Cytotoxic T lymphocytes in AIDS pathogenesis: lessons to be learned from the macaque model of simian immunodeficiency virus infection

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Introduction

Increasing evidence indicates a protective role for cytotoxic T lymphocytes (CTL) in the host defence against human immunodeficiency virus (HIV). CTL reactive against HIV antigens have been detected in persons exposed to the virus but lacking evidence of infection. These include a small number of female prostitutes in Africa, sexual partners of infected persons, children born to infected mothers and health care workers exposed to infectious body fluids (reviewed by Rowland-Jones & McMichael, 1995). Based upon the assumption that CTL induction requires endogenous synthesis of viral proteins, these findings suggest that transient HIV infection and virus clearance by CTL are indeed possible. During acute HIV infection, the detection in circulation of specific CTL coincides with the fall in viraemia that follows the initial virus burst (Borrow *et al.*, 1994; Koup *et al.*, 1994). In the subsequent stages, persistence of antiviral CTL may be associated with a prolonged asymptomatic period, whereas their decline is usually coincident with disease progression (Klein *et al.*, 1995). Nevertheless, a few patients with advanced disease may maintain relatively strong CTL responses against the core HIV Gag protein (Geretti *et al.*, 1996). Studies on the relationship between virus load and CTL responses in chronically infected patients have also yielded conflicting results, as both direct (Ferbas *et al.*, 1995) and inverse (Klein *et al.*, 1995) correlations have been reported. While further work is needed to clarify such discrepancies, it is clear that antiviral CTL responses can only be interpreted in the framework of the dynamics of virus replication, recognition and clearance of infected cells, and virus attempts to evade immune surveillance.

Infection of macaques with simian immunodeficiency virus (SIV) offers a valuable model for studying the complex interaction between lentiviruses and the host immune system. The aim of this review is to outline how this model has contributed to our understanding of the role of CTL in the control of lentiviral infections, and how its full potential may be exploited in the future.

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General aspects of SIV infection of macaques

SIV of macaques (SIVmac) was first isolated in 1985 from animals held in captivity in the United States. Macaques, however, are not natural hosts of the virus. It is believed that cross-species transmission from sooty mangabeys, which are healthy natural carriers of SIV (SIVsm), was the source of infection. Fighting and biting among animals co-housed in outdoor corrals was the most likely route of horizontal transmission (Gardner, 1996). SIVmac shares with HIV many biological and structural features, including its tropism for the CD4 receptor and CCR5 co-receptor (Chen *et al.*, 1997), a similar genomic organization and extensive genetic homology. The virus establishes persistent infection in macaques and causes an immunodeficiency syndrome closely resembling human AIDS. As in humans, prominent features of the disease include CD4^{+} cell loss, constitutional symptoms, lymphadenopathy, skin and neurological disorders, opportunistic infections and neoplasias. Although the average time to disease is 1–2 years, the course of infection varies considerably among macaques. Even after infection with the same viral molecular clone, some animals rapidly develop disease and die within months, whereas others may remain asymptomatic for a few years, thus resembling long-term non-progressors with HIV infection. This variability, combined with the opportunity to define parameters of infection such as strain, dose and route of virus inoculation, may prove valuable for clarifying the role of CTL immunity in lentivirus containment.

Detection of SIV-specific CTL

Target cells

SIV-specific CTL responses are currently measured against autologous B lymphoblastoid cell lines (B-LCL) immortalized by herpesvirus papio. After infection with recombinant vaccinia virus vectors (rVV) encoding SIV proteins, these cells express endogenously processed antigen in the context of MHC class I molecules. The use of rVV expressing different regions of the SIV genome has shown that SIV-specific CTL target both structural and regulatory viral proteins, although the strength and magnitude of the response vary among animals (Venet *et al.*, 1992; Geretti *et al.*, 1997 *a*). Alternatively,

B-LCL can be sensitized for lysis by incubation with short synthetic viral peptides that bind directly to MHC class I molecules on the cell surface, thus allowing the definition of epitope specificities.

