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Functional analyses of natural killer cells in macaques infected with neurovirulent simian immunodeficiency virus

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Clearance of HIV and SIV from the peripheral blood by the cellular immune system lessens the viral burden in infected individuals and may have an impact on virus infection of the CNS and the development of CNS lesions. However, the role of immune responses in preventing or limiting CNS infection has not been clearly defined. We investigated the role of natural killer cells in the outcome of SIV infection of macaques as a model for humans with AIDS and HIV encephalitis. In our study, six pig-tailed macaques were infected with the neurovirulent virus, SIV/17E-Fr, and the immunosuppressive virus, SIV/DeltaB670, in a model system that causes rapid progression to AIDS and a high frequency of CNS lesions. NK lytic activity in each macaque was monitored longitudinally. In addition, we enumerated NK cells and tested macaque PBMC for the ability to lyse SIV-infected target cells. We found that there was a significant inverse correlation (P=0.02) between the robustness of NK response and the development of CNS lesions. Animals lacking strong NK cell responses developed more severe CNS lesions than those with robust NK responses did. Furthermore, pre-infection levels of NK activity were predictive of CNS lesion severity. The macaque with the most robust pre-infection NK activity developed no CNS lesions. In these infected macaques, NK activity was shown to be directed against SIV-infected cells. We extended these *in vivo* findings to delineate precisely which cell type was mediating this SIV-directed lysis. We used both macaque and human cells to demonstrate that the population that mediated anti-SIV and anti-HIV cytolytic effects was NK cells. Furthermore, we showed that this anti-SIV and anti-HIV cytolytic effect was directed at the envelope protein and not *gag* proteins. Thus, NK cells have the capacity to recognize and lyse cells expressing SIV and HIV antigens. These data support a role for NK cells in the modulation of CNS disease. *Journal of NeuroVirology* (2001) **7**, 11–24.

Keywords: natural killer cells (NK cells); simian immuno-deficiency virus (SIV); central nervous system (CVS)

Introduction

In approximately 70–90% of patients infected with human immunodeficiency virus (HIV), there are pathological changes in the brain (Budka, 1989; McArthur *et al*, 1993). These changes include HIV leukoencephalopathy, characterized by loss of myelin in the deep white matter of the cerebral hemispheres, and HIV encephalitis, characterized by accumulations of multinucleated giant cells and microglial nodule (Bouwman *et al*, 1998; Conant *et al*, 1998; Kelder *et al*, 1998; McArthur *et al*, 1997). HIV encephalitis is thought to be associated with the trafficking and subsequent residence in the brain of infected monocytes/macrophages, cytokine production in the CNS, excitotoxic injury, and resultant neurodegeneration (Kelder *et al*, 1998; Nottet *et al*, 1996; Persidsky *et al*, 1997).

There are three branches of the immune system that act at different stages of viral infection. Innate immunity (NK cells) acts very early in infection, while cellular immunity (cytotoxic T lymphocytes

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or CTL) develops at the same time or shortly after the innate response. Humoral immunity (virus-specific antibodies from B cells) develops after the cell-mediated immune responses. Among these three types of immune responses, the least amount of attention has been focused on innate immunity and the natural killer (NK) cell. There have been previous reports demonstrating that NK cells are important in the defense against HSV-1 encephalitis (Adler *et al*, 1999), and NK cells are important in the protection against Theiler's murine encephalitis virus (TMEV) and demyelination (Paya *et al*, 1989). In HIV infections, Schwarz and Nair (1997) have demonstrated that a deficiency of NK cell activity could play a significant role in the progression of HIV infections.

Natural killer cells are large, granular lymphocytes comprising about 15 – 30% of primate peripheral blood mononuclear cells (PBMC). NK cells may provide the first line of defense against invading pathogens. They require neither prior sensitization to target cells nor the participation of professional antigen presenting cells. NK cells are able to lyse tumor and virus-infected cells (See *et al*, 1997; Welsh, 1986) in a non-MHC-restricted manner.

It is well documented that both CTL and NK lytic activity decline during HIV progression (Bariou *et al*, 1997; Carmichael *et al*, 1993; Jin *et al*, 1998; Ullum *et al*, 1995). While much attention has been focused on the role of CTL in HIV progression, the role of NK cells in HIV progression, especially in rapid progressors, is largely unknown. The importance of the NK cells of the immune system is illustrated in studies demonstrating that HIV-infected patients with lower NK or no NK cells progress to disease faster than those patients with normal levels of NK cell numbers and lytic ability (Bruunsgaard *et al*, 1997; Ullum *et al*, 1999). We set out to determine the impact of NK cells in the course of AIDS and CNS disease using a model of rapid progression in SIV-infected macaques. We found that there was a strong inverse correlation between the severity of CNS lesions and NK lytic activity or NK cell numbers. In addition, we found that the pre-infection robustness of NK activity of each macaque was critical in predicting the severity of the CNS lesions. Furthermore, we demonstrated that NK cells mediate anti-SIV and anti-HIV cytolytic responses.

Results

In previous studies, natural killer cell activity has not been monitored longitudinally in a systematic manner during the course of SIV infection. In this study, a model of rapid progression of AIDS and CNS lesions in SIV-infected macaques was employed to monitor different aspects of NK lytic activity longitudinally during infection to determine the immunological contribution that NK cells play in the outcome of CNS infection.

Development of CNS lesions, CD4$^+$ cell counts, and plasma viral load

The number of CD4 positive lymphocytes was quantitated throughout infection to measure progression to AIDS. The number of CD4$^+$ T cells declined rapidly during the course of infection for all macaques (Figure 1). The mean starting CD4 count for all six infected macaques was 1142 CD4 cells/μl blood; this dropped to 618 CD4 cells/μl within 3 days of infection. CD4 counts continued to decline throughout the course of infection. By the time of necropsy, the mean CD4 count was 116 CD4 cells/μl.

Viral RNA in peripheral blood peaked at ten days post-infection with an average of 2.3×10^8 copy Equivalents (Eq.)/ml plasma (Figure 1). After the peak at ten days post-infection, the average virus RNA levels remained high and fluctuated between 4.8×10^7 copy Eq./ml to 1×10^8 viral RNA copy Eq./ml for the remainder of the study. The levels of viral RNA were also measured in the brain of the infected macaques at necropsy. Five out of six macaques had high levels of viral RNA in the brain and these corresponded to the five infected macaques that developed CNS encephalitis (Zink *et al*, 1999).

