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霊長類ゲノム情報を利用した抗エイズウイルス自然免疫因子の探索およびその新規エイズ治療法への応用

平成２２年度総括報告書

研究代表者　武内　寛明

前所属先：東京大学医科学研究所　感染症国際研究センター
現所属先：東京医科歯科大学　医歯学総合研究科　ウイルス制御学分野

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研究代表者　武内　寛明　　前所属：東京大学医科学研究所　特任助教
現所属：東京医科歯科大学　助教

研究要旨　世界的に流行しているエイズの原因であるヒトエイズウイルス(HIV)は、サルエイズウイルス（SIV）が「種の壁」を乗り越え病原性を示すHIVへと変貌を遂げた歴史的背景が明らかとなってきている。しかし、SIVがヒトに感染し、病原性を示すようになった原因は、未だ解明がなされていない部分が多い。当該研究では、SIVが「種の壁」を乗り越えてヒトへ感染伝播する際に関わる自然免疫因子（群）およびHIV感染制御ヒト宿主因子（群）を同定することにより、新規エイズ治療法に向けての基盤確立に寄与すること、および今後の新興感染症に対するヒト宿主防御機構に対する理解を深めることが目的である。本研究では（１）RNA干渉法を用いてヒトおよびサル機能遺伝子抑制T細胞ライブラリーを樹立し、HIVおよびSIV感染制御宿主因子群の探索および、（２）エイズウイルス感染過程に影響をおよぼすウイルス側要因の探索を行った。その結果、（１）HIV感染制御宿主因子群とSIV感染制御宿主因子群とを多数見出すことが出来た。これら宿主因子群の中には、既知のHIV感染制御因子だけでなく、未知の宿主因子群も相当数含まれていることが判明した。また、（２）ヒトT細胞内における感染前期過程に影響をおよぼすSIV側要因として、構造蛋白であるCA蛋白のC末端側(CTD)に位置するアミノ酸部位を同定するに至った。このSIV CA-CTD変異体のサルT細胞での感染増殖能は野生SIV株と同等であることから、ヒト宿主因子にのみ影響を受けるものであることが示唆された。

A. 研究目的

世界的に流行しているエイズの原因であるヒトエイズウイルス（HIV）は、サルエイズウイルス（SIV）が「種の壁」を乗り越え病原性を示すHIVへと変貌を遂げた歴史的背景が明らかとなってきている。しかしながら、SIVがヒトに感染し病原性を示すようになった原因は、未だ解明がなされていない部分が多い。当該研究では、SIVが「種の壁」を乗り越えてヒトへ感染伝播する際に関わる自然免疫因子（群）およびHIV感染制御ヒト宿主因子（群）を同定することにより、新規エイズ治療法に向けての基盤確立に寄与すること、および今後の新興感染症に対するヒト宿主防御機構の理解を深めることを目的とした。

現在までにヒトゲノム情報に立脚したエイズウイルス制御宿主因子探索法として、RNA干渉法によるgenome-wide screening法による研究成果が幾つか報告されているが、エイズウイルス標的細胞を用いたものではなく、そのため自然感染におけるエイズウイルス感染伝播での役割については不明な点が多い。本研究では、エイズウイルス標的細胞であるTリンパ球を用いて機能遺伝子発現抑制Tリンパ球ライブラリーを構築し、これらライブラリーと正常Tリンパ球との間でのウイルス感染効率を比較検討し、HIV感染制御抑制因子群とSIV感染制御因子群とを同定することを試みる。また、SIVがヒトT細胞における感染増殖能に影響を及ぼすウイルス側要因の同定も

試みる。平成２２年度は、RNA 干渉法による機能遺伝子発現抑制 T 細胞ライブラリーを構築し、ウイルス感染制御因子群の探索およびヒト T 細胞内における感染増殖能に影響をおよぼす SIV 側責任領域の同定を試みた。

B. 研究方法

1. 宿主機能遺伝子発現抑制 T 細胞ライブラリーの作製

宿主遺伝子（約１万８千遺伝子）を標的とした short hairpin RNA（shRNA)ライブラリーを発現するカセットを組み込んだレンチウイルスベクターを用いて T 細胞ゲノムへ shRNA 発現カセットを組み込むことにより、機能遺伝子発現抑制 shRNA T 細胞ライブラリーを作製した。

具体的には、CD4 陽性ヒト T 細胞株である MT-4/CCR5 細胞をライブラリー作製材料とし、shRNA-レンチウイルスライブラリーを MOI=10 で感染させた。shRNA 発現カセットには、ピューロマイシン耐性遺伝子が含まれていることから、shRNA 発現カセットが組み込まれた細胞を選択する目的で、感染４８時間後にピューロマイシン（1 μg/ml）を含む培地と交換し、２週間選択培養を行った後に T 細胞株ライブラリーを樹立した。

2. HIV および SIV 感染制御宿主因子群の探索

（１）にて作製したヒト T 細胞株ライブラリーを用いて、HIV および SIV 感染耐性 T 細胞株を選択した。具体的には、HIV-1 NL4-3 株および SIVmac239 株感染感受性 T 細胞株である MT-4/CCR5 細胞ライブラリーに、HIV（MOI=0.001）および SIV（MOI=0.1）を感染させた。感染 12 日後に HIV/SIV 感染耐性細胞を限界希釈し、更に２週間培養することで、HIV/SIV 感染耐性 T 細胞ライブラリーを得た。その後、これら細胞株が保持している shRNA 配列を解析し、shRNA 配列が標的としている宿主機能遺伝子を同定した。

3. SIV 遺伝子変異株の作製

Site-directed mutagenesis 法を用いて、SIV キャプシド(CA)領域内において HIV 遺伝子と異なる遺伝子配列をもとに点変異導入を行い、SIV/HIV-CA キメラウイルスを作製した。

4. ウイルス増殖能の解析

各 T 細胞株に、HIV-1 もしくは SIV を感染させた。その後、経時的に培養上清を回収し、それらに含まれるウイルス由来の逆転写酵素（RT）活性を測定した。

（倫理面への配慮）

本研究における遺伝子組み換え生物等を用いる実験については、必要に応じた東京大学医科学研究所の機関承認および文部科学大臣承認を既に取得済みである。

C. 研究結果

1. HIV および SIV 感染制御因子探索システムの確立

ヒト機能遺伝子（約１万８千遺伝子）を標的とした shRNA ライブラリーによる機能遺伝子発現抑制 T 細胞株を作製するため、SIV および HIV の双方に対して感染感受性細胞である MT-4/CCR5 細胞をライブラリー作製細胞として選択した。様々な感染条件を検討した結果、1 x 10^5 個の MT-4/CCR5 細胞に MOI=10 に相当する shRNA-レンチウイルスライブラリーを 48 時間感染させた。shRNA 発現カセットには、ピューロマイシン耐性遺伝子が含まれていることを利用して、shRNA 発現カセットが組み込まれた細胞を選択する目的で、感染 48 時間後にピューロマイシン（1 μg/ml）を含む培地と交換し、更に２週間選択培養を行った後に、T 細胞株ライブラリーを樹立した。

2. 機能遺伝子発現抑制T細胞株ライブラリーを用いたHIV/SIV感染制御因子の探索

樹立したT細胞株ライブラリーを用いて、HIV NL4-3 (MOI=0.001) およびSIVmac239 (MOI=0.1) を感染させた。感染12日後にHIV/SIV感染耐性細胞を限界希釈し、更に2週間培養することで、HIV/SIV感染耐性T細胞株を得た(図1)。

