Journal of General Virology (2010), **91**, 2974–2984 **DOI** 10.1099/vir.0.022772-0

No difference in Gag and Env immune-response profiles between vaccinated and non-vaccinated rhesus macaques that control immunodeficiency virus replication

Ivonne Nieuwenhuis,[1] Niels Beenhakker,[1] Willy M. J. M. Bogers,[1] Nel Otting,[2] Ronald E. Bontrop,[2] Patrice Dubois,[3] Petra Mooij,[1] Jonathan L. Heeney[1,4] and Gerrit Koopman[1]

Correspondence
Gerrit Koopman
koopman@bprc.nl

[1]Department of Virology, Biomedical Primate Research Centre (BPRC), Rijswijk, The Netherlands
[2]Department of Comparative Genetics & Refinement, BPRC, Rijswijk, The Netherlands
[3]ImmunoVacc Consulting, Brussels, Belgium
[4]Department of Veterinary Medicine, University of Cambridge, Cambridge, UK

Recent advances in human immunodeficiency virus (HIV) vaccine design have resulted in induction of strong CD4 T-cell proliferative and polyfunctional cytokine responses, which are also characteristic for long-term non-progressing (LTNP) HIV-infected individuals. However, limited information is available on the persistence of these responses after infection. Results from studies in non-human primates indicate that vaccine-induced immune responses are partially maintained upon viral infection and differ from the responses seen in non-vaccinated animals that typically progress to disease. However, it is unclear how these partially preserved responses compare to immune responses that are acquired naturally by LTNP animals. In this study, immune-response profiles were compared between vaccinated animals that, upon $SHIV_{89.6p}$ challenge, became infected but were able to control virus replication, and a group of animals having spontaneous control of this viral infection. Both groups were found to develop very similar immune responses with regard to induction of CD4 and CD8 T-cell polyfunctional cytokine responses, proliferative capacity and cytotoxic capacity, as measured by a standard ^{51}Cr release assay and more direct *ex vivo* and *in vivo* CTL assays. Hence, vaccinated animals that become infected, but control infection, appear to establish immune responses that are similar to those elicited by long-term non-progressors.

Received 15 April 2010
Accepted 1 September 2010

INTRODUCTION

Since the onset of the human immunodeficiency virus (HIV) epidemic more than three decades ago, more than 60 million persons are estimated to have become infected, of which the large majority typically progress to AIDS in the absence of anti-retroviral treatment. However, a small group of so-called 'long-term non-progressors' (LTNP) have been identified who, without treatment, remain clinically and immunologically stable for years (Cao *et al.*, 1995; Muñoz *et al.*, 1995; Pantaleo *et al.*, 1995; Sheppard *et al.*, 1993), although some progression was found to occur on longer-term follow-up (Lefrère *et al.*, 1997; Rodés *et al.*, 2004). More recently, an even smaller cohort (<1 % of HIV-infected people), termed 'ELITE controllers', was defined. They share a durable control of HIV replication, leading to plasma viral loads that are consistently <50 RNA copies ml^{-1} (Deeks & Walker, 2007; Hubert *et al.*, 2000). Studies directed at identifying the mechanisms underlying viral control have shown no direct relationship with the presence of viral variants (Miura *et al.*, 2008), indicating that host factors are of primary importance. Indeed, the association of certain MHC-class I and II alleles with low virus load (Giraldo-Vela *et al.*, 2008; Harari *et al.*, 2007; Kiepiela *et al.*, 2004; Loffredo *et al.*, 2007; Migueles *et al.*, 2000; Yant *et al.*, 2006) highlights a role for adaptive immune responses in the control of viral replication, although innate natural killer cell responses, involving killer-cell immunoglobulin-like receptor–MHC class I interactions (Martin *et al.*, 2002), as well as triggering of type I interferon release from plasmacytoid DC might play a key role as well (Soumelis *et al.*, 2001). A central role for CTL function in suppression of virus replication can be inferred from: (i) the observation that, in the acute phase

A supplementary figure and a supplementary table are available with the online version of this paper.

of the infection, suppression of virus replication is preceded by an increase in HIV-specific CD8 T-cells, (ii) from observations on viral escape and (iii) from CD8 T-cell depletion experiments in simian immunodeficiency virus (SIV)–macaque infection models (Goulder *et al.*, 1997; Schmitz *et al.*, 1999). In LTNP, CD8 responses are increased both in magnitude and breadth, and in part are directed to conserved epitopes or to epitopes that can only be mutated at a cost of viral fitness (Addo *et al.*, 2003; Altfeld *et al.*, 2003, 2006; Betts *et al.*, 2001; Martinez-Picado *et al.*, 2006). Functional characterization of HIV-specific T-cell responses in LTNP has demonstrated an increased cytotoxic potential in the CD8 T-cell population. Also demonstrated was an increased proliferative capacity, and polyfunctional cytokine expression patterns, in CD4 as well as CD8 T-cells, relative to individuals characterized by progressive disease (Betts *et al.*, 2006; Emu *et al.*, 2005; Harari *et al.*, 2004; Horton *et al.*, 2006a; Migueles *et al.*, 2002, 2008; Pereyra *et al.*, 2008; Younes *et al.*, 2003; Yue *et al.*, 2004).

These characteristic immune features encountered in LTNP have been taken as a guide to set reference standards for HIV-vaccine-induced T-cell responses. Indeed, recent vaccine trials have reported induction of polyfunctional CD4 and CD8 T-cell responses (Harari *et al.*, 2007; Precopio *et al.*, 2007). However, preservation of these responses after challenge is not well studied and is difficult to interpret since new responses emerge on a continuous basis (Horton *et al.*, 2006b). Studies in vaccinated rhesus macaques have been more extensive, reporting preservation of polyfunctionality in animals that could partially suppress virus replication (Acierno *et al.*, 2006; Sun *et al.*, 2008), but also reporting a loss-of-function or skewing of immune responses through induction of multiple new specificities (Mattapallil *et al.*, 2006; Vogel *et al.*, 2001, 2002). In none of these studies, however, have post-infection immune responses in vaccinated macaques been compared directly to responses seen in 'natural' controllers infected with exactly the same viral isolate. Here a group of rhesus macaques, which without antiviral treatment were able to suppress $SHIV_{89.6p}$ replication to below 500 copies ml^{-1} for more than 140 weeks, were directly compared to a group of 'vaccine controller' animals that had been subjected to T-cell-directed HIV vaccine strategies. Both groups displayed not only similar HIV/SIV-specific CD4 and CD8 polyfunctional cytokine responses, but also similar proliferative responses and comparable CTL function.

RESULTS

Over the past decade, many vaccine-evaluation studies in macaques have been performed, at various centres, in which efficacy against viral challenge was evaluated by using recombinant $SHIV_{89.6p}$ (Amara *et al.*, 2001; Barouch *et al.*, 2001; Doria-Rose *et al.*, 2003; Egan *et al.*, 2004; Koopman *et al.*, 2008; Letvin *et al.*, 2004; Mooij *et al.*, 2004, 2008; Shiver *et al.*, 2002). Table 1 summarizes the general outcome of these studies at the BPRC, specifying the relative proportion of animals able to control virus load to <500 copies ml^{-1} by week 40 after challenge, as well as the number of animals that developed symptoms of AIDS during the 40–70 week period of follow-up. Naive macaques of both Indian and Chinese origin progressed to disease, although, as also reported by others (Reimann *et al.*, 2005), macaques of Indian origin were less capable of controlling virus replication and more readily progressed to AIDS. In contrast, vaccinated animals showed, in both the Indian and Chinese origin groups, a large level of control of virus replication and rare disease development. It has been hypothesized that the immune-response patterns observed in naturally infected non-progressing individuals could similarly provide control of infection in vaccinated persons. However, it is not known whether immune responses are qualitatively and quantitatively comparable between unvaccinated 'natural' controllers and vaccinated controllers. To study this, a small group of animals (described in Table 2) was selected from the cohort for further follow-up (ranging from 65 to 195 weeks) and immunological analysis. Ten vaccinated animals that controlled infection, of which six were of Indian origin, were compared with seven 'natural' controllers, of which three were of Indian origin (Table 2). For comparison, five progressor animals (all of Indian origin) were also included.

Table 1. Overview of control of virus load as well as disease development in rhesus macaques after challenge with $SHIV_{89.6p}$

Summary of the results obtained from vaccinated and control animals of Indian and Chinese origin (see Methods) that were followed for a 40–70 week period after challenge.