Longitudinal NK activity in infected macaques

To assay the NK lytic activity of each infected macaque, PBMC were sampled prior to and at intervals during the course of infection and used in standard NK assays. The lysis that is measured by this assay can be attributed to NK cells because K562 cells are xenogenic and MHC-mismatched with respect to macaque PBMC. Since each macaque had a different genetic composition, the number and lytic activity of the NK cells within

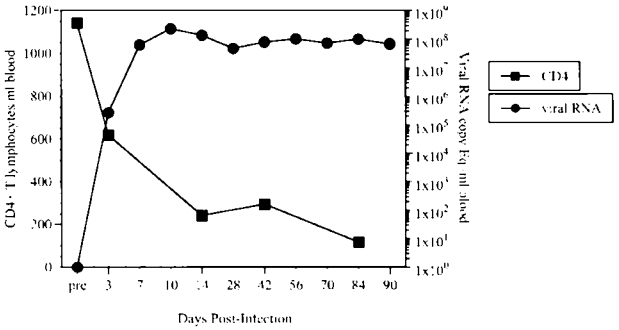

Figure 1 Viral load and CD4 lymphocytes in infected pig-tailed macaques. The average of both viral RNA copies and CD4 counts for all six infected pig-tailed macaques are shown. Viral RNA was quantitated by RT – PCR. CD4 counts were determined by staining PBMC with CD4 monoclonal antibody directly conjugated to a fluorochrome and subsequent analysis on a flow cytometer.

each macaque was different. The pre-infection NK activity ranged from 25 to 70% (Figure 2). Within 1 week of infection, the NK activity dropped sharply and then rebounded to reach peak lytic activity at day 14 post-infection (Figure 2). Macaques 18031 and 17834 displayed the highest levels of NK activity at 50 and 36%, respectively. Macaques 18242 and 18292 showed moderate levels of NK killing while macaques 18033 and 17850 had low levels of NK killing. Following the peak of NK activity at day 14, the NK activity fell off sharply and was completely undetectable by 42 days post-infection. The average NK lysis of human PBMC was 11.5±2.6%.

NK activity prior to and during acute infection predicted CNS lesion severity

To determine a pre-inoculation value for NK activity, all macaques were assayed for NK activity 1 week prior to SIV inoculation. Macaque 17834 had the highest NK activity (70%). Macaques 18031 and 18242 had intermediate NK activity levels of 47 with 46% lysis respectively (Figure 2). The remaining macaques, 18033, 18292, and 17850 had lower NK activity levels of 38, 28, and 25%, respectively. When we assigned ordinal rankings to the pre-infection NK activity levels of each macaque to perform a statistical correlation test with CNS lesion severity, we found that there was a perfect correlation between the pre-infection levels of NK activity and the severity of CNS lesion that developed. In particular, macaque 17850 started this study with the lowest NK activity (25%) and after inoculation with SIV, this macaque never mounted a significant NK response and subsequently developed the most severe CNS lesions. In contrast, macaque 17834 started with the highest NK activity (70%) of all the macaques, nearly three times as high as macaque 17850. Macaque 17834 mounted a strong NK response during the acute phase infection, developed no CNS lesions, and had no detectable viral RNA in the brain.

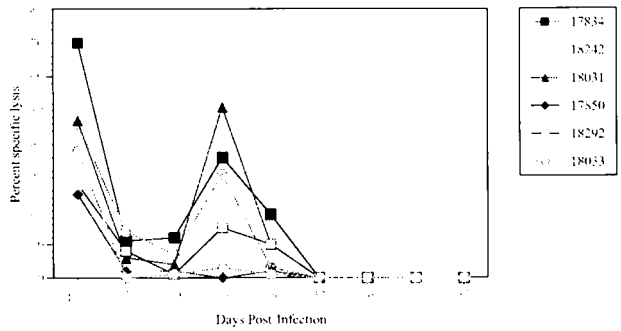

Figure 2 Longitudinal profile of lytic NK activity for all infected macaques. The NK lytic activity was determined by standard chromium release of K562 target cells at E:T ratio of 50:1. PBMC for each timepoint for each macaque was assessed at one time to minimize assay variability. Human PBMC from one donor was used as effector cells as an internal control of NK activity. The lytic activity of the internal control was 11.5±2.6%.

At necropsy, sections from each macaque brain were assessed for CNS lesions and scored by two pathologists independently. The severity of CNS lesions ranged from none (macaque 17834) to moderate (macaques 18031 and 18242) to severe (macaques 18033, 18292, and 17850). Detailed descriptions of CNS lesions is described elsewhere (Zink *et al*, 1999). The severity of CNS lesions were assigned ordinal rankings for statistical analysis with one indicating least lesion severity and six indicating most lesion severity. NK activity levels at the peak of lytic activity at day 14 post-infection were also assigned ordinal rankings (one=highest NK activity, six=lowest NK activity). A significant correlation (P=0.02) was found between the severity of CNS lesions at necropsy and the level of peak NK activity. Macaque 17834 had one of the most robust NK lytic activities (peak of 36% specific lysis at 14 days post-infection) and developed no CNS lesions. Macaque 17850 with the lowest NK lytic activity (peak of 3% specific lysis at day 14) developed many severe lesions.

Furthermore, the levels of pre-infection NK activity also showed a significant correlation (P=0.02) with peak NK activity during acute phase. Macaques 17834 and 18031, which had inherently high pre-infection NK activity, were able to mount more vigorous responses to SIV infection during the acute phase. These two macaques developed the mildest CNS lesions. Thus, the levels of NK lytic activity prior to and after infection were predictive of the severity of CNS lesion that later developed.

Absolute number of NK cells in peripheral blood in infected macaques predicts CNS lesion severity

To determine whether the number of NK cells present in the animals played a role in the development of CNS lesions, absolute numbers of peripheral blood NK cells at the time of peak NK activity (14 days p.i.) were enumerated in each infected macaque using CD3, CD8, and CD16 markers (Carter *et al*, 1999) (Table 1).

At the time of peak NK activity, macaque 17834, which did not develop CNS lesions, had three times the number of NK cells as the next highest macaque 18031. Macaques 18033 and 17850 had poor peak NK activity and the fewest numbers of NK cells, 253 and 232 absolute NK cells/μl, respectively. Both of these macaques developed severe CNS lesions. Macaques 18242 and 18292 had modest numbers of NK cells and developed moderate CNS lesions. Thus, the absolute number NK cells was another factor in limiting CNS lesions.

Composition of inflammatory cells in brain

The percentages of NK cells, cytotoxic T lymphocytes, and $CD4^+$ T cells in the parenchyma of the basal ganglia and parietal cortex of the brains of the

SIV-infected macaques above were determined by double immunohistochemical staining. The percentages of inflammatory cells in the brains of two uninfected macaques were used as negative controls. The majority of cells (86–89%) in the brain of uninfected macaques or macaques without neurological lesions were $CD4^+$ T lymphocytes (Table 2). In these animals, NK cells were rare, and CTLs comprised a small percentage of the cells in the brain parenchyma.

In contrast, in macaques with moderate or severe neurological lesions, 40–47% of the cells in the brain parenchyma were CTLs. Increased number of NK cells were also detected in the brain parenchyma of macaques with neurological lesions, the highest numbers were observed in the animals with severe lesions. TIA-1 antibody detects the constitutively expressed cytotoxic granules in the cells, however, this does not identify functionally active NK cells. Thus, the detection of increased NK cells in the brain parenchyma of animals with CNS lesions suggests that these cells were recruited into the CNS. However, the lack of functional activity of these cells in the periphery may reflect their inability to control virus infection in either compartment.