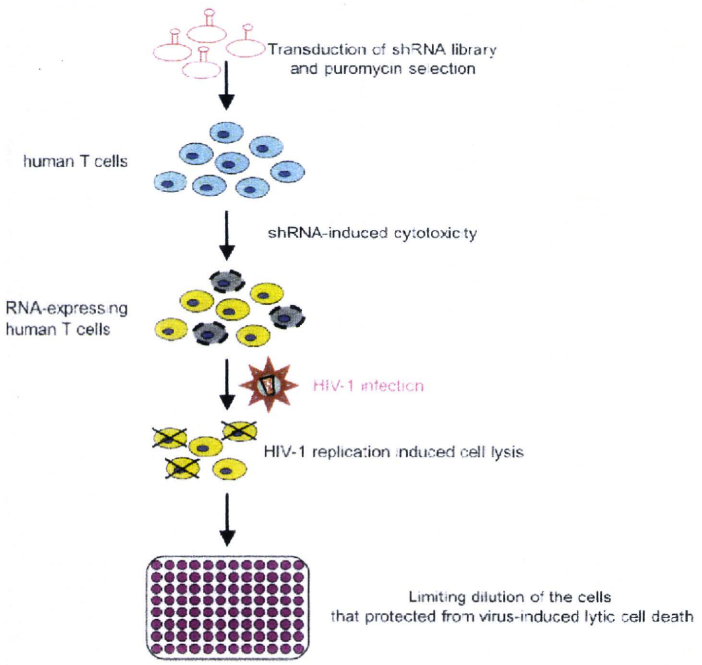


図1. ヒトT細胞株ライブラリーの樹立およびHIV感染耐性細胞株の選択法

次に、これら細胞株が保持しているshRNA配列を解析し、shRNA配列が標的としている宿主機能遺伝子群を同定した。これら遺伝子群の機能解析を行うために、細胞内局在分布をINGENUITY社データベースによるYFG search (SIGMA-ALDRICH)を行った。その結果、HIVおよびSIV感染制御因子群共に、細胞膜および細胞質に局在するものが50%前後を占める結果となった(図2-1および図2-2)。

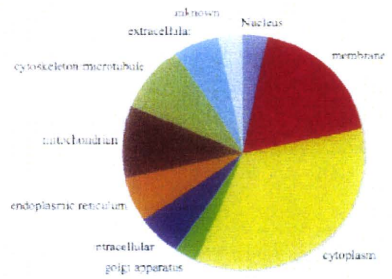


図2-1. HIV感染制御因子群の細胞内局在分布

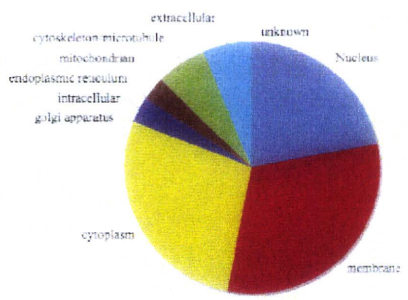


図2-2. SIV感染制御因子群の細胞内局在分布

3. HIV 感染増殖効率におよぼす機能遺伝子群の解析

HIV 感染耐性 T 細胞ライブラリーを希釈して再培養を行った後に得られた細胞集団（sub-clonal cell population）のウイルス既感染の有無、および shRNA の off-target 効果によるウイルス感染必須レセプターである CD4/CXCR4/CCR5 の細胞膜表面発現レベルへの影響について、正常 MT-4/CCR5 細胞とともに比較検討を行った。具体的には、ウイルス感染の有無については、細胞内ウイルス DNA の定量および培養上清中の逆転写酵素活性を測定した。その結果、細胞内ウイルス DNA および培養上清中の逆転写酵素活性共に認められなかった。また、細胞膜表面上の各上記レセプターの発現レベルを細胞表面解析装置（FACS）にて検討したところ、発現レベルの差異は認められなかった。これらの結果より、HIV 感染耐性細胞は HIV 非感染細胞であり、かつ HIV 感染標的細胞としての性状を維持していることが判明した（data not shown）。

次に、これら T 細胞ライブラリーと正常 T 細胞とを用いてウイルス感染効率を比較検討するために、T 細胞ライブラリー集団および正常 T 細胞とに、HIV（MOI=0.001）を感染させた。感染効率については、定期的に培養上清中の RT activity を測定することにより検討した。その結果、HIV 感染効率が顕著に低下した T 細胞ライブラリー集団を複数確認した。その代表的なライブラリー集団３つの結果を示す（図 3-1, 3-2, 3-3）。

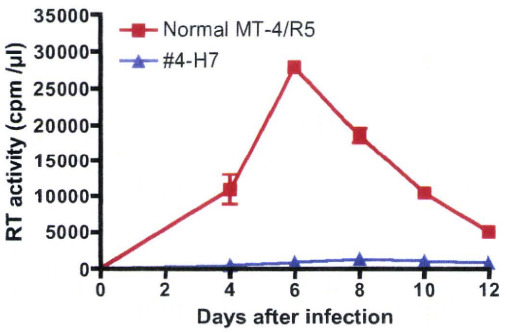


図 3-1. HIV 感染増殖効率に対する感染制御機能遺伝子群の影響. 4 種の機能遺伝子を標的とした T 細胞ライブラリー集団(#4-H7)および正常 T 細胞（Normal MT-4/R5）に NL4-3 を感染させ、経時的に培養上清中の RT activity を測定した。

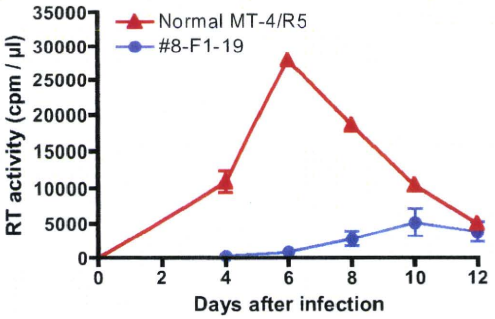


図 3-2. HIV 感染増殖効率に対する感染制御機能遺伝子群の影響. 7 種の機能遺伝子を標的とした T 細胞ライブラリー集団(#8-F1-19)および正常 T 細胞（Normal MT-4/R5）に NL4-3 を感染させ、経時的に培養上清中の RT activity を測定した。

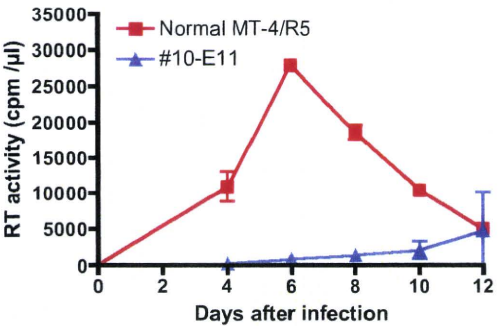


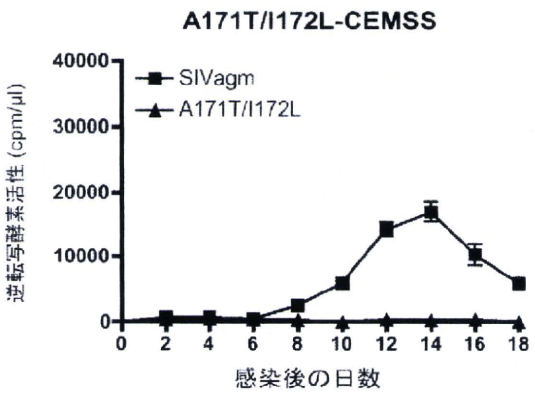
図 3-3. HIV 感染増殖効率に対する感染制御機能遺伝子群の影響. 6 種の機能遺伝子を標的とした T 細胞ライブラリー集団(#10-E11)および正常 T 細胞（Normal MT-4/R5）に NL4-3 を感染させ、経時的に培養上清中の RT activity を測定した。

4. ヒト T 細胞内における SIV 増殖必須領域の同定と解析

研究代表者は先行研究にて、SIV-CA 領域において、ヒト T 細胞での HIV と SIV との感染指向性（トロピズム）を規定する部位が存在することを見出している

(Takeuchi, *Vaccine*, 2010)。本研究では、SIV と HIV との CA 領域内におけるアミノ酸配列が異なる領域について、SIV を骨格とした SIV/HIV-CA キメラウイルスを作製し、ヒト細胞内での増殖能に影響をおよぼす領域を同定することを試みた。その結果、SIV-CTD 領域内である Helix8 (図 4-1) および Helix11 (図 4-2) 領域内のアミノ酸配列が、サル T 細胞では野生 SIV 株と同等の増殖能を保持しているにも関わらず、ヒト細胞内で増殖出来なくなることが示された。

(A)



(B)

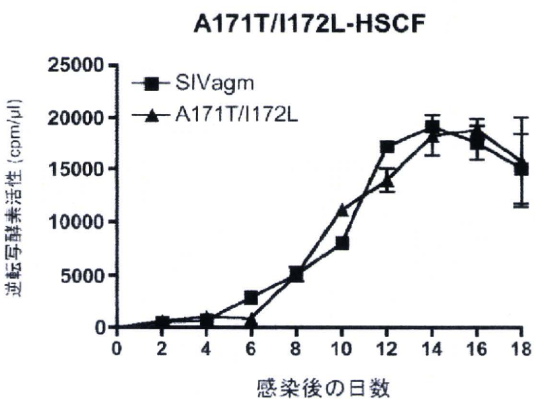
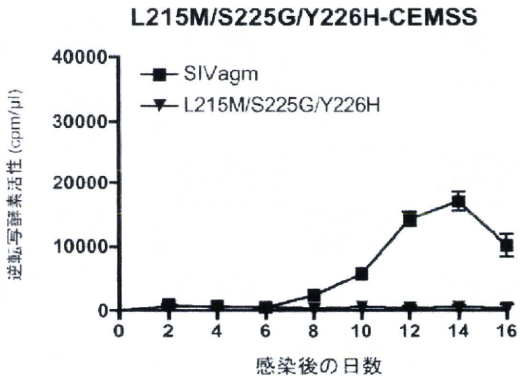