Animals	Viral load <500 copies ml^{-1}	AIDS
Vaccinated		
Indian	19/28 (70 %)	1/28 (4 %)
Chinese	70/80 (87 %)	3/80 (4 %)
Control		
Indian	4/19 (21 %)	8/19 (42 %)
Chinese	16/35 (46 %)	8/35 (23 %)

Vaccinated and 'natural' controllers of infection have comparable cytokine and proliferative responses during chronic infection

In the chronic phase of the infection (weeks 39–150 after challenge), six of ten vaccinated controllers and all seven 'natural' controllers showed a positive response against SIV Gag, as measured by an interferon gamma (IFN-γ) ELISpot assay, while none of the progressor animals showed a positive response (Table 2). Although the group of 'natural' controllers might be considered to have more uniform positive responses against SIV Gag, the differences from the group of vaccinated controllers are not significant ($P=0.10$; Fisher's exact test), while both vaccinated and 'natural' controllers had significantly higher responses than the progressors ($P=0.04$ and $P=0.001$, respectively). Responses against HIV-1 Env were less prevalent and were

Table 2. History of the animals included in this study and immune responses in the chronic phase of the infection as measured by IFN-γ ELISpot assay

Origins of the animals are indicated (Ind, Indian; Chi, Chinese; Bur, Burmese rhesus macaque). The vaccine type used is also given (DNA–ISCOM, DNA prime–immune stimulating complex; DNA–MVA, DNA prime–modified vaccinia Ankara; DNA–NYVAC, and also the antigen that was incorporated in the vaccine (T, HIV-1 tat; E, HIV-1 env; G, SIV gag; P, SIV pol). Responses against SIV Gag peptides and HIV-1 Env peptides were determined in the chronic phase of the infection (39–150 weeks post-challenge) and are presented semi-quantitatively: –, negative with <50 spots per 10^6 PBMC; +, positive with 50–100 spots per 10^6 PBMC; ++, strongly positive with >100 spots per 10^6 PBMC.

Animal	Origin	Vaccine type	Antigen	Follow-up (weeks)	Chronic phase response	
					SIV Gag	HIV-1 Env
Vaccinated controllers						
DVT	Ind	DNA–ISCOM	T/E/G/P	143	−	−
VH2	Bur	DNA–ISCOM	T/E/G/P	139	−	−
ECX	Ind	DNA–ISCOM	T	143	+	−
Ri178	Chi	DNA–ISCOM	T/E/G/P	130	+	−
EEG	Ind	DNA–ISCOM	T/E/G/P	191	++	++
EKK	Ind	DNA–ISCOM	T/E/G/P	195	−	−
WJ8	Ind	DNA–ISCOM	T/E/G/P	195	++	++
Ri191	Chi	DNA–MVA	T/E/G/P	173	+	−
Ri226	Chi	DNA–MVA	T/E/G/P	173	++	++
Ri253	Chi	DNA–MVA	T/E/G/P	173	−	−
'Natural' controllers						
94053	Ind	DNA–ISCOM	Empty	195	++	+
R120	Ind	−	−	195	+	−
Ri009	Chi	DNA–ISCOM	Empty	195	++	−
Ri081	Chi	−	−	147	++	−
Ri094	Chi	−	−	165	++	−
Ri206	Chi	−	−	173	+	+
R00045	Ind	DNA–NYVAC	Empty	65	++	−
Progressors						
9068	Ind	DNA–NYVAC	Empty	65	−	−
96025	Ind	DNA–NYVAC	Empty	52	−	−
R00001	Ind	DNA–NYVAC	Empty	30	−*	−*
R99013	Ind	DNA–NYVAC	Empty	39	−	−
R99041	Ind	DNA–NYVAC	Empty	26	−*	++*

*Animal had died of AIDS before week 40. The responses shown were recorded in the acute phase of the infection 4–8 weeks post challenge.

seen in 3/10 vaccinated and 2/7 'natural', controller animals. To define the nature of these responses further, an intracellular cytokine staining (ICS) assay was performed on samples obtained during the chronic phase of the infection, 39–150 weeks post-challenge. In this assay, antigen-specific induction of three cytokines was measured, i.e. (IFN-γ), tumour necrosis factor alpha (TNF-α) and interleukin 2 (IL-2). These cytokines are considered to be important immune-response indicators, and simultaneous production of two or three cytokines, i.e. polyfunctional responses, has been associated with the effective control of virus replication (Betts *et al.*, 2006; Harari *et al.*, 2004). As shown in Fig. 1, SIV Gag-specific cytokine responses were seen both in the vaccinated and 'natural' controller groups, while the progressor animals were totally negative. In both of the controller groups, virus-specific responses were observed to be mediated by CD4 as well as CD8 T-cells. HIV Env-specific responses were only observed occasionally, both in the vaccinated as well as in the 'natural' controller group. There was no statistically significant difference between the vaccinated and 'natural' controller groups in either CD4 or CD8 T-cell-mediated SIV Gag or HIV-1 Env responses (Mann–Whitney test). However, in both controller groups, SIV Gag responses were significantly higher than in the progressor group. For HIV-1 Env, only the difference between the vaccinated controllers versus the progressors was statistically significant. Further analysis of cytokine expression (Fig. 2) demonstrated polyfunctional responses with a comparable distribution of single, double and triple IFN-γ, IL-2 and TNF-α cytokine-producing CD4 and CD8 subsets in both the vaccinated and 'natural' controller groups (Mann–Whitney test). IFN-γ single and IFN-γ, IL-2 double cytokine-producing CD4 T-cells, as well as IFN-γ single-producing CD8 T-cells, were significantly higher for the vaccinated and 'natural' controller group relative to the group of progressor animals while for other cytokine

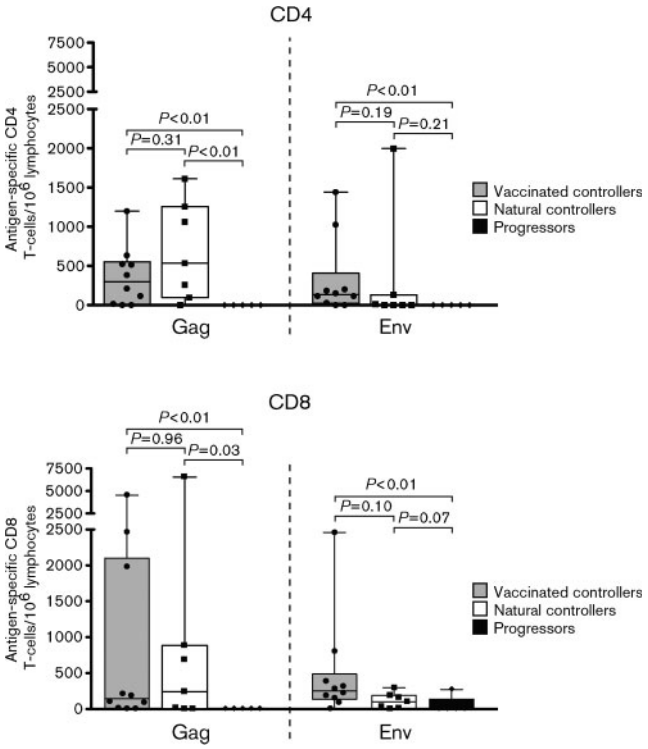

Fig. 1. Antigen-specific T-cell cytokine responses as measured by ICS assay during the chronic phase of the infection, 26–150 weeks post-challenge. The magnitude of the combined IL-2, IFNγ and TNFα cytokine response is plotted for SIV Gag and HIV-1 Env in CD4 and CD8 T-cells, for the vaccinated controller, 'natural' controller and progressor groups of animals. Box-whisker plots indicate the interquartile ranges and the medians (horizontal lines) of the groups; each dot represents a single animal. Assay responses are plotted as the number of positive cells per 10^6 lymphocytes with the background subtracted. The background reading was taken from cells cultured without antigen but in the presence of anti-CD28, anti CD49d and Golgiplug.

combinations, whose levels were often of lower magnitude, these differences were not significant (Fig. 2). Preservation of lymphoproliferative capacity is an important feature observed in human LTNP (Migueles *et al.*, 2002) and was therefore analysed in this cohort on samples obtained during the chronic phase of the infection. Although the responses were rather low, the vaccinated and 'natural' controller animals did display similar levels of proliferation induction, upon SIV Gag as well as HIV-1 Env peptide stimulation, in the CD4 as well as CD8 T-cell populations (Fig. 3). Proliferative capacity was difficult to establish in the progressor animals due to the small number of CD4 T-cells present, which further deteriorated during culture. In conclusion, the magnitude and composition of the cytokine responses, as well as induction of CD4 and CD8 T-cell proliferation, were not significantly different between the groups of vaccinated versus 'natural' controllers of virus replication.