Table 1 Enumeration of NK cells in infected pig-tailed macaques. At time of peak NK lytic activity, PBMC from infected macaques were analyzed for their absolute lymphocyte count. A combination of CD3, CD8, and CD16 antibodies were used to delineate macaque NK cells because this combination of markers most accurately identifies macaque cells. The NK cells were $CD3^-CD8^+CD16^+$ cells within the lymphocyte gate when analysis was performed on a flow cytometer. From flow cytometric analysis, the percentage of $CD3^-CD8^+CD16^+$ NK cells was obtained. Absolute NK cell numbers were calculated by multiplying the per cent NK cells by the absolute lymphocytes. Peak NK activity was assessed at 14 days post-infection by lysis of K562 target cells.

Macaque	*Absolute lymphocytes*	*% NK cells*	*Absolute NK cells*
17834	3520	39	1373
18031	1769	25	442
18242	1988	25	497
18292	2722	15	408
18033	725	35	253
17850	705	33	232

NK cells exhibited SIV-directed killing

Although lysis of K562 is the standard by which NK activity is measured, an important issue is whether the NK cells in these macaques could lyse SIV-infected cells. In order to determine whether the NK lytic activity observed at day 14 reflected macaque NK cells lysing SIV-infected target cells, we performed NK assays with PBMC from infected macaques taken at day 14 using SIV-infected U937 cells as targets. U937 cells are MHC-mismatched and xenogenic, thus, we ruled out the possibility that any observed lysis was mediated by cytotoxic T cells (CTL).

Because uninfected U937 cells are targets for NK cells, there were background levels of killing of uninfected U937 cells. This background level was subtracted from killing of SIV-infected U937 to obtain levels of SIV-directed lysis. Five out of the six infected macaques were capable of SIV-directed lysis at an E:T ratio of 50 : 1 (Table 3). However, macaque 17834 showed such vigorous killing against U937 alone that its E:T ratio had to be reduced to 25 : 1 to reduce background levels. Even though the number of macaque 17834 PBMC effectors was half of the amount of PBMC effectors used for other macaques, macaque 17834 still showed levels of SIV specific killing comparable to the other macaques. The ability of macaque 17834 to lyse SIV-infected targets cells efficiently at lower E:T ratios combined with its intrinsically high NK activity and its high NK cell numbers provided strong argument that robust NK responses are an important factor in limiting the development of CNS lesions. Macaques 18031, 18242, 18033, and

Table 2 Cytolytic cells in the brain of macaques with severe CNS lesions. The NK and CTL cells in the brain of SIV infected macaques were enumerated by immunohistochemistry and image analyses.

Macaque	*SIV-directed killing*
17834	12% ±1.4%
18031	18% ±2.1%
18242	19% ±4.7%
18033	16% ±0.7%
18292	13% ±0.2%
17850	0%

Table 3 SIV-directed lysis by infected macaques. PBMC taken from infected macaques at time of peak NK activity against K562 cells were used in standard chromium release assay against U937 cells infected with SIV.

Seronegative human donors	*K562+vacc-wt*		*K562+vacc-env*		*HIV-1 directed lysis*	
	(5 h)	*(20 h)*	*(5 h)*	*(20 h)*	*(5 h)*	*(20 h)*
Human 5	50% ±0.1%	68% ±1%	55% ±0.1%	78% ±1%	5% ±1%	10% ±1.2%
Human 6	21% ±2.2%	61% ±1%	27% ±1.1%	76% ±1.2%	6% ±3.3%	15% ±2.2%
Human 7	33% ±0.1%	60% ±1%	38% ±1%	77.7% ±1%	5% ±1%	17.7% ±1.2%
Human 10	37% ±0.1%	63% ±1%	43% ±1%	76% ±1%	6% ±1%	13% ±1.2%

18292 were also able to mediate SIV-directed lysis. Macaque 17850 did not exhibit any SIV-directed killing. In addition to the lack of SIV-directed killing, this macaque displayed poor pre-infection and post-infection NK activity, and had the fewest NK cells. This macaque had the most rapid progression to AIDS and the most severe CNS lesions.

In summary, several factors combined to generate effective NK responses: absolute number of NK cells, pre-infection NK activity, peak NK activity during the acute phase, and the ability to lyse targets in a SIV-directed manner. Macaque 17834 with the highest number of NK cells as well as the highest pre-infection NK activity and high acute phase NK activity did not develop any neurological lesions. In contrast, the other macaques that had fewer numbers of NK cells, less robust pre-infection NK activity, and lower acute phase NK activity developed moderate to severe CNS lesions.

To examine whether uninfected macaques were able to mediate SIV-directed lysis similar to the SIV-directed lysis seen in the infected macaques, PBMC from uninfected macaques were used as effectors in NK assays. Using SIV-infected U937 cells as targets, PBMC from two normal macaques exhibited SIV-directed lysis of 8 and 11% after 5 h of incubation that increased to 13 and 14% respectively after 20 h of incubation (Figure 3). These levels of SIV-directed lysis were not statistically different from those seen in infected macaques ($P > 0.05$).

PBMC from normal macaques mediated lysis of SIV-envelope expressing Raji target cells

Our *in vivo* results suggested that NK cells from infected and uninfected macaques mediated anti-SIV cytolytic effects. To extend this observation, PBMC from uninfected macaques were used in NK assays against targets expressing different SIV-antigens. The specific lysis of the *env*-expressing cells by uninfected macaque PBMC was 18±1.5% after 5 h incubation and 24±2.5% after 20 h incubation (Figure 4a). Lysis of SIV *gag*-expressing targets was not above background level. Thus, this non-MHC-restricted killing observed was directed against the *env* and not *gag* antigen.

PBMC from uninfected humans mediated SIV-directed lysis

Macaque NK cells are difficult to work with for several reasons. First, because of the animal size, only small amounts of PBMC can be obtained with one bleed. Second, macaque NK cells require three different markers, CD3, CD8, and CD16 for phenotypic identification (Carter *et al*, 1999).

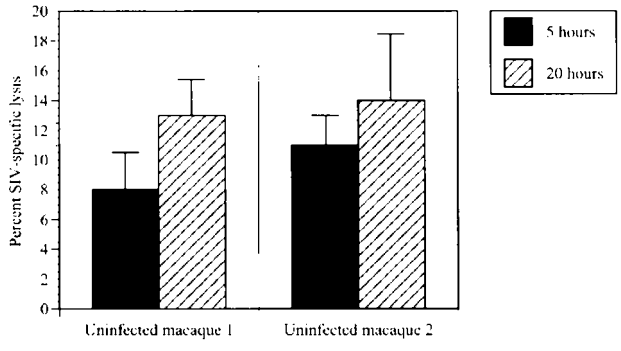

Figure 3 SIV-directed lysis was mediated by normal macaques. PBMC from normal, uninfected macaques were used as effector cells in a standard chromium release assay against U937 target cells that were infected with SIV. SIV-directed lysis was calculated as described in the previous figure. Lysis of U937 cells alone ranged from 21–28% for uninfected macaque 1 and from 14–21% for uninfected macaque 2. SIV-directed lysis by uninfected macaque 1 was 8±2% standard deviation after 5 h incubation and 13±1.49% after 20 h incubation. SIV-directed lysis by uninfected macaque 2 was 11±1.3% after 5 h and 14±4.8% after 20 h.

