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# Poxvirus interleukin-4 expression overcomes inherent resistance and vaccine-induced immunity: Pathogenesis, prophylaxis and antiviral therapy

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## Abstract

In 2001, Jackson *et al*. reported that murine IL-4 expression by a recombinant ectromelia virus caused enhanced morbidity and lethality in resistant C57BL/6 mice as well as overcame protective immune memory responses. To achieve a more thorough understanding of this phenomenon, and to asses a variety of countermeasures, we constructed a series of ECTV recombinants encoding murine IL-4 under the control of promoters of different strengths and temporal regulation. We showed that the ECTV-IL-4 recombinant expressing the highest level of IL-4 was uniformly lethal for C57BL/6 mice even when previously immunized. The lethality of the ECTV-IL-4 recombinants resulted from virus-expressed IL-4 signaling through the IL-4 receptor, but was not due to IL-4 toxicity. A number of treatment approaches were evaluated against the most virulent IL-4 encoding virus. The most efficacious therapy was a combination of two antiviral drugs (CMX001® and ST-246®) that have different mechanisms of action.

### Keywords

immunosuppression; poxvirus; ectromelia virus; lethal infection; IL-4

## Introduction

IL-4 is a prototypic $T_h2$ cytokine produced by T cells, mast cells, basophils and eosinophils. IL-4 plays a central role in shaping the immune response, promoting the development of $T_h2$ responses and inhibiting those of $T_h1$ character. IL-4 is also an important regulator of immunoglobulin (Ig) class switch to IgG1 and IgE in mice, and IgG4 and IgE in humans



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(Gessner & Rollinghoff, 2000). Although ectopically expressed IL-4 from recombinant viruses would be expected to enhance the $T_h2$ character of the immune response, and result in enhanced pathogenesis, this is not a universal observation. Expression of IL-4 by retroviruses (Benedetti, et al., 1998), adenoviruses (Lukacs, et al., 1997), vesicular stomatitis (Fernandez, et al., 2002) and herpes simplex virus (Andreansky, et al., 1998;Ghiasi, et al., 1999;Ghiasi, et al., 2001;Ikemoto, et al., 1995) resulted in little effect on viral pathogenesis, whereas IL-4 expression by influenza virus (Moran, et al., 1996), respiratory syncytial virus (Fischer, et al., 1997) and vaccinia virus (VACV) (Andrew & Coupar, 1992;Bembridge, et al., 1998;Sharma, et al., 1996) delayed viral clearance or enhanced pathogenesis in mice. The most striking phenotypes were observed with IL-4 expressing recombinants generated from poxviruses ectromelia (ECTV) and myxoma in their natural hosts. Expression of the rabbit IL-4 gene from the SLS strain of myxoma virus resulted in fatal myxomatosis in genetically resistant wild rabbits (Kerr, et al., 2004). Similarly, expression of the murine IL-4 gene by a recombinant ECTV caused enhanced lethality for inherently resistant C57BL/6 mice, and overcame protective immune responses induced by vaccination (Jackson, et al., 2001). The enhanced lethality of the ECTV-IL-4 recombinant was likely due to immunosuppression, although the exact mechanism of action remains to be determined (Mullbacher & Lobigs, 2001;Norazmi, 2001;Stanford & McFadden, 2005).

The Jackson *et al.* study raised a number of questions. The utilized recombinant may have underestimated the potential of an ECTV-IL-4 recombinant to overcome vaccine-induced immunity as it lacked a functional *thymidine kinase* (*tk*) gene (ORF 078) due to insertion of the *Escherichia coli lacZ* gene (Jackson, et al., 1998;Jackson, et al., 2001). A second possible loss of gene function occurred through insertion of the IL-4 gene and herpes simplex virus *tk* gene into ORF 030, an orthologue of the VACV F7L gene of unknown function. The herpes simplex virus *tk* gene produced thymidine kinase activity to only 10% of the ECTV WT level (Coupar, et al., 2000). Furthermore, in the Jackson *et al*. study memory immunity was generated using an attenuated ECTV recombinant instead of the standard VACV smallpox vaccine. This homologous vaccine/challenge protocol therefore fails to model a variola virus (VARV) challenge of humans vaccinated with the smallpox vaccine. And finally, the study did not evaluate the ability of treatment modalities, such as immune serum and antiviral drugs to protect against the fatal infection by the ECTV-IL-4 recombinant.

To confirm and extend the studies of Jackson *et al*., we constructed a series of ECTV recombinants with a murine IL-4 gene under the control of promoters of differing strengths and temporal regulations. The ECTV-IL-4 recombinants posed no risk to humans as ECTV does not cause human disease, and murine IL-4 does not efficiently signal through the human IL-4R (Buller & Fenner, 2007;Mosmann, et al., 1987). We showed that ECTV-IL-4 recombinants expressing high levels of IL-4 were uniformly lethal for naïve, disease resistant mice or mice previously immunized with the smallpox vaccine. We evaluated a number of treatment approaches including anti-IL-4 antibody therapy to reduce systemic IL-4 levels, and a treatment with two antivirals (CDV/CMX001® and ST-246®) (Painter & Hostetler, 2004;Parker, et al., 2008;Parker, et al., 2009;Yang, et al., 2005).

## Results

### Construction and characterization of ECTV recombinants encoding murine IL-4 and non-functional IL-4$^{D116D119}$ from early and late promoters of differing strengths

To evaluate the maximum potential of ECTV-IL-4 recombinants to cause lethal mousepox in normally resistant mice, we recombined the murine IL-4 gene into the CHO gene of WT ECTV. The CHO gene (~20,500 bases from the left-hand terminus) is one of 26 naturally disrupted ORFs present in the ECTV genome (Chen, et al., 2003). A recombinant ECTV

expressing *lac z* from this locus was previously shown to be as virulent as ECTV-wild type (WT) for A/NCR mice validating this site for expression of the IL-4 gene without affecting the natural life-cycle of the virus (data not shown). IL-4 expression cassettes were constructed with the IL-4 gene under the control of four promoters with different strengths and patterns of temporal regulation. Early (E) and late (L) promoters are active before and after DNA synthesis, respectively. The 7.5E promoter consists of the early element of the 7.5 E/L promoter, which expresses both early and late in the replication cycle. The 7.5 E/L promoter was used in the Jackson *et al* study to express IL-4 (Jackson, et al., 2001). The strong synthetic early (SSE) promoter is an early promoter whose activity has been increased based on a mutational analysis of several early promoters (Davison & Moss, 1989). The 11KL promoter is a strong late promoter that regulates expression of a VACV structural protein F17 (Bertholet, et al., 1985). As a control, the WT IL-4 gene was mutated by substituting aspartic acid residues for the glutamine and tyrosine residues at positions 116 and 119, respectively. This IL-$4^{D116/D119}$ mutant bound to the IL-4 receptor α subunit with similar affinity as wild type IL-4 without inducing cellular responses (Grunewald, et al., 1997). The IL-4 and IL-$4^{D116/D119}$ genes controlled by the various promoters were cloned into a CHO gene targeting vector, and recombined into the ECTV genome by the transient dominant selection system.

## In vitro evaluation of ECTV recombinants encoding murine IL-4

The recombinant viruses were evaluated for IL-4 protein expression levels and activity. Western blot analysis of culture supernatants from CV-1 cells infected with ECTV-IL-4 recombinants detected species that migrated as major and minor bands at 16.6–17.8 and 13 kDa, respectively. The minor band of 13 kDa was also present in the baculovirus produced IL-4 (BV-IL-4) and probably represented the nonglycoslylated protein since its size is similar to the predicted size of unmodified IL-4 (13.6 kDa). The major band is a doublet that consisted of two closely migrating bands of 16.6 and 17.8 kDa. These apparent molecular weights were larger than the 15.8 kDa of the baculovirus produced IL-4, but smaller than the 20 kDa size reported for natural IL-4 (Figure 1B) (Paul & Ohara, 1987). These differences are likely caused by glycosylation. Glycoslylation is not required for IL-4 bioactivity (Paul & Ohara, 1987). The rank order of accumulation of IL-4 was -11KL-IL-4 >-7.5E/L-IL-$4^{D116/D119}$ or -7.5E/L-IL-4 >SSE-IL-4 >-7.5 E/IL-$4^{D116/D119}$ or -7.5E-IL-4. The amount of IL-4 or IL-$4^{D116/D119}$ expressed in CV-1 cells by the various recombinants was further quantified by ELISA and bioassay (Figure 1C). Supernatants from infections with ECTV-7.5E/L-IL-4 and ECTV-SSE-IL-4 recombinants contained 5.2 and 3.4 times as much IL-4 by ELISA, respectively, as a comparable ECTV-7.5E-IL-4 recombinant infection. The highest level of IL-4 expression was achieved with the 11KL promoter that was 2.1-fold higher than the 7.5E/L promoter. The level of IL-$4^{D116/D119}$ was ~ 3-fold lower than a comparable promoter construct (7.5E or 7.5E/L) expressing IL-4. This was likely due to different affinities of the ELISA capture antibody for IL-4 and IL-$4^{D116/D119}$ as no difference in expression level was noted in the western blot analysis that used the same detection antibody as the ELISA (Figure 1B). As verified by bioassay, IL-4 was biologically active, whereas the IL-$4^{D116/D119}$ was not.