図 4-1. SIV 感染増殖能に対する SIV-CA Helix8 領域の影響. アフリカミドリザルを宿主とする SIVagm の CA-Helix8 領域を HIV-1 の Helix8 領域に置換したキメラウイルスを用いて、CEM-SS ヒト T 細胞株(A)および HSC-F サル T 細胞株(B)に感染させ、培養上清中の逆転写酵素活性を経時的に測定した。

(A)



(B)

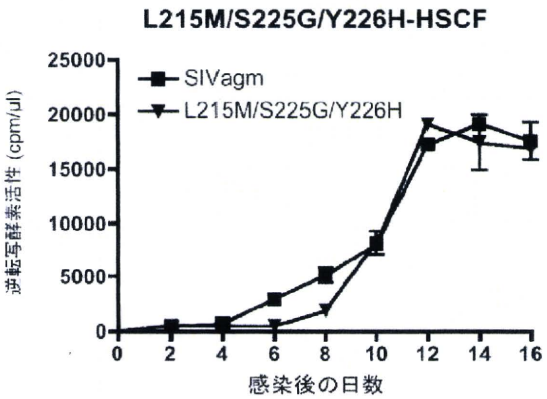


図 4-2. SIV 感染増殖能に対する SIV-CA Helix11 領域の影響. アフリカミドリザルを宿主とする SIVagm の CA-Helix11 領域を HIV-1 の Helix11 領域に置換したキメラウイルスを用いて、CEM-SS ヒト T 細胞株(A)および HSC-F サル T 細胞株(B)に感染させ、培養上清中の逆転写酵素活性を経時的に測定した。

D. 考察

現在までに、ヒトゲノム情報に立脚したエイズウイルス制御宿主因子探索は既に幾つかの成果が報告されているが、エイズウイルス標的細胞を用いたものではなく、そのため自然感染におけるエイズウイルス感染伝播での役割については不明な点が多い。そこで当該研究では、エイズウイルス標的細胞である T リンパ球を用いた機能遺伝子発現抑制ヒ

トT細胞ライブラリーを構築した。次に、これらライブラリーと正常T細胞との間でのウイルス感染効率を比較検討した結果、HIV感染制御宿主因子群とSIV感染制御宿主因子群とを同定するに至った。これら宿主因子群は、shRNAにより発現抑制されることで、HIV感染耐性に至ったことから、HIV感染必須因子であることが推察出来る。細胞からこれら宿主因子群の中には、HIV感染必須因子として既に知られているTransportin-3およびCyclophilin Aも含まれていたことから、本研究で樹立した機能遺伝子発現抑制T細胞ライブラリーおよびウイルス感染必須遺伝子群の探索法の妥当性が認められたと考えられる。その上で、既知のHIV感染制御因子だけでなく、未知の宿主因子群も多数含まれていることが判明したことからも、新たなHIV感染制御因子探索にも使用可能であると考えられる。また、本研究で同定したHIVおよびSIV感染制御機能遺伝子群を比較検討した結果、それら遺伝子群が大きく異なることが判明したが、HIVおよびSIV感染に共通した未知の感染制御因子群も明らかとなった。これらは、ウイルス普遍的な宿主因子群であることが考えられる。

SIV変異株を用いた研究では、ヒトT細胞への感染増殖能には、CA-CTDのHelix8およびHelix11領域が必須であることが明らかとなった。ところが、このSIV変異株のサルT細胞における感染増殖能は野生SIV株と同等であることから、変異によるウイルス粒子形成能およびウイルス産生効率の低下よりもむしろヒトT細胞への新たな感染段階において、ヒト宿主因子にのみ影響を受けるものであると考えられる。

E. 結論

当該研究事業初年度で機能遺伝子発現抑制T細胞ライブラリーの構築に成功し、HIVおよびSIV感染制御宿主因子候補群を多数見出す事が出来た。またSIVのヒトへの感染にはSIV CA-CTDが重要であることを見出した。これらの成果は、世界中で他の研究グループが成し得ていないものであり、継続する価値が非常に高いものと思われる。

F. 健康危険情報

該当なし

G. 研究発表

1. 論文発表

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H. 知的財産権の出願・登録状況

該当なし

別紙４

研究成果の刊行に関する一覧表レイアウト（参考）

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
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Inagaki N., **Takeuchi H**., Yokoyama M., Sato H., Ryo A., Yamamoto H., Kawada M., Matano T	A structural constraint for functional interaction between N-terminal and C-terminal domains in simian immunodeficiency virus capsid proteins	***Retrovirology***	7	90	2010
Takeuchi H	Contribution of Cyclophilin A to determination of simian immunodeficiency virus tropism: A progress update	***Vaccine***	28 Suppl 2	B51-4	2010

Broadening of CD8$^+$ cell responses in vaccine-based simian immunodeficiency virus controllers

Nami Iwamoto[a], Tetsuo Tsukamoto[a], Miki Kawada[a], Akiko Takeda[a], Hiroyuki Yamamoto[a], Hiroaki Takeuchi[a] and Tetsuro Matano[a,b]

Objective: In our prior study on a prophylactic T-cell-based vaccine, some vaccinated macaques controlled a simian immunodeficiency virus (SIV) challenge. These animals allowed viremia in the acute phase but showed persistent viral control after the setpoint. Here, we examined the breadth of postchallenge virus-specific cellular immune responses in these SIV controllers.

Design: We previously reported that in a group of Burmese rhesus macaques possessing the MHC haplotype *90-120-Ia*, immunization with a Gag-expressing vaccine results in nonsterile control of a challenge with SIVmac239 but not a mutant SIV carrying multiple cytotoxic T lymphocyte (CTL) escape *gag* mutations. In the present study, we investigated whether broader cellular immune responses effective against the mutant SIV replication are induced after challenge in those vaccinees that maintained wild-type SIVmac239 control.

Methods: We analyzed cellular immune responses in these SIV controllers ($n = 8$).

Results: These controllers elicited CTL responses directed against SIV non-Gag antigens as well as Gag in the chronic phase. Postvaccinated, prechallenge CD8$^+$ cells obtained from these animals suppressed wild-type SIV replication *in vitro*, but mostly had no suppressive effect on the mutant SIV replication, whereas CD8$^+$ cells in the chronic phase after challenge showed efficient antimutant SIV efficacy. The levels of in-vitro antimutant SIV efficacy of CD8$^+$ cells correlated with Vif-specific CD8$^+$ T-cell frequencies. Plasma viremia was kept undetectable even after the mutant SIV super-challenge in the chronic phase.

Conclusion: These results suggest that vaccine-based wild-type SIV controllers can acquire CD8$^+$ cells with the potential to suppress replication of SIV variants carrying CTL escape mutations.

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Keywords: AIDS vaccine, cytotoxic T lymphocyte, major histocompatibility complex, mutation, simian immunodeficiency virus

Introduction

Virus-specific CD8$^+$ cytotoxic T lymphocyte (CTL) responses are crucial for the control of HIV and simian immunodeficiency virus (SIV) replication [1–6]. Cumulative studies on HIV-infected individuals have shown association of HLA genotypes with rapid or delayed AIDS progression [7,8]. For instance, most of the HIV-infected individuals possessing *HLA-B*57* have been indicated to show a better prognosis with lower viral loads, implicating HLA-B*57-restricted epitope-specific CTL responses in this viral control [9–11]. Indian rhesus macaques possessing particular major histocompatibility complex class I (MHC-I) alleles such as Mamu-B*17 tend to show SIV control [12–14]. These imply possible HIV control by induction of particular effective CTL responses.

[a]International Research Center for Infectious Diseases, The Institute of Medical Science, The University of Tokyo, and [b]AIDS Research Center, National Institute of Infectious Diseases, Tokyo, Japan.

Correspondence to Tetsuro Matano, International Research Center for Infectious Diseases, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan.