Effector cells

In vitro restimulation has almost universally been required to amplify SIV-specific CTL responses to detectable levels. As an interesting exception, direct cytotoxicity against the envelope protein (Env), Gag and the regulatory protein Nef has been recently observed with intestinal intraepithelial lymphocytes of two macaques infected with SIVmac$_{251}$ (Coudel-Courteille *et al.*, 1997). One of the animals showed signs of advanced disease, including a marked colitis. Although a high proportion of intestinal intraepithelial lymphocytes express the CD8 marker, the exact nature and function of the activated intestinal effector cells, and their contribution to host defence or immunopathogenesis, remain to be determined. The same authors found little or no evidence of direct cytotoxicity in peripheral blood, spleen and lymph nodes. This is in line with the observation that the frequencies of circulating CTL precursors (CTLp) detected in SIV-infected or vaccinated macaques are generally lower than those often measured in asymptomatic HIV-infected adults (Geretti *et al.*, 1997 *a*). While the requirement for appropriate methods of CTLp restimulation may explain the apparent lack of CTL induction in some vaccine studies, it also highlights the need for standardized assays which would facilitate comparison of different experiments, often including only small numbers of animals.

One successful method for the expansion *in vitro* of HIV- or SIV-specific CTLp is based on stimulation with paraformaldehyde-fixed autologous B-LCL infected with rVV expressing HIV or SIV antigens (Van Baalen *et al.*, 1993). Compared with non-specific methods of stimulation, this approach enhances CTL detection in macaques by inducing selective CTL expansion, with reduced interference from background lysis (Geretti *et al.*, 1997 *a*). Cell culture under limiting dilution conditions appears to increase further the sensitivity of CTL measurement, as it allows CTLp detection, albeit at low frequencies, in macaques lacking significant responses in standard bulk CTL assays (Geretti *et al.*, 1997 *a*). Autologous blasts infected with SIV have also been used successfully as stimulator cells (Gallimore *et al.*, 1995). Alternatively, synthetic peptides spanning defined regions of the SIV genome can be used to stimulate the growth of epitope-specific CTL.

Should vaccine-induced CTL prevent or limit virus replication?

Early vaccine studies in macaques aimed primarily at preventing infection through the induction of virus-neutralizing antibodies. As a result, a protective role of CTL in some of the successful vaccination strategies reported, such as those based upon live attenuated SIV vaccines (reviewed by Ruprecht *et al.*, 1996), can only be hypothesized. More recently,

the prevailing view that it may be desirable for a vaccine to prevent disease if not infection, and the appreciation that SIV infection of macaques provides a valuable model for studying HIV pathogenesis, coupled with the development of reliable means of detecting SIV-specific CTL, have drawn considerable attention to antiviral cell-mediated immunity.

SIV Env-, Gag- or Nef-specific CTL have been induced in macaques through several immunization strategies, including live attenuated viruses (Cranage *et al.*, 1997), proteins either expressed by live vectors such as rVV (Gallimore *et al.*, 1995; Kent *et al.*, 1996), incorporated into iscoms (Hulskotte *et al.*, 1995) or adjuvated by QS-21 saponin (Newman *et al.*, 1994), non-infectious virus-like particles (Klavinskis *et al.*, 1997), peptides in various formulations (Bourgault *et al.*, 1994; Hulskotte *et al.*, 1995; Yasutomi *et al.*, 1995) and DNA plasmids (Lu *et al.*, 1996). In those studies that have tested the outcome of subsequent challenge, the detection of vaccine-induced CTL has usually failed to predict complete resistance to infection. In one early report, vaccine-induced CTL against pC11, a well-defined epitope in Gag, failed to protect macaques from intravenous challenge with SIV *Macaca nemestrina* (SIVmne) (Yasutomi *et al.*, 1995). The induction of relatively low frequencies of CTLp of limited specificity, and the absence of antibodies, were believed to have contributed to the lack of protection. However, in a subsequent report, immunization with Env- and Gag-iscoms and three lipopeptides spanning the central region of Nef induced both virus neutralizing antibodies and Env-, Gag or Nef-specific CTLp, but similarly failed to protect macaques from intravenous challenge with SIVmac$_{32H\text{-}J5}$ (Hulskotte *et al.*, 1995). Furthermore, no correlation was found between the frequencies of SIV-specific CTLp detected before challenge and the levels of cell-associated virus load measured after infection. It is of note, however, that CTLp frequencies were in most cases below $26/10^6$ peripheral blood mononuclear cells (PBMC). Nevertheless, even the animal with a relatively high frequency of Env-specific CTLp ($105/10^6$ PBMC) was not protected from infection. Recently, intravenous, intramuscular and gene gun inoculations of SIV DNA plasmids have produced similar results: despite the induction of virus neutralizing antibodies and Env-specific CTL, no protection was induced against intravenous challenge with SIVmac$_{251}$ (Lu *et al.*, 1996).

