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Expression of rabbit IL-4 by recombinant myxoma viruses enhances virulence and overcomes genetic resistance to myxomatosis

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Abstract

Rabbit IL-4 was expressed in the virulent standard laboratory strain (SLS) and the attenuated Uriarra (Ur) strain of myxoma virus with the aim of creating a Th2 cytokine environment and inhibiting the development of an antiviral cell-mediated response to myxomatosis in infected rabbits. This allowed testing of a model for genetic resistance to myxomatosis in wild rabbits that have undergone 50 years of natural selection for resistance to myxomatosis. Expression of IL-4 significantly enhanced virulence of both virulent and attenuated virus strains in susceptible (laboratory) and resistant (wild) rabbits. SLS-IL-4 completely overcame genetic resistance in wild rabbits. The pathogenesis of SLS-IL-4 was compared in susceptible and resistant rabbits. The results support a model for resistance to myxomatosis of an enhanced innate immune response controlling virus replication and allowing an effective antiviral cell-mediated immune response to develop in resistant rabbits. Expression of IL-4 did not overcome immunity to myxomatosis induced by immunization.
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Keywords: Myxoma virus; Genetic resistance; IL-4; Rabbits; Coevolution

Introduction

One of the best-studied examples of the coevolution of a species with a novel pathogen is the introduction of myxoma virus into the Australian wild population of European rabbits (*Oryctolagus cuniculus*) and the subsequent 50 years of evolution of both pathogen and host. Myxoma virus is a poxvirus (genus: *Leporipoxvirus*). The native host of myxoma virus is the South American tapeti (forest rabbit; *Sylvilagus brasiliensis*) or the North American brush rabbit (*Sylvilagus bachmani*). In its native hosts, the virus causes a cutaneous fibroma at the inoculation site and minimal signs of clinical disease. However, in European rabbits, myxoma virus causes the highly lethal disease myxomatosis.

Because of the high lethality of myxoma virus, the standard laboratory strain (SLS) was experimentally introduced into the Australian wild rabbit population in 1950. This release triggered a series of lethal epidemics of myxomatosis, which are estimated to have reduced the Australian rabbit population by as much as 95% (Williams et al., 1995). Since this introduction, there has been ongoing coevolution of virus and host. This rapidly led to the virus population being dominated by strains of reduced virulence that were more efficiently transmitted by the mosquito vectors, and to strong selection for genetic resistance to myxoma virus in the rabbit population (reviewed in Fenner and Fantini, 1999; Kerr and Best, 1998).

This coevolution of host and pathogen has provided a unique system to understand how introduction of a novel pathogen into a naive mammalian host, in an outbred natural system, on a continent-wide scale can drive the evolution of both the host and the pathogen. This system is unique in that the originally released strain of virus is

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available, laboratory rabbits can be used as naturally susceptible hosts because these have never been selected for resistance to myxoma virus, and wild rabbits can be bred in captivity to study resistance. In addition, the highly attenuated field strain of myxoma virus Uriarra-2-53/1 (Ur) (Mykytowycz, 1953; Russell and Robbins, 1989) isolated in Australia in 1953, only 2 years after the release of SLS, and derived from SLS, has been characterized as a model attenuated virus.

The pathogenesis of the virulent SLS and attenuated Ur strain of myxoma virus has been characterized in laboratory (genetically susceptible) and wild (genetically resistant) rabbits (Best and Kerr, 2000; Best et al., 2000). SLS kills 100% of laboratory rabbits with an average survival time of 10–12 days (Fenner et al., 1957; Robinson et al., 1999). However, in wild rabbits, SLS killed only a small proportion of infected rabbits although still causing severe clinical myxomatosis. Similarly, Ur infection of laboratory rabbits was characterized by a wide range of clinical signs from severe to moderate and the occasional death, whereas in wild rabbits it caused few or no clinical signs of myxomatosis apart from the primary lesion at the inoculation site (Best and Kerr, 2000).

In both resistant and susceptible rabbits, myxoma virus replicated initially in MHC-II-positive cells in the upper dermis and then spread to the epidermal cells and to the draining lymph node. From this site, virus was disseminated to other lymphoid tissues, lungs, testis, and secondary skin sites. Lymphocytes were probably important for dissemination of the virus. The key features of resistance were the ability of the wild rabbits to delay spread of the virus to distal tissues and to limit replication of the virus at these sites, particularly the lymph node draining the inoculation site. Replication in the skin was not initially different in resistant or susceptible rabbits for SLS or Ur; however, the subsequent inflammatory response at the site of inoculation was quite different (Best and Kerr, 2000; Best et al., 2000).

A model for resistance was proposed postulating that the initial innate immune response to the virus in resistant rabbits controls virus replication distal to the skin inoculation site sufficiently for the rabbit to develop an effective cell-mediated immune response. The model postulates that resistant wild rabbits mount an effective innate antiviral immune response dominated by natural killer (NK) cells, interferons α/β, and the Th1 cytokines such as interferon-γ (IFN-γ), TNF, and IL-12. In contrast, susceptible rabbits are unable to control viral replication sufficiently to allow an effective early cell-mediated response and that this would be seen as an initial innate immune response dominated by Th2 cytokines such as IL-4 and IL-10 (Best and Kerr, 2000; Best et al., 2000). This model was based to a large extent on studies of ectromelia virus in resistant and susceptible mice where resistant animals tend to produce Th1 cytokines such as IL-2 and IFN-γ while susceptible mice produced Th2 cytokines such as IL-4 and IL-10 (Karupiah, 1998). It has subsequently been demonstrated that expression of IL-4 by a recombinant ectromelia virus enables the virus to overcome genetic resistance by suppressing both NK and cytotoxic-T-lymphocyte (CTL) responses to ectromelia virus (Jackson et al., 2001).

To test this model for genetic resistance to myxoma virus, we constructed recombinant myxoma viruses expressing rabbit IL-4 either in a virulent SLS genetic background or an attenuated Ur genetic background. These viruses were each tested in laboratory and wild rabbits, and the pathogenesis of SLS-IL-4 characterized. We demonstrated that expression of rabbit IL-4 by myxoma virus enhanced the virulence of the virus and overcame genetic resistance in rabbits, and suggest a mechanism by which this is occurring.

Results

Virus construction and characterization

The plasmids used for construction of recombinant viruses are shown in Fig. 1. The recombinant viruses SLS-slp-IL-4, SLS-p28m-IL-4, and Ur-ZPB-p28m-IL-4 were isolated by repeated plaque purification until only recombinant virus was present when tested by PCR. These plaques were then amplified for virus stocks in RK13 cells. Fig. 2 shows the results of PCR analysis for SLS-p28m-IL-4. Sequence specific for the recombinant virus is amplified in lane 2 using primers MJ2a-R2 and IL-4-start-Bgl that will generate a 900-bp product from the recombinant virus and primers MJ2a-R2 and TKF2 that will generate a 700-bp product from the wild-type virus. Lane 2 shows that the recombinant virus sequence is amplified while lane 3 indicates that no wild-type virus is present. Lane 4 is a positive control for wild-type virus.

