Evasion of Cytotoxic T Lymphocyte (CTL) Responses by Nef-dependent Induction of Fas Ligand (CD95L) Expression on Simian Immunodeficiency Virus-infected Cells

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Summary

Inoculation of macaques with live attenuated SIV strains has been shown to protect against subsequent challenge with wild-type SIV. The protective mechanism(s) remain obscure. To study the effect in more detail, we have investigated the role of virus-specific CTL responses in macaques infected with an attenuated SIV strain (pC8), which has a four-amino acid deletion in the nef gene, as compared with the wild-type SIVmac32H clone (pJ5). Cynomolgus macaques infected with pC8 were protected against subsequent challenge with pJ5 and did not develop any AIDS-like symptoms in the 12 months after infection. The pC8-induced protection was associated with high levels of virus-specific CTL responses to a variety of viral antigens. In contrast, pJ5-infected macaques had little, if any, detectable CTL response to the viral proteins after three months. The latter group of macaques also showed increased Fas expression and apoptotic cell death in both the CD4+ and CD8+ populations. In vitro, pJ5 but not pC8 leads to an increase in FasL expression on infected cells. Thus the expression of FasL may protect infected cells from CTL attack, killing viral-specific CTLs in the process, and providing a route for escaping the immune response, leading to the increased pathogenicity of pJ5. pC8, on the other hand does not induce FasL expression, allowing the development of a protective CTL response. Furthermore, interruption of the Fas-FasL interaction allows the regeneration of viral-specific CTL responses in pJ5-infected animals. This observation suggests an additional therapeutic approach to the treatment of AIDS.

Live attenuated SIV or HIV with a deletion of the nef gene exhibits reduced pathogenicity and does not cause an AIDS-like illness (1–3). Moreover, macaques immunized with the nef-mutant SIV are protected against superinfection with wild-type pathogenic SIV (2, 4). However, the mechanism(s) of protection induced by the attenuated virus are not known.

Accumulated evidence suggests that CTL play a critical role in controlling HIV replication (5). HIV-specific CTL activity has been observed at different stages of the disease in infected individuals. In particular, the early induction of an HIV-specific CTL response has been associated with the initial control of viremia and may influence the subsequent clinical outcome (6–8). However during disease progression the fall in CD4+ T cells is associated with a decrease in CD8+ T cell numbers and an associated reduction in the virus-specific CTL activity (9–11). Although several mechanisms are known to cause depletion of CD4+ T cells in HIV infection (12–15), less is known about the mechanisms leading to the fall in numbers and dysfunction of CD8+ T cells, which probably result from several factors in addition to the reduction of CD4+ T cell help. In particular, HIV-associated programmed cell death (apoptosis) has been well documented in infected individuals (16–18).
terestingly, these apoptotic cells include not only uninfected CD4+ cells but also CD8+ (bystander) cells (18, 19).

Apoptosis of lymphocytes can be triggered by several cell surface receptors including TNF-R1, Fas, and a newly cloned molecule WSL-1/DR3 (20, 21). Each of these molecules will trigger apoptosis when it contacts its counter-receptor or ligand, TNF and FasL for TNF-R1 and Fas, respectively (22). Interaction between Fas and FasL plays an important role in the homeostatic regulation of normal immune responses (23). The expression of Fas is quite diffuse being found on a variety of extra-lymphoid tissues such as liver, ovary, and heart. The expression of FasL on the other hand is much more tightly controlled being restricted to activated lymphocytes and selected sites enjoying immune privilege (24, 25).

Fas is upregulated on both CD4+ and CD8+ cells from HIV-infected individuals (26, 27). However Fas expression per se does not lead to cell death as ligation of Fas by FasL is required to trigger apoptosis. There are therefore two crucial questions relating to the death of CD8+ cells in HIV infection, first where is FasL expressed, and secondly what induces its expression.