Characterization of T-cell cytotoxic function in vaccinated versus 'natural' controllers of infection

The characteristic preservation of T-cell cytotoxic function in human LTNP (Harari *et al.*, 2009; Migueles *et al.*, 2008) prompted us to investigate this in an indirect culture-based assay, i.e. a ^{51}Cr-release assay, as well as in direct *ex vivo* and *in vivo* cytotoxicity assays. SIV Gag-specific CTL activity, measured in a ^{51}Cr-release assay where peptide-stimulated *in vitro*-expanded cultures are tested against autologous peptide-loaded B-lymphoblastoid cell lines (B-LCL), was detected in 5/10 vaccinated controllers and 3/6 'natural' controllers (Table 3). Although this assay has been used extensively to assess CTL function, the use of B-LCL as the target cell as well as the *in vitro*-expansion protocol may not accurately represent the actual level of CTL-mediated elimination that virus-infected cells encounter *in vivo*. Therefore, a more direct *in vivo* elimination assay was developed in which autologous PBMC that were labelled with fluorescent dye were loaded with viral peptides and reinfused into the animal. Subsequently, elimination of the cells within a 24 h period was evaluated against a control of autologous PBMC loaded with control peptides (herpes simplex virus gD) and labelled with a different dye or with the same dye at a different intensity. In this '*in vivo* cytotoxicity assay', high-intensity-labelled antigen-pulsed cells as well as low-intensity-labelled control cells could be detected immediately (10 min) after injection at approximately similar frequencies, whereas, 24 h later, in animals with specific CTL responses, the antigen-loaded population was diminished relative to the control population (an example of this analysis is shown in Supplementary Fig. S1, available in JGV Online). As shown in Table 3, the *in vivo* CTL assay confirmed the positive responses of the ^{51}Cr-release assay but generally showed higher levels of specific cell lysis, which may indicate that the *in vivo* assay is more sensitive. Furthermore, some of the negative cases in the ^{51}Cr-release assay did exhibit antigen-specific lysis in the *in vivo* CTL evaluation, resulting in detection of CTL activity in 6/7 tested vaccinated controllers and all six 'natural' controllers. Since these studies were performed with peptide pools covering the entire Gag sequence, which could include peptides with varying killing-triggering capacity, the studies were extended to the peptide-specific level, using a few select animals. Peptide mapping studies, which were based on the ^{51}Cr-release assay because it most closely resembles the *in vivo* CTL assay, demonstrated specific responses against GNYVHLPLSP in animal DVT and against YNTNILDVK in animal VH2 (Fig. 4). Subsequently, *in vivo* CTL activity was evaluated in these animals using specific peptides (GNYVHLPLSP in animal DVT, and YNPNILDVK in animal VH2). For comparison, this technique was also used in an *ex vivo* setting where 'effector' PBMC were incubated for 24 h with autologous dye-labelled and peptide-loaded 'target' PBMC. PIPVGNIYR was used as a control peptide. In addition, ECX was included as a control animal that had shown no responses against either of these peptides in the ^{51}Cr-release assay (Fig. 4). As shown in Fig. 5, peptide-

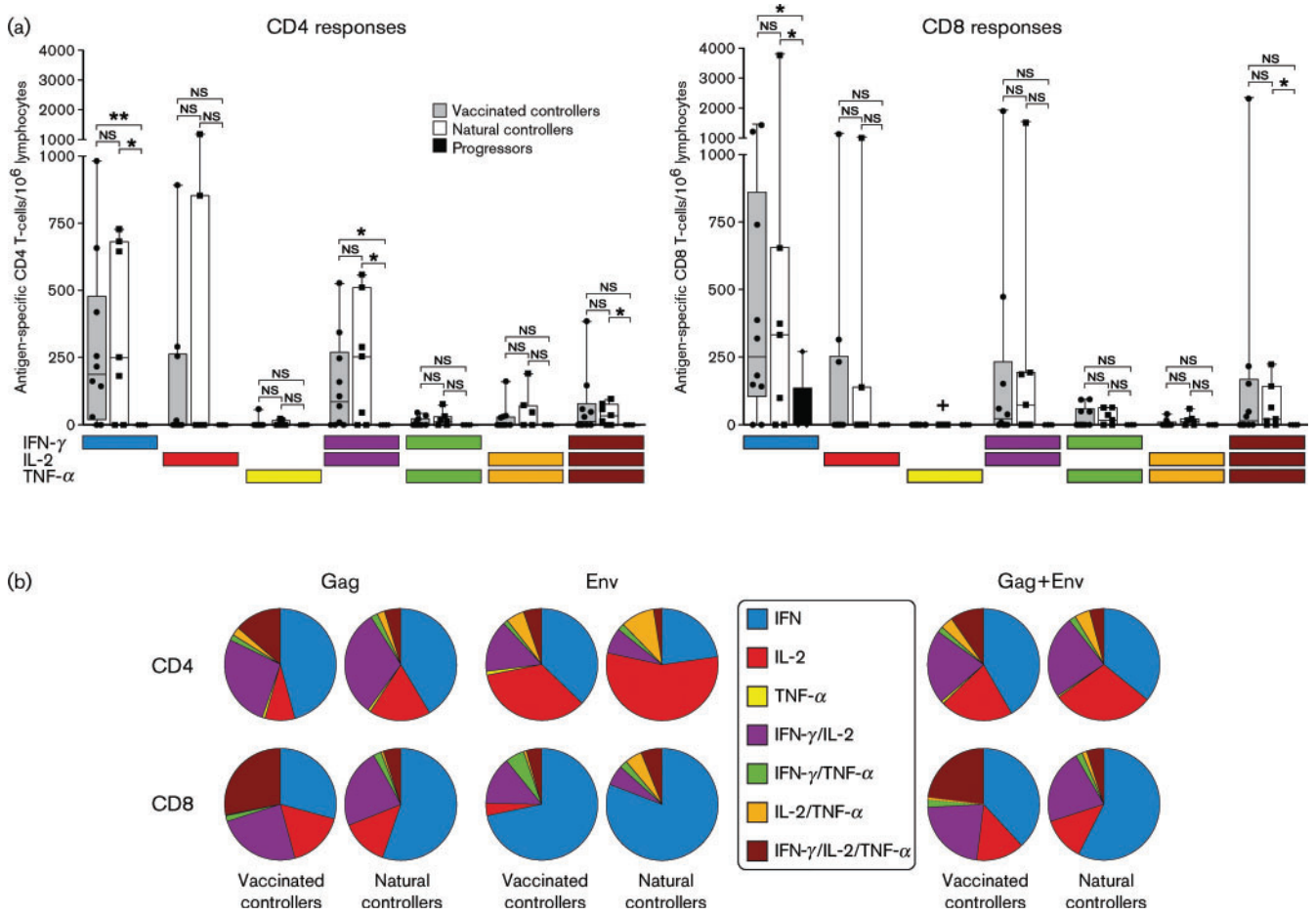

Fig. 2. Cytokine-response patterns of antigen-specific CD4 and CD8 T-cells. The number of IL-2, IFNγ and TNFα, single-, double- and triple-expressing cells for HIV-1 Env and SIV Gag combined (a), as well as the individual antigen-specific responses (b), is shown for CD4 and CD8 T-cells and for all three study groups. Box-whisker plots indicate the interquartile ranges and the medians (horizontal lines) of the groups; each dot represents a single animal. Responses are expressed as the number of positive cells per 10^6 lymphocytes with the background subtracted. The background reading was taken from cells cultured without antigen but in the presence of anti-CD28, anti-CD49d and Golgiplug. *, Significant $P<0.05$; **, significant $P<0.01$; NS, not significant; +, no statistical evaluation possible because all values were zero.

specific cytotoxic activity could be demonstrated in DVT and VH2, but not in the control animal ECX. However, in animal VH2, the cells loaded with the control peptide PIPVGNIYR were also lysed. A re-evaluation of the ^{51}Cr-release data showed some reaction against this peptide as well (Fig. 4), which could have become more prominent in the *ex vivo* and *in vivo* CTL assays because of their relatively higher sensitivities. In conclusion, in both groups, vaccinated controllers and 'natural' controllers, T-cell cytotoxic function was evident and comparable with regard to the strength of the responses as well as to the proportion of the animals that showed a positive response. These comparable results were obtained by the standard ^{51}Cr-release assay as well as by the more sensitive *in vivo* CTL assays.

