Title: The Ectromelia Virus SPI-2 Protein Causes Lethal Mousepox by preventing NK Cell Responses

Running Title: The Effect of ECTV SPI-2 on NK Cell Responses

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Abstract
Ectromelia virus (ECTV) is a natural pathogen of mice, causing mousepox and many of its genes have been implicated in the modulation of the host immune responses. The serine protease inhibitor-2 (SPI-2) is one of these putative ECTV host response modifier proteins. SPI-2 is conserved across orthopoxviruses, but results defining its mechanism of action and in vivo function are lacking or contradictory. We studied the role of SPI-2 in mousepox by deleting the SPI-2 gene or its serine protease inhibitor reactive site. We found that SPI-2 does not affect viral replication or cell intrinsic apoptosis pathways since mutant viruses replicate in vitro as efficiently as wild-type virus. However, in the absence of SPI-2 protein, ECTV is attenuated in mousepox-susceptible mice, resulting in lower viral loads in the liver, decreased spleen pathology and substantially improved host survival. This attenuation correlates with more effective immune responses in the absence of SPI-2, including earlier serum IFN-γ response, raised serum IL-18, increased numbers of granzyme B+ CD8+ T cells and, most notably, increased numbers and activation of NK cells. Both virus attenuation and the improved immune responses associated with SPI-2 deletion from ECTV are lost when mice are depleted of NK cells. Consequently, SPI-2 renders mousepox lethal in susceptible strains by preventing protective NK cell defences.

Introduction
Ectromelia virus (ECTV) is a large DNA virus and the causative agent of mousepox. This poxvirus has been long established as a classical model to study acute viral pathogenesis. ECTV encodes 175 genes and approximately
25% of the gene products are thought to be mediators of host immune evasion by targeting diverse processes such as cellular signalling, intrinsic and extrinsic cell death pathways and components of the innate immune response (6).

Host immune response modulation by poxviruses is essential for virulence and progeny production, with gene deletions of many of the immune host response modifiers resulting in virus attenuation (1, 47-49, 54). The first orthopoxvirus gene product found to be associated with evasion of the host immune system was the *cytokine response modifier A* (*crmA*) from cowpox virus (CPXV) (41). CrmA is one of three serine proteinase inhibitors (or serpins or SPI proteins) encoded by such viruses and is hereafter referred to as SPI-2. SPI-2 is highly conserved among orthopoxviruses (~90% amino acid sequence identity) such as ECTV, vaccinia virus (VACV) and variola virus (VARV) (51) and orthologues can also be found outside the genus, for example in myxoma virus (MYXV). Despite this conservation, a single common role for SPI-2 in poxvirus biology has so far not been identified, given that SPI-2 deletion in CPXV and VACV have varying effects *in vivo*, depending on the virus, host and mode of infection (25, 28, 30, 41, 49); and orthologues from CPXV and MYXV are not functionally interchangeable (39).

Different serpins inhibit distinct types of proteinases. Thus far caspase-1 (24, 27, 45), caspase-8 (11, 24) and granzyme B (44) have been shown to be targets of CPXV and/or VACV SPI-2 proteins. Yet ECTV SPI-2 was observed to inhibit *in vitro* caspase-1 and caspase-8, but not granzyme B, despite the high level of conservation among orthopoxviruses SPI-2 (50). The amino acids responsible for this apparent difference in specificity remain
to be identified, as is the question whether they contribute to differences seen
*in vivo*. Thus, despite extensive studies the role of SPI-2 in poxviruses
infection is still poorly understood. There is contradictory evidence as to
whether SPI-2 affects virus replication *in vivo* (28, 30). In addition, the wide
range of targets identified for SPI-2 *in vitro* suggest a number of possible
roles, from interference with cytolytic lymphocyte-mediated killing of infected
cells (37) to inhibition of cleavage activation of pro-inflammatory cytokines
such as IL-1β (24, 27, 45, 50) and IL-18.

In the present work, we generated ECTV SPI-2 mutants and
examined the *in vivo* function of this viral gene product in the pathogenesis of
mousepox, i.e. in a natural host-pathogen relationship. We found that SPI-2
is an important virulence factor that mediates its effects primarily via
prevention of NK cell responses.

**Material and Methods**

**Ethics Statement**

This study was carried out in strict accordance with the recommendations in
the Australian Code of Practice for the Care and Use of Animals for Scientific
Purposes. The protocol was approved by the Animal Experimentation Ethics
Committee (AEEC) of the Australian National University (Protocol Number:
J.IG.66.08). All efforts were made to minimize suffering.

**Mice and cells**

8 to 10-week-old female C57BL/6 and BALB/c mice were obtained from the
specific pathogen-free facility at the John Curtin School of Medical Research
(JCSMR Canberra, Australia) or the Animal Resources Centre (Perth, Australia) and used according to institutional experimentation approval. BS-C-1, a continuous African green monkey kidney cell line, L929, a continuous fibroblast line from the C3H mouse, and mouse embryonic fibroblasts (MEF) (26) were maintained in Eagle’s minimal essential medium (EMEM) plus 5% fetal bovine serum and antibiotics at 37°C in a humidified atmosphere with 5% CO₂.

**Viruses**

Plaque purified ECTV (Moscow strain) and recombinant viruses were propagated in murine L929 cells as previously described (7). Virus titres were determined by plaque assay on BS-C-1 monolayers. For virus multistep growth curves, L929 and MEF cell lines were infected at a MOI of 3 for 1 h. Unabsorbed virus was washed off and fresh media was added. At various times post-infection (PI), cell culture and cell-associated material were harvested separately and virus titres were determined by plaque assay using BS-C-1 monolayers.

**Recombinant virus generation**

Two ECTV SPI-2 deficient viruses containing different genetic modifications in the SPI-2 gene (locus ECTVgp162 of ECTV Moscow, NCBI accession NC_004105.1; also known as EVM161 and serpin C7L) were generated. The ECTV SPI-2Δ was generated by a general transient-dominant selection method used for constructing poxvirus mutants based on β-gal gene expression and *Escherichia coli* gpt with mycophenolic acid selection (12, 13).
The complete SPI-2 gene sequence was deleted. The ECTV SPI-2 SAD\(^\Delta\) and ECTV SPI-2 REV were generated by a novel transient-dominant selection method using GFP fluorescence and blasticidin resistance as selection markers (53). The nucleotides +271 to +698 were deleted in the open reading frame of the ECTV SPI-2 SAD\(^\Delta\). The deletion process inserts the AATGACGGCGAT exogenous sequence into the gene, generating a frame shift and an early stop codon in the mutant predicted protein. As a consequence, only the aminoacids 1 to 91 of the wt SPI-2 protein are expressed in the ECTV SPI-2 SAD\(^\Delta\). The ECTV SPI-2 REV was generated from the ECTV SPI-2 SAD\(^\Delta\) mutant. Blasticidin was purchased from Invivogen. Gene deletion/restoration was confirmed by PCR and sequencing.