To address this question, we have investigated CTL responses and apoptosis in macaques infected with the attenuated phenotype in vivo (4). Four cynomolgus macaques were infected intravenously with pC8 (106 TCID50) for 12 mo and then challenged with a pathogenic SIV mac32H clone, pJ5 (50 M ID50) for an additional 3 mo. Another group of four naive macaques was infected with pJ5 (50 M ID50) only.

In vitro infection of PBMCs or CEM cells. PBMCs (1 × 10^6) were stimulated with Con A (5 µg/ml) for 12 h and superinfected with 200 µl of pC8 or pJ5 supernant containing 5 × 10^5 TCID50 for 2 h at 37°C under 5% CO2. After washing three times with RPMI 1640, cells were adjusted to a concentration of 2.0 × 10^6/ml and incubated in R10H medium for another 48 h. For infection of CEM cells, 1 × 10^5 cells were infected with 400 µl of pC8 or pJ5 supernant containing 5 × 10^5 TCID50 for 2 h and incubated in R10H for another 24 h. Mock infection was performed under the same conditions using a supernatant generated from Con A-stimulated or unstimulated uninfected cells.

Virus infection in M acacnes

The presence of SIV-specific DNA sequences in the blood of SIV-challenged macaques was performed as described previously (4). In brief, this involves the amplification of a region of the SIV nef gene. Restriction analyses of PCR products using the enzyme RsAl allows the differentiation of products derived from pJ5 or pC8 virus.

Detection of Virus-specific CTL Activities

SIV-specific CTL activity was measured in bulk cultures as previously described (30). In brief, PBMC were isolated on Ficoll-Hypaque and one-tenth of the autologous PBMC were stimulated with Con A (5 µg/ml) for 24 h. Cells were infected with 100 µl SIV pC8 supernant for 2 h, washed, and then added back to the remaining cells. Infected cells were then cultured in R10H medium for 3 d and maintained for another 7–14 d in medium supplemented with 10 U/ml IL-2. H. papio-transformed autologous B cell lines infected with recombinant vaccinia viruses carrying the SIV mac nef, gag, pol, env, RT, rev, tat, or control (influenza NP) gene were used as target cells. In some experiments soluble Fas-Fc fusion protein (5 µg/ml) was added initially in bulk cultures and the cells were then washed before using as effector cells in CTL assay. Cytotoxicity was determined by culturing 51Cr-labeled target cells with effector cells at various E/T ratios for 4 h in 96-well U-bottomed plates. Maximum and spontaneous release were determined by incubating target cells with 5% Triton X-100 or media, respectively. Percentage lysis was calculated as [(experimental release − spontaneous release)/ (maximum release − spontaneous release)] × 100. Spontaneous release varied from 10% to 25%. Specific lysis was calculated by
subtracting background killing of influenza NP-infected target cells.

**Detection of Apoptotic cells by DNA Fragmentation**

PBMC were cultured in R10H media for 4 or 16 h and fragmented DNA was extracted according to the method previously described (31). In brief, pelleted cells (0.5–1 \(10^6\) cells) were lysed with 100 \(\mu\)l lysis buffer (1% NP-40, 20 mM EDTA, 50 mM Tris-HCL, pH 7.5) for 1 min and centrifuged at 1,600 \(g\) for 5 min. The supernatant was collected and treated with 1% SDS and RNAseA (5 \(\mu\)g/ml) at 56 °C for 2 h. After digestion with proteinase K (2.5 \(\mu\)g/ml) for 2 h at 37 °C, the DNA was precipitated by adding 1/2 volume of 10 M ammonium acetate and 2.5 vol of cold ethanol, and then analyzed by electrophoresis on 1.5% agarose gels. To quantitate the percentage DNA fragmentations Southern blot was hybridized with \(^{32}P\)-labeled probe generated by random priming of whole monkey genomic DNA. The percentage DNA fragmentation was calculated by dividing the total counts by the counts found on DNA fragments below 23,000 bp (32). DNA extracted from naive controls had <20% of the counts as determined by this method.