## In vivo evaluation of ECTV recombinants encoding murine IL-4

Although IL-4 is notoriously difficult to detect in blood, ECTV expressed IL-4 was detected by ELISA in sera of C57BL/6 mice 6 days post footpad (FP) infection with ECTV-IL-4 recombinants. The rank order of IL-4 concentrations was identical to that observed in tissue culture (Figure 1D). The reduced concentration of IL-$4^{D116/D119}$ in sera of mice infected with ECTV-7.5E-IL-$4^{D116/D119}$ as compared to ECTV-7.5E-IL-4 was due in part to the differential sensitivity of the ELISA for IL-4 as compared to IL-$4^{D116/D119}$ (above), and to the more restricted disease as exemplified by lower virus titers in spleens and livers of mice,

which were similar to those observed in ECTV-WT infections (Table 1). The sustained levels of IL-4 detected in sera of C57BL/6 mice infected with ECTV-IL-4 recombinants ranged from 107 to 1075 ng/ml. These levels far exceeded that detected in the ECTV-WT infections where IL-4 concentrations were below the level of detection of the ELISA (<2 ng/ml) or a CBA assay (<25 pg/ml, data not shown).

### Pathogenesis of fatal disease caused by ECTV-IL-4 recombinants in C57BL/6 mice

Each recombinant virus was tested by the FP route in a lethal dose 50 ($LD_{50}$) assay in C57BL/6 mice (Reed & Muench, 1938). C57BL/6 mice infected via the FP route are resistant to ECTV-WT infection up to at least 9000 PFU (Parker, et al., 2009). As shown in Table 1, infections with ECTV-IL-4 recombinants yielded higher infectivity levels in spleens and livers, $LD_{50}$ values of less than 1 PFU/mouse, and mean time to death (MTD) values of ~7 days at a virus dose of $4 \times 10^3$ PFU (Table 1, footnote 2). Because the particle/PFU ratio for orthopoxviruses is typically ~20–30 to 1, an $LD_{50}$ value of < 1 indicates that the mouse is a more sensitive assay for ECTV infectivity than tissue culture. The lethal effects were observed with promoters that expressed at early, late, and early and late times during the virus replication cycle. There were no deaths in mice inoculated with ECTV-WT, ECTV-7.5E-IL-$4^{D116/D119}$, or ECTV-7.5E/L-IL-$4^{D116/D119}$ at virus doses exceeding $10^5$ PFU/mouse. The lethality of crude, sucrose-cushion-purified or sucrose-band-purified preparations of ECTV-IL-4 recombinants were similar, suggesting that any contaminating host molecules did not contribute to virulence (data not shown).

Furthermore, the lethality of ECTV-IL-4 recombinants was due to virus expressed IL-4 and not the induction of host IL-4 gene expression as ~100 PFU of ECTV-7.5E-IL-4 was 100% lethal (with an identical MTD) for C57BL/6 mice and C57BL/6-*Il4*$^{tm1Nnt}$ mice, which lacked the ability to make IL-4 (data not shown). And finally, the IL-4 (IL-4Rα-γc) and/or IL-13 (IL-4Rα-IL-13Rα1) receptor(s) was required for lethal disease of ECTV-IL-4 recombinants as ECTV-SSE-IL-4 caused a lethal infection in VACV (Dryvax™) immunized BALB/cJ mice, but not immunized BALB/c-*Il4ra*$^{tm1Sz}$, which lack a functional IL-4/IL-13 receptor (Table S1).

To prove that the observed lethality of the ECTV-IL-4 recombinants was not caused by direct toxicity from high levels of IL-4, we administered twice daily (BID) intraperitoneal injections of 3.1 to 100 μg of IL-4 to mock and ECTV-infected C57BL/6 mice during the first 5 days of infection. Uniformly lethal disease was observed in ECTV-infected C57BL/6 mice dosed with 6.3 to 100 μg of IL-4, whereas administration of 100 μg of IL-4 for the same duration to mock-infected animals caused no deaths or weight loss (Figure S1 and Table S2). This data show that the observed lethality of ECTV-IL-4 recombinants infected animals is not directly due to IL-4 toxicity.

To examine in greater detail the effect of virus-expressed IL-4 on pathogenesis, C57BL/6 mice were infected by the FP route with ECTV-WT or ECTV-7.5E/L-IL-4 and tissue viral titers and histopathological changes were monitored at various days p.i. At 3 days p.i., similar virus titers were detected in spleen and livers of ECTV-7.5E/L-IL-4 as compared to ECTV-WT (Table 2). This changed dramatically at day 4 in the liver and day 5 in the spleen. By day 6 p.i. virus titers in spleen and liver homogenates of ECTV-7.5E/L-IL-4 infected C57BL/6 mice had reached $4 \times 10^6$ PFU/ml as compared to ECTV-WT values of ~$3 \times 10^2$ PFU/ml. Histopathologic examination of tissue from sacrificed mice revealed prominent lesions in popliteal and cervical lymph nodes (LNs), bone marrow, thymus, liver, and spleen. Hepatic lesions consisting of focal necrosis were prominent among ECTV-7.5E/L-IL-4 infected C57BL/6 mice by 6 days p.i., and extensive by 7 days p.i. with large areas of the liver showing confluent necrosis. There were no inflammatory cells associated within any areas of necrosis (Figure 2, panel A). In contrast, ECTV-WT infected C57BL/6 mice

had fewer numbers and smaller sizes of lesions, which were infiltrated by small lymphocytes (Figure 2, panel C). Another major difference observed in the liver was the presence of large numbers of macrophages in the hepatic vessels of ECTV-7.5E/L-IL-4 infected mice, where ECTV-WT-infected mice showed only a scattering of cells (Figure 2, panels B and D, respectively). The increase in myeloid lineage cells in ECTV-7.5E/L-IL-4 infected C57BL/6 mice was even more prominent in the sternal bone marrow where there was a progressive replacement of normal hematopoietic cells with macrophages. In comparison, the ECTV-WT infected C57BL/6 mice had only a moderate increase in myeloid cells in the sternal bone marrow. In the spleen, histiocytosis and hemorrhage in the red pulp were more prominent in ECTV-7.5E/L-IL-4 than ECTV-WT infected C57BL/6 mice. Although more difficult to evaluate, it appeared that lymphopoiesis (reactivity) was more pronounced in ECTV-WT than ECTV-7.5E/L-IL-4 infected C57BL/6 mice. In the thymus, cortical necrosis was a prominent feature of ECTV-7.5E/L-IL-4 infections, but was undetectable with ECTV-WT. Of the three LNs examined, only the popliteal LN that drained the site of infection showed severe necrotic changes that by day 4 p.i. resulted in complete obliteration of the nodal structure in both ECTV-WT and ECTV-7.5E/L-IL-4 infections. This finding was expected based on the high level of infectivity initially detected in the popliteal LN (data not shown). The cervical and axillary LNs exhibited moderate lymphopoiesis in the ECTV-WT infection with the medulla containing large numbers of immunoblast-like progenitors as well as small lymphocytes. In contrast, similar samples from the ECTV-7.5E/L-IL-4 infections showed little lymphopoietic activity. Table 3 summarizes these observations. In summary, ECTV recombinants expressing IL-4 from promoters of varying strength and temporal regulation were lethal for normally resistant C57BL/6 mice over a wide-range of viral doses. These ECTV-IL-4 recombinants replicated to high titers in spleens and livers correlating with extensive tissue necrosis, and the lack of a detectable inflammatory infiltrate.

## Transmissibility of ECTV-11KL-IL-4

ECTV-WT is efficiently transmitted from an infected mouse to naïve cage mates over ~ 10 day period (Wallace & Buller, 1985). The transmissibility of ECTV-11KL-IL-4 between C57BL/6 mice is shown in Figure 3. ECTV-WT infected mice transmitted efficiently between 3 and 19 days post-infection. Unexpectedly during the 3 to 5 day p.i. time period, and under conditions where ECTV-WT infected mice transmitted to 3 of 6 (50%) contact mice, ECTV-11KL-IL-4 infected mice failed to transmit to contacts. During the 5 to 7 day p.i. time interval, ECTV-11KL-IL-4 infected mice transmitted to 1 of 6 (17 %) contact mice, while ECTV-WT infected mice transmitted to 6 of 6 (100%) mice. By day 7 the ECTV-11KL-IL-4 infected mice had all died, and therefore from days 7–19 transmissibility data are shown only for ECTV-WT infected mice.

