The Ectromelia Virus SPI-2 Protein Causes Lethal Mousepox by Preventing NK Cell Responses

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Ectromelia virus (ECTV) is a natural pathogen of mice that causes mousepox, and many of its genes have been implicated in the modulation of host immune responses. 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 an earlier serum gamma interferon (IFN-γ) response, raised serum interleukin 18 (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 mouspox lethal in susceptible strains by preventing protective NK cell defenses.

MATERIALS AND METHODS

Ethics statement. This study was carried out in strict accordance with the recommendations of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. The protocol was approved by the Animal Research Ethics Committee of the Australian National University.
were deleted in the open reading frame of ECTV SPI-2 SAD
Moscow; NCBI accession NC_004105.1; product also known as EVM161 and
material were harvested separately, and virus titers were determined by plaque
growth curves, L929 cell and MEF lines were infected at a multiplicity of infec-
tion (MOI) of 3 for 1 h. Unabsorbed virus was washed off, and fresh medium was
added. At various times postinfection (p.i.), the cell culture and cell-associated
material were harvested separately, and virus titers were determined by plaque
assay using BS-C-1 monolayers.

Recombinant virus generation. Two ECTV SPI-2-defective viruses containing
different genetic modifications in the SPI-2 gene (locus ECTVgp162 of ECTV
Moscow; NCBI accession NC_004105.1, previously known as EVM161 and
serpin C7L) were generated. ECTV SPI-2 Δwas generated by a general transient-
dominant selection method used for constructing poxvirus mutants based on
β-galactosidase (β-Gal) gene expression and Escherichia coli gpt with mycophe-
monic acid selection (12, 13). The complete SPI-2 gene sequence was deleted. ECTV SPI-2 REV was generated by a novel transient-
dominant selection method using green fluorescent protein (GFP) fluorescence
and blasticidin resistance as selection markers (53). Nucleotides +271 to +698 were
deleted in the open reading frame of ECTV SPI-2 SADΔ. The deletion process inserts the AATGACGGCGAT exogenous sequence into the gene, generating a frameshift and an early stop codon in the mutant predicted protein. As a consequence, only amino acids 1 to 91 of the wild-type (wt) SPI-2 protein are expressed in ECTV SPI-2 SADΔ. ECTV SPI-2 REV was generated from the ECTV SPI-2 SADΔ mutant. Blasticidin was purchased from Invivogen. Gene deletion/restoration was confirmed by PCR and sequencing.

Western blotting. For analysis of SPI-2 expression, 1 × 107 MEF were infected at
an MOI of 10 for 4 h at 35°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; catalog no. 65921A; dilution, 1/400) as the primary antibody, a secondary anti-mouse IgG1 peroxidase-conjugated antibody (BD Phar-
ingen), and enhanced chemiluminescence substrate (Amersham). For actin detection, a rabbit anti-actin polyclonal antibody (Santa Cruz) was used, fol-
lowered anti-rabbit IgG peroxidase-conjugated antibody (BD Pharmingen).

Infections. In for in vivo studies of ECTV pathogenesis, mice were infected with
105 PFU or 106 PFU subcutaneously (s.c.) in both hind legs in a total volume of
30 μl phosphate-buffered saline (PBS) (25 μl per leg). The mice were monitored daily by blinded examiners for signs of micepox, such as coat condition, con-
junctivitis, 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. Scores of 1 to 3 were assigned to each disease parameter, and mice with a score of 3 in one parameter or 5 in cumulative parameters were
sacrificed. Coat condition scores were as follows: 1, slightly rough; 2, disheveled;
3, bleeding or irritated wounds/severe hair loss. Eye condition scores were as follows: 1, mild; 2, severe conjunctivitis. Movement scores were as follows: 1, normal/uncoordinated; 2, walking on tiptoe or reluctant to move; 3, staggering/paralysis. Limb condition scores were as follows: 1, swell-
ing; 2, abnormal limbs; 3, severe necrosis of limbs.