The expression of IL-4 by the recombinant viruses was confirmed by immunoblotting using antipeptide antisera to IL-4. Fig. 3 shows that three molecular weight species of IL-4 were produced and that the IL-4 is produced in much larger amounts under the control of the synthetic late promoter in SLS compared to the p28m promoter in Uriarra. However, no difference could be demonstrated between the amounts of IL-4 produced by the synthetic late promoter and the p28m promoter in the SLS background using further dilutions of protein or antibody (data not shown).

The growth kinetics in cultured RK13 cells were examined for each of SLS, SLS-slp-IL-4, SLS-p28m-IL-4, and Ur-ZPB-p28m-IL-4 to determine if the properties of the recombinant viruses had been significantly altered in culture compared to SLS. Fig. 4 shows that there was essentially no difference in the growth kinetics of the three recombinant viruses and SLS at either a multiplicity of infection (moi) of 3 or a moi of 0.1. The infected cell monolayers showed virus-induced cytopathic effect (cpe) but there was less cpe

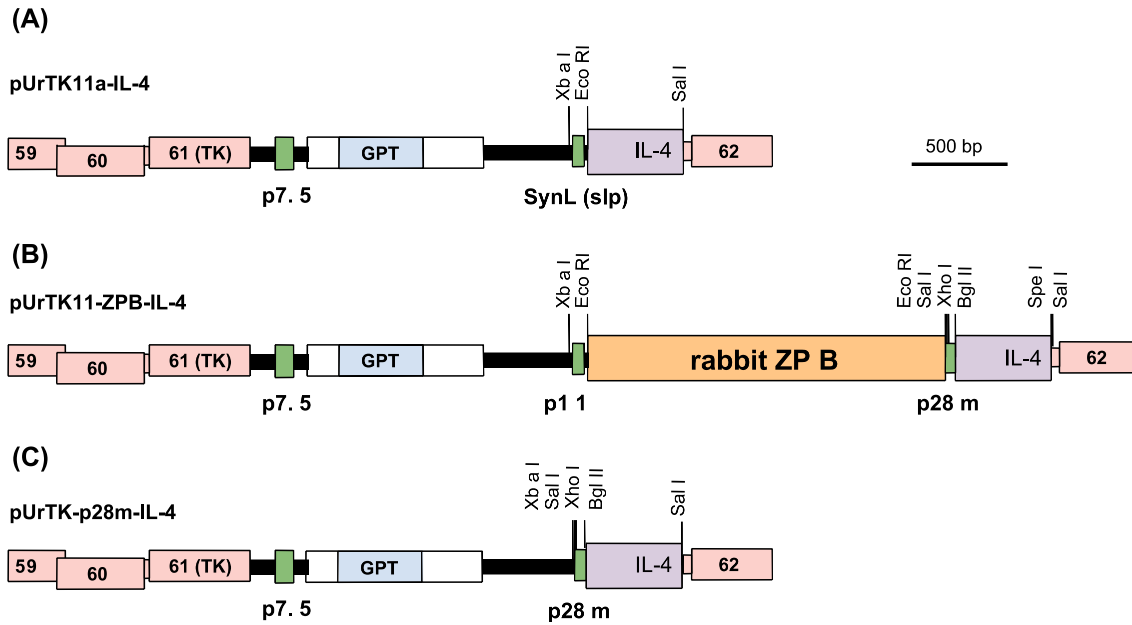

Fig. 1. Plasmid constructs used as transformation vectors. The IL-4 cDNA is under control of the strong synthetic late promoter (slp) in pUrTK11a-IL-4 (A), which was used to construct SLS-slp-IL-4 recombinant virus. The IL-4 gene is under control of the modified p28 (p28m) promoter in pUrTK11-ZPB-IL-4 (B), which was used to construct Ur-ZPB-p28m-IL-4 recombinant virus. The IL-4 gene is also under control of the p28m promoter in pUrTK-p28m-IL-4 (C), which was used to construct SLS-p28m-IL-4 recombinant virus. Flanking myxoma virus sequences from the M059R to M062R genes are identified by their abbreviations 59 to 62. TK = thymidine kinase. Unshaded areas in the GPT gene indicate noncoding sequence; black bars around the promoters indicate noncoding sequences from vaccinia virus contained within the background plasmid. In each case, insertion of the foreign DNA will be intergenic between the M061 and M062 genes. See text for further details.

at 24 h at a moi of 3 with Ur-ZPB-p28m-IL-4 infection compared to the other infections (data not shown).

Virulence assays in laboratory and wild rabbits

The virulence of each of the three IL-4-expressing viruses was characterized in both laboratory rabbits (highly susceptible to myxomatosis) and wild rabbits (highly resistant to myxomatosis). To confirm the resistant phenotype of the wild rabbit colony, a group of five wild rabbits was also infected with SLS. Virulence was measured as the proportion of rabbits that died and the average survival time (AST). These data are summarized in Table 1. As a comparison, the results of recent testing of the SLS stock in laboratory rabbits are shown (Robinson et al., 1999). Only one of five wild rabbits infected with SLS died and this was at 19 days (rabbit was euthanized). However, the expression of IL-4 in SLS under the control of the synthetic late promoter or the p28m late promoter was completely lethal for both susceptible and resistant rabbits. Ur-ZPB-p28m-IL-4 recombinant virus was completely lethal for laboratory rabbits but only 1/6 wild rabbits infected with this virus died.

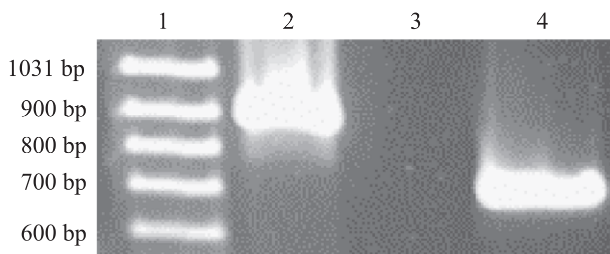

Fig. 2. PCR analysis of SLS-p28m-IL-4. Individual plaques were amplified in 96-well plates of RK13 cells. DNA from the resulting virus was analyzed for purity by PCR. Lane 1: 100-bp markers. Lane 2: SLS-p28m-IL-4 virus amplified to demonstrate recombinant virus amplification product of 900 bp. Lane 3: SLS-p28m-IL-4 virus amplified to demonstrate residual wild-type virus (note no wild-type virus was detectable). Lane 4: SLS amplified to demonstrate wild-type virus amplification product of 700 bp.

The ASTs for those viruses that were lethal in 100% of rabbits were compared by t test. SLS-slp-IL-4 and SLS-p28m-IL-4 viruses had significantly lower survival times compared to SLS and to Ur-ZPB-p28m-IL-4 ($P < 0.001$). SLS had a lower AST than Ur-ZPB-p28m-IL-4 in laboratory rabbits ($P < 0.002$). SLS-slp-IL-4 had a significantly lower AST in laboratory rabbits compared to wild rabbits ($P < 0.01$) but there was no significant difference in AST between wild and laboratory rabbits infected with SLS-p28m-IL-4.

