The ectromelia virus virulence factor C15 facilitates early viral spread by inhibiting NK cell contact

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Highlights
- Ectromelia virus C15 protein facilitates early viral replication and dissemination
- Early effect of ECTV C15 dependent on NK cells
- ECTV C15 inhibits NK cell number and cytolytic function
- ECTV C15 limits contact between NK cells and infected cells in vivo

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SUMMARY
The success of poxviruses as pathogens depends on their antagonism of host responses by multiple immunomodulatory proteins. The largest of these expressed by ectromelia virus (the agent of mousepox) is C15, one member of a well-conserved poxviral family previously shown to inhibit T cell activation. Here, we demonstrate by quantitative immunofluorescence imaging that C15 also limits contact between natural killer (NK) cells and infected cells in vivo. This corresponds to an inhibition in the number of total and degranulating NK cells, ex vivo and in vitro, with no detectable impact on NK cell cytokine production or the transcription of factors related to NK cell recruitment or activation. Thus, in addition to its previously identified capacity to antagonize CD4 T cell activation, C15 inhibits NK cell cytolytic function, which results in increased viral replication and dissemination in vivo. This work builds on a body of literature demonstrating the importance of early restriction of virus within the draining lymph node.

INTRODUCTION
The extraordinary virulence of poxviruses can be attributed to their extensive antagonism of the host immune response (reviewed1,2). The orthopoxvirus (OPXV) genus includes variola virus (VARV, the agent of smallpox), vaccinia virus (VACV, the gold standard smallpox vaccine) and multiple viruses that pose threats from zoonotic transmission including monkeypox virus (MPXV). OPXVs are the largest mammalian viruses with genomes containing around 200 open reading frames (ORFs).3 Many of these ORFs encode immunomodulatory proteins that are exquisitely tuned to the immune response of the host. Thus, their functions are best studied during natural host-pathogen relationships. Mousepox, the murine poxviral disease caused by ectromelia virus (ECTV), provides a unique opportunity to study natural OPXV infection in a tractable system.4

One notable ECTV immunomodulatory protein is C15. It is a member of the B22 protein family, which are the largest OPXV proteins at nearly 2000 amino acids. B22 family members are well conserved across the virulent OPXVs (including VARV, MPXV and ECTV), but the C15 ORF is truncated in VACV. The size and conservation of B22 family members suggest a large contribution to virulence, and this has been demonstrated for the homologs in MPXV and ECTV.5–7 Functionally, B22 family members have been shown to modulate T cell responses via complex mechanisms that target both CD4 and CD8 T cells or only CD4 T cells.5,7

The function of C15 appears to extend beyond inhibiting adaptive immunity. Work by others indicated that C15 may impact viral spread in the draining lymph node (LN) of susceptible BALB/c mice as early as 4 days post infection (dpi),8 suggesting antagonism of the innate immune response that contributes to virulence. This finding is in line with the concept that innate host defense mechanisms in the draining LN are pivotal in controlling ECTV dissemination early in infection.2 The innate mechanisms of control relevant in ECTV infection are type 1 interferons (IFN-α/β), phagocytes including macrophages and monocytes, and natural killer (NK) cells.2 NK cells are necessary for resistance to ECTV infection in the first 4 dpi8 through the production of antiviral IFN-γ and by perforin-mediated cytolyis.8,9 Because of their importance in the early control of infection, NK cell function is targeted by many viruses, including OPXV, to promote viral spread (reviewed10–12).
Few viral proteins have been reported to antagonize the function of both innate (NK cell) and adaptive (T and B cell) lymphocytes (example 13). Here, we demonstrate that the B22 family member of ECTV, C15, inhibits NK cell cytolytic function by limiting target cell contact in addition to its previously identified capacity to antagonize CD4 T cell activation. The impact of this inhibition allows for increased viral replication and dissemination within the draining LN early after infection.

RESULTS

C15 facilitates early ECTV replication and dissemination in an NK cell-dependent manner

To investigate the apparent impact of C15 on early stages of ECTV infection, we analyzed viral titers in the draining popliteal LN at 1–3 days after footpad infection of B6 mice with eGFP-expressing WT ECTV (WT) and C15-deficient ECTV (ΔC15). Although there was no impact on replication in vitro, expression of C15 profoundly increased viral titers in the LN by 3 dpi, evident both by traditional titering (Figure S1A) and focus forming assay (Figure 1A). There was a minimal and not significantly different amount of WT and...
ΔC15 ECTV detected in the LN before 48 h post infection, by viral titer or flow cytometric analysis of infected cells (Figures S1A and S3A), indicating that the two viruses are initially similarly capable of accessing the draining LN but that the impact of C15 manifests early after viral entry to the LN. Given the magnitude of the impact at early timepoints, we hypothesized that C15 targets an aspect of innate immune control.

