Natural killer cells as novel helpers in anti-HSV immune response

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Running title: NK cell as helpers

Key words: NK cell, memory T cell, Virus, avidity and rescue

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Abstract

Innate defenses help eliminate infection, but some of them also play a major role in shaping the magnitude and efficacy of the adaptive immune response. With regard to influencing subsequent adaptive immunity, NK cells aided by DCs may be the most relevant components of the innate reaction to HSV infection. We confirm that mice lacking or depleted of NK cells are susceptible to HSV induced lesions. The quantity and quality of CD8+ CTLs generated in the absence of NK cells were diminished; thereby contributing to susceptibility to HSV induced encephalitis. We demonstrate a novel helper role for NK cell in that, NK cells compensate for the loss of CD4 helper T cells and NK cell supplementation enhanced the function of wild type anti-HSV CD8 T cells. In addition, NK cells were able to partially rescue the dysfunctional CD8+ T cells generated in the absence of CD4 T helper cells, there by performing a novel rescue function. Hence, NK cells may well be exploited for enhancing, rescuing T cell response in situations where CD4 helper response is affected.
Introduction

We have known since the pioneering studies of Lopez that NK cells influence susceptibility to HSV infection (4). Most notably, susceptibility to severe herpes infection occurs in humans with genetic defects in the NK cell response (3), and in mice genetic susceptibility differences may be explained by NK cell functional differences (39), but details of this genetic control remain confused (28). We know that the NK cell response and perhaps also NK-T cells (15), helps prevent initial infection, but for HSV it is not clear if the virus-NK cell interaction affects the pattern of subsequent adaptive immune response. It has become quite evident that the NK cell system is diverse and that several phenotypic and some functional subsets exist (41). The basis of the phenotypic diversity is explained by the multiple receptors expressed on NK cells, some of which are expressed on all NK cells and others limited to different subsets (41). Some of them are also expressed on CD8+ alpha beta T cells (34).

The NK cell receptors fall into two major functional groups: inhibitory receptors and activating. The inhibitory receptors mainly recognize MHC proteins, and when such ligands are diminished, such as can occur on a virus infected or tumor cell, the inferior signal stimulation results in NK cell activation (31). This “missing self” hypothesis could explain why HSV infected cells, which does down regulate MHC molecules (37), result in NK cell activation. NK cells additionally express many activating receptors which may be engaged directly by viral proteins as has been observed with MCMV. Here it was shown that the CMV M157 protein binds to the activating receptor Ly-49H and that mice strains that encode Ly-49H are resistant to infection (1). It is not known if other herpes viruses such as HSV encode proteins that engage NK activating receptors but doubtless the search for such molecules is ongoing. Since HSV expresses both TLR9 and TLR2 ligand activity(23, 27) conceivably HSV could also interact with NK cells by acting as a ligand for TLR2 or TLR9, both of which are present on NK cells (45). Additionally the ability of HSV to induce IL-12 or other pro-inflammatory cytokines could act as means of NK cell activation. These NK promoting activities may in turn promote skewing of adaptive immune response to a protective Th1 phenotype. Furthermore, activated NK products may contribute towards avidity maturation of responding T cells such as inducing them to become multiple cytokine producers (9).
human and mouse studies suggest that NK cells may also contribute to adaptive immune responses by modulating DC function or by producing effector cytokines (7, 33). In mice, NK cells were shown to be important for inducing T\textsubscript{H}1 responses and for early resistance to infection (50). Depletion of NK cells inhibited the generation of Toxoplasma specific CD8+ T cell immunity in CD4/-/- mice and adoptive transfer of NK cells restored their CD8+ T cell response (6). However, it is not clear if a similar series of events occur with HSV infection.

The enhanced NK activities after HSV-1 infection have been well documented both in vitro and in vivo (10, 12, 16, 26); but, the molecular mechanism(s) responsible for this innate immune response has not been fully investigated. Moreover, NK cell functional biology is much more complex than previously thought [reviewed in (18)]. NK cell are for example highly interactive, with several cell type that include DCs, B cells and perhaps T cells. Accordingly, in CMV infection NK cells were shown to differentially modulate different type of DCs (42). While the function of plasmacytoid DCs (pDCs) was limited by the NK cells, the conventional DC (cDCs) compartment was promoted or preserved leading to accelerated antiviral CD8 T cell responses (42). Our study extends the findings to the direct helper role by NK cells that skews the immune response to Th1 during priming and also highlights their ability to compensate for the loss of CD4 helper T cell mediated grooming of CD8 T cells.

Our results presented in this article suggest that the resistance pattern observed in C57BL/6 can be altered if NK cell were depleted. In addition, we demonstrate a novel helper role for NK cells in that NK cell produced cytokines (IFN\textgamma and/or IL-15, IL-12) contributes to effective adaptive immune response generation and helps in CD8+ CTL avidity maturation. Although the site of interaction and the actual viral protein ligand that activate the NK cell is not known, the data strongly supports the notion that HSV induced productive interaction of DC-NK cells as the probable mechanism. Increase in NK cell number at the site of immune induction and its additional novel non-cytotoxic helper role in during immune induction and memory recall augments the anti-HSV immune response.
Materials and Methods:

Mice

C57BL6 mice were purchased from Harlan Sprague Dawley, Indianapolis, Indiana and maintained according to the Guide for the Care and Use of laboratory Animals (National Academy Press, Washington DC, 1996). Animals were kept in specific pathogen-free conditions in the Division of Animal Resources, College of Medicine, East Tennessee State University which is fully accredited by the American Association for Accreditation of Laboratory Animal Care.

Virus:

HSV-1 KOS and HSV-1 17 strains of viruses were titrated after growing them on Vero (CCL81; American Type Culture Collection, Manassas, VA) cell lines. The virus was stored in aliquots at -80 until required.

Cell Lines:

Vero and Yac cell lines were obtained from ATCC. The Vero cells were maintained in DMEM supplemented with 10% heat inactivated FBS, 100u of penicillin G/ml, 100 μg of streptomycin sulfate/ml and 2mM L-glutamine. The YAC cells were maintained in RPMI supplemented with 10% FBS, HEPES, sodium bicarbonate and 2.25g Glucose/liter.

Infection of mice:

The zosteriform infection was performed as described earlier(20). Briefly, hair was depleted from the skin dorsal to the posterior tip of the spleen, corresponding to the tenth thoracic dermatome by using Veet hair removal gel cream after anesthetizing the mice using avertin. The skin was then scarified using Dremel Variable Speed Rotary Tool and 20µl of HSV-1 17 containing $10^5$ PFU of virus was applied to hair depleted area of the skin and massaged. The other route of infection adopted was the tail vein, wherein a mouse restrainer was used. The footpad and the intra-peritoneal routes of infection were also adopted based on the need of the experiment. All animal
experiments were performed in agreement with American Association for Accreditation of Laboratory Animal Care.

