Early suppression of SIV replication by CD8+ *nef*-specific cytotoxic T cells in vaccinated macaques

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In order to develop a successful subunit vaccine against infection with the human immunodeficiency virus (HIV), protective immune effector functions must be identified. Until now, there has been only indirect evidence that HIV-specific cytotoxic T lymphocytes (CTLs) fulfill this role. Using the macaque simian immunodeficiency virus (SIV) model, the protective potential of *nef*-specific CTLs, stimulated by vaccination, was examined in animals challenged with a high intravenous dose of the pathogenic simian immunodeficiency virus, SIVmac251(32H)(pJ5). An inverse correlation was found between the vaccine-induced *nef*-specific CTL precursor frequency and virus load measured after challenge. In addition, the early decline in viraemia, observed in both vaccinated and unvaccinated control animals was associated with the development of virus-specific CTL activity and not with the presence of virus-specific neutralizing antibodies. The results imply that vaccines that stimulate strong CTL responses could protect against HIV infection.

There is currently considerable interest in whether virus-specific cytotoxic T lymphocyte (CTL) responses, induced by vaccines, could protect against infection with human immunodeficiency virus (HIV). The virus-specific CTL responses have been shown to be a protective host defence against many other virus infections in a number of animal models[1,2]. Similarly, there is accumulating evidence that CTLs play a critical role in controlling established HIV infection in humans: CTL responses are associated with the initial reduction of the early viraemia[3,4], CTL can inhibit HIV replication *in vitro*[5–8] and some apparently protected individuals make CTL responses[9].

Simian immunodeficiency virus (SIV) has a genomic organization similar to that of HIV (ref. 10) and causes acquired immune deficiency syndrome (AIDS) in infected macaques[11]. Thus the SIV-macaque model offers the chance to explore the role of CTLs in detail and in particular to determine whether induction of specific CTL responses by vaccination gives any prophylaxis. To date, the most effective way to protect macaques from high doses of pathogenic SIV has been to infect them first with attenuated molecular clones of SIV, SIVmac239 *nef*-deletion[12] or SIVmac251/32H/pC8 (SIVmacC8)[13–15]. Both of these attenuated viruses contain deletions within the *nef* gene, four amino acids in the case of SIVmacC8, which damage or destabilize the nef protein and reduce pathogenicity of the virus considerably. Although the mechanism of protection in these animals is not understood, SIV-specific CTL responses, predominantly against nef, were detected in SIVmacC8-infected cynomolgus macaques, which were protected from infection with the wild-type virus, SIVmac251/32H/pJ5 (SIVmacJ5) (N.A. *et al.* manuscript submitted). This result suggested that *nef*-specific CTLs could contribute, perhaps in a major way, to the protection seen in this model. In order to examine this question further, cynomolgus macaques were immunized with recombinant vaccinia expressing SIV nef. The nef protein has a theoretical attraction as a target for virus-specific CTLs, because it is produced early in the virus life cycle[16] and has the experimental advantage that it is a non-structural protein, so that nef-immunization cannot generate neutralizing antibody; this allows critical examination of the role of T-cell responses alone. In the study described here, a high-dose SIV challenge was given to immunized animals and controls. A strong inverse correlation was found between the *nef*-specific CTL level induced by vaccination and the initial virus load after challenge with SIV.

Results

Correlation between CTLp and bulk culture CTL activity

Seven cynomolgus macaques were immunized with either recombinant vaccinia virus (rVV) expressing the nef protein

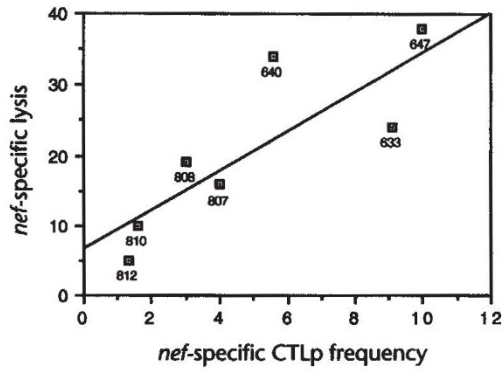

Fig. 1 Correlation between *nef*-specific CTL precursor frequencies and *nef*-specific lysis determined using 'bulk'-cultured CTLs. *Nef*-specific CTL lysis is shown for a 30:1 effector:target ratio; *nef*-specific CTL frequency is shown as precursors per 10^5 PBMCs.

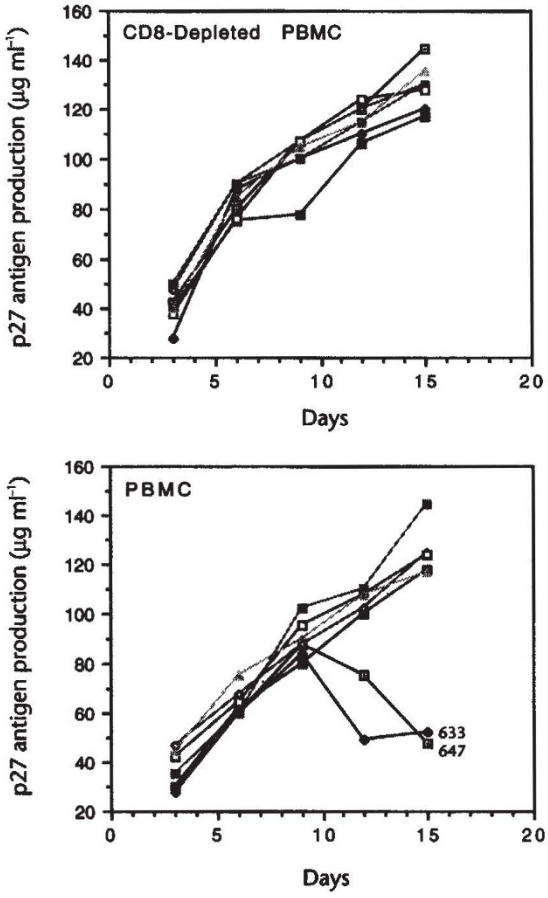

Fig. 2 p27 antigen production in CD8⁺ T-cell-depleted PBMCs (upper panel) and in unfractionated PBMCs (lower panel) from each vaccinated macaque, before *in vivo* challenge, cultured for the periods shown after infection *in vitro* with SIV J5.

