

Primary naïve and interleukin-2-activated natural killer cells do not support efficient ectromelia virus replication

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Natural killer (NK) cells are known for their ability to lyse tumour cell targets. Studies of infections by a number of viruses, including poxviruses and herpesviruses, have demonstrated that NK cells are vital for recovery from these infections. Little is known of the ability of viruses to infect and complete a productive replication cycle within NK cells. Even less is known concerning the effect of infection on NK cell biology. This study investigated the ability of ectromelia virus (ECTV) to infect NK cells *in vitro* and *in vivo*. Following ECTV infection, NK cell gamma interferon (IFN- γ) production was diminished and infected cells ceased proliferating and lost viability. ECTV infection of NK cells led to early and late virus gene expression and visualization of immature and mature virus particles, but no detectable increase in viable progeny virus. It was not unexpected that early gene expression occurred in infected NK cells, as the complete early transcription system is packaged within the virions. The detection of the secreted early virus-encoded immunomodulatory proteins IFN- γ -binding protein and ectromelia inhibitor of complement enzymes (EMICE) in NK cell culture supernatants suggests that even semi-permissive infection may permit immunomodulation of the local environment.

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INTRODUCTION

Natural killer (NK) cells constitute 5–15 % of the lymphocyte population and are distinguished by their large morphology, the presence of germline NK cell receptors and a lack of recombined receptors, such as the T-cell receptor (Seaman, 2000; Yokoyama *et al.*, 2004). Studies have shown NK cells to play a pivotal role in the innate immune response to viral infections, particularly murine cytomegalovirus (MCMV) and human herpesviruses (Biron *et al.*, 1989). In the case of MCMV, the autosomal dominant *Cmv1* locus encoding the NK cell activation receptor Ly49H has been shown to be responsible for enhanced resistance of C57BL/6 mice to a lethal MCMV challenge (Brown *et al.*, 2001). Further studies identified the MCMV viral protein m157 as the ligand responsible for triggering the Ly49H receptor and the subsequent specific proliferation and activation of the Ly49H⁺ subset of NK cells (Smith *et al.*, 2002). It is not known whether MCMV productively infects NK cells.

Previous studies have demonstrated an important role for NK cells in recovery from mousepox, a fulminant disease of selected mouse strains that is used as a small-animal model of smallpox. Depletion of NK cells through the use of anti-asialo GM₁ gammaglobulin or anti-NK1.1 antibody treatment increased the mortality and tissue infectivity titres following infection of C57BL/6 mice with ectromelia virus (ECTV); however, interpretations of these studies were limited by the observation that treatment with anti-asialo GM₁ also depletes CD8⁺ T cells, thus diminishing a virus-specific cytotoxic lymphocyte response, and anti-NK1.1 treatment depleted both NK and NKT cells (a subset of T cells that co-express an $\alpha\beta$ T-cell receptor and NK cell receptors) (Delano & Brownstein, 1995; Jacoby *et al.*, 1989; Karupiah *et al.*, 1996). Recent studies of NK1.1-depleted mice and mice genetically deficient in NK cells (NKD mice) have confirmed and extended these studies (Parker *et al.*, 2007).

These past studies have raised a number of additional questions. Are NK cells susceptible to infection? Does

infection affect NK cell antiviral function? Does infection result in the production of host response modifiers, such as gamma interferon (IFN- γ)-binding protein (IFN- γ BP) and ectromelia inhibitor of complement enzymes (EMICE), that target innate responses to viral infection? Are infections productive or abortive?

In this study, we assessed the effect of viral infection on lymphokine-activated killers (LAKs) and naïve NK cells and demonstrated that ECTV is capable of infecting NK cells and suppressing NK cell function. Although the production of viable progeny was not efficient, ECTV-infected NK cells produced viral proteins, including immunomodulatory proteins, which have the potential to contribute to immune dysfunction in the local microenvironment.

METHODS

Mice. C57BL/6 female mice were purchased from Charles River Laboratories/NCI. All mice were used at 6–10 weeks of age.

Media. LAKs and freshly isolated NK cell cultures were prepared in complete medium consisting of RPMI 1640 supplemented with L-glutamine (300 $\mu\text{g ml}^{-1}$) and 10% heat-inactivated fetal calf serum (FCS; HyClone). BS-C-1, a green monkey kidney epithelial cell line, was cultured in Dulbecco's modified Eagle's medium supplemented with L-glutamine (2 mM), penicillin (100 U ml^{-1}), streptomycin (100 $\mu\text{g ml}^{-1}$) and 10% FCS.

Flow cytometry. Leukocytes were blocked in anti-mouse CD16/CD32 (BD Biosciences) for 10 min at 4 °C to prevent non-specific antibody binding. Samples were then stained for 30 min, washed and fixed in 1% formaldehyde or stained for 30 min, washed and subjected to cell sorting. Phycoerythrin (PE)-labelled PK136 (anti-NK1.1), PE-labelled DX5 (anti-CD49b), peridinin-chlorophyll protein complex-labelled Cy5.5-145-2C11 (anti-CD3), allophycocyanin (APC)-labelled 30-F11 (anti-CD45) and APC-labelled XMG1.2 (anti-IFN- γ) were obtained from BD Biosciences. Intracellular cytokine staining of IFN- γ was performed as described by the manufacturer (BD Biosciences). Samples were run on a BD FACSCalibur. Cell sorting was performed on a BD FACSAria.