**Flow Cytometry**

For analysis of Fas expression, PBMCs (5 \(\times\) \(10^5\)) were incubated first with anti-Fas IgM monoclonal antibody and then with a secondary FITC-conjugated rabbit anti–mouse IgM (Sigma Chem. Co., St. Louis, MO). Fas stained cells were then counterstained with a PE-conjugated anti-CD4 or CD8 mAbs. For intracytoplasmic staining of SIV nef antigen, the infected cells were incubated with the anti-SIV nef mAb together with 0.3% saponin (Sigma) and then stained with a secondary FITC-conjugated rabbit anti–mouse Ig (Sigma). Labeled cells were analyzed on a FACScan®.

**Isotype-specific mAbs of irrelevant specificity were used as negative controls (Dako Corp.).**

**A nalys of FasL Expression**

Functional FasL was assessed using a bioassay for FasL (33). SIV-infected cells were cocultured with \(^{51}\)Cr-labeled Fas-sensitive Jurkat cells at various E/T ratios in the presence or absence of the human Fas–Fc fusion protein (10 \(\mu\)g/ml) or blocking anti-FasL mAbs (5 \(\mu\)g/ml) for 12–16 h. The level of chromium release into the supernatant was determined using a \(\beta\)-plate counter.

To determine the level of FasL expression on the cell surface, SIV-infected cells were incubated with 20 \(\mu\)l of biotin-conjugated anti-human FasL mAb (NOK-1; PharMingen), followed by 5 \(\mu\)l of PE-conjugated streptavidin (Sigma). Negative controls were either infected cells stained with PE-streptavidin only or uninfected cells stained with anti-FasL mAb and PE-streptavidin.

**Results**

**Protective Effects of pC8 on Challenge of the Macaques with pJ5.** After infection with the attenuated strain of SIV pC8, all macaques became infected but did not develop the characteristic clinical manifestations of AIDS over the subsequent 12 mo. These pC8-infected macaques and four naive animals were then challenged with pJ5. After 8 wk, the viral load was assessed. Table 1 indicates that the attenuated clone pC8 protects against subsequent challenge with pJ5.

| Group    | Abs to SIV gp140 | Virus detected by V/P | Abs to SIV gp140 | Virus detected by V/P |
|----------|------------------|-----------------------|------------------|-----------------------|
| pC8-infected |                  |                       |                  |                       |
| N 113    | 4.0              | −/?                   | 4.0              | −/pC8                 |
| N 114    | 3.5              | −/pC8                 | 3.0              | −/pC8                 |
| N 115    | 3.6              | −/pC8                 | 3.6              | −/?                   |
| N 116    | 3.6              | −/pC8                 | 3.9              | −/pC8                 |
| Naive    |                  |                       |                  |                       |
| N 174    | <1.5             | −/ND                  | 3.7              | +/pJ5                 |
| N 175    | <1.5             | −/ND                  | 3.5              | +/pJ5                 |
| N 176    | <1.5             | −/ND                  | 3.5              | +/pJ5                 |
| N 177    | <1.5             | −/ND                  | 3.3              | +/pJ5                 |

*Monkeys N113–116 were infected intravenously with 10⁴ TCID₅₀ of SIVpC8 for 35 mo and then together with naive N174–177 monkeys challenged intravenously with 50 M ID₅₀ SIV pJ5 clone.

†Antibody to SIV gp140 was determined by ELISA using anti-SIV gp140 mAbs and data is end point titers expressed as log₁₀.

‡V, virus detected by virus isolation; P, virus detected by PCR. + indicates virus recovered from \(5 \times 10⁶\) PBMCs; − indicates no virus recovered from \(5 \times 10⁶\) PBMCs. pC8 indicates PCR product with characterization of pC8; pJ5 indicates PCR product with characterization of pJ5; ? indicates indeterminate PCR product unlike pJ5; ND indicates not determined.