Although disappointing, these findings are consistent with the view that CTL may not be able to prevent or control SIV infection, unless stringent qualitative and quantitative requirements are met. This finds indirect support in the observation that macaques immunized with whole inactivated SIVmac$_{32H}$, and protected from intravenous challenge with cell-associated virus, share the Mamu-A26 MHC class I allele with the donor of the infected cells (Osterhaus *et al.*, 1992; Heeney *et al.*, 1994). Mamu-A26 positive animals also behave as long-term survivors (Bontrop *et al.*, 1996), suggesting that gene products of this allele may be correlated with the ability to mount protective CTL responses against SIV. More direct evidence of

a protective role of defined CTL subpopulations is provided by a study of macaques immunized with SIV Nef rVV (Gallimore *et al.*, 1995). Although six of seven animals became infected upon intravenous challenge with SIVmac$_{32H-J5}$, the frequencies of vaccine-induced Nef-specific CTLp measured before challenge were inversely correlated with the levels of virus load measured after infection. In addition, the animal with the highest CTLp frequency was protected from infection. As these promising findings indicate, the protective role of CTL responses targeting regulatory proteins expressed early during the virus replication cycle deserves further investigation.

Recent findings appear to support the concept that protective CTL may limit rather than completely prevent virus replication. Vaccinated macaques lacking both detectable virus and antibody responses after either intravenous or intrarectal challenge with SIVmne showed CTL responses against SIV proteins present in the challenge virus but not in the vaccine (Kent *et al.*, 1996). Similarly, macaques immunized with Env-iscoms lacked both detectable virus and anamnestic antibody responses after intravenous challenge with a chimeric simian–human immunodeficiency virus (SHIV), but had CTLp against antigens other than Env, including the non-virion regulatory proteins Rev and Tat (E. G. J. Hulskotte, unpublished). These data indicate that CTL responses provide a highly sensitive marker of transient or low-level virus replication. It is tempting to speculate that similar silent or abortive infections may have remained undetected in some of the early vaccine studies.

What is the role of antiviral CTL in mucosal immunity?

As most HIV infections are acquired by mucosal routes, current efforts are directed at inducing immune responses that may prevent or limit virus spread after mucosal exposure. The SIV model offers the opportunity to explore issues related to mucosal immunity and infection that are difficult to address in humans. Indeed, evidence that protection from sexually transmitted SIV and, by inference, HIV may be induced by effectively stimulating genital and systemic antiviral CTL is increasing. In female macaques inoculated intravaginally with SIVmac$_{251}$, at least some (1 in 2425 to 1 in 26 686) of the CD8$^+$ cells recovered from the vaginal epithelium are SIV-specific CTLp directed against Env or Gag. In line with the view that progressive CTL compartmentalization occurs during persistent infection, the frequencies of genital CTLp are higher in chronically infected monkeys than in animals with recent infection (Lohman *et al.*, 1995). SIV-specific CTL have also been detected in both peripheral blood and gut-associated lymph nodes of macaques infected intravenously with live attenuated SIVmac$_{C8}$, and resistant to intrarectal challenge with either SIVmac$_{32H-J5}$ or SHIV (Cranage *et al.*, 1997). Furthermore, inoculation of macaques with SIV p27:Ty virus-like particles by either the rectal-oral and vagino-oral route, or subcutaneous immunization targeting the iliac lymph nodes, has induced specific CTL in the rectal and cervico-vaginal mucosa, as well as in regional lymph nodes, spleen and peripheral blood (Klavinskis *et al.*, 1997). Finally, intra-vaginal inoculations with attenuated SHIV have induced at least partial resistance to intravaginal challenge with pathogenic SIVmac$_{239}$ (Miller *et al.*, 1997). Although the presence of SIV-specific CTL in the genital tract was not determined in this study, protected animals had circulating Gag-specific CTL at the time of challenge, with or without specific antibodies in genital secretions. To explain the generation of local and systemic primary immune responses upon vaccine delivery to mucosal sites, antigen uptake by resident dendritic cells is hypothesized, followed by their migration to regional lymph nodes where naive T lymphocytes are stimulated. These would then enter the circulation and migrate to the genital site, where they may be restimulated upon re-exposure to the antigen, thus providing a first line of defence against sexually transmitted viruses.

What is the relationship between SIV-specific CTL and virus replication?