**HSV and peptide-specific lymphoproliferation**

Splenocytes from experimental mice were restimulated in vitro for assessing proliferative ability as described earlier (22). In brief, responders were stimulated with either peptide-pulsed or virus-infected APCs for 3 days. The controls included stimulators with anti-CD3 and naïve stimulators. The last 18 h of the incubation was done in the presence of [³H]thymidine. After incubation, plates were harvested and read using the Inotech cell harvester and reader (Inotech, Biosystems International, Lansing, MI). Proliferative responses tested in quadruplicate wells were expressed as mean cpm ± SD.

**In Vitro CTL Assay:**

The assay was performed as described previously (21). In brief, the splenocytes were serially diluted and added to 96-well U-bottomed plates containing ⁵¹Cr-labelled SSIEFARL-pulsed MHC-matched MC-38 (H-2⁵b), which served as the target cells. Uninfected MC-38 and HSV infected EMT6 (H-2d) were used as syngeneic uninfected and allogeneic infected controls. The effector: target ratio was 90:1, 30:1, 10:1 and 3:1. After incubation for 4 hrs at 37°C, the culture supernatant was collected and analyzed for radioactivity by scintillation counter. Spontaneous release of ⁵¹Cr was determined by incubating the target cells with medium alone, and maximum release was determined by adding Triton X-100 to a final concentration of 5%. The percentage of specific lysis was calculated as: 100 x [(experimental release - spontaneous release) / (maximum release - spontaneous release)].

**Tetramer staining and flow cytometry:**

MHC class I (H-2⁵b) tetramers to measure SSIEFARL-specific T cells were provided by the NIAID MHC Tetramer Core Facility (Atlanta, GA). A total of 10⁶ cells obtained from various mice were stained with a mixture of fluorescein isothiocyanate (FITC)-labeled,
PerCp labeled appropriate markers as needed in combination with phycoerythrin (PE)-labeled tetramers for 45 min at 4°C. The controls included isotype control, stained cells, and unstained cells. They were then analyzed by using a FACScalibur machine and FCS software. The cells were gated on HSV-gB tetramer positive cells and analyzed for various markers to determine HSV specific events.

**Flow Cytometric Analysis:**

10^6 Splenocytes /ml were incubated with antibody to lysosome associated membrane proteins CD107a and CD107b (FITC labeled) for an hour, followed by the activation with the SIEFFARL peptide along with the addition of the secretion inhibitor monensin (Golgistop), followed by incubation for another 4-5 hours. After incubation, the cells were stained for surface markers using anti-CD8 (FITC, PerCP or APC), anti-CD4 (FITC), anti-CD3 (FITC), anti-NK1.1 (PE), anti-Granzyme(alexaflour 647) and anti-CD69 (FITC). The cells were then fixed and permeabilized using perm-fix and perm wash, followed by 2x washing. The cells were then stained for intracellular markers with anti-IFNγ (PE), IL-2 (PE), TNFα (FITC) and IL-15Ra (PE). The samples were analyzed using FACScalibur 4 color flow cytometer and the data were analyzed using Cellquest or FCS Express software.

**NK Cell purification for adoptive Transfer:**

Splenocytes of C57Bl/6 mice were RBC lysed and washed with RPMI and plated on Petri dish for 1 hr at 37°C. The non-adherent cells were collected and further purified for NK cells using the magnetic bead columns (BD Biosciences) by negative selection principle. The fraction eluted out of the column was > 95% pure for NK cells (CD3^- NK1.1^+ cells). The NK cells were exposed to 10 μL (/1x10^6 cells) of rIL-2, washed thoroughly and then adoptively transferred to B6 mice via the tail vein.

**In vivo CD4 T Cell and NK Cell Depletion:**

The CD4 T cells depletion was performed as described earlier (13). To deplete CD4 T cells, mice received 250 μg of clone GK1.5 monoclonal antibody (GK 1.5 hybridoma;
ATCC TIB207, Rockville, MD) on day 0, 12 and 15. Ascitic fluid containing rat IgG2b antibody served as isotype control. FACS staining was performed to determine the efficacy of CD4 T cell depletion. The NK cells were depleted using a polyclonal antibody against the asialo ganglio-N-tetraosylceramide (asialo GM1) (Wako), expressed at high levels on NK cells or anti-NK1.1 monoclonal antibody. The mice were given a 40 µl dose of the antibody i.p. 5 days p.i the ability of the antibody to deplete the NK cells was measured by assessing NK cell functional activity on YAC cells.

In vivo assay for CTL activity measure:

Splenocytes from the different groups of mice were stained with CFSE (Molecular Probes, Eugene, OR) and 5 x 10⁶ cells of each population adoptively transferred via the tail vein route into the corresponding groups of mice. The spleen was isolated from the mice after 5 hours and the lymphocytes were isolated from the spleen as previously described (20). Target cells were distinguished from recipient cells based on CFSE staining. Histogram plots were used to demonstrate the difference in separation pattern based on intensity of CFSE staining. The recovery and percent killing of the various CFSE-labeled peptide-pulsed targets were calculated as follows: 100 - {[(percentage of peptide pulsed in immunized/percentage of unpulsed in immunized) / (percentage of peptide pulsed in unimmunized/percentage of unpulsed in unimmunized)] x 100}.

Statistical analysis:

The statistical analysis was performed using the SPSS package version 1.5 and Graphpad Prism 4. Mann-Whitney U test and anova methods were used to arrive at the statistical significance. The p values are provided in each figure and table.
Results

Effect of NK cell depletion on susceptibility to HSV:

