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Virus replication and evolution drive the kinetics and specificity of SIV-specific cytotoxic T lymphocytes

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Summary: SIV (simian immunodeficiency virus) infection of cynomolgus macaques provides an excellent model for investigating the basis of protective immunity against HIV (human immunodeficiency virus). We explored the protective role of cytotoxic T lymphocytes (CTL) against the pathogenic molecular clone SIVmac-J5. Vaccine-induced CTL precursors (CTLp) against Env, Gag or Nef did not protect macaques against intravenous challenge. However, detection of Rev-specific CTLp in infected macaques was associated with effective virus containment. Furthermore, CTL against an immunodominant Gag/p26 epitope (amino acids 242–250) resulted in the emergence of a mutant virus that uniformly replaced wild-type virus in the spleen and partially escaped recognition. During primary infection, CTLp detection in blood coincided with decreasing viremia. After 12 months, two outcomes emerged. In one group of macaques, persistent viremia was associated with high viral load in lymphoid organs and declining $CD4^+$ T-cell counts. CTLp were maintained in asymptomatic macaques, but declined in the symptomatic phase of infection. In a second group, loss of detectable viremia was associated with low-level virus reservoirs in lymphoid organs, asymptomatic status and maintained $CD4^+$ T-cell counts. CTLp peaked in the first 4 months of infection and subsequently declined in this group. These studies provide insights into the complex interplay between virus replication and host immunity.

Detection of SIV-specific CTLp

Cytotoxic responses were measured in ^{51}Cr-release assays, which provide a direct measurement of CTL function, and were quantified by limiting dilution analysis (LDA). Effector cells were either mononuclear cells isolated from peripheral blood (PBMC), lymph nodes (LNMC) and spleen (SPMC), or positively selected $CD8^+$ cells (1). Autologous B lymphoblastoid cell-lines (B-LCL) immortalised by herpes virus papio were used as antigen presenting cells (2). B-LCL were either infected with recombinant vaccinia viruses expressing SIV antigens or sensitised with synthetic peptides. This approach induced highly selective expansion of $CD8^+$ and MHC class I-restricted CTL (3). The same B-LCL were used as target cells. It has recently emerged that LDA may underestimate the size of the CTL pool when compared to MHC-peptide tetramer

assays. However, the use of tetramer assays is limited by the fact that only a few MHC alleles and SIV-specific CTL epitopes have been identified in cynomolgus macaques. Most importantly, LDA has been shown to provide reliable information on the kinetics and duration of memory CTL (4).

Vaccine-induced CTL do not protect macaques from intravenous challenge

CTL are believed to be important correlates of protection against SIV (5, 6). To date, however, vaccine studies have generally failed to provide clear-cut evidence of their ability to protect from challenge (reviewed in 7 and 8). We examined the protective role of SIV-specific CTL in macaques immunised with Env glycoproteins incorporated into immune-stimulating complexes (Env-iscoms), Gag/p26-iscoms and three Nef lipopeptides containing CTL epitopes (9). After four sequential intramuscular immunisations, the macaques were challenged intravenously with SIVmac-J5 (50 monkey 50% infectious doses). On the day of challenge, high titres of SIV-neutralising antibodies were detected in the vaccinated macaques. In addition, the macaques had Env- (3/4 tested), Gag- (4/4) or Nef- (3/4) specific CTLp at levels ranging between 6 and 122/10^6 PBMC, with a mean value of 49/10^6.

After challenge, all macaques became infected. Serial viral load measurements over the following 16 weeks showed no significant difference between immunised and control macaques. No correlation was found between CTLp levels on the day of challenge and peak viral load after challenge. Furthermore, after infection, CTLp levels of immunised macaques were similar to those of control macaques, indicating that immunisation did not enhance secondary CTL responses. Although disappointing, these findings are not inconsistent with the view that CTL may be able to prevent infection if stringent quantitative and qualitative requirements are met. This is supported by the finding that CTL against multiple SIV antigens, including the non-virion proteins Rev and Tat, can be detected in macaques lacking both detectable virus and antibody responses after challenge with SIV (10) or SHIV (simian-human immunodeficiency virus) (E. G. J. Hulskotte, unpublished data).