## Evaluation of VACV vaccination against infection with ECTV-IL-4 recombinants

Vaccination with VACV was evaluated in mousepox resistant C57BL/6, susceptible outbred Swiss Cr:NGP (Swiss), and susceptible inbred A/NCR mice all infected via the FP route (note that C57BL/6 mice are susceptible to infection, but are resistant to lethal mousepox disease as described by (Marchal, 1930). The mousepox susceptible mouse strains are better models for smallpox as human populations are susceptible to disease, and not resistant, to morbidity and mortality following VARV infection. Because the smallpox vaccine is currently the only prophylaxis for controlling an outbreak of smallpox or human monkeypox our first experiment tested the efficacy of a vaccine memory response against challenge with ECTV IL-4 recombinants expressing various amounts of IL-4. Susceptible Swiss and resistant C57BL/6 mice were immunized with $2 \times 10^3$ PFU (low dose) or $2 \times 10^4$ PFU (high dose) of the Dryvax™ vaccine followed 7 wk later with a challenge of $4 \times 10^4$ PFU of

ECTV-WT or ECTV-IL-4 recombinants. On a per weight basis the high dose of vaccine is ~100 times greater than the standard vaccine dose used for humans (~$2.5 \times 10^5$ PFU/dose).

Table 4 summarizes the results of this challenge experiment. As expected the vaccinated mice were completely protected from a lethal challenge with ECTV-WT at both doses of Dryvax™ vaccine. The low dose of vaccine partially protected Swiss mice (5/8 deaths) and fully protected C57BL/6 mice from lethal challenge with ECTV-7.5E-IL-4, which produced the lowest level of IL-4 (Figure 1, 107±16 ng/ml); however, the high dose of vaccine was almost completely protective for Swiss mice (1/8 deaths) and also fully protected C57BL/6 mice. By comparison ECTV-7.5E/L-IL-4 and ECTV-11KL-IL-4, which expressed medium (218±71 ng/ml) and high (1075±413 ng/ml) levels of IL-4 (Figure 1), respectively, were uniformly lethal for Swiss and C57BL/6 mice regardless of the dose of vaccine employed. We also found that immunizing naïve A/NCR mice or C57BL/6 mice with Dryvax™vaccine 7 days prior to challenge with ECTV-11KL-IL-4 did not prevent a uniformly lethal infection (Table S3).

Dryvax™ immunization followed by ECTV challenge is a heterologous test system in which the vaccine and challenge viruses are different viral species, and therefore not matched antigenically. To test the effectiveness of a homologous test system, C57BL/6 mice were FP "immunized" with ECTV-WT or Dryvax™ and challenged 49 days later with 1,600, 160 or 16 PFU of ECTV-11KL-IL-4 (Table S4). In response to all three ECTV-11KL-IL-4 challenge doses, 18/18 (100%) mock, 15/18 (83%) Dryvax™ and 0/11 (0%) ECTV-WT vaccinated mice died. As compared to mock vaccinated mice, ECTV-WT provided significant protection against death ($p=0.0001$), but Dryvax™ did not ($p=0.23$).

### Evaluation of prophylaxis with vaccinia immune globulin (VIG)

Vaccinia immunoglobulin is licensed for the treatment of complications of VACV vaccinations in humans, although its efficacy in humans is not well documented (CDC, 2009;Vora, et al., 2008). A/NCR mice were intraperitoneally injected with 2 g/Kg of VIG 4 days prior to FP infection with 130 PFU of ECTV-WT or ECTV-11KL-IL-4. The VIG treatment did not prevent lethal infection with each virus, and did not delay the mean time to death (MTD) as compared to controls receiving placebo (Table S5).

### Evaluation of prophylaxis with the antiviral cidofovir (CDV)

CDV is a nucleoside analog of cytosine, and is effective in inhibiting replication of a broad range of DNA viruses including poxviruses through targeting the virus-encoded DNA polymerase (De Clercq & Holy, 2005;De Clercq, 2007;Parker, et al., 2008). A plaque reduction assays yielded a 50% inhibitory concentration of ~10 μM CDV for both ECTV and ECTV-7.5E-IL-4. In addition, the administration of CDV at the time of infection of susceptible A/NCR mice protected against a lethal aerosol challenge of ECTV-WT (Buller, et al., 2004). Table 5 shows CDV protected susceptible A/NCR mice against death following a FP challenge with ECTV-WT and the low IL-4 expressing recombinant, ECTV-7.5E-IL-4 (1/8 mortality), but not recombinants with the higher strength promoters, although the MTD was extended.

### Evaluation of combinations of treatments

The failure of single modality treatments lead us to evaluate combination treatments. Treatment of A/NCR or C57BL/6 mice with CDV (12.5 mg/kg daily for 5 days) and a monoclonal antibody (mAB; 2 mg/mouse at T=−1, +1, +3 and +5) that specifically neutralizes IL-4 activity protected against an ECTV-11KL-IL-4 lethal challenge, although the anti-IL-4 mAB treatment alone was ineffective for A/NCR mice and only partially

effective for C57BL/6 mice (Table S6). Substitution of VIG for the anti-IL-4 mAB did not prevent lethal ECTV-11KL-IL-4 infections when administered with CDV (data not shown).

Mice could be protected from lethal ECTV-11KL-IL-4 infections with CDV if they were first immunized with Dryvax™ and had the memory immunity activated by a second immunization. As shown in Table S7 only mice receiving the prime and boost immunizations and CDV treatment were completely protected from lethal disease at the low (25 PFU) virus challenge dose. Up to 75% protection from death and a significant extension of the MTD was observed at the higher virus challenge doses of 250 and 2,500 PFU.

The above findings suggested that CDV might be effective against the lethal ECTV-11KL-IL-4 infections, if combined with at least one other type of antiviral therapy. Combination antiviral therapy would provide rapid synergistic antiviral activity, whereas a primary or secondary vaccine immunization would have the prerequisite time lag. Preliminary studies evaluated a combination of CDV and ST-246®. ST-246® is an orally available antiviral that is in Phase II clinical trials. ST-246® targets the F13L protein, a major envelope protein required for the production of extracellular virus and consequently virion transport to the surface and virus release (Parker, et al., 2008;Yang, et al, 2005). Administration of CDV and ST-246® consistently provided significant protection against infections with ECTV-11KL-IL-4 (Table S8 and data not shown). Although CDV is suitable for the demonstration of a proof-of-principle, its inherent nephrotoxicity and requirement to be administered intravenously makes it an unsuitable option for wide-scaled human administration. Fortunately, CMX001®, a lipid conjugate of CDV, has an identical intracellular molecular mode of action, is orally administered, is in Phase II clinical trials, and has been shown to be more efficacious against ECTV than CDV. Administration of CMX001® and ST-246® protects ECTV-11KL-IL-4 intranasally infected A/NCR mice from mortality (Table 6) and weight loss (Figure 4).

## Discussion

We constructed a series of ECTV recombinants expressing murine IL-4 or non-functional IL-4$^{D116/D119}$ mutant under the control of different promoters with varying strengths and temporal regulation. The expression of the murine IL-4 gene was consistent with the reported strengths of the tested promoters. Accordingly, the ECTV-7.5E-IL-4 and ECTV-11KL-IL-4 recombinants were observed to produce *in vitro* and *in vivo* the lowest and the highest levels of IL-4, respectively. There was no obvious difference in the pathogenesis of disease that could be attributed to promoter strength, unless virus replication was reduced by prophylaxis. In ECTV-7.5E-IL-4 infections, protection from death in the inherently susceptible A/NCR and Swiss strains could be reduced by Dryvax™ immunization or CDV treatment. Based on this observation, we selected the ECTV-11KL-IL-4 recombinant, which produced the highest level of IL-4, as the challenge virus for evaluation of the robustness of the various treatments.

The IL-4 levels generated *in vitro* by the Jackson *et al.* ECTV-IL-4 recombinant were not reported, but would likely be comparable to our ECTV-7.5E/L-IL-4 that utilized an identical promoter, but lacked a fully functional *tk* gene and contained an insertion in the orthologue of the VACV F7L ORF. The IL-4 levels induced by all ECTV recombinants far exceeded that achieved by other vectors. For example, adenovirus and *P. aeruginosa* vectors achieved maximal systemic levels of 150 pg/ml and ~100ng/ml of IL-4, respectively, following intravenous infection of mice (Giampietri, et al., 2000;Kim, et al., 2000). For the following reasons we conclude the lethality of the ECTV-IL-4 recombinants results from virus-expressed IL-4 signaling through the IL-4 and/or IL-13 receptor and not due to IL-4 toxicity or the contribution of host-expressed IL-4: 1. immunized BALB/c-*Il4ra*$^{tm1Sz}$ mice lacking a

functional IL-4 receptor were resistant to lethal infection; 2. administration of 6.3 to 100 μg of exogenous IL-4 was lethal for ECTV-WT-infected, but not mock-infected, C57BL/6 mice; 3. the non-signaling ECTV-IL-4$^{D116/D119}$ recombinants behaved as ECTV-WT; and 4. C57BL/6-*Il4*$^{tm1Nnt}$ mice lacking the ability to make IL-4 were equally susceptible to lethal infection.