derived from SIVmacJ5 or with rVV expressing the SIVmacC8-derived nef. All animals were immunized by scarification three times at five-week intervals with 5×10^8 plaque-forming units (PFU) of rVV-nef. Two weeks before virus challenge and four weeks after the third vaccination, MHC class I-restricted *nef*-specific CD8⁺ CTL responses were measured in bulk cultures and by limiting dilution, to determine precursor frequencies of specific CTLs in each immunized animal. In the bulk cultures, cytotoxicity could be inhibited by at least 80% by preincubating effector cells with anti-CD8 monoclonal antibodies or target cells with the anti-MHC class I monoclonal antibodies W6/32 (data not shown), implying classical CTL activity. A wide range of CTL activity was observed, despite identical immunization protocols with the two recombinant vaccinias; no difference could be discerned between the CTL responses stimulated by the J5 and C8 constructs. The differences in response may have been due, in part, to the different MHC class I types of the seven animals determined by isoelectric focusing of W6/32 immunoprecipitates. A good correlation was obtained between *nef*-specific CTL precursor frequencies and bulk culture *nef*-specific CTL activity (Fig. 1). No *nef*-specific antibodies were detectable in any animal by enzyme-linked immunosorbent assays (ELISA) from plasma taken one week before challenge (data not shown).

Inhibition of SIV replication *in vitro* by CD8⁺ T cells

At the same time as the above CTL responses were measured, lymphocytes from vaccinated animals were tested for inhibition of SIV replication in autologous CD4⁺ cells *in vitro*. Cultures of unfractionated and CD8⁺ T-cell-depleted peripheral blood mononuclear cells (PBMC) from each animal were infected with SIVmacJ5, and the concentration of p27 antigen was measured every three days. It was found that virus grew in all cultures but was inhibited by CD8⁺ lymphocytes in two animals, macaques 633 and 647 (Fig. 2). These animals also demonstrated the highest *nef*-specific CTL precursor frequencies. It is likely that the CD8⁺ cells responsible for the inhibition of virus growth are closely related to or identical to the lytic CTL.

Correlation between CTL activity, IFN-γ production

Three weeks after the last vaccination, each immunized monkey and four unimmunized control animals (macaques 215, 848, 849 and 862) were challenged intravenously with 50 MID_{50} (half minimal infecting dose) of the pathogenic molecular clone SIVmacJ5. With the exception of macaque 647, each animal became infected, as determined by virus cocultivation from PBMC and by a positive result after polymerase chain reaction (PCR) amplification. Virus was titred by coculture techniques before challenge (week 0) and at weeks 2, 4, 6 and 8 after challenge. Virus titres peaked between weeks 2 and 4 in all animals. When prechallenge *nef*-specific CTL activity was plotted against peak virus titres and total virus loads (Fig. 3*a*, *b*), strong inverse correlations were found ($r < -0.8$). Precursor CTL frequencies were not measured in the four control animals, but bulk culture CTL activity was not detectable before challenge. Virus levels in these unvaccinated animals were very similar to those with low CTL activity after vaccination (Fig. 3*c*). Interferon-γ (IFN-γ) levels in the supernatants of the CTL bulk cultures were also measured and when these were plotted against peak virus titres, an inverse correlation was also found (Fig. 3*d*).

Clearance of initial viraemia correlates with CTL responses

Six weeks after challenge virus burdens were reduced in each infected animal. This finding may be attributed to the total virus-specific CTL response, activated following SIV infection.