Table 1. Challenge with Wild-type SIV pJ5 in pC8-infected and Naive Macaques
**Table 2. SIV-specific CTL Responses after SIV pJ5 Challenge in pC8-infected and Naive Macaques**

| Target cell infection<sup>a</sup> | pC8-infected | Naive  |
|----------------------------------|--------------|--------|
| N 113                            | N 114        | N 115  | N 116  | N 174 | N 175 | N 177 |
| rVV-nef                          | 7.2<sup>i</sup> | <1.0    | 19.3   | 19.4   | <1.0  | <1.0  | <1.0  |
| rVV-gag/pol                      | <1.0         | 11.8    | <1.0   | 17.8   | 2.0   | <1.0  | <1.0  |
| rVV-env                          | 22.9         | <1.0    | 12.5   | 16.6   | <1.0  | <1.0  | <1.0  |
| rVV-RT                           | <1.0         | <1.0    | <1.0   | 25.5   | <1.0  | <1.0  | <1.0  |
| rVV-rev                          | <1.0         | 18.7    | 13.6   | <1.0   | <1.0  | <1.0  | <1.0  |
| rVV-tat                          | <1.0         | 31.5    | <1.0   | 9.1    | <1.0  | <1.0  | <1.0  |

<sup>*</sup>pC8-infected (N 113–N 116) or naive (N 14, N 175, and N 177) macaques were intravenously injected with 50 MID<sub>50</sub> SIV pJ5 clone as shown in Table 1 and bulk cultured CTL activities were determined at 3 mo after infection.

<sup>†</sup>Herpes papio-transformed autologous B cell lines were infected with recombinant vaccinia viruses (10 PFU/cell) expressing SIV proteins for 2 h at 37°C. After washing the cells were cultured in RPMI 10% FCS for 12 h and then used as target cells.

<sup>§</sup>Specific lysis was calculated by subtracting the background killing of rVV fluN P-infected target.

SIV-specific CTL activities. To investigate the mechanism of protection induced by pC8, SIV-specific CTL responses were measured in PBMC bulk cultures 3 mo after challenge with pJ5. All pC8-infected macaques showed multiple virus-specific CTL responses to nef, gag/pol, env, RT, rev, and/or tat (Table 2). By contrast, no detectable virus-specific CTL activity was observed in PBMC from pJ5-infected animals, at an E/T of 30:1, although a weak CTL response was found in a lymph node from one of these animals.

Induction of Apoptosis In Vivo by SIV pJ5 Compared with pC8. To characterize the loss of CTL responses in pJ5-infected animals further, we analyzed the viability of PBMCs in both groups. Freshly isolated PBMCs were cultured for 4 or 16 h in R10H medium at 37°C and apoptosis was assessed by DNA fragmentation (Fig. 1). Spontaneous apoptosis was significantly higher in pJ5-infected macaques than in pC8-infected animals after 16 h (47.2% vs. 18.1%, P < 0.001). Apoptosis was more profound in the CD<sup>8+</sup> population (CD4 vs. CD8; 28.3% vs. 41.4%, P < 0.05, respectively) (Fig. 2). Thus T cells, in particular CD8<sup>+</sup> T cells, from pJ5-infected animals are more vulnerable to apoptosis than those from pC8-infected animals.

To explain this increased susceptibility to apoptosis we analyzed Fas expression on lymphocyte subsets obtained from pJ5 and pC8-infected animals (Fig. 3). Although Fas expression was significantly increased in both infected groups (pJ5: 36.9 ± 12.7%, pC8: 17.6 ± 4.2%) compared with naive animals (6.2 ± 1.2%), pJ5 induced a significantly higher level of Fas expression than pC8 (P < 0.05) (Fig. 3A). Moreover, while Fas expression was found to be upregulated on both CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the pJ5 group, it was significantly higher in the CD8<sup>+</sup> T cells (P < 0.05).