Emergence of CTL coincides with virus clearance during primary SIV infection

PBMC-associated viral load (assayed by virus culture on C8166 cells under limiting dilution conditions), p26 antigenemia, and SIV-specific CTLp were measured sequentially in eight macaques during the first 16 weeks of infection with SIVmac-J5 (3). Levels of viremia peaked between weeks 1 and 4 and subsequently declined to set points that differed considerably between macaques. All macaques (8/8 tested) developed Gag-specific CTLp and most had also CTLp against Tat (6/7 tested), Env (4/7), Pol (4/7) and Nef (4/7). CTLp were first detected between weeks 1 and 4 (3, 9, 11, 12). This coincided with decreasing viremia, supporting a role for CTL in containing virus replication. Over time, CTLp levels rose to 331/10^6 PBMC, with a mean value of 96/10^6. However, CTLp levels did not correlate with cell-associated viral load. In SIV-infected rhesus macaques, a similar lack of correlation has been described between tetramer-binding $CD8^+$ T cells and cell-associated RNA load (13).

CTLp accumulate preferentially in blood and spleen rather than lymph nodes

Primary infection

In two macaques, data from peripheral blood were compared to those from sequential axillary and inguinal lymph node biopsies. Lymph node mononuclear cells (LNMC) showed higher CD4/CD8 ratios (range 1.9–2.6) than those observed in PBMC (range 0.5–0.7). The kinetics of both cell-associated viral load and SIV-specific CTLp were similar in peripheral blood and lymph nodes (11, 12). CTLp levels, however, were lower in LNMC than in PBMC. This observation was confirmed after adjustment of CTLp frequencies to the $CD8^+$ percentages of the respective mononuclear cell populations. In SIV-infected rhesus macaques, the percentage of tetramer-binding $CD8^+$ T cells is also lower in lymph nodes than in PBMC during primary infection (14). Similarly, HIV-specific CTL accumulate preferentially in peripheral blood rather than lymph nodes during primary HIV infection (15).

Chronic infection

In five macaques, data from peripheral blood were compared to those from lymph nodes and spleen after 22 months of infection. $CD8^+$ percentages and CTLp levels remained lower in lymph nodes than in peripheral blood. In contrast, those in the spleen were either similar or higher than those measured in blood (12, and A.M. Geretti, unpublished data). In SIV-infected rhesus macaques, tetramer-binding $CD8^+$ T cells have also been shown to localise preferentially to the spleen and bone marrow (13). These observations suggest that the spleen may provide a milieu particularly conducive to CTL expansion. This may in turn reflect persistent antigenic expression in this organ.

Long-term virus containment results in CTLp decline

We monitored virus replication and CTL responses in seven SIV-infected macaques for 22 months (12). By 12 months after infection, two virological and immunological patterns emerged. Four macaques, which can be regarded as progressors, had persistent viremia associated with a significant decline in $CD4^+$ T-cell counts. Three remained asymptomatic, whereas one developed simian AIDS. In contrast, three macaques, which can be regarded as non-progressors, lost detectable virus in peripheral blood and remained asymptomatic with preserved $CD4^+$ T-cell counts.

The most rapid loss of detectable viremia was observed in macaque K71. A burst of virus replication had occurred in the first 2 weeks after infection (>1,000 infected cells/10^6 PBMC), followed by a rapid decline (3, 12). After 3 months, we failed to recover virus from PBMC, even after removing potential virus-suppressive factors by depletion of $CD8^+$ T cells. Proviral DNA (assayed by a nested Gag-PCR using PBMC, cultured blasts and positively selected $CD4^+$ T cells) persisted in peripheral blood during the first 6 months of infection, but became undetectable after 12 months. Loss of detectable viremia in the non-progressors, however, was not an indicator of virus clearance, as 22 months after infection, proviral DNA was detected in both lymph nodes and spleen (12). Cell-associated virus load, however, was either below (lymph nodes) or barely above (spleen) detection limits. In contrast, progressors had high virus load in lymphoid organs, especially in the spleen (A.M. Geretti, unpublished data).

All macaques had CTLp against Gag and Tat, and most macaques also had CTLp against Env (5/7), Nef (4/7) or Pol (4/7). However, only the three non-progressors had Rev-specific CTLp (12). This observation supports the hypothesis that CTL directed against early regulatory proteins may play a crucial role in virus containment. In both SIV-infected macaques (16) and HIV-infected humans (17), Rev- and Tat-specific CTL have been shown to correlate with lack of disease progression. Furthermore, at least partial protection against intravenous virus challenge has been observed in macaques immunised with Rev- or Tat-based vaccines (18, 19).