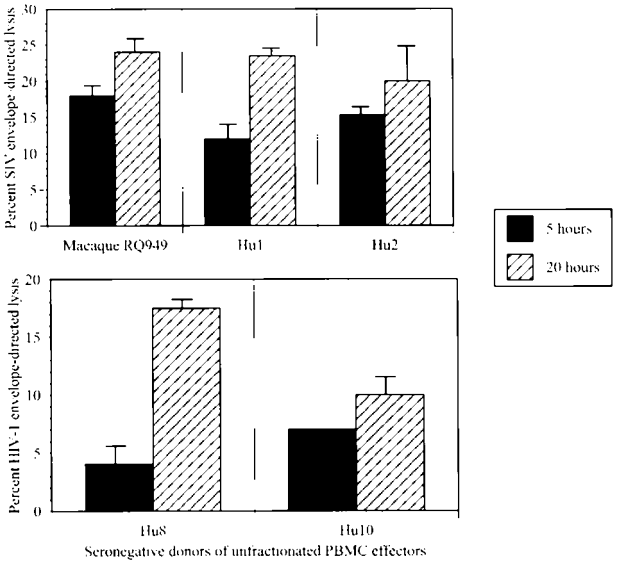

Figure 4 (**a**) SIV envelope-directed lysis by PBMC from several seronegative donors. Unfractionated PBMC were used in chromium release assays against Raji target cells that expressed SIV envelope using vaccinia virus constructs. Envelope-directed lysis was calculated by subtracting vaccinia wild type control lysis from vaccinia envelope lysis. Envelope-directed lysis mediated by macaque RQ949 was 18±1.5% after 5 h incubation and 24±2.5% after 20 h incubation. Envelope-directed lysis mediated by human 1 was 12±2.8% after 5 h incubation and 23.5±0.7% after 20 h incubation. Envelope-directed lysis mediated by human 2 was 15.3±1.5% after 5 h incubation and 20±5% after 20 h incubation. Lysis of vaccinia wild type-infected Raji cells (background lysis) was as follows: Human 1 13–18%, Human 2 14–18%, Macaque RQ949 42–76%. (**b**) HIV envelope-directed lysis mediated by PBMC from HIV seronegative humans. The envelope-directed lysis mediated by human 8 was 4±1.4% after 5 h incubation and 17.5±0.7% after 20 h incubation. The envelope-directed lysis mediated by human 10 was 7±0% after 5 h incubation and 10±1.4% after 20 h incubation. Envelope-directed lysis was calculated by subtracting vaccinia wild type control lysis (range 13–20%) from vaccinia-envelope lysis.

Because of these limitations, PBMC from leukophoresis of four normal human donors were used as effector cells. When unstimulated PBMC from healthy HIV seronegative blood donors were used as effector cells in NK assays against SIV-envelope expressing targets, SIV *env*-directed lysis by human 1 was 12±2.8% after 5 h incubation and 23.5±0.7% after 20 h incubation. Human 2 cells mediated SIV envelope-directed lysis at levels of 15.3±1.5% after 5 h and 20±5% after 20 h incubation (Figure 4a).

Since the human PBMC effector cells exhibited SIV-directed lysis that was similar to macaque PBMC effector cells, human PBMC were used in subsequent experiments to determine whether NK cells were the cell type mediating anti-SIV and anti-HIV cytolytic effects.

PBMC from HIV seronegative human donors mediated HIV-1 envelope-directed lysis

To investigate a possible role for NK cells in non-MHC restricted killing of HIV-1 envelope-expressing cells, we used PBMC from healthy HIV seronegative blood donors as effector cells against targets expressing HIV *env* (HIV-1 IIIB). Effector cells from two donors exhibited killing of target cells infected with recombinant vaccinia-HIV envelope above the level of killing observed in control cells infected with vaccinia wild type. These effector cells preferentially lysed the envelope-expressing target cells; envelope-directed killing above background level (i.e. vaccinia wild type) for seronegative human donor 8 was 4±1.4% after 5 h incubation and increased to 17.5±0.7% after 20 h incubation. Seronegative donor human 10 mediated HIV-envelope-directed lysis of 7% after 5-h incubation and increased to 10±1.4% after 20-h incubation (Figure 4b).

Envelope-directed lysis was not mediated by MHC class I-peptide complex

To test the hypothesis that T cell conventional envelope peptide-MHC complex was being recognized by the NK cell, we used K562, a well-established NK target cell that lacks MHC class I molecules on its cell surface, as the target 3cell for expressing HIV-envelope using a vaccinia expression system. PBMC from four HIV seronegative donors were used as effector cells. Four out of four humans mediated HIV envelope-directed lysis (Figure 5). These levels of *env*-directed killing were significantly higher than background killing of K562 cells infected with vaccinia-wild type alone. The background levels of vaccinia-wt killing as well as vaccinia-HIV *env* killing are summarized in Table 4. Thus, HIV-1 envelope-directed lysis was mediated efficiently in target cells lacking MHC class I molecules. This ruled out the possibility that *env*-directed lysis was due to peptide-MHC class I complexes.

T cells were not responsible for envelope-directed lysis

To exclude the possibility that lysis of HIV/SIV envelope-expressing cells was mediated by cytolytic T cells, we depleted CD3 positive cells using flow cytometric cell sorting. CD3 is expressed at high levels on all mature TCR alpha/beta and gamma/delta bearing T cells, but only on a trace population of NK cells (the 'NK-T' cells). PBMC that have been depleted of T cells lysed *env*-expressing Raji cells less efficiently than unfractionated PBMC, exhibiting 11% specific lysis compared to the 18% specific lysis of unfractionated PBMC at 20 h (Figure 6). We also obtained highly purified T cells by flow cytometric cell sorting and used this population in the chromium release assay. The highly purified T cells did not lyse SIV envelope-expressing Raji target cells or vaccinia wild type infected Raji target cells or K562 control target cells (data not shown). Taken together these data indicate that the lysis observed is not a T cell-mediated process.

Depletion of CD56 positive cells from PBMC completely abrogated envelope-directed killing

To determine whether NK cells were responsible for the non-MHC-restricted *env*-directed killing, we depleted the NK cell population from human PBMC using flow cytometry and anti-CD56 monoclonal antibody. Depletion of the $CD56^+$ NK cells from normal human PBMC completely abrogated *env*-directed killing (Figure 6). This result provided strong support that the NK cell is the effector cell responsible for *env*-directed killing. In a subsequent series of experiments we obtained highly purified NK (i.e. CD56 positive) cells from normal donor

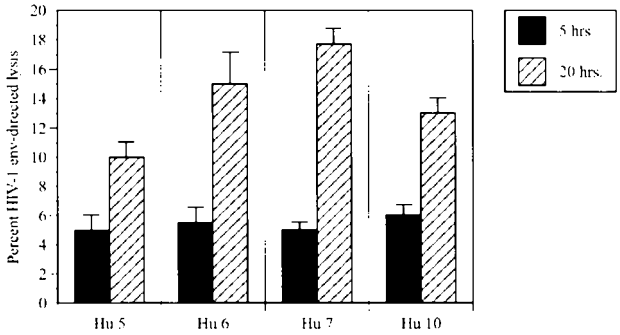

Figure 5 HIV envelope-directed lysis was not mediated by MHC class I/peptide complex. Unfractionated PBMC from four seronegative donors were used as effectors against class I-deficient K562 cells expressing HIV-1 envelope protein. E:T ratios used were as follows: Human 5: 25:1; Human 6: 25:1; Human 7: 50:1 and Human 10: 12:1. Levels of *env*-directed lysis were as follows: Human 5: 5±1% after 5 h incubation; 10±1.22% after 20 h incubation; Human 6: 5.5±3.3% after 5 h incubation; 15±2.2% after 20 h incubation; Human 7: 5±0.14% after 5 h incubation; 17.7±1.23% after 20 h incubation and Human 10: 6±0.69% after 5 h incubation; 13±1.23% after 20 h incubation.