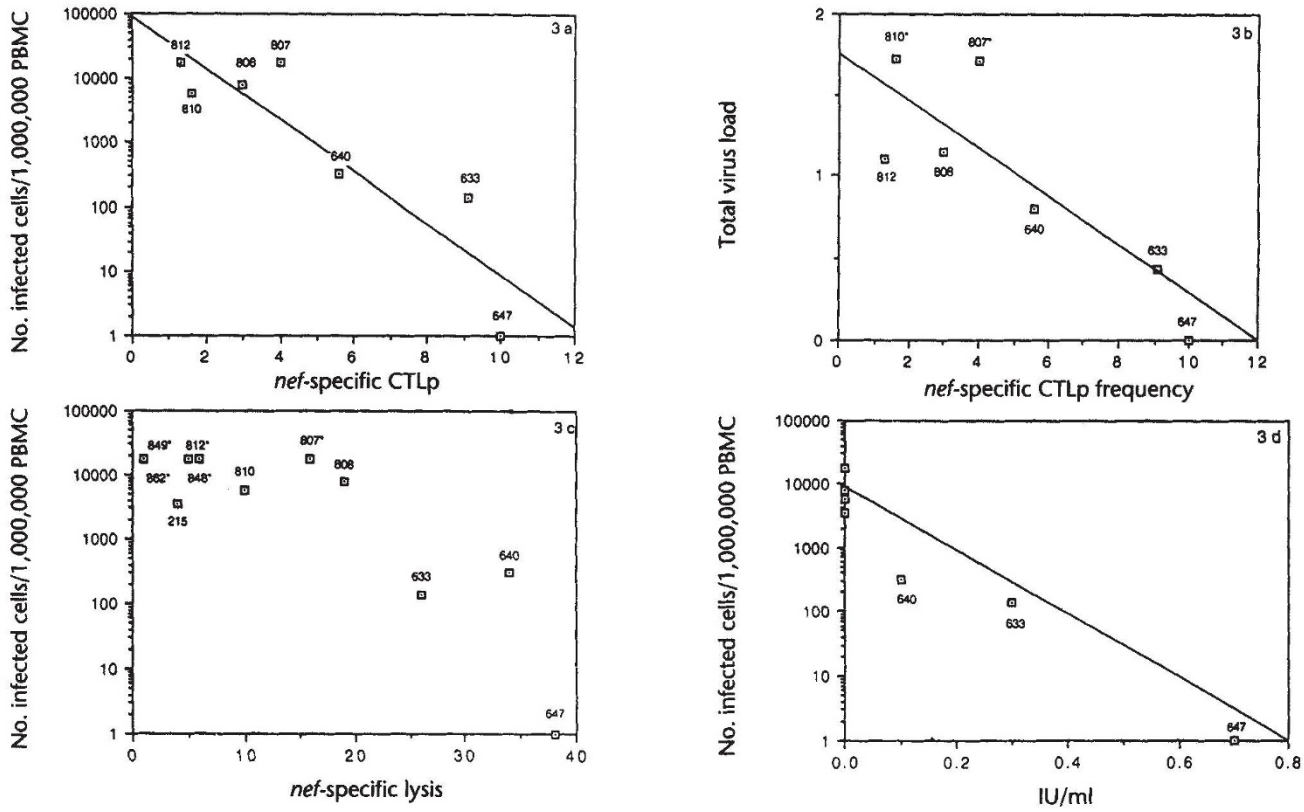

Fig. 3 Correlation between *nef*-specific CTL activity, IFN-γ production and virus loads measured following challenge. Animals were challenged intravenously with 50 MID_{50} of cell-free supernatant of SIVmacJ5 grown in simian PBMCs. From each animal, PBMCs were separated and virus titred by cocultivation with C8166 cells, as described in Methods. *a*, Correlation between *nef*-specific CTL precursor (CTLp) frequencies (*x*-axis) and peak virus titres (*y*-axis). For macaques 807 and 812 the titres were at the top of the scale and therefore at least as high as shown. The correlation coefficient *r* was estimated as –0.9, which is smaller than the true value of the correlation coefficient, –0.875, which is significant at the 1% level. *b*, Correlation between *nef*-specific CTLs and total virus load determined using a Bioimage Analyser; estimated correlation coefficient –0.86, which is smaller than the true correlation coefficient –0.754 and is significant at the 5% level. *c*, A scatter diagram showing the relationship between maximum virus titres (*y*-axis) and *nef*-specific lysis detected using bulk cultured CTLs from each animal before challenge. Included in this plot are the unvaccinated control animals 849, 862, 215 and 848. Points representing animals 807, 812, 848, 849 and 862 are marked with an asterisk (*) to indicate that virus load was at least as high as the value shown in the graph. *d*, Peak virus load is plotted against IFN-γ titres measured in the bulk CTLs culture supernatants. The estimate of *r* was calculated as –0.96, which is smaller than the true value of –0.847, significant at the 0.1% level. In animals 807, 808, 810 and 812, no interferon was measurable.

Both neutralizing antibody responses and CTL responses to the SIV gag, nef, tat and pol antigens were evaluated at weeks 2, 4, 6 and 8 after the SIVmacJ5 challenge. Neutralizing antibodies only became detectable in two animals (633 and 215) at week 8, after the decline in virus load occurred. In contrast, the specific CTL response became detectable (above the background value of 10% specific lysis) at the time the virus load began to decline in each animal (Fig. 4). These results are very similar to findings reported in acutely infected humans, although there CTLs were only detected by limiting dilution assay[4]. It is also noteworthy that one animal (812) that failed to make a CTL response to nef after vaccination did so after challenge with SIV. Animal 647 made CTL responses above the background level of 10% only to the nef antigen. The response to nef did not decay noticeably by eight weeks after challenge, which is not surprising since it has been reported (ref. 19 and data not shown) that following immunization of mice or macaques CTLs remain detectable for at least three months.

Proviral loads

Eight weeks after challenge, proviral DNA burdens in the PBMC of each macaque were measured by PCR end-point dilution (Table 1). Table 1 shows that the highest levels of SIV proviral burden, ranging from 1 out of 117 to 1 out of 937 cells infected, were found in control animals 848, 849, 862 and 215 and in vaccinated animals 807 and 810. The remaining macaques showed at least a reduction to 1/10 (macaques 808, 633 and 812) and at most a reduction to 1/40 (macaque 640) in proviral load as compared with the controls. No proviral DNA was detected in macaque 647. These data show that proviral burdens were lowest in animals where viraemia was significantly reduced by week 4 (633, 640, 808 and 812, see Fig. 3). In HIV-2-infected humans, proviral loads show an inverse correlation with CTL activity[20]; low HIV-1 proviral levels are associated with non-progression[18,19]. Thus, the low proviral loads observed in vaccinated animals, 633, 640, 808 and 812, may be important predictors of long-term survival; this will be explored in longer-term experiments.