**Western blot**

For analysis of SPI-2 expression, 1 x 10\(^7\) MEF cells were infected at a MOI of 10 for 4 h at 35\(^\circ\)C. Cell lysates were prepared by cell pellet resuspension with radioimmunoprecipitation assay lysis buffer (25 mM Tris-HCl pH 8, 137 mM sodium chloride, 2 mM EDTA, 1% glycerol, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)). Lysates were cleared using QIAshredder columns (Qiagen), separated by SDS-polyacrylamide gel electrophoresis with 12% gels, and blotted against nitrocellulose membranes (Bio-Rad). SPI-2 protein was detected using a mouse anti-SPI-2 monoclonal antibody (BD-Pharmingen cat no 65921A, dilution 1/400) as primary antibody, a secondary anti-mouse IgG1 peroxidase-coupled antibody (BD Pharmingen), and enhanced chemiluminescence substrate (Amersham). For actin
detection, a rabbit anti-actin polyclonal antibody (Santa Cruz) was used followed by anti-rabbit IgG peroxidase-coupled antibody (BD Pharmingen).

Infections

For in vivo studies of ECTV pathogenesis, mice were infected with $10^3$ PFU or $10^6$ PFU subcutaneously (s.c.) in both hind legs in a total volume of 50 µL PBS (25 µL per leg). Mice were monitored daily by blinded examiners for illness signs of mousepox such as coat condition, conjunctivitis, body paralysis and limb swelling. Mice were euthanized if disease manifestations were extremely severe in one or more illness parameters based on a numeric score. Score grades of 1 to 3 were attributed to each disease parameter and mice with score 3 in one parameter or 5 in cumulative parameters were sacrificed. Coat condition: 1 = slightly rough, 2 = dishevelled/wounds forming, 3 = bleeding or irritated wounds/severe hair loss. Eyes condition: 1 = mild discharge, 2 = severe conjunctivitis. Movement: 1 = abnormal/uncoordinated, 2 = walking on tiptoe/reluctant to move, 3 = staggering/paralysis. Limbs condition: 1 = swelling, 2 = abnormal limbs, 3 = severe necrosis/loss of limbs.

Virus titration of organs, histological evaluation, and liver enzymes and cytokines levels in the serum

Mice were infected s.c. with $10^3$ PFU and sacrificed at indicated times PI for organ and serum collection. Liver, spleen and lungs samples were homogenised in media volume proportional to the sample weight (grams x 9 = mL). Popliteal draining lymph node samples were homogenised in a fixed volume of media. The viral load was quantified by plaque assay using BS-C-1
monolayers. Histology samples were fixed in 10% formalin and paraffin-
embedded tissue sections were stained with hematoxylin and eosin (H&E) or
with 4'-6-diamidino-2-phenylindole (DAPI) (Sigma) and Dead End™
Fluorometric TUNEL system (Promega), according to manufacturer’s
protocols. The percentages of apoptotic cells measured by terminal
deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining were
manually counted as the green fluorescent (TUNEL⁺) colocalized with blue
fluorescent (DAPI⁺) cells within the total DAPI⁺ cell population. For each
mouse sample, >500 cells were analysed in 5 different regions of the tissue
section visualized in a Leica TCS SP5 Confocal microscope. Liver enzyme
(alanine aminotransferase and aspartate aminotransferase) levels in the
serum were determined at the ACT Pathology unit of the Canberra Hospital,
Australia. The cytokine levels of mouse IFN-γ, TNF-α, IL-2, IL-4, IL-10, IL-6
and IL-17A in the serum were quantified by the BD CBA mouse Th1/Th2/Th17
cytokine kit (BD Biosciences). The levels of IL-18 and IL-1β in the serum of
infected mice were determined by the mouse IL-18 ELISA (Bender
MedSystems) and the BD OptEIA mouse IL-1β ELISA set (BD Biosciences),
respectively. For IFN-β detection, rabbit polyclonal antibody against mouse
IFN-β (PBL interferon source 32400-1) and rat monoclonal antibody against
mouse IFN-β (RMMB-1) (PBL interferon source 22400-1) were used in a
capture ELISA.

Flow cytometry
Infected mice were sacrificed at given times PI, and spleens and blood
collected. Single splenocyte suspensions were obtained by pressing organs
through cell strainers followed by hypotonic red blood cell lysis. Total splenocyte numbers were determined by counting, and $10^6$ cells per sample were analysed by FACS. 25 $\mu$L of each blood sample treated with heparin followed by red blood cell lysis was used for FACS staining. Splenocytes and blood cells were incubated in 7-amino-actinomycin D (7AAD) and stained for CD8$\alpha$ APC-conjugated clone 53-6.7 (BD Pharmingen), CD8$\alpha$ FITC-conjugated clone 53-6.7 (BD Pharmingen), CD4 FITC-conjugated clone H129.19 (BD Pharmingen), B220 PE-conjugated clone RA3-6B2 (BD Pharmingen), F4-80 FITC-conjugated clone CI:A3-1 (AbD Serotec), and CD49b Alexa Fluor 647-conjugated clone DX5 (BioLegend). Cells were then fixed with Fixation buffer (BioLegend), permeabilized with 0.5% saponin, and stained with polyclonal rabbit anti-granzyme B serum (a gift from Prof. Markus Simon, Max Planck Institute, Freiburg, Germany) or pre-immune rabbit serum, followed by Alexa Fluor 647-conjugated (Molecular Probes) or PE-conjugated (CALTAG Laboratories) rat anti-rabbit IgG. For apoptosis assays, MEF cells were infected as described before and, at given hours PI, cells were stained with AnnexinV-PE Apoptosis Detection kit I (BD Pharmingen) or with DiOC$_6$ (Molecular Probes). Cells were analysed on a FACSCalibur flow cytometer (BD Biosciences) and on WEASEL FACS software (Walter and Eliza Hall Institute of Medical Research) or FlowJo (Flow Cytometry Analysis Software).