ECTV expressed IL-4 could exert its effect on cells in two distinct ways. The large amounts of biologically active IL-4 secreted from ECTV-IL-4 infected cells could induce physiological changes through the IL-4 receptor ubiquitously found on a large number of cell types. In addition, virus expressed IL-4 could be non-specifically trapped within the virion during assembly and released within the cell's cytoplasm in the subsequent replication cycle as previously shown for chloramphenicol acetyl transferase (Franke & Hruby, 1987). Indeed western blot analysis of sucrose gradient, band-purified virions from ECTV-IL-4 infected cells detected ~ $7 \times 10^5$ molecules of IL-4/virion (Figure S2); however, for the virion associated IL-4 to have physiological effects, it would need to have access to recently synthesized IL-4 receptor or receptor on the cell surface as no IL-4 receptor independent pathways for IL-4 function has yet to be described.

The Jackson *et al.* study reported ECTV-IL-4 recombinant infections associated with increased viral titers in spleens, and diminished splenic NK and T cell lytic and IFN-γ responses. In our studies we also observed increased viral titers in assayed tissues. Histopathologic examination of sacrificed mice revealed large areas of the liver showing confluent necrosis without associated inflammatory cells. Another major difference from ECTV-WT infections was the presence in the hepatic vessels of large numbers of macrophages and a progressive replacement of normal hematopoietic cells with macrophages in the bone marrow. This is consistent with a recent study that has shown that exogenously provided IL-4 can also drive expansion of macrophage populations in uninfected mice (Milner, et al., 2010). In ECTV-7.5E/L-IL-4 infections, histiocytosis and hemorrhage in the red pulp of the spleen were more prominent and in general lymphopoiesis was reduced in examined lymphoid tissue. In summary, ECTV-IL-4 recombinants replicated to high titers in lymphoid organs and the liver correlating with extensive tissue necrosis, and the lack of a detectable protective inflammatory infiltrate. We speculate that a major factor contributing to the lethality of ECTV-IL-4 recombinants is its lymphoid tissue tropism, resulting in focal production of extremely high levels of IL-4 at the sites of primary and memory adaptive immune responses. This hypothesis may explain the failure of other virus-IL-4 recombinants to cause similar disease.

In spite of replicating to higher titers in examined tissues, surprisingly ECTV-11KL-IL-4 infected mice were less efficient in transmission to contacts suggesting an effect of IL-4 on virus replication in the skin, the primary source of transmissible virus. Based on severe immunosuppression induced in the ECTV-IL-4 recombinant infections and reduced transmissibility, we speculate that a VARV expressing human IL-4 may cause a disease similar to, or more severe than hemorrhagic smallpox. Hemorrhagic smallpox is thought to occur in subjects with varying degrees of immunodeficiency. As compared to ordinary smallpox, it presents with a shorter time to death and more severe disease manifestations that may result in earlier hospitalization and/or isolation, which could result in less efficient person-to-person transmission of VARV.

Treatments using the VACV-based Dryvax™ smallpox vaccine (prime or prime/boost formats) and VIG were thoroughly evaluated and found to be ineffective against ECTV-IL-4 recombinants producing the greatest amounts of IL-4. Prime/boost immunizations failed to protect even when challenges were initiated during a period when high levels of virus specific T cells and antibodies were present in blood. It was of interest to note that prior

infection with ECTV-WT completely protected mice from lethal ECTV-IL-4 recombinant infections. This protective effect was also observed by Jackson *et al.* where an attenuated, recombinant ECTV (ECTV-602) was able to protect 40% of immunized C57BL/6 or BALB/c mice from challenge with an ECTV-IL-4 recombinant employing the 7.5E/L promoter. This differential effect might reflect the differences in antigenicity of VACV as compared to ECTV (even though the genomes of the viruses share ~95% nucleotide identity) and as a consequence of the significantly greater systemic replication competence of ECTV as compared to VACV in mice (Chen, et al., 2003;Bray, et al., 2000;Neyts, et al., 2010;Smee, et al., 2004).

Although it can be problematic to extrapolate conclusions from one system to another, the biology of IL-4 appears to be quite similar in mouse and human species (Okada, et al., 2003). Therefore, it is possible that a VARV-IL-4 recombinant would also break through the immunity generated by a vaccine based on VACV (*e.g.* Dryvax™, ACAM2000, MVA or Lister). It is possible that a vaccine composed of key protective antigens from VARV could provide improved efficacy against such a VARV-IL-4 recombinant.

We also evaluated small molecule inhibitors of specific steps in the virus replication cycle for their efficacy against ECTV recombinants expressing high levels of IL-4. We confirmed the finding of Robbins *et al* that the DNA polymerase inhibitor, CDV, was ineffective at protecting inherently susceptible mice from lethal ECTV-IL-4 infections (Robbins, et al., 2005). Similarly, an oral lipid conjugate of CDV, CMX001®, or the virus transport and release inhibitor, ST-246®, failed to protect against lethal ECTV-11KL-IL-4 infections at doses and regimens used in this study; however, the combined administration of CDV or CMX001® and ST-246® significantly protected against lethal IN or FP challenges with ECTV-11KL-IL-4. Although this combination of antivirals worked well, the added presence of memory immunity provided by the licensed smallpox vaccine might provide an additional margin of protection. Also useful would be a third antiviral that specifically targets transcription, which would prevent expression of all transgenes regardless of activity.

The failure of antiviral monotherapy to protect mice from ECTV-IL-4 recombinants is consistent with experimental and clinical data that suggests a functioning immune system is necessary for successful antiviral treatment of robust poxvirus infection. ST-246 protected against lethal challenges in all tested immunocompetent animal models, and provided full protection in some, but not all immunodeficient hosts infected with VACV strain WR (Grosenbach, et al., 2010). Similarly, CDV treatment of vaccinia or cowpox virus infections of athymic nude, SCID, or cyclophosphamide treated mice failed to clear infections (Bray, et al., 2000;Neyts, et al., 2010;Smee, et al., 2004). In 2007, a child with eczema vaccinatum, a life-threatening complication of VACV infection, was treated with VIG alone and then in combination with CDV and finally with a second antiviral, ST-246, prior to clinical progress and recovery was observed (Vora s et al 2008). Similarly, in 2008, a case of progressive vaccinia, a rare and often fatal adverse event to vaccination with smallpox vaccine, was treated with VIG and then in combination with ST-246, Imiquimod, and CMX001 (CDC, 2009). In both cases a monotherapy evolved into a combination therapy due to the lack of clinical response. These two clinical cases, which involved patients with varying degrees of immunodeficiency, the available antiviral studies using immunodeficient animal hosts, and our work here with a virus that induces a profound immunodeficient state suggest that more research is needed to evaluate combination antiviral therapies against poxviruses. This is particularly relevant as a portion of the population is immunosuppressed or immunocompromised (Handley, et al., 2009). Combination antiviral therapy with compounds that target different pathways in virus replication have the added benefit of mitigating against the evolution of resistance, which is the inevitable consequence of monotherapy.

ECTV-IL-4 recombinants could be useful tools for evaluating the potency of candidate antivirals, since they induce a profound immunosuppression in all tested mouse strains whether genetically resistant or susceptible to severe mousepox. Furthermore, our studies indicate it should be possible to develop effective countermeasures against poxviruses expressing IL-4 or other molecules. And finally, the reduced transmissibility of ECTV-11KL-IL-4 for mice suggests that poxviruses expressing IL-4, while lethal for those infected, may or may not initiate an epidemic.

## Materials and methods

### Cells and viruses

BSC-1 cells (ATCC CCL 26) were grown in Eagle's minimum essential medium (MEM) containing 10% fetal calf serum (FCS) (Hyclone III, Logan, UT), 2 mM L-glutamine (GIBCO, Grand Island, NY), 100 U/ml penicillin (GIBCO, Grand Island, NY), and 100 μg/ml streptomycin (GIBCO, Grand Island, NY). CT4R cells were the kind gift of William Paul and were grown as described in Gros *et al.* (Le Gros, et al., 1990). A plaque-purified isolate of the MOS strain of ECTV (ATCC VR-1374) designated MOS-3-P2, was propagated in an African green monkey kidney cell line, BSC-1 (Chen, et al., 1992). Virus was purified through a sucrose cushion as described elsewhere (Moss, 1998). Virus infectivity was estimated as described previously (Wallace & Buller, 1985). Briefly, virus suspensions were serially diluted in PBS +1% Fetal Calf Serum (Fetal Clone II, HyClone), absorbed to monolayers for 1 hr at 37° C, and overlaid with a suspension of 1% carboxyl methyl cellulose in DMEM +5 % Fetal clone II. After 4 days at 37°C, virus plaques were visualized and virus inactivated by the addition to each well of 0.5 ml of a 0.3% crystal violet/10% formalin solution.