Production of LAKs. Spleens from C57BL/6 mice were harvested and single-cell suspensions (1×10^8 cells ml^{-1}) were prepared in PBS containing 10% FCS. NK cells were positively selected using DX5 magnetic beads and magnetic cell sorting (Miltenyi Biotech) according to manufacturer's instructions. NK cells were grown in interleukin (IL)-2 (800 U ml^{-1}) containing RPMI 1640 for 6–8 days in T75 flasks at a density of 1×10^6 cells ml^{-1} . The purity of NK1.1⁺ CD3⁺ cells was determined by flow cytometry to be >90% after 6 days culture.

Freshly isolated NK cell preparation. C57BL/6 spleens were harvested and single-cell suspensions were prepared in PBS containing 10% FCS. NK cells were negatively selected from splenocyte suspensions using magnetic cell sorting (Miltenyi Biotech). The negatively selected NK cell population was subjected to cell sorting of CD49b⁺ CD3⁺ to increase the purity of the NK cell population. Purity after cell sorting was determined to be >90%.

Construction of the targeting plasmid pNCEVdrVT-NP-S-eGFP. The left and right flanking sequences of the disrupted region V in the ECTV Moscow strain (ECTV-MOS) genome were PCR amplified using ECTV-MOS genomic DNA as the template and primers 5'-GCGGGCGCCGACTTGTAGATAGAGATGAGATG-3' (*NarI* site

underlined) and 5'-GCGAGATCTGCTAGCGGTACCGTCGACCACTATCTTACGCATCTCTAG-3' (*BglII* site underlined) for the left flank, and 5'-GCGGGATCCGCGGCCGCAAGCTTGGTAAACGGTCATGTGACG-3' (*BamHI* site underlined) and 5'-GCGGAATTCGTAGCTATATAATGTATGGTAACATG-3' (*EcoRI* site underlined) for the right flank. The left and right flanks were digested with *BglII* and *BamHI*, respectively, and ligated together using T4 DNA ligase in the presence of *BglII* and *BamHI* to prevent self-ligation. The gel-purified fragment containing both left and right flanks was then digested with *NarI* and *EcoRI*, and cloned into pUCP7.5gpt-1 cut with the same enzymes (kindly provided by Dr Kangla Tsung, Washington University, MO, USA) to yield pNCEVdrVT. The plasmid pSC11-NP-SIINFEKL-eGFP (a gift from John Yewdell, NIH, MD, USA) was cut with *XbaI* and *NotI*. The fragment containing the NP-SIINFEKL-eGFP fusion gene (NP-S-eGFP, comprising the influenza nucleoprotein, SIINFEKL and enhanced green fluorescent protein sequences) under the control of the vaccinia virus (VACV) p7.5 promoter was gel purified and cloned into pNCEVdrVT digested with *NheI* and *NotI* to obtain the final targeting plasmid pNCEVdrVT-NP-S-eGFP. The sequences of the left and right flank and of the NP-S-eGFP expression cassette were verified by sequencing.

Generation of recombinant ECTV expressing NP-S-eGFP. The NP-S-eGFP cassette was recombined into ECTV-MOS as described previously (Falkner & Moss, 1990). Briefly, CV-1 cells in a six-well plate (2.5×10^6 cells ml^{-1}) were infected with ECTV-MOS and then transfected with 2 μg pNCEVdrVT-NP-S-eGFP using Lipofectamine 2000 (Invitrogen). After 48 h, cell lysates were prepared and recombinant isolates were subject to two rounds of plaque purification in the presence of mycophenolic acid followed by three rounds of plaque purification without mycophenolic acid. The genotypes of the final isolates were determined by PCR and expression of GFP was detected by fluorescence microscopy. One recombinant ECTV-eGFP was used in subsequent experiments.

Virus. Plaque purified ECTV-MOS was propagated in murine L929 cells as described previously (Chen *et al.*, 1992). Virus was purified through a sucrose cushion as described previously (Earl *et al.*, 1998). In strain A mice, wild-type ECTV and the recombinant ECTV-eGFP had similar LD₅₀ values of 0.02 and 0.04 p.f.u., respectively.

Infection of NK cells. LAKs and naïve NK cells were cultured in 24-well plates at a concentration of 2.5×10^5 cells ml^{-1} . Medium was removed by aspiration and cells were infected with wild-type ECTV or recombinant ECTV-eGFP under the p7.5 early/late promoter at an m.o.i. of 5 in 0.1 ml. Virus was allowed to adsorb for 1 h at 37 °C, followed by the addition of 1–2 ml complete RPMI 1640.

Trypan blue exclusion. Infected and uninfected cultures (1.5 ml containing 3.3×10^5 cells ml^{-1}) were centrifuged and resuspended in 10 μl PBS, combined with 10 μl 0.4% trypan blue and viewed in a haemocytometer. Cells were assessed in 16 squares and the number of cells excluding trypan blue was used to determine viability.