Table 4 Levels of background lysis (K562 cells infected with vaccinia-wt) and envelope lysis (K562 cell infected with viccinia-*env*) are shown.

Seronegative human donors	*K562+ vacc-wt*		*K562+vacc-env*		*HIV-1 directed lysis*	
	(5 h) (%)	*(20 h) (%)*	*(5 h) (%)*	*(20 h) (%)*	*(5 h) (%)*	*(20 h) (%)*
Human 5	50±0.1	68±1	55±0.1	78±1	5±1	10±1.2
Human 6	21±2.2	61±1	27±1.1	76±1.2	6±3.3	15±2.2
Human 7	33±0.1	60±1	38±1	77.7±1	5±1	17.7±1.2
Human 10	37±0.1	63±1	43±1	76±1	6±1	13±1.2

These levels of background lysis is representative of the background lysis seen in other experiments in this study. HIV-directed killing was calculated as the difference between envelope lysis and background lysis. HIV-directed killing by seronegative donors is shown in Figure 5.

PBMC. We examined the ability of these cells to mediate *env*-directed lysis of Raji cells. Highly purified NK cells were unable to lyse the *env*-expressing target cells better than vaccinia-wt control targets. However, other groups have seen similar results with NK cells killing virus-infected targets (Fitzgerald-Bocarsly *et al*, 1988; Howell *et al*, 1993; Howell and Fitzgerald-Bocarsly, 1991).

These results suggested that some accessory factor(s), either cellular or humoral, was required to mediate *env*-directed lysis. The fact that our experiments were all carried out with cells from healthy HIV seronegative donors eliminated anti-HIV antibody as the accessory factor and antibody-dependent cell-mediated cytotoxicity (ADCC) as the killing mechanism.

Highly purified CD56$^+$ NK cells and unfractionated PBMC kill targets coated with whole HIV-1 gp120

To test the hypothesis that whole gp120 protein was being recognized on the target cells, U937 cells, which express high levels of CD4, were used for targets. The high level of CD4 expression on U937 cells allowed us to bind purified HIV-1 gp120 directly to the U937 cells resulting in high levels of the envelope glycoprotein on the cell surface. Unfractionated PBMC from three seronegative donors were used in a lytic assay against U937 cells coated with HIV gp120 (derived from HIV IIIB). All gp120-coated U937 showed high levels of surface gp120 binding as measured by flow cytometry. PBMC from multiple donors exhibited gp120 specific target cell lysis of 9.3±2.5% (Figure 7).

We next tested the ability of highly purified NK cells from healthy donors to mediate gp120-specific lysis. The CD56 positive NK cells efficiently lysed gp120 coated U937 cells with 17±1.8% specific lysis (Figure 7). When CD56 positive NK cells were depleted, gp120-specific lysis was completely abrogated. This experiment directly demonstrated that purified NK cells can recognize the gp120 envelope protein of HIV on cells and mediate anti-HIV cytolytic response.

NK lysis of gp120 coated autologous target cells

We tested the ability of bulk PBMC effector cells from three different normal human donors to HIV-1 gp120 bearing target cells without *in vitro* stimulation, and found that none of the donors exhibited killing of autologous targets, with or without HIV

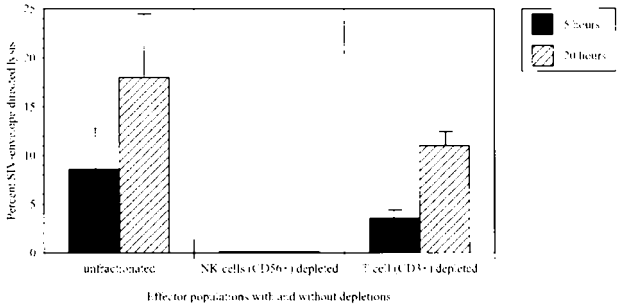

Figure 6 Depletion of CD56$^+$ NK cells abrogated SIV envelope-directed lysis, however, depletion of CD3$^+$ T cells did not abrogate envelope-directed lysis. Flow cytometric depletions of CD56$^+$ NK cells as well as CD3$^+$ T cells were performed. Unfractionated PBMC, NK cell depleted cell population, and T cell depleted population were used as effectors in NK assay. SIV-directed lysis by unfractionated PBMC was 8.6±4.1% after 5 h incubation and 18.5±6.4% after 20 h incubation. Depletion of T cells resulted in SIV envelope-directed lysis of 3.5±0.7% after 5 h incubation and 11.0±1.4% after 20 h incubation. Depletion of NK cells completely abrogated envelope-directed lysis.

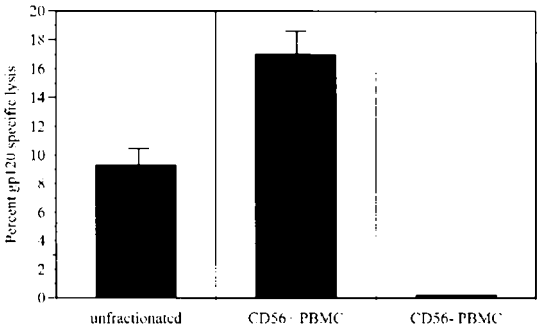

Figure 7 Purified CD56$^+$ NK cells from HIV seronegative humans mediated gp120-specific lysis. Unfractionated cells as well as CD56$^+$ NK cells mediated gp120-specific lysis of U937 target cells coated with gp120. gp120-specific lysis was calculated by subtracting background lysis of U937 cells alone from lysis of U937 cells coated with gp120. The coating of U937 cells with gp120 was confirmed by flow cytometry. Unfractionated PBMC lysed gp120 specifically 9.3±2.5%. Purified CD56$^+$ NK cells lysed gp120 specifically 17±1.8%. When CD56$^+$ NK cells were depleted, all gp120-specific lysis was abrogated.

gp120 (data not shown). Next we generated short-term NK cell lines from highly purified peripheral blood NK cells from one of the normal human donors, and tested the ability of these cells to lyse HIV gp120-pulsed autologous PBMC target cells. The results of the lytic assays are shown in Table 5. The NK cell line displayed robust lysis of gp120-pulsed target cells (approximately 86%). Interestingly, this cell line also demonstrated significant lysis of autologous PBMC target cells devoid of HIV antigen (44%). Still, the lysis of gp120 pulsed target cells was roughly 42% above the non-pulsed target cells. The NK cell line also displayed remarkable lysis of K562 cells (approximately 93%).

