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Virology 385 (2009) 11–21

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Mousepox in the C57BL/6 strain provides an improved model for evaluating anti-poxvirus therapies

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ARTICLE INFO

Article history:
Received 1 May 2008
Returned to author for revision
26 May 2008
Accepted 10 November 2008
Available online 18 December 2008

Keywords:
Antiviral
Bioterrorism
Cidofovir
Ectromelia
Oral drug
Smallpox

ABSTRACT

The intranasal lethal mousepox model employing the A/Ncr mouse strain is used to evaluate anti-orthopoxvirus therapies. These infections mimic large droplet transmission and result in 100% mortality within 7-10 days with as little as 1 PFU of ectromelia virus. Unlike the A/Ncr model, humans are less susceptible to lethal respiratory infections with variola virus and monkeypox virus as demonstrated by their lower mortality rates. In this study we show that a low dose intranasal infection of C57BL/6 mice results in 60-80% mortality and better models smallpox. Comparing CMX001 (HDP-cidofovir) efficacy in the A/Ncr strain and the C57BL/6 strain revealed that delayed treatment with CMX001 is more efficacious at preventing severe disease in the C57BL/6 strain. The increased efficacy of CMX001 in C57BL/6 over A/Ncr following an intranasal infection with ectromelia appears to be mediated by a stronger Th1 cell mediated response. Following footpad infection we show that the C57BL/6 strain has earlier and more robust transcriptional activity, Th1 cytokine secretions, antigen presenting activity and IFNγ splenic CD8+ T cell responses as compared to the A/Ncr strain. As a result of the enhanced immune response in the C57BL/6 strain, non-lethal intradermal ectromelia infections can therapeutically protect up to 3 days following a homologous, lethal intranasal infection – much like how smallpox vaccination can protect humans for up to 4 days following intranasal variola infection.

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Introduction

We are currently faced with the potential use of natural or recombinant variola virus (VARV) and monkeypox virus (MPXV) as biological weapons, as well as the emergence of human monkeypox in Africa (Mortimer, 2003a; Parker et al., 2007b). The majority of the population have no protection against these viruses due to the cessation of vaccination in the smallpox-free epoch (Parker et al., 2008). Furthermore, a growing section of the community cannot be vaccinated due to their immuno-compromised status (Baker et al., 2003; Bray, 2003). Currently, the only antiviral treatment for such diseases is intravenous cidofovir (CDV) administration, which is limited by its inherent nephrotoxicity and intravenous delivery method (De Clercq, 2002; Safrin et al., 1997). To address this, an orally bioavailable hexadecyloxypropyl ester of CDV (CMX001) and ST-246 have has been produced and are in phase 1/2 clinical trials (Buller et al., 2004; Kern et al., 2002; Parker et al., 2008a; Quenelle et al., 2004, 2007; Yang et al., 2005). CMX001 and ST-246 have demonstrated antiviral efficacy against a number of orthopoxviruses and CMX001 also has activity against a number of viruses that encode their own DNA polymerase.

We have previously shown that delayed oral administration of CMX001 can be used to treat a lethal mousepox infection in the A/Ncr strain (Buller et al., 2004; Parker et al., 2008a). The mousepox model is arguably the best small animal model for the evaluation of VARV/MPXV therapeutics due, in part, to the low dose of virus required for a lethal infection, which produces a disease course that accurately reflects the progress of natural smallpox. The A/Ncr strain is susceptible to lethal mousepox when infected intranasally (IN) or intradermally via breaks in the skin of the footpad (FP) with extremely low dose inoculums. However, human respiratory infections with VARV or MPXV have much lower mortality rates compared to that of the A/Ncr mousepox model (Fenner et al., 1988; Parker et al., 2007b). Furthermore, healthy humans infected with VARV via breaks in the skin have a greatly reduced frequency of severe disease or mortality (approx. 0.5-1%). Therefore the A/Ncr strain may be too susceptible to mousepox, making it difficult to fully evaluate the therapeutic efficacy of candidate antivirals. For this reason we tested the efficacy of CMX001 in the C57BL/6 strain, which is highly susceptible to lethal infections by the IN route but resistant following infections through the FP route, similar to VARV in humans (Schell, 1960). Here we show that the CMX001 therapeutic treatment window is extended in the C57BL/6 strain compared to the A/Ncr, and that the reason for this is likely to be the increased early magnification of a Th1 biased cell mediated response and more efficient antigen presentation at the draining lymph node.