In agreement with observations made in HIV-infected humans (20), CTLp declined over time in the macaque that developed symptomatic disease, but were maintained in the asymptomatic progressors. A surprising finding was that CTLp levels in the three non-progressors reached a plateau at 4 months but declined subsequently (8, 12). CTLp loss associated with loss of detectable viremia and low-level virus burden in lymphoid organs suggests that SIV-specific memory CTL decline under conditions of limited antigenic stimulation. CTL responses against HIV antigens have also been shown to disappear in a long-term non-progressor carrying a defective HIV provirus (21) and in HIV-infected patients receiving highly active antiretroviral therapy (22). In view of these data, it is perhaps not surprising that the degree of protection conferred by live attenuated SIV vaccines, and the strength of the CTL responses they induce, appear to be inversely correlated with the level of virus attenuation (23). These observations have implications for the design of vaccination strategies aimed at inducing lasting CTL responses against HIV.

Rechallenge leads to re-expansion of SIV-specific CTLp

Immunological dysfunction may be proposed as an alternative explanation for the loss of CTLp observed in the three non-progressors. However, both $CD4^+$ T-cell counts and allogeneic T-cell responses were preserved in the three macaques during nearly 2 years of observation. Most importantly, an increase in the frequency of CTLp was observed in macaque K71 after intravenous rechallenge (12), arguing against a compromised immune function. Rechallenge with SIVmac-J5 (200 monkey 50% infectious doses) was followed by transient viremia, decreasing $CD4^+$ T-cell counts, and increasing $CD8^+$ T-cell counts, antibody titres and CTLp levels. Interestingly, both the kinetics and the levels of CTLp were remarkably similar to those observed during primary infection (12). These findings imply that, despite effective virus containment, the macaque remained susceptible to re-infection. However, virus replication was transient and very limited in comparison with the virus burst observed during primary infection (12). It is tempting to speculate that restimulated CTLp, rather than being a mere reflection of antigenic load, played a role in mediating virus containment.

Fine specificities of CTL responses in one non-progressor

CTL responses were defined in detail in macaque K71 (24). CTL lines were derived from PBMC by stimulation with SIV Gag. The target epitope was first identified with sets of 20-mer overlapping peptides spanning the p26, p17 and p15 sequences of SIV Gag. Using shorter peptides, the epitope was minimally defined as a 9-mer region spanning amino acids 242–250 (SVDEQIQWM) of p26. Two truncated peptides lacking either the N-terminal (VDEQIQWMY) or the C-terminal residue (SSVDEQIQW) were considerably less efficient

***Table 1.* Recognition of wild-type and variant peptide by p26A5-specific CTL lines**[a]

	Peptide recognition	
Time after infection	Wild-type only	Wild type and variant
6 weeks	17	9
10 months	10	4
22 months	11	5
Total	38 (68%)	18 (32%)

[a] CTL lines were generated by stimulation of PBMC in limiting dilution with peptide p26A5. Results are presented as number of CTL lines recognising targets sensitised with 30 μM of either wild type (SVDEQIQWM) or variant (SVEEQIQWM) peptide.

***Table 2.* CTL responses against wild-type and variant peptide after effector cell stimulation with wild-type peptide**

	Target[a]	
Effector cells	Wild-type peptide	Variant peptide
PBMC	50%	19%
LNMC	14%	8%
SPMC	29%	18%

[a] Targets were sensitised with 100 μM of either wild-type or variant peptide. Results are presented as mean percentages of specific lysis (after subtraction of background lysis) at an effector to target cell ratio of 10:1.

***Table 3.* CTL responses against wild-type and variant peptide after effector cell stimulation with variant peptide**

	Target[a]	
Effector cells[a]	Wild-type peptide	Variant peptide
PBMC	8%	8%
LNMC	5%	6%
SPMC	20%	40%

[a] Targets were sensitised with 100 μM of either wild-type or variant peptide. Results are presented as mean percentages of specific lysis (after subtraction of background lysis) at an effector to target cell ratio of 10:1.

in sensitising target cells for CTL lysis. Further truncation at either terminus abolished recognition. The newly defined CTL epitope was designated p26A5. The epitope sequence is conserved in several SIVmac strains. However, amino acid substitutions in corresponding HIV-1 group M (TLQEQIGWM) and HIV-2_{ROD} (TVEEQIQWM) sequences abolished CTL recognition (24). p26A5-specific CTL therefore cannot be regarded as cross-reactive.