**NK cell cytotoxic assay**

Mice were infected s.c. with $10^3$ PFU and sacrificed at 4 and 6 days PI. Spleens were collected and tested for NK cytotoxicity in standard $^{51}$Chromium-release assay (34), using uninfected YAC cells as targets.
Uninfected mice were used as controls. P values were calculated from specific lysis from a four-point logarithmic regression curve, interpolated at an e/t ratio of 30.

NK depletion

For NK depletion, 20 µL of anti-Asialo GM1 anti-serum (Wako Pure Chemical Industries, Ltd.) or 100 µL of pre-immune rabbit serum (Sigma), each diluted to 200 µL with PBS, were injected intraperitoneally (i.p.) one day before virus infection, 2 days PI, and 5 days PI. Three mice per group were injected in each of two independent experiments. In one experiment PBS was injected as negative control and in the other experiment pre-immune rabbit serum was used as negative control for NK depletion.

Results

1. ECTV SPI-2 mutants and revertant

We generated two ECTV SPI-2 deficient viruses containing different genetic modifications in the SPI-2 gene. The ECTV SPI-2 SAD^∆ was produced by deletion of 427 bp of the coding sequence, resulting in the absence of the carboxy-terminal region in the SPI-2 protein. Most of the SPI-2 mRNA will not be translated due to a frame-shift mutation in the coding sequence, however, a truncated form of SPI-2 protein lacking the serpin reactive site (LVSD) could be possibly expressed. The second mutant, referred to as ECTV SPI-2^∆, was generated by deletion of the complete gene. A revertant virus (ECTV SPI-2 REV) was generated by restoring the wild-type (wt) SPI-2 gene on the ECTV SPI-2 SAD^∆ background. The genetic profile of the recombinant viruses was
confirmed by PCR and sequencing. All the remaining SPI-2 gene coding sequence plus 39 upstream nucleotides and 101 downstream nucleotides were determined by sequencing of the ECTV SPI-2 SAD\(^{\Delta}\). 240 upstream nucleotides and 410 downstream nucleotides were determined by sequencing of the ECTV SPI-2\(^{\Delta}\).

The SPI-2 or a truncated form of this protein were not detected by Western blot analysis of uninfected cells or cells infected with the ECTV SPI-2 SAD\(^{\Delta}\) or ECTV SPI-2\(^{\Delta}\) whereas lysates of cells infected with wt ECTV or ECTV SPI-2 REV presented a band of expected size for the full-length SPI-2 protein (Supplemental Figure 1). All samples expressed actin, including uninfected cells.

2. Deletion of SPI-2 does not compromise virus growth in vitro

To determine whether SPI-2 is required for efficient replication in vitro, virus growth curves were established using two different types of mouse cell lines. The recombinant viruses tested had similar replication efficiencies compared to wt ECTV in L929 and MEF cells (Figure 1A and B, respectively).

In agreement with this observation, the appearance of apoptotic features triggered by infection was similar between wt virus and mutants measured by phosphatidylserine exposure (Figure 1C) and production of reactive oxygen species (data not shown). Both SPI-2 mutant viruses induced lower disruption of the mitochondrial membrane potential than wt virus 24h post-infection (PI), but the difference was not statistically significant (Figure 1D).

3. ECTV SPI-2 protein is a potent virulence factor in susceptible mice
To study the importance of SPI-2 in viral pathogenesis, C57BL/6 and BALB/c mice were infected subcutaneously (s.c.) with either wt ECTV or recombinant viruses. Disease signs, such as dishevelled coat, conjunctivitis, foot swelling and movement impairment, were recorded daily and scored. It is well established that C57BL/6 mice are highly resistant (LD$_{50}$ > 10$^5$ PFU) whereas BALB/c mice are susceptible (LD$_{50}$ < 10 PFU) to s.c. ECTV infection (2). Therefore, C57BL/6 mice were infected with a high (10$^6$ PFU) or a low dose (10$^3$ PFU) of each virus. BALB/c mice were infected with 10$^3$ PFU, which results in 100% mortality when wt ECTV is used.

All C57BL/6 mice infected with ECTV SPI-2 SAD$^\lambda$ or wt virus survived for at least 4 weeks PI and showed few or no disease signs, regardless of the dose given (data not shown). As was expected, BALB/c mice infected with ECTV or ECTV SPI-2 REV virus were highly susceptible to classical mousepox disease, and most animals succumbed to infection, with a median survival time of 9 days PI. Severe manifestations of disease were frequently observed before mice were euthanized. In contrast, 80 to 90% of mice infected with SPI-2-deficient viruses survived the infection (Figure 2), although some signs of illness including dishevelled coat, conjunctivitis and limb swelling were observed throughout the course of infection. About half of the mice surviving ECTV SPI-2 mutants infection still had detectable virus by 4 weeks PI (mean +/- SD of 3.8 x 10$^3$ +/- 1.6 x 10$^3$ in the spleen and 5.2 x 10$^2$ +/- 1.9 x 10$^2$ in the liver), suggesting that clearance of the virus took longer than in mousepox infection of resistant strains. By 8 weeks PI, mutant viruses were not detectable any more.
4. Diminished viral loads in livers of mice infected with ECTV in the absence of SPI-2

As the final cause of death in ECTV-infected mice has been described in the literature as a consequence of acute hepatitis followed by multi-organ failure (18), it was of interest to know whether the virus loads in various target organs, especially the liver, were affected by deletion of SPI-2 from ECTV. Surprisingly, viral loads in the spleen and popliteal draining lymph nodes were similar for all viruses (Table 1). A remarkably high viral load in the spleen was observed on day 4 and day 6 PI in mice infected with ECTV or ECTV-SPI-2 mutants, and only a modest but significant (P > 0.05) reduction in titre was seen for ECTV SPI-2 SAD\(^\Delta\) at 4 days PI relative to wt virus (Table 1). On the other hand, the viral loads in the liver increased more slowly and at both days 4 and 6 PI ECTV SPI-2 mutants had replicated to lower levels than ECTV (Table 1). The liver viral load decrease 6 days PI was about 100-fold in ECTV-SPI-2 SAD\(^\Delta\)-infected mice and 10-fold in ECTV SPI-2 SAD\(^\Delta\)-infected mice, compared to wt virus. In another independent experiment, we found higher viral loads (10-fold) in the livers of mice infected with 10\(^3\) PFU of ECTV SPI-2 REV compared to mice infected with the ECTV SPI-2 SAD\(^\Delta\) (P = 0.0403, data not shown). We also found lower viral loads in the lungs of ECTV SPI-2 SAD\(^\Delta\)-mutant-infected mice compared to ECTV-infected mice (Table 1).