NK cells provide critical early control of ECTV infection in the draining LN8,9,14; therefore, we next asked whether C15 targets this cell type to gain a replicative advantage. We depleted B6 mice of NK cells using anti-NK1.1 antibody (Figure S1 B) and found that, in comparison to isotype control-treated mice, NK cell depletion largely eliminated the replicative advantage conferred by C15 expression (Figure 1B). In addition, NK depletion did not statistically impact viral titers in the LN of mice infected with WT virus, whereas replication of ΔC15 virus was significantly increased in the absence of NK cells (Figure 1B). These results suggest that C15 acts on NK cells to facilitate replication at this early timepoint.

We were next interested in identifying whether C15 impacts viral dissemination within draining LNs of B6 mice. We harvested LNs 3 dpi with WT or ΔC15 ECTV, imaged these tissues by confocal microscopy and analyzed the amount and distribution of infected cells (GFP+) in relation to the LN stroma (ERTR7+) (Figures 1C and 1D). Mirroring the titer data, there was an increase in GFP expression in NK-intact (isotype control-treated) mice infected with WT versus ΔC15 (Figures 1C and 1D), and we noted differential distribution of GFP+ virus-infected cells in the two infection conditions (Figure 1C). Similar to previous observations in BALB/c mice,6 the viral GFP signal in NK-intact B6 mice was restricted to the LN periphery in ΔC15 infection compared to a larger area of GFP expression during WT infection, suggesting that C15 facilitates increased viral replication and spread in vivo.

We also performed this analysis in NK cell-depleted mice and found that, mirroring the titer data in Figure 1B, the difference in GFP expression between WT and ΔC15 infection was lessened in NK-versus isotype control-depleted animals (Figure 1D), further supporting the conclusion that C15 exerts an immunomodulatory function on NK cells. In NK cell-depleted mice, visual analyses showed that depletion provided a more disseminated phenotype even in the absence of C15 expression (Figure 1C), though infection disrupted the LN architecture precluding categorization of nodal distribution. Considering the variation between biological replicates, there appeared to be increased nodal spread in WT infected tissues, both NK cell-depleted and-intact, compared to NK cell-intact ΔC15 infected LNs (Figures 1C and S2), suggesting that NK cells are able to restrict virus specifically in the absence of C15 expression. These data are consistent with targeting of NK cell responses by C15 to facilitate early viral replication and dissemination in the LN and suggest that C15 is a major contributor to ECTV-mediated inhibition of NK cells. Our findings are consistent with previous work6 showing that during ΔC15 infection, increasing the inoculating dose paradoxically resulted in decreased titers in all central sites but not at the site of infection (contrary to WT infection where increased dose was correlated with increased titer at all sites), suggesting that in ΔC15 infection, increased viral loads are more easily detected by the host and spread is better controlled by the immune response.

Viral expression of C15 reduces numbers of total and degranulating NK cells in infected tissue
To assess the impact of C15 on the NK cell response in vivo, we next analyzed WT-versus ΔC15-infected LNs 3 dpi by flow cytometry. There was no difference in the total number of live cells recovered from the two infection conditions (Figure 2A), despite the substantial increase in virus present in WT versus ΔC15 infected tissues at this time (Figure 1A) that was reflected in the number of GFP+ (infected) cells (Figure 2B). Nevertheless, NK cells made up a significantly increased percentage of the live cells in the LN during ΔC15 infection (Figure 2C) and there was a similar, though less profound, difference in the absolute number of NK cells identified (Figure 2D). We also analyzed the percentage and number of an unrelated innate cell type, F4/80+ macrophages, which followed the opposite trend, where there was a similar number (Figure 2D) but increased percentage (Figure 2C) in WT versus ΔC15 ECTV infection; this trend fits more closely with the expected immune cell response based on viral titer alone.

We next assessed the functional responses of NK cells in the LNs of WT- and ΔC15-infected mice by flow cytometry. Surface CD107a staining is a marker for cytolytic degranulation; there was a significant increase in the number of CD107a+ NK cells in ΔC15 versus WT infection (Figure 2E), but notably, there was no
change in the proportion of CD107a+ degranulating NK cells between infection conditions (Figure 2F). This suggests that the difference in NK cell cytolytic activity is directly attributable to the increased frequency of NK cells in the LN. There was also no difference in the median fluorescence intensity (MFI) of CD107a on positive cells (Figure 2G), suggesting that C15 does not impact NK cell degranulation on a per cell basis.

NK cells not only act as important cytolytic effectors, but are also crucial early sources of IFNγ, which orchestrates the antiviral immune response to ECTV in the LN.8,15,16 Although there was no difference in the number (Figure 2E) or the MFI (Figure 2G) of IFNγ+ NK cells in WT versus ΔC15 infection, the percentage of IFNγ+ NK cells was higher in WT versus ΔC15 infection (Figure 2F), in marked contrast with CD107a. This is consistent with the finding that early IFNγ production by NK cells is in response to infected cells,16 which are increased in WT versus ΔC15 infection (Figure 2B). Thus, C15 does not impact NK cell degranulation or IFNγ production on a per cell basis; instead, C15 reduces the total number of NK cells and thereby the number of degranulating NK cells in infected LNs on a population level.