Virus titration of organs, histological examination, and liver enzyme and cyto-
line levels in the serum. Mice were infected s.c. with 105 PFU and sacrificed at the indicated times p.i. for organ and serum collection. Liver, spleen, and lung samples were homogenized in a medium volume proportional to the sample weight (grams × 9 ml). Pojilat draining lymph node samples were homog-

Effector cell responses. Spleen cell suspensions were obtained by pressing organs through cell strainers, followed by hypotonic red blood cell lysis. Total spleenocyte numbers were determined by counting, and 106 cells per sample were analyzed by fluorescence-activated cell sorter (FACS). Twenty-five

Virus infection of mice. Eight- to 10-week-old female C57BL/6 and BALB/c mice were infected s.c. with 103 PFU and sacrificed at 2, 4, and 6 days p.i. for organ and serum collection. Liver, spleen, and lung samples were homogenized in a medium volume proportional to the sample weight (grams × 9 ml). Pojilat draining lymph node samples were homog-

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RESULTS

ECTV SPI-2 mutants and revertant. We generated two ECTV SPI-2-deficient viruses containing different genetic modifications in the SPI-2 gene. ECTV SPI-2 SADΔ 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 is not translated due to a frameshift mutation in the coding sequence; however, a truncated form of SPI-2 protein lacking the serpin reactive site (LVSD) could be expressed. The second mutant, referred to as ECTV SPI-2 Δ, was generated by deletion of the complete gene. A revertant virus (ECTV SPI-2 REV) was generated by restoring the wt SPI-2 gene on the ECTV SPI-2 SADΔ back-
The genetic profile of the recombinant viruses was confirmed by PCR and sequencing. All of the remaining SPI-2 gene coding sequence plus 39 upstream nucleotides and 101 downstream nucleotides was determined by sequencing of ECTV SPI-2 SAD/H9004. Two hundred forty upstream nucleotides and 410 downstream nucleotides were determined by sequencing of ECTV SPI-2/H9004.

SPI-2 or a truncated form of the protein was not detected by Western blot analysis of uninfected cells or cells infected with ECTV SPI-2 SAD/H9004 or ECTV SPI-2/H9004, whereas lysates of cells infected with wt ECTV or ECTV SPI-2 REV presented a band of the expected size for the full-length SPI-2 protein (see Fig. S1 in the supplemental material). All samples expressed actin, including uninfected cells.

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 replication efficiencies similar to that of wt ECTV in L929 cells and MEF (Fig. 1A and B, respectively). In agreement with this observation, the appearances of apoptotic features triggered by infection were similar in wt virus and mutants as measured by phosphatidylserine exposure (Fig. 1C) and production of reactive oxygen species (data not shown). Both SPI-2 mutant viruses induced less disruption of the mitochondrial membrane potential than wt virus 24 h p.i., but the difference was not statistically significant (Fig. 1D).

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 s.c. with either wt ECTV or recombinant virus. Disease signs, such as disheveled coat, conjunctivitis, foot swelling, and movement impairment, were recorded daily and scored. It is well established that C57BL/6 mice are highly resistant (50% lethal dose [LD<sub>50</sub>] < 10<sup>5</sup> PFU) whereas BALB/c mice are susceptible (LD<sub>50</sub> < 10<sup>3</sup> PFU) to s.c. ECTV infection (2). Therefore, C57BL/6 mice were infected with 10<sup>3</sup> PFU, which results in 100% mortality when wt ECTV is used.

All C57BL/6 mice infected with ECTV SPI-2 SAD/H9004 or wt virus survived for at least 4 weeks p.i. 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 were highly susceptible to classical mousepox disease, and most animals succumbed to infection, with a median survival time of 9 days p.i. 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 (Fig. 2), although some signs of illness, including disheveled coat, conjunctivitis, and limb swelling were observed throughout the course of infection. About half of the mice that survived ECTV SPI-2 mutant infection still had detectable virus by 4 weeks p.i. (mean ± standard deviation [SD], 3.8 × 10<sup>3</sup> ± 1.6 × 10<sup>3</sup> in the spleen and 5.2 × 10<sup>2</sup> ± 1.9 × 10<sup>2</sup> in the liver), suggesting that clearance of the virus took longer than in mousepox infection of resistant strains. By 8 weeks p.i., mutant viruses were no longer detectable.

![Figure 1](image-url)
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 acute hepatitis followed by multiorgan 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, the 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 p.i. in mice infected with ECTV or ECTV SPI-2 mutants, and only a modest, but significant (*P < 0.05; **, *P < 0.001 in relation to both wt virus and ECTV SPI-2 REV. The error bars indicate standard errors of the means (SEM)).