NK cells have been known as killers of virus infected cells but their ability to assist the T cell responses especially those mediated by CD8+ T cells has not been evaluated in detail. To measure the influence of NK cells on shaping the primary T cell responses to HSV, we compared normal B6 mice with those depleted of NK cells using the antibody anti-NK1.1 (PK136) or anti-asialo GM1 Ab. The efficacy of depletion was measured both by FACS staining (FITC-anti-CD3 and PE-NK1.1 antibodies) and killing assay to measure NK cell mediated cytolytic activity. As shown in figure 1a, the NK cell activity, as measured by its ability to lyse YAC cells, was significantly diminished in both the anti-asialo GM1 mAb and PK136 administered groups compared to controls given IgG or untreated control animals. In addition, flow cytometric analysis of the FACS stained cells revealed that the CD3−NK1.1+ cells, that are indicative of NK cells, were below detection limits in anti-asialo GM1 and PK136 administered mice (figure 1b). In contrast, the NK cell (CD3−NK1.1+) numbers were 3±1.5% and 4± 2% in the untreated mice and control IgG recipients respectively. The data presented above was observed 48hr after administration of 50ul of anti-asialo GM1 Ab (Wako) or 1mg of PK136 and was the optimal time and dose, established by dose response and kinetics of depletion studies. Both antibodies were effective in depleting NK cells; however we shall use PK136 mAb in experiments involving CD8+ T cell responses since some of them are known to express GM1 on their surface (14, 46). In addition, wherever applicable and meaningful, PK 136 transgenic mice (PK136 Tg mice) that are chronically depleted of NK cells were used in parallel in support of our findings.
Based on the kinetics and dose ascertained previously, the C57BL/6 mice were either depleted of NK cells (1mg of anti-NK1.1) or given control IgG 2 days before being challenged with a sub-lethal dose of HSV using the Zosteriform skin infection model (21). This model, as previously demonstrated (20, 21), depended on all arms of immunity for complete protection. Additional controls included mice that received adoptive transfer of one million MACS column purified NK cells and PK 136 Tg mice. Low dose and high dose challenge of the virus (HSV-1 17) were performed. The low dose did not cause any fatalities in control mice but may induce mild skin lesions (lesion score of 2). However, the high dose will cause death in naïve control mice but should be resisted by HSV immune and NK cell supplemented mice. These doses were chosen based on our previously published dose response studies (30). The NK cell depleted mice showed early onset of diseases (skin lesions-pustules-lesion score <2) and as shown in figure 1c, all of them succumbed to encephalitis by day 10. The control group suffered only minor lesions except for one which developed severe encephalitis and was sacrificed on day 12. The lesion score in NK depleted animals compared to control IgG administered animals was always significantly higher on any day until day 10 (p <0.0001).

To further confirm the protective effect, Wild type B6 mice were adoptively transferred with varying numbers of magnetic column purified NK cells (1x10^4 – 5x10^6) and challenged 15 hours later with high dose of HSV1-17 virus. The mice that did not receive any NK cells succumbed to challenge with the high dose of virus, while animals that received additional NK cells showed a decrease in lesion score with increase in NK cell transferred (figure 1d). A million NK cells provided the optimum protection and
hence this number was routinely used in subsequent experimentation. Taken together
the data indicate that the PK136 mAb effectively depleted NK cells and that the absence
of NK cell lowers the threshold of protection. Increasing the number of NK cells by
adoptive transfer also increased their innate resistance and possibly also contributed to
better or augmented anti-HSV adaptive immune responses.

**NK cell orchestrates anti-HSV adaptive immune response**

*NK cell stimulated by HSV polarizes primary immune responses to a protective Th1
type:*

Results accrued from the challenge model described earlier portray the negative impact
of NK cell loss on immune protection. To analyze the influence of NK cell on the
adaptive immune response, the frequency and function of T cells responding to HSV
were compared in intact mice to those depleted of NK cells prior to infection with HSV.
Splenocytes from the control or depleted animal were stimulated in vitro with uv
inactivated HSV for 16 hr or stimulated for 5 hr with HSV gB_{498-505} peptide (SSIEFARL)
respectively to analyze the HSV specific CD4+ T cells or CD8+ T cell phenotype and
function. As depicted in figure 2a, the frequency of CD4+ IFN$\gamma$+ T cells was reduced
about 3-4 fold in NK cell depleted mice compared to control IgG treated animals. A
similar effect was also observed with the HSV specific CD8+ T cell response. While the
frequency of CD8+ IFN$\gamma$+ T cell was 8±1.9 % in control animals, it was approximately
two-fold less (3.18±1%) in NK depleted mice. The frequency of CD8+ IL-2+ T cells was
1±0.3 % in the absence of NK cells compared to 2.8±0.7 in control animals. The
frequency of HSV specific CD4+IL-4+ and CD4+IL-10+ T helper type 2 (Th2) cells were
also analyzed in NK cell depleted and control mice. There was a slight decrease in the frequency of Th2 type cells in NK depleted mice in response to HSV infection (data not shown). However, the difference was only significant in CD4+IL-10+ Th2 cells. The frequency of anti-HSV specific CD4+IL-10+ cells was 1.13 ± 0.06% in NK depleted compared to 0.39 ± 0.03 in control animals. Although, it is tempting to suggest a role for these CD4+IL-10+ cells but its reduced number in comparison to Th1 type cells may not entirely contribute to diminished Th1 response in the absence of NK cells. Nevertheless, in all instances the TH1 and Tc1 cytokine secreting cells was significantly less in NK depleted mice.

**NK cell contribute to efficacious CD8+ CTL generation:** Since, there was an overall dampening effect in the immune response by NK cell depletion, it was presumed that the cytolytic ability of the virus specific CD8+ T cell would also be affected in vivo. Hence, an in vivo CTL assay was adapted to measure the ability of the HSV infected mouse (NK depleted and NK intact) to clear or lyse peptide pulsed syngeneic targets that were adoptively transferred. As expected (figure 2b) the cytolytic ability was 48±6 % in NK cell depleted mouse compared to 74±9 % in control IgG given mouse. This indicates that the CD8+ CTLs were less efficient in their cytolytic ability in vivo if they were primed in the absence of NK cells. These data further lend support to our hypothesis that NK cells play a pivotal role in setting the stage for adaptive immune response and possibly also affect the subsequent memory generation as well.

**Diminished response to HSV in PK 136 Tg mice:**

The PK 136 transgenic mice (49), were infected with HSV and the subsequent acute and memory CD8+ T cell response compared to control animals. The mice were
sacrificed on day 8 for acute phase analyses and for memory recall studies primed animal were given a low recall dose of virus at day 40 and analyzed 5 days later (d45). Flow cytometry based assays were performed on single cell suspensions. As shown in figure 3, significant differences in the frequency of HSV specific CD4+ and CD8+ T cells was observed between the wild type and PK 136 Tg mice indicating that the expansion of T cells was not optimal in the absence of NK cells (PK136 Tg mice). Similar difference could be observed when the quality of the T cell was analyzed. Accordingly, 50 % more CD4+ T cells responded by producing both IL-2 and IFNγ upon in vitro stimulation with UV inactivated HSV in the wild type mice compared to PK 136 Tg mice (figure 3a).