C15 does not impair transcription of factors involved in NK cell recruitment and activation

We next sought a potential explanation for the increased number of NK cells in ΔC15 infected tissues 3 dpi by assessing earlier transcriptional differences in molecules involved in NK cell activation and recruitment. We collected total RNA from infected LNs 40 h pi, a time before obvious divergence of virus replication both by titer (Figure 1A) and the percent of infected cells (Figure S3A). In addition, there was not yet an obvious difference in the number of NK cells present in the LN at this time, as analyzed by flow cytometry (Figure S3B). Despite these findings, reverse transcription followed by quantitative PCR revealed significant increases in viral RNA, indicated by the unrelated viral transcript Evm003, in WT versus ΔC15 infected LNs even at this early time (Figure 3A). This demonstrates that, using a highly sensitive method of quantification, C15 facilitates viral replication at very early times post infection.

We then analyzed the transcription of various cytokines and chemokines reported to be important during the early stages of ECTV infection and relevant to NK cell recruitment to ECTV-infected tissue and to NK cell activation. Recruitment of NK cells to the LN involves a coordinated response by different cell types

Figure 2. Viral expression of C15 reduces numbers of total and degranulating NK cells in infected tissue

B6 mice were infected with GFP-containing WT or ΔC15 ECTV or mock infected with PBS and sacrificed 3 dpi. The dissociated popliteal LN cells were analyzed by flow cytometry.

(A) Total number of cells isolated from each LN, (B) total number of GFP+ cells per LN, (C) total number and (D) percent of NK1.1+CD3− NK cells and of F4/80+ macrophages are shown. The number (E), percent (F) and median fluorescence intensity (G) (MFI, single representative of 3 independent experiments shown) of NK cells expressing CD107a on the surface and IFNγ intracellularly. n = 9 per group, data pooled from 3 independent experiments. A one-way ANOVA with multiple comparisons was performed and p values are reported. Bars = mean, error bars = SEM.
beginning with infection-induced CCL2 and CCL7 production leading to increased IFN-γ and finally CXCL9- and CXCL10-mediated recruitment of circulating NK cells to the LN. 15–17 Although chemokine transcription was clearly induced by infection, we detected no statistically significant increases in transcription of selected chemokines in the absence of C15 (Figures 3B–3E). Consistent with our findings by flow cytometry (Figures 2E–2G) and aligning with the increase in detected viral transcripts (Figure 3A), we detected a significant increase in IFN-γ transcripts in WT infection (Figure 3F), again demonstrating that C15 does not inhibit the IFN-γ response at this time. C15 had no impact on the transcription of NK cell-activating cytokines IL-15 and IL-18 (Figures 3G and 3H), the NK cell effector molecule TNFα (Figure 3I) or the type I-IFNs (Figures 3J–3L) which are also important NK cell activators. 18 Overall, there were no instances in which transcripts increased in D.C15 versus WT infection. Therefore, we conclude that C15 does not directly target the transcription of factors in the LN involved in NK cell recruitment or activation. Results of the transcript analyses (Figure 3) together with the ex vivo flow data (Figure 2) support a model in which C15 selectively protects infected cells from NK cytolysis.

C15 inhibits NK cell degranulation in vitro

To test our model, we sought to investigate whether the expression of C15 on infected cells impacts their ability to be killed by NK cells in vitro. Because of viral cytopathic effects, inhibition of NK cells by other ECTV immune modulators, 10 and poor infectability of traditional murine target cell lines, we were unable to achieve reliable results when using traditional readouts of target cell death. Therefore, we explored differences in the functions of NK cells following co-culture with infected targets in vitro by evaluating surface CD107a staining and intracellular IFNγ production. We used a B6-derived primary skin fibroblast line as targets and found that these cells induced high levels of NK cell degranulation at baseline, but that this was selectively inhibited by C15-expressing WT infected target cells (Figure 4A). In contrast, and concordant with our model, we saw no significant differences in the production of IFNγ by NK cells when co-incubated with WT or ΔC15 infected targets (Figure 4A). We also performed this assay in the presence of blocking antibody for NKG2D, an NK cell activating receptor that has been shown to be necessary...
for optimal NK cell responses to ECTV infection. We found that blocking NKG2D inhibited the overall NK cell response to target cells, consistent with previous findings, but that it had no impact on the inhibitory effect of C15, suggesting that C15 does not target NKG2D-mediated NK cell activation (Figure S4A). These data demonstrate that expression of C15 on target cells results in a significant inhibition of NK cell cytolytic degranulation and support our model in which C15 selectively interferes with NK cell cytolysis.
**C15 inhibits NK cell contacts with virus-infected cells**

Our finding that C15 inhibits NK cell cytolytic function but not cytokine production can be explained by literature showing that NK cell cytology and IFNγ production are differentially regulated. NK cells can be activated by cytokines, which signal through the JAK-STAT pathway leading to IFNγ production and promoting cell replication. Separately, NK cells recognize target cells at contact points called immunological synapses by the integration of NK cell activating and inhibitory receptors through DAP-10/DAP-12 adaptors; this can trigger cytotoxic, proliferation and IFNγ release via a mechanism more similar to T and B cell receptor signaling. Therefore, while IFNγ production can be triggered by both cytokine-mediated activation and by target recognition, NK cell cytolytic degranulation occurs only following contact with a target cell resulting in formation of an activating immunologic synapse. Because we determined that C15 reduces the number of degranulating NK cells (Figures 2E and 4A) but does not inhibit IFNγ (Figures 2E–2G and 4A) or inhibit the transcripts for NK cell-activating cytokines (Figures 3F–3L), our data support a model in which C15 interferes with the target cell contact-dependent activation of NK cells.