Upregulation of Fas Ligand Expression on SIV-infected Cells. Engagement of FasL is required for Fas-induced apoptosis, so to search for the source of FasL we used a sensitive bioassay. The assay exploits the sensitivity of Jurkat cells to Fas-mediated killing which can be blocked by the addition of an excess of soluble Fas-Fc fusion protein or anti-FasL mAbs.

PBMC or CEM cells were infected in vitro with either pC8 or pJ5 and then cocultured with 3<sup>Cr</sup>-labeled Jurkat cells (Fig. 4). The results for PBMC and CEM are equivalent, and demonstrate that pJ5-infected but not pC8-infected cells induce killing of Jurkat cells which is abrogated by Fas-Fc fusion protein, implying upregulation of FasL in the pJ5-infected cells. To confirm the results of the bioassay we analyzed FasL expression on infected CEM cells, 24 h after infection, with an anti-FasL mAb. As expected FasL was upregulated in the pJ5 infected cells to a greater degree than cells infected with pC8 (Fig. 5A).

This phenomenon does not result from a difference in the infectivity of the two viral strains. The percentage of infected cells at 3 d, pC8 (98.3%; 76.5 MFI) and pJ5 (98.1%; 78.8 MFI), are equivalent when assessed with anti-nef mAb (data not shown). Moreover, the lysis of Jurkat cells is not due to direct viral invasion as Jurkat cells are resistant to SIV infection (our unpublished observation and others [34]). Similar results were also made with fresh PBMC isolated from macaques 3 mo after infection with pJ5 (Fig. 6). Fractionation of T cells from these macaques demonstrates that although the CD8<sup>+</sup> population expresses more Fas, the
majority of FasL activity resides in the CD4$^+$ population, probably on SIV-infected cells.

CTL responses can be restored by blocking Fas–FasL interactions. One explanation for the poor CTL responses mounted by the pJ5-infected macaques is that SIV-infected CD4$^+$ cells, which we have demonstrated to express FasL, paradoxically kill cognate cytotoxic T cells. If this is the case, we should be able to restore CTL responses by blocking the interaction of FasL expressed on infected CD4$^+$ T cells with Fas expressed on CTL.

We tested this hypothesis with a bulk culture CTL assay in the presence or absence of Fas-Fc fusion protein. No CTL responses were elicited from cells cultured with medium alone or soluble CD4 protein, in agreement with our previous results on the pJ5-infected macaques. However, in the presence of soluble Fas-Fc fusion protein a nef-specific CTL response was established (Fig. 7).

Figure 1. Spontaneous DNA fragmentation of PBMCs from pC8-induced protected (N113-N114) or pJ5-infected (N174-N177) macaques. Fresh isolated PBMCs (1 x 10$^6$) were cultured in medium containing 10% human AB serum for 4 or 16 h. Fragmented DNA was extracted as described in Materials and Methods. (A) DNA was analyzed by electrophoresis on 1.5% agarose gels. (B) Southern blot analysis of the DNA by hybridization with $^{32}$P-labeled genomic macaque DNA. (C) The percentage of DNA fragmentation in B was calculated as dividing the total counts of each sample by the number of counts in the bottom 85-90% of the gel (below 23,000 bp). The percentage of DNA fragmentation of PBMCs from naive macaques was always <20% (data not shown). P value for the two groups <0.005 after 16 h.