Rectal temperatures were measured daily as an indication of the progress of the disease. Fig. 5 shows that wild rabbits infected with SLS had a sustained mean rectal temperature of ≥40 °C from day 4 to day 16 after which mean temperature dropped to the normal range. In contrast, both wild and laboratory rabbits infected with SLS-

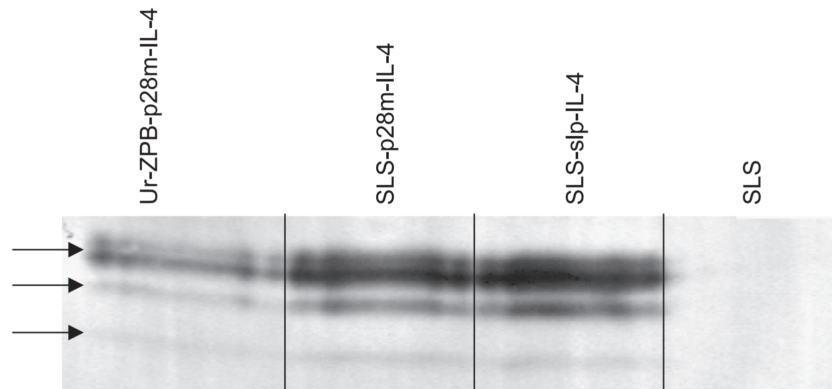

Fig. 3. Immunoblot analysis of concentrated supernatants from RK13 cells infected with Ur-ZPB-p28-IL-4, SLS-p28m-IL-4, SLS-slp-IL-4, and SLS, showing a complex of three bands (arrows) at approximately 15–17 kDa. Equivalent amounts of concentrated supernatant were loaded in each lane.

slp-IL-4 were unable to maintain rectal temperature above normal by day 7. Mean rectal temperatures for wild and laboratory rabbits infected with SLS-p28m-IL-4 were similar to those for SLS-slp-IL-4 (data not shown). The group of laboratory rabbits infected with Ur-ZPB-p28m-IL-4 had only 1 day when mean rectal temperature was ≥40 °C and mean temperatures fell below normal after day 10. Wild rabbits infected with Ur-ZPB-p28m-IL-4 maintained mean clinical temperatures within the normal range and although individual rabbits developed fevers the mean rectal temperature was never ≥40 °C.

All five wild rabbits infected with SLS developed myxomatosis and became quite ill, but from about day 12 after infection four of the five began to make a rapid recovery. The fifth rabbit, while controlling many of the signs of virus infection, continued to lose weight and was euthanized at day 19. Autopsy revealed pneumonia in the anterior lobe of one lung. The clinical signs in laboratory and wild rabbits infected with SLS-slp-IL-4 are summarized in Table 2. Most laboratory rabbits developed early signs of myxomatosis, but late in infection some also showed other clinical signs such as intense muscle trembling, particularly on handling, loss of placing reflexes in the front legs, respiratory difficulty, and coma. The classical signs of closed, pus-filled eyes and mucopurulent discharge from the nose were absent. There were fewer clinical signs in wild rabbits than in laboratory rabbits and these developed later. Those that survived long enough developed similar clinical signs to the laboratory rabbits infected with SLS-slp-IL-4 (Table 2). At autopsy, the most prominent features were fluid dripping from the lungs in some cases, and in some rabbits small 1–2 mm gastric ulcers and 2–5 mm pyloric ulcers, as well as the more typical findings of myxomatosis such as enlarged lymph nodes and the glistening mucoid swelling in the cut surface of the dermis and thickening of the epidermis at the inoculation site. The clinical and autopsy findings for SLS-p28m-IL-

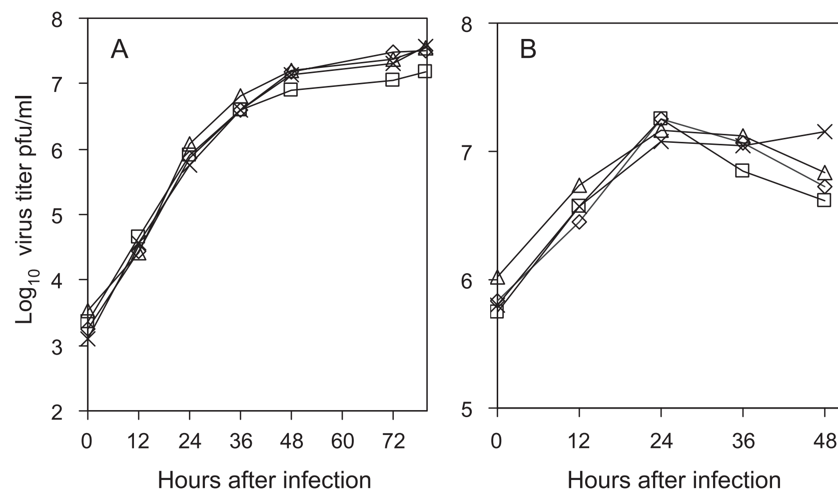

Fig. 4. Growth kinetics in RK13 cells of SLS, SLS-slp-IL-4, SLS-p28m-IL-4 and Ur-ZPB-p28m-IL-4. (A) moi = 0.1. (B) moi = 3. △, SLS-p28m-IL-4; ×, Ur-ZPB-p28m-IL-4; □, SLS-slp-IL-4; ◇, SLS.

Table 1
Virulence of recombinant viruses in laboratory (susceptible) and wild (resistant rabbits)

	Laboratory rabbits			Wild rabbits		
	Mortality	AST[a] (SD) (days)	Range (days)	Mortality	AST (SD) (days)	Range (days)
SLS	30/30[b]	11.1 (0.9)	9.6–12.6	1/5	19	n/a
SLS-slp-IL-4	6/6	7.1 (0.6)	6.5–8	10/10	8.4 (0.8)	7.5–9.75
SLS-p28m-IL-4	6/6	7.1 (0.5)	6–7.5	6/6	7.7 (1.2)	6–9.5
Ur-ZPB	0/12[c]	n/a	n/a	nt	nt	nt
Ur-ZPB-p28m-IL-4	9/9	12.6 (1.7)	10.5–15.5	1/6	16.5	n/a

nt—not tested; n/a—not applicable.
[a] Average survival time (AST). Standard deviation (SD) is shown in brackets.
[b] Data for laboratory rabbits are from a recent test of this virus stock (Robinson et al., 1999).
[c] Data from Kerr et al. (1999).

4 in laboratory and wild rabbits were similar to those for SLS-slp-IL-4 (data not shown).

Laboratory rabbits infected with Ur-ZPB-p28m-IL-4 developed clinical myxomatosis and all died. However, wild rabbits retained resistance to this virus. Infection of six wild rabbits caused moderate to severe myxomatosis with only one death, at day 16.5, and recovery of the remaining five rabbits. Table 3 compares the clinical signs of Ur-ZPB-p28m-IL-4 infection of laboratory and wild rabbits.

SLS and Ur have been previously compared in laboratory and wild rabbits and clinical and pathological descriptions are given in Best and Kerr (2000).