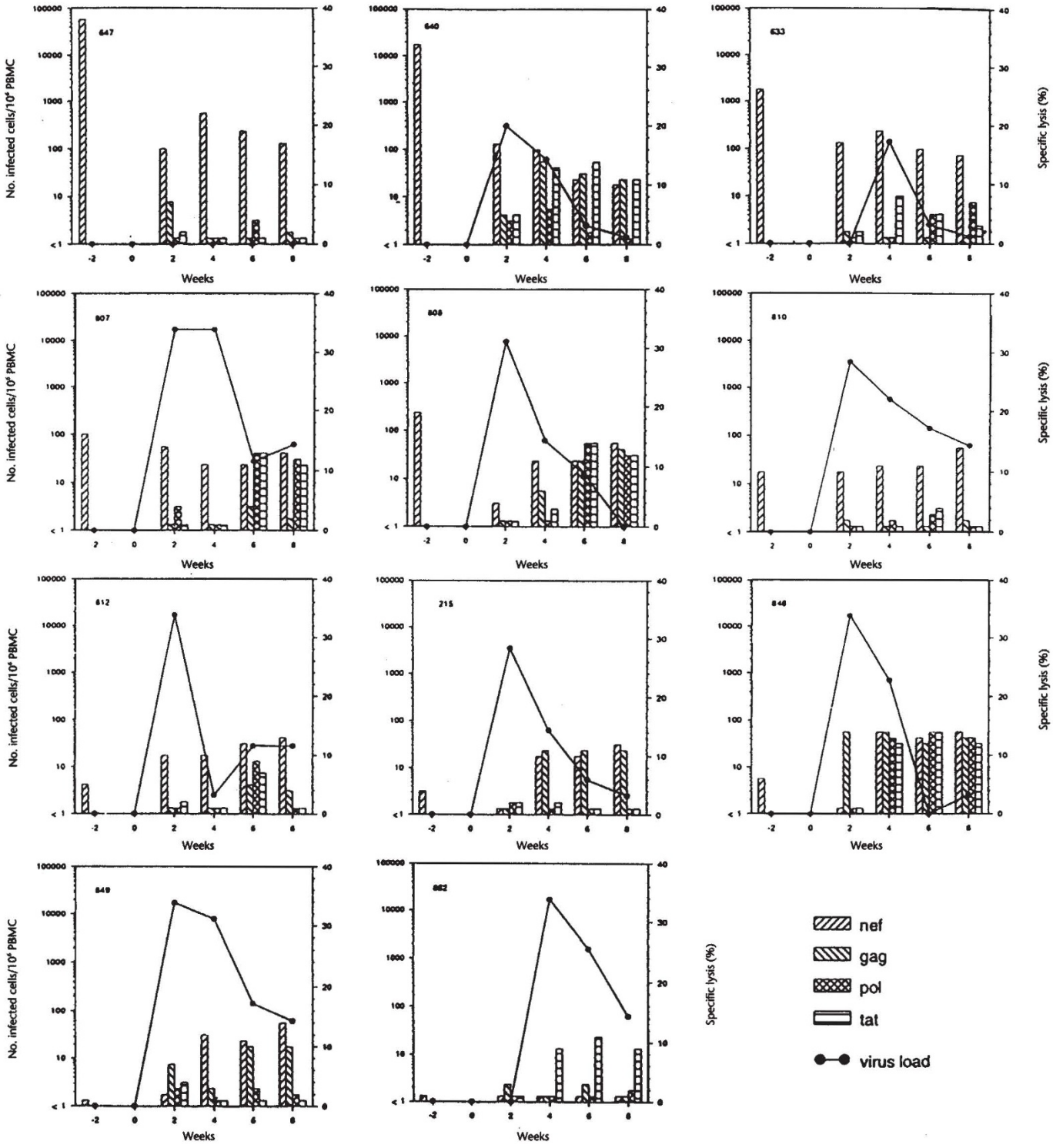

Fig. 4 Decreasing viraemia is associated with virus-specific CTL activity. CTL responses to SIV gag, nef, tat and pol antigens were analysed at 2, 4, 6 and 8 weeks after virus challenge. Cytotoxicity, shown in the histograms, was measured from bulk cultures as described in Methods. CTLs from these cultures were measured for virus-specific CTL activity at an effector:target ratio of 30:1 using autologous B cell lines, which were infected with the appropriate recombinant vaccinia virus. Background ^{51}Cr release was below 20%. The percent lysis of targets infected by rVV-fluNP (vaccinia–influenza A nucleoprotein) was subtracted from the values for targets infected by rVV-nef, -gag , -pol and -tat. Lysis values below 10% may be nonspecific.

Discussion

This report provides direct evidence in support of a role for specific CTLs in containing primary viraemia following SIV infection of macaques. In unimmunized animals, and animals unprotected or only partially protected by the vaccine, CTLs greatly reduce early viraemia without eliminating virus. In all an-

imals early virus load had greatly declined before neutralizing antibody was detectable. This is analogous to the few, but extremely important, reports that reduction of early viraemia in humans occurs when the CTL response appears, before detectable neutralizing antibody[4,21]. Peak and total virus loads were substantially reduced in vaccinated animals with strong CTL responses and detectable IFN-γ production in the CTL cultures. In the animal with the strongest CTL response, virus was not detectable at any time. These effects could not be attributed to neutralizing antibody. Although $CD4^+$ T-helper (Th1) cells as well as $CD8^+$ T cells produce IFN-γ, the *in vitro* experiments, on reduction of challenge virus replication, imply that the $CD8^+$ T cells are themselves responsible. Taken as a whole the data strongly implicate $CD8^+$ T cells in the protective effect observed, although it is not clear whether the $CD8^+$ T cells protect by killing infected cells and/or by releasing cytokines such as IFN-γ (refs 5, 8, 22).