Single-cycle growth curves. LAKs, naïve NK cells and BS-C-1 cells were cultured in 24-well plates at a concentration of 2.5×10^5 cells ml^{-1} and infected as described above with ECTV at an m.o.i. of 5. Virus growth was assessed at 1, 12, 24, 48 and 72 h post-infection (p.i.) by plaque assay on BS-C-1 cells. Virus titre was expressed as p.f.u. ml^{-1} .

SDS-PAGE and Western blotting. Proteins from supernatants and cell pellets (2.5×10^5 cells) were separated by SDS-PAGE in a 4–12% NuPAGE Bis/Tris gel prior to electrophoretic transfer onto nitrocellulose filters as described previously (Bai *et al.*, 2005). Briefly, filters were blocked for at least 1 h at room temperature in 0.1% Tween 20/5% non-fat dried milk in PBS followed sequentially by a 1 h

incubation at room temperature with anti-E3 (diluted 1:1000), anti-ATI (1:5000), anti-IFN γ BP (1:10 000) or anti-EMICE (1:5000). Reactive proteins were detected with horseradish peroxidase-conjugated goat anti-rabbit IgG (H+L) (Kirkegaard & Perry Laboratories) using an ECL Plus protein identification system according to the manufacturer's instructions (Amersham Pharmacia Biotech). Total protein loaded onto gels varied less than twofold for all samples. Films were scanned into Adobe Photoshop using an Epson Perfection 4990 Photo Scanner.

Electron microscopy. Virus-infected cells (1×10^6) were washed with PBS and fixed with 2% glutaraldehyde in phosphate buffer (pH 7.2) overnight and then post-fixed in a 1% OsO $_4$ solution. Cells were dehydrated in a series of graded ethanol solutions and embedded in plastic. Sections were viewed with a JOEL 100CX transmission electron microscope.

NK1.1 cross-linking assay. Twelve-well polystyrene plates were coated with mouse monoclonal antibody (mAb) PK136 as described previously (Kim *et al.*, 2005). LAKs (1×10^6 cells ml $^{-1}$) were infected with ECTV-eGFP at an m.o.i. of 1 for 24 h and incubated with PK136-coated plates for 1 h at 37 °C. Brefeldin A (0.12% final concentration; BD Biosciences) was added and the plates were allowed to incubate for an additional 6–8 h. Cells were stained for NK1.1, CD3 and IFN- γ to determine the activation state of the infected NK cells.

RESULTS

ECTV infects NK cells *in vivo* and *in vitro*

To determine whether NK cells were capable of being infected *in vivo*, we utilized an ECTV recombinant

expressing eGFP and flow cytometry. C57BL/6 mice were infected with 1000 p.f.u. ECTV-eGFP by the footpad route of infection. At 24, 48 and 72 h p.i., draining popliteal lymph nodes were collected and assessed for GFP-positive NK cells (NK1.1 $^+$ CD3 $^-$). Infection of C57BL/6 mice in three experiments resulted in mean \pm SEM values of $0.5 \pm 0.2\%$ GFP $^+$, $2.0 \pm 0.4\%$ GFP $^+$ and $5.8 \pm 1.0\%$ GFP $^+$ NK cells at 24, 48 and 72 h p.i., respectively (Fig. 1a and b).

We next assessed the ability of ECTV to infect naïve NK and LAKs *in vitro*. LAKs were generated by culturing splenocytes with exogenous IL-2 and were used to model the behaviour of activated NK cells in the mouse following exposure to a variety of stimuli including infectious agents such as ECTV. A 24 h infection with ECTV at an m.o.i. of 5 resulted in $33.9 \pm 2.0\%$ GFP $^+$ and $64.9 \pm 6.1\%$ GFP $^+$ naïve NK cells and LAKs, respectively (Fig. 2a and b). Not only were more LAKs infected than naïve NK cells, but the mean fluorescence intensity was also nearly 1 log higher. Together, these results indicated that NK cells could be infected as a consequence of an experimental *in vivo* or *in vitro* infection, and that activated NK cells may be more permissive to ECTV infection than naïve NK cells.

ECTV infection kills LAKs

We next determined the effect of ECTV infection on the viability of IL-2-activated LAK cells. Uninfected and infected LAKs were assayed for viability by trypan blue exclusion at 24, 48 and 72 h p.i. (Fig. 2c and d). The