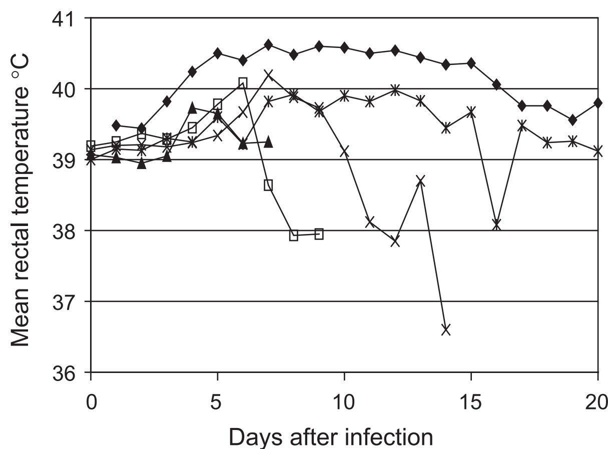

Fig. 5. Mean rectal temperatures of groups of wild rabbits infected with SLS and wild and laboratory rabbits infected with SLS-slp-IL-4 or Ur-ZPB-p28m-IL-4. ×, Ur-ZPB-p28-IL-4 laboratory rabbits; *, Ur-ZPB-p28m-IL-4 wild rabbits; □, SLS-slp-IL-4 wild rabbits; ▲, SLS-slp-IL-4 laboratory rabbits; ♦, SLS wild rabbits.

Table 2
Clinical signs in laboratory and wild rabbits infected with SLS-slp-IL-4

SLS-slp-IL-4	Laboratory rabbits	Wild rabbits
Day 2	5/6 rabbits had 0.5–1.5-cm-diameter primary lesion at inoculation site.	3/6 rabbits had a red spot at the inoculation site. At day 3, all rabbits had a raised, pale pink 1-cm-diameter swelling at the inoculation site.
Day 4	6/6 rabbits had a 1–2.5-cm-diameter lesion at inoculation site. 3/6 rabbits had rectal temperatures ≥40 °C.	Swellings at inoculation sites were 1.5–2 cm in diameter.
Day 5	2/6 rabbits had thickened ears; 2/6 rabbits had rectal temperatures ≥40 °C.	Similar to day 4.
Day 6	One rabbit had an extended head and neck with deep slow respiration, a wet chin and thick drooping ears. One rabbit had temperature ≥40 °C and one <38 °C. Several rabbits had small secondary lesions on the eyelids and slight inflammation of the anogenital region. Two rabbits died overnight and a third was moribund and euthanized.	Slight redness of eyelids noted in two rabbits; 5/6 had rectal temperatures ≥40 °C.
Day 7	All rabbits died between day 6 and 8. Clinical signs included slight eyelid swelling, muscular trembling of face and jaw, and precipitous drop in rectal temperature. One rabbit was semiconscious sitting on his chest with head on floor of cage. Handling induced severe muscular twitching and jerking.	Lesions at inoculation site 3–4 cm in diameter. Some redness of the eyelids. Two rabbits were uncoordinated and trembling; both had rectal temperatures below normal. Both were found dead the next morning.
Day 8 to day 10	All rabbits dead.	Two rabbits had subnormal temperatures. Others had rapid respiration, were uncoordinated, and had shivering and muscle twitching. One rabbit was euthanized and another died overnight. All rabbits dead by day 10.

Pathogenesis trials of SLS-slp-IL-4

Nine laboratory and 12 wild rabbits were injected with 1000 pfu of SLS-slp-IL-4 in the dorsal skin of the right hind foot. Three rabbits from each group were killed at 2, 4, 6, and for wild rabbits, 8 days after inoculation. The major autopsy findings were the typical mucoid thickening of the dermis and swelling of the epidermis at the inoculation site and the swelling of the draining and contralateral lymph

Table 3
Clinical signs in laboratory and wild rabbits infected with Ur-ZPB-p28m-IL-4

Ur-ZPB-p28m-IL-4	Laboratory rabbits	Wild rabbits
Day 2	Slight pinkness at inoculation site in 3/9 rabbits at 48 h; by 72 h, all rabbits had 0.5–1.0-cm-diameter primary lesions.	On day 3, 2/6 rabbits had a slight skin thickening and pinkness at the inoculation site.
Day 4	Primary lesions up to 3 cm in diameter.	All rabbits had some reaction at the inoculation site. One rabbit had a rectal temperature of 40 °C.
Day 6	Increased rectal temperature in 1/9 rabbits on day 6. By day 7, 5/9 rabbits had temperatures ranging from 40–41.4 °C. Two rabbits had slight swelling of the anogenital area and one rabbit had red eyelid margins. Primary lesions were up to 3.5 cm in diameter.	Primary lesions were 1.5–3 cm in diameter. 1/6 rabbits had slightly swollen eyelid margins. By day 7, 5/6 rabbits had mildly thickened eyelid margins and 3/6 had rectal temperatures ≥40 °C.
Day 8	All rabbits showing some signs of disseminated myxomatosis: red eyelid margins, small secondary lesions, thickened ears, anogenital swelling, and slight ocular discharge. By day 9, these signs were more severe.	All rabbits had clinical signs of mild myxomatosis such as swollen eyelids and anogenital swelling. Primary lesions were up to 4.5 cm in diameter. By day 9, primary lesions were up to 6 cm in diameter.
Day 10	Nasal obstruction was occurring; slight scrotal oedema was noted in the males. One rabbit died between day 10 and day 11 and clinical signs became more severe. Four rabbits had subnormal temperatures and died overnight.	Primary lesions were very swollen-up to 8–10 cm in diameter. Rabbits were starting to group into those with mild and those with moderate clinical signs of myxomatosis. By day 11, eyelids were very swollen but there was little or no ocular discharge.
Day 12	One rabbit with severe clinical myxomatosis and subnormal temperature (35.3 °C) was euthanized. Survivors ranged from alert to very depressed.	Clinical signs started to resolve; primary lesions were becoming circumscribed and starting to scab. Slight snuffling in one rabbit. At day 13, this slow resolution of clinical signs was more obvious but one rabbit still had severe myxomatosis.
Day 14	Two surviving rabbits were very quiet with shallow, slow respiration, subnormal temperatures (34 and 35.8 °C), quite severe myxomatosis but responsive to handling.	One rabbit still very ill although anogenital swelling was resolving, another snuffling and quiet. Primary lesions still very large but resolving.
Day 16	All rabbits dead or euthanized.	Sick rabbit had temperature of 37.1 °C and was euthanized. Primary lesions resolving but still 3.5–7 cm in diameter.
Day 20		Remaining sick rabbit clinically recovering. Other rabbits recovering. Primary lesions resolved to scabs.

nodes from 4 days after infection. The only finding that was not typical of myxomatosis was the presence of small 1–2 mm ulcers scattered in the gastric mucosa, pylorus, and occasionally in the mucosa of the upper duodenum. Similar ulceration had been noted in autopsies performed on rabbits that died during the virulence tests with recombinant viruses expressing IL-4.

To allow a direct indication of the range of results, titers of virus in each tissue for each individual rabbit at each time point are shown in Fig. 6. To avoid cluttering the graph, mean titers have not been shown but are easily interpolated by inspection. At day 2 after infection, virus titers in the skin at the inoculation site of laboratory rabbits infected with SLS-slp-IL-4 ranged from 6.1 to 7.0 $\log_{10}$ pfu/g of tissue. In wild rabbits infected with SLS-slp-IL-4, virus was undetectable at this time in two rabbits and a third rabbit had a titer similar to the laboratory rabbits. By day 4, there were essentially no differences in skin titers at the inoculation site between laboratory and wild rabbits (Fig. 6A). In laboratory rabbits, virus was detectable in distal skin at all time points but at low titers (2.9–4.7 $\log_{10}$ pfu/g). In contrast, in wild rabbits, no virus was detectable in distal skin before day 6 and at this time the titers were similar to laboratory rabbits. However, at day 8 in wild rabbits, distal skin titers ranged from 5.3 to 7.3 $\log_{10}$ pfu/g of skin (Fig. 6B).