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0042-6822/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.virol.2008.11.015

Results and discussion

The C57BL/6 strain is resistant to footpad ECTV challenges

To compare the relative susceptibilities of A/Ncr and C57BL/6 strains, groups of A/Ncr and C57BL/6 mice were infected IN (Figs. 1A and B) or via the FP (Figs. 1C and D) routes with increasing ectromelia (ECTV) doses ranging from 0.009 to 9000 PFU. All A/Ncr mice died with a minimum of 0.9 and 0.09 PFU for IN and FP infections, respectively (LD_{50}=0.3 and <0.009 PFU, respectively). A minimum dose of 900 PFU was required to kill all the C57BL/6 mice with an IN challenge (LD_{50}=90 PFU). The 90 PFU IN dose killed 50% of the mice. The maximum dose of 9000 PFU was not sufficient to kill any C57BL/6 mice with a FP infection (LD_{50}>9000 PFU). These findings confirmed those of Schell who showed that the closely related C57BL strain was susceptible to a lethal infection by the respiratory route but not by the subcutaneous route (Schell, 1960). Tissue infectivity titres reflected the relative susceptibilities of mouse strains to lethal infection following FP inoculation with 1000 PFU (Figs. 1E and F). Virus was detected in spleens and livers of A/Ncr mice from day 4 post-infection (p.i.) and the titres increased until day 7. Conversely, we found significantly lower titres in the C57BL/6 tissues compared to those in the A/Ncr and found that splenic and liver titres were absent or reduced by day 6 p.i. The low titres in C57BL/6 tissues indicate that this strain begins to respond and control the infection from very early time points, possibly within the first 24-48 hours. The susceptibility of the C57BL/6 mouse to an IN challenge, but not to a FP challenge, is reminiscent of variola infections in humans.