Table 1 Assessment of proviral DNA burden in PBMC by PCR end-point dilution

Animal	Vaccine	PCR end-point	DNA concentration at PCR end-point (ng)	Reciprocal frequency of infected cells
633	J5NEF	1/32	50	7,500
647	J5NEF	<1/2	>800	>120,000
812	J5NEF	1/32	50	7,500
640	C8NEF	1/8	200	30,000
807	C8NEF	1/512	6.25	937
808	C8NEF	1/8	50	7,500
810	C8NEF	1/128	3.1	465
862	None	1/512	3.1	465
849	None	1/512	4.6	690
848	None	1/512	0.78	117
215	None	1/512	1.6	240

These results imply that the level of CTL precursors required for protection must be at least as high as 1 in 10,000 PBMC, a level comparable to that found in HIV infected humans[23] and Δ-nef SIV-infected animals (data not shown), but not reliably achieved by the recombinant vaccinia virus used here. This may explain why a clear association between vaccine-induced CTLs and low virus load after SIV challenge has not been reported before (for example, ref. 24). We emphasize here the importance of measuring both CTL levels induced and virus loads at frequent intervals after challenge. It is apparent from this study that identically immunized animals make quite different CTL responses, some not responding at all, despite previous observations by other groups that the nef protein is highly immunogenic in both macaques and humans[25–28]. This may in large part be due to the different MHC types in these animals, selecting different immunodominant epitopes. We have discussed elsewhere[29] how anti-viral CTL responses tend to concentrate on a few immunodominant epitopes, if these happen to be in the large vaccinia component of the vaccine, there may be no anti-nef response at all. This could explain why one animal failed to respond to nef after nef-vaccinia challenge but did so after SIV challenge.

The implication of this study is that vaccines should be designed to generate high levels of virus-specific CTLs before SIV or HIV exposure. Specific CTLs have the potential to reduce early virus load and may even protect against the establishment of infection, probably by clearing early infection because CTLs cannot neutralize infecting virus. Although there are no human/HIV data to compare, there are recent reports of individuals highly exposed to HIV who make virus-specific CTL responses in the absence of antibody and who appear to be protected[9,30]. These findings would be consistent with the ability of specific CTLs to clear virus as implied here.

It remains to be determined whether the CTL-mediated protection observed here is unique to nef. Although there has been no previous indication that an anti-nef CTL response is more advantageous, the protein may be a particularly good candidate for protective CTLs, because it is expressed early in the SIV/HIV infectious cycle. It is quite likely that any strong preinduced CTL response could have the beneficial effect observed here on the early reduction in virus load. It is not known yet whether substantial reductions in early virus loads will have long-term benefits; this needs testing.

We are aware that the numbers of animals used here is small, as is inevitable in all macaque SIV-vaccine experiments. Therefore we must be cautious about the implication for HIV vaccines, but point out that the nature of protective immunity after SIV/HIV vaccination may only be defined in this way, by careful quantitation of both the immune response and the virus load after challenge.

In these experiments, the nef sequence of the challenge virus was either identical to (rVV-J5nef), or only four amino acids different from (rVV-C8nef), that of the vaccine. Nevertheless CTLs will often crossreact between strains because of shared stretches of conserved amino acid sequence. If the principle of vaccine-induced CTL protection can be confirmed, it should be relatively easy to generate vaccines that focus the response on conserved epitopes so that immunity can be achieved against different virus strains. This is especially important in the HIV context.

Methods

Immunization. Seven cynomolgus macaques (macaques 633, 640, 647, 807, 808, 810, 812) were immunized with either recombinant vaccinia virus (rVV) expressing the nef protein derived from SIVmacJ5 or with rVV expressing the SIVmacC8-derived nef (E. Rud and M. Mackett, unpublished observations). The rVV-nef constructs were regulated by the VV 7.5K promoter. All animals were immunized by scarification on the mid-line of the back three times at five-week intervals with 5×10^8 PFU of rVV-nef. Care of non-human primates was in accordance with UK Home Office Guidelines. Animals were anaesthetized with a single dose of ketamine at 10 mg per kg body weight given intramuscularly before vaccination or bleeding.

Measurement of CTL activity. Two weeks before virus challenge and four weeks after the third vaccination, MHC class I-restricted *nef*-specific $CD8^+$ CTLs were measured in bulk cultures and by limiting dilution, to determine precursor frequencies of specific CTLs, in each immunized animal.

The restimulation protocol of 'bulk'-cultured CTLs was a modification of methods previously described[31]. Briefly, PBMC were isolated on Ficoll-Hypaque and one-tenth of the cells were stimulated for 24 hours with PHA, infected with 100 μl SIVmacC8 supernatant, washed and then added back to the remaining cells. Cells were cultured in RPMI 1640 (Gibco) supplemented with 10% human serum (blood transfusion centre, John Radcliffe Hospital, Oxford, UK) and penicillin/streptomycin. Cultures were supplemented with 10 U ml^{-1}

IL-2 from day 3. At days 10–14, CTLs in these cultures were tested for *nef*-specific cytotoxicity at an effector/target (E/T) ratio of 30:1 with autologous B cell lines, which were labelled with chromium-51 and infected with either rVV-J5nef or an irrelevant rVV-influenza A nucleoprotein (rVV-fluNP) described previously[32]. In order to determine maximum and spontaneous release, target cells were incubated in quadruplicate with 5% Triton X-100 or media, respectively. Following a 5-hour incubation at 37 °C, specific lysis was calculated as described previously[31]. Background chromium release was below 20%. Percentage lysis of rVV-fluNP-infected target cells, which was always >10%, was subtracted from percentage lysis of rVV-J5*nef*-infected targets.