### Construction of ectromelia recombinants expressing murine IL-4

The IL-4 gene was PCR-amplified from the plasmid p2A-E3 (ATCC, 37561) using Deep Vent DNA polymerase (New England Biolabs, Inc., Beverly, MA), and a pair of primers: 5'-GCGCTCGAGGGATCCGAATTCACCATGGGTCTCAACCCCCAGCTAG-3' (5'-primer) and 5'-GCGCCCGGGAAGCTTCTACGAGTAATCCATTTGC-3' (3'-primer), which created *Xho* I, *BamH* I and *EcoR* I (underlined) at the 5' terminus, and *Sma* I and *Hind* III (underlined) at the 3' terminus of the gene. The IL-$4^{D116/D119}$ mutant of was similarly amplified using the same 5'-primer and new 3' primer (5'-GCGCCCGGGAAGCTTCTACGAGTCATCCATATCCATGATGCTCTTTAGG-3'), resulting in substitution of both glutamine 116 and tyrosine 119 by aspartic acid. The PCR-amplified IL-4 was cloned into pKT1012 (*gpt*⁻) regulated by Pkt10, a synthetic strong early promoter (SSE) (5'-AAAAATTGAAAAACTAGCGTCTTTTTTTGCTCGA of VACV to yield pKT1012 (gpt⁻)-IL-4 (Tsung, Yim et al., 1996). pKT1012 (*gpt*⁻) was derived from pKT1012 (kindly provided by Dr. Tsung) by digesting it with *Nde* I and *EcoR* V to remove the *Escherichia coli* guanine phosphoribosyltransferase (*E. coli* gpt) gene followed by Klenow-blunting and self-ligation. The PCR-amplified IL-4 was digested with *BamH* I and *Sma* I, and cloned into pKT0334 under the control of VACV 11K late promoter (11KL) (5'-TAGTAGAATTTCATTTTGTTTTTTTCTATGCTATAAATA. Bold indicates G to A change (Meko, Yim et al., 1995). The SSE- and 11KL-IL-4 expression cassettes were obtained from their respective plasmids and cloned into the *Bgl* II and *Hind* III cut ECTV transfer vector pMBEV1.1 (Figure 1A). To optimize the expression of IL-4 in pMBEV-11KL-IL-4, a "C" in the sequence TTTTCTT was changed to a "T", and a "G" to an "A" in TAAATG by a two-step PCR protocol. pMBEV.1 contains unique *Bgl* II and *Hind* III sites flanked by the sequences from the ECTV CHO host range (hr) homologue with the *E. coli* gpt gene driven by the VACV P7.5 early promoter as a transient dominant selectable marker outside of the flanking sequences. The VACV P7.5 early promoter (5'-

AAAAGTAGAAAATATATTCTAATTTATTGCACGG. was synthesized by annealing the following oligonucleotides: 5'-GATCTAGAAAAGTAGAAAATATATTCTAATTTATTGCACG GATCCGAATTCA-3' (sense strand) and 5'-AGCTTGAATTCGGATCCGTGCAATAAATT AGAATATATTTTCTACTTTTCTA-3' (anti-sense strand), and ligated into pMBEV.1 which was cut by *Bgl* II and *Hind* III. The PCR-amplified IL-$4^{D116/D119}$ gene digested by *EcoR* I and *Hind* III were cloned into the same-cut pMBEV-7.5E downstream from the promoter 7.5E to yield pMBEV-7.5E-IL-4 and pMBEV-7.5E-IL-$4^{D116/D119}$, respectively. The 11KL-IL-$4^{D116/D119}$ construct was generated by replacing the IL-4 gene of pMBEV-11KL-IL-4 with the IL-$4^{D116/D119}$ gene from pMBEV-7.5E-IL-$4^{D116/D119}$. The VACV P7.5 early/late promoter (7.5EL) (5'-GGTAGTTGCGATATACATAAACTGATCACTAATTCCAAACCCACCCGCTTTTTAT AGTAAGTT TTTCACCCATAAATAATAAATACAATAATTAATTTCTCGTAAAAGTAGAAAATA TATTCTAA TTTATTGCACGG. was PCR-amplified from VACV WR using following pair of primers: 5'-GCGAGATCTGGTAGTTGCGATATACATAAACTG-3' and 5'-GCGGAATTCGGATCCGTGCAATAAATTAGAATATATTTTCTAC-3' which created *Bgl* II (underlined) at the 5' terminus, and *EcoR* I and *BamH* I (underlined) at the 3' terminus. This p7.5EL was cloned into pMBEV-7.5E-IL-4 and pMBEV-7.5E-IL-$4^{D116/D119}$ replacing the p7.5E. The DNA sequence of all final constructs was verified by sequencing. The methods for generation and screening of recombinant ECTV have been described previously for VACV (Falkner & Moss, 1990).

### Quantitation of IL-4 expression by recombinant ectromelia viruses

IL-4 in culture supernatant was detected by Western blot using a 15% SDS-PAGE, PVDF membrane, and a biotinylated anti-mouse IL-4 antibody (Cat# BAF404, R&D Systems, Inc., Minneapolis, MN). The signal was developed with an ECL plus Western Blotting Detection System (Amersham Pharmacia Biotech Inc., Piscataway, NJ). The ELISA utilized a rat anti-IL-4 monoclonal and biotinylated goat anti-IL-4 polyclonal as a capture and detection antibodies, respectively (Cat# MAB404 (clone 30340) and BAF404, R & D Systems, Inc.). The IL-4 bioassay utilized a CT.4R cells which proliferate in response to IL-4. Proliferation was measured by $^{3}$H-thymidine incorporation. For each assay, IL-4 was quantitated using a standard curve generated from purified baculovirus-expressed recombinant mouse IL-4 (Pharmingen, San Diego, CA).

### Animals

Four to six week old female (C57BL/6, A/NCr, Cr:NGP, BALB/c, C57BL/6-*Il4*$^{tm1Nnt}$ and BALB/c-*Il4ra*$^{tm1Sz}$ mice were obtained from Charles River Laboratories, Inc., Wilmington, MA or from Jackson Laboratory, Bar Harbor, MA., housed in filter-top microisolator cages and fed commercial mouse chow and water, ad libitum. The mice were housed in an animal biosafety level 3 containment areas. Indicated viruses were introduced into the mice by needle inoculation into a single FP. All mice were housed under pathogen-free condition in the AAALAC accredited Comparative Medicine Department of Saint Louis University School of Medicine. All experimentation was performed in a BSL-3 facility within the vivarium in order to protect the mouse stocks from inadvertent infection with ECTV. All experiments were carried out in accordance with regulations set forth by Saint Louis University, complying with all laws and regulations of USDA and PHS (OLAW/NIH).

### Viral infections, tissue assays and histopathology

Mice were anesthetized with a $CO_2$ 80%/$O_2$ 20% gas mixture, and inoculated in the right rear footpad with 10–25 μl of ECTV-WT or recombinant viruses in the form of crude cellular, sucrose cushion purified or sucrose band purified preparations as described before.

Mice were observed daily for disease signs. In certain experiments mice were weighed daily or every other day to record weight change.

Mice were bled via the orbital sinus with micro-hematocrit capillary tubes to measure serum levels of IL-4 and/or sacrificed at various times after infection in a $CO_2$ 80%/$O_2$ 20% gas mixture to examine tissue for pathology and to measure virus infectivity levels in spleen, and liver tissues. Tissue was ground in PBS (10% w/v), frozen and thawed three times, and sonicated for 20 seconds followed by titration on BSC-1 monolayers. Tissues were fixed in phosphate-buffered formalin, embedded in paraffin, sectioned into 4-μm sections, and stained with hematoxylin and eosin before being examined by light microscopy. The following tissues were examined: lung (inflated), heart, esophagus, thymus, salivary glands, cervical LNs, kidney, liver, spleen, ileum, sternal bone marrow, popliteal LN, axillary LN and pancreas.

Remaining mice were observed for clinical signs of disease (morbidity) and mortality. Mice found in a moribund condition (hunched posture and labored breathing) were sacrificed. On termination of certain experiment, mice were sacrificed, and spleen and liver tissues were harvested and tested for residual virus infectivity.

### Treatments

The Dryvax™ vaccine was obtained from CDC and reconstituted as recommended by the manufacturer. Mice were vaccinated at the base of the tail or in the FP. CDV (CDV; [S]-1-[3-hydroxy-2-phosphonylmethoxypropyl]cytosine, HPMPC) was provided as a gift by Gilead Sciences, Inc. (Foster City, CA) under the trademark name Vistide®. It was injected by the intraperitoneal route. A lipid conjugate of CDV, CMX001® (Chimerix Inc. Durham, NC), and/or ST-246® (SIGA Technologies Inc, New York, NY) were administered by oral gavage in a volume of 100 μl. On the first day ST-246® (100 mg/kg) or ST-246® vehicle was administered at ~ 4 PM, 6 hrs after infection. On days 1–14, CMX001® (4 mg/kg) or CMX001® vehicle was administered at ~8:00 AM and ST-246® (100 mg/kg) or ST-246® vehicle was administered at ~4:00 PM. A rat anti-IL-4 mAB (clone 11B11, IgG1) or a rat isotype control mAB (anti-muLVgag30, clone R187, IgG1) were injected by the intraperitoneal route. The antibodies were produced in Integra CL 350 growth chambers (INTEGRA Biosciences, Inc.) purified by ion-exchange and size exclusion chromatography, and determined to be ~ 80% pure by SDS-PAGE followed by silver staining.