Kinetics studies have shown that the detection of Gag- or Nef-specific CTL by week 1 or 2 after intravenous SIV infection is coincident with the decrease in virus load and p26 antigenaemia that follows the initial virus burst (reviewed by Letvin *et al.*, 1994). In the early phase of infection, virus load and SIV-specific CTL responses show similar kinetics in peripheral blood and lymph nodes. In contrast, during chronic infection, virus sequestration within lymphoid organs (Chakrabarti *et al.*, 1994) is mirrored by CTL compartmentalization to the sites of infection (A. M. Geretti, unpublished). These observations are consistent with CTL being the effector cells of the *in vivo* immune response against SIV. Further support for a protective role of antiviral CTL comes from the observation that macaques with strong CTL responses against multiple SIV antigens, including the regulatory proteins Nef, Rev and Tat, remained free of disease for at least 2 years after intravenous infection with SIVmac$_{251}$. In contrast either absent, transient or weak CTL responses were observed in animals rapidly progressing to overt disease (Venet *et al.*, 1992). Preliminary findings also indicate that the detection of CTL responses against Rev and Tat in the early phase of infection may be associated with effective virus containment and long-term survival in both HIV-infected humans (Van Baalen *et al.*, 1997) and SIV-infected macaques (A. M. Geretti, unpublished). These data are again in agreement with the hypothesis that CTL targeting early gene products may be effective in virus control before release of progeny virus occurs. Another point of interest is that the same CTL may also recognize latently infected cells that, although not actively producing virus, are known to express multiple spliced viral messenger RNAs encoding the regulatory proteins Nef, Rev and Tat (Seshamma *et al.*, 1992; Embretson *et al.*, 1993).
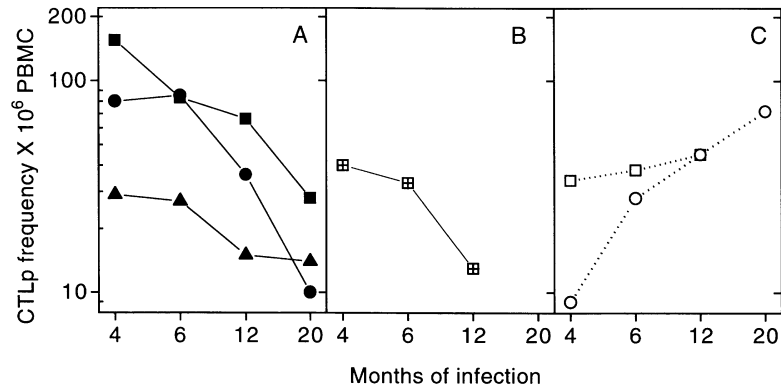
Fig. 1. Kinetics of circulating SIV-specific CTL precursors (CTLp) in macaques infected intravenously with SIVmac$_{32H-J5}$. After the initial burst of virus replication, three monkeys (A) lost evidence of either culturable virus or PCR-detectable provirus in peripheral blood, while maintaining low-level virus reservoirs in spleen and lymph nodes; these animals remained asymptomatic with stable CD4$^+$ cell counts throughout 22 months of observation. Three other monkeys had persistent virus in peripheral blood and high virus load in lymphoid organs: one (B) developed AIDS 18 months after infection, whereas two others (C) remained asymptomatic but over time showed a significant CD4$^+$ cell decline. CTLp frequencies were measured by limiting dilution assays. Autologous B-LCL infected with rVV expressing SIV antigens were used *in vitro* as stimulator and target cells. The cumulative frequencies of circulating SIV-specific CTLp, after reaching a plateau, declined significantly in the three macaques lacking detectable virus in peripheral blood (A) and in the animal which progressed to overt disease (B), but were maintained in the two asymptomatic macaques with persistent infection in peripheral blood (C).