Tel: +81 3 6409 2078; fax: +81 3 6409 2076; e-mail: matano@ims.u-tokyo.ac.jp

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Recent trials of prophylactic T-cell-based vaccines in macaque AIDS models have indicated a possibility of reduction in postchallenge viral loads [15–20]. We previously developed a prophylactic AIDS vaccine consisting of a DNA prime followed by a boost with a Sendai virus (SeV) vector expressing SIVmac239 Gag (SeV-Gag) [21,22]. Our trial showed vaccine-based control of a SIVmac239 challenge in a group of Burmese rhesus macaques sharing the MHC-I haplotype *90–120-Ia*; these *90-120-Ia*-positive vaccinees dominantly elicited $Gag_{206-216}$ (IINEEAADWDL) epitope-specific and $Gag_{241-249}$ (SSVDEQIQW) epitope-specific CTL responses and contained SIVmac239 replication after challenge [15,23]. In contrast, *90-120-Ia*-positive vaccinees failed to control a challenge with a mutant virus, SIVmac239Gag216S244E247L312V373T (referred to as SIV-G64723mt), which carries five *gag* mutations resulting in escape from recognition by Gag-specific CTLs including $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTLs. This indicates that these CTL responses play a crucial role in the vaccine-based primary control of wild-type SIVmac239 replication [24]. Furthermore, in a SIVmac239 challenge experiment of *90-120-Ia*-positive rhesus macaques that received a prophylactic vaccine expressing the $Gag_{241-249}$ epitope fused with enhanced green fluorescent protein (EGFP), this single epitope vaccination resulted in control of SIVmac239 replication with dominant induction of $Gag_{241-249}$-specific CTL responses in the acute phase after challenge [25]. We refer to these vaccinated animals that controlled viral replication after wild-type SIVmac239 challenge as SIV controllers in the present study.

Administration of SIV controllers with a monoclonal anti-CD8 antibody (i.e., CD8 depletion after the establishment of primary viral control) has suggested that $CD8^+$ cell responses play an important role in maintaining the viral control in the chronic phase [26,27]. Then, it is of great concern whether these wild-type SIV controllers can acquire $CD8^+$ cells effective against replication of SIV variants escaping from dominant CTL responses. In the present study, we have analyzed *90-120-Ia*-positive vaccinees controlling a SIVmac239 challenge in order to examine whether *90-120-Ia*-positive animals can elicit cellular immune responses effective against the mutant SIV, SIV-G64723mt, carrying multiple CTL escape *gag* mutations. Our analyses in these vaccine-based SIV controllers revealed dynamics of virus-specific cellular immune responses during persistent viral control and suggested postchallenge induction of $CD8^+$ cells able to suppress replication of SIV variants carrying CTL escape mutations.

Materials and methods

SIV-G64723mt

The SIV-G64723mt (SIVmac239Gag216S244E247L 312V373T) carries five *gag* mutations, GagL216S (leading to a leucine [L]-to-serine [S] substitution at the 216th amino acid in Gag, GagD244E (aspartic acid [D]-to-glutamic acid [E] at the 244th amino acid), GagI247L (isoleucine [I] to L at the 247th amino acid), GagA312V (alanine [A] to valine [V] at the 312th amino acid), and GagA373T (A to threonine [T] at the 373rd amino acid), which were selected, at the cost of viral fitness, in a SIVmac239-infected macaque possessing the MHC-I haplotype *90-120-Ia*, as described previously [23,28]. GagL216S, GagD244E, GagI247L, and GagA373T mutations, which became dominant mostly in SIVmac239-infected *90-120-Ia*-positive rhesus macaques, result in viral escape from recognition by Gag206-216-specific, Gag241-249-specific, and Gag373-380-specific CTLs, respectively, whereas it remains unclear whether GagA312V was selected for by CTLs.

Animal experiments

Eight Burmese rhesus macaques (*Macaca mulatta*) possessing the MHC-I haplotype *90-120-Ia*, which showed vaccine-based control of a SIVmac239 challenge, were used in this study and divided into two groups (Fig. 1a). Five macaques, R06-015, R03-014, R03-012, R02-002, and R02-003, in group I received a prophylactic DNA prime/SeV-Gag boost vaccine (referred to as DNA/SeV-Gag vaccine) and contained SIVmac239 challenge as reported previously [15,24,29]. The DNA used for the vaccination, CMV-SHIVdEN [15], was constructed from *env*-deleted and *nef*-deleted simian–human immunodeficiency virus $SHIV_{MD14YE}$ [30] 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 [31,32]. At week 1 after SIV challenge, macaque R03-014 was inoculated with nonspecific immunoglobulin G (IgG), and macaques R03-012 and R02-002 with IgG purified from neutralizing antibody-positive plasma of chronically SIV-infected macaques in our previous study [29]. Two macaques R04-016 and R06-007 in group II received a prophylactic prime-boost vaccine eliciting single $Gag_{241-249}$ epitope-specific CTL responses (referred to as DNA/SeV-$Gag_{236-250}$-EGFP vaccine) and contained SIVmac239 challenge as reported previously [25]. In this vaccine protocol, animals were primed with 5 mg of pGag$_{236-250}$-EGFP-N1 DNA expressing a $Gag_{236-250}$-EGFP fusion protein, followed by a boost with 6×10^9 CIU of F-deleted SeV expressing the $Gag_{236-250}$-EGFP fusion protein (SeV-$Gag_{236-250}$-EGFP). Macaque R04-015 in group II received a prophylactic prime-boost vaccine eliciting $Gag_{206-216}$ epitope-specific and $Gag_{241-249}$ epitope-specific CTL responses (referred to as DNA/SeV-$Gag_{202-216}$-EGFP and DNA/SeV-$Gag_{236-250}$-EGFP vaccine); this animal was primed with pGag$_{202-216}$-EGFP-N1 and pGag$_{236-}$

(a)

Group	Macaques	Vaccination	SIVmac239 challenge	SIV-G64723mt superchallenge	Anti-SIV NAb
I	R06-015	DNA/SeV-Gag	week 0	week 40	Undetectable
	R03-014	DNA/SeV-Gag	week 0	week 53	Undetectable
	R03-012	DNA/SeV-Gag	week 0	week 53	Undetectable
	R02-002	DNA/SeV-Gag	week 0	week 156	Undetectable
	R02-003	DNA/SeV-Gag	week 0	week 208	Undetectable
II	R04-015	DNA/SeV-Gag202-216-EGFP & DNA/SeV-Gag236-250-EGFP	week 0	week 116	Undetectable
	R04-016	DNA/SeV-Gag236-250-EGFP	week 0	week 116	Undetectable
	R06-007	DNA/SeV-Gag236-250-EGFP	week 0	week 123	Undetectable

(b)

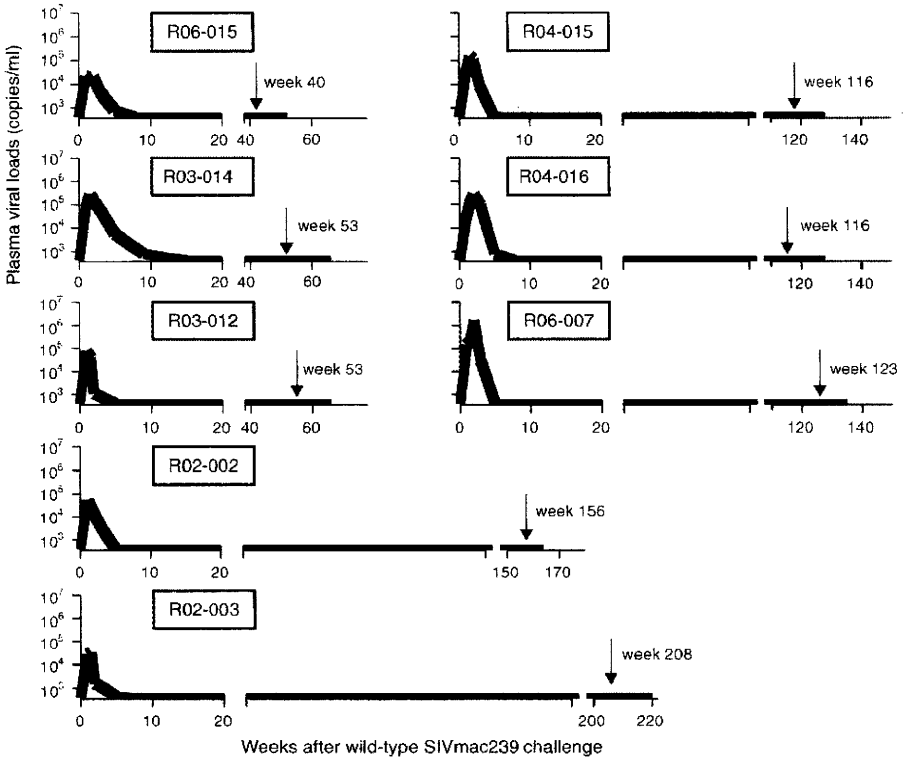

Fig. 1. Plasma viral loads in simian immunodeficiency virus controllers. (a) The list of rhesus macaques used in this study. All are *90-120-Ia*-positive. SIVmac239-specific neutralizing antibody (anti-SIV NAb) responses just before the mutant SIV superchallenge were undetectable. (b) Plasma viral loads (SIV *gag* RNA copies/ml plasma) determined as described previously [15]. The lower limit of detection is approximately 4×10^2 copies/ml. The arrows indicate the time points of SIV-G64723mt superchallenge. SIV, simian immunodeficiency virus.