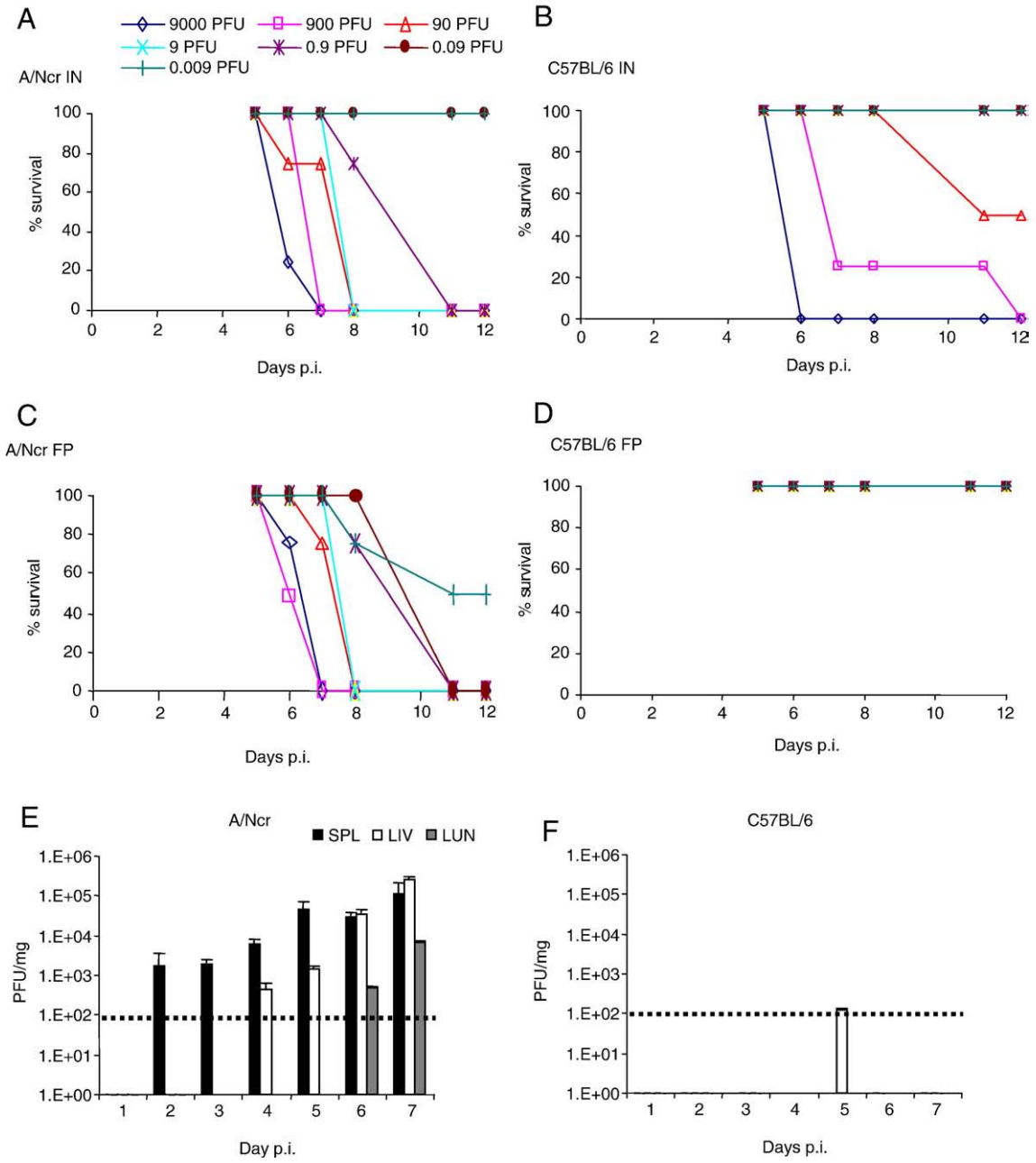

Fig. 1. The A/Ncr strain is more susceptible to ECTV than the C57BL/6 strain. Groups of A/Ncr and C57BL/6 mice were infected with ECTV doses ranging from 0.009 to 9000 PFU via the IN route (A and B) and the FP route (C and D). Spleen, liver and lung tissues were titred from A/Ncr mice (E) and C57BL/6 (F) mice on days 1-7 p.i. following a 100 PFU FP infection. The limit of detection is indicated by a dashed line.

Mousepox is less severe in the C57BL/6 strain compared to the A/Ncr strain following intranasal infections

The cause of death in mice infected with ECTV is unknown, but liver necrosis is a contributing factor. To compare the severity of mousepox in each strain, we sacrificed groups of infected A/Ncr and C57BL/6 mice everyday for 7 days following an IN infection. The infectious dose of 1500 PFU was used to ensure both strains had 100% mortality, thus, allowing us to dissect how each strain responded to a lethal infection. Virus particles were detected in liver samples at 2 and 3 days p.i. in the A/Ncr (Fig. 2A) and C57BL/6 (Fig. 2B) strain, respectively. On day 4 p.i. the A/Ncr liver had an average of almost 1×10^6 PFU/mg, the C57BL/6's liver did not reach a comparable level until 5 days p.i.. Both strains demonstrated an increase in liver titres as time increased following infection. Viral titers in the A/Ncr strain's liver were almost 2 logs higher than that of the C57BL/6 on day 6 p.i. ($p=0.0115$). As expected, high levels of virus were detected in the spleens and lungs of both strains, but the A/Ncr strain consistently achieved higher titres several days earlier than the C57BL/6 strain.

To better compare the severity of disease, we also measured blood levels of AST and ALT as biomarkers of acute liver damage and hepatic disease (Figs. 2C and D). We found that levels of AST and ALT in the A/Ncr strain were significantly elevated by day 6 p.i. compared to the background control and corresponded closely with the average day of death for the A/Ncr strain, which was 7.2±0.2 (Fig. 2E). The average day of death for the C57BL/6 strain was 9.2±0.2; two days prior to this the levels of AST and ALT were variably elevated. Similarly, data from patients infected in the Midwestern United States MPXV outbreak of