The popliteal lymph node drains the inoculation site; at day 2, virus titers ranged from undetectable in one rabbit to 5.1 $\log_{10}$ pfu/g in laboratory rabbits but virus was undetectable in all three wild rabbits. At day 4, these figures were 6.8–7.2 $\log_{10}$ pfu/g in laboratory rabbits and 6.2–6.4 $\log_{10}$ pfu/g in wild rabbits. At day 8, the titers in wild rabbit draining lymph nodes were 9 $\log_{10}$ pfu/g (Fig. 6C). Similarly, virus was detectable in contralateral lymph nodes at all time points in laboratory rabbits and reached 6.8 $\log_{10}$ pfu/g by day 6. In wild rabbits, virus was not detectable until day 4 but reached 6 $\log_{10}$ pfu/g at day 8 (Fig. 6D).

Laboratory rabbits infected with SLS-slp-IL-4 had virus detectable in the lung by day 4. In contrast, it was day 8 before virus was detected in the lung of two of three wild rabbits infected with SLS-slp-IL-4, although these titers were high (Fig. 6E). Virus was not found in the spleen before day 6 (Fig. 6F). Virus was occasionally detectable in brains of infected laboratory rabbits but the titers were low and inconsistent. However, in one wild rabbit, which died before autopsy on day 8, high titers were found in the brain (Fig. 6G). This rabbit also had similar titers in lung and spleen.

Testing whether SLS-slp-IL-4 could overcome pre-existing immunity

Expression of murine IL-4 in ectromelia virus has been shown to overcome pre-existing immunity, from vaccination, to mousepox (Jackson et al., 2001). To determine whether myxoma virus expressing IL-4 was able to overcome pre-existing immunity to myxoma virus, eight labo-

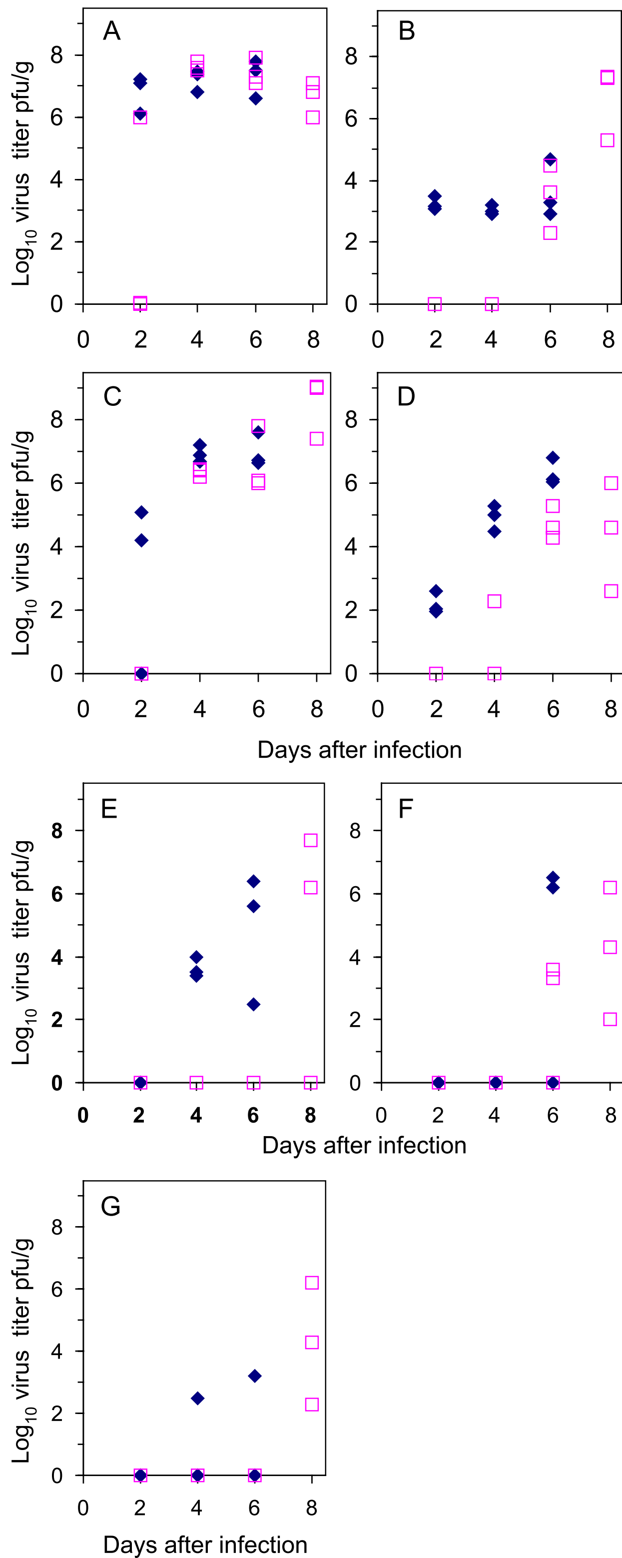

Fig. 6. Virus titers in tissues from wild or laboratory rabbits infected with SLS-slp-IL-4. Log_{10} virus titers for each individual rabbit are shown in each panel. (A) Skin at inoculation site. (B) Contralateral skin. (C) Draining lymph node. (D) Contralateral lymph node. (E) Lung. (F) Spleen. (G) Brain. Solid diamonds (◆) indicate laboratory rabbits and open squares (□) indicate wild rabbits.

ratory rabbits were immunized with the attenuated but immunogenic recombinant virus Ur-HA (Kerr and Jackson, 1995). All rabbits developed a primary lesion at the inoculation site and one rabbit developed disseminated small secondary lesions over the face. None of the rabbits appeared clinically ill. Four of these rabbits were challenged 6 weeks later with 1000 pfu of SLS-slp-IL-4 and four with 1000 pfu of SLS and all rabbits were monitored for clinical signs of myxomatosis. There were no differences between the rabbits challenged with SLS and the rabbits challenged with SLS-slp-IL-4. All rabbits developed a 1–1.5 cm lesion at the challenge inoculation site within 24–48 h. This increased in size up to 2-cm diameter by day 3 but had resolved by day 5. No clinical signs of myxomatosis were observed, and there was no rise in rectal temperatures following either challenge.

Four of the rabbits were retained and challenged again with SLS-slp-IL-4 29 weeks after the original vaccination. This time, the rabbits developed a raised lesion 0.5–1 cm in diameter at the inoculation site 24–48 h after challenge but there were no signs of myxomatosis. The swelling at the inoculation site began to scab and resolve by day 4 after challenge.

Thus, expression of IL-4 was not able to overcome immunity to myxomatosis in outbred laboratory rabbits at doses at the high end of what might be delivered by a mosquito (Fenner and Ratcliffe, 1965). The use of higher doses was not investigated.