$_{250}$-EGFP-N1 DNAs, followed by a boost with SeV-Gag$_{202-216}$-EGFP and SeV-Gag$_{236-250}$-EGFP. Both pGag$_{202-216}$-EGFP-N1 and SeV-Gag$_{202-216}$-EGFP express a Gag$_{202-216}$-EGFP fusion protein [33]. These vaccinated animals were challenged intravenously with 1000 50% tissue culture infective doses ($TCID_{50}$) of SIVmac239 [34] approximately 3 months after the boost and were superchallenged intravenously with 1000 $TCID_{50}$ of SIV-G64723mt in the chronic phase. The challenge virus stocks were prepared by virus propagation on rhesus macaque peripheral blood mononuclear cells (PBMCs). All animals were maintained in accordance with the guidelines for animal experiments at the National Institute of Infectious Diseases.

In-vitro viral suppression assay

To evaluate in-vitro anti-SIVmac239 or anti-SIV-G64723mt efficacy of $CD8^+$ cells, we examined SIVmac239 or SIV-G64723mt replication on CD8-depleted PBMCs in the presence of $CD8^+$ cells positively selected from macaque PBMCs as described previously [27,35]. In brief, PBMCs were separated into $CD8^+$ and

$CD8^-$ cells by using Macs CD8 MicroBeads (Miltenyi Biotec, Tokyo, Japan). For preparing target cells, the $CD8^-$ cells selected from PBMCs obtained before SIVmac239 challenge were cultured in the presence of 2 μg/ml phytohemagglutinin L and 20 IU/ml recombinant human interleukin-2 (Roche Diagnostics, Tokyo, Japan) and infected with SIVmac239 at a multiplicity of infection (MOI) of $1:10^3$ $TCID_{50}$/cell or with SIV-G64723mt at MOI of $1:10^2$ $TCID_{50}$/cell, using the virus stocks prepared by virus propagation on HSC-F cells (herpesvirus saimiri-immortalized macaque T-cell line) [36]. SIV-G64723mt with lower replicative ability was added at higher MOI to show similar replication kinetics with SIVmac239 replication in the control culture without $CD8^+$ cells. Target cells were cultured for 2 days and then effector $CD8^+$ cells selected from PBMCs obtained 1 week after boost or at several time points after the challenge were added to the target cells at an effector : target ($E:T$) ratio of 1 : 4. Reverse transcriptase activities in these culture supernatants were measured [37] to determine the peak of viral production in the control culture of target cells without $CD8^+$ cells. RNA was extracted from culture supernatants at the peak using the high pure viral RNA Kit (Roche Diagnostics) and viral RNA levels were measured by LightCycler system (Roche Diagnostics) using SIV *gag*-specific primers (GTAGTATGGGCAGCAAATGA and TGTTCCTG TTTCCACCACTA) and probes (GCATTCACGCA GAAGAGAAAGTGAAACA-Flu and LCRed-ACTG AGGAAGCAAAACAGATAGTGCAGAGA) (Nihon Gene Research Laboratories Inc., Sendai, Japan). Reduction in viral production by addition of each group of $CD8^+$ cells was shown as reduction (fold) in viral RNA level compared with that in the supernatant from virus-infected $CD8^-$ cell culture without $CD8^+$ cells.

Analysis of virus-specific $CD8^+$ T-cell responses

We measured virus-specific $CD8^+$ T-cell levels by flow cytometric analysis of gamma interferon (IFN-γ) induction after specific stimulation as described previously [15]. In brief, PBMCs were cocultured for 6 h with autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines pulsed with 1 μmol/l SIVmac239 $Gag_{206-216}$, $Gag_{241-249}$, or $Gag_{367-381}$ peptides for $Gag_{206-216}$-specific, $Gag_{241-249}$-specific, or $Gag_{367-381}$-specific stimulation. Alternatively, PBMCs were cocultured with 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 using a CytofixCytoperm kit (BD, Tokyo, Japan) and fluorescein isothiocyanate-conjugated antihuman CD4, peridinin chlorophyll protein-conjugated antihuman CD8, allophycocyanin-conjugated antihuman CD3, and phycoerythrin-conjugated antihuman IFN-γ monoclonal antibodies (BD). Specific $CD8^+$ T-cell levels were calculated by subtracting nonspecific IFN-γ^+ $CD8^+$ T-cell frequencies from those after peptide-specific stimulation. Specific $CD8^+$ T-cell levels lower than 100 per million PBMCs were considered negative.

Analysis of virus-specific neutralizing antibody responses

SIVmac239-specific neutralizing antibody responses were examined by determining the end point plasma titers for inhibiting 10 $TCID_{50}$ virus replication as described previously [26]. Serial two-fold dilutions of heat-inactivated plasma were prepared in quadruplicate and mixed with 10 $TCID_{50}$ of SIVmac239. In each culture, 5 μl of virus was incubated with 5 μl of plasma for 45 min and was added to 5×10^4 MT4 cells. Reverse transcriptase activities in the culture supernatants on day 12 were measured to determine the 100% neutralizing endpoint. The lower limit of detection is a titer of 1 : 2.

Statistical analysis

Statistical analysis was performed using Prism software version 4.03 (GraphPad Software Inc., San Diego, California, USA) with significance levels set at a P value of less than 0.05. Specific $CD8^+$ T-cell frequencies and in-vitro anti-SIV efficacy levels (fold of reduction in viral production) were log transformed and correlation was analyzed by the Pearson test.

Results

Anti-SIVmac239 and anti-SIV-G64723mt efficacy *in vitro* of $CD8^+$ cells in simian immunodeficiency virus controllers

We analyzed eight *90-120-Ia*-positive rhesus macaques that showed vaccine-based control of a SIVmac239 challenge (Fig. 1a). These SIV controllers were divided into group I consisting of five animals (R06-015, R03-014, R03-012, R02-002, and R02-003) vaccinated with DNA/SeV-Gag [15] and group II consisting of one animal (R04-015) vaccinated with DNA/SeV-$Gag_{202-216}$-EGFP and DNA/SeV-$Gag_{236-250}$-EGFP and two (R04-016 and R06-007) vaccinated with DNA/SeV-$Gag_{236-250}$-EGFP [25]. After an intravenous challenge with SIVmac239, all of these macaques showed viremia in the acute phase, but then controlled viral replication; plasma viremia was undetectable after the setpoint (Fig. 1b).

First, we investigated the potential of macaque $CD8^+$ cells obtained at several time points, after boost but before SIVmac239 challenge (referred to as postboost) and after challenge, to suppress SIVmac239 (Fig. 2) or SIV-G64723mt (Fig. 3) replication by in-vitro viral suppression assay [27,38–40]. In this assay, PBMC-derived $CD8^-$ target cells infected with SIVmac239 or SIV-G64723mt were cocultured with effector $CD8^+$ cells from PBMCs obtained at several time points at an E/T