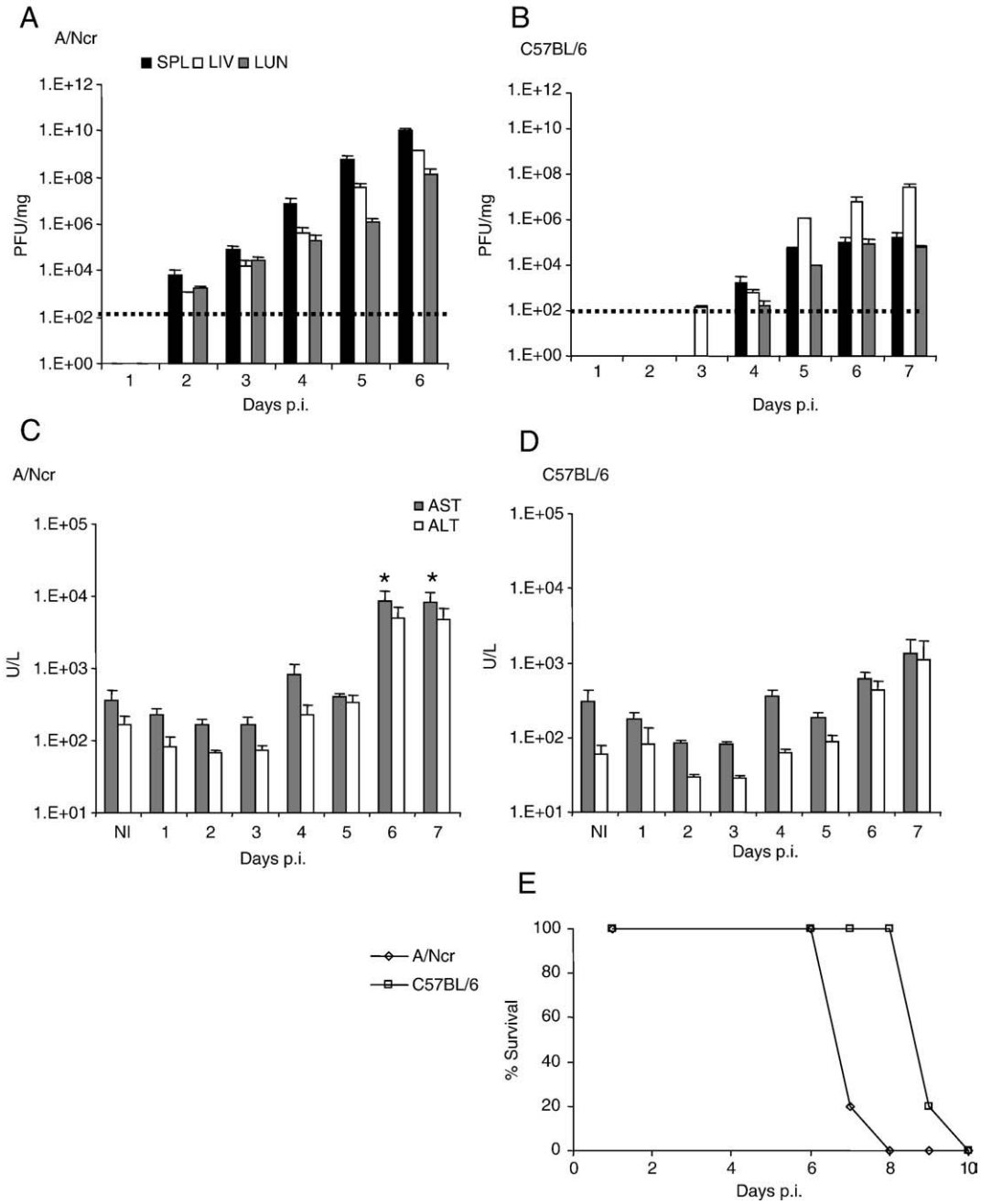

Fig. 2. C57BL/6 mice have reduced tissue titres and liver enzyme levels. On days 1-7 p.i., spleen, liver and lung tissue titres were calculated from A/Ncr (A) and C57BL/6 (B) mice intranasally infected with 1500 PFU. The limit of detection is indicated by a dashed line. AST and ALT liver enzymes from A/Ncr (C) and C57BL/6 (D) bleeds were monitored from days 1 to 7 p.i. (E) The A/Ncr and C57BL/6 survival curves are shown (* indicates $P<0.055$).

2003 revealed that 50% and 59% of analysed individuals had abnormally high AST and ALT levels, respectively (Huhn et al., 2005). In previous studies, we found that ALT levels were less variable and gave a better indication of the stage of disease compared to AST levels (Parker et al., 2008b).

Efficacy of delayed intervention with CMX001

Mousepox was less severe in C57BL/6 mice than A/Ncr mice at a matched IN dose. To see if this difference resulted in an increased therapeutic window we IN inoculated A/Ncr and C57BL/6 with 100 PFU of ECTV, and treated mice on various days p.i. with a 10 mg/kg loading dose followed by every-other-day dosing of 2.5 mg/kg of CMX001, a hexadecyloxypropyl ester of cidofovir. Treatments were initiated on days 0 to 6 p.i. All of the A/Ncr vehicle treated mice died by day 8 p.i. compared to 60% of the C57BL/6 mice by day 11 (Figs. 3A and B). 100% survival was observed in A/Ncr mice whose CMX001 treatment was delayed until 2 days p.i. ($P=0.008$), 80% survival was achieved in A/Ncr mice whose treatment was initiated immediately or was delayed until days 3 and 4 ($P=0.05$)(Fig. 3A). Delaying intervention until 5 days p.i. resulted in 100% mortality in the A/Ncr strain. All treated C57BL/6 mice survived even when treatment was delayed until 6 days p.i (Fig. 3B) and had minimal weight-loss compared to the A/Ncr strain (Figs. 3C and D). In comparison, all infected A/Ncr mice had significant weight loss, even in the group with no mortality (day 2) (Fig. 3C). In studies where A/Ncr mice were inoculated with a low LD_{100} (2 PFU/mouse) dose by the IN route, we never observed 100% CMX001 mediated protection at 6 days p.i. (data not shown). Thus, we conclude that the C57BL/6 strain responds better to CMX001 treatment compared to the A/Ncr strain at an equivalent challenge dose, and the A/Ncr mousepox model may understate the efficacy of CMX001 as a human therapeutic.