### Statistics

An unpaired two-tailed t-test was used to compare the means of two groups of mice. P values below 0.05 were considered statistically significant. Mortality rates were compared using Fisher's exact test. $LD_{50}$ values were calculated as described (Reed & Muench, 1938).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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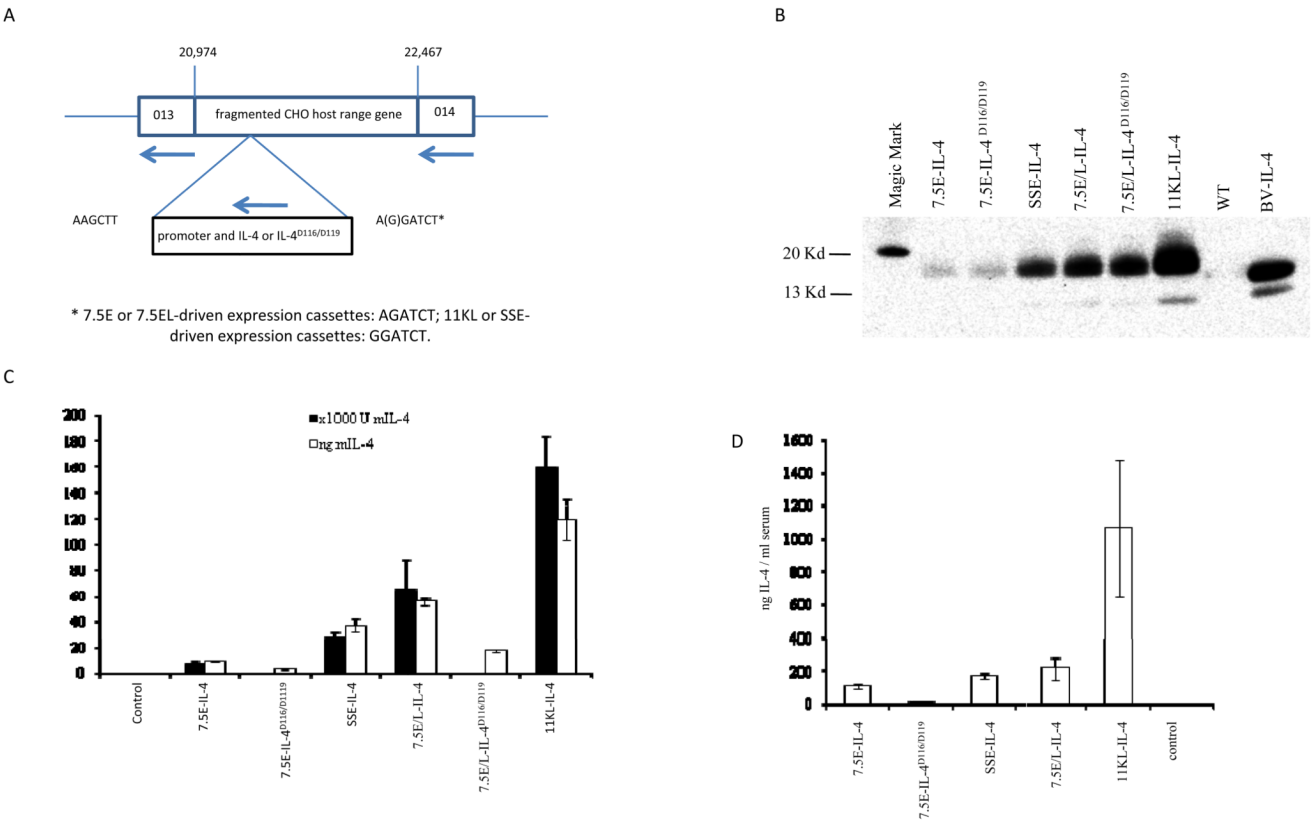


**Figure 1.**

IL-4 expression by ECTV recombinant viruses as a function of promoter strength. **(A)** A schematic of the pMBEV1.1 plasmid indicating the location of the fragmented CHO gene insertion site flanked by EV014 and EV015 genes. Numbers represent the position in bp of the respective sites. *Hind* III and *Bgl* I insertion sites are also indicated (Chen, Danila, Feng, Buller, Wang, Han, Lefkowitz, & Upton, 2003). (**B and C**) $3 \times 10^4$ CV-1 cells were infected with each ECTV recombinant at 10 PFU/cell. The supernatants were collected at 24 hr p.i. Comparable volumes of supernatants were quantified for IL-4 by Western blot (B) and ELISA and bioassay with CT.4R cells **(C)**. BV-IL-4 indicates baculovirus produced IL-4. **(D)** Groups of 6 C57BL/6 mice were infected by the FP route with 3,000 PFU of the indicated viruses. At 6 days p.i., the mice were bled from the orbital sinus and sera were assayed for IL-4 by ELISA.

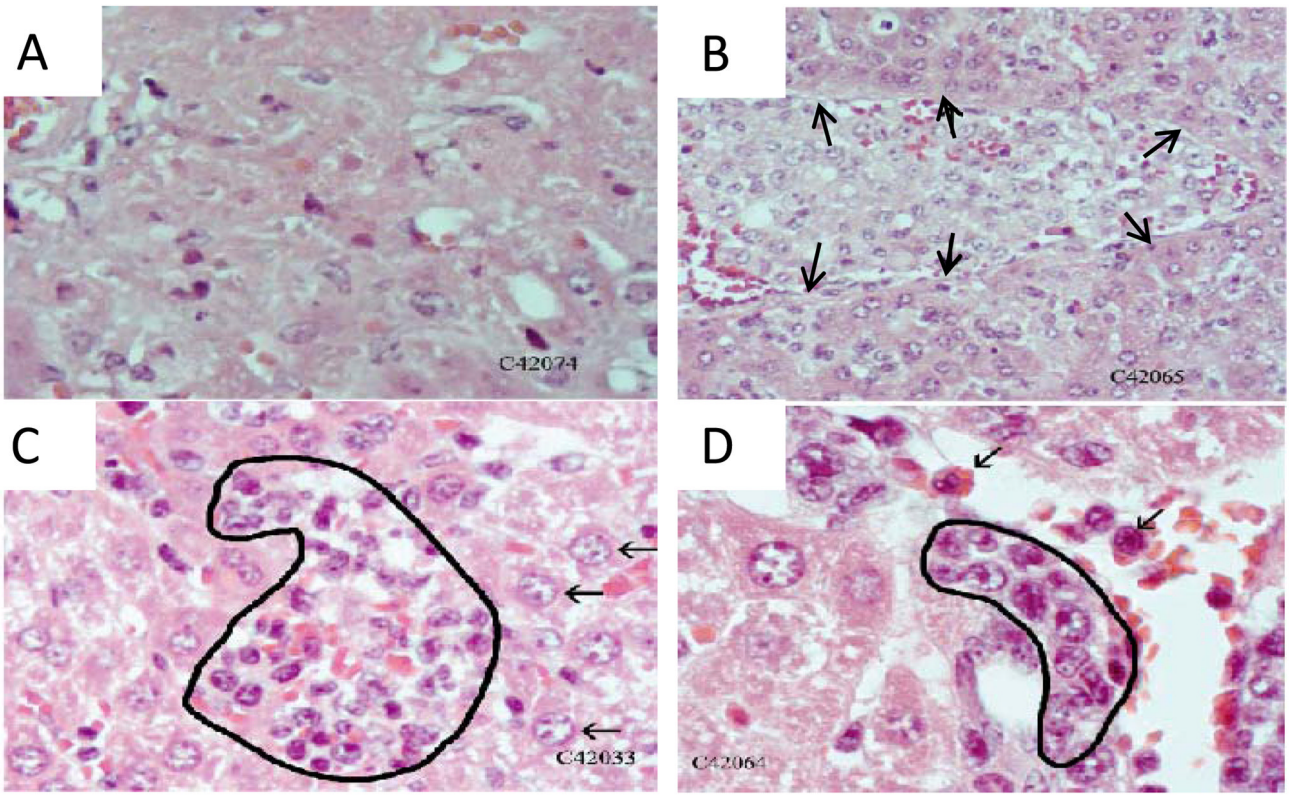


**Figure 2.**

Histopathology of liver from ECTV-7.5E/L-IL-4 infected C57BL/6 mice at 7 days p.i. ECTV-7.5E/L-IL-4 infection. **Panel A.** Confluent area of necrosis. Mag. 400×; **Panel B.** A hepatic vessel filled with macrophages. The arrows delineate the boundary of the vessel. Mag. 200×; ECTV-WT infection. **Panel C.** Discrete focus of necrosis (irregular line) containing inflammatory/immune cells and surrounded by intact hepatocytes (arrows), Mag. 400×; **Panel D.** The irregular line encompasses a focus of lymphocytes. Arrows show macrophages containing phagocytized erythrocytes. Mag 600×.