Oral delivery trials

Oral delivery of myxoma virus does not normally lead to successful infection. However, because of the high lethality of SLS-slp-IL-4 and its ability to overcome genetic resistance, we investigated whether this virus had changed its pathogenesis sufficiently to infect rabbits following oral delivery. This would potentially allow the use of bait containing the virus as a rabbit-specific biocide. We dosed six laboratory rabbits with 50,000 pfu of SLS-slp-IL-4 in a 200-μl volume into the cheek pouch. None of the rabbits developed clinical signs of myxomatosis or increased rectal temperatures (data not shown). The trial was repeated, this time using the same dose of virus in a 10-μl volume delivered inside the lower lip. Again, no signs of myxomatosis were observed and none of the rabbits sero-converted (data not shown). Thus expression of IL-4 did not enhance oral infection by myxoma virus. Therefore, we concluded that this virus would have no practical role as a rabbit bait unless used on highly abrasive foodstuffs that inoculated virus directly into the tissues of the mouth or pharynx.

Discussion

Australian wild rabbits have developed substantial resistance to myxomatosis over 50 years of coevolution (Best

and Kerr, 2000; Best et al., 2000; Kerr et al., 2003; Marshall and Douglas, 1961; Marshall and Fenner, 1958). The pathogenesis of this resistance has previously been examined using a model of virulent virus SLS and attenuated virus Ur (Best and Kerr, 2000; Best et al., 2000). Based on these pathogenesis studies, it was predicted that resistance depended on the ability of the innate immune response in wild rabbits to control the virus sufficiently for the rabbit to develop an effective adaptive immune response (Kerr and McFadden, 2002). It was also suggested that this might manifest as a Th1 cytokine response in resistant rabbits and a Th2 cytokine phenotype in susceptible rabbits.

To test these predictions, we conducted a manipulative experiment to shift the cytokine environment in resistant or susceptible rabbits toward a Th2 state. This was done using recombinant myxoma viruses expressing the Th2 cytokine rabbit IL-4 under two different late promoters and in a virulent SLS background and a highly attenuated Ur-ZPB background. As would be predicted from the results of previous studies with poxviruses expressing IL-4 (Andrew and Coupar, 1992; Jackson et al., 2001; Sharma et al., 1996), the expression of IL-4 enhanced the virulence of SLS and the recombinant SLS viruses expressing IL-4 were able to overcome genetic resistance in wild rabbits. In contrast, most wild rabbits infected with the parental SLS virus were able to survive the infection.

Although Ur-ZPB is a very attenuated virus (Kerr et al., 1999), Ur-ZPB-p28m-IL-4 was lethal in all infected laboratory rabbits. However, in contrast to the results with SLS-p28m-IL-4, most wild rabbits survived infection with Ur-ZPB-p28m-IL-4, albeit with clinical signs of severe myxomatosis. Thus, in wild rabbits, expression of IL-4 in the Ur-ZPB background caused similar mortality to wild-type SLS.

Expression of foreign genes between the M061 and M062 genes in Ur has resulted in further attenuation of this virus (Kerr and Jackson, 1995; Kerr et al., 1999) but whether this was caused by the insertion at this site or by the particular gene expressed has not been determined. It has also been demonstrated that expression of foreign genes in this site in SLS leads to significant attenuation of the virus (S. Junankar, P.J. Kerr, B.H. van Leeuwen, unpublished data). Thus, IL-4 expression has been able to overcome any attenuation due to genetic modification of Ur or SLS, increase virulence beyond that of the parental virus, and overcome genetic resistance to myxoma virus.

Our prediction when starting this study was that IL-4 would diminish the ability of the resistant rabbits to mount an effective cell-mediated immune response to myxoma virus. However, if as we predicted, resistance was mediated by the innate immune response or other cellular factors, we expected that wild rabbits would have lower titers of virus than laboratory rabbits at early time points after infection but this difference would be lost later in infection due to the deviation away from a cell-mediated immune response induced by IL-4.

When virus titers were measured in tissues of wild and laboratory rabbits infected with SLS-slp-IL-4, it was clear that at early time points wild rabbits did have lower titers of virus than laboratory rabbits (see Fig. 6).

Taken together, the titers of virus tend to confirm that wild rabbits were able to control SLS-slp-IL-4 replication at early time points more effectively than laboratory rabbits. This is similar to previous results for trials with SLS or Ur (Best and Kerr, 2000). However, in the skin, at the inoculation site, and in the draining lymph node, these differences were largely overcome by day 4 after infection with SLS-slp-IL-4. Similarly, SLS-slp-IL-4 was slower to disseminate to distal tissues in wild rabbits compared to laboratory rabbits but once at the distal tissues, it was able to replicate to similar titers. This is in strong contrast to the previous results with SLS where the virus was not only slower to disseminate to distal tissues in wild rabbits compared with laboratory rabbits, but then replicated to lower titers at these sites (Best and Kerr, 2000).

These results support a conceptual model for resistance to myxomatosis whereby myxoma virus replication is initially limited at tissues distal to the skin inoculation site by an enhanced innate immune response or other cellular factors that are able to partially overcome the modulation of the immune response normally induced by myxoma virus (Kerr and McFadden, 2002). This would favor the development of an effective cell-mediated immune response involving Th1 cells and cytotoxic T cells that control virus replication from day 4 onwards (Best et al., 2000; Kerr and McFadden, 2002).

Similar results have been reported in mice when murine IL-4 was expressed in recombinant ectromelia virus. The recombinant virus caused lethal infection in inbred strains of mice that were normally resistant to mousepox and able to control replication of ectromelia virus distal to the skin inoculation site. Expression of IL-4 by ectromelia virus induced both downregulation of innate NK cell activity and suppression of CTL activity (Jackson et al., 2001). Because there are as yet no convenient methods for examining CTL and NK responses in outbred rabbits, direct measurement of immune deviation could not be done in our study.

A quite striking finding reported by Jackson et al (2001) was that the ectromelia virus expressing IL-4 was able to overcome immunity induced by vaccination of mice by an attenuated virus strain. Following challenge with ectromelia virus expressing IL-4, 60% of vaccinated mice died. A myxoma virus that could overcome pre-existing immunity as well as genetic resistance might have major advantages as a biological control agent for rabbits. However, SLS-slp-IL-4 was unable to overcome pre-existing immunity when injected in a dose at the extreme high end of what might be delivered by a mosquito. This indicated that there was either a major difference in the immune deviation caused by myxoma virus expressing IL-4 in rabbits and ectromelia virus expressing IL-4 in mice or that the ectromelia vacci-

nation did not induce comparable immunity to the myxoma virus vaccine. There is also a formal possibility that the use of a late promoter to drive IL-4 expression in myxoma virus and the use of an early or late promoter in ectromelia virus may have led to a difference at challenge. Whether other poxviruses expressing IL-4 can overcome vaccination has not been examined.

The use of myxoma virus as a biological control for rabbits in Australia has been well reviewed (Fenner and Fantini, 1999). It is clear from the biology of the disease that selection for successful field strains of myxoma virus is driven by arthropod transmission of virus and that transmission is most successful with virus strains that are able to cause significant disease with high mortality rates but which allow rabbits to survive for long periods before succumbing (Fenner, 1983; Kerr et al., 2003; Mead-Briggs and Vaughan, 1975). Although recombinant SLS viruses expressing IL-4 are highly lethal in Australian wild rabbits, despite genetic resistance, and therefore potentially attractive for biological control, they would be unlikely to compete successfully in the field because the rabbits die very quickly following infection, thus preventing further transmission.