Figure 2. Spontaneous DNA fragmentation of CD4 and CD8 subpopulations from pJ5-infected control (N174-N177) macaques. PBMCs were cultured in the medium for 16 h and CD4$^+$ or CD8$^+$ T cells was then purified by positive selection using anti-CD4 or CD8 magnetic beads, respectively. The purity of each subset was >95% as assessed by flow cytometry. After extracting the DNA, Southern blot was performed (A) and analyzed as described in Fig. 1 (B). P value for CD4 vs CD8 <0.05. These data are representative of three separate experiments.
To date, the most effective vaccination strategy in macaques has been the use of live attenuated nef mutant SIV such as pC8 (2, 4). Our results show that one possible contributing mechanism to such protection is the induction of strong virus-specific CTL responses with multiple specificities. Some CTL responses can be elicited from pJ5-infected macaques within 8 wk of infection (37), but at 3 mo these appear to be lost revealing a striking difference in the responses of the animals to challenge with pC8 and pJ5. In addition, the strong CTL activity observed in the protected animals correlates with a lower frequency of apoptotic cell death of both CD4+ and CD8+ T cells. Fas, which is upregulated in infected cells from HIV patients is a candidate for the induction of apoptosis seen in the uninfected cells (26, 32). Our study in SIV corroborates these results showing increased expression of Fas in both CD4+ and CD8+ T cells. Interestingly, animals infected with nef-attenuated SIV express less Fas antigen on their cell surface. This may reflect the generalized state of immune activation seen in SIV/HIV infection.

FasL expression is tightly regulated being confined to activated lymphocytes, Sertoli cells, stromal cells of the anterior chamber of the eye, and neurons (22). The expression at these nonlymphoid sites of immune privilege suggests that FasL may play a role in protection from immunological attack (38). Indeed, it has recently been shown that allogeneic transplanted testes from gld mice that lack FasL expression are rapidly rejected (39). Tumor cells may also express FasL, gaining immune privilege and escaping an anti-tumor immune response (25, 40, 41). HIV has been shown to upregulate FasL expression on macrophages (42).
and HIV tat/gp120 can enhance anti-CD3-induced apoptosis by increasing the expression of FasL on CD4+ cells (43). In our studies we have assessed the effects of FasL up-regulation upon apoptosis and the course of infection in vivo. We demonstrate that freshly isolated PBMC show increased FasL expression and kill Fas-sensitive targets which is blocked by soluble Fas–Fc fusion protein or anti-FasL mAb. The activity of FasL is contained within the CD4+ cell subset.
population, possibly SIV-infected cells. Our demonstration at the protein level of FasL induction by SIV infection is consistent with the demonstration of FasL induction on HIV-infected CD4\(^+\) cells shown by RT-PCR (44). On the other hand, nef mutant SIV-infected cells do not upregulate FasL expression. The nef gene codes for a protein that is not essential for viral growth in vitro, but which is essential to the development of AIDS (45). Nef leads to the downregulation of CD4 expression and is believed to increase the state of T cell activation through interactions with proteins involved in cellular activation and signaling such as Src family tyrosine kinases (46). T cell activation via several modalities leads to an increase in FasL expression (33), so nef through enhancing T cell activation may similarly lead to the expression of FasL. The mechanisms underlying the failure of the nef-mutant SIV pC8 to induce FasL expression require clarification. In preliminary experiments, we have shown that full-length nef expression by vaccinia does not upregulate FasL. This may be due to either counter-activity induced by vaccinia gene products or other HIV/SIV genes may be involved.

We propose that the increased expression of FasL is the cause for the increased pathogenicity of wild-type SIV pJ5. The FasL expression by infected CD4\(^+\) cells can trigger apoptosis of virus-specific CTL, which themselves express Fas. This situation thus mimics the expression of FasL at sites of immune privilege, or the upregulation of FasL by certain tumors. In this way the virus can evade the immune response by preventing the development of an effective CTL response. The effective CTL response developed by macaques infected with pC8, which does not cause FasL expression in CD4\(^+\) cells, suggests that inhibition of the FasL activity on infected cells may restore CTL responses. This is indeed the case; our results show that incubation of cells from the infected macaque with soluble Fas leads to the generation of an efficient anti-nef CTL response. These results suggest a new therapeutic intervention in the treatment of SIV/HIV which we are testing on infected macaques in vivo.

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