Since *Mamu-A*001*, *Mamu-B*008* and *Mamu-B*017* have been associated with containment of SIV replication (Hel *et al.*, 2002; Loffredo *et al.*, 2007; Yant *et al.*, 2006), the animals were typed for MHC class I alleles (Supplementary Table S1). *Mamu-A*001* alleles were found in four animals: two vaccinated controller (EKK and WJ8) and two progressor animals (9068 and 96025). *Mamu-B*008* was seen in one animal belonging to the progressor group (9068). *Mamu-B*017* was not observed. On the basis of this distribution, it is unlikely that these particular alleles are confounding factors in this study.

DISCUSSION

Previous vaccine-evaluation studies in macaques have demonstrated preservation of CD4 T-cells and, more recently, maintenance of high-magnitude polyfunctional immune responses upon challenge with pathogenic

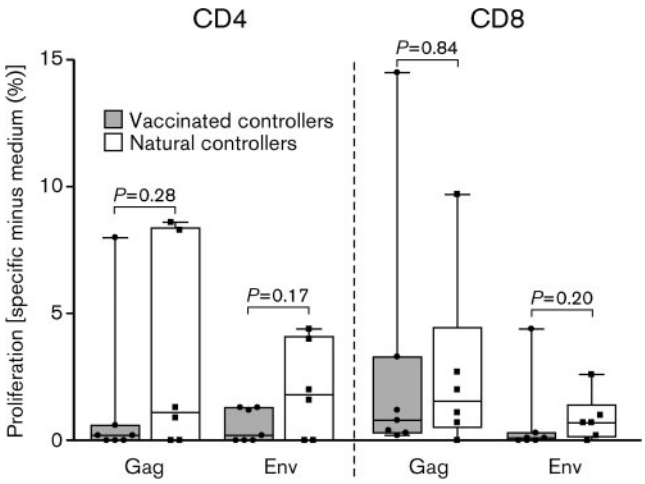

Fig. 3. Antigen-specific lymphoproliferative responses during the chronic phase of the infection, 39–150 weeks post-challenge. Depicted are the HIV-1 Env- and SIV Gag-specific, CD4 and CD8 T-cell divisions, as determined for seven vaccinated controllers and six 'natural' controllers. Box-whisker plots indicate the interquartile ranges and the medians (horizontal lines) of the groups; each dot represents a single animal. The percentage of divided cells is calculated as (fraction of cells with reduced CFSE labelling after 6 days incubation with antigen) – (background). The background was the fraction of cells with reduced CFSE labelling in the medium control. Background responses were as follows: 2.81 ± 2.70 and 2.82 ± 2.40 in the CD4 T-cells of the vaccinated and 'natural' controllers, respectively, and 1.87 ± 2.92 and 0.80 ± 0.78 in the CD8 T-cells of the vaccinated and 'natural' controllers, respectively. Animals with a ConA response of <20 % above background were excluded from the analysis and are not shown.

$SHIV_{89.6P}$, relative to unvaccinated SHIV-infected control animals (Acierno *et al.*, 2006; Sun *et al.*, 2006, 2008). However, as in HIV-1-infected humans, some animals have spontaneous control of this infection without previous immunization or treatment (Reimann *et al.*, 2005). Here, we describe a similar group of so-called 'natural' controllers and compare their immune-response profiles with a group of vaccinated animals that control infection. Detailed analysis showed that Gag- and Env-specific cytokine induction, proliferative responses and cytotoxic T-cell function were comparable between the groups of 'natural' and vaccinated controllers.

The animals used in this study were selected from previous vaccine-evaluation studies and had received a combination of HIV-1 Tat, HIV-1 Env and SIV Gag/Pol immunogens or HIV-Tat alone, or had served as challenge control animals in the same study. SIV Gag-specific responses were readily detectable in almost all vaccinated animals when SIV Gag virus-like particles (VLP) were used as an antigen (not shown). Clear responses against the SIV_{mac251} Gag peptides were observed after challenge (Table 2) and were shown to be mediated by CD8, as well as CD4, T-cells (Fig. 1).

Table 3. Cytotoxic capacity measured by ^{51}Cr release and *in vivo* CTL assays

Indicated is the antigen used for stimulation of the cells in the ^{51}Cr release assay as well as for loading of target cells. Assays were performed during the chronic phase of the infection, 100–150 weeks post-challenge. Antigen that was used for stimulation of the cells in the ^{51}Cr-release assay as well as for loading of the target cells is indicated. The percentage of cell lysis for the ^{51}Cr-release assay was calculated as (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100. The percentage of cell lysis for the in vivo CTL assay was calculated as 100−[(number of CFSE-positive cells, specific peptide 24 h) × (number of CFSE-positive cells, unpulsed 10 min)/(number of CFSE-positive cells, unpulsed 24 h) × (number of CFSE-positive cells, specific peptide 10 min) × 100]. Specific lysis <10% is considered negative.

Animals	Antigen	Lysis (%)	
		^{51}Cr release	*In vivo* CTL
Vaccinated controllers			
DVT	Gag	30	*
VH2	Gag	29	*
ECX	Gag	4	*
Ri178	Gag	7	44
EEG	Gag	13	32
EKK	Gag	3	94
WJ8	Gag	28	54
Ri191	Gag	4	84
Ri226	Gag	80	64
Ri253	Env	5	1
'Natural' controllers			
94053	Gag	20	95
R120	Gag	23	68
Ri009	Gag	1	57
Ri081	Gag	7	68
Ri094	Gag	1	60
Ri206	Gag	76	99

*Performed only against specific peptides and described in Fig. 5.

Our data, compiled from several vaccine evaluation studies, showed that spontaneous control of virus replication was relatively rare in unvaccinated rhesus macaques of Indian origin (21 %). However, control of virus replication to <500 copies ml^{-1} was found in 46 % of the macaques of Chinese origin, thus partially supporting previous findings of reduced virus replication in these animals (Reimann *et al.*, 2005). In vaccinated animals, suppression of virus replication was relatively common, reaching 70 and 87 % in macaques of Indian and Chinese origin, respectively, and progression to AIDS was rare. Although it is conceivable that some of the animals in the vaccinated controller group that was studied here might have spontaneously controlled their infection, the data presented above indicate that this could only be expected for three out of the ten animals, which, in view of the presented data, would not have changed the outcome of the analysis.

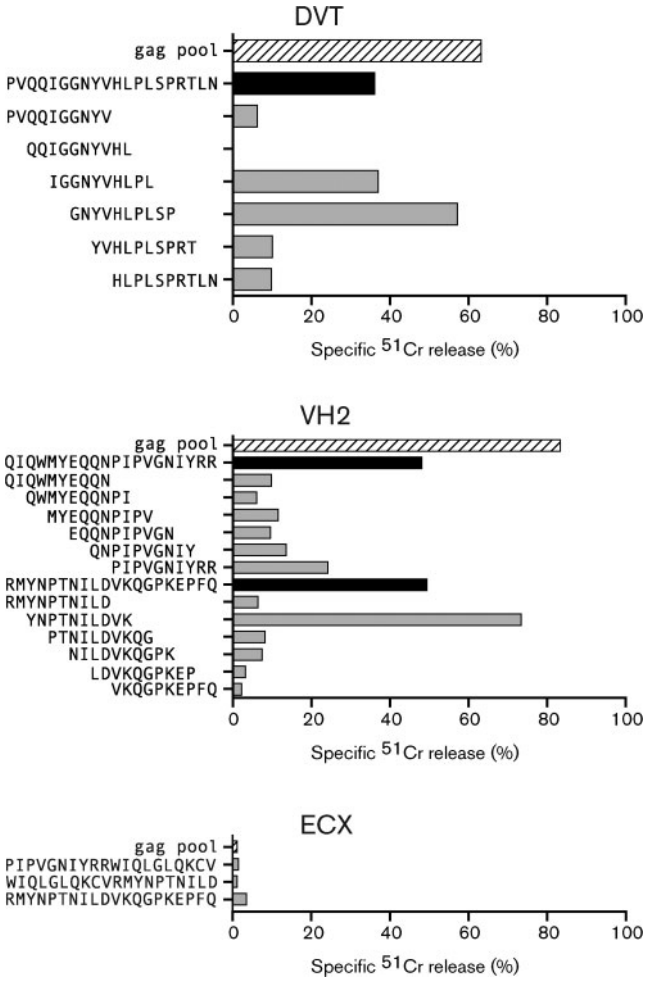

Fig. 4. Peptide epitope mapping in animals DVT, VH2 and ECX. Responses against individual 20-mer SIV Gag peptides were evaluated using a standard ^{51}Cr-release assay, giving a positive response against one peptide in animal DVT and two peptides in VH2. Further fine mapping by using 10-mer peptides showed responses in DVT to be directed against GNYVHLPLSP and in VH2 to be directed against YNPNILDVK.