5. Liver and spleen pathology caused by mousepox is reduced in the absence of SPI-2 protein

Next we asked whether the higher viral loads seen in livers of ECTV-compared with ECTV SPI-2 SAD\(^\Delta\)-infected mice was associated with a
difference in overt liver pathology. Examination of liver sections collected at
days 6 and 9 PI revealed intense lymphocyte infiltration and several necrotic
foci throughout the entire organ in both groups, but no obvious differences
between the two groups (Supplemental Figure 2). In addition, the overall liver
structure was preserved, unlike the pathology recorded after ECTV infection
of highly susceptible perforin-deficient C57BL/6 mice (38). However, levels of
two serum markers of liver pathology, aspartate aminotransferase (AST) and
alanine aminotransferase (ALT), were increased in ECTV-, but not ECTV SPI-
2 SAD\(^\Delta\)-infected mice (Figure 3A and B). The reduced viral loads in the liver
and improved liver function in mice infected with ECTV mutant lacking SPI-2
suggest that the increased survival of these mice is linked to an attenuation of
the hepatitis caused by the wt ECTV infection in BALB/c mice (18).

In contrast to liver, a significant difference in histopathology was found
between spleens of mice infected with wt virus and mice infected with ECTV
SPI-2 SAD\(^\Delta\). Wt ECTV-infected mice presented spleens with massive tissue
destruction at day 6 PI whereas ECTV SPI-2 SAD\(^\Delta\)-infected mice presented
markedly less tissue damage (Figure 4B and C, respectively). In concordance
with this histological evidence of widespread necrotic damage, the sizes of
spleens from mice infected with wt ECTV or ECTV SPI-2 REV were greatly
reduced macroscopically, and total splenocytes numbers were at least 2-fold
lower, compared to those from mice infected with ECTV SPI-2 SAD\(^\Delta\) or ECTV
SPI-2\(^\Delta\) (Figure 4D).

As more direct measure of the damage caused by infection to liver
and spleen, tissue sections were analysed for the presence of apoptotic cells
presenting DNA fragmentation measured by TUNEL staining (Figure 5).
Similar levels of apoptosis were found in organs of mice infected with wt or mutant virus at day 4 PI. At both liver and spleen tissues, the wt ECTV induced higher proportions of TUNEL$^+$ cells than the ECTV SPI-2 SAD$^+$ 6 days PI, even though the differences were not statistically significant.

Although similar viral loads were observed in spleens of ECTV- and ECTV SPI-2 mutant-infected mice (Table 1), indicating that on a per volume basis virus titres are higher in wt virus infected mice, the overall spleen destruction caused by mousepox is greatly exacerbated in the presence of the SPI-2 protein. In view of the comparable growth properties of the mutant and wt viruses, the spleen protection observed in mutant-infected mice suggests that the virulence factor SPI-2 thwarts immune-mediated control of ECTV infection.

6. **IL-18 and IFN-γ production are enhanced in mice infected with ECTV SPI-2 mutants**

Host recovery from a primary mousepox infection depends on the induction of a Th1-type immune response and requires effective antiviral cytotoxic T and NK cell immunity (3, 5, 33, 43). To test if these immune responses were differentially elicited by ECTV in the presence or absence of SPI-2, the cytokine profile in the serum of infected mice was examined. The levels of IL-2, IL-4, IL-17A, IL-10, IL-6, IFN-γ and TNF-α were assessed at days 2, 4, 6 and 9 PI. Given their often rapid induction, IFN-β, IL-1β and IL-18 were analysed in addition at 0, 6, 12 and 24 hours PI. All infected mice generated a predominantly Th1-type of cytokine response, characterized by IFN-γ, IL-18 and TNF-α production. However, IL-10 was also detected in
several mice throughout the infection (data not shown). Levels of IFN-β, IL-1β, IL-2, IL-4 and IL-17A were not markedly raised at any of the time points tested compared to uninfected mice.

Most notably, ECTV-infected mice had considerably lower levels of serum IFN-γ than mice infected with ECTV SPI-2 mutants at 4 days PI, while two days later all infected mice reached similar peak levels of IFN-γ, decreasing by 9 days PI (Figure 6A). IL-18 release was only detected in the serum between 4 and 9 days PI, and at 6 days PI the levels of this cytokine were higher in ECTV SPI-2 mutants-infected mice than in ECTV-infected mice (Figure 6B). IL-18 was not detected in the blood at any time tested before day 4 PI. In contrast, increased levels of TNF-α in the serum of mice infected with ECTV expressing the SPI-2 protein were found at 6 days PI (Figure 6C). No difference in IL-10 and IL-6 levels in the serum was detected between wt virus- and mutant virus-infected mice. Thus, in the absence of SPI-2, the host Th1 cytokine responses, in particular IFN-γ and IL-18, are enhanced.

7. NK cell and CD8+ T cell responses are enhanced in the absence of SPI-2

Given the increased protection of the splenic tissue and the Th1 cytokine response enhancement observed in mutant-infected mice, we investigated the development and activation of lymphocyte populations during ECTV infection with or without SPI-2. The relative percentages of B cells, CD4+ and CD8+ T cells and NK cells in the spleen and circulating in the blood 6 days PI did not markedly differ between ECTV- and ECTV SPI-2 SAD∆-infected mice (Figure 7B, C and F; and data not shown). However, because
the total number of splenocytes in ECTV SPI-2 SAD^Δ-infected mice was around double that in wt virus-infected mice (Figure 4D), the absolute number of each lymphocyte population was markedly reduced following wt virus infection (Figure 7A and E, and data not shown).

Granzyme B is an essential effector molecule for recovery from mousepox (38, 42). Therefore, granzyme B expression in CD8^+ T cells and NK cells from ECTV- and ECTV SPI-2 SAD^Δ-infected mice was assessed. At 6 days PI, the percentage of CD8 and granzyme B-positive cells in the spleen was low (around 1%) in both ECTV- and ECTV SPI-2 SAD^Δ-infected mice. In blood, a ~2-fold greater percentage of cells were CD8^+ and expressed granzyme B in ECTV SPI-2 SAD^Δ-infected mice compared to ECTV-infected mice (Figure 7D) although this difference did not reach statistical significance.