CTL responses to the SIV gag, nef, tat and pol antigens were analysed at weeks 2, 4, 6 and 8 after virus challenge. Cytotoxicity was measured from 'bulk' cultures as described above. Vaccinia recombinants expressing SIV *gag*, *tat*, *pol* and *env* were obtained from the Medical Research Council AIDS Directed Programme and used to prepare target cells as above.

Limiting dilution assays. *Nef*-specific CTL precursor frequencies were measured by limiting dilution assays (LDA) as previously described[23]. Effector cells were distributed at varying numbers from 5,000 to 20,000 cells per well in round-bottomed microtitre plates. Twenty-seven replicate wells were set up at each effector cell number. Autologous PHA-stimulated, SIVmacC8-infected and irradiated (3000 rad) PBMCs (5×10^3) were added to each well. Irradiated PBMCs, cultured in triplicate at each input cell number with the stimulator cells, were used as feeder controls. Microcultures were fed at days 3 and 10 by the addition of 50 µl R10 supplemented with 10 U ml^{-1} of IL-2. After 14 days of culture, the effector cells were mixed in each individual well, and equal volumes were transferred to wells in two new 96-well plates. The effectors were assayed for cytotoxic activity on 10^4 ^{51}Cr-labelled, rVV-J5nef- and rVV-fluNP-infected autologous B-cell lines. Specific lysis was determined as described above and values >10% lysis were considered positive. Standard limiting dilution plots were produced by comparing the percentage of negative wells for a target at each input cell number, with the number of PBMCs initially cultured. The best straight line was determined by the method of least squares[33], with linear regression analysis[34]. *Nef*-specific CTL precursor frequencies were estimated as the input cell number that would produce 37% negative cultures against rVV-J5*nef*-infected target cells. Statistical analysis was performed using GLIM (ref. 33). The correlation coefficient estimate (r) between LDA and bulk assay was calculated as 0.84. This value is larger than the true correlation coefficient ($P = 0.754$) and thus is significant at the 5% level. We calculated 95% confidence intervals and if these are included the inverse relationship between CTL precursors and viral load was still statistically significant (data not shown).

Measurement of interferon-γ. IFN-γ was measured in 24-hour 'bulk' culture supernatants prepared as above at a concentration of 10^6 PBMCs ml^{-1}. Measurements were made by ELISA according to the manufacturer's instructions (AMS Biotechnology Ltd). Cytokine levels were quantitated by reference to standard curves of known amounts of rIFN-γ (Boehringer Mannheim).

Inhibition of SIV replication *in vitro*. (Percentage suppression of virus replication by $CD8^+$ cells in SIVmacJ5-infected unfractionated PBMC). $CD8^+$ cells were depleted from 3×10^6 PBLs using CD8-conjugated dynabeads (Dynal). PBMC with and without $CD8^+$ cells were pelleted and SIV-infected by incubation with 25 µl SIVmacJ5 supernatant for 1 h at 37 °C. The cells were washed twice and resuspended in RPMI 1640, supplemented with 10% fetal calf serum (Gibco) and antibiotics, at a concentration of 10^6 ml^{-1} and plated at 10^5 $CD4^+$ cells per well of flat-bottomed 96-well microtitre plates. Cells were harvested by centrifugation from two wells of each culture at days 3, 6, 9, 12 and 15. Cells were lysed in 0.5% NP-40 and lysates were assessed in duplicate for *p27-gag* expression in a p27 antigen capture ELISA as described previously[35] using antibodies obtained from Karen Kent (NIBSC). SIV p27 antigen concentration was measured for 15 days at 3-day intervals.

SIV challenge. Animals were challenged intravenously with 50 MID_{50} of cell-free supernatant of SIVmacJ5 grown in simian PBMCs. The SIVmacJ5 molecular clone, with which each animal was challenged, was derived from uncloned SIVmac32H and has been shown to induce AIDS in infected macaques (G. Hall, pers. commun.). The *in vivo* intravenous titre was determined by titration in cynomolgus macaques[13].

SIV load. From each challenged animal, PBMCs were separated from whole blood by centrifugation of Ficoll-Hypaque (Pharmacia). Separated cells were diluted from 10^6 to 4×10^5 and subsequently in 5-fold steps to 130 cells, and duplicate cultures were cocultivated with the human T-cell line, C8166 in 25 cm^2 flasks. Medium and C8166 were replenished every 3 to 4 days, and the total culture volume was maintained at approximately 15 ml. All cultures were maintained for 30 days or until cytopathic effect was apparent. Virus isolation was confirmed by indirect immunofluoresence using simian polyclonal anti-SIV serum. Fifty percent end points were calculated using the Karber formula, and the results were expressed as the number of infected cells per 10^6 PBMCs.