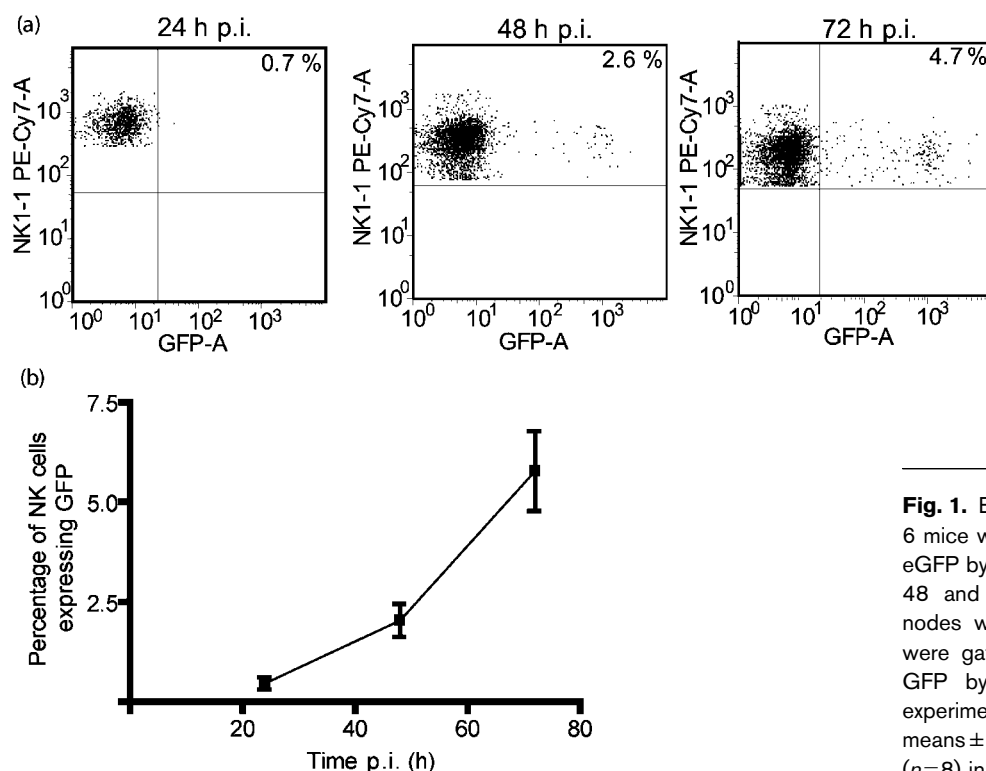


Fig. 1. ECTV infects NK cells *in vivo*. C57BL/6 mice were infected with 1000 p.f.u. ECTV-eGFP by the footpad route of infection. At 24, 48 and 72 h p.i., draining popliteal lymph nodes were harvested. NK1.1 $^+$ CD3 $^-$ cells were gated and assessed for expression of GFP by flow cytometry. A representative experiment is shown in (a) and the means \pm SEM of three independent experiments ($n=8$) in (b).