Ex-vivo cytotoxicity against virus-infected, MHC-I matched target cells was marginal by splenocytes from mice infected with either virus for 4 and 5 days (data not shown). In contrast, splenocytes from mice infected with the ECTV SPI-2 SAD^Δ for 6 days were able to induce target cell lysis and were more cytotoxic than splenocytes from mice infected with the wt (P < 0.0077) or revertant (P < 0.0008) virus (Supplemental Figure 3).

The proportion and total number of NK cells expressing granzyme B in the spleen and blood were higher in mice infected with ECTV SPI-2 SAD^Δ than in mice infected with wt virus at 6 days PI (Figure 7G, H and I). This increased granzyme B^+ NK cell population accounted for approximately 25% of the total circulating leukocytes in the blood following mutant virus infection (Figure 7G). The increased expression of granzyme B in NK cells of ECTV SPI-2 SAD^Δ-infected mice suggests an increase in cytotoxic potential of these
cells compared to NK cells of wt ECTV-infected mice. At day 4 PI, the ex-vivo cytotoxicity of splenocytes against YAC-1 cells was similar for ECTV- and ECTV SPI-2 SAD\(^{Δ}\) mutant-infected mice (Figure 8A). However, at day 6 PI, the ex-vivo cytotoxicity of splenocytes against YAC-1 cells was higher for ECTV SPI-2\(^{Δ}\) (P = 0.0097) and ECTV SPI-2 SAD\(^{Δ}\) (not statistical significant, P = 0.0526) mice compared to mice infected with wt or revertant virus (Figure 8B). Even though the increase in NK cytotoxicity induced by the ECTV SPI-2 SAD\(^{Δ}\) infection was not statistical significant with the sample size used, both SPI-2 mutant viruses presented a similar trend and, therefore, we believe that the increased levels of target cell lysis mediated by ex-vivo splenocytes are representative for both mutant-infected mice compared to mice infected with ECTV expressing SPI-2. This result correlated with the lower granzyme B expression in NK cells and the lower levels of IL-18 in the blood of wt ECTV-infected mice at this time PI relative to ECTV SPI-2 SAD\(^{Δ}\)-infected mice, suggesting that SPI-2 may prevent optimal NK cell activation and cytotoxicity.

8. SPI-2 abrogates the protective NK cell response in mousepox infection

To test if the vigorous NK cell response is essential for resistance of BALB/c mice against infection with ECTV SPI-2 mutants, NK cells were depleted in vivo. Both CD4\(^{+}\) (data not shown) and CD8\(^{+}\) lymphocyte populations (Supplemental Figure 4) were not markedly affected by anti-Asialo GM1 treatment, whereas the DX5\(^{+}\) cell population was effectively depleted (Figure 9A). Lack of NK cells rendered BALB/c mice as susceptible to ECTV SPI-2 SAD\(^{Δ}\) infection (all animals died by day 7 PI) as non-depleted
mice to wt ECTV (Figure 9B). The liver viral loads were ~10-fold and ~1000-fold increased in NK cell-depleted mice compared to control mice when infected with wt virus or mutant virus, respectively (Figure 9C). Importantly, following NK cell depletion, mice infected with mutant and wt viruses presented similar liver viral loads in magnitude at 6 days PI. Moreover, the depletion of NK cells in ECTV SPI-2 SADΔ-infected mice resulted in pronounced tissue destruction in spleen and loss of splenocytes (Figure 9D), similar to that seen in NK-sufficient mice infected with wt virus. The NK depletion treatment was not intrinsically cytotoxic because equivalent splenocyte numbers were obtained from NK cell-depleted and non-depleted uninfected mice.

The levels of IFN-γ found in the serum of NK cell-depleted mice infected with the mutant virus were reduced to the levels found in NK sufficient mice infected with the wt virus at 4 days PI (Figure 9E). In contrast, the levels of TNF-α in the serum of NK-depleted mice were remarkably high compared to non-depleted mice at 6 days PI (Figure 9F).

Collectively, the decreased survival, increased liver viral load and reduced levels of serum IFN-γ of NK cell-depleted mice infected with ECTV SPI-2 SADΔ support the conclusions that NK cells are the key cell executioner of protection in mutant virus-infected mice against mousepox and that SPI-2 deletion counteracts this disease ameliorating immune pathway. Furthermore, NK cell are the likely predominant source of early IFN-γ. Thus, the main downstream effect of SPI-2 expression is the prevention of NK cell responses in susceptible strains.
Discussion

In the present study we establish that the ECTV SPI-2 protein is a virulence factor that prevents the induction of a protective NK cell response in susceptible mouse strains. This conclusion is based on the striking similarity seen for multiple aspects of pathogenesis in NK cell-depleted mice infected with the SPI-2 mutant virus compared with NK-sufficient animals infected with wt ECTV. These similarities include viral loads in the liver, delayed IFN-γ response, spleen pathology and levels of mortality (Figure 9). In short, all the hallmarks of attenuation due to loss of SPI-2 activity from ECTV are absent in mice lacking NK cells.

We also found that the proportions of CD8⁺ T cells that express granzyme B and the ex-vivo cytotoxicity of these cells were elevated in mutant-infected mice (Figure 7D and Supplemental Figure 3). However, it seems more likely that the improved CD8⁺ T cell responses observed in mice infected with the SPI-2 mutant virus are a consequence of the enhanced NK cell function rather than a direct effect of SPI-2 on CD8⁺ T cells. This is because previous work has shown that lack of an efficient NK response leads to uncontrolled virus replication and compromises the development of CD8⁺ T cells (14, 15). Our data support these findings as we also found that NK cell-depleted, ECTV-infected mice have severely lymphopenic spleens (Figure 9D) with reduced numbers and proportions of CD8⁺ T cells expressing granzyme B (Supplemental Figure 4), even though the proportion of all CD8⁺ T cells in the spleen is not affected by the absence of NK cells.

While virus attenuation was also found in other orthopoxviruses SPI-2 mutants (28, 30, 31, 41, 49), this is the first time that SPI-2 effects on NK cell
function have been observed. The only previous work to suggest an inhibitory effect on in vivo cell mediated immune responses by this viral protein was an increased influx of CD3+ cells into the ear pinnae of C57BL/6 mice 8 days PI with a CPXV crmA mutant (30). In contrast, one study attributed the attenuation of the VACV SPI-2 mutant virus to in vivo replication deficiency and suggested that the mutant virus elicits humoral and cell-mediated responses as efficiently as the parental virus (28). Livers and spleens of mice infected with wt ECTV presented similar or slightly increased numbers of apoptotic foci compared to mice infected with mutant virus at 6 days PI (Figure 5), suggesting that an anti-apoptotic function for SPI-2 is unlikely. These observations reinforce the notion that caution must be exercised when extrapolating findings across different infectious models even within closely related viruses (36).