The C57BL/6 strain upregulates TLR13 and CD55 expression in response to a footpad infection

To determine the basis for the enhanced resistance of the C57BL/6 mouse to lethal infection, as compared to the A/Ncr strain, we measured a number of components of the host immune response. First, as a general measure of the early response to infection we monitored changes in gene transcription levels in the popliteal lymph nodes (PLN)

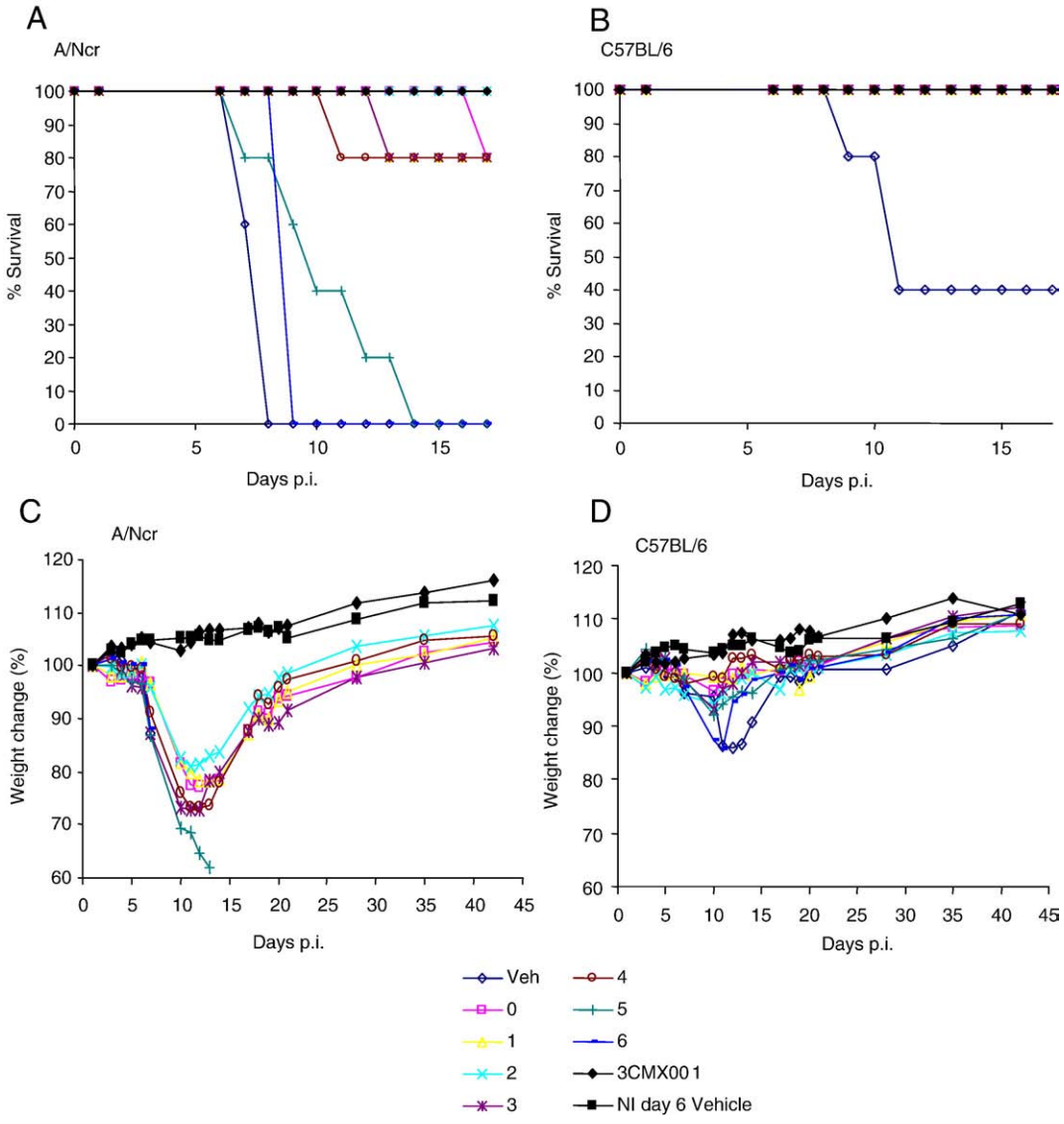

Fig. 3. Efficacy of intervention with CMX001. A/Ncr and C57BL/6 mice were infected with 100 PFU of ECTV and treated with CMX001 starting on day 0 to 6 p.i. A/Ncr and C57BL/6 survival curves are shown in A and B, weight change is shown in C and D, respectively. Veh are infected mice receiving vehicle; 3CMX001 are non-infected mice receiving CMX001 on day 3 p.i; NI 6 vehicle are non-infected mice receiving vehicle at day 6 p.i. The numbers 1-6 indicate the day p.i. that therapy was initiated.