To test this model, we analyzed NK cell interactions with infected cells by immunofluorescence imaging of LNs. Using the same images as in Figures 1 and S2, cell-surface NK1.1+ cells were first identified using automated computer imaging analysis software and then classified according to the distance of each individual cell from GFP+ virus-infected cells (Figures 4B and 4C). When normalized to the total LN area, we observed an increase in the overall number of NK cells in ΔC15 versus WT infected LNs, despite the restricted replication of virus (Figure 4D), recapitulating our findings by flow cytometry (Figure 2C). We then measured the distance between each NK cell and the next closest NK cell, finding that C15 does not substantially impact the overall distribution of NK cells within the LN (Figure 4E). To evaluate the interaction of NK cells with infected cells, we next compared the distance of NK cells to sites of infection between conditions, classifying cells located within <5 μm of each other as contacting (Figure 4C). This revealed that C15 profoundly decreased the number of NK cells contacting infected cells when normalized both by LN area and by GFP expression (Figures 4F and 4G). Overall, we conclude that C15 limits NK cell contacts with ECTV-infected cells, decreasing opportunities for activation-induced cytolytic degranulation.

**DISCUSSION**

Previous work has shown that B22 family members of the OPXV genus interfere with T cell function,5–7 a fundamental aspect of adaptive immunity. Here we demonstrate that the ECTV member of the B22 family, C15, also compromises innate host responses, specifically NK cell-target cell contact and cytolyis. Thus, C15 is one of few viral immunomodulatory proteins (example 13) that targets both innate and adaptive host responses.

Our finding that C15 inhibits NK cell cytolytic function but not cytokine production can be explained by significant biologic variation during this natural murine infection, we found the data implicating NK cells as a target of C15 were less clear by titer (Figure 1B) than when visualized in the draining LN by immunofluorescence imaging (Figure 1C). Even when the magnitude of viral GFP expression was low, we still observed an increased distribution of virus in the presence of C15 or in the absence of NK cells. Similarly, imaging provided crucial spatial context in our analysis of the NK cell response. Using imaging, we detected a profound difference in the contacts between NK cells and virus-infected cells (Figures 4F and 4G) in comparison to the more modest difference in the overall number of NK cells identified in the LN by imaging (Figure 4D) as well as by flow cytometry (Figures 2C and 2D). In whole organ analyses such as flow cytometry, the decrease in viral replication because of absence of a virulence factor confounds detection of an increase in the response that the factor inhibits. In contrast, in our imaging analysis, the spatial context instead highlighted that inverse relationship and provided potential mechanistic insight, allowing us to observe that C15 profoundly decreases NK cell-target cell engagement proportional to the amount of virus (Figure 4G).

We also used an in vitro assay to confirm that C15 selectively inhibits NK cell degranulation. Based on the difficulty we had using assays that readout target lysis, we resorted to an in vitro NK cell degranulation assay but found that there was a high level of background degranulation in response to uninfected target cells and only a modest inhibitory effect of C15 (Figure 4A). We conclude that this assay does not accurately reveal the full-scale impact of C15 on NK cells and speculate that traditional cytolysis assays are not optimized to reveal differences in the context of infected targets. In addition, because ECTV is known to encode many other factors responsible for inhibiting NK cells, these other NK cell modulators have likely influenced the results of our in vitro and in vivo assays and interfered with our detection of the impact of C15. There is a need for in vitro assays better able to reveal impacts of virally expressed factors on NK cell cytology and accurately predict their
effects on NK cell biology in vivo. Nevertheless, this assay allowed us to reveal that C15 was capable of selectively inhibiting NK degranulation in a closed in vitro system (Figure 4A). We also observed in vivo that C15 had no impact on NK cell degranulation on a per cell level (demonstrated by MFI, Figure 2G), and instead appeared to impact NK cell cytolytic function on a population level in the LN. Despite finding that C15 did not impact the percentage of NK cells degranulating in the LN by flow cytometry (Figure 2F), we found that there were in fact a significantly greater number of total (Figure 2C) and degranulating (Figure 2E) NK cells in the face of a significantly decreased number of virus-infected cells (Figure 2B). This data could be explained by differential recruitment and/or retention of NK cells in the site of infection but, integrating our in vitro and imaging data (Figure 4), we believe this best supports a hypothesis in which C15 instead interferes with the interaction of NK cells with infected cells at immunologic synapses, thereby blocking contact-dependent NK cell cytolysis and downstream effects such as replication. Thus, C15 joins a battery of OPXV proteins that target NK cells, further underscoring the threat these cells pose to members of the genus. The reduction in percentage of degranulating NK cells following ΔC15 versus mock infection (Figure 2F) may reflect the activities of these other NK cell modulating proteins.