Discussion

An SIV model of rapid disease progression in pig-tailed macaques was used to address the role of NK cells in limiting the development of CNS lesions. In SIV-infected macaques, robust NK activity, both pre-infection and post-infection, as well as absolute NK cell numbers during the acute phase were important factors in limiting the development of CNS lesions. We found a perfect correlation between the robustness of pre-infection NK activity and the severity of CNS lesions that developed. Furthermore, macaques with high NK activity during acute infection developed no or only mild CNS lesions while macaques that did not mount significant NK responses during the acute phase developed severe CNS lesions. In addition to NK functional activity, the absolute number of NK cells and the ability of NK cells to mediate SIV-directed killing was found to be related to CNS disease progression. Macaques with higher numbers of NK cells had less severe CNS lesions than macaques with few NK cells. Likewise, macaques able to lyse their targets in a SIV-directed manner developed milder CNS lesions. The combination of these factors: pre-existing levels of NK lytic activity prior to infection, peak NK activity during the acute phase, absolute NK cell numbers in peripheral blood, and the ability to lyse target cells in a SIV-directed manner, were found to correlate directly with the severity of CNS lesions in infected macaques. These results suggest that NK cells play a role in controlling virus replication in the CNS.

There was a profound difference in the composition of lymphocytes within the brain parenchyma of animals with and without neurological lesions. The majority of cells in the brain of macaques with severe neurological lesions were NK cells and CTLs, as compared to uninfected macaques or macaques with no neurological lesions. In the latter groups the major lymphocyte subtype was the $CD4^+$ T lymphocyte. Since macaques with moderate to severe neurological lesions also had increased total numbers of lymphocytes and higher viral loads, this suggests that active viral replication in the brain may result in increased influx of inflammatory cells (NK and CTL) into the brain from the peripheral blood (Zink *et al*, 1999). The NK cells in the peripheral blood were not functionally active at the point of terminal infection. However, from this study we do not know whether the NK cells in the brain reflected those in the periphery at the time of sacrifice or whether these were cells that had entered the CNS at earlier points when the NK cells displayed lytic activity. If the NK cells were active, together with the CTL, their cytotoxic effects would contribute to the development of the typical CNS lesions observed. However, at earlier times after infection high levels of NK activity and the expansion of the NK cell population may be important in limiting virus replication in the CNS.

Our findings suggested that NK cells play an important role in limiting the development of CNS lesions, there are other factors that contribute to the development of CNS disease. Inherent genetic susceptibility and differing immunological composition are likely additional important factors that determine response to viral infection. In our macaque model, we focused our attention on one aspect of the immunological composition, namely, the NK cell and how it impacted the development of CNS lesions. The ability to eradicate NK cells over the course of infection would enable us to determine the full extent of NK cells' impact in CNS disease, however, the technology to deplete NK cells over a long period of time does not yet exist. In addition to eliminating NK cells in macaques, the genetic makeup of each infected macaque would have to be the same in order to rule out genetic susceptibility to SIV infection and subsequent immune response. In light of these limitations, we extended our *in vivo* findings to *in vitro* studies that were conducted to examine the phenomenon of non-MHC restricted cytolysis directed against the HIV/SIV envelope.

Table 5 A human NK cell line lyses autologous gp120-coated PBMC. NK activity of human NK cell lines against autogous PBMC uncoated and coated with gp120.

Effector & Target cell	*Experiment 1*	*Experiment 2*	*Mean* ± SD
Hu9 NK cell line vs. Hu9 PBMC	44.5	44.5	44.5 ± 0.0
Hu9 NK cell line vs. Hu9 PBMC+gp120	84.6	88.5	86.5 ± 2.7
Hu9 NK cell line vs. K562	92.5	93.9	93.2 ± 1.0

NK cell line lysis of autologous PBMC derived of HIV antigen (A) or pulsed with HIV gp120(B) measured by ^{51}Cr release effector to target ration (EIT) for each group was 9:1.

Only occasional studies of non-MHC restricted killing of HIV envelope-expressing target cells have been reported (Vowels *et al*, 1990; Yamamoto *et al*, 1990; Yin *et al*, 1999). In most instances, MHC class I-restricted responses are the primary focus. In those studies, however, the identity of the cell type mediating the phenomenon is frequently not clearly established. Although the MHC haplotypes expressed by our effector and target cells are unknown, the target cells were allogenic and xenogenic with respect to the human and macaque effector cells, respectively. Therefore, the *env*-directed killing observed was non-MHC restricted. The lytic activity was preferentially directed against the HIV or SIV viral *env* and not the *gag* gene product. These studies demonstrated that NK cells were responsible for the lytic activity because *env*-directed killing was observed by cells from HIV/SIV antigenically naïve subjects without *in vitro* stimulation to the effector cells. Furthermore, experiments combining flow cytometric cell sorting and functional cytolytic assays showed conclusively that the NK cell is the predominant mediator of this phenomenon. These studies demonstrated that the NK cell recognizes gp120 glycoprotein in a whole form rather than as peptide-MHC complexes.

Although NK activity is frequently demonstrated *in vitro* using MHC disparate effector cells and target cells, the physiologic role of NK cells *in vivo* in the context of viral infection is to lyse autologous virus-infected cells. Therefore, we addressed whether NK cells are capable of lysing autologous HIV *env*-bearing target cells. We found that 'resting' bulk PBMC from HIV seronegative donors were unable to lyse autologous target cells with or without HIV antigen. We thought that this result might be due at least in part to inhibitory influences exerted by other cell populations within the bulk PBMC (e.g. monocytes). We therefore successfully generated an NK cell line from one of the normal donors. We found that this cell line exhibited robust lysis of HIV gp120 bearing autologous target cells. The cell line also killed autologous target cells devoid of HIV antigen. We speculate that the culture conditions (e.g. removal of other cell populations, K562 feeder cells, and high levels of IL-2) may have provided activating signals to the NK cells that overcame the normal inhibitory signals provided by self MHC class I. We also suggest that this unusual activation state is reflected in the unusually high killing of the MHC class I negative K562 cells. The physiologic relevance of such an activated state of NK cells achieved in this experiment is not certain, but it seems plausible that viral infection may lead to such an activated state of NK cells.