Even more dramatic effects on the quality of CD8+ T cells could be seen in the PK136 Tg mice compared to wild type mice. As illustrated in figure 3b, the frequency of CD8+ T cells that were induced to produce both IFNγ and TNFα was about 40% more in the wild type mouse (%CD8+ IFNγ+ TNFα+ = 10%) and it differed significantly from the PK 136 Tg mice (%CD8+ IFNγ+ TNFα+ = 5.8%). There was a similar difference in the quality at the acute phase of infection as well, but it was more pronounced during memory recall response.

The cytolytic ability of the CD8+ T cells as measured by the expression of CD107a and b and granzyme B was not significantly different between the two groups at the acute phase (figure 3c). Nevertheless, a dramatic difference was observed during memory recall response. Accordingly, 3.5 times more wild type HSV specific CD8+ T cells expressed CD107a and b compared to HSV specific CD8+ T cells obtained from PK 136 Tg mice. Similarly about 4% of HSV specific PK 136 Tg CD8+ T cells expressed
granzyme B compared to about 12% of wild type CD8+ T cells. This kind of distinct
distinction in the diminished quality of memory CD8+ T cells in the PK 136 Tg mouse
implies the need for NK cell mediated effects for optimal memory anti-HSV CD8+ T cell
response.

**NK cell play pertinent role during recall responses and can partially compensate
for CD4+ T cell absence:**

The data analyzed at primary phase suggested that the lack of NK cells affected
the T cell responses and it is possible that it would have a ripple effect on establishment
of memory. In addition, no information exists as to the helper role of NK cells during
recall or secondary response or in the event of reactivation from latency. To analyze if
NK cell function impinges on secondary recall responses, especially the protective
CD8+ T cell compartment, mice that were primed in the absence of NK cells or
supplemented with NK cells (IL-2 activated) were analyzed at the memory phase along
with appropriate controls. In addition, the role of NK cells during recall with low dose
virus (1x10^5 pfu) challenge was also assessed in HSV memory mice by depleting NK
cells before challenge. As an initial step, fate of adoptively transferred NK cells was
assessed. Accordingly, donor NK cells were labeled with CFSE before transfers into
naïve, HSV infected and NK depleted hosts. The recipient animals were sacrificed at
different time points and their spleens harvested. The single cell suspensions were
analyzed for CFSE^+ NK1.1^+ cells. The NK cells expanded (CFSE dilution) depending on
‘NK space’. In that, NK cells expanded when it had space to do so (for example in NK
depleted and naïve mice). The expansion was about 20% of the original numbers. NK
cells \((10^6)\) transferred to NK depleted mice expanded 2-3 fold. However, the number remained static in wild type mice with ongoing HSV infection. Therefore, there was no significant expansion in HSV infected, but they still remained viable and were able to secrete cytokines (data not shown). The behavior was similar to a T cell in this regard as reported earlier\((40)\). The responding CD8+ T cells were analyzed in these animals for their cytolytic activity, cytokine secretion, cytokine receptor expression and avidity maturation. The cytolytic ability (Table. 1), as measured by in vitro CTL assay at 90:1 effector: target ratio was 1.8 fold reduced in NK cell depleted mice compared to control animals. The percentage lysis in the NK supplemented mice was 1.2 fold higher compared to control animals. The greatest impact was seen in mice that were depleted of CD4+ T cells. It was 1.4, 2.7 and 3.1 fold reduced compared to NK depleted, control and NK supplemented groups respectively. The differences between the groups were more pronounced at 30:1 effector:target ratio. Besides, confirming our previous finding that lack of concomitant anti-HSV CD4 response affects the quality of HSV specific CD8+ CTLs \((20, 22)\), it also extends the observation that a similar effect on CD8+ T cells may also be observed with the loss of NK cells. Hence, supplementing or activating NK cells may help during re-immunization of primed animals.

To test this idea, spleens cells isolated from control, NK cell depleted (NK\(^{-}\)), CD4 depleted (CD4\(^{-}\)), CD4 depleted NK supplemented (CD4\(^{-}\)NK\(^{++}\)) and normal mice were stimulated with SSIEFARL peptide to measure the outcome in terms of frequency and function of HSV specific CD8+ T cells. The CD8+ T cells that were stimulated in the presence of cognate peptide and IL-2 for 5 hours were stained for IFN\(\gamma\) intracellularly. As seen in figure 4, the percentage of CD8+ IFN\(\gamma\)+ T cells was 4.1 ± 1.2, 5.2 ± 1.5, 8 ±
2.1, and 11.2 ± 2 in CD4\(^{-}\), NK\(^{-}\), CD4\(^{-}\)NK\(^{++}\), and NK\(^{++}\) groups respectively. (NK\(^{++}\) Vs CD4\(^{-}\) and NK\(^{-}\) = P <0.005; NK\(^{++}\) Vs CD4\(^{-}\)NK\(^{++}\) = P<0.01). Although, the frequency of CD8+ IFN\(_\gamma\)+ was higher in NK\(^{++}\) group compared to CD4 NK\(^{++}\) group the difference was not significant. The frequency of CD8+IFN\(_\gamma\)+ cells was 8.8 ± 2 in animals that received the control IgG. The results suggests the following, NK cell contribute to the functional efficacy of virus specific memory CD8+ T cells; NK cell supplementation has an enhancing effect on memory CD8+ T cell function and lastly NK cell may also contribute to partially compensate for the loss of CD4+ T cell.

**NK cell cytokines may contribute towards functional improvement and avidity maturation of CD8+ CTLs:** In an attempt to further support this notion, HSV induced memory mice were divided into four groups; 1. NK cell depleted (NK\(^{-}\)) 2. CD4+ T cell depleted and NK supplemented (CD4\(^{-}\)NK\(^{++}\)), 3. NK cell supplemented (NK\(^{++}\)) and 4. given control IgG. These mice were then given HSV (1x10\(^5\) pfu) to recall CD8+ T cells. Five days later the spleen cells were harvested and analyzed for their CD8+ T cell function that included IFN\(_\gamma\) production, cytokine receptor expression (IL-15R\(_\alpha\)) and avidity maturation (CD8\(^\beta\) expression). As can be seen in figure 5, the percentage of CD8+ IFN\(_\gamma\)+ T cells was 4±1.3% in NK depleted mice; 7.3±1.7 % in CD4 depleted and NK cell supplemented; 11±2.4 % in NK supplemented group; and 9.2±2 % in controls given IgG. In comparison to the control group there was a 50% reduction in CD8+ T cell function in NK depleted mice. However, upon compensation (adoptive transfer) with additional NK cells, the CD4 depleted mice showed an increase of 55% in CD8+ T cell function but this was still 20% below the control group. Interestingly, normal memory mice supplemented with NK cells showed an approximate 20% increase in CD8+ T cell function when compared to
control mice. Additionally, the CD8+ T cells were separated as high, intermediate and low producers of IFNγ in the same FACS dot plot. A quarter of the CD8+ IFNγ+ T cells were high producer of IFNγ in the group that received the NK cell supplement (NK++), whereas in NK depleted group it was less than 10%. The percentages of high producer of IFNγ are provided in parenthesis for each group in representative plot (figure 4). Hence, the functional avidity of CD8+ T cells measured as the capacity to produce IFNγ (8) was highest in NK supplemented groups (both CD4 depleted and normal memory mice). Taken together, the results support ours and other previous observation of the importance of CD4+ T cell during recall. In addition, it also highlights the additive effect or may be synergistic effect of NK cell supplement on recall of functionally effective CD8+ T cells.