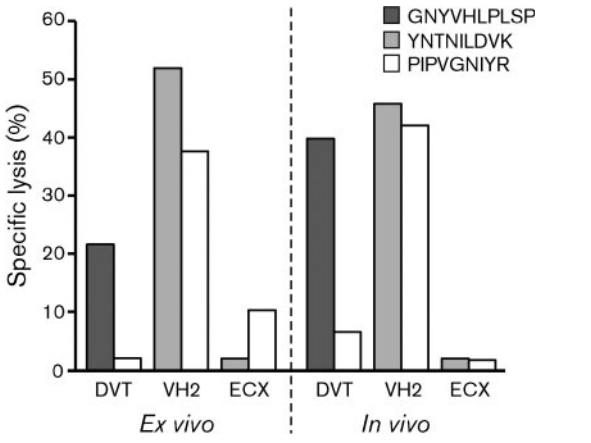

Fig. 5. Peptide-specific cytotoxic capacity measured by *ex vivo* CTL and *in vivo* CTL assays. Animals received either autologous CFSE-high-labelled cells loaded with specific CTL epitopes (GNYVHLPLSP for DVT and YNPNILDVK for VH2), or CFSE-low labelled cells loaded with the PIPVGNIYR control peptide. ECX received autologous CFSE-labelled PBMC loaded with either YNPNILDVK or PIPVGNIYR, which could both be considered control peptides (as they were not previously detectable by ^{51}Cr-release assay). The percentage of cell cytotoxicity in the *ex vivo* and *in vivo* CTL assays was calculated as 100 − [(number of CFSE-positive cells, specific peptide$_{24\ h}$ × number of CFSE-positive cells, unpulsed$_{10\ min}$)/(number of CFSE-positive cells, unpulsed$_{24\ h}$ × number of CFSE-positive cells, specific peptide$_{10\ min}$) × 100]. Specific cytotoxic activity <10 % was considered negative.

Previous studies have implicated reduced CD8 T-cell effector function in HIV-disease progression but shown high proliferative capacity, polyfunctional cytokine production, granzyme and perforin expression, and cytotoxic function in LTNP individuals (Appay & Rowland-Jones, 2002; Betts *et al.*, 2006; Migueles *et al.*, 2002, 2008; van Baarle *et al.*, 2002). Interestingly, these capacities are thought to be mediated by different memory subsets, with preponderant proliferation in the central memory compartment and cytotoxic function in the effector memory and effector cell compartments (Champagne *et al.*, 2001; Sallusto *et al.*, 1999). The data presented here show similar levels of CD4 and CD8 T-cell proliferation and cytokine production in 'natural' versus vaccinated controllers. In order to compare CTL effector function between these groups, two fundamentally different assays were applied: firstly, a classical *in vitro* ^{51}Cr-release assay, which requires prior cell expansion and that monitors effector function elicited from the central memory compartment, and secondly, a direct *ex vivo* and *in vivo* cytotoxicity assay that monitors immediate effector function and which is probably mediated by the effector and effector memory compartment (Oehen & Brduscha-Riem, 1998). In both the 'natural' controller group and the vaccinated controller group about half of the animals showed a positive response in the ^{51}Cr-release assays, while in both groups almost all of the animals exhibited cytotoxic activity as measured by the *in vivo* CTL assay. These data further support the overlapping response patterns already observed between 'natural' and vaccinated controllers. The high response levels observed in the *in vivo* CTL assay could be indicative of a dominant effector plus effector memory compartment. These data were further confirmed at the peptide-specific level (Fig. 5). Detailed analysis of the MHC class I repertoire did not show a strong association between *Mamu-A*001*, *-B*008* and *-B*017* and resistance to disease, as was reported for several SIV strains (Hel *et al.*, 2002; Loffredo *et al.*, 2007; Yant *et al.*, 2006). Apart from the fact that we used a slightly different virus, most of our studies were conducted using animals of Chinese origin. Therefore, it is possible that in our cohort other MHC class I alleles contributed to resistance.

In conclusion, the previously documented relative preservation of immune function in vaccinated animals upon $SHIV_{89.6p}$ challenge (Acierno *et al.*, 2006; Sun *et al.*, 2006, 2008) can also be obtained at comparable levels in animals that are able to control infection without prior immunization. Hence, the premise of using immunological analysis of LTNP cohorts to set standards for human vaccine trials seems valid.

METHODS

Animals. Mature captive-bred rhesus monkeys (*Macaca mulatta*) of Chinese and Indian origin were used in this study and one animal was of Burmese origin. The origin of the animals was confirmed by animal import permits, purchase records or pedigrees and by mitochondrial 12S rRNA analysis. They were housed in environmentally controlled conditions at the BPRC. The animals were negative for antibodies to SIV-1, simian type D retrovirus and simian T-cell lymphotropic virus at the initiation of the study. All animals were challenged with 50–100 MID_{50} (50% monkey infectious dose) of the pathogenic cell-free SHIV stock '89.6p' by the intravenous route (1 ml per monkey). Of the 22 animals that were studied in detail, ten had received previous immunizations consisting of either a combination of HIV-1 Env, HIV-1 Tat and SIV Gag/Pol antigens or HIV-1 Tat alone, given either in a DNA prime–immune stimulating complex (ISCOM)-formulated protein combination or a DNA prime–modified vaccinia Ankara (MVA) viral-vector booster combination (Mooij *et al.*, 2004) (Table 2). The control animals had either received control DNA, ISCOM or NYVAC vectors or were completely naive controls (Table 2). Five animals developed disease; the other animals were able to control viral load at low levels and maintain stable $CD4^+$ T-cell counts. During the course of the study, the animals were checked twice daily for appetite and general behaviour, and stools were checked for consistency. With each sedation for blood collection or immunization, the body weight and body temperature were measured. Animals developing opportunistic infections, a body weight loss of >10 %, persistently low CD4 counts and high viral loads were considered to be progressing to AIDS and were euthanized. The Institutional Animals Care and Use Committee approved study protocols developed according to strict international ethical and scientific standards and guidelines.

Determination of virus load and CD4 T-cell count. Plasma virus load was determined using a quantitative competitive-RNA RT-PCR using plasma from EDTA-treated blood samples as described previously by Ten Haaft *et al.* (1998). To monitor changes in lymphocyte subset composition and CD4 T-lymphocyte count, flow cytometry was performed after $SHIV_{89.6p}$ challenge as described by Koopman *et al.* (2004).

MHC class I typing and restriction. Originally the MHC type (Mamu-A and -B) of the animals was determined using polyclonal sera raised by active immunizations. A cluster of positive typing reactions defines the serotypes. The nucleotide profiles and transcription status of the corresponding Mamu class I alleles which segregate with particular serotypes were described by Otting *et al.* (2005, 2007, 2008).

ELISpot assay for IFNγ secretion. PBMC (4×10^6 ml^{-1}) were stimulated with either SIV_{mac239} Gag VLP (prepared by Dr R. Wagner, Regensburg University, Germany) as protein antigen or with SIV_{mac239}-Gag (NIH 6204) or HIV-$1_{89.6}$-Env (NIH 4827) pooled peptide sets (15-mers, 11 aa overlap) at a concentration of 2 μg ml^{-1} in RPMI 1640 medium supplemented with 2 mM glutamine, 50 U penicillin ml^{-1}, 50 μg streptomycin ml^{-1} and 5 % pooled rhesus serum in a 24-well plate for 16 h at 37 °C. The negative control was medium alone, while PMA–ionomycin (used at 20 ng ml^{-1} and 1 μg ml^{-1}, respectively) was used as a positive control. After stimulation, the non-adherent cells were collected and plated at 2×10^5 cells per well in triplicate in 96-well flat-bottomed plates (Nunc) coated with anti-monkey IFN-γ mAb (U-CyTech) and incubated for an additional 5 h with the same antigens added again. At the end of the incubation period, cells were lysed by adding 200 μl of ice-cold water and leaving the plates on ice for 10 min before cell debris was washed away. IFN-γ production was detected using biotinylated rabbit-anti-rhesus IFN-γ. Spots were visualized using a gold staining–silver enhancement technique (U-CyTech). Spots were counted using an automated ELISpot counter (AELVIS).