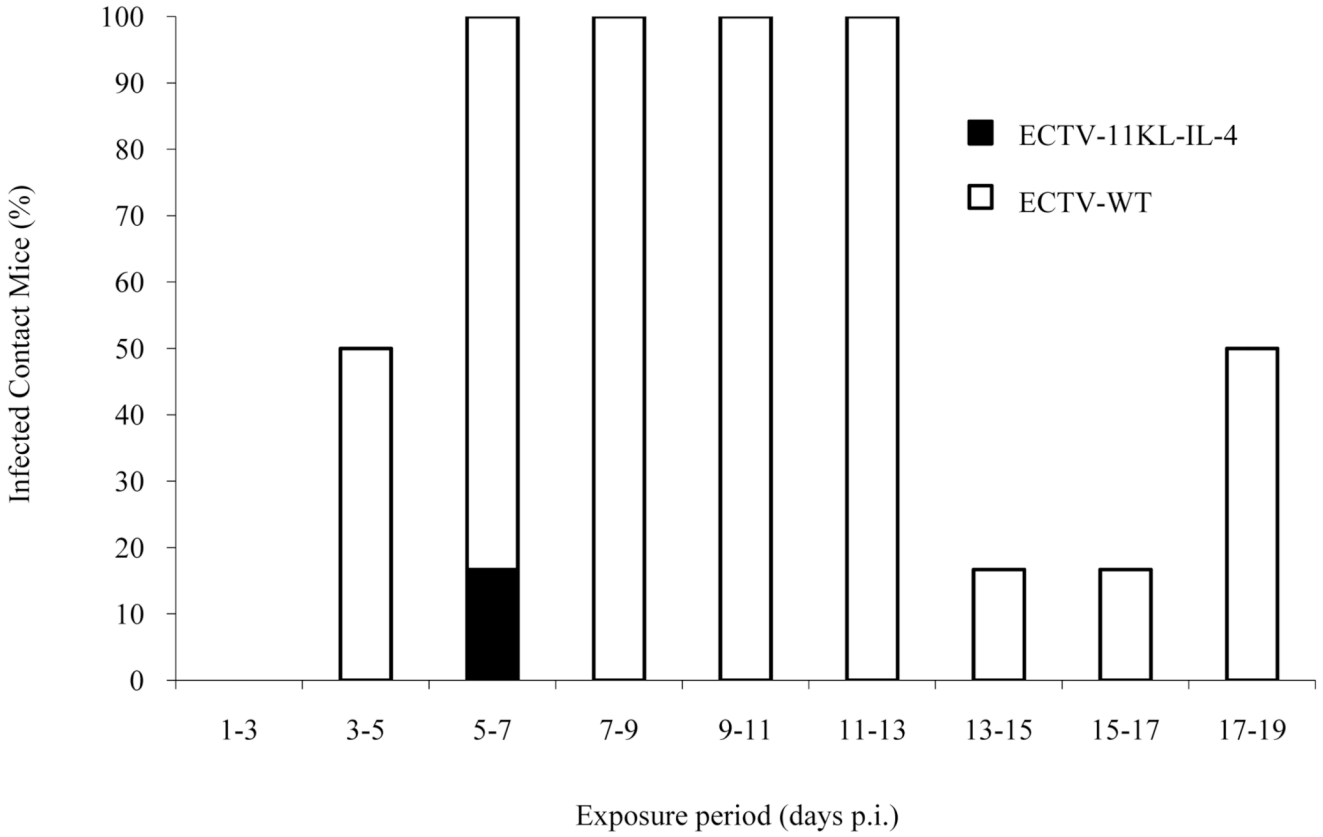


**Figure 3.**

Transmissibility of ECTV-11KL-IL-4 in C57BL/6 mice. Four C57BL/6 mice were infected with $5 \times 10^3$ PFU of ECTV-WT or ECTV-11KL-IL-4 in the left rear FP. The infected mice were transferred every 2 days into a new cage containing 6 naïve, disease susceptible A/NCR mice. Infection was monitored by death or seroconversion.

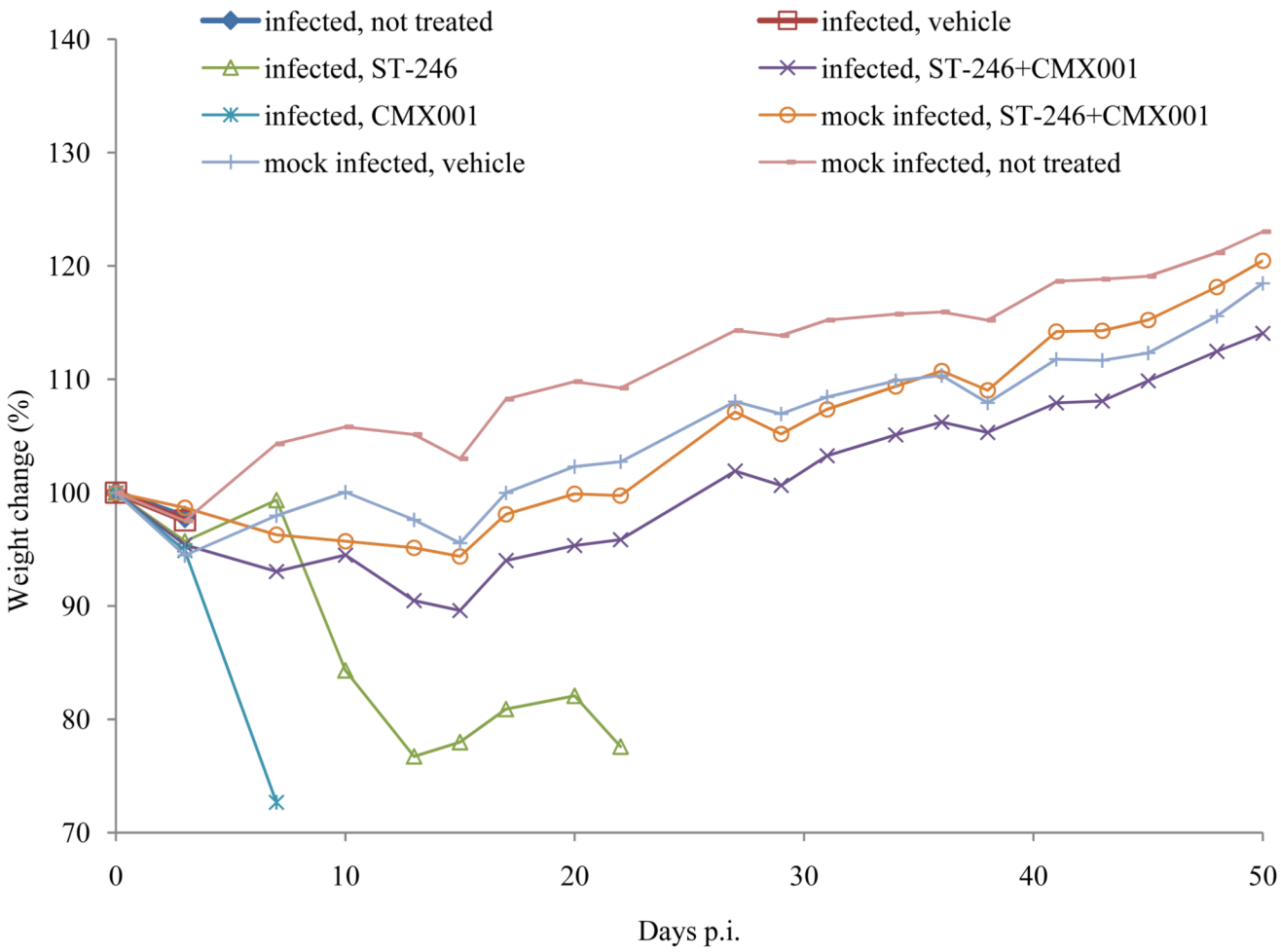


**Figure 4.**

Efficacy of ST-246® and CMX001® combination treatment against ECTV-11KL-IL-4 in A/NCR mice. Female A/NCR (n=5) mice were inoculated intranasally with 200 PFU of ECTV-11KL-IL-4. Commencing at 6 hrs p.i., mice were treated by gavage daily for 14 days with placebo, ST-246® (100 mg/kg) and/or CMX001® (4 mg/kg). Mice were weighed individually 3 times a week. For clarity, error bars have not been shown; however, they do indicate similar kinetics of weight change.