The supportive role of NK cell was more evident upon analysis of IL-15Rα and CD8β expression on SSIEFARL peptide specific CD8+ T cells. The molecular connection between high IL-15Rα levels and CD8β expression in the context of high avidity CD8+ CTLs has been addressed earlier (35). Since NK cells are a source of IL-15 and in addition posses the ability to transpresent IL-15(5), it was hypothesized that NK cells may contribute to enhanced IL-15Rα and CD8β expression on HSV specific CTLs. To test this, splenocytes harvested from various groups: 1. NK cell depleted (NK⁻), 2. CD4+ T cell depleted and NK supplemented (CD4⁻NK++), 3. NK cell supplemented (NK++) and 4. control IgG given) were briefly stimulated (2 hours) with SSIEFARL peptide and immediately analyzed by FACS staining for the expression of IL-15Rα, CD8β, CD69, and CD8α. HSV specific CD8+ T cell expressed the early activation marker CD69 as a consequence of brief exposure to the cognate peptide. Thus, peptide
stimulated splenocytes were gated on CD8<sup>+</sup> CD69+ cells and analyzed for the expression of IL-15R<sub>α</sub> or CD8β. As shown in figure 6, the IL-15 receptor expression was highest on cells that were obtained from mice supplemented with NK cells (38%) followed by the control IgG administered group (24%). The group that was depleted of their CD4+ T cells but given additional NK cells was within the error margin of the control group (21%). The lowest expression was found on cells collected from mice that were depleted of NK cells. The difference observed, based on the staining of this receptor, support the conclusion that NK cell mediated effects (cytokines IL-15 or IFN<sub>γ</sub>) induced high avidity CTLs that expressed higher levels of IL-15R<sub>α</sub>. This enables them to respond to homeostatic proliferation by efficiently capturing IL-15 (5).

With regards to the CD8β expression the pattern was in line with the IL-15R<sub>α</sub> expression. As expected the HSV specific CD8+ CTLs isolated from the NK supplemented mice showed the highest expression of the co-receptor- CD8β (41%), the expression in control group was around 50% reduced (24%). About 28% of the HSV specific CD8+ T cell in CD4 depleted-NK supplemented group expressed CD8β and the least expression (9%) was observed in the NK cell depleted group (figure 6). The expression levels were about 2 fold more in the NK supplemented group compared to controls and 3 fold when compared to unhelped CD8+ CTLs.

High avidity CTLs can kill specific targets even when their co-receptor- CD8β is blocked. Hence, to further confirm the above observation, an in vitro CTL assay was performed in the presence of anti-CD8β antibody during the co-incubation of CTLs and targets. The blocking of the CD8β did not hamper the lytic ability of the higher avidity
CTLs isolated from NK supplemented mice in stark contrast to CTLs isolated from NK depleted where the cytolysis was reduced around 55% (data not shown).

In conclusion, our results show that the NK cells contribute to avidity maturation of CD8+ CTLs by their production and probably trans-presentation of IL-15 and in addition the IFNγ produced by them may also be able to partially rescue the CTLs generated in the absence of a helper response.

Discussion:

Our results confirm that NK cells contribute to innate resistance of C57BL/6 mice and that depletion of NK cell results in greater susceptibility to HSV infection. In addition, the NK cells also help shape the adaptive immune response by probably promoting effective interaction between the APC and T cells by licensing the APCs and lowering the threshold of activation of the responding T cells. Further, in a novel helper role the NK cell produced cytokine IFNγ may contribute towards better differentiation and avidity maturation of CD8+ CTLs either directly or in conjunction with IL-15. NK cells because of their expression of high IL-15Rα may help towards this process by trans-presentation of IL-15. Incidentally, the CD8+ CTLs isolated from NK supplemented groups showed the highest expression of IL-15Rα and CD8β which is indicative of their high quality. Although, there are multiple studies highlighting the importance of NK cells in the primary anti-viral response, we believe that our results show for the first time the critical role that NK cells play during recall of anti-viral memory responses. Our experiments with NK supplementation during recall response to HSV demonstrated their helper role in augmenting the quality of T cell responses especially CD8+ T cells. Thus NK cell besides performing innate immune functions could also be exploited during memory recall by specifically targeting them to make key cytokines or even help in direct costimulation.

The general response to viral infections is often an increase in NK activity (2, 43). Our results have confirmed by a number of approaches that NK cell depletion leads to greater susceptibility to HSV infection. The PK136 mAb effectively depleted NK cells in C57BL/6 mice and that the absence of NK cell lowered the threshold of protection. Additionally, PK136
transgenic mice (49) that lacks NK cells also mounted an inferior response to HSV infection. Increasing the number of NK cells in wild type mice by adoptive transfer increased their innate resistance and possibly also contributed to better or augmented anti-HSV immune response. On detailed analysis it is evident that NK cell depletion resulted in reduced adaptive immune response to HSV and a partial impairment of T cell differentiation towards a Th1 or Tc1 phenotype. Furthermore, NK cell depletion had several effects on the quantity and quality of anti-HSV CD8+ and CD4+ T cell response. Evidence from numerous studies in human and mice have suggested that NK cells play a relevant role in the establishment of adaptive immune responses (6, 7, 32, 48).

Our results further lend support to the finding that NK cells play a pivotal role in setting the stage for adaptive immune response and possibly also affect the subsequent memory generation as well. In addition, our study for the first time highlights the importance of NK cell as novel helpers in the rescue of CD8+ T cells in the absence of conventional CD4+ T helper cells. NK cell helper function may be manifested in several ways. Evidence from earlier studies indicate that NK cells can be induced to function as non-cytotoxic helper cells following stimulation with IL-18 (29). NK cells are also known to perform costimulation of T cells (17), and lastly, NK cells may enable effective antigen presentation by killing antigen bearing migratory DCs and making it available for lymphoid resident DCs to cross present to the T cells. Hence, NK cell appropriately stimulated may play a prominent role in the protection and subsequent modulation of the immune responses to HSV during re-immunization or secondary recall.