Intracellular cytokine staining. PBMC were incubated at 37 °C for 2 h with 2 μg anti-CD28 ml^{-1} and 2 μg anti-CD49d antibodies ml^{-1} (BD Pharmingen) and appropriate peptides of interest at a concentration of 1.25 μg peptide ml^{-1}. Medium alone was used as the negative control and staphylococcal enterotoxin B served as the positive control. To inhibit protein trafficking, cells were treated with Golgiplug (BD Pharmingen) and incubated for 16 h at 37 °C. After the incubation period, cells were washed with PBS (pH 7.4) with 1 % BSA and stained for surface markers by incubation with anti-$CD3^{APC}$, anti-$CD8^{APC\text{-}Cy7}$ and anti-$CD4^{PerCP}$ for 30 min at 4 °C in the dark. Subsequently, cells were washed with PBS (pH 7.4) with 1 % BSA and fixed with Cytofix/Cytoperm solution (BD Pharmingen) for 20 min at 4 °C. The cells were then washed with permeabilization buffer (BD Pharmingen) and resuspended in permeabilization buffer containing anti-IL-2^{PE}, anti-IFN-$\gamma^{Alexa700}$ and TNF-$\alpha^{PE\text{-}Cy7}$ (BD Biosciences). After 30 min of incubation at 4 °C cells were washed with permeabilization buffer and fixed in 2 % paraformaldehyde solution for 16 h. Samples were measured on a FACSAria and analysed using FACS Diva software (Becton Dickinson).

Proliferation assay. Proliferative capacity upon antigen stimulation of PBMC was investigated using the 5(and 6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE; Molecular Probes) proliferation assay as described previously (Lyons & Parish, 1994; Oehen & Brduscha-Riem, 1998). Cells were stimulated with either medium alone, peptides of interest at a concentration of 1.25 μg peptide ml^{-1}, or 5 μg concanavalin A (ConA) ml^{-1} , and incubated for 6 days at 37 °C and 5 % CO_2. Cells were stained for viability using the LIVE/DEAD Fixable Violet Dead Cell dye (Molecular Probes) and subsequently with a mixture of $CD3^{APC}$, $CD4^{PerCP\text{–}Cy5.5}$, $CD8^{APC\text{–}Cy7}$ and cells were fixed overnight in 2 % paraformaldehyde at 4 °C and measured on a FACSAria (Becton Dickinson). Animals in which the ConA response was less than 20 % above background were excluded from the analysis. Viability was above 95 % for all animals except DVT, for which it was 60 %.

^{51}Cr-release assay. Effector cells were generated from PBMC via co-cultivation for 10–14 days with autologous, peptide-pulsed, feeder cells and regularly replenished with culture medium containing IL-2 during the culture period. Cytotoxicity was assessed in a classical 5 h ^{51}Cr-release assay. Briefly, target cells were autologous or allogeneic herpes papio-transformed B-LCL labelled with 40 μCi $Na_2{}^{51}CrO_4$ (Amersham International) and peptide pools or individual peptides at 3 μg ml^{-1} for 16 h at 37 °C. B-LCL without peptides were used as a control. Subsequently, target cells were washed and plated at 5×10^3 cells well^{-1} in U-shaped 96-well plates (Costar). Effector cells were added at several effector/target cell ratios in triplicate. After 5 h incubation at 37 °C, supernatants were harvested and ^{51}Cr release was measured using a gamma counter (Cobra 5; Packard). Spontaneous ^{51}Cr release was determined from control wells containing target cells with medium alone. Maximum ^{51}Cr release was obtained from target cells incubated with 1 % Triton X-100 (Sigma). Percentages of specific

^{51}Cr release were calculated as [(experimental release−spontaneous release)/(maximum release−spontaneous release)] × 100.

***In vivo* and *ex vivo* cytotoxicity assay.** *In vivo* and *ex vivo* cytotoxic activity was determined by using a previously described method with modifications and using PBMC as target cells (Aichele *et al.*, 1997). The total amount of PBMC was split into three equal parts. Each part was labelled with a different concentration of CFSE for 5 min at room temperature. The staining reaction was stopped by adding an equal volume of FCS and the cells were washed twice and left overnight at 37 °C. Subsequently, the two populations that had been labelled at intermediate and high staining intensities were pulsed with peptide of interest at 10 μg ml^{-1} for 1 h at 37 °C. The third part of the cells served as a control and was not pulsed. For intravenous injection all cells were pooled and injected at 20×10^6 cells ml^{-1}. Blood samples were collected 10 min and 24 h after injection. *Ex vivo* cytotoxicity was measured by co-incubation of the three different pulsed-cell populations (5×10^5 cells for each population) for 10 min and 24 h. CFSE-labelled cells were measured using a FACSsort and analysed using CellQuest software (Becton Dickinson). Percentages of specific *in vivo* and *ex vivo* lysis were calculated as 100−[(number of CFSE-positive cells, specific peptide$_{24\ h}$ × number of CFSE-positive cells, unpulsed$_{10\ min}$/number of CFSE-positive cells, unpulsed$_{24\ h}$ × number of CFSE-positive cells, specific peptide$_{10\ min}$) × 100].

Statistical evaluation. A Mann–Whitney test was used for comparison of cytokine and proliferative responses in CD4 and CD8 T-cells between the group of vaccinated versus 'natural' controllers. Fisher's exact test was used for comparison of SIV Gag ELISpot responses between the group of vaccinated versus 'natural' controllers in the chronic phase of the infection. Statistical analysis was performed with InStat version 3.0 (GraphPad).

ACKNOWLEDGEMENTS

This work was supported by funding of the EuroVacc (European Vaccine Effort Against HIV/AIDS) project QLK2-CT-1999-01321.

REFERENCES

Acierno, P. M., Schmitz, J. E., Gorgone, D. A., Sun, Y., Santra, S., Seaman, M. S., Newberg, M. H., Mascola, J. R., Nabel, G. J. & other authors (2006). Preservation of functional virus-specific memory CD8$^+$ T lymphocytes in vaccinated, simian human immunodeficiency virus-infected rhesus monkeys. *J Immunol* **176**, 5338–5345.

Addo, M. M., Yu, X. G., Rathod, A., Cohen, D., Eldridge, R. L., Strick, D., Johnston, M. N., Corcoran, C., Wurcel, A. G. & other authors (2003). Comprehensive epitope analysis of human immunodeficiency virus type 1 (HIV-1)-specific T-cell responses directed against the entire expressed HIV-1 genome demonstrate broadly directed responses, but no correlation to viral load. *J Virol* **77**, 2081–2092.

Aichele, P., Brduscha-Riem, K., Oehen, S., Odermatt, B., Zinkernagel, R. M., Hengartner, H. & Pircher, H. (1997). Peptide antigen treatment of naive and virus-immune mice: antigen-specific tolerance versus immunopathology. *Immunity* **6**, 519–529.

Altfeld, M., Addo, M. M., Rosenberg, E. S., Hecht, F. M., Lee, P. K., Vogel, M., Yu, X. G., Draenert, R., Johnston, M. N. & other authors (2003). Influence of HLA-B57 on clinical presentation and viral control during acute HIV-1 infection. *AIDS* **17**, 2581–2591.

Altfeld, M., Kalife, E. T., Qi, Y., Streeck, H., Lichterfeld, M., Johnston, M. N., Burgett, N., Swartz, M. E., Yang, A. & other authors (2006). HLA alleles associated with delayed progression to AIDS contribute strongly to the initial CD8$^+$ T cell response against HIV-1. *PLoS Med* **3**, e403.

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

Appay, V. & Rowland-Jones, S. L. (2002). Premature ageing of the immune system: the cause of AIDS? *Trends Immunol* **23**, 580–585.

Barouch, D. H., Santra, S., Kuroda, M. J., Schmitz, J. E., Plishka, R., Buckler-White, A., Gaitan, A. E., Zin, R., Nam, J. H. & other authors (2001). Reduction of simian-human immunodeficiency virus 89.6P viremia in rhesus monkeys by recombinant modified vaccinia virus Ankara vaccination. *J Virol* **75**, 5151–5158.

Betts, M. R., Ambrozak, D. R., Douek, D. C., Bonhoeffer, S., Brenchley, J. M., Casazza, J. P., Koup, R. A. & Picker, L. J. (2001). Analysis of total human immunodeficiency virus (HIV)-specific CD4$^+$ and CD8$^+$ T-cell responses: relationship to viral load in untreated HIV infection. *J Virol* **75**, 11983–11991.

Betts, M. R., Nason, M. C., West, S. M., De Rosa, S. C., Migueles, S. A., Abraham, J., Lederman, M. M., Benito, J. M., Goepfert, P. A. & other authors (2006). HIV nonprogressors preferentially maintain highly functional HIV-specific CD8$^+$ T cells. *Blood* **107**, 4781–4789.

Cao, Y., Qin, L., Zhang, L., Safrit, J. & Ho, D. D. (1995). Virologic and immunologic characterization of long-term survivors of human immunodeficiency virus type 1 infection. *N Engl J Med* **332**, 201–208.

Champagne, P., Ogg, G. S., King, A. S., Knabenhans, C., Ellefsen, K., Nobile, M., Appay, V., Rizzardi, G. P., Fleury, S. & other authors (2001). Skewed maturation of memory HIV-specific CD8 T lymphocytes. *Nature* **410**, 106–111.

Deeks, S. G. & Walker, B. D. (2007). Human immunodeficiency virus controllers: mechanisms of durable virus control in the absence of antiretroviral therapy. *Immunity* **27**, 406–416.

Doria-Rose, N. A., Ohlen, C., Polacino, P., Pierce, C. C., Hensel, M. T., Kuller, L., Mulvania, T., Anderson, D., Greenberg, P. D. & other authors (2003). Multigene DNA priming-boosting vaccines protect macaques from acute CD4$^+$-T-cell depletion after simian-human immunodeficiency virus SHIV89.6P mucosal challenge. *J Virol* **77**, 11563–11577.

Egan, M. A., Chong, S. Y., Rose, N. F., Megati, S., Lopez, K. J., Schadeck, E. B., Johnson, J. E., Masood, A., Piacente, P. & other authors (2004). Immunogenicity of attenuated vesicular stomatitis virus vectors expressing HIV type 1 Env and SIV Gag proteins: comparison of intranasal and intramuscular vaccination routes. *AIDS Res Hum Retroviruses* **20**, 989–1004.

Emu, B., Sinclair, E., Favre, D., Moretto, W. J., Hsue, P., Hoh, R., Martin, J. N., Nixon, D. F., McCune, J. M. & Deeks, S. G. (2005). Phenotypic, functional, and kinetic parameters associated with apparent T-cell control of human immunodeficiency virus replication in individuals with and without antiretroviral treatment. *J Virol* **79**, 14169–14178.

Giraldo-Vela, J. P., Rudersdorf, R., Chung, C., Qi, Y., Wallace, L. T., Bimber, B., Borchardt, G. J., Fisk, D. L., Glidden, C. E. & other authors (2008). The major histocompatibility complex class II alleles *Mamu-DRB1*1003* and *-DRB1*0306* are enriched in a cohort of simian immunodeficiency virus-infected rhesus macaque elite controllers. *J Virol* **82**, 859–870.

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

Harari, A., Petitpierre, S., Vallelian, F. & Pantaleo, G. (2004). Skewed representation of functionally distinct populations of virus-specific CD4 T cells in HIV-1-infected subjects with progressive disease: changes after antiretroviral therapy. *Blood* **103**, 966–972.

Harari, A., Cellerai, C., Enders, F. B., Kostler, J., Codarri, L., Tapia, G., Boyman, O., Castro, E., Gaudieri, S. & other authors (2007). Skewed association of polyfunctional antigen-specific CD8 T cell populations with HLA-B genotype. *Proc Natl Acad Sci U S A* **104**, 16233–16238.

Harari, A., Enders, F. B., Cellerai, C., Bart, P. A. & Pantaleo, G. (2009). Distinct profiles of cytotoxic granules in memory CD8 T cells correlate with function, differentiation stage, and antigen exposure. *J Virol* **83**, 2862–2871.

Hel, Z., Nacsa, J., Tryniszewska, E., Tsai, W. P., Parks, R. W., Montefiori, D. C., Felber, B. K., Tartaglia, J., Pavlakis, G. N. & Franchini, G. (2002). Containment of simian immunodeficiency virus infection in vaccinated macaques: correlation with the magnitude of virus-specific pre- and postchallenge $CD4^+$ and $CD8^+$ T cell responses. *J Immunol* **169**, 4778–4787.

Horton, H., Frank, I., Baydo, R., Jalbert, E., Penn, J., Wilson, S., McNevin, J. P., McSweyn, M. D., Lee, D. & other authors (2006a). Preservation of T cell proliferation restricted by protective HLA alleles is critical for immune control of HIV-1 infection. *J Immunol* **177**, 7406–7415.

Horton, H., Havenar-Daughton, C., Lee, D., Moore, E., Cao, J., McNevin, J., Andrus, T., Zhu, H., Rubin, A. & other authors (2006b). Induction of human immunodeficiency virus type 1 (HIV-1)-specific T-cell responses in HIV vaccine trial participants who subsequently acquire HIV-1 infection. *J Virol* **80**, 9779–9788.

Hubert, J. B., Burgard, M., Dussaix, E., Tamalet, C., Deveau, C., Le Chenadec, J., Chaix, M. L., Marchadier, E., Vilde, J. L., Delfraissy, J. F., Meyer, L. & Rouzioux, C. (2000). Natural history of serum HIV-1 RNA levels in 330 patients with a known date of infection. The SEROCO Study Group. *AIDS* **14**, 123–131.

Kiepiela, P., Leslie, A. J., Honeyborne, I., Ramduth, D., Thobakgale, C., Chetty, S., Rathnavalu, P., Moore, C., Pfafferott, K. J. & other authors (2004). Dominant influence of HLA-B in mediating the potential co-evolution of HIV and HLA. *Nature* **432**, 769–775.

Koopman, G., Mortier, D., Hofman, S., Niphuis, H., Fagrouch, Z., Norley, S., Sutter, G., Liljestrom, P. & Heeney, J. L. (2004). Vaccine protection from $CD4^+$ T-cell loss caused by simian immunodeficiency virus (SIV) mac251 is afforded by sequential immunization with three unrelated vaccine vectors encoding multiple SIV antigens. *J Gen Virol* **85**, 2915–2924.

Koopman, G., Mortier, D., Hofman, S., Mathy, N., Koutsoukos, M., Ertl, P., Overend, P., van Wely, C., Thomsen, L. L. & other authors (2008). Immune-response profiles induced by human immunodeficiency virus type 1 vaccine DNA, protein or mixed-modality immunization: increased protection from pathogenic simian-human immunodeficiency virus viraemia with protein/DNA combination. *J Gen Virol* **89**, 540–553.

Lefrère, J. J., Morand-Joubert, L., Mariotti, M., Bludau, H., Burghoffer, B., Petit, J. C. & Roudot-Thoraval, F. (1997). Even individuals considered as long-term nonprogressors show biological signs of progression after 10 years of human immunodeficiency virus infection. *Blood* **90**, 1133–1140.

Letvin, N. L., Huang, Y., Chakrabarti, B. K., Xu, L., Seaman, M. S., Beaudry, K., Korioth-Schmitz, B., Yu, F., Rohne, D. & other authors (2004). Heterologous envelope immunogens contribute to AIDS vaccine protection in rhesus monkeys. *J Virol* **78**, 7490–7497.

Loffredo, J. T., Friedrich, T. C., Leon, E. J., Stephany, J. J., Rodrigues, D. S., Spencer, S. P., Bean, A. T., Beal, D. R., Burwitz, B. J. & other authors (2007). $CD8^+$ T cells from SIV elite controller macaques recognize Mamu-B*08-bound epitopes and select for widespread viral variation. *PLoS ONE* **2**, e1152.

Lyons, A. B. & Parish, C. R. (1994). Determination of lymphocyte division by flow cytometry. *J Immunol Methods* **171**, 131–137.

Martin, M. P., Gao, X., Lee, J. H., Nelson, G. W., Detels, R., Goedert, J. J., Buchbinder, S., Hoots, K., Vlahov, D. & other authors (2002). Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. *Nat Genet* **31**, 429–434.

Martinez-Picado, J., Prado, J. G., Fry, E. E., Pfafferott, K., Leslie, A., Chetty, S., Thobakgale, C., Honeyborne, I., Crawford, H. & other authors (2006). Fitness cost of escape mutations in p24 Gag in association with control of human immunodeficiency virus type 1. *J Virol* **80**, 3617–3623.

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

Migueles, S. A., Sabbaghian, M. S., Shupert, W. L., Bettinotti, M. P., Marincola, F. M., Martino, L., Hallahan, C. W., Selig, S. M., Schwartz, D. & other authors (2000). HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors. *Proc Natl Acad Sci U S A* **97**, 2709–2714.

Migueles, S. A., Laborico, A. C., Shupert, W. L., Sabbaghian, M. S., Rabin, R., Hallahan, C. W., Van Baarle, D., Kostense, S., Miedema, F. & other authors (2002). HIV-specific $CD8^+$ T cell proliferation is coupled to perforin expression and is maintained in nonprogressors. *Nat Immunol* **3**, 1061–1068.

Migueles, S. A., Osborne, C. M., Royce, C., Compton, A. A., Joshi, R. P., Weeks, K. A., Rood, J. E., Berkley, A. M., Sacha, J. B. & other authors (2008). Lytic granule loading of $CD8^+$ T cells is required for HIV-infected cell elimination associated with immune control. *Immunity* **29**, 1009–1021.

Miura, T., Brockman, M. A., Brumme, C. J., Brumme, Z. L., Carlson, J. M., Pereyra, F., Trocha, A., Addo, M. M., Block, B. L. & other authors (2008). Genetic characterization of human immunodeficiency virus type 1 in elite controllers: lack of gross genetic defects or common amino acid changes. *J Virol* **82**, 8422–8430.

Mooij, P., Nieuwenhuis, I. G., Knoop, C. J., Doms, R. W., Bogers, W. M., Ten Haaft, P. J., Niphuis, H., Koornstra, W., Bieler, K. & other authors (2004). Qualitative T-helper responses to multiple viral antigens correlate with vaccine-induced immunity to simian/human immunodeficiency virus infection. *J Virol* **78**, 3333–3342.

Mooij, P., Balla-Jhagjhoorsingh, S. S., Koopman, G., Beenhakker, N., van Haaften, P., Baak, I., Nieuwenhuis, I. G., Kondova, I., Wagner, R. & other authors (2008). Differential $CD4^+$ versus $CD8^+$ T-cell responses elicited by different poxvirus-based human immunodeficiency virus type 1 vaccine candidates provide comparable efficacies in primates. *J Virol* **82**, 2975–2988.

Muñoz, A., Kirby, A. J., He, Y. D., Margolick, J. B., Visscher, B. R., Rinaldo, C. R., Kaslow, R. A. & Phair, J. P. (1995). Long-term survivors with HIV-1 infection: incubation period and longitudinal patterns of $CD4^+$ lymphocytes. *J Acquir Immune Defic Syndr Hum Retrovirol* **8**, 496–505.

Oehen, S. & Brduscha-Riem, K. (1998). Differentiation of naive CTL to effector and memory CTL: correlation of effector function with phenotype and cell division. *J Immunol* **161**, 5338–5346.

Otting, N., Heijmans, C. M., Noort, R. C., de Groot, N. G., Doxiadis, G. G., van Rood, J. J., Watkins, D. I. & Bontrop, R. E. (2005). Unparalleled complexity of the MHC class I region in rhesus macaques. *Proc Natl Acad Sci U S A* **102**, 1626–1631.

Otting, N., de Vos-Rouweler, A. J., Heijmans, C. M., de Groot, N. G., Doxiadis, G. G. & Bontrop, R. E. (2007). MHC class I A region

diversity and polymorphism in macaque species. *Immunogenetics* **59**, 367–375.

Otting, N., Heijmans, C. M., van der Wiel, M., de Groot, N. G., Doxiadis, G. G. & Bontrop, R. E. (2008). A snapshot of the *Mamu-B* genes and their allelic repertoire in rhesus macaques of Chinese origin. *Immunogenetics* **60**, 507–514.

Pantaleo, G., Menzo, S., Vaccarezza, M., Graziosi, C., Cohen, O. J., Demarest, J. F., Montefiori, D., Orenstein, J. M., Fox, C. & other authors (1995). Studies in subjects with long-term nonprogressive human immnodeficiency virus infection. *N Engl J Med* **332**, 209–216.

Pereyra, F., Addo, M. M., Kaufmann, D. E., Liu, Y., Miura, T., Rathod, A., Baker, B., Trocha, A., Rosenberg, R. & other authors (2008). Genetic and immunologic heterogeneity among persons who control HIV infection in the absence of therapy. *J Infect Dis* **197**, 563–571.

Precopio, M. L., Betts, M. R., Parrino, J., Price, D. A., Gostick, E., Ambrozak, D. R., Asher, T. E., Douek, D. C., Harari, A. & other authors (2007). Immunization with vaccinia virus induces polyfunctional and phenotypically distinctive $CD8^+$ T cell responses. *J Exp Med* **204**, 1405–1416.

Reimann, K. A., Parker, R. A., Seaman, M. S., Beaudry, K., Beddall, M., Peterson, L., Williams, K. C., Veazey, R. S., Montefiori, D. C. & other authors (2005). Pathogenicity of simian-human immunodeficiency virus SHIV-89.6P and SIVmac is attenuated in cynomolgus macaques and associated with early T-lymphocyte responses. *J Virol* **79**, 8878–8885.

Rodés, B., Toro, C., Paxinos, E., Poveda, E., Martinez-Padial, M., Benito, J. M., Jimenez, V., Wrin, T., Bassani, S. & Soriano, V. (2004). Differences in disease progression in a cohort of long-term non-progressors after more than 16 years of HIV-1 infection. *AIDS* **18**, 1109–1116.

Sallusto, F., Lenig, D., Forster, R., Lipp, M. & Lanzavecchia, A. (1999). Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* **401**, 708–712.

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

Sheppard, H. W., Lang, W., Ascher, M. S., Vittinghoff, E. & Winkelstein, W. (1993). The characterization of non-progressors: long-term HIV-1 infection with stable $CD4^+$ T-cell levels. *AIDS* **7**, 1159–1166.

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

Soumelis, V., Scott, I., Gheyas, F., Bouhour, D., Cozon, G., Cotte, L., Huang, L., Levy, J. A. & Liu, Y. J. (2001). Depletion of circulating natural type 1 interferon-producing cells in HIV-infected AIDS patients. *Blood* **98**, 906–912.

Sun, Y., Schmitz, J. E., Buzby, A. P., Barker, B. R., Rao, S. S., Xu, L., Yang, Z. Y., Mascola, J. R., Nabel, G. J. & Letvin, N. L. (2006). Virus-specific cellular immune correlates of survival in vaccinated monkeys after simian immunodeficiency virus challenge. *J Virol* **80**, 10950–10956.

Sun, Y., Santra, S., Schmitz, J. E., Roederer, M. & Letvin, N. L. (2008). Magnitude and quality of vaccine-elicited T-cell responses in the control of immunodeficiency virus replication in rhesus monkeys. *J Virol* **82**, 8812–8819.

Ten Haaft, P., Verstrepen, B., Uberla, K., Rosenwirth, B. & Heeney, J. (1998). A pathogenic threshold of virus load defined in simian immunodeficiency virus- or simian-human immunodeficiency virus-infected macaques. *J Virol* **72**, 10281–10285.

van Baarle, D., Kostense, S., van Oers, M. H., Hamann, D. & Miedema, F. (2002). Failing immune control as a result of impaired $CD8^+$ T-cell maturation: CD27 might provide a clue. *Trends Immunol* **23**, 586–591.

Vogel, T. U., Allen, T. M., Altman, J. D. & Watkins, D. I. (2001). Functional impairment of simian immunodeficiency virus-specific $CD8^+$ T cells during the chronic phase of infection. *J Virol* **75**, 2458–2461.

Vogel, T. U., Horton, H., Fuller, D. H., Carter, D. K., Vielhuber, K., O'Connor, D. H., Shipley, T., Fuller, J., Sutter, G. & other authors (2002). Differences between T cell epitopes recognized after immunization and after infection. *J Immunol* **169**, 4511–4521.

Yant, L. J., Friedrich, T. C., Johnson, R. C., May, G. E., Maness, N. J., Enz, A. M., Lifson, J. D., O'Connor, D. H., Carrington, M. & Watkins, D. I. (2006). The high-frequency major histocompatibility complex class I allele *Mamu-B*17* is associated with control of simian immunodeficiency virus SIVmac239 replication. *J Virol* **80**, 5074–5077.

Younes, S. A., Yassine-Diab, B., Dumont, A. R., Boulassel, M. R., Grossman, Z., Routy, J. P. & Sekaly, R. P. (2003). HIV-1 viremia prevents the establishment of interleukin 2-producing HIV-specific memory $CD4^+$ T cells endowed with proliferative capacity. *J Exp Med* **198**, 1909–1922.

Yue, F. Y., Kovacs, C. M., Dimayuga, R. C., Parks, P. & Ostrowski, M. A. (2004). HIV-1-specific memory $CD4^+$ T cells are phenotypically less mature than cytomegalovirus-specific memory $CD4^+$ T cells. *J Immunol* **172**, 2476–2486.