Overall, we demonstrated that C15 selectively impacts NK cell contact with target cells and cytolytic function without impacting IFNγ production, both in vitro and ex vivo, and that this facilitates early viral replication and spread in vivo. Our data support a model in which C15 protects infected cells by interfering with engagement of target cells and subsequent activating/inhibitory receptor-based activation of NK cell cytolysis. We eliminated NKG2D (an activating receptor important to NK control of ECTV and a well-studied viral target as the mechanism (Figure 5A), but there are many other activating and inhibitory NK cell receptors that C15 could target. We found that WT ECTV had no early replicative advantage in β2 microglobulin-deficient mice (Figure 5B), suggesting a nonclassical MHC class I-based mechanism of NK cell activation as a potential target of C15.

The findings shown here are particularly interesting when considered in the context of what else is known about B22 family proteins. With the addition of this NK-inhibitory function, we have now revealed that in murine cells C15 is able to inhibit NK and CD4 T cell but not CD8 T cell contact and function. Although the specific molecular mechanism of C15 antagonism of CD4 T cells remains to be determined, it appears to impact the formation of immunological synapses. Although there are significant distinctions between T cell and NK cell synapses, these contact-dependent interaction sites are also implicated by results shown here. Together, our data prompt a nuanced exploration of how B22 family proteins selectively impact CD4 and CD8 T cell and NK cell activation. Further work is necessary to explore the molecular mechanism(s) of this protein, which will reveal important underlying biology about similarities and differences of lymphocyte immunological synapses.

Limitations of study
A major limitation of these studies is that C15 deletion considerably enhances viral proliferation in vivo (Figure 1), consequently eliciting a more potent host response. This obscures the true impact of C15 in vivo. In addition, the many other poxviral immunomodulatory proteins that inhibit NK cell responses limited our ability to detect the overall NK cell response and the effect of C15 in vivo and in vitro. In vitro assays would allow for investigation of the impacts of C15 outside of the confounding effects of viral load, but we were unable to identify a cytolysis assay that worked reliably in the ECTV system, due to viral cytopathic effect and poor infectability of traditional murine target cell lines. In the future, this could be addressed by expression of C15 without viral infection, an endeavor we found challenging because of the very large size of C15. Other limitations of this study include the significant biologic variation in this natural murine infection system, despite the use of all female mice, and the incomplete depletion of NK cells in Figure 1B (as shown in Figure 5B) that underestimated the impact of NK cell depletion, due in large part to the lack of a single marker expressed by all NK cells. A final limitation of this work is that the quantitative PCR performed in Figure 3 was performed at a time before we could identify a divergence in titer or NK response (Figure 5). Nevertheless, there was a difference in viral load when quantified by the more sensitive qPCR (Figure 3). Therefore, the data in Figure 3 likely also underestimate the impacts of C15 in vivo.

STAR Methods
Detailed methods are provided in the online version of this paper and include the following:
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QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105510.

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AUTHOR CONTRIBUTIONS
Conceptualization: E.M.P., S.D.C., H.D.H., L.C.E.; Formal analysis: E.M.P., L.P.; Funding acquisition and project administration: E.M.P., L.C.E.; Investigation: E.M.P.; Methodology: E.M.P., G.V.R., L.P.; Supervision: L.C.E., H.D.H.; Validation: E.M.P., L.P.; Visualization: E.M.P., L.P.; Writing – original draft: E.M.P.; Writing – review and editing: E.M.P., S.D.C., L.P., G.V.R., H.D.H., L.C.E.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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### iScience Article