There are several pathways by which SPI-2 might inhibit a protective NK cell response in the mousepox model. One possibility is the inhibition of infected-cell killing by either blocking the granule exocytosis pathway, proposed to be mediated mainly by granzyme B (16), or the death receptor pathway mediated by caspase-8. However, in vitro studies have suggested that ECTV SPI-2 may only weakly inhibit mouse granzyme B (50) and this serine protease restricts ECTV replication even in the presence of SPI-2 (42). We have shown previously that the CPXV SPI-2 protein inhibits the death receptor pathway (37) and recently we also found that ECTV SPI-2 blocks target cell killing by virus-immune splenocytes in the absence of perforin (C. R. Melo-Silva et al., unpublished data). However, perforin and the granule exocytosis pathway are essential for mousepox recovery (35, 38) and mice
deficient in Fas Ligand or Fas molecule are not overtly susceptible to ECTV infection (C. R. Melo-Silva et al., unpublished data, (33)), suggesting that SPI-2 effect on NK cell responses is not only or not at all connected to inhibition of target cell lysis.

Interestingly, we found that at 6 days PI ECTV-infected mice present increased levels of TNF-α in the blood compared to ECTV SPI-2 mutants-infected mice (Figure 6C). TNF-α is a cytokine classically associated with induction of apoptosis in TNFR-expressing infected cells (29, 33, 46). This cytokine binds to death receptors expressed in the plasma membrane of target cells and the result is activation of pro-apoptotic caspases and cell death. However, this cytokine has also been implicated in immunopathology (8, 17, 55). Most ECTV-infected mice die by day 10 (Figure 2), whereas ECTV SPI-2 mutant-infected mice survive. Moreover, ECTV-infected mice have higher viral load in the liver than mutant-infected mice at 6 days PI (Table 1). Taken together, these data suggests that, even if the TNF-α circulating in the blood induces apoptosis in infected cells, this is not sufficient to protect ECTV-infected mice. Therefore, we speculated that the increased levels of TNF-α in blood of ECTV-infected mice could be contributing to disease by immunopathology. Liver and lungs exert specialized and essential functions and excessive unspecific tissue damage could contribute to multi-organ failure and death. In agreement with this notion, NK-depleted infected mice presented very high levels of this cytokine 6 days PI, one day before their death (Figure 9F).

Another putative mechanism of action is inhibition of caspase-1, a property which was the first function assigned to an orthopoxvirus-encoded
SPI-2 protein (45). Caspase-1 converts IL-18 and IL-1β from their inactive precursors to the biologically active cytokines (10). IL-18 is an inducer of IFN-γ and a stimulator of NK cell activation and cytotoxicity (16, 19, 20, 40, 52). The SPI-2 inhibition of caspase-1 in, for instance, infected macrophages or dendritic cells (DC) might deprive developing NK cells of an environment containing IL-18, which could lead to reduced production of IFN-γ, a cytokine that is important for recovery from mousepox (23), or to reduced NK cell cytotoxicity (16). The increased levels of IL-18 in the blood of mice infected with the ECTV SPI-2 mutants (Figure 6B) correlated with increased NK and CD8+ T cell numbers, increased granzyme B expression by these cells (Figure 7), and higher NK cytotoxicity (Figure 8B) suggesting that the SPI-2 inhibition of caspase-1 activity may indeed partially affect the NK cell response.

Similar in vivo responses were found between the present work and studies using the ECTV p13 mutant. The p13 protein encoded by ECTV is an IL-18 BP capable of competing with this cytokine cellular receptor reducing IL-18 mediated NF-κB signalling (4). The ECTV p13 mutant does not seem to affect intrinsic virus replication and the major consequence of reduced IL-18 signalling is a defect in NK cell responses. Taken together, these studies highlight the importance of IL-18 in the development of an efficient NK cell antiviral response.

Given that caspase-1 also activates pro-IL-1β, its inhibition may also lead to a reduction of the pyrogenic inflammatory response mediated by this cytokine. We could not detect systemic IL-1β at any time tested. In addition, we failed to detect any IL-1β secretion by mouse macrophage cells infected in
vitro with ECTV SPI-2 mutants. For instance, IL-1β secretion induced by LPS and ATP treatment of peritoneal exudate cells (32) was efficiently inhibited by ECTV infection, regardless of SPI-2 (Supplemental Figure 5). We therefore believe that this is unlikely to be a dominant pathway of initiating a cascade ultimately leading to a protective NK cell response.

The finding that NK cells are important in recovery from mousepox in a susceptible strain reflects previous observations of their importance in resistant C57BL/6 mice (14, 15, 43). NK cells are essential to curb virus spread and allow the development of antiviral CD8+ T cell and B cell responses in resistant mice and NK cell deficiency has been associated with susceptibility in DBA/2J mice (9, 21). The important difference between resistant and susceptible strains in a s.c. infection with ECTV is that, while C57BL/6 mice mount a strong NK cell response even in the presence of SPI-2, in the susceptible BALB/c strain, SPI-2 prevents NK cell development and/or induction. The resistant phenotype may be characterized by redundancy or stronger responses that are either inhibited or present at lower levels in susceptible mice, as has indeed been suggested for inherent ability for IFN-γ production (5, 22). The notion of redundancy is supported by the fact that BALB/c mice do not express IL-12p40 mRNA (22), another activator of NK cells, suggesting that the NK cell response in BALB/c mice may be more dependent on IL-18 than are NK cells from C57BL/6 mice and this is probably one of the reasons why the virulence mediated by SPI-2 is exacerbated in the BALB/c strain.

In conclusion, the data presented here demonstrate that the ECTV SPI-2 protein is a virulence factor in susceptible mice infection and plays a
previously unexpected role in the prevention of NK cell responses. This adds
to the repertoire of known biological functions of SPI-2 homologues and
suggests that these genes may have been retained by different
orthopoxviruses to counter different aspects of the host response to infection.

Acknowledgments

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References


52. **Wang, Y., G. Chaudhri, R. J. Jackson, and G. Karupiah.** 2009. IL-12p40 and IL-18 play pivotal roles in orchestrating the cell-mediated immune response to a poxvirus infection. J Immunol **183**:3324-31.