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-infected mice than in those of ECTV SPI-2 SAD + -infected mice were associated with a difference in overt liver pathology. Examination of liver sections collected at days 6 and 9 p.i. revealed intense lymphocyte infiltration and several necrotic foci throughout the organ in both groups but no obvious differences between the two

| Table 1. Viral loads from target organs of mice infected with wt or mutant virus |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Organ           | Day 4 p.i.      | Day 6 p.i.      |
|                 | ECTV            | SPI-2 SAD +     | SPI-2 A         | ECTV            | SPI-2 SAD +     | SPI-2 A         |
| Lymph node      | 6.2 × 10^5 ± 1.9 × 10^5 | 1.7 ± 1.1 × 10^5 | 4.9 ± 1.7 × 10^5 | 2.8 ± 8 × 7.8 × 10^5 | 2.2 ± 4.6 × 10^5 | 2.2 ± 3.8 × 10^5 |
| Spleen          | 4.3 ± 10^7 ± 1.0 ± 1.0^6 | 1.1 ± 5.8 × 10^5 | 1.5 ± 8.0 × 10^5 | 2.0 ± 7.2 ± 10^5 | 1.15 ± 2.9 × 10^7 | 2.7 ± 9.4 × 10^7 |
| Liver           | 2.8 × 10^7 ± 1.1 ± 10^7 | 2.0 ± 6.3 × 10^7 | 6.0 ± 9.0 × 10^7 | 1.3 × 10^8 ± 3.3 × 10^8 | 9.9 ± 5.8 × 10^8 | 1.1 ± 10^8 ± 1.0 × 10^8 |
| Lung            | 3.5 ± 10^4 ± 1.2 ± 10^4 | 4.1 ± 2.5 × 10^4 | NT              | 1.1 × 10^8 ± 2.8 × 10^8 | 6.7 ± 10^8 ± 1.4 × 10^6 | NT              |

* BALB/c mice were infected s.c. with 10^7 PFU of ECTV or ECTV SPI-2 mutants. Viral loads in various organs at 4 and 6 days p.i. were determined by plaque assay. The viral loads are given in PFU/lymph node or PFU/g of tissue for spleen, liver and lung. The assay detection limit was ≤ 10^2 PFU/g. The data shown are means ± standard errors of the mean (SEM) pooled from two independent experiments (n = 3 for each experiment), *, *P < 0.05, and **, 0.001 ≤ *P ≤ 0.01 in relation to ECTV. NT, not tested.
groups (see Fig. S2 in the supplemental material). 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, the levels of two serum markers of liver pathology, AST and ALT, were increased in ECTV- but not ECTV SPI-2 SAD³-infected mice (Fig. 3A and B). The reduced viral loads in the liver and improved liver function in mice infected with an 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 the liver, a significant difference in histopathology was found between the spleens of mice infected with wt virus and those of mice infected with ECTV SPI-2 SAD³. Wt ECTV-infected mice presented spleens with massive tissue destruction at day 6 p.i., whereas ECTV SPI-2 SAD³-infected mice presented markedly less tissue damage (Fig. 4B and C). The reduced viral loads in the liver and improved liver function in mice infected with an 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).

As a more direct measure of the damage to the liver and spleen caused by infection, tissue sections were analyzed for the presence of apoptotic cells presenting DNA fragmentation measured by TUNEL staining (Fig. 5). Similar levels of apoptosis were found in organs of mice infected with wt ECTV or ECTV SPI-2 REV were greatly reduced macroscopically, and total splenocyte numbers were at least 2-fold lower, compared to those from mice infected with ECTV SPI-2 SAD³ or ECTV SPI-2² (Fig. 4D).

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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 sera 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 p.i. Given their often rapid induction, IFN-β, IL-1β, and IL-18 were analyzed in addition at 0, 6, 12, and 24 h p.i. 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 p.i., while 2 days later, all infected mice reached similar peak levels of IFN-γ, decreasing by 9 days p.i. (Fig. 6A). IL-18 release was detected in the serum only between 4 and 9 days p.i., and at 6 days p.i., the levels of the cytokine were higher in ECTV SPI-2 mutant-infected mice than in ECTV-infected mice (Fig. 6B). IL-18 was not detected in the blood at any time tested before day 4 p.i. In contrast, increased levels of TNF-α in the sera of mice infected with ECTV expressing the SPI-2 protein were found at 6 days p.i. (Fig. 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.