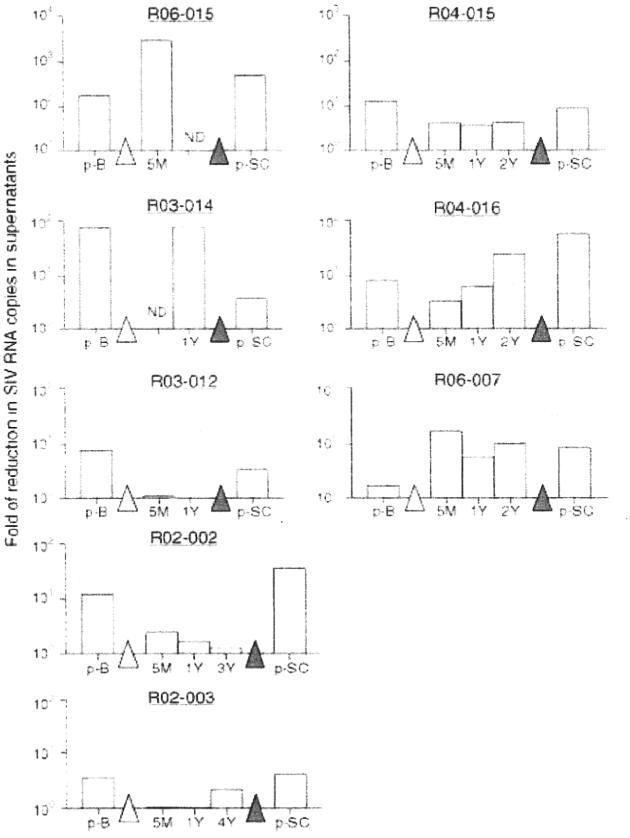

Fig. 2. Anti-SIVmac239 efficacy *in vitro* of CD8$^+$ cells in simian immunodeficiency virus controllers. PBMC-derived CD8$^-$ (target) cells infected with SIVmac239 were cultured alone or cocultured with autologous PBMC-derived CD8$^+$ (effector) cells at several time points at an $E:T$ ratio of 1 : 4. The ratios of viral RNA levels in the supernatants from the coculture to those without CD8$^+$ cells are shown. ND: not determined. p-B: 1 week after boost; 5M, 1Y, 2Y, 3Y, and 4Y: 5 months, 1, 2, 3, and 4 years after challenge, respectively; p-SC: 1 or 2 months after superchallenge. Open triangles indicate the time points of SIVmac239 challenge and closed triangles SIV-G64723mt superchallenge. PBMC, peripheral blood mononuclear cell; SIV, simian immunodeficiency virus.

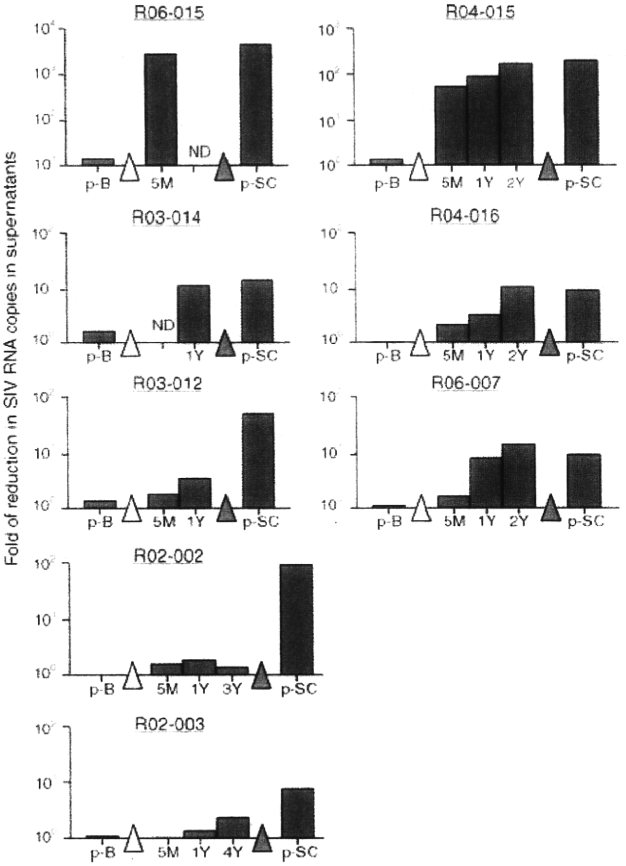

Fig. 3. Anti-SIV-G64723mt efficacy *in vitro* of CD8$^+$ cells in simian immunodeficiency virus controllers. PBMC-derived CD8$^-$ cells infected with SIV-G64723mt were cultured alone or cocultured with autologous PBMC-derived CD8$^+$ cells at several time points at an $E:T$ ratio of 1 : 4. The ratios of viral RNA levels in the supernatants from the coculture to those without CD8$^+$ cells are shown. ND: not determined. p-B: 1 week after boost; 5M, 1Y, 2Y, 3Y, and 4Y: 5 months, 1, 2, 3, and 4 years after challenge, respectively; p-SC: 1 or 2 months after superchallenge. Open triangles indicate the time points of SIVmac239 challenge and closed triangles SIV-G64723mt superchallenge. PBMC, peripheral blood mononuclear cell; SIV, simian immunodeficiency virus.

ratio of 1 : 4, and viral production in culture supernatants was examined to assess suppressive effect of CD8$^+$ cells on viral replication in vitro.

CD8$^+$ cells 1 week after boost mostly suppressed wild-type SIVmac239 replication efficiently. In contrast, these postboost CD8$^+$ cells failed to show efficient suppressive effect on SIV-G64723mt replication. These results suggest that $Gag_{206-216}$-specific, $Gag_{241-249}$-specific, and $Gag_{367-381}$-specific CTL responses play a central role in the suppression of SIVmac239 replication by postboost CD8$^+$ cells.

After SIVmac239 challenge, all these animals showed efficient in-vitro anti-SIV-G64723mt efficacy (more than two-fold reduction in viral production) of CD8$^+$ cells, sooner or later, in the chronic phase. The levels of in-vitro anti-SIV-G64723mt efficacy of CD8$^+$ cells tended to become higher in the chronic phase. Anti-SIVmac239 efficacy of CD8$^+$ cells was not associated with anti-SIV-G64723mt efficacy. For instance, some CD8$^+$ cells efficiently suppressed SIV-G64723mt but not SIVmac239 replication. After all, all SIV controllers acquired CD8$^+$ cells able to suppress the mutant SIV-G64723mt replication *in vitro* in the chronic phase.

Control of a mutant simian immunodeficiency virus superchallenge

These animals were superchallenged with a mutant SIV, SIV-G64723mt, that has five *gag* mutations resulting in

escape from recognition by $Gag_{206-216}$-specific, $Gag_{241-249}$-specific, and $Gag_{367-381}$-specific CTLs around 1 year (R06-015, R03-014, and R03-012), 2 years (R04-015, R04-016, and R06-007), 3 years (R02-002), or 4 years (R02-003) after SIVmac239 challenge. The replicative ability of SIV-G64723mt is significantly lower than that of wild-type SIVmac239, but SIV-G64723mt challenge of naive *90-120-Ia*-negative rhesus macaques can result in persistent viral replication and AIDS progression [23,28]. It has previously been shown that *90-120-Ia*-positive macaques vaccinated with DNA-prime/SeV-Gag-boost are unable to contain a SIV-G64723mt challenge, whereas they can control replication of wild-type SIVmac239 [24]. Indeed, we confirmed that $CD8^+$ cells obtained from these *90-120-Ia*-positive vaccinees before challenge efficiently suppressed wild-type SIVmac239 but not SIV-G64723mt replication *in vitro*. In the present study, however, all eight wild-type SIV controllers contained the SIV-G64723mt superchallenge without detectable viremia (Fig. 1b). SIVmac239-specific neutralizing antibody responses were undetectable around the superchallenge in any of these controllers (Fig. 1a). These results indicate that, after SIVmac239 challenge, the SIV controllers acquired the potential to control SIV-G64723mt replication in the absence of neutralizing antibody responses, although to what extent $CD8^+$ cell responses may contribute to this containment of SIV-G64723mt superchallenge remains unclear. Postsuperchallenge $CD8^+$ cells suppressed both SIVmac239 and SIV-G64723mt replication *in vitro* efficiently (Figs. 2 and 3).

Simian immunodeficiency virus Gag-specific cytotoxic T lymphocyte responses in simian immunodeficiency virus controllers

Then, in these SIV controllers, we examined $Gag_{206-216}$-specific, $Gag_{241-249}$-specific, and $Gag_{367-381}$-specific CTL responses, which have previously been indicated responsible for control of SIVmac239 replication in *90-120-Ia*-positive vaccinees [24] (Fig. 4a). In DNA/SeV-Gag vaccinated animals (R06-015, R03-014, R03-012, and R02-002), SIV-specific CTL responses were undetectable before SeV-Gag boost (data not shown), but $Gag_{206-216}$-specific, $Gag_{241-249}$-specific, and $Gag_{367-381}$-specific responses were efficiently induced 1 week after the boost. After SIVmac239 challenge, these animals showed efficient responses of these CTLs in the acute phase. These CTL levels were reduced in the chronic phase, but $Gag_{241-249}$-specific CTL responses were detectable even 1 year after challenge. In macaque R04-015 vaccinated with DNA/SeV-$Gag_{202-216}$-EGFP and DNA/SeV-$Gag_{236-250}$-EGFP, $Gag_{206-216}$-specific CTL responses were induced dominantly 1 week after boost and 2 weeks after SIVmac239 challenge, whereas $Gag_{241-249}$-specific CTL responses were detected predominantly in the chronic phase. In macaques R04-016 and R06-007 vaccinated with DNA/SeV-$Gag_{236-250}$-EGFP, $Gag_{241-249}$-specific CTL responses were induced dominantly 1 week after boost and 2 weeks after SIVmac239 challenge and were maintained in the chronic phase. No significant enhancement of these CTL responses was observed after SIV-G64723mt superchallenge.