The advances in our understanding of the mechanisms of activation/inhibition of NK cells have revealed complexity that was not originally expected(24). Thus, while NK cell activation occurred due to lack of surface MHC class I on targets, the actual functionality and target lysis requires a multitude of signals that include stress proteins, cytokines and involvement of other sentinel cells(24). Although NK cells were originally thought to act independently, accumulating evidence indicates that NK cells also respond to stimuli from other immune effectors, especially DC (51), During the writing of this manuscript, a recent study on the role of NK cells in promoting early CD8 T cell responses against cytomegalovirus was published by Robbins et al.(42). The mechanism proposed by this group is by the ability of NK cells to limit pDC IFN-α/β production to levels not immunosuppressive to the host so
permitting promotion of early CD8 T cell response. Our preliminary studies also suggest that the reciprocal cross-talk between NK cells and DCs that is induced by HSV products not only promotes rapid innate responses against the virus but also favors the generation of appropriate downstream adaptive responses. Detailed studies are underway and may offer better clues as to the site and sequence of events immediately after HSV infection.

Although signaling through activating and inhibitory receptors seems to be important, cytokines also play a critical role in dictating NK cell behavior. Cytokines such as IL-12, IL-15, IL-18, IL-21 and interferons (IFN α/β) can induce NK cell survival, proliferation, cytotoxicity and/or IFN-γ production (25, 47). In fact IL-12 and IL-18 may also prevent the NK inhibition induced by inhibitory receptor signaling(38). Of all the cytokines IL-15 may probably play a dominant role in the NK cell mediated effects. A strong support for this contention comes from a very recent study by Medzhitov et al. (19). Accordingly the signaling through an activating NK receptor (NKG2D) and IL-15Rα are tied together affecting the function and response to each other(19). Additionally, NK cells are also capable of transpresenting IL-15(5) efficiently and as also shown in human studies could possibly present antigen to stimulate T cells besides producing stimulatory cytokines(17).

The molecular connection between high IL-15Rα levels and CD8β expression in the context of high avidity CD8+ CTLs has been demonstrated earlier in a different context (35). Since NK cells are a potent source of IL-15 and additionally posses the ability to transpresent IL-15, it was hypothesized that they may contribute to enhanced CD8β expression on HSV specific CTLs. The supportive role of NK cell was more evident upon analysis of IL-15Rα and CD8β expression on SSIEFARL peptide specific CD8+ T cells. The 2 fold increase based on the staining of this receptor in NK supplemented group support the conclusion that NK cell mediated effects (cytokines IL-15 or IFNγ) induced high avidity CTLs that expressed higher levels of IL-15Rα. This enables them to better respond to homeostatic proliferation by efficiently capturing IL-15. Thus, NK cells probably maintains the frequency of anti-HSV specific CD8+IFNγ + T cells by producing IFNγ, which elicits secretion of IL-15 and IL-18 by other cells. These cytokines in turn favor expansion of Tc1 CD8+ T cells. This conclusion finds support from earlier studies done in other systems. In a study in persons infected with M. tuberculosis(48), it was shown that the frequency of CD8+IFNγ + T cells was restored by
activated NK cells. The mechanism for such restoration was mediated by soluble factors such as IFN\(\gamma\), IL-15 and IL-18 (anti-IFN\(\gamma\) abrogated the positive effect), and/or through CD40/CD40L interaction (48). Therefore, NK cells utilize at least two distinct mechanisms to contribute towards effective CD8+ T cell generation.

Our earlier studies on the rescue of defective CD8+ T cells indicated that CD4+ T cells producing IFN\(\gamma\) was better at restoring the anti-HSV CD8+ T cell function (20). In addition, IFN\(\gamma\) has been shown to increase the production of IL-15 in phagocytic cells (11). IL-15 for its part enhances virus specific CD8+ T cells (44) and IL-18 also contributes towards the development of Tc1 CD8+ T cells (36). We speculate that during the initial interaction at immune induction site, activated NK cells produce IFN\(\gamma\) that induces the production of IL-15 in DCs. In turn, the IL-15 is now taken up and transpresented by NK cells facilitating the expansion of HSV specific CD8+ T cells that becomes efficient effector cell. Classically, CD8+ T effector cells that have been optimally activated have been considered to both produce IFN\(\gamma\) and exhibit CTL activity. Results from assays measuring IFN\(\gamma\), CD107 a and b and granzyme B in HSV specific CD8+ CTL brings out the superior quality in NK cell competent mice.

Thus we conclude that NK cells activated by the virus directly or by an intermediary cell contribute to initial reduction in viral load, enhance the stimulatory ability of the DCs by enabling effective antigen processing and presentation. In addition, activated NK cells are able to rescue defective CD8+ T cells that were generated in the absence of cognate help and could compensate for loss in CD4+ T cells. These findings have the following implications: a) NK cell could help the DCs to increase their stimulatory ability, b) The quality of CD8+ T cells are better if NK cells are involved, c) NK cells can compensate for the loss in CD4 help, d) NK cell can be exploited in situations where CD4+ T cell is depleted and in individuals who are incapable of mounting a strong CD4 response, e) In elderly individuals that are better responders to vaccine (e.g. Flu) have efficient NK cells. Hence, therapeutic strategy involving immunization protocols exploiting NK cells specifically could be adopted especially during priming and recall of adaptive immune response. We plan to selectively expand different subsets of NK cells prior to or early or late in infection to see if this would shape the response. Several drugs, adjuvants and antibodies do serve to activate and expand NK cells and it is worthwhile to test some of these for their influence on HSV immunity.
Acknowledgements:

Funding: Startup funds, Department of Microbiology, Quillen College of Medicine, ETSU and NIH grant # AI 106336501 to BTR.
Figure Legends:

Figure 1. Lack of NK cells contributes to susceptibility, while supplementation enhances resistance

a. Mice given anti-asialoGM1 and anti-NK 1.1 antibodies lack detectable NK cell lytic activity

Sample animals from anti-asialoGM1 or control IgG administered groups were sacrificed 48 hr later. Spleen cells were analyzed for in vitro NK cell cytotoxic activity. A suspension of 10^7/ml of spleen cells was serially diluted with 100ml in each triplicate well of a 96 well round bottomed plate. Target ^51^Cr-labelled YAC-1 mouse lymphoma cells were added to give a effector:target ratio of 200-100-50-25. After 4 hour incubation at 37°C, supernatants containing released ^51^Cr were collected and counted by an automatic scintillation counter. Specific lysis= (experimental release-spontaneous release)/(total release-spontaneous release) x 100%, where spontaneous release was derived from wells without effectors and total release from wells with 3% triton-X added to it. @ Asialo GM1 vs control IgG or untreated p=<0.0001; # PK136 vs control IgG and untreated controls p=<0.0001.

b. Depletion of NK cells by anti-NK1.1 and asialo GM1 antibodies.