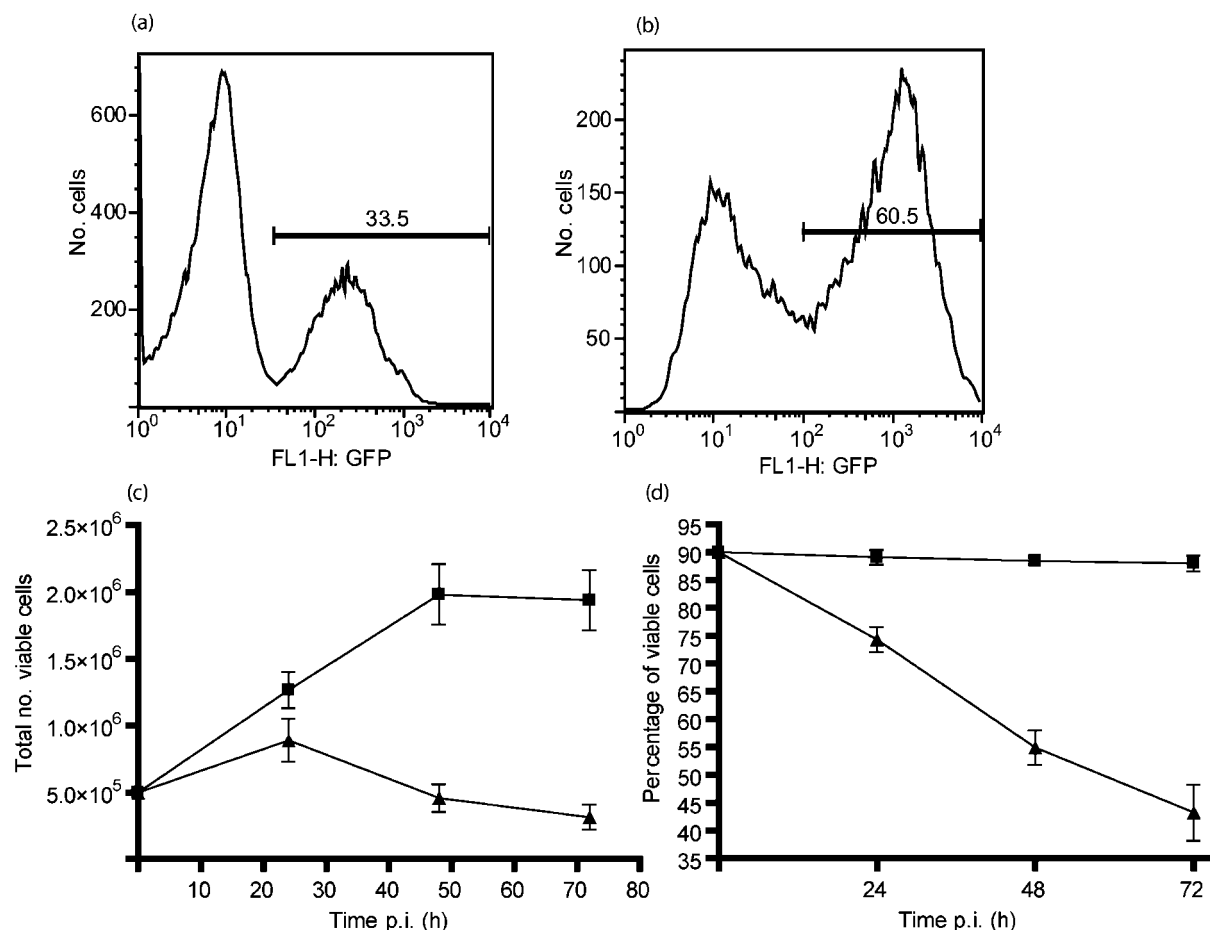


Fig. 2. ECTV infects LAKs and naïve NK cells *in vitro*. (a) Naïve NK cells were prepared by sorting NK1.1⁺ CD3⁻ splenocytes with a non-activating antibody, PE-DX5, and then infected with ECTV-eGFP (m.o.i.=5). At 24 h p.i., cells were harvested and GFP was detected by flow cytometry. Experiments were carried out in triplicate; representative results are shown. (b) LAKs were infected with ECTV-eGFP (m.o.i.=5) for 24 h and GFP expression was detected by flow cytometry. Experiments were carried out in triplicate; representative results are shown. (c, d) LAKs were mock-infected (■) or infected with ECTV (▲) for 24, 48 or 72 h. Viability was determined by trypan blue exclusion. The total number of viable cells (c) and the percentage of viable cells (d) were measured over time. Experiments were repeated four times and the results are shown as means \pm SEM.

population of uninfected LAKs maintained 90 % viability and increased in concentration fourfold over the course of the experiment. In contrast, ECTV-infected LAKs did not increase in number and exhibited ~40 % viability at the termination of the experiment at 72 h p.i. These results revealed that infection with ECTV can decrease the viability of LAK cells.

ECTV affects IFN- γ synthesis by LAKs

To determine further the effect of ECTV infection on NK cell effector function, we assessed the ability of NK cells to produce IFN- γ after a 24 h ECTV infection. Uninfected and ECTV-infected LAK cells were stimulated to produce IFN- γ through binding of mAb PK136 to the NK1.1 activation receptor (Kim *et al.*, 2002, 2005). For uninfected LAKs incubated with anti-NK1.1-coated plates for 8 h,

14.7 ± 1.2 % produced IFN- γ , whilst for similarly treated LAKs from cultures pre-infected with ECTV for 24 h, 2.2 ± 0.3 % of the GFP⁺ and 3.9 ± 0.4 % of the GFP⁻ LAKs produced IFN- γ (Fig. 3, $P < 0.001$). Uninfected and ECTV-infected LAKs incubated on plates coated with no mAb or with an isotype control (anti-MAR18.5) demonstrated only 1.0–2.3 % NK1.1⁺ IFN- γ ⁺ cells. These results revealed that infection of LAKs with ECTV significantly eliminated the ability of LAKs to be stimulated to produce IFN- γ ($P < 0.02$), possibly by decreasing viability. The decrease in the percentage of infected NK cells producing IFN- γ was not due to the virus-encoded IFN- γ BP interfering with the IFN- γ assay, as similar results were obtained with an ECTV IFN- γ BP⁻ mutant (data not shown). In addition, uninfected LAKs (GFP⁻) in the presence of ECTV-infected LAKs also displayed significantly reduced IFN- γ production ($P < 0.001$).

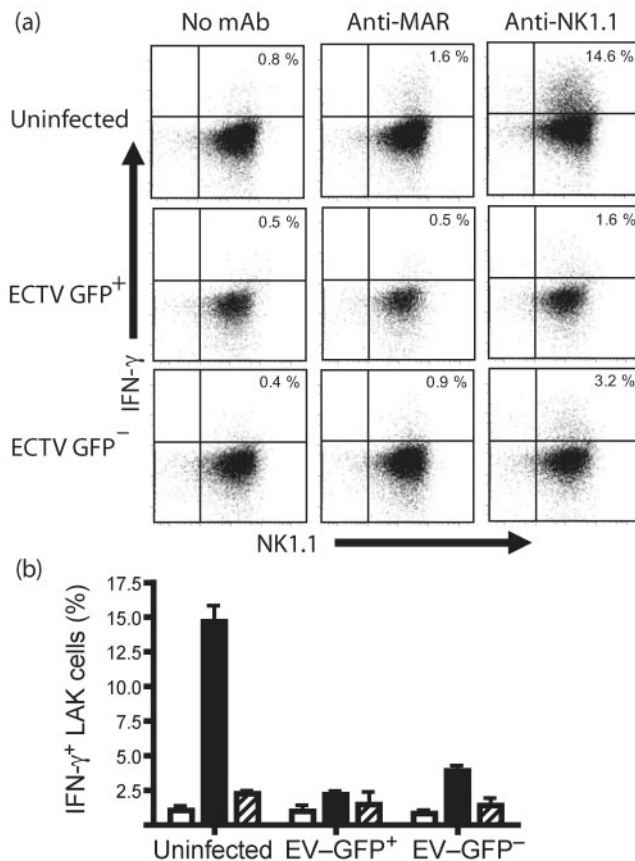


Fig. 3. ECTV infection diminishes the ability of NK cells to produce IFN- γ . LAKs were infected with ECTV-eGFP (m.o.i.=1) for 24 h. Uninfected and ECTV-infected LAKs were harvested and plated in six-well plates coated with anti-NK1.1, anti-MAR18.5 (isotype control) or no mAb. After 8 h incubation, cells were analysed for GFP expression and IFN- γ by flow cytometry. A representative experiment is shown in (a) and the means \pm SEM of four independent experiments is shown in (b). Empty bars, no mAb; filled bars, anti-NK1.1; hatched bars, anti-MAR18.5.