The direct recognition of gp120 by NK cells was one of the most intriguing findings in this study. Like gamma/delta T cells, NK can directly bind and be activated by microbial antigens. One well-known example is that NK cells can directly bind to and inhibit the growth of cryptococcus (Hidore *et al*, 1991; Levitz and Dupont, 1993; Murphy, 1993; Murphy *et al*, 1993). Gamma/delta T lymphocytes are known to have the ability to directly recognize and be activated by 'unconventional' antigens, such as mycobacterial glycolipids, low molecular weight lipids, and nucleotides (Poquet *et al*, 1996; Sciammas and Bluestone, 1998). Of particular relevance is the ability of gamma/delta T cells to directly recognize whole HSV glycoprotein in a conformation-dependent fashion (Johnson *et al*, 1992). Studies by others have previously identified at least two mechanisms by which non-MHC restricted, envelope-directed killing may occur. Tyler *et al* (1989) demonstrated gp120-specific antibody dependent cellular cytotoxicity (ADCC) mediated by NK cells from HIV seropositive individuals. Yamamoto *et al* (1990) also reported such killing in SIV-infected rhesus macaques. The killing was detected specifically against B-lymphoblastoid cell lines infected with vaccinia-SIV envelope recombinant virus and using PBMC as effector cells. The killing was completely abrogated by depletion of the NK population. However, since they could only observe *env*-directed killing using NK cells from SIV seropositive animals, their conclusion was that the killing was mediated by antibody-armed NK cells (i.e. direct ADCC). The other mechanism is the induction of apoptosis when primary CD4 positive lymphocytes come in contact with HIV envelope-expressing cells, leading to the death of both participating cell populations (Heinkelein *et al*, 1995; Ohnimus *et al*, 1997). This is a non-cytotoxic mechanism that is perforin independent, fas/fas ligand independent, and TNF alpha/TNF receptor independent. It is dependent on a direct interaction between gp120 and the CD4 molecule (Ohnimus *et al*, 1997). A recent study extends these observations in the SIV/macaque system, and suggests that this may be the basis of non-MHC restricted, *env*-directed killing (Yin *et al*, 1999). While interesting in itself, this phenomenon appears unrelated to the one that we describe in the present study, as we clearly showed that depletion of CD3 positive T cells (which depletes both the CD4 as well as the CD8 positive lymphocytes) had little effect on *env*-directed killing.

In conclusion, this study demonstrated that NK cells can preferentially kill HIV/SIV envelope expressing target cells. This killing most likely occurs through direct binding of the NK cell to gp120 on the target cells. The NK receptor that is involved as well as the precise molecular mechanism of the receptor-gp120 interaction remains to be elucidated. Attempts to identify correlates of immune protection against HIV/SIV have been disappointing overall. Neither antibody nor con-

ventional CTL responses adequately predict clinical disease course, and vaccine approaches that focus on antibody and CTL induction have been largely unsuccessful. Future studies should address a possible role for the NK cell in restraining virus growth, and retarding HIV disease progression. In addition, augmentation of innate immunity in the context of vaccine development might be desirable. These studies demonstrate that early levels of NK cell activity in the peripheral blood may be one important factor in limiting the severity of CNS lesions perhaps by controlling virus replication in the CNS. However, the presence of increased numbers of NK cells in the brain of macaques with CNS lesions also suggests that these cells may contribute to the damaging inflammatory responses in the brain once virus replication is established. Thus, the impact of NK cell activity in the CNS may be both protective and inflammatory depending on the stage of infection. It is clear that the functional role of NK cells in the CNS during HIV and SIV infection needs to be further investigated.

Materials and methods

Inoculation of macaques

A total of nine pig-tailed macaques (*Macaca nemestrina*) were used in this study. Six were co-inoculated with SIV/Delta B670 (50 AID_{50}) and SIV/17E-Fr (10 000 AID_{50}). SIV/DeltaB670 is a swarm of dual-tropic viruses and SIV/17E-Fr is a cloned, recombinant, neurovirulent virus that was constructed by inserting the *env* and *nef* genes as well as the 3'LTR of SIV/17E-Br, a virus isolated from a macaque with encephalitis, into the backbone of $SIV_{mac}239$ (Anderson *et al*, 1993; Flaherty *et al*, 1997). Recombinant virus stocks were made by transfecting SIV/17E-Fr DNA into CEMx174 cell lines. The remaining three animals were mock inoculated with saline solution and used as virus-negative controls. Macaques were anesthetized with ketamine-HCl (Parke-Davis, Morris Plains, NJ, USA) and 10 ml of blood was drawn after infection at days 3, 7, 10, 14, 28, and every 2 weeks thereafter.

CD4 counts

Complete blood counts with differentials were performed on every blood sample and the absolute number of lymphocytes determined using a CellDyn 3200 hematology analyzer (Abbott). Mononuclear cells were separated on Percoll discontinuous gradient and labeled with fluorochrome-conjugated monoclonal antibodies (CD3-clone PR34, Pharmingen; CD4-Leu3a, CD8-Leu2, Becton Dickinson) to identify $CD4^+$ lymphocytes as previously described. Absolute $CD4^+$ cell counts were determined by multiplying the percentage of $CD4^+$ cells by the absolute lymphocyte count. The remaining PBMC were cryopreserved at each time point.

Viral RNA in plasma and brain

Viral RNA in the plasma was measured as an index of ongoing viral replication, using real time RT–PCR on an Applied Biosystems Prism 7700 Sequence Detection System, as described (Hirsch *et al*, 1996; Suryanarayana *et al*, 1998). Detection of RNA in the brain is described elsewhere (Zink *et al*, 1999).

Histopathology

Sections of CNS including frontal, parietal, temporal and occipital cortex, basal ganglia, thalamus, midbrain, medulla, cerebellum, and cervical spinal cord were examined microscopically in a blinded fashion by two pathologists (Dr M Christine Zink and Dr Joseph L Mankowski). To quantitate the severity of lesions, sections of frontal and parietal cortex, basal ganglia, thalamus, midbrain, and cerebellum were each given numerical scores of 1 (mild), 2 (moderate) or 3 (severe) using the following semiquantitative system. Sections with more than 30 perivascular macrophage-rich cuffs were given a score of 3, sections with 10–30 perivascular cuffs were given a score of 2, and those with less than 10 perivascular cuffs were given a score of 1. The scores for all sections were totaled and divided by 6 (six regions were graded for each brain) to give a mean score (out of a maximum of 3) for severity of CNS lesions.

Immunohistochemical detection of lymphocytes in brain

Polyclonal anti-CD3 (Dako) and monoclonal anti-TIA-1 (Coulter) were used in combination to identify lymphocyte subsets in sections of basal ganglia from the SIV-infected macaques by immunohistochemical staining. Cells that stained with TIA-1 (which identifies constitutive expression of cytotoxic granules in both NK cells and cytotoxic T lymphocytes) but not CD3 were classified as NK cells (Carter *et al*, 1999). Cells that stained with both TIA-1 and CD3 were classified as cytotoxic T lymphocytes. Cells that stained with CD3 but not TIA-1 were classified as CD4 cells. For each SIV-infected macaque, 200 cells in the basal ganglia and subcortical white matter of the parietal cortex were counted and the percentage of each type of cell calculated.

Immunohistochemical staining was performed using an Optimax Plus automated cell stainer (BioGenex, San Ramon, CA, USA). Briefly, Streck-fixed, paraffin-embedded tissue sections were deparaffinized and rehydrated, then post-fixed in Streck tissue fixative for 20 min. For antigen retrieval, tissues were rinsed in water and heated in a microwave in EDTA (0.001 M, pH 8.0) for 8 min. Endogenous peroxidase was quenched with 3% H_2O_2 in water for 10 min, then sections were blocked with buffered casein for 8 min. Anti-TIA-1 antibody was applied to the tissues for 30 min at

room temperature, the tissues were washed in wash buffer, and secondary biotinylated goat anti-mouse antibody (BioGenex) was applied for 20 min. The tissues were washed and streptavidin-HRP was added for another 20 min. Diaminobenzidine tetrahydrochloride (DAB) in buffer containing H_2O_2 was applied to the sections for 10 min. Anti-CD3 antibody was next applied to the tissues for 30 min at room temperature, the tissues were washed in wash buffer, and secondary biotinylated goat anti-rabbit antibody (BioGenex) was applied for 20 min. The sections were then washed and streptavidin-alkaline phosphatase was added for 20 min. Fast red in Tris buffer containing naphthol phosphate was applied to the sections for 10 min. The sections were washed in water and mounted with aqueous mounting medium.