The relatively low frequencies of SIV-specific CTLp usually detected in SIV-infected macaques seem in contrast with the strong CTL responses often detected in asymptomatic HIV-infected persons. Besides the influence of host genetic factors, or the effects of progressive immunodeficiency, relatively low CTLp frequencies may reflect a low degree of antigenic stimulation *in vivo*, due to rapid down-regulation of SIV replication after infection. Consistent with this view, high CTLp frequencies can be detected occasionally in animals with a high virus load in their PBMC (Geretti *et al.*, 1997a). Conversely, the frequencies of circulating SIV-specific CTLp may decline significantly over time in asymptomatic SIV-infected macaques lacking detectable virus in PBMC and showing low-level virus reservoirs in lymphoid organs (Fig. 1; A. M. Geretti, unpublished). In view of these findings, it is not surprising that the degree of protection conferred by live attenuated SIV vaccines, and the strength of the Env- and Gag-specific CTL responses they induce, appear to be inversely correlated with the level of virus attenuation (Lohman *et al.*, 1994). These data have direct implications for vaccine designs aimed at inducing long-lasting protective CTL responses: a successful vaccine will need to achieve a difficult balance between safety, which is dependent upon a high degree of attenuation, and efficacy, which appears directly related to the ability of the virus to replicate.

Are virus-specific CTL deleterious to the host?

It has been suggested that in a physiological attempt to eradicate persistent infection, CTL may also induce pathological changes deleterious to the host. This is supported by the finding that HIV-specific CTL are present in both the bronchoalveolar lavage of AIDS patients with lymphocytic alveolitis and in the cerebrospinal fluid (CSF) of patients with AIDS dementia complex (reviewed by Zinkernagel, 1995). The detection of SIV-specific CTL in the skin rash of SIV-infected macaques (Yamamoto *et al.*, 1992) also suggests a role for CTL in mediating tissue damage. SIV-specific CTL have been also detected in the CSF and brain of SIV-infected macaques as early as 1 week after infection and concomitant with the detection of virus (Von Herrath *et al.*, 1995). Interestingly, different SIV proteins were targeted by CTL recovered from the brain, CSF and peripheral blood, suggesting that these can be separate compartments in SIV infection. As these interesting data indicate, the SIV model provides an excellent system to explore further the role of CTL in HIV-associated neurological disorders.

Can SIV variants escape CTL recognition?

Selection of mutant viruses resistant to specific CTL may be proposed as one mechanism whereby highly variable viruses such as HIV or SIV escape from immune recognition. Single amino acid substitutions within CTL epitopes can abrogate recognition by affecting either MHC binding or T cell receptor (TCR) interaction (Rothbard *et al.*, 1989), whereas mutations within epitope flanking regions may reduce presentation by affecting peptide processing and transport (Eisenlohr *et al.*, 1992). Recent studies also suggest that mutated epitopes may arise, which still interact with the TCR, but inhibit CTL function by inducing T cell anergy, or by showing partial agonistic or antagonistic activity (reviewed by Klenerman *et al.*, 1996). In this case, even virus variants that do not become

the predominant viral species may affect immune surveillance, as they can block CTL recognition of viruses that do not contain mutated epitope sequences. Finally, rapid virus evolution and strong antigenic stimulation may also favour immune evasion by causing CTL exhaustion (Von Boehmer, 1993).

Although mutant viruses which are resistant to CTL have been detected in persons infected with HIV (reviewed by Koup, 1994; Koenig *et al.*, 1995; Borrow *et al.*, 1997; Goulder *et al.*, 1997), and despite evidence that multiple mechanisms may be operative in HIV attempts to escape recognition (Couillin *et al.*, 1994), the biological relevance of these findings remains somewhat controversial. The fact that HIV-infected persons, or SIV-infected macaques, generally mount CTL responses against multiple viral antigens suggests that despite the loss of CTL recognition of one epitope, complete evasion from immune surveillance should be a rare occurrence. Nevertheless, qualitative aspects of antiviral CTL, such as the nature of the targeted epitope (Moskophidis & Zinkernagel, 1995) and the affinity of effector cell–target cell interactions (Tsomides *et al.*, 1994) may be crucial determinants of effective CTL pressure. It is therefore conceivable that under stringent circumstances, strong CTL responses targeting key epitopes may promote selection of virus mutants which, at least temporarily, can evade immune recognition. The finding that the presence of strong CTL responses against Env is associated with pronounced HIV genetic heterogeneity (Wolinsky *et al.*, 1996) appears to support this view.

The effects of CTL pressure on SIV evolution have so far received limited attention. In a study of macaques infected with SIVmac$_{251}$, the presence of strong p11C-specific CTL responses was associated with the detection of amino acid mutations within the CTL epitope (Chen *et al.*, 1992). Although two of four mutated epitope sequences were recognized less efficiently than the prototype sequence, statistical analysis failed to indicate a higher rate of mutations in the epitope compared with other regions of Gag, and selection of the mutated viruses could not be demonstrated.