Table 1
Transcriptional changes in genes involved in innate immunity, signalling (MAPK), defence response and cellular defence response

	Probe set ID	Expression profile	Gene symbol	Gene title	Gene ontology and pathway
Innate immune response	1457753_at	B6 6↑ 12↑ 24↓	Tlr13	Toll-like receptor 13	Innate immune response/inflammatory response
(GO:0045087)	1418762_at	B6 24↓	Cd55	CD55 antigen	Innate immune response/complement activation, classical pathway
	1455560_at	AJ 6↓ 12↓ 24↓	Ecsit	Signaling intermediate in toll pathway (evolutionarily conserved)	Innate immune response/MAPK signaling pathway/ transcriptional regulation
MAPK signaling pathway	1453851_a_at	B6 24↑	Gadd45g	Growth arrest and DNA-damage-inducible 45 gamma	Activation of MAPKK activity/apoptosis/interferon-gamma biosynthetic process/T-helper 1 cell differentiation
(KEGG:mmu04010)	1420895_at	B6 24↓	Tgfbr1	Transforming growth factor, beta receptor I	TGF-beta receptor signaling pathway/gene expression/ induction of apoptosis/negative regulation of apoptosis
	1415834_at	B6 24↓	Dusp6	Dual specificity phosphatase 6	MAPK signaling (phosphatase activity)
Defense response	1457753_at	B6 6↑ 12↑ 24↓	Tlr13	Toll-like receptor 13	Innate immune response/inflammatory response
(GO:0006952)	1418762_at	B6 24↓	Cd55	CD55 antigen	Innate immune response/complement activation, classical pathway
	1422758_at	B6 24↓	Chst2	Carbohydrate sulfotransferase 2	Carbohydrate (N-acetylglucosamine) metabolic process/ inflammatory response
	1455560_at	AJ 6↓ 12↓ 24↓	Ecsit	Signaling intermediate in toll pathway (evolutionarily conserved)	–
	1427752_a_at	AJ 6↑ 12↑ 24↑	Tcrb-J	T-cell receptor beta, joining region	Cellular defense response
	1422188_s_at	AJ 6↓ 12↓ 24↓	Tcrg-V3	T-cell receptor gamma, variable 3	Cellular defense response
	1422189_x_at, 1450521_a_at	AJ 6↓ 12↓ 24↓	Tcrg-V4	T-cell receptor gamma, variable 4	Cellular defense response

at 6, 12 and 24 hours p.i. following a 1000 PFU FP infection. All the strain/time interaction effects were analyzed in accordance with the 2-way ANOVA model, thereby resulting in 10 simultaneous pair-wise statistical comparisons (6 strain-matched/p.i. time and 4 p.i. time matched/between-strain). Emphasis was placed on the 6 strain-matched/p.i. time comparisons in order to determine the differential gene expression profiles due specifically to ECTV infection and not due to consistent baseline strain-specific transcript differences. Statistically significant differentially expressed genes were partitioned into 13 distinct, infection time course-specific co-expression groups: 9 C57BL/6 (80 genes in total) and 4 A/Ncr (22 genes in total) groups (Supplemental table S1). Notably, there was no overlap between the C57BL/6 and A/Ncr groups, suggesting that the genetic responses enabling the C57BL/6 to overcome a FP infection is completely absent in the A/Ncr strain. Of particular interest was the innate immune response biological process gene ontology (Table 1). We detected two differentially expressed genes in the C57BL/6 response to ECTV infection which are involved in the innate response: TLR13 (toll-like receptor 13) and CD55 (decay accelerating factor, DAF). Overall, we found that the C57BL/6 strain was more pro-active at adjusting transcriptional regulation than the A/Ncr strain (Supplemental table 1).

In the C57BL/6 strain, the level of TLR13 significantly increased over the first 12 hours p.i. but returned to below naïve levels by 24 hours p.i. However, TLR13 levels in the A/Ncr strain did not change significantly over the first 12 hours but, similar to the C57BL/6 strain, decreased significantly below naïve levels by 24 hours p.i. (Fig. 4A). Unfortunately, little is known about TLR13 in mammals, including its ligands, adaptors and location; but, it is known that humans do not have a homologue of TLR13 (Wu et al., 2008).

Unlike TLR13 levels, CD55 was only significantly down regulated at 24 hours p.i. in the C57BL/6 strain. In contrast, the levels of CD55 did not significantly change at any of the time points in the A/Ncr strain (Fig. 4B). In the complement pathway, CD55 (also know as decay accelerating factor, DAF) binds C3b, C4b, C3bBb and C4b2a to inhibit C3 convertase formation and accelerate its decay (Kameyoshi et al., 1989). This action of CD55 prevents inappropriate activation of the complement arm of the immune system. The failure of the A/Ncr strain to upregulate TLR13 within the first 12 hours of infection and to downregulate CD55 by 24 hours p.i. may be important to its inability to recover from infection. In the latter case, the non-reduction of CD55 in the A/Ncr strain might be responsible for the A/Ncr's sensitivity due to failure of the activation of the complement system – we are currently exploring this hypothesis.