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 p.i. did not markedly differ between ECTV- and ECTV SPI-2 SAD³-infected mice (Fig. 7B, C, and F and data not shown). However, because the total number of splenocytes in ECTV SPI-2 SAD³-infected mice was around double that in wt virus-infected mice (Fig. 4D), the absolute number of each lymphocyte population was markedly reduced following wt virus infection (Fig. 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^3-infected mice was assessed. At 6 days p.i., the percentages of CD8- and granzyme B-positive cells in the spleen were low (around 1%) in both ECTV- and ECTV SPI-2 SAD^3-infected mice. In blood, an ~2-fold-greater percentage of cells were CD8^+ and expressed granzyme B in ECTV SPI-2 SAD^3-infected mice than in ECTV-infected mice (Fig. 7D), although this difference did not reach statistical significance. Ex vivo cytotoxicity against virus-infected, major histocompatibility complex class I (MHC-I)-matched target cells was marginal for splenocytes from mice infected with either virus for 4 and 5 days (data not shown). In contrast, splenocytes from mice infected with ECTV SPI-2 SAD^3 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 (see Fig. S3 in the supplemental material).

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^3 than in mice infected with wt virus at 6 days p.i. (Fig. 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 (Fig. 7G). The increased expression of granzyme B in NK cells of ECTV SPI-2 SAD^3-infected mice suggests an increase in the cytotoxic potential of these cells compared to NK cells of wt ECTV-infected mice. At day 4 p.i., the ex vivo cytotoxicities of splenocytes against YAC-1 cells were similar for ECTV- and ECTV SPI-2 SAD^3 mutant-infected mice (Fig. 8A). However, at day 6 p.i., the ex vivo cytotoxicity of splenocytes against YAC-1 cells was higher for ECTV SPI-2^3 (P = 0.0097) and ECTV SPI-2 SAD^4 (not statistically significant; P = 0.0526) mice than for mice infected with wt or revertant virus (Fig. 8B). Even though the increase in NK cytotoxicity induced by the ECTV SPI-2 SAD^4 infection was not statistically significant with the sample size used, the two SPI-2 mutant viruses presented similar trends, 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 p.i. relative to ECTV SPI-2 SAD^3-infected mice, suggesting that SPI-2 may prevent optimal NK cell activation and cytotoxicity.

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 to infection with ECTV SPI-2 mutants, NK cells were depleted in vivo. Both CD4^+ (data not shown) and CD8^+ lymphocyte populations (see Fig. S4 in the supplemental material) were not markedly affected by anti-asialo GM1 treatment, whereas the DX5^+ cell population was effectively depleted (Fig. 9A). Lack of NK cells rendered BALB/c mice as susceptible to ECTV SPI-2 SAD^3 infection (all animals died by day 7 p.i.) as nondepleted mice were to wt ECTV (Fig. 9B). The liver viral loads were ~10-fold and ~1,000-fold increased in NK cell-depleted mice compared to control mice when infected with wt virus or mutant virus, respectively (Fig. 9C). Importantly, following NK cell deple-
tion, mice infected with mutant and wt viruses presented liver viral loads that were similar in magnitude at 6 days p.i. Moreover, the depletion of NK cells in ECTV SPI-2 SAD\textsuperscript{5}-infected mice resulted in pronounced tissue destruction in the spleen and loss of splenocytes (Fig. 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 nondepleted, uninfected mice.

The levels of IFN-\(\gamma\) found in the sera 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 p.i. (Fig. 9E). In contrast, the levels of TNF-\(\alpha\) in the sera of NK-depleted mice were remarkably high compared to nondepleted mice at 6 days p.i. (Fig. 9F).