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**Figure 1. In vitro replication and apoptosis induction of ECTV SPI-2 mutants is similar to wt virus.** L929 (A) and MEF (B) cells were infected at a MOI of 3 with ECTV or ECTV SPI-2 mutants. The cell-associated viral load was quantified by plaque assay. Graphs show means +/- SEM of one representative of three independent experiments. (C and D) MEF cells were infected as in (B). Infected cell apoptosis (C) and mitochondrial potential disruption (D) were quantified by phosphatidylycerine exposure (Annexin^+ 7AAD^-) and DiOC₆ staining of MEF cells, respectively, at given times PI.
Figure 2. ECTV SPI-2 mutants are strongly attenuated in vivo. (A) BALB/c female mice were infected s.c. with $10^3$ PFU of either ECTV, ECTV SPI-2 SAD$^3$, ECTV SPI-2$^4$, or ECTV SPI-2 REV. Illness signs were scored daily, % survival excludes dead and euthanized mice. Data combined from two independent experiments. (B-C) Illness signs of the two experiments pooled in the survival graph shown in (A). Mice that have been euthanized or found dead were given the score at the day of euthanasia or 5, respectively, for the days following their death. * $P < 0.05$, ** $0.001 \leq P \leq 0.01$ and *** $P < 0.0001$ in relation to both ECTV and ECTV SPI-2 REV.

Figure 3. Liver damage induced by mousepox is reduced in the absence of SPI-2. BALB/c mice were infected s.c. with $10^3$ PFU of ECTV, ECTV SPI-2 SAD$^3$, or mock infected. Serum was collected at 6 days PI and the liver enzyme aspartate aminotransferase (A) and alanine aminotransferase (B) levels determined. Graphs represent means +/- SEM of data combined from three independent experiments. * $P < 0.05$ in relation to mock-infected mice. NS: not statistically significant ($P > 0.05$).

Figure 4. Spleen pathology caused by mousepox is reduced in the absence of SPI-2 protein. BALB/c mice were mock-infected (A) or infected s.c. with $10^3$ PFU of wt ECTV (B) or ECTV SPI-2 SAD$^3$ (C). The animals were sacrificed at 6 days PI and spleens were sectioned and stained with H&E, arrows indicate the presence of pyknotic cells. (D) Total splenocyte numbers are increased in mice infected with ECTV lacking SPI-2. Mice were infected as in (A-C) with each virus. At day 6 PI, spleens were processed for cell...
counting. The graph represents means +/- SEM of data combined from two independent experiments (N = 3 for each experiment). *** P < 0.0001 in relation to both wt ECTV and ECTV SPI-2 REV.

Figure 5. ECTV lacking SPI-2 presents similar proportions of TUNEL+ cells in spleen and liver compared to wt ECTV. BALB/c mice were infected s.c. with 10^3 PFU of wt ECTV or ECTV SPI-2 SADΔ. Organs collected at given days PI were sectioned, formalin-fixed and analysed for DNA fragmentation with TUNEL staining. Cells presenting green fluorescence (TUNEL+) colocalized with blue fluorescence (DAPI) were counted on spleen (A) and liver (B) sections. (C-F) Representative pictures of spleen (C and D) and liver (E and F) sections of mice infected with wt ECTV (C and E) or ECTV SPI-2 SADΔ (D and F) for 6 days, stained for TUNEL and DAPI.

Figure 6. IFN-γ and IL-18 cytokine levels are increased in the serum of SPI-2 mutant-infected mice. BALB/c mice were infected s.c. with 10^3 PFU of ECTV, ECTV SPI-2 SADΔ, or ECTV SPI-2Δ. Sera were collected at given time points; IFN-γ (A), IL-18 (B) and TNF-α (C) levels determined by cytometric bead array or ELISA. Graphs show means +/- SEM from combined data from three independent experiments (N = 3 for each experiment). * P < 0.05, ** 0.001 ≤ P ≤ 0.01 in relation to ECTV.

Figure 7. SPI-2 protein reduces the size of cytotoxic lymphocyte populations in mousepox infection. BALB/c mice infected s.c. with 10^3 PFU of each virus were sacrificed at 6 days PI. Spleens and blood were harvested...
for FACS staining. (A) Total numbers of CD8+ splenocytes. (B and C) Percentages of CD8+ cells found in the spleen and blood, respectively. (D) Percentages of granzyme B+ CD8+ cells. (E) Total numbers of DX5+ splenocytes. (F) Percentages of DX5+ cells in the spleen and blood. (G and H) Percentages of granzyme B+ DX5+ cells. (I) Total numbers of DX5+ and granzyme B+ splenocytes. Graphs are the means +/- SEM of data pooled from three independent experiments (A, B, D-G) or two independent experiments (C, H, I) (N = 3 for each experiment). * P < 0.05, ** 0.001 ≤ P ≤ 0.01 and *** P < 0.0001 in relation to both wt ECTV and ECTV SPI-2 REV. (J-M) Representative flow cytometry plots of blood lymphocyte samples from mice infected with ECTV SPI-2 SAD (J, L and M) or ECTV (K) shown in column graph G. The background staining of pre-immune rabbit serum was used for gating granzyme B+ populations (J) and NK-cell depleted blood lymphocytes were used for gating DX5+ populations (M).

**Figure 8. Increased NK cell cytotoxicity in the absence of SPI-2.** Mice were infected s.c. with 10³ PFU of each virus, spleens were harvested at 4 (A) and 6 (B) days PI, and standard ⁵¹Cr-release assay against YAC cell targets was performed for 6h. Splenocytes from uninfected mice were used as controls.