Infection of macaques with molecular clones of SIV may help to clarify the significance of virus escape in pathogenesis and disease progression, as any mutation detected in the viral genome must have originated during the course of the infection. In a long-term non-progressor macaque infected with the molecular clone SIVmac$_{32H\text{-}J5}$, the detection of 'high affinity' immunodominant CTL targeting a 9-mer epitope in Gag designated p26A.5 (Geretti *et al.*, 1997 *b*), coincided with the emergence of a variant virus carrying a mutated epitope sequence (A. M. Geretti, unpublished). Surprisingly, the mutated sequence was detected in the spleen, where it had uniformly replaced the challenge virus, but not in peripheral blood or lymph nodes. One explanation for this finding is that co-existence of viral expression and CTL expansion in splenic white pulp creates a highly favourable microenvironment for immunological pressure (Cheynier *et al.*, 1994). The absence of epitope mutations in animals lacking detectable p26A.5-
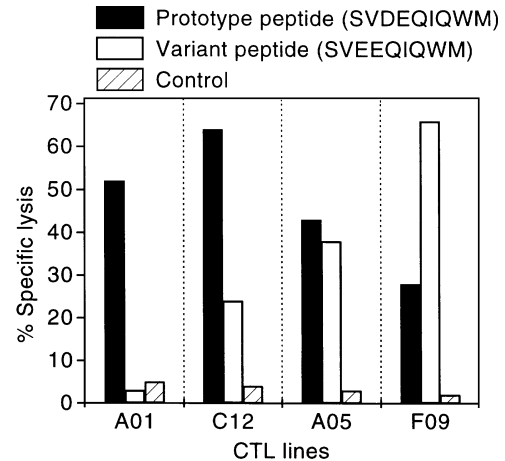

Fig. 2. Different patterns of epitope-specific CTL recognition. In a long-term non-progressor macaque infected with the molecular clone SIVmac$_{32H\text{-}J5}$, the detection of CTL targeting a 9-mer epitope in Gag designated p26A.5 (amino acids 242–250 of p26) coincided with the emergence in the spleen of a variant virus carrying an aspartic acid to glutamic acid substitution (D → E) at position 244. Short-term CTL lines generated *in vitro* by stimulation of PBMC with a peptide representing the prototype p26A.5 epitope were tested for their ability to lyse chromium-labelled B-LCL pulsed with either the prototype p26A.5 peptide, or a variant peptide carrying the mutated epitope sequence. Four patterns of reactivity were identified: most (38 of 56) CTL lines (e.g. A01) did not recognize the variant peptide even at the highest peptide concentration tested (100 µM); eight (e.g. C12) recognized the prototype peptide with higher efficiency than the variant peptide; seven (e.g. A05) recognized both the prototype and the variant peptide with a similar efficiency; and three (e.g. F09) recognized the variant peptide with higher efficiency than the prototype peptide.

specific CTL favours immunological pressure over simply growth advantage as the cause of virus mutation. The variant virus escaped recognition by most, but not all p26A.5-specific CTL (Fig. 2). The latter observation suggests a certain redundancy of CTL responses targeting the same epitope, also described for pC11-specific CTL (Shen *et al.*, 1994; Chen *et al.*, 1996), which may represent a first-line safeguard mechanism against emerging virus variants. Remarkably, CTL subpopulations targeting the variant sequence also localized preferentially in the spleen. The low virus load detected in this compartment suggests effective virus containment. However, the idea that a variant virus with even a slight advantage might have eventually replaced the challenge virus also in other compartments and gradually affected the control of the infection remains a valid hypothesis.

Conclusions

Over the past decade, the macaque model of SIV infection has provided significant new information on the host–virus interplay taking place during the course of lentiviral infections. Increasing evidence indicates a role for antiviral CTL in the containment of SIV replication, and supports the view that the quality of antiviral CTL is as important as their quantity in

determining both the outcome of infection and the course of the disease. It is now believed that the ability to induce antiviral CTL that would limit, if not completely prevent virus spread, particularly after mucosal exposure, is an important prerequisite of an effective HIV vaccine. The concept that the design of such a vaccine will require improved understanding of what may constitute a protective CTL response is also now widely accepted. The SIV model offers valuable opportunities to better understand the mechanisms of lentivirus-induced disease and to clarify the basis for protective immunity. Hopefully, it will fulfil its promise – helping the development of a successful vaccination strategy against HIV.

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