ECTV gene expression is delayed and progeny virus production is inefficient in LAKs and naïve NK cells

To determine whether ECTV infection of LAKs and naïve NK cells was a productive infection, we infected LAKs, naïve NK cells and BS-C-1 cells with ECTV and collected samples at 1, 12, 24 and 48 h p.i. Under single-cycle replication conditions, little or no ECTV replication was detected in LAKs or naïve NK cells (Fig. 4a and b).

To determine the stage of the block in the virus life cycle, we measured early and late gene expression at various times p.i. by Western blotting and assessed virus assembly by electron microscopy. LAKs, naïve NK cells and fully permissive BS-C-1 cells were infected at an m.o.i. of 5. Early gene expression was assessed by measuring expression of the E3 orthologue (a double-stranded RNA-binding protein; Kibler *et al.*, 1997), IFN- γ BP and EMICE (Esteban

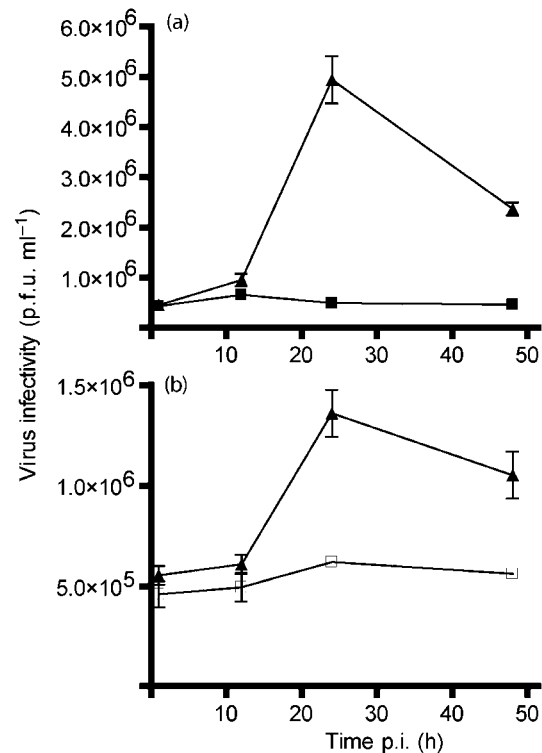


Fig. 4. LAKs and naïve NK cells do not support a complete virus replication cycle. LAKs (a) and naïve NK cells (b) were infected with ECTV (m.o.i.=5) and harvested at 0, 12, 24 or 72 h p.i. The virus titre of each sample was determined by plaque assay on BS-C-1 cells. Experiments were carried out in triplicate and results are shown as means \pm SEM. ■, LAK; □, naïve NK; ▲, BS-C-1.

& Buller, 2005). Late gene expression was followed through synthesis of a major late non-structural protein, ATI (A-type inclusion protein; Patel *et al.*, 1986). BS-C-1 cells showed E3 expression as early as 4 h p.i. (data not shown) and this increased in intensity up to the 48 h p.i. time point. LAKs exhibited delayed expression of E3, but nowhere near the level of that seen with BS-C-1 cells (Fig. 5a). Naïve NK cells showed results similar to those seen with LAKs (data not shown). Expression of the immunomodulators EMICE and IFN- γ BP was also seen in LAKs. EMICE could be detected by 12 h p.i. in the supernatant of BS-C-1 cells and the cell pellet of LAKs (Fig. 5b). Although EMICE is a secreted protein, previous studies have shown its presence in cell pellets, which may indicate binding to the cell surface via a heparin motif (Liszewski *et al.*, 2006). The IFN- γ BP was also expressed by both BS-C-1 cells and LAKs; however, the secreted immunomodulator was found in BS-C-1 cell supernatants earlier than in LAK supernatants (Fig. 5c). The apparent delay in secretion of IFN- γ BP in LAKs is not understood. The late viral protein ATI appeared at approximately 12 h p.i. in BS-C-1 cells. In both LAKs and naïve NK cells, ATI gene expression appeared to be decreased and delayed compared with that seen in BS-C-1 cells (Fig. 5d).