We found that p26A5 was also the only target of CTL expanded from PBMC by mitogenic stimulation, suggesting a narrowly focussed CTL response. p26A5-specific CTL were $CD8^+$ and restricted by the Mafa A2 MHC class I allele (24). Interestingly, their lytic activity was not significantly inhibited by blockade with anti-CD8 monoclonal antibodies (A. M. Geretti, unpublished data), suggesting high affinity effector–target cell interactions (25).

Split-well LDA assays were used to measure Gag- and p26A5-specific CTLp frequencies simultaneously (12). The proportion of Gag-specific CTLp directed against p26A5 was 77% in the first 16 weeks after infection and 40% between 4 and 7 months, suggesting that additional Gag epitopes emerged over time. Subsequently, Gag-specific CTLp declined more rapidly than p26A5-specific CTLp. As a result, between 12 and 20 months of infection, >90% of Gag-specific CTLp were directed against p26A5. These findings are consistent with the observation that CTL against immunodominant epitopes persist longer than CTL against minor epitopes (26). These CTL may be triggered by infected cells displaying very low epitope density (27), be relatively independent of T-cell help (28), and ultimately be more effective in virus containment (29).

p26A5-specific CTL select for a virus variant that partially escapes recognition

The immunodominance of p26A5-specific CTL and their susceptibility to amino acid substitutions within the epitope, suggested that they may select for virus variants that escape recognition. Infection with a molecular clone of SIV provides an optimal background for exploring this issue, as any mutation detected in the viral genome must have originated during the course of the infection. We sequenced (by both direct sequence and clone analysis) a 500 bp Gag fragment of proviral DNA from PBMC, axillary, inguinal and mesenteric LNMC, and SPMC of macaque K71. A macaque lacking p26A5-specific CTL served as control. In macaque K71, the Gag region was conserved in both blood and lymph nodes. In contrast, all splenic sequences carried two single point mutations. One (D→E) substitution was found within the epitope region at position 244. A second (N→S) substitution occurred outside the epitope region at position 143 (A. M. Geretti, unpublished data). The lack of epitope mutations in the control macaque favours CTL pressure over simply growth advantage as the cause of virus mutation. These findings confirm that co-existence of viral expression and CTL expansion in the spleen creates a highly favourable microenvironment for immunological pressure (30).

A variant peptide spanning the mutated sequence (SVEEQIQWM) was used to sensitise target cells for lysis by bulk CTL, short-term CTL lines and CTL clones derived from PBMC

by stimulation with either Gag or peptide p26A5 (8, and A. M. Geretti, unpublished data). In most cases, CTL recognition was abolished or diminished by the mutation, although a range of responses was observed.

Interestingly, 2/7 CTL clones and 18/56 CTL lines recognised both the wild-type and the variant peptide (*Table 1*). This suggests the existence of multiple CTL populations directed against the same region in p26, and may reflect either the induction of new CTL by the variant epitope or a certain redundancy of CTL responses targetting the same epitope (31–33). The latter may represent a first-line safeguard mechanism against virus escape.

The variant peptide was also used to stimulate the expansion of CTL from PBMC, LNMC and SPMC. In bulk assays, the peptide was poorly immunogenic for both PBMC and LNMC. In contrast, it was effective in stimulating CTL growth from SPMC (*Tables 2 and 3*). Consistent with this observation, the frequencies of CTLp specific for the variant peptide were higher in SPMC than in PBMC or LNMC (A. M. Geretti, unpublished data). These findings indicate that the virus mutant and CTL specific for the mutated sequence co-localised within the spleen, supporting the concept that CTL are important effectors of the immune response against SIV.

Conclusions

SIV infection of cynomolgus macaques results in a wide range of outcomes. At the two ends of the spectrum, some animals show a precipitous loss of $CD4^+$ T cells and develop symptomatic disease within a few months, whereas others show no evidence of disease progression for 2 years or longer. This dichotomy provides an excellent opportunity for studying CTL responses in relation to virus replication and disease outcome. Although the host–virus interplay that characterises SIV and HIV infection appears to be complex, increasing evidence indicates a role for CTL in virus containment. There is also strong support for the view that qualitative aspects of CTL responses are important in determining the outcome of infection. Indeed, the bulk of evidence suggests that a broad range of potent CTL responses, which focus on conserved epitopes and target early viral proteins such as Rev and Tat, may be able to mediate effective virus containment.

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