NK lytic assay

To assess each macaque's longitudinal NK profile, PBMC were thawed for all time points from each macaque and then used in NK assays and flow cytometry. To control for day to day variation in the NK assays, an internal control of human PBMC from a single donor was used as effectors in every assay. K562 cells were obtained from the American Type Culture Collection (ATCC). 2×10^6 K562 cells were labeled with 250 μCi of chromium (Na_2CrO_4, Amersham, St. Louis, MO, USA) for 2 h, washed twice, and adjusted to 2×10^5 cells/ml in complete media. Effector PBMC were thawed for all time points for one animal and then rested overnight at a concentration of 2×10^6 cells/ml in RPMI-1640 supplemented with 10% FBS. Effectors were counted and resuspended at a concentration of 1×10^7 cell/ml. One hundred μl of effectors were combined with 100 μl of target cells for E:T of 50 : 1 and incubated at 37°C for 5 h. After 5-h incubation, 80 μl were removed and counted on a gamma counter (LKB Wallac, St. Louis, MO, USA). Total release was determined by lysing target cells with 0.1 N HCl and spontaneous release was determined by incubating target cells with RPMI-1640 media supplemented with 10% FBS. Specific lycis was calculated as follows: (experimental-spontaneous)/(total-spontaneous). Specific lysis was then expressed as percentage by multiplying by 100%. Spontaneous release was less than 15% of the total release.

Vaccinia infection of target cells

Raji cells and K562 cells were obtained from the ATCC. HIV-1 envelope (HIV-1 IIIB) vaccinia virus construct and its corresponding wild type construct used for background lysis were obtained from the AIDS Reagent program. SIV-envelope ($SIV_{mac}239$) vaccinia virus construct and its corresponding wild type construct used for background lysis were a generous gift from Dr Yilma at UC Davis. The methods used to make the SIV-envelope recombinant are described elsewhere (Ahmad and Menezes, 1996). 2×10^6 Raji cells or K562 cells were infected with vaccinia virus construct at a MOI=10 for 16 h and then labeled with chromium for use as targets in a standard NK assay. Envelope-directed lysis was calculated by subtracting the lysis of vaccinia wild type control cells from lysis of envelope expressing target cells. Because NK-mediated killing of target cells infected with certain viruses required incubation of effector and target cells for extended periods, we measured lysis following a 20-h incubation as well as a standard 5-h incubation (Ahmad and Menezes, 1996; Fitzgerald *et al*, 1982; Fitzgerald-Bocarsly *et al*, 1988).

SIV-directed lysis

U937 cells, obtained from the ATCC, were transfected with SIV/17E-Fr DNA and infectious virus was allowed to spread throughout the flask. Once U937 cells were infected, they were used as target cells in a standard chromium release assay, as described above. Lysis of U937 target cells alone was used as background level and was subtracted from the lysis of SIV-infected U937 to obtain SIV-directed lysis. An E:T ratio of 50 : 1 was used in NK assays with all pig-tailed macaque PBMC effector cells except for macaque 17834. This macaque exhibited such high background lysis of U937 cells (>95%) that the SIV-directed lysis could not be measured accurately. The E:T ratio was thus reduced to 25 : 1.

Enumeration of NK cells

Macaque NK cells was enumerated using CD3, CD8, and CD16 markers. Although CD56 is a reliable marker for human NK cells, CD56 does not mark macaque NK cells (Carter *et al*, 1999). PBMC were stained with directly conjugated monoclonal antibodies CD3-FITC (Pharmingen), CD8-PE, CD16-PECy5 (Becton Dickinson, San José, CA, USA) for 15 min, washed with PBS, and analyzed on FacsCalibur flow cytometer (Becton Dickinson, San José, CA, USA) using the Cell Quest program. NK cells were defined as CD3-negative, CD8-positive, CD16-positive cells within the lymphocyte gate.

Sorting purified NK cells and other populations

CD56 (NCAM) – PE monoclonal antibody (Becton Dickinson, San José, CA, USA) was used to detect human NK cells. $CD56^+$ cells within the lymphocyte gate were sorted on a Coulter Epics ELITE flow cytometer with ELITE software. The purified NK cells (>98% purity) were cultured overnight in complete RMPI media without activation and used the next day as effector cells. Populations of $CD3^+$ and $CD3^-$ lymphocytes (>95% purity) were obtained in the manner described above by using CD3-FITC monoclonal antibody (Becton Dickinson, San José, CA, USA).

Depletion of NK cells
CD56-PE (Becton Dickinson, San José, CA, USA) was used to detect human NK cells within the lymphocyte gate. $CD56^+$ NK cells were discarded and the remaining $CD56^-$ lymphocyte population (>98% purity) was cultured overnight in complete RPMI media without activation (without PHA) and used the next day as effector cells.

HIV-1 gp120-coating of targets
U937 target cells were obtained from the ATCC and recombinant gp120 with proper conformation and glycosylation was obtained from Advanced Biotechnology Incorporated (ABI). U937 cells were coated with gp120 in serum-free RPMI-1640 with gentle shaking at 4°C to prevent internalization for 30 min followed by two washes with serum-free RMPI-1640. HIV gp120-binding was detected using polyclonal sera from a seropositive patient as a source for primary antibodies and goat anti-human FITC-conjugated secondary (gift from James Hildreth, Johns Hopkins School of Medicine) and analyzed on a Becton Dickinson FACSCalibur flow cytometer using Cell Quest software. The HIV gp120 binding population was brighter than the control population stained with secondary antibody only, thus ruling out non-specific binding.

Human NK cell line
A short term NK cell line was generated from a normal human donor. Peripheral blood mononuclear cells were obtained from peripheral blood by centrifugation over Ficoll-Hypaque. Highly purified NK cells were obtained by flow cytometric cell sorting of CD56 positive; CD3 negative lymphocytes. The purity of the resulting NK cells was 96%. The purified NK cells were then cultured in RPMI containing 1000 U/ml recombinant human IL-2 (Sigma, St. Louis, MO, USA) and 10% fetal calf serum. On the third day of culturing and every 72 h thereafter, $1 \times 10^{(5)}$ gamma irradiated (5000 Rads) K562 cells were added to the cultures as feeder cells with 100 U/ml fresh IL-2. The cultures were maintained in this fashion for approximately 3 weeks before use in lytic assays.

Statistical analysis
To determine whether there was correlation between CNS lesion severity and NK activity prior to and after infection, the six macaques were ranked for level of NK activity and severity of CNS lesions. Spearman's rank correlation test was used to determine the degree of correlation between each measure of viral load and the severity of CNS lesions. Kendall's tau test was used to determine the significance of each correlation. The Mann-Whitney U test was used to determine whether the level of SIV-directed lysis was significantly different between uninfected and infected macaques.

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