### STAR★METHODS

#### KEY RESOURCES TABLE

| REAGENT or RESOURCE                        | SOURCE                        | IDENTIFIER  |
|--------------------------------------------|-------------------------------|-------------|
| **Antibodies**                             |                               |             |
| Rabbit polyclonal anti-VACV                | ThermoFisher                  | Cat# PA1-7258|
| HRP-conjugated goat anti-rabbit monoclonal | Cell Signaling Technology    | Cat# 7074S  |
| InVivoMab mouse anti-NK1.1 (PK136)         | Bio X Cell                    | Cat# BE0036, RRID:AB_1107737|
| InVivoMab, mouse IgG2a isotype control (C1.18.4) | Bio X Cell                    | Cat# BE0085, RRID:AB_1107771|
| GFP AF488 (clone FM2-64G)                  | BioLegend                     | Cat# 338007, RRID:AB_2563287|
| NK.1.1 AF647 (clone PK136)                 | BioLegend                     | Cat# 108719, RRID:AB_493186|
| Lyve-1 eFluor450 (clone ALY7)              | Thermo Fisher Scientific      | Cat# 48-0443-80, RRID:AB_2784722|
| ER-TR7 AF594 Fibroblast marker (clone ER-TR7) | Santa Cruz Biotechnology      | Cat# sc-73355, RRID:AB_1122890|
| B220 AF700 9clone RA3-6B2)                | BioLegend                     | Cat# 103232, RRID:AB_493171|
| CD107a PE (clone EDB4)                    | BioLegend                     | Cat# 121612, RRID:AB_2134487|
| Anti-mouse CD16/32 (FC receptor blockade)  | Bio X Cell                    | Cat# BE0307, RRID:AB_2736987|
| NK.1.1 Per-CP-Cy5.5 (clone PK136)          | BioLegend                     | Cat# 108727, RRID:AB_2132706|
| F4/80 AF594 (clone BM8)                    | BioLegend                     | Cat# 123140, RRID:AB_2563241|
| CD3e APC-Cy7 (clone 145-2C11)             | BD Biosciences                | Cat# 557596, RRID:AB_396759|
| Granzyme B BV510 (clone GB11)             | BD Biosciences                | Cat# 563388, RRID:AB_2738174|
| Ki-67 APC (clone SolA5)                    | Thermo Fisher Scientific      | Cat# 17-5698-82, RRID:AB_2688057|
| IFN gamma AF700 (clone XMG1.2)            | BD Biosciences                | Cat# 557998, RRID:AB_396797|
| Mouse monoclonal anti-NKG2D               | R and D Systems               | Cat# MAB1547, RRID:AB_2133391|
| CD3 BV650 (clone 145-2c11)                | BD Biosciences                | Cat# 564378, RRID:AB_2738779|
| **Bacterial and virus strains**            |                               |             |
| Ectromelia virus eGFP – ECTV 189898-p7 S-EGFP | (Fang et al., 2008)          | N/A         |
| C15-deficient Ectromelia virus – ΔC15 ECTV | (Forsyth et al., 2020)       | N/A         |
| **Chemicals, peptides, and recombinant proteins** |                               |             |
| KPL TrueBlue peroxidase substrate         | SeraCare                      | Cat# 5510-0030|
| Live/Dead Blue                             | Invitrogen                    | Cat# L23105 |
| **Critical commercial assays**             |                               |             |
| EasySep mouse NK isolation kit             | STEMCELL Technologies         | Cat# 19855  |
| BD Cytofix/Cytorperm kit                   | BD biosciences                | Cat# 554714 |
| RNeasy Plus Mini Kit                       | Qiagen                        | Cat# 74104  |
| High Capacity cDNA Reverse Transcription Kit | Applied Biosystems          | Cat# 4368814|
| PowerUp SYBR Green Master Mix              | Applied Biosystems            | Cat# A25742 |
| **Experimental models: Cell lines**        |                               |             |
| Mouse: Immortalized primary B6 skin fibroblasts | (Sinnathamby et al., 2004)   | N/A         |
| Human: TK-; 143b TK- osteosarcoma line     | ATCC                          | CRL-8303    |
| Monkey: BS-C-1                             | ATCC                          | CCL-26      |
| **Experimental models: Organisms/strains** |                               |             |
| Mouse: B6; C57BL6/J                       | The Jackson Laboratory        | RRID:IMSR_JAX:000664|
| Mouse: β2M; B6.129P2-B2mTm1UM1/DcrJ       | The Jackson Laboratory        | RRID:IMSR_JAX:002087|

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to the lead contact, Dr. Laurence C. Eisenlohr (eisenlc@pennmedicine.edu).

Materials availability
This study did not generate any new unique reagents.

Data and code availability
Data reported in this paper will be shared by the lead contact upon request. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice
All of the experimental protocols involving animals were approved by the Children’s Hospital of Philadelphia Institutional Animal Care and Use Committee. C57BL/6J (B6) mice were bred in house or purchased from The Jackson Laboratory and were source matched within each study. B6.129P2-B2m<sup>tm1Unc</sup>/DcrJ (B2 microglobulin<sup>−/−</sup>) mice were purchased from the Jackson Laboratory. All mice used for this study were females from 6–14 weeks of age, aged matched within each study. All mice were group housed in experimental groups in conventional barrier cages and experiments were carried out an isolated room in the quarantine suite of the animal facility.

Cells
NK cells used in in vitro assays were purified from spleens of B6 mice infected for 5–6 days with ΔC15 ECTV, using the EasySep mouse NK isolation kit (Stemcell, 19,855). These cells were used directly in assays, in RPMI supplemented with 5% FBS, penicillin, streptomycin, 2 mM L-glutamine and 50 μM 2-mercaptoethanol. The
primary B6 skin fibroblast cell line was derived in our laboratory and has been described previously. These cells and TK-143b osteosarcoma cells were maintained in DMEM supplemented with 5% FBS, penicillin, streptomycin and 2 mM L-glutamine. BS-C-1 cells were maintained in DMEM supplemented with 10% FBS, penicillin, streptomycin and 2 mM L-glutamine. All cells were cultured at 37°C at 5% CO₂.