Collectively, the decreased survival, increased liver viral load, and reduced levels of serum IFN-\(\gamma\) of NK cell-depleted mice infected with ECTV SPI-2 SAD\textsuperscript{5} support the conclusion that NK cells are the key effector cells for protection against mousepox in mutant-virus-infected mice and that SPI-2 deletion counteracts this disease-ameliorating immune pathway. Furthermore, NK cells are the likely predominant source of early IFN-\(\gamma\). 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/H9253 response, spleen pathology, and levels of mortality (Fig. 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+/H11001 T cells that express granzyme B and the ex vivo cytotoxicity of these cells...
were elevated in mutant-infected mice (Fig. 7D; see Fig. S3 in the supplemental material). 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 (Fig. 9D) with reduced numbers and proportions of CD8\(^+\) T cells expressing granzyme B (see Fig. S4 in the supplemental material).
receptor pathway (37), and recently, we also found that ECTV previously that the CPXV SPI-2 protein inhibits the death replication even in the presence of SPI-2 (42). We have shown suggested that ECTV SPI-2 may only weakly inhibit mouse way, mediated by caspase 8. However, 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 (33; Melo-Silva et al., unpublished), suggesting that effect of SPI-2 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 p.i., ECTV-infected mice present increased levels of TNF-α in the blood compared to ECTV SPI-2 mutant-infected mice (Fig. 6C). TNF-α is a cytokine classically associated with induction of apoptosis in TNF receptor (TNFR)-expressing infected cells (29, 33, 46). This cytokine binds to death receptors expressed in the plasma membranes of target cells, and the result is activation of pro-apoptotic caspases and cell death. However, the cytokine has also been implicated in immunopathology (8, 17, 55). Most ECTV-infected mice die by day 10 (Fig. 2), whereas ECTV SPI-2 mutant-infected mice survive. Moreover, ECTV-infected mice have higher viral loads in the liver than mutant-infected mice at 6 days p.i. (Table 1). Taken together, these data suggest that, even if the TNF-α circulating in the blood induces apoptosis in infected cells, it is not sufficient to protect ECTV-infected mice. Therefore, we speculated that the increased levels of TNF-α in the blood of ECTV-infected mice could contribute to disease by immunopathology. The liver and lungs exert specialized and essential functions, and excessive non-specific tissue damage could contribute to multiorgan failure and death. In agreement with this notion, NK-depleted infected mice presented very high levels of the cytokine 6 days p.i., 1 day before their death (Fig. 9F).

Another putative mechanism of action is inhibition of caspase 1, a property that 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 (Fig. 6B) correlated with increased NK and CD8+ T cell numbers, increased gran -

![Figure 8](https://example.com/fig8.png)

**FIG. 8.** Increased NK cell cytotoxicity in the absence of SPI-2. Mice were infected s.c. with 10⁶ PFU of each virus, spleens were harvested at 4 (A) and 6 (B) days p.i., and a standard ⁵¹Cr release assay against YAC cell targets was performed for 6 h. Splenocytes from uninfected mice were used as controls.

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 killing of infected cells by blocking either 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, J. Pardo, M. Lobigs, A. Koskinen, D. C. Tscharke, R. M. Buller, A. Müllbacher, and M. Regner, 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 (33; Melo-Silva et al., unpublished), suggesting that effect of SPI-2 on NK cell responses is not only or not at all connected to inhibition of target cell lysis.

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**NK cell lysis - day 4 PI**

![Graph A](https://example.com/graphA.png)

**NK cell lysis - day 6 PI**

![Graph B](https://example.com/graphB.png)
FIG. 9. NK cells mediate survival of ECTV SPI-2 SAD^3-infected mice. BALB/c mice were treated with anti-asialo GM1 antibody or preimmune serum injected i.p. at days 1, 2, and 5 in relation to infection with 10^3 PFU of ECTV or ECTV SPI-2 SAD^3. (A) Percentage of DX5^+ cells in the spleen and blood at 6 days p.i. The 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 p.i., mice were sacrificed, liver samples were collected for plaque assay (C), and total splenocyte numbers were determined (D). The mice were bled for serum collection at days 4 and 6 p.i., and IFN-γ (E) and TNF-α (F) levels in the serum were determined by cytometric bead assay. Panels C, D, E, and F show combined data from two independent experiments (n = 3 for each experiment). The graphs show means ± SEM. *, P < 0.05; **, 0.001 ≤ P ≤ 0.01; ***, P < 0.001 in relation to the nondepleted group.
Given that caspase 1 also activates pro-IL-1β, its inhibition may also lead to a reduction of the pyrogentic inflammatory response mediated by the cytokine. We could not detect systemic IL-1β at any time tested. In addition, we failed to detect any IL-1β secretion by mouse macrophages infected in vitro with ECTV SPI-2 mutants. For instance, IL-1β secretion induced by lipopolysaccharide (LPS) and ATP treatment of peritoneal exudate cells (32) was efficiently inhibited by ECTV infection, regardless of SPI-2 (see Fig. S5 in the supplemental material). We therefore believe that this is unlikely to be a dominant pathway for 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 an 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 the inherent ability to produce IFN-γ (5, 22).

The notion of redundancy is supported by the fact that BALB/c mice do not express mRNA for IL-12p40 (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 the infection of susceptible mice and that it 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 the genes may have been retained by different orthopoxviruses to counter different aspects of the host response to infection.

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REFERENCES

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