anti-SeV NAbs and suggested the potential of this vector to induce antigen-specific CD8⁺ T-cell responses even in humans.

2. Materials and methods

2.1. Animal experiments

The animal experiments were conducted through the Cooperative Research Program in Tsukuba Primate Research Center (TPRC), National Institute of Biomedical Innovation with the help of the Corporation for Production and Research of Laboratory Primates. All animals were maintained in accordance with the guidelines for laboratory animals of the National Institute of Infectious Diseases. Blood collection and vaccination were performed under ketamine anesthesia. Cynomolgus macaques (*Macaca fascicularis*) of the TPRC breeding colonies derived from Indonesia, Malaysia, and the Philippines were used for this experiment. All animals received a DNA vaccine followed by a single boost with a replication-defective (non-transmissible) F-deleted SeV expressing SIVmac239 Gag, F(−)SeV-Gag, as described previously [9]. The DNA, CMV-SHIVdEN, used for the vaccination was constructed from an *env*- and *nef*-deleted $SHIV_{MD14YE}$ molecular clone DNA [27] and has the genes encoding SIVmac239 Gag, Pol, Vif, and Vpx, SIVmac239-HIV-1 chimeric Vpr, and HIV-1 Tat and Rev [19]. At the DNA vaccination, animals received 5 mg of CMV-SHIVdEN DNA intramuscularly. Six weeks after the DNA prime, animals intranasally or intramuscularly received a single boost with 6×10^7, 6×10^8, or 6×10^9 cell infectious units (CIU) of F(−)SeV-Gag [22,28]. Group II and IV animals were intranasally infected with 1×10^8 CIU of replication-competent (transmissible) V-knocked-out SeV [18,21] nine weeks before the DNA prime.

2.2. Measurement of Gag-specific CD8⁺ T-cell responses

We measured Gag-specific CD8⁺ T-cell levels by flow-cytometric analysis of gamma interferon (IFN-γ) induction after specific stimulation as described previously [9]. Peripheral blood mononuclear cells (PBMCs) were cocultured with autologous herpesvirus papio-immortalized B lymphoblastoid cell lines (B-LCLs) pulsed with peptide pools using panels of 117 overlapping peptides (mostly 15-mer) spanning the entire SIVmac239 Gag amino acid sequences [25] (Fig. 1) or a vaccinia virus vector expressing SIVmac239 Gag (Figs. 3 and 4) for Gag peptide pool-specific or Gag-specific stimulation. Intracellular IFN-γ staining was performed using CytofixCytoperm kit (BD, Tokyo, Japan) and the following monoclonal antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-human CD4 (BD, #556615, M-T477), peridinin chlorophyll protein (PerCP)-conjugated anti-human CD8 (BD, #347314, SK1), allophycocyanin (APC)-conjugated anti-human CD3 (BD, #557597, SP34-2), and phycoerythrin (PE)-conjugated anti-human IFN-γ antibodies (BD, #557074, 4S.B3). Specific CD8⁺ T-cell levels were calculated by subtracting non-specific IFN-γ⁺ CD8⁺ T-cell frequencies from those after Gag peptide pool-specific or Gag-specific stimulation. Specific CD8⁺ T-cell levels less than 0.02% of CD8⁺ T lymphocytes were considered negative.

2.3. Measurement of anti-SeV IgG levels

The plasma anti-SeV immunoglobulin G (IgG) levels were measured by an enzyme-linked immunosorbent assay (ELISA) (Denka Seiken, Tokyo, Japan) using whole inactivated SeV (HVJ Z strain) particles and a peroxidase-conjugated anti-monkey IgG antibody [29].

2.4. Measurement of anti-SeV neutralizing titers

We measured plasma SeV-specific neutralizing titers on LLC-MK2 cells using a recombinant SeV expressing enhanced green