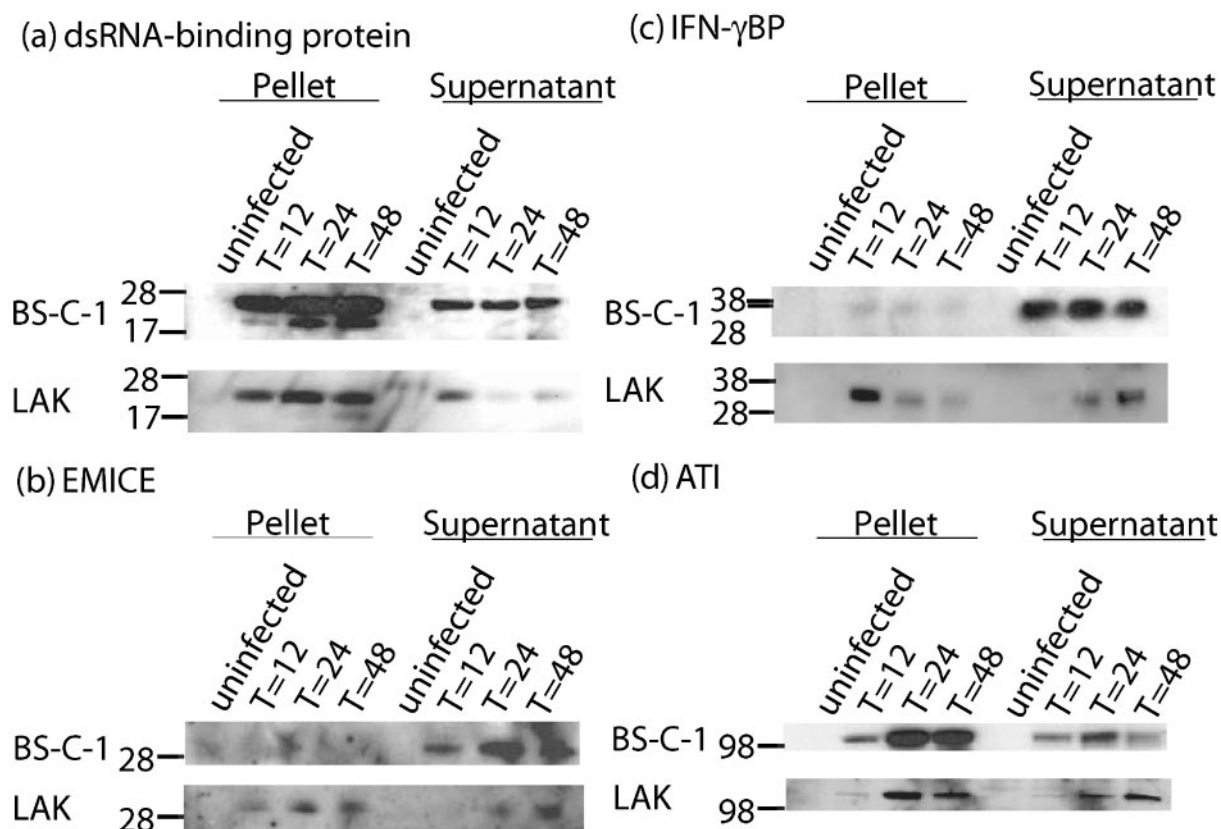


Fig. 5. Infected LAKs express early and late genes. Supernatants and cell pellets from ECTV-infected and uninfected cells were harvested at various time points following infection and analysed by Western blotting for expression of dsRNA-binding protein (a), EMICE (b), IFN- γ BP (c) and ATI (d). Molecular mass standards (kDa) are shown on the left of each blot.

Intracellular viral proteins E3 and ATI were detected in the culture supernatants from both cell types and may result from membrane permeability changes following infection, which may be cell-type specific. As both early and late viral proteins were expressed, but viable progeny were not produced, we next examined virion assembly.

Virion assembly in LAKs was examined by electron microscopy. In BS-C-1 control cells, we found that $88.3 \pm 5.2\%$ of cells infected at an m.o.i. of 10 demonstrated both immature and mature virions by 24 h p.i. (Fig. 6). In infected LAKs, we observed that $36.1 \pm 8.2\%$ of cells contained immature and/or mature virions by 24 h p.i. (Fig. 6). LAKs containing virions demonstrated significantly lower numbers of particles, with 6.1 ± 0.9 immature particles and 8.1 ± 1.8 mature particles per infected cell compared with BS-C-1 cells, which contained 28.9 ± 4.5 and 35.1 ± 7.1 immature and mature particles per infected cell, respectively (Fig. 6). The lower levels of viral proteins, the lack of infectious progeny following single-cycle replication conditions in naïve NK cells and LAKs, and the presence of fewer immature and intracellular mature virions following infection indicated that NK cells

are not as fully permissive to ECTV replication as BS-C-1 cells.

DISCUSSION

NK cells play an important role in controlling virus infections. Upon activation, these cells secrete cytokines, including IFN- γ , and kill virus-infected cells via the perforin/granzyme pathway. The importance of these cells during virus infections has become apparent in several systems. In the MCMV system, the genetic resistance of the C57BL/6 mouse is mediated through the NKC (NK cell gene complex) locus (Brown *et al.*, 2001; Daniels *et al.*, 2001; Scalzo *et al.*, 1990). The importance of NK cells during viral infections is also demonstrated in the poxvirus system, where NK cells are crucial for early control and recovery from ECTV infection in C57BL/6 mice (Parker *et al.*, 2007).