**METHOD DETAILS**

**Viruses and infection**

All viruses used in this work have been previously described: ECTV expressing eGFP (Moscow strain background) was a kind gift of Dr. Luis Sigal. eGFP expressing ΔC15 ECTV (Moscow strain background) was generated as previously described; the C15 revertant ECTV that was constructed was demonstrated to behave similarly to WT ECTV and was not used in this work. All viruses were grown in house in TK-143b osteosarcoma cells at high MOI for 3 days and virus was harvested from cells by cycles of freeze-thawing and sonication. Virus was then purified from the cell lysate by ultracentrifugation (20,000 rpm, 1 h) through a 36% sucrose cushion and resuspended in 10 mM Tris at pH 9.0. To ensure titers were comparable, all viruses were titered simultaneously by traditional plaque assay on BS-C-1 cells under an overlay of 1% methylcellulose in DMEM media supplemented with 5% FBS, penicillin, streptomycin and L-glutamine for 5 days.

Mice were infected with 3 × 10³ plaque-forming units of ECTV by injection in a 30 μL volume of PBS through an insulin syringe into the hind footpad.

**Viral titering in tissue**

Mice were infected in the hind footpad for 1–3 days with 3000 pfu of eGFP WT or ΔC15 ECTV for 1–3 days. The whole draining popliteal LN was collected, weighed and homogenized. LNs were homogenized by manual disruption between two frosted glass slides and homogenates were then sonicated to release virions and cellular debris was pelleted by spinning at 400 xg for 10 s. The homogenate supernatant was titered by serial dilution on BS-C-1 cells utilizing either a traditional plaque assay, as described above, or a focus forming assay as first described by Forsyth: 2.5 × 10⁴ BS-C-1 cells were plated per well in a flat 96-well plate. The next day, serially diluted homogenates were added to monolayers in technical triplicate. Virus was allowed to adhere for 1 h and then an overlay of 1.25% avicel in complete DMEM was added and infection was left to proceed for 18 h. Then virus was removed, cell monolayers were fixed for 1 h using 4% paraformaldehyde in PBS, permeabilized for 7 min using 0.5% Triton X-100 in PBS and then blocked for 1 h with 5% BSA in Tris-buffered saline with 0.2% Tween 20. Virus infected cells were detected by staining with a primary rabbit anti-VACV antibody (1:1000 in blocking buffer for 1 h, Thermo Fisher PA1-7258) and a secondary horseradish peroxidase-conjugated goat anti-rabbit antibody (1:1000 in blocking buffer for 1 h, Cell Signaling Technology 70475) with the peroxidase substrate KPL TrueBlue (30 min, SeraCare 5510-0030). Virus-infected foci representing one Focus Forming Unit (FFU) were imaged and counted spots using a CTL ImmunoSpot S6 Universal Analyzer with ImmunoSpot software (ImmunoSpot). When results were pooled from separate animal experiments, all samples were re-titered together on the same day with the same settings to account for any variation in the titering assay.

**In vivo NK cell depletion**

B6 mice were depleted of NK cells by i.p. injection of 200 μg of anti-NK1.1 mAb (PK136, BioXCell BE0036) in 100 μL of PBS, compared to injection of IgG2a isotype control mAb (C1.18.4, BioXCell BE0085), 24 h prior to infection. Depletion was confirmed by flow cytometric analysis of splenocytes for viral titering experiments and visually for immunofluorescence imaging experiments.

**Confocal microscopy of frozen LN sections**

Popliteal LNs were harvested 3 days after infection with eGFP WT or ΔC15 ECTV, following isotype control or NK1.1 depletion. LNs were fixed overnight in periodate-lysine-paraformaldehyde buffer, equilibrated in 30% sucrose in PBS overnight and then embedded in Optimal Cutting Temperature medium (Sakura 4583) and frozen in liquid nitrogen cooled isopentane, as previously described. Sixteen micron sections were cut and blocked in 0.01% Triton X-100 with 2% of FBS and donkey serum including TrueStain Monocyte Blocker (Biolegend 426101) for 1 h and then stained overnight with the following directly conjugated mAbs: GFP AF488 (clone FM2-64G, BioLegend 338007), NK1.1 AF647 (clone PK136, BioLegend 108719), Lyve-1 eFluor 450 (clone ALY7, Invitrogen 48-0443-80), ER-TR7 AF594 (Santa Cruz Biotechnology...
sc-73355), B220 AF700 (clone RA3-6B2, BioLegend 103232). Slides were washed in .1% Tween20 in PBS, mounted using Pro-Long Glass Antifade Mountant (Invitrogen P36984) and imaged using a Leica TCS SP8 WLL confocal microscope. Images were acquired using identical laser and HyD detector settings and scans were taken of an entire popliteal LN section using a 40x 1.30 NA objective, with a z step of 1.5 µm and a total z size of 9.0 µm; individual fields were then merged into a single image.