Cytokine responses at the PLN are different between the A/Ncr and C57BL/6 strains

To further evaluate the innate immune response at the PLN we measured the levels of 23 cytokines and chemokines at 6, 12, 24 and 48 hours following a 1000 PFU FP challenge. Three mediators

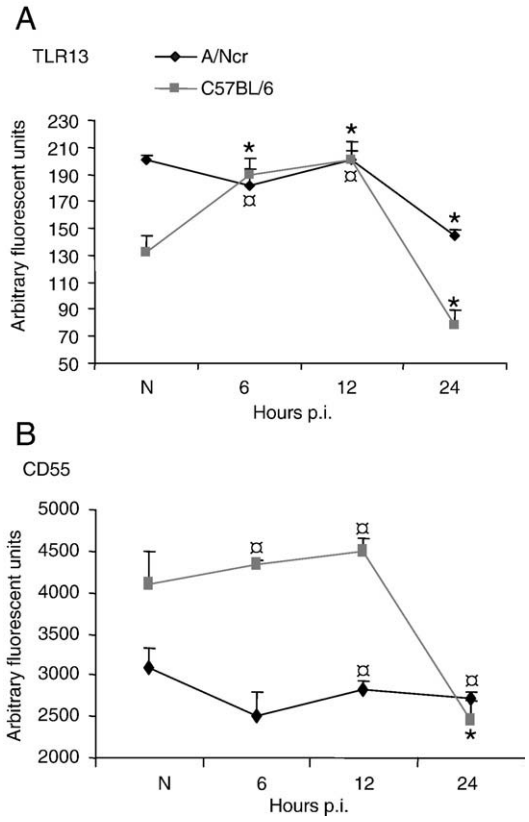

Fig. 4. Changes in TLR13 and CD55 transcription in the PLN. Following a 1000 PFU FP infection TLR13 (A) and CD55 (B) were the only innate immune genes from the C57BL/6 PLNs to change transcription levels compared to naïve controls (N). Transcription levels were evaluated from infected PLNs at 6, 12 and 24 hours p.i. (*$P<0.003$ and ¤$P>0.05$).

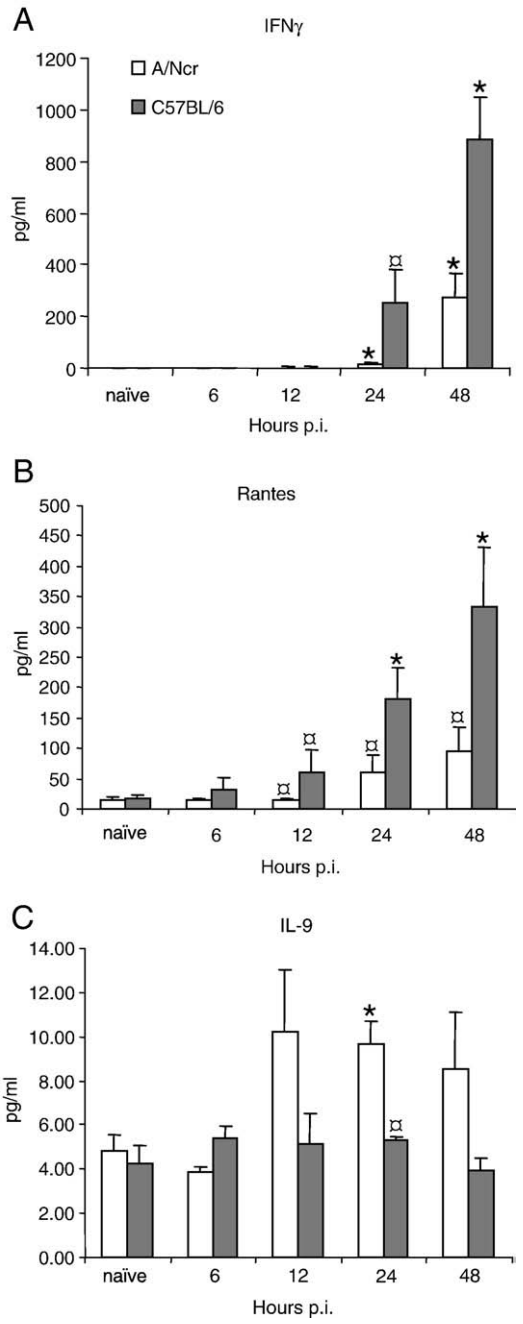

Fig. 5. Early cytokine changes at the PLN following infection. PLNs were harvested at 6, 12, 24 and 48 hours p.i. and 27 cytokines were monitored for changes. (A) IFNγ and (B) Rantes (CCL5) increased more rapidly in PLNs from the C57BL/6 strain. (C) The level of IL-9 remained constant in the C57BL/6 strain but was rapidly upregulated from 12 hours p.i. in the A/Ncr strain (*$P<0.05$ and ¤$P>0.05$).