B6 mice given anti-asialoGM1 and PK136 were analyzed for the absence of NK cell by flow cytometry. Single cell suspensions of spleens were stained with anti-NK1.1 (PE) and anti-CD3 (FITC). The plots represent data obtained from a mouse in each group. The numbers within the plot are the mean ± SD of 5 mice in each group. The controls included untreated mice and mice given control IgG. Cells that are CD3^- NK1.1^+ are taken to be the NK cells.
c. Early onset of lesion upon Zosteriform challenge in NK depleted mice.

Zoster challenge experiment was performed as described elsewhere (20). Before challenge the left flank area was depilated by a combination of hair clipping and use of chemical Nair (Carter-Wallace, New York, NY). The animals were anesthetized with avertin and scarifications were made in a ~4-mm² area. To such scarifications, 10 µl containing 10⁴ PFU of HSV-1 (strain 17) were added and gently massaged. Animals were inspected daily for the development of zosteriform ipsilateral lesions, general behavior changes, encephalitis, and mortality. The severity of the lesions was scored as follows: 1+ = vesicle formation; 2+ = local erosion and ulceration of the local lesion; 3+ = mild to moderate ulceration; 4+ = severe ulceration, hind limb paralysis, and encephalitis; and 5+ = ultimate death († mice that were moribund and hence euthanized). The experiments were repeated three times with 5 mice in each group and the outcome was similar. The figure shows lesion score of all mice within a group at day 10 post challenge from one such experiment. * Asialo GM1 vs control IgG and untreated p=<0.0001; # PK136 vs control IgG and untreated p=<0.0001.

d. NK cell transfer augments protection against HSV

Wild type B6 mice were divided into six groups of three mice each. Group 1 and 2 were challenged (zosteriform) with low dose (5x10³) and high dose of virus (1x10⁵). Groups 3 to 6 (NK-1,2,3,4) were adoptively transferred with increasing numbers of purified NK cells as indicated 15 hours before challenge with high dose of virus. The animals were handled as mentioned in figure 1c and lesion score recorded. The experiment was repeated three times with 3 mice per group. The figure show data collected on day 10 post challenge of one such experiment. * Animals in this group were moribund and
hence sacrificed. Statistically significant difference between NK-3 (1 million NK cells) and Wt-Hi- \( p=0.05 \).

**Figure 2. NK cell contributes to skewing towards protective Th1 type.**

*a. Reduced Th1 cytokine production in anti-HSV CD4 and CD8 T cells: C57BL6 mice were divided into two groups. One group was depleted of NK cells; the other group was administered control IgG and was infected with HSV by the footpad route. Ten days post-infection, their splenocytes were harvested and stimulated with uv-inactivated HSV or SSIEFARL peptide and analyzed for the CD4 and CD8 T cell activity respectively. Samples were processed individually and a similar pattern was observed within the same group. The figures represent the IFN-\( \gamma \) producing CD4+ T cells and IFN-\( \gamma \) and IL-2 producing CD8+ T cells in the control IgG, NK-depleted and uninfected control animals. The experiment was repeated three times with similar outcome. The dot plot shows results obtained from one such experiment and the numbers within the plot is the mean \( \pm \text{SD} \) of 5 mice.*

*b. In vivo cytolytic ability is reduced in NK depleted mouse: Splenocytes from wild type B6 mice were stained with CFSE and loaded with SSIEFARL peptide. 5 x 10^6 cells were adoptively transferred through the tail vein into control mice and NK depleted mice. The spleens were isolated from these mice after 5 hours and the splenocytes isolated. Target cells were distinguished from recipient cells based CFSE staining. Histogram plots were used to demonstrate the difference in separation pattern based on intensity of CFSE staining. The recovery and percent killing of the various CFSE-labeled peptide-pulsed targets were calculated as follows: 100 - ((percentage of peptide pulsed in...
immunized/percentage of unpulsed in immunized)/ (percentage of peptide pulsed in unimmunized/percentage of unpulsed in unimmunized)] x 100). The experiment was repeated two times with 3 mice in each group. The histogram plot shows the data obtained from one such experiment and the number within the plot is the mean ± SD of killing ability in 3 individual mice.

**Figure 3: Reduced frequency and diminished quality of anti-HSV specific CD4+ T helper and CD8+ T cell numbers in PK136 Tg mouse.**

C57BL6 mice and PK136 mice were infected with HSV-1 17 strain by the skin route and analyzed for CD4+ and CD8+ T cell activity 7 days p.i. to study the primary immune response and 45 days p.i. to study the memory response. Splenocytes were harvested at the indicated time points and analyzed for polyfunctional CD4+ T cells (IFNγ+ and IL-2+) and CD8+ T cells (IFNγ+ and TNFα+). In addition, the cytolytic ability of CD8+ T cell was assessed by measuring granzymes and degranulation (CD107a/b). The experiment was done multiple times with similar outcome. Data shown is representation of one such experiment.

**a. Reduced number of poly-functional CD4+ T-cells during memory phase of infection:**

Single cell suspensions were processed as described in materials and methods section. The sample was gated on IL-2 and IFNγ double positive cells and analyzed for CD4 expression. The histogram plot shows the number of CD4+ T cells that are IFNγ+ IL-2+ in C57BL/6 and PK136 Tg mice. The pattern was similar in all of the mice within the group. * The difference is statistically significant.

**b. Reduced number of anti-HSV polyfunctional CD8+ T cells during acute and memory infection in PK 136 Tg mouse:** Single cell suspensions was stained as described earlier.
The cells were gated on IFN-γ+ and TNF-α+ cells as indicated and analyzed for HSV-gB tetramer positivity. The figure depicts a representative plot showing the number of gB tetramer+ CD8+ T cells producing both IFN-γ and TNF-α (poly-functional CD8+ T cells) during the primary and memory immune response. The difference between the wild type and PK136 Tg mice were significant.

c. PK 136 Tg mice develop reduced CD8+ T cells with cytotoxic ability to HSV infection:
Single cell suspensions were processed as described in materials and methods section. The analysis for cytolytic activity was done on CD8+ HSV-gB tetramer+ gated population. The figure is a pair of graphs representing the CD107 a/b (left) and granzyme (right) expressing HSV-gB tetramer+ CD8+ T cells in PK136 Tg mice and wt mice during primary and recall immune response. Although, the response was lower in PK136 Tg mice, the difference was statistically significant only during the memory phase analysis.