**Image analysis**

Raw data were processed using a Gaussian filter to remove background noise, then processed into 3D maximum intensity projection. Spots were created for the NK cell (NK1.1+) channel using the automated Imaris function “spots” with manual correction as needed. Lymph node surface was created using the Lyve-1 channel. Surface for the virus infected cells was created based on the eGFP channel. The number of NK cells was calculated based on the number of spots generated. To calculate distance to the nearest neighbors, Imaris XT plugin “nearest neighbor” was applied to the NK spots and the average value was extracted from each image. The number of NK cells in contact with virus infected cells was calculated using the Imaris XT plugin “distances spots to surface” with a filtered distance of 5 µm or less between NK cells and virus-infected cell surface. Statistics were exported from Imaris. NK cell numbers were normalized to lymph node area. Values were then plotted in Prism (GraphPad).

**Flow cytometric analysis of infected tissue**

Popliteal LNs were harvested 3 days after infection with eGFP WT or ΔC15 ECTV and incubated at 37°C in media containing monensin (Invitrogen 00-4505-51) and PE labeled CD107a antibody (1:100, clone EDB4, BioLegend 121,612) for 1 h. LNs were then dissociated using frosted glass slides, filtered through a 40 µm strainer, and entire LN populations were counted and stained for flow cytometric analysis. Cells were first stained in Live/Dead Blue (1:500, Invitrogen L23105), treated for Fc-receptor blockade with anti-CD16/32 (100 µg/mL, BioXCell BE0307) and then stained for surface antibodies at 0.1 µg/mL unless indicated: NK1.1 PerCP-Cy5.5 (clone PK136, BioLegend 108727), F4/80 AF594 (clone BM8, BioLegend 123140), CD3e APC-Cy7 (clone 145-2C11, BD 557596). Cells were then fixed and permeabilized for intracellular staining with the BD Cytofix/Cytoperm kit (BD 554714) and then stained for intracellular antibodies at .1 µg/mL unless indicated: GzB BV510 (1:10, clone GB11, BD 563388), Ki-67 APC (clone SolA5, Thermo Fisher 17-5698-82), IFNγ AF700 (clone XMG1.2, BD 557998). Flow cytometry data were acquired on the CytoFLEX LX instrument using CytExpert software (Beckman Coulter) and analyses were conducted using FlowJo software (FlowJo LLC). Live singlet cells were gated, and infected, NK and macrophage cells were identified as GFP+, CD3 “NK1.1” and F4/80” respectively. Reported cell counts are cytometer-based counts, and median fluorescence intensity (MFI) was analyzed for the positive staining populations.

**RNA isolation and RT-PCR**

Total RNA was extracted from LNs by bead homogenization and purified using the Qiagen RNeasy Plus Mini Kit (Qiagen 74104) and cDNA was prepared from 750 ng of RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems 4368814). Quantitative PCR was performed using the PowerUp SYBR Green Master Mix (Applied Biosystems A25742) and measured using a StepOnePlus Real Time PCR Machine with StepOnePlus software (Applied Biosystems). Expression was quantified relative the housekeeping gene Gapdh normalized to the mean of the mock-infected group, using the ΔΔC_T method. Fold change values, 2^{-ΔΔC_T}, were pooled from 3 independent experiments. The forward and reverse primer pairs used are listed in Table S1.

**In vitro NK degranulation assay**

Primary B6 skin fibroblast cells were mock infected with PBS or were infected with an MOI of 3 of eGFP WT or ΔC15 ECTV for 1 h in minimal volume and then infection was allowed to proceed for a total of 6 h. At this time, these target cells were plated at the indicated effector:target ratios with NK cells that were isolated as described above. These cocultures were conducted for 5 h in the presence of monensin (Invitrogen 00-4505-51) and PE labeled CD107a antibody (1:100, clone EDB4, BioLegend 121,612), and in the indicated experiments with 20 µg/mL of blocking anti-NKG2D antibody (R&D systems MAB1547). Cells were then washed, stained for Live/Dead Blue (1:100 Invitrogen L23105) and extracellular antigens (1:200 CD3 BV650, clone 145-2c11, BD 564378; 1:200 NK1.1 PerCP-Cy5.5, clone PK136, BioLegend 10727) for 30 min in PBS, fixed and permeabilized using the BD Cytofix/Cytoperm kit (BD 554714) and then stained intracellularly (1:200 IFNγ AF700, clone XMB1.2, BD 557998) for one hour. Data were acquired on the CytoFLEX LX instrument
using CytExpert software (Beckman Coulter) and analyses were conducted using FlowJo software (FlowJo LLC). NK cells were identified as CD3^-NK1.1+ live singlets.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Information about n (number of animals used), test used, and measurement of center and dispersion are included in figure legends. Statistics analyses were performed in Prism 9. Wherever possible, all data from three experimental replicates is pooled for display and analysis. Data were tested for normality, using the D’Agostino & Pearson test, prior to selecting parametric analyses. In instances in which an outlier test revealed that non-normality was driven by a single data point, the data was considered normal and parametric tests were selected. Where possible, lognormal data was log transformed to enable use parametric tests. Any manipulation of the data, such as log transformation, and the statistical test used are indicated in the figure legend and p values are shown for transparency. Individual biological or technical repeats are shown as points for transparency and all bars correspond to means and error bars represent SEM.