We also examined Gag-specific CTL responses in SIV controllers at several time points by using a panel of overlapping peptides (Gag peptide pools 1–10) spanning the entire SIVmac239 Gag (Fig. 4b). Group I macaques vaccinated with DNA/SeV-Gag elicited CTL responses directed against not only Gag peptide pool 5 (including $Gag_{206-216}$ and $Gag_{241-249}$) and 7 (including $Gag_{367-381}$) but also other Gag peptide pools after boost and after challenge; some peptide pool-specific CTLs were diminished, whereas others appeared in the chronic phase. In contrast, group II macaques eliciting CTL responses directed against single $Gag_{206-216}$ (R04-015) or $Gag_{241-249}$ (R04-016 and R06-007) epitope after boost showed predominant Gag peptide pool 5-specific CTL responses after challenge and accumulated multiple Gag epitope-specific CTL responses in the chronic phase. These results indicate dynamics of postchallenge Gag-specific CTL responses in vaccine-based SIV controllers. After SIV-G64723mt superchallenge, changes in the pattern of Gag-specific CTL responses were observed in some animals.

Simian immunodeficiency virus non-Gag antigen-specific cytotoxic T lymphocyte responses in simian immunodeficiency virus controllers

Next, in SIV controllers, we examined CTL responses directed against SIV non-Gag antigens by using panels of overlapping peptides spanning the entire SIVmac239 antigens other than Gag (Fig. 5a). These SIV controllers showed SIV non-Gag-specific CTL responses from the early phase after challenge. After SIV-G64723mt superchallenge, broadening or changes in the pattern of these CTL responses were observed in some animals; Vif-specific or Nef-specific CTL responses were detected predominantly, although we did not find common CTL epitopes in Vif or Nef.

Correlation of antigen-specific cytotoxic T lymphocyte levels with in-vitro antivirus efficacy levels

Finally, we analyzed correlation of antigen-specific CTL levels with in-vitro anti-SIVmac239 or anti-SIV-G64723mt efficacy levels of $CD8^+$ cells (Fig. 5b). We found a correlation of anti-SIVmac239 efficacy levels with $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL levels but not with total Gag-specific CTL levels. The anti-SIVmac239 efficacy levels did not correlate with either $Gag_{206-216}$-specific or $Gag_{241-249}$-specific CTL levels alone (data not shown), although our previous study [25] indicated inverse correlation between peak plasma viral loads and the levels of $Gag_{241-249}$-specific CTLs dominantly induced in DNA/SeV-$Gag_{236-250}$-EGFP-vaccinated animals in the acute phase after

Fig. 4. Gag-specific CD8$^+$ T-cell responses in simian immunodeficiency virus controllers. (a) $Gag_{206-216}$-specific (206), $Gag_{241-249}$-specific (241), and $Gag_{367-381}$-specific (367) CD8$^+$ T-cell frequencies at several time points are shown. Regarding macaque R02-003, we confirmed efficient responses of these CTLs after boost and in the acute phase as reported previously [24] but did not have enough PBMC samples for the analyses in the chronic phase. (b) A panel of 117 overlapping peptides (15–17 amino acid in length and overlapping by 10–12 amino acid) spanning the entire SIV Gag amino acid sequence was divided into the following 10 pools (each consisting of 11 or 12 peptides): pool 1, first to 65th amino acid in SIV Gag; pool 2, 55th to 114th amino acid; pool 3, 104th to 165th amino acid; pool 4, 155th to 213th amino acid; pool 5, 202nd to 265th amino acid; pool 6, 255th to 316th amino acid; pool 7, 306th to 364th amino acid; pool 8, 354th to 416th amino acid; pool 9, 406th to 464th amino acid; and pool 10, 453rd to 510th amino acid. These Gag peptide pool-specific CD8$^+$ T-cell frequencies at several time points are shown. ND: not determined. p-B: 1 week after boost; 2W, 5M, 1Y, and 2Y: 2 weeks, 5 months, 1, and 2 years after challenge, respectively; p-SC: 1 or 2 months after superchallenge. Open triangles indicate the time points of SIVmac239 challenge and closed triangles SIV-G64723mt superchallenge. CTL, cytotoxic T lymphocyte; PBMC, peripheral blood mononuclear cell; SIV, simian immunodeficiency virus.