differed between the strains. Higher levels of IFNγ were detected at 24 and 48 hours p.i., compared to those of the A/Ncr strain (Fig. 5A). In addition to elevated IFNγ, the C57BL/6 had a rapid increase in Rantes (CCL5) as early as 6 hours p.i. After 6 hours p.i. the level of Rantes continued to increase to significantly higher levels, and more rapidly than those of the A/Ncr strain at 12, 24 and 48 hours p.i. (Fig. 5B). This chemotactic chemokine is responsible for recruitment of T cells, eosinophils, basophils and NK cells (Kuna et al., 1992; Maghazachi et al., 1994; Schall et al., 1990). Interestingly, Parker et al have previously observed that NK cells are crucial for recovery from mousepox and the trafficking of IFNγ secreting NK cells to the PLN peaks at 56 hours p.i. following an ECTV footpad infection in C57BL/6 mice (Parker et al., 2007a).

We found no significant differences in the response of all other cytokines from both strains except for the response of IL-9 in the A/Ncr strain which increases from 12 hours p.i., in contrast, the C57BL/6's levels of IL-9 do not change (Fig. 5C). The increase in IL-9 is suggestive of a Th2 biased response (Spellberg and Edwards, 2001) in the A/Ncr strain, this hypothesis is supported by the observation that A/Ncr mice produce significantly less IFNγ following an ECTV infection and do not upregulate the Th1 cytokine Rantes to the same level as the C57BL/6 strain does (Figs. 5A and B). Indeed, previous studies have shown that a distinct cytokine profile is employed by the resistant C57BL/6 strain that includes the upregulation of IFNγ and TNF in the PLN compared to the susceptible BALB/c (Chaudhri et al., 2004).

The C57BL/6 strains responds to antigen presentation more efficiently than the A/Ncr strain

In a number of diverse infectious disease models the C57BL/6 mouse has been shown to have radically different susceptibilities to severe disease compared to other inbred mouse strains (Heinzel et al., 1991; Wallace and Buller, 1985; Wu-Hsieh, 1989). In the ECTV model, data have suggested that this resistance is based partly on a more robust Th1 biased immune response (Chaudhri et al., 2004). To determine if the C57BL/6 mouse was immunologically more responsive than the A/Ncr strain, we evaluated the antigen presenting cells (APCs) of the PLN at various times p.i.

We found that APCs from the C57BL/6 strain incubated with splenic CD3+ T cells from vaccinated C57BL/6 mice generated significantly higher ^{3}H-thymidine counts at 24, 48 and 72 hours p.i. compared to the non-infected controls. In contrast, the ^{3}H-thymidine counts generated by incubating A/Ncr APCs with vaccinated splenic A/Ncr CD3+ T cells were not significantly different to the non-infected controls at any time points (Fig. 6A).

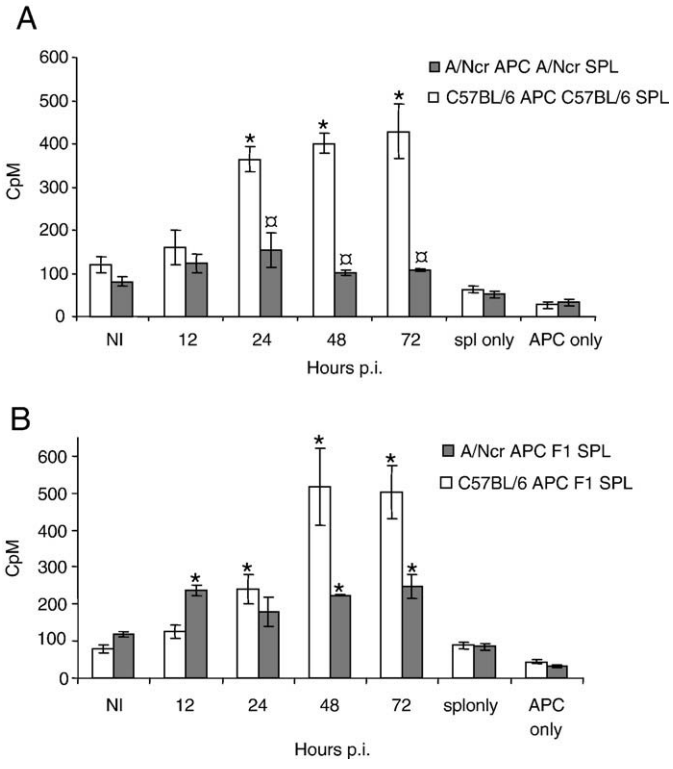

Fig. 6. C57BL/6 and A/Ncr responder cells react differently to antigen presentation by PLN cells. Antigen