## Table 1

ECTV recombinants expressing IL-4 from promoters of different strength and temporal regulation are uniformly lethal for disease resistant C57BL/6 mice.

| Virus | Virus infectivity[1] | | Mean time to death[2] | $LD_{50}$[3] |
|---|---|---|---|---|
| | Spleen | Liver | | |
| -WT | $4.3\times10^{3}\times\div9.9$ | $1.3\times10^{3}\times\div8.5$ | NA[4] | $>1\times10^{5}$ |
| -7.5E-IL-4 | $9.4\times10^{5}\times\div1.3$ | $5.5\times10^{5}\times\div3.3$ | 7.3 ± 0.5 | $\leq7\times10^{-2}$ |
| -7.5E-IL-$4^{D116/D119}$ | $3.0\times10^{3}\times\div36$ | $5.5\times10^{3}\times\div67$ | NA | $\geq1\times10^{5}$ |
| -SSE-IL-4 | $3.4\times10^{6}\times\div6.3$ | $3.8\times10^{6}\times\div0.2$ | 7 ± 0 | $<2.5\times10^{-1}$ |
| -7.5E/L-IL-4 | ND[5] | ND | 7 ± 1.2 | $2.8\times10^{-2}$ |
| -7.5E/L-IL-$^{D116/D119}$ | ND | ND | NA | $>3.0\times10^{5}$ |
| -11KL-IL-4 | ND | ND | 7 ± 0 | $2.5\times10^{-2}$ |

[1] Groups of 4 C57BL/6 mice were infected with the indicated virus in the left FP with 12 PFU. At 7 days p.i. mice were sacrificed, spleen and liver tissues were isolated, and virus titers were measured.

[2] Mean time to death was calculated at a dose of approximately $4\times10^{3}$ PFU/mouse.

[3] Groups of mice were infected with increasing doses of the indicated virus and $LD_{50}$ values were calculated by the Reed and Muench method.

[4] NA – not applicable.

[5] ND – not done.

## Table 2

ECTV recombinants expressing IL-4 in resistant C57BL/6 mice replicate to higher titers in spleens and livers than in ECTV-WT infections.

| Virus[1] | Days p.i. | Virus infectivity (PFU/ml) | |
|---|---|---|---|
| | | Spleen | Liver |
| -WT | 3 | $1.1\times10^3 \times\div 2.8$ | $1.0\times10^3 \times\div 0.4$ |
| | 4 | $2.3\times10^3 \times\div 1.9$ | $3.5\times10^3 \times\div 0.3$ |
| | 5 | $2.7\times10^3 \times\div 2.0$ | $4.3\times10^3 \times\div 0.06$ |
| | 6 | $3.5\times10^2 \times\div 8.3$ | $3.8\times10^2 \times\div 11.3$ |
| | 7 | $7.6\times10^2 \times\div 0.2$ | $1.3\times10^3 \times\div 0.9$ |
| | | | |
| -7.5E/L-IL-4 | 3 | $7.1\times10^2 \times\div 2.2$ | $2.2\times10^3 \times\div 0.9$ |
| | 4 | $4.2\times10^3 \times\div 0.2$ | $4.2\times10^4 \times\div 0.08$ |
| | 5 | $1.4\times10^5 \times\div 1.9$ | $3.7\times10^5 \times\div 0.2$ |
| | 6 | $4.1\times10^6 \times\div 0.07$ | $4.5\times10^6 \times\div 0.03$ |
| | 7 | $4.2\times10^6 \times\div 0.03$ | $4.4\times10^6 \times\div 0.002$ |

[1]Groups of 4 C57BL/6 mice were infected in the left FP route with $1\times10^3$ PFU of indicated viruses. At specific time points 4 mice from each infection were bled and sacrificed. Tissues were collected for measurement of virus infectivity and histopathology.

## Table 3

Tissues from ECTV-7.5E/L-IL-4 infected C57BL/6 mice have extensive tissue necrosis without lymphopoiesis.

| Description[1] | -WT | -7.5E/L-IL-4 |
|---|---|---|
| Focal necrosis in liver | ++[2] | ++++[3] |
| Monocytes: | | |
| -hepatic vessel | ± | +++ |
| -bone marrow | ± | +++ |
| Splenic necrosis: | | |
| -lymphopoiesis | +++ | + |
| -hemorrhage | − | ++ |
| -red pulp histocytosis | ++ | ++++ |
| Thymic necrosis | ± | ++ |
| Popliteal lymph node necrosis | ++++ | ++++ |
| Cervical lymph node lymphopoiesis | +++ | − |

[1] Four mice were infected with ECTV-WT or ECTV-7.5E/L-IL-4 and were sacrificed at 7 days p.i. as described in Table 2. Tissue sections were prepared as described in the Methods and one wax section from each mouse was examined by light microscopy. Only reproducible histological changes are reported (see Methods for complete list of tissues examined).

[2] Infiltrated with lymphocytes.

[3] Not infiltrated with lymphocytes.

**Table 4**

ECTV-IL-4 recombinants expressing the highest levels of IL-4 cause lethal infections of Swiss and C57BL/6 mice immunized with the Dryvax™ smallpox vaccine.

| Challenge virus | Strain | Dose of Dryvax™[1] | | | |
|---|---|---|---|---|---|
| | | 2×10$^3$ PFU/mouse | | 2×10$^4$ PFU/mouse | |
| | | Mean time to death | Mortality at 21 days p.i. (%) | Mean time to death | Mortality at 21 days p.i. (%) |
| -WT | Swiss | NA[2] | 0/8 (0) | NA | 0/8 (0) |
| -7.5E-IL-4 | Swiss | 6 | 5/8 (63)[3] | 7 | 1/8 (13)[4] |
| -7.5E/L-IL-4 | Swiss | 6.6 ± 2.0 | 8/8 (100) [3] | 7.0 ± 0.9 | 8/8 (100)[3] |
| -11KL-IL-4 | Swiss | 6.0 ± 0.08 | 8/8 (100) [3] | 6.4 ± 0.8 | 7/7 (100)[3] |
| | | | | | |
| -WT | C57BL/6 | NA | 0/8 (0) | NA | 0/8 (0) |
| -7.5E-IL-4 | C57BL/6 | NA | 0/8 (0)[4] | NA | 0/8 (0)[4] |
| -7.5E/L-IL-4 | C57BL/6 | 7.4 ± 0.7 | 8/8 (100) [3] | 7.1 ± 0.8 | 8/8 (100)[3] |
| -11KL-IL-4 | C57BL/6 | 7.4 ± 0.5 | 8/8 (100) [3] | 6.9 ± 0.4 | 8/8 (100)[3] |

[1] Swiss and C57BL/6 mice were infected with the indicated dose of Dryvax™ vaccine in the right-rear FP. Seven weeks later mice were challenged in the left-rear FP with the indicated viruses at 2×10$^4$ PFU/mouse.

[2] NA – not applicable.

[3] P<0.05 compared to ECTV-WT control.

[4] P>0.05 compared to ECTV-WT control.

## Table 5

CDV treatment of susceptible A/NCR mice infected with ECTV –IL-4 recombinants.

| Challenge virus | CDV[1] | Mean time to death | Mortality at day 21 p.i. (%[3]) |
|---|---|---|---|
| -WT | + | NA[2] | 0/8 (0) |
| | − | 7.3 ± 0.5 | 4/4 (100) |
| -7.5E-IL-4 | + | 13 | 1/8 (13)[5] |
| | − | 6.3 ± 0.5 | 4/4 (100)[3] |
| -7.5E/L-IL-4 | + | 10. 6± 1.3 | 8/8 (100)[4] |
| | − | 6.0 ± 0 | 4/4 (100)[3] |
| -11KL-IL-4 | + | 9.5 ± 1.4 | 8/8 (100)[4] |
| | − | 6.0 ±0 | 4/4 (100)[3] |

[1] Mice were IP injected on T=0, 1, 2, and 3 days p.i. with 100 µl of CDV (12.5 mg/kg). Mice were challenged with the indicated virus at $3\times10^3$ PFU/mouse at T=0.

[2] NA - not applicable.

[3] P=1.0 compared to mice challenged with ECTV-WT and not receiving CDV.

[4] P<0.05 compared to mice challenged with ECTV-WT and receiving CDV.

[5] P=1.0 compared to mice challenged with ECTV-WT and receiving CDV.

**Table 6**

Combination therapy with ST-246 and CMX001 protects mice against infection with ECTV-11KM-IL-4

| Virus[1] | ST-246[2] | CMX001[3] | ST-246® Veh[4] | CMX001® Veh[5] | Mean time to death | Mortality (%) |
|---|---|---|---|---|---|---|
| -11KL-IL-4 | – | – | – | – | 6±0 | 8/8 (100) |
| | – | – | + | + | 6±0 | 8/8 (100)[6] |
| | + | – | – | + | 14.1±1.6[7] | 8/8 (100)[6] |
| | + | + | – | – | 100.5±16.5[7] | 2/8 (25)[7] |
| | – | + | + | – | 7.9±0.1[7] | 8/8 (100)[6] |
| Mock | + | + | – | – | NA[8] | 0/8 (0)[7] |
| | – | – | + | + | NA | 0/8 (0)[7] |
| | – | – | – | – | NA | 0/8 (0)[7] |

[1] Mice were infected IN with 200 PFU of ECTV-11KL-IL-4 or were mock infected.

[2] Mice were dosed with 100 mg/kg of ST-246 for 14 days.

[3] Mice were dosed with 4 mg/kg of CMX001 for 14 days.

[4] ST-246® vehicle controls received 0.75% methylcellulose and 1% tween.

[5] CMX001® vehicle controls received saline.

[6] P=1.0 compared to infected non-treated controls.

[7] P<0.05 compared to infected non-treated controls.

[8] Not applicable.