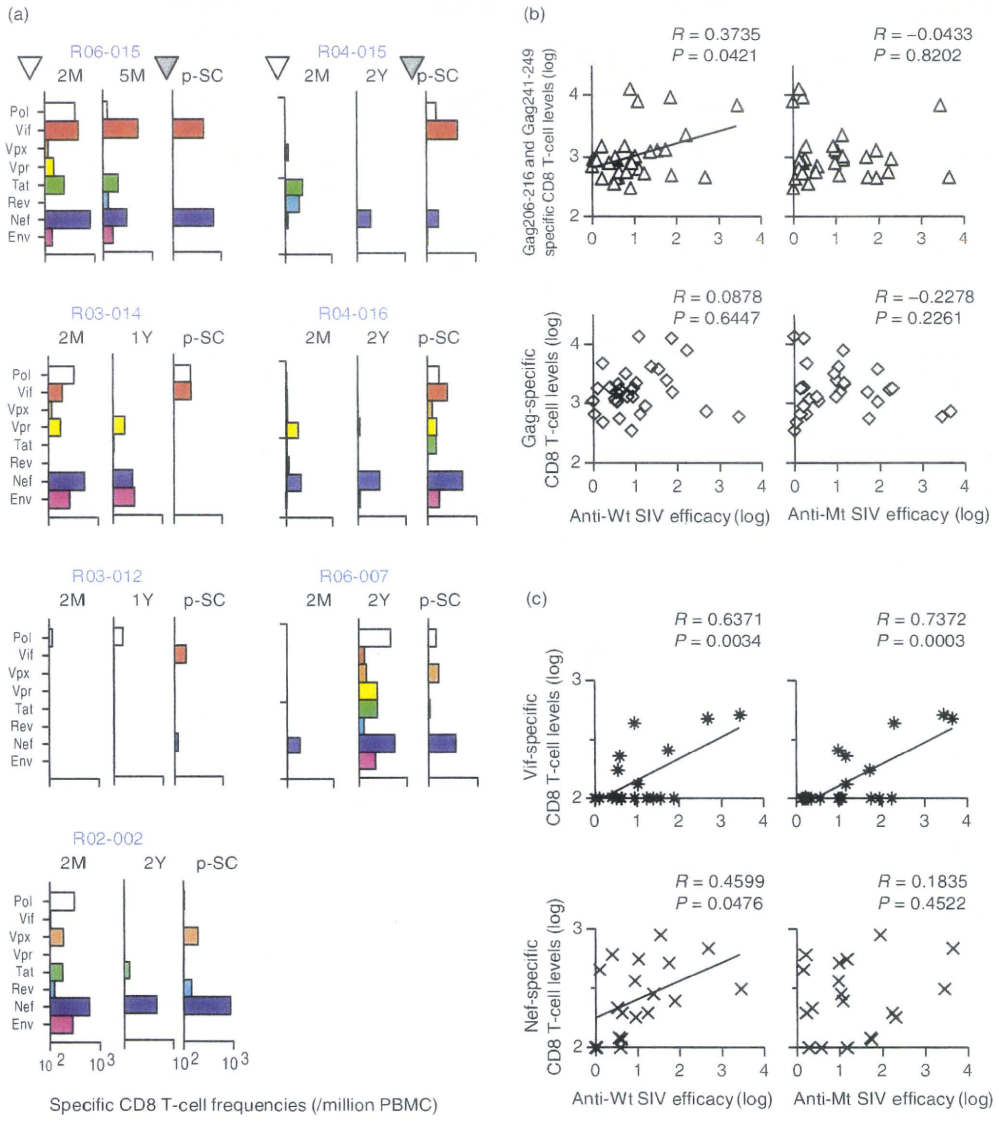

Fig. 5. Analysis of correlation between anti-SIVmac239 or anti-SIV-G64723mt efficacy *in vitro* and simian immunodeficiency virus antigen-specific CD8$^+$ T-cell levels in simian immunodeficiency virus controllers. (a) SIV non-Gag antigen-specific CD8$^+$ T-cell responses. Pol-specific, Vif-specific, Vpx-specific, Vpr-specific, Tat-specific, Rev-specific, Nef-specific, and Env-specific CD8$^+$ T-cell frequencies at several time points were measured by using panels of overlapping peptides spanning the entire SIVmac239 Pol, Vif, Vpx, Vpr, Tat, Rev, Nef, and Env amino acid sequences, respectively. R02-003 PBMC samples were unavailable. 2M, 5M, 1Y, and 2Y: 2, 5 months, 1, and 2 years after challenge, respectively; p-SC: 1 or 2 months after superchallenge. Open triangles indicate the time points of SIVmac239 challenge and closed triangles SIV-G64723mt superchallenge. (b) Analysis of correlation between anti-SIVmac239 (Wt SIV) efficacy (left panels) or anti-SIV-G64723mt (Mt SIV) efficacy (right panels) levels and $Gag_{206\text{-}216}$-specific plus $Gag_{241\text{-}249}$-specific CTL (upper panels) or Gag-specific CTL (lower panels) levels ($n = 30$ in each panel). A correlation between anti-SIVmac239 efficacy levels and $Gag_{206\text{-}216}$-specific plus $Gag_{241\text{-}249}$-specific CTL levels is indicated ($P = 0.0421$, $R = 0.3735$). (c) Analysis of correlation between after challenge anti-SIVmac239 efficacy (left panels) or anti-SIV-G64723mt efficacy (right panels) levels and Vif-specific CTL (upper panels) or Nef-specific CTL (lower panels) levels ($n = 19$ in each panel). Correlations of anti-SIVmac239 efficacy levels with Vif-specific CTL ($P = 0.0034$, $R = 0.6731$) and with Nef-specific CTL levels ($P = 0.0476$, $R = 0.4599$) and a strong correlation between anti-SIV-G64723mt efficacy levels and Vif-specific CTL levels ($P = 0.0003$, $R = 0.7372$) are indicated. CTL, cytotoxic T lymphocyte; SIV, simian immunodeficiency virus.

challenge. Correlations of anti-SIVmac239 efficacy levels after challenge with Vif-specific CTL levels and with Nef-specific CTL levels were indicated. On the contrary, anti-SIV-G64723mt efficacy levels after challenge strongly correlated with Vif-specific CTL levels, although any correlation of these levels with other SIV antigen-

specific CTL levels was not indicated. These results suggest that Vif-specific CTL induction may contribute in part to acquisition of the potential to suppress SIV-G64723mt replication efficiently.

Discussion

We have previously shown that *90-120-Ia*-positive macaques eliciting Gag-specific CTL responses by vaccination can control SIVmac239 replication but are unable to contain a challenge with a mutant SIV, SIV-G64723mt, carrying multiple *gag* mutations that result in escape from recognition by $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTLs [24]. The present study revealed, by in-vitro viral suppression assay, that those *90-120-Ia*-positive vaccinees can acquire, after wild-type SIVmac239 challenge, $CD8^+$ cells able to suppress the mutant SIV replication. Induction of these $CD8^+$ cell responses may have some supportive effect on the maintenance of viral control after the initial viral containment [4,26,27]. Such dynamics of anti-SIV responses have not been shown clearly even in live attenuated SIV infection [41–44]. Recently, HIVs have been suggested to accumulate mutations escaping from dominant CTL responses [45–51], but our results imply a possibility of induction of cellular immune responses effective against even those HIV variants escaping from dominant CTL responses.

The group I animals induced multiple Gag epitope-specific CTL responses after boost (before challenge) and after challenge, whereas the group II animals elicited only $Gag_{206-216}$-specific or $Gag_{241-249}$-specific CTL responses before challenge and showed induction of additional CTL responses directed against Gag epitopes other than $Gag_{206-216}$ and $Gag_{241-249}$ after challenge. Furthermore, both groups elicited SIV non-Gag-specific CTL responses after challenge. These results indicate post-challenge accumulation of broader CTL responses. The in-vitro anti-SIVmac239 efficacy levels correlated with Vif-specific and Nef-specific CTL as well as $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL levels but not with total Gag-specific or total SIV-specific CTL levels, suggesting that not all but some particular epitope-specific CTL responses were involved in suppression of SIVmac239 replication. Nef-specific CTL responses were detected more frequently than Vif-specific ones, whereas the latter showed stronger correlation with antiviral efficacy levels (Fig. 5). We did not find common CTL epitopes in Vif or Nef. These may imply higher frequencies of effective CTLs in Vif-specific ones; conversely, Nef-specific CTLs may include effective ones but with higher frequencies of ineffective ones.

Postboost $CD8^+$ cells able to suppress SIVmac239 replication failed to show suppressive effect on SIV-G64723mt replication. We confirmed it also in two *90-120-Ia*-positive vaccinated animals that had failed to control the mutant SIV challenge in our previous studies [24] (data not shown). However, $CD8^+$ cells in the chronic phase suppressed SIV-G64723mt replication efficiently. This indicates postchallenge induction of $CD8^+$ cells with the potential to suppress SIV-G64723mt replication in vaccine-based SIVmac239 controllers, although it remains unclear whether these $CD8^+$ cells with antimutant SIV efficacy are responsible for the control of mutant SIV superchallenge *in vivo*. The in-vitro anti-SIV-G64723mt efficacy levels correlated with Vif-specific CTL levels and $CD8^+$ cells with detectable Vif-specific CTL responses showed suppressive effect on SIV-G64723mt replication. These results implicate Vif-specific CTL responses in the suppression of SIV-G64723mt replication *in vitro* by $CD8^+$ cells in the chronic phase, although other factors may also be involved in this suppression. Preservation of memory $CD4^+$ T cells by vaccine-based SIV control [26] may contribute to induction of these effective CTL responses.

We found dynamics of cellular immune responses during viral control in vaccine-based SIV controllers, but the exact mechanism for broadening or changes in dominance patterns of CTL responses remains unclear. All the group I animals and macaque R04-015 showed rapid selection of a CTL escape *gag* mutation, L216S, at week 5 after challenge, whereas no *gag* mutations were selected at week 5 in macaques R04-016 or R06-007 (data not shown). We failed to recover viral genome cDNAs for sequencing from plasma after week 5 due to undetectable viral loads, but selection of viral CTL escape mutations and reversions [23,28,52–57] under undetectable levels of viral replication may contribute to induction of broader CTL responses in SIV controllers.

It is difficult to directly compare anti-SIVmac239 and anti-SIV-G64723mt efficacy of $CD8^+$ cells because of difference in their replicative ability, but the ratios of the latter level to the former 1 year after challenge were higher than those after boost in all animals. Indeed, $CD8^+$ cells 1 year after challenge in macaques R03-012 and R02-003 showed suppressive effect on SIV-G64723mt but not on wild-type SIVmac239 replication, although R03-012 $CD8^+$ cells at 5 months and 1 year after challenge efficiently suppressed SIVmac239 replication at higher E/T ratio of 1 : 1 (R02-003 $CD8^+$ cells in the chronic phase for this analysis were unavailable). Because no SIV controllers elicited CTL responses specific for peptides with mutated amino acid sequences (data not shown), all CTLs specific for SIV-G64723mt antigens in SIV controllers are expected to recognize SIVmac239 antigens also. Thus, our observation that some post-challenge $CD8^+$ cells showed efficient suppressive effect on SIV-G64723mt but not on SIVmac239 replication *in vitro* may be explained by higher replicative ability of SIVmac239 compared with SIV-G64723mt; it could

be more difficult for $CD8^+$ cells to suppress replication of the wild-type SIVmac239 than the mutant SIV-G64723mt, implying a possible requirement of more potent CTL responses for SIVmac239 control than for SIV-G64723mt control.

In summary, this study showed dynamics of postchallenge cellular immune responses in vaccine-based SIV controllers. Our results suggest that, during persistent viral control, vaccine-based SIV controllers can acquire $CD8^+$ cells with the potential to suppress replication of SIV variants carrying CTL escape mutations. Elucidation of the mechanism for induction of broader responses in these controllers may contribute to development of a vaccine effective against highly diversified HIV infection.

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