Another orthopoxvirus, VACV, readily infects human peripheral blood lymphocytes including NK cells, monocytes and B cells (Kirwan *et al.*, 2006; Sanchez-Puig *et al.*,

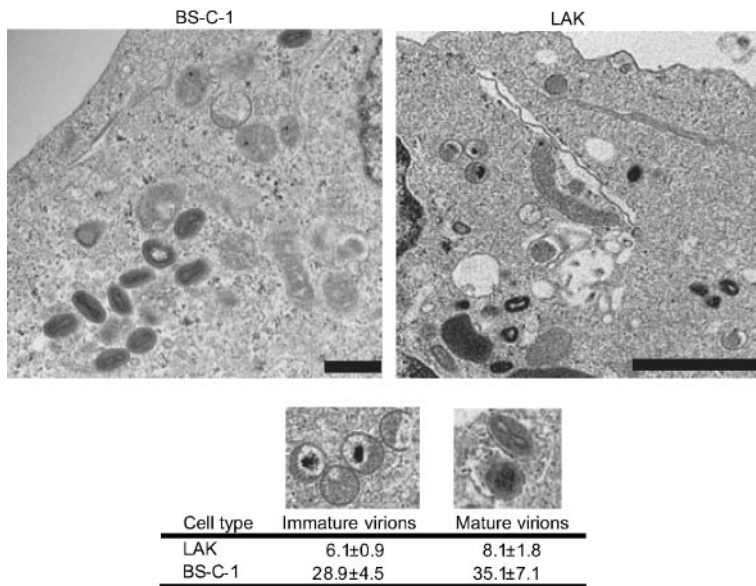


Fig. 6. LAKs contain immature and mature virions. LAKs and BS-C-1 cells were mock-infected or infected with ECTV (m.o.i.=5) for 24 h and harvested for electron microscopy. A total of 20 cells was scored to determine the number of mature and immature virion particles per section. Bars, 500 nm (left); 2 μ m (right).

2004). Kirwan *et al.* (2006) demonstrated VACV infection of human NK cell lines, naïve peripheral blood NK cells and blood-derived LAKs by both direct infection and co-culture with infected cells. Here, we have shown that, like VACV, ECTV infects both resting and activated NK cells (Fig. 2). Assessment of late viral gene expression and progeny virus infectivity following infection revealed comparable results for VACV and ECTV. Both VACV and ECTV were capable of infecting and expressing late genes weakly, which did not lead to efficient production of infectious virus particles (Figs 4 and 5) (Kirwan *et al.*, 2006). Consistent with a minimal increase in progeny, fewer immature and mature virion particles were detected in LAKs compared with BS-C-1 cells (Fig. 6). Studies of NK cell function following poxvirus infection also yielded analogous results. VACV infection of NK cells leads to decreased NK cell lytic activity, whilst ECTV infection resulted in decreased IFN- γ production following stimulation through the NK1.1 receptor (Fig. 3) (Kirwan *et al.*, 2006). Our studies also demonstrated decreased viability of LAKs following ECTV infection; the effect of VACV on NK cell viability has not been reported in the literature.

Although NK cells have been tested for infectability with a number of non-poxviruses, most studies did not monitor the entire replication cycle. A recombinant Epstein-Barr virus expressing GFP under the control of the HCMV immediate-early promoter demonstrated reporter gene expression in human NK cell clones and peripheral blood NK cells, but infected cultures were not monitored for virus DNA synthesis, virion assembly and progeny virus infectivity. These infected NK cells underwent apoptosis as early as 72 h.p.i. (Isobe *et al.*, 2004). In the case of human herpesvirus 6 (HHV-6), NK cell clones that do not kill HHV-6-infected peripheral blood mononuclear cells (PBMCs) are infectable and express an early/late viral

phosphoprotein, whilst NK cells capable of killing HHV-6-infected PBMCs appear refractile to virus replication (Lusso *et al.*, 1993). Clonal and polyclonal populations of NK cells from peripheral blood can be infected with human immunodeficiency virus type 1, as indicated by p24 antigen production, and show decreased viability similar to that seen with ECTV (Chehimi *et al.*, 1991).

Other cell types have been shown to be semi-permissive for poxvirus infections. VACV infection of dendritic cells (DCs) and monocytes results in abortive infection, with only early virus gene expression (Drillien *et al.*, 2000; Engelmayer *et al.*, 1999; Jenne *et al.*, 2000). Further studies have revealed that VACV infection of immature DCs blocks maturation, whilst infection of mature DCs impairs antigen-presenting abilities, suggesting yet another means of dampening the immune response (Engelmayer *et al.*, 1999; Jenne *et al.*, 2000). Semi-permissive poxvirus infections of DCs, monocytes and NK cells all appear to express early viral genes. In agreement with these studies, we detected synthesis of early proteins, including the immunomodulatory proteins EMICE and IFN- γ BP, which may skew the cytokine milieu in the surrounding microenvironment. A possible demonstration of this principle may be observed in the experiment in which infected LAKs failed to produce IFN- γ upon stimulation and a significant proportion of the uninfected LAKs within the same culture failed to synthesize IFN- γ , possibly through virus immunomodulation (Fig. 3).

Studies have shown the ability of NK cells to kill VACV-infected cells (Baraz *et al.*, 1999; Chisholm & Reyburn, 2006), as well as the importance of the cytokine IFN- γ in recovery from ECTV infection (Chaudhri *et al.*, 2004). Diminution of NK cell antiviral activities and disruption of the innate immune response by infection may be advantageous to the virus, particularly for a virus that, unlike poxviruses, does not

produce a large range of immunomodulators. For poxviruses, the ability to rapidly transcribe early genes, a number of which have immunomodulatory properties, may provide an additional mechanism to modify the local environment through semi-permissive infection of NK cells.

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