There was a formal possibility that expression of IL-4 by recombinant myxoma viruses was lethal because of direct toxic effects of the cytokine on tissues such as liver and lungs rather than its action in immune deviation (Andrew and Coupar, 1992; Guillot et al., 2001; Huaux et al., 2003). We attempted to address this in two ways, firstly by placing the IL-4 gene under the control of the p28m promoter rather than the synthetic late promoter (slp). Based on the results reported for these two promoters by Davison and Moss (1989), we expected that this would reduce expression of IL-4 to 20% of the levels obtained with the slp. However, in the SLS background, we could not detect any difference in the amounts of IL-4 produced by SLS-slp-IL-4 and SLS-p28m-IL-4 and there was no difference in the lethality of the two viruses. The second approach was to express IL-4 in a very highly attenuated strain of myxoma virus Ur-ZPB under the control of the p28m promoter. This led to a reduction in IL-4 expression compared to the SLS background. The Ur-ZPB-p28m-IL-4 virus was highly lethal in laboratory rabbits but was much less lethal in wild rabbits although causing severe myxomatosis. However, the lower virulence in wild rabbits could also be associated with reduced viral replication in wild rabbits compared to laboratory rabbits and thus potentially less IL-4 production in wild rabbits. Thus, we could not formally discard the hypothesis that IL-4 was having a direct toxic effect.

However, comparing the titers of SLS-slp-IL-4 in wild rabbits and those from SLS-slp-IL-4-infected laboratory rabbits, it was clear that IL-4 was suppressing virus control mechanisms and allowing replication of myxoma virus to high titers in otherwise resistant wild rabbits. This is the best direct evidence that IL-4 expression is overcoming genetic resistance through an immune deviation mechanism rather than through direct toxicity. Interestingly, titers of virus in laboratory rabbits were not increased above what would have been expected from infection with SLS. This may well be because the significant tissue destruction of lymphoid tissues that occurs with SLS infection in laboratory rabbits (Best et al., 2000) limits the amount of viral replication that can occur but this has not yet been examined for SLS-slp-IL-4.

This model of enhanced viral virulence due to immune deviation is indirectly supported by clinical descriptions of infections of rabbits with the highly lethal Californian strains of myxoma virus (Fenner and Ratcliffe, 1965). These viruses cause death in laboratory rabbits with an AST of 9.2 days and very few clinical signs of myxomatosis but signs of central nervous system dysfunction similar to those described here. Similar clinical signs of central nervous system dysfunction, coma, and pulmonary edema are also reported from laboratory rabbits infected with highly lethal Australian strains of myxoma virus that have evolved to transmit in populations of genetically resistant rabbits and are more virulent than SLS (Kerr et al., 2003).

In conclusion, we have demonstrated that expression of the Th2 cytokine IL-4 by myxoma virus enhances virulence and overcomes genetic resistance to myxoma virus. This is most likely due to immune deviation induced by IL-4 preventing development of an effective CTL response and possibly due to decreased NK cell activity. The predicted role of the innate immune system in resistance is also supported by these studies. Thus, the use of immune deviation has provided a useful tool for further detailed studies into the mechanism of genetic resistance selected in a naive outbred species following the introduction of a novel pathogen.

Methods

Virus strains and cell lines

The SLS stock used in this study was derived from a freeze-dried rabbit tissue stock produced in 1953 that was obtained from Professor Frank Fenner, John Curtin School of Medical Research, Australian National University. This virus was subsequently passaged twice in RK13 cells and then twice in rabbits, and a rabbit testis stock was produced. SLS has Grade 1 virulence for rabbits with an average survival time of <13 days (Fenner et al., 1957). This has been confirmed for virus from this stock (Robinson et al., 1999). Ur was derived from the Uriarra/2-53/1 isolate (Mykytowycz, 1953) by plaque purification (Russell and Robbins, 1989). The virus is of grade 5 virulence, with all laboratory rabbits developing myxomatosis but most eventually recovering from infection (Best and Kerr, 2000; Kerr et al., 1999). Ur-HA is a recombinant Ur strain of myxoma virus expressing the influenza virus hemagglutinin and which does not cause clinical myxomatosis (Kerr and Jackson, 1995).

Virus stocks were cultured in RK13 cells in minimal essential medium (MEM) (GibcoBRL) supplemented with 10% fetal calf serum, 200 units/ml penicillin, and 100 μg/ml streptomycin in an atmosphere of 5% CO_2 in air at 37 °C. Viruses were titered by plaque assays in duplicate on Vero cell monolayers cultured in the same medium plus 2.5 μg/ml of amphotericin B. Titers were expressed as plaque forming units per milliliter of culture (pfu/ml).

Transfer plasmid constructs

The plasmid pUrTK11a-IL-4 was constructed using pUrTK11a as the starting plasmid. pUrTK11a (Jackson et al., 1998) is a modification of pUrTK11 (Kerr and Jackson, 1995) in which the poxvirus p11 late promoter is replaced with the optimized vaccinia virus strong synthetic late promoter (slp) (Davison and Moss, 1989). The virus sequence in this plasmid is from the Ur strain of myxoma virus (Jackson and Bults, 1992). There is only one nucleotide difference between the Ur sequence in this plasmid and the equivalent region in the published Lausanne (Brazil/Campinas 1949) strain of myxoma virus (Cameron et al., 1999). This is an A > T transversion in the third position of codon 41 in the M061 gene leading to a conservative Lys > Asn amino acid change. Given this degree of conservation and that Ur is directly derived from SLS, it was assumed that the SLS and Ur sequence were highly unlikely to differ in this region.

The rabbit IL-4 cDNA (Perkins et al., 2000) was inserted into *Eco*RI–*Sal*I-cut pUrTK11a. The resulting plasmid (Fig. 1A), and those described below, also contain the *E. coli gpt* gene under the control of the poxvirus p7.5 early or late promoter to enable selection for mycophenolic acid resistance. Flanking sequences from the myxoma virus TK (M061R) and M062R genes enable insertion of the foreign sequences intergenically between these two genes. This plasmid was used to make the SLS-slp-IL-4 recombinant virus.

pUrTK11-ZPB (Kerr et al., 1999) was used to construct pUrTK11-ZPB-IL-4 by insertion of the rabbit IL-4 cDNA under the control of a modified p28 vaccinia virus late promoter (p28m). This modified promoter (CACTTTTTTTTCTTCTCTAAAT) is described by Davison and Moss (1989) as 20 times as strong as the native p28 promoter and about a fifth the strength of the synthetic late promoter. The p28m promoter, made by annealing two complementary oligonucleotides with restriction site overhangs, was cloned immediately upstream of the IL-4 cDNA contained in another plasmid in use in our laboratory (unpublished results). The p28m-IL-4 cassette was excised from this plasmid and cloned into the *Sal*I site of pUrTK11-ZPB. The resulting plasmid (Fig. 1B) also contains the rabbit zona pellucida B gene under the control of the p11 late promoter (Davison and Moss, 1989), and was used to make the Ur-ZPB-p28m-IL-4 recombinant virus.