**Figure 9. NK cells mediate survival of ECTV SPI-2 SAD-infected mice.** BALB/c mice were treated with anti-asialo GM1 antibody or pre-immune serum injected i.p. at days -1, 2, and 5 in relation to the infection with 10³ PFU of ECTV or ECTV SPI-2 SAD. (A) Percentage of DX5+ cells in the spleen and
blood at 6 days PI. Graph shows means +/- SEM of one representative of two independent experiments. (B) Illness signs and survival were scored daily for 20 days. (C and D) At day 6 PI, mice were sacrificed, liver samples were collected for plaque assay (C), and total splenocyte numbers were determined (D). Mice were bled for serum collection at days 4 and 6 PI and IFN-γ (E) and TNF-α (F) levels in the serum were determined by cytometric bead assay. C, D, E and F show combined data from two independent experiments (N = 3 for each experiment). Graphs show means +/- SEM. * P < 0.05, ** 0.001 ≤ P ≤ 0.01, *** P < 0.001 in relation to non-depleted group.
Table 1. Viral loads from target organs of mice infected with wt or mutant viruses

<table>
<thead>
<tr>
<th></th>
<th>ECTV</th>
<th>SPI-2 SAD⁺</th>
<th>SPI-2⁻</th>
<th>ECTV</th>
<th>SPI-2 SAD⁺</th>
<th>SPI-2⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lymph node</strong></td>
<td>6.2 x 10⁵ (±1.9 x 10⁵)</td>
<td>1.7 x 10⁵ (± 1.1 x 10⁵)</td>
<td>2.4 x 10⁵ (± 1.7 x 10⁵)</td>
<td>2.8 x 10⁵ (± 7.8 x 10⁵)</td>
<td>2.2 x 10⁶ (± 4.6 x 10⁵)</td>
<td>2.2 x 10⁶ (± 3.8 x 10⁵)</td>
</tr>
<tr>
<td><strong>Spleen</strong></td>
<td>4.3 x 10⁷ (± 1.0 x 10⁷)</td>
<td>1.1 x 10⁷ * (± 5.8 x 10⁶)</td>
<td>1.5 x 10⁷ (± 8.0 x 10⁶)</td>
<td>2.0 x 10⁹ (± 7.2 x 10⁷)</td>
<td>1.15 x 10⁹ (± 2.9 x 10⁷)</td>
<td>2.7 x 10⁹ (± 9.4 x 10⁷)</td>
</tr>
<tr>
<td><strong>Liver</strong></td>
<td>2.8 x 10⁵ (± 1.1 x 10⁵)</td>
<td>2.0 x 10⁴ * (± 6.3 x 10⁴)</td>
<td>6.0 x 10⁴ (± 9.0 x 10⁴)</td>
<td>1.3 x 10⁸ (± 3.3 x 10⁷)</td>
<td>9.9 x 10⁸ ** (± 5.8 x 10⁷)</td>
<td>1.1 x 10⁹ ** (± 1.0 x 10⁷)</td>
</tr>
<tr>
<td><strong>Lung</strong></td>
<td>3.5 x 10³ (± 1.2 x 10³)</td>
<td>4.1 x 10³ (± 2.5 x 10³)</td>
<td>nt</td>
<td>1.1 x 10⁸ (± 2.8 x 10⁷)</td>
<td>6.7 x 10⁶ ** (± 1.4 x 10⁷)</td>
<td>nt</td>
</tr>
</tbody>
</table>

a BALB/c mice were infected s.c. with 10⁴ PFU of ECTV or ECTV SPI-2 mutants. Viral loads in various organs at 4 and 6 days PI were determined by plaque assay. Viral loads are described as PFU/lymph node or PFU/g of tissue for spleen, liver and lung. The assay detection limit was ≤ 10² PFU/g.

Data show means (+/- SEM) pooled from two independent experiments (n = 3 for each experiment). * P < 0.05, ** 0.001 ≤ P ≤ 0.01 in relation to ECTV.

Abbreviation: nt = not tested.
Figure 1

A

\( \text{Log}_{10} \text{PFU/cells} \)

\begin{align*}
\text{Hours post-infection}
\end{align*}

B

\( \text{Log}_{10} \text{PFU/cells} \)

\begin{align*}
\text{Hours post-infection}
\end{align*}

C

\% Asrin+ TAD*cells

\begin{align*}
\text{Hours post-infection}
\end{align*}

D

\% DIOcells

\begin{align*}
\text{Hours post-infection}
\end{align*}
Figure 2

A

% survival

ECTV (N = 20)

SPI-2 SAD\(^4\) (N = 20)

SPI-2 \(^6\) (N = 10)

SPI-2 REV (N = 10)

Days post-infection

B

Morbidity score per mouse (N = 10)

ECTV

SPI-2 SAD\(^4\)

SPI-2 REV

Days post-infection

C

Morbidity score per mouse (N = 10)

ECTV

SPI-2 SAD\(^4\)

SPI-2

***
**
*

Days post-infection

Figure 3

A

Day 6 PI

ECTV

SPI-2 SAD\(^4\)

mock

Unit/mL

B

Day 6 PI

ECTV

SPI-2 SAD\(^4\)

mock

Unit/mL

NS

NS

Unit/mL

on August 30, 2017 by guest
http://jvi.asm.org/ Downloaded from
Figure 4

A

B

C

D

![Image of Figure 4]

- A: Histological view showing... (description)
- B: Close-up with arrows indicating... (description)
- C: Additional detail with arrows... (description)
- D: Bar chart illustrating number of... (description)
Figure 5

A: Spleen

% TUNEL* cells

ECTV SPI-2 SAD

ECTV SPI-2 SAD

Day 4 Day 6

B: Liver

% TUNEL* cells

ECTV SPI-2 SAD

ECTV SPI-2 SAD

Day 4 Day 6

C

D

E

F
Figure 6
Figure 7

A

Number of DCS

B

% CD8 cells

C

% CD8 cells

D

% CD8 cells

E

Number of DCS

F

% CD8 cells

G

% CD8 cells

H

% CD8 granzyme B

I

% CD8 granzyme B

J

mock ECTV SPI-2 SAD

K

mock ECTV SPI-2 SAD

L

mock ECTV SPI-2 SAD

M

mock ECTV SPI-2 SAD

ECTV SPI-2 SAD

ECTV SPI-2 SAD

ECTV SPI-2 SAD

mock ECTV SPI-2 SAD

mock ECTV SPI-2 SAD

mock ECTV SPI-2 SAD

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mock ECTV SPI-2 SAD

mock ECTV SPI-2 SAD

mock ECTV SPI-2 SAD

mock ECTV SPI-2 SAD

mock ECTV SPI-2 SAD
Figure 8

A) NK cell lysis - day 4 PI

B) NK cell lysis - day 6 PI

% specific lysis vs. e/t ratio for different strains: ECTV, SPI-2 SADΔ, SPI-2 ΔECTV, SPI-2 REV, uninfected.
Figure 9

Figure 9A: % D* cells

Figure 9B: % survival (N = 6)

Figure 9C: Log10 PFU/g

Figure 9D: Number of spinocytes (x 10^7)

Figure 9E: Serum IFN-γ (pg/mL)

Figure 9F: Serum TNF-α (pg/mL)