Proviral loads. DNA was extracted from 1 ml whole blood[35] 8 weeks after challenge. Serial dilutions of DNA were assayed by nested PCR (40 + 40 cycles) using primers complementary to a region of the *env* gene of SIVmac (SE 6656N + SE776C followed by SE7054N + SE7695) in a 50-µl reaction volume[36] containing 1.0 mM $MgCl_2$ and 0.25 mM of each primer. A portion (5 µl) of product from the first-round PCR was used as a template for the second-round PCR. Post-PCR material (10 µl) was visualized by ethidium bromide staining following 1.75% agarose gel electrophoresis. PCR products were confirmed by Southern blot hybridization[37] by using either α-^{32}P-labelled[38] or dioxygenin-labelled[39] probes. The most dilute sample of DNA giving a positive signal was assumed to contain 1 copy of SIV genome: this was designated the PCR end point. The total DNA concentration of each sample was measured using a fluorescence assay[37] and the DNA concentration at the PCR end point calculated. Based on the assumption that 1 µg DNA = 150,000 cells, the frequency of infected PBMCs in each macaque was determined. All coprocessed extraction controls and PCR H_2O controls were negative as confirmed by hybridization.

A set of SIV standards containing 1.10 and 100 copies of the molecular clone BK28 linearized using *Nde*I and diluted in 100 ng of carrier DNA prepared from PBMC from an uninfected cynomolgus macaque was used to assess inter-assay variability between PCR runs. These standards were prepared and validated in the manner described for equivalent HIV.1 DNA standards used in international collaborative studies of PCR (refs 36, 40). The results of each individual assay were accepted only if these standards produced the expected results. Using the methods described in this paper, the 1 molecule standard yields a positive result in 2 out of 3 assays, as would be predicted by a simple probability model.

The PCR was designed to minimize problems in the efficiency of amplification. Each round of 40 cycles should, in theory and in practice using the standards, be sufficient to amplify a single copy of template DNA to detectable levels. The combination of nested PCR primers and two rounds of 40 cycles means that considerable redundancy is built into the assay, which would allow for the inefficiency

of having relatively high amounts of total DNA in the tube. Furthermore, for each sample a dilution series of the DNA was prepared from 1/2 to 1/1084, and each of the sample dilutions was tested by PCR to ensure that any negatives obtained at low dilutions were not due to the presence of excess DNA. Furthermore, in our hands, we have found that the method of DNA extraction from the blood (Proteinase K/SDS digestion followed by phenol and chloroform extraction and ethanol precipitation) does not result in the carryover of inhibitory molecules through to the PCR.