To make a recombinant SLS-IL-4 virus in which IL-4 expression is controlled by the p28m late promoter, we constructed the plasmid pUrTK-p28m-IL-4 (Fig. 1C). This was achieved by deleting p11-ZPB from pUrTK11-ZPB and replacing it with p28m-IL-4, which was amplified by PCR from pUrTK11-ZPB-IL-4 above.

Preparation of recombinant myxoma viruses

Isolation of recombinant myxoma viruses was as previously described (Kerr et al., 1999). Briefly, RK13 cells were infected with either SLS (for SLS-slp-IL-4, SLS-p28m-IL-4) or Ur (for Ur-ZPB-p28m-IL-4) at a multiplicity of infection (moi) of 1. The infected cell cultures were transfected with plasmid using Lipofectin or Lipofectamine (GibcoBRL) as per the manufacturer's instructions. Recombinant viruses expressing GPT were selected for mycophenolic acid resistance in MEM containing 10% dialyzed fetal calf serum (or no fetal calf serum), 5–20 μg/ml mycophenolic acid, 250 μg/ml xanthine, 15 μg/ml hypoxanthine, 2 μg/ml aminopterin, and 10 μg/ml thymidine. After the initial transformations, three to six rounds of plaque purification were undertaken and the plaques checked for recombinant virus by PCR using primers and conditions that provided an amplification product specific to the recombinant virus or wild-type virus. The following primers and conditions were used for screening for the SLS-p28m-IL-4 recombinant virus purity. Wild-type virus detection used primers designated MJ2a-R2 from the M062 gene (ATCGTAGGGTATTCGCACG) and TK-F2 (GATATCCGAACACGTCGAAG) from the M061 gene. These primers produce a 700-bp amplification product from wild-type virus sequence. The extension times used did not allow amplification across the recombinant sequence from these primers. Primers MJ2a-R2 and IL-4-start-Bgl (GCAAGATCTA<u>TGGGGCTCCCTGCCCAG</u> -sequence matching IL-4 is underlined the remaining sequence is a *Bgl*I site used for other cloning steps) were used to detect recombinant virus. These primers produce a 900-bp amplification product. Amplification conditions were as follows: cycle 1 (1×) 95 °C/2 min; cycle 2 (35×) 95 °C/30 s; 56 °C/30 s; 72 °C/45 s; cycle 3 (1×) 72 °C/3 min). For Ur-pZPB-p28m-IL-4 detection and SLS-slp-IL-4 detection the following primers were used: IL-4-start-Bgl and IL-4-stop-spe (TTAACTAGT<u>TCAGCTCTGACGCTTTGAG</u>). This primer pair produces a 462-bp product. Wild-type virus was detected using primers BI2 (CAACCTAATCGTAAATATCCG) from just 5′ to the M062 start codon and TK-F2, which produce a 358-bp product. The PCR cycle conditions for these primer pairs were: cycle 1 (1×) 95°/3min; cycle 2 (×35) 95°/30 s; 55°/30 s; 72°/90 s; cycle 3 (1×) 72°/5 min.

Recombinant virus from plaques that were free of wild-type virus by PCR analysis was amplified in RK13 cells and used for subsequent experiments.

Immunoblotting

To determine that each recombinant virus was expressing IL-4 in cell culture, RK13 cells were infected (moi of 1) in MEM without bovine serum. After 24 h, culture supernatants were concentrated by acetone precipitation and resuspended in SDS-PAGE loading buffer for gel electrophoresis. Following electrophoretic separation, the proteins were transferred to PVDF membranes using a semidry transfer apparatus and probed with an antiserum to a rabbit IL-4 synthetic peptide raised in rats. Electrophoresis and immunoblotting were performed as previously described (Kerr et al., 1999).

Replication kinetics of recombinant viruses

The replication kinetics of each recombinant virus were measured by infecting confluent monolayers of RK13 cells at moi of 3 or 0.1 in duplicate. Cell monolayers were incubated for 75 min (37 °C, 5% CO_2 in air) with the virus inoculum, washed twice, and the growth medium replaced. Cells and supernatant were harvested at appropriate time points; the combination was freeze–thawed twice and plaque-assayed on Vero cell monolayers in duplicate.

Rabbits

Laboratory rabbits were bred and housed in the CSIRO Sustainable Ecosystems animal facility. Wild rabbits were bred for at least two generations in the same animal facility from breeding stock originally obtained as 4–6-week-old kittens from the field and unexposed to myxoma virus. Animal experimentation was approved by the CSIRO Sustainable Ecosystems Animal Experimentation Ethics Committee. All experiments with recombinant viruses were conducted under containment level 2 conditions under license from the Commonwealth of Australia Office of the Gene Technology Regulator.

Virulence trials

Groups of 5–12 rabbits >4 months of age were housed in individual cages and inoculated intradermally in the thigh with 1000 pfu of virus in a volume of 100 μl. Rabbits were examined twice a day. Clinical signs and rectal temperatures were recorded at 24-h intervals after inoculation. Survival times were allocated based on the nearest 0.25 days. Rabbits that were moribund were euthanized with intravenous barbiturate. If a rabbit was euthanized, an extra 0.5 day was arbitrarily added to the survival time. Results were calculated as average survival times (AST) and statistical differences between AST for each group were analyzed by *t* test.

Pathogenesis trials

To examine the pathogenesis of SLS-slp-IL-4, groups of laboratory and wild rabbits were inoculated with 1000 pfu of SLS-slp-IL-4 into the dorsal skin of the right hind foot (Best and Kerr, 2000). At 2, 4, 6 and, for wild rabbits, 8 days, after infection, three rabbits were killed with intravenous barbiturate and autopsied. The following tissues were collected into preweighed vials on dry ice: skin from the inoculation site, distal skin from the equivalent site on the left (contralateral) foot, the right (draining) popliteal lymph node, the left (contralateral) popliteal lymph node, spleen, lung, and brain. These tissues were frozen at −70 °C until processing. After weighing, tissues were chopped finely and homogenized using hand-held homogenizers. Homogenized tissues were freeze–thawed twice and sonicated to release virus as previously described (Best and Kerr, 2000) before being diluted to a 10% w/v suspension in MEM. Virus titers were measured by plaque assay on Vero cell monolayers and expressed as pfu/g of tissue. The lowest level of detection was 100 pfu/g.

Immunity trials

Eight laboratory rabbits were immunized with 5000 pfu of Ur-HA injected intradermally into the right thigh. Rabbits were allowed to recover and at 6 weeks after immunization, four rabbits were challenged with 1000 pfu of SLS-slp-IL-4 by injection into the skin opposite the original vaccination site. The other four were challenged with wild-type SLS. Following this challenge, four rabbits were retained for a further 29 weeks and then rechallenged.

Oral delivery trials

Six laboratory rabbits were dosed with 50,000 pfu of SLS-slp-IL-4 in 200 μl of 1:1 saline/glycerol delivered into the mouth using a Gilson Pipetman. Clinical signs of disease and rectal temperatures were recorded daily. In a second trial, with the same rabbits, 50,000 pfu of SLS-slp-IL-4 were delivered, in a 10-μl volume of saline, behind the lower lip and the rabbits monitored for clinical signs of myxomatosis. After this trial, serum was collected from each rabbit and examined for antibodies to myxoma virus by ELISA (Kerr, 1997).

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