Figure 4: NK cell affects CD8+ T cell IFNγ production, but addition can compensate for the loss of CD4+ T cell help.
C57BL6 mice with HSV memory were divided into five groups: 1. depleted of NK cells (NK−), 2. depleted of CD4+ T cells (CD4−), 3. depleted of CD4+ T cells but supplemented with NK (CD4−NK++), 4. supplemented with NK cells (NK++) and 5. control and received control IgG. All the mice were given 1x10^5 pfu of HSV and 5 days later, analyzed for the IFN-γ production by the CD8+ T cell to measure recall responses. The pattern was similar in all three experiments. The figure shows a representative plot of intracellular interferon-γ staining in peptide stimulated CD8+ T cells. The data represents one mouse
from each of the following groups uninfected control, control IgG, CD4\(^-\), NK\(^-\), CD4\(^+\)NK\(^++\) and NK\(^++\). The number within the plot is the mean\(\pm SD\) of 5 mice in each group. The difference between the NK\(^++\) Vs CD4\(^-\) and NK\(^-\) groups were statistically significant (P <0.005). The difference between NK\(^++\) Vs CD4 NK\(^++\) was significant (P<0.01).

**Figure 5: NK cell contributes to higher IFN\(\gamma\) production by anti-HSV CD8\(^+\) T cells.**

As described earlier in figure 4, C57BL6 HSV memory mice were divided into four groups wherein mice from one group were depleted of NK cells (NK\(^-\)), the other were depleted of CD4 T cells and supplemented with NK (CD4\(^-\)NK\(^++\)) while the 3\(^{rd}\) group was supplemented with NK cells (NK\(^++\)). The 4\(^{th}\) group served as control and received control IgG. All the mice were given \(1 \times 10^5\) pfu of HSV and 5 days later, analyzed for the IFN-\(\gamma\) production. The figure depicts representative dot plots showing the CD8 T cells producing IFN-\(\gamma\) in the NK depleted (NK\(^-\)), CD4 depleted –NK supplemented (CD4\(^-\)NK\(^++\)), control IgG administered (control) and NK supplemented (NK\(^++\)) mice. The FACS plot has been compensated such that the cells get skewed as high, intermediate and low IFN-\(\gamma\) producing CD8 T cells. The numbers indicate mean \(\pm SD\) of percentage of CD8+IFN\(\gamma\)+ T cells from 5 individual mice. The number in parenthesis is the percentage of high IFN\(\gamma\) producers within the positive population. One-way analysis of variance indicate the difference to be statistically significant (p<0.0001).

**Figure 6 NK cell mediates functional improvement and avidity maturation of anti-HSV CD8\(^+\) T cells.**

HSV memory C57BL/6 mice were grouped and treated as described in figure 5. The splenocytes were briefly stimulated (2hr) with the HSV immuno-dominant SSIEFARL
(gB 498-505) peptide. It was followed by staining with anti-IL-15Rα and anti-CD8β antibody. HSV-gB peptide specific CD8+ T cells were analyzed for the expression of IL-15 Rα and CD8β by gating CD69 positive CD8+ T cells. The dot plot represents the IL-15Rα and CD8β expressing HSV specific CD8+ T cells in the 4 groups mentioned above. The experiment was repeated three times with similar pattern of results. The number within the plot is mean ± SD from one such experiment. One-way analysis of variance indicate the difference between the groups to be significant (p<0.0001).

**Footnotes for table:**

Table 1. **In vitro CTL activity is reduced in memory mice that lacked NK cells during priming.**

C57BL6 mice were either depleted or supplemented with NK and were primed with HSV. Groups of mice depleted of CD4 T cells and another group of unmanipulated mice served as controls. On day 45, all the mice were infected with a low dose (1x10⁵ pfu) of HSV-Kos. The CD8+ T cells from these mice were purified and incubated with MHC-matched HSV infected (MC38-HSV); MHC matched mock infected (MC38-Mock); MHC mismatched HSV infected (EMT6-HSV) and YAC cell targets in the ratio of 90:1, 30:1, 10:1 and 3:1 (effector: target ratio) and an in vitro CTL assay was performed. The table represents the mean ± SD of lysis percentage obtained at an E:T target ratio of 90:1 from 5 mice in each group. The date presented here is one of the 3 experiments with similar pattern of results. The YAC cell killing was insignificant. The difference between the groups a, b, c, d are statistically significant (p=<0.0001).
1a

![Graph showing % Lysis vs NK:Target ratio for different treatments.](chart)

- **PK136**
- **Asialo GM1**
- **Control IgG**
- **Untreated**

1b

![Flow cytometry plots comparing NK1.1 and CD3 expression.](chart)

- **anti-asialo GM1**
- **PK 136**
- **Untreated**
- **Control IgG**

**Legend:**
- PE-anti-NK1.1
- FITC anti-CD3

**Graph Details:**
- % Lysis ranges from 0 to 40%
- NK:Target ratios: 25:1, 50:1, 100:1, 200:1
- Error bars indicate standard deviation

**Flow Cytometry Details:**
- <0.1% lysis
- 3±1.2% lysis
- 4±2% lysis

**Note:**
- Figures represent data from untreated and treated samples with various concentrations of antibodies.
3a:

![Diagram showing IL-2 and IFN-γ](image)

3b:

![Diagram showing TNF-α and IFN-γ](image)

**CD4+ T cells (IFNγ+, IL-2+)**

**CD8+ T (IFNγ+, TNFα+) cells**

**Acute Phase**

**Memory Phase**

* C57BL/6
  - Black line
  - PK136
  - Gray line

**IFN-γ**

**TNF-α**
3c

Number of CD8+CD107a/b+  Number of CD8+Granzyme B+

%gB Tetramer+

Acute Phase  Memory Phase  Acute Phase  Memory Phase

C57BL6  PK136  C57BL6  PK136

*
controls
infected

CD4
NK
CD4 NK
NK

PE-IFNγ
FITC-CD8

4.1 ±1.2
5.2 ±1.5
8 ±2.1
11.2 ±2
Table 1. In vitro CD8+ CTL activity is reduced in memory mice that lacked NK cells during priming.

<table>
<thead>
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<th>Targets</th>
<th>MHC Matched</th>
<th>MHC Mismatched</th>
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<td>MC-38-Mock</td>
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References:


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