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1. Bangham, C.R.M. & McMichael, A.J. in *T Cells* (eds Feldmann, M., Lamb, J. & Owen, M.J.) 281310 (Wiley, New York, 1989).
2. Lukacher, A.E., Braciale, V.L. & Braciale, T.J. In vivo effector function of influenza virus-specific cytotoxic T lymphocyte clones is highly specific. *J. exp. Med.* **160**, 814–826 (1984).
3. McMichael, A.J. & Walker, B. Cytotoxic T lymphocyte epitopes: Implications for HIV vaccines. *AIDS (suppl.)* **8**, S155-S173 (1994).
4. Koup, R. *et al.* Temporal association of cellular immune responses with the initial control of viraemia in primary human immunodeficiency virus type 1 syndrome. *J. Virol.* **68**, 46504655 (1994).
5. Walker, C.M., Moody, D.J., Stites, D.P. & Levy, J.A. CD8$^+$ lymphocytes can control HIV infection in vitro by suppressing virus replication. *Science* **234**, 1563–1566 (1986).
6. Tsubota, H.C., Lord, C.I., Watkins, D.I., Morimoto, C. & Letvin, N.L. A cytotoxic T lymphocyte inhibits acquired immunodeficiency syndrome virus replication in peripheral blood lymphocytes. *J. exp. Med.* **169**, 1421–1434 (1989).
7. Mackewicz, C.E., Ortega, H.W. & Levy, J.A. CD8$^+$ cell anti-HIV activity correlates with the clinical state of the infected individual. *J. clin. Invest.* **87**, 1462–1466 (1991).
8. Kannagi, M., Chalifoux, L.V., Lord, C.I. & Letvin, N.L. Suppression of simian immunodeficiency virus replication in vitro by CD8$^+$ lymphocytes. *J. Immun.* **140**, 2237–2242 (1988).
9. Rowland-Jones, S. *et al.* HIV-specific cytotoxic T-cells in HIV-exposed but uninfected Gambian women. *Nature Med.* **1**, 59–64 (1995).
10. Chakrabarti, L. *et al.* Sequence of simian immunodeficiency virus from macaque and its relationship to other human and simian retroviruses. *Nature* **328**, 543–547 (1987).
11. Letvin, N. *et al.* Induction of AIDS-like disease in macaque monkeys with T-cell tropic retrovirus STLV-III. *Science* **230**, 71–73 (1985).
12. Kestler, H. *et al.* Importance of the nef gene for maintenance of high virus loads and for development of AIDS. *Cell* **65**, 651–662 (1991).
13. Rud, E. *et al.* Molecular and biological characterization of simian immunodeficiency virus macaque strain 32H proviral clones containing nef size variants. *J. Gen. Virol.* **75**, 529–543 (1994).
14. Rud, E. *et al.* Infectious molecular clones of SIVmac32H: Nef deletion controls ability to re-isolate virus from rhesus macaques. *Vaccines 92: Modern Approaches to New Vaccines including AIDS* (eds F.Brown, R.M.Chanock, H.S.Ginsberg, and R.A.Lerner) 239–245 (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1992).
15. Daniel, M.D., Kirchhoff, F., Czajak, S.C., Sehgal, P.K. & Desrosiers, R.C. Protective effects of a live attenuated SIV vaccine with a deletion in the *nef* gene. *Science* **258**, 1938–1941 (1992).
16. Robert-Guroff, M. *et al.* Structure and expression of tat-, rev-, and nef-specific transcripts of human immunodeficiency virus type 1 in infected lymphocytes and macrophages. *J. Virol.* **64**, 3391–3398 (1990).
17. Bachmann, M.-F. *et al.* Induction of protective cytotoxic T cells with viral proteins. *Eur. J. Immun.* **24**, 2228–2236 (1994).
18. Cao, Y., Qin, L., Zhang, L., Safrit, J. & Ho, D. Virologic and immunologic characterisation of long-term survivors of human immunodeficiency virus type 1 infection. *New Engl. J. Med.* **332**, 201–208 (1995).
19. Pantaleo, G. *et al.* Studies in subjects with Long-term nonprogressive human immunodeficiency virus infection. *New Eng. J. Med.* **332**, 209–216 (1995).
20. Ariyoshi, K. *et al.* HIV-2-specific cytoxic T-lymphocyte activity is inversely related to proviral load. *AIDS* **9**, 555–559 (1995).
21. Yasutomi, Y., Reimann, K.A., Lord, C.I., Miller, M.D. & Letvin, N.L. Simian immunodeficiency virus-specific CD8$^+$ lymphocyte response in acutely infected rhesus monkeys. *J. Virol.* **67**, 1707–1711 (1993).
22. Brinchmann, J.E., Gaudernack, G. & Vartdal, F. CD8+ T cells inhibit HIV replication in naturally infected CD4$^+$ T cells: Evidence for a soluble inhibitor. *J. Immun.* **144**, 2961–2966 (1990).
23. Gotch, F.M., Nixon, D.F., Alp, N., McMichael, A.J. & Borysiewicz, L.K. High frequency of memory and effector gag specific cytotoxic T lymphocytes in HIV seropositive individuals. *Int. Immun.* **2**, 707–712 (1990).
24. Abimuku, A.G. *et al.* HIV-1 recombinant poxvirus vaccine induces cross-protection against HIV-2 challenge in rhesus macaques. *Nature Med.* **1**, 321–329 (1995).
25. Culmann, B., Gomard, E. & Kieny, M.-P. Six epitopes reacting with human cytotoxic CD8+ T cells in the central region of the HIV-1 nef protein. *J. Immun.* **146**, 1560-1565 (1991).
26. Culmann-Penciolelli, B. *et al.* Identification of multirestricted immunodominant regions recognised by cytolytic T lymphocytes in the human immunodeficiency virus type 1 nef protein. *J. Virol.* **68**, 7336–7343 (1994).
27. Bourgault, I. *et al.* Simian immunodeficiency virus as a model for vaccination against HIV. *J. Immun.* **152**, 2530–2537 (1994).
28. Lamhamedi-Cherradi, S.C. *et al.* Qualitative and quantitative analysis of human cytotoxic T lymphocyte responses to HIV-1 proteins. *AIDS* **6**, 1249–1258 (1992).
29. Nowak, M.A. *et al.* Antigenic oscillations and shifting immunodominance in HIV-1 infections. *Nature* (in the press).
30. Rowland-Jones, S. & McMichael, A.J. Cell-mediated immune responses in HIV-exposed individuals: Have they cleared the virus? *Curr. Topics Immun.* (in the press).
31. Gotch, F., Hovell, R., Delchambre, M., Silvera, P. & McMichael, M. Cytotoxic T-cell responses to simian immunodeficiency virus by cynomolgus macaque monkeys immunized with recombinant vaccinia virus. *AIDS* **5**, 317–320 (1991).
32. Townsend, A. *et al.* Defective presentation to class 1 restricted cytotoxic T lymphocytes in vaccinia infected cells is overcome by enhanced degradation of antigen. *J. exp. Med.* **168**, 1211–1224 (1988).
33. Norusis, M.J. SPSS/PC+ V2.0 (SPSS Inc., Chicago, Illinois, 1988).
34. Lefkovits, I. & Waldmann, H. Limiting dilution analysis of cells in the immune system (Cambridge University Press, Cambridge, 1979).
35. Rose, J., Silvera, P., Flanagan, B., Kitchin, P. & Almond, N. The development of PCR-based assays for the detection and differentiation of simian immunodeficiency virus in vivo. *J. Virol. Methods* **51**, 229–239 (1995).
36. Bootman, J. & Kitchin, P. An international collaborative study to assess a set of reference reagents for HIV-1 PCR. *J. Virol. Methods* **37**, 23–42 (1992).
37. Sambrook, J., Fritsch, E. & Maniatis, T. *Molecular Cloning: A Laboratory Manual*, 2nd edn (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989).
38. Saluz, H. & Jost, J. A simple high-resolution procedure to study DNA methylation and in vivo DNA-protein interactions on a single copy gene level in higher eukaryotes. *Proc. natn. Acad. Sci. U.S.A.* **86**, 2602–2626 (1989).
39. An, S., Franklin, D. & Fleming, K. Generation of dioxygenin-labelled double-stranded probes using the polymerase chain reaction. *Molec. Cell Probes* **6**, 193-200 (1992).
40. Bootman, J.S. & Kitchen, P.A. Reference preparations in the standardisation of HIV.1 PCR: An international collaborative study. *J. Virol. Methods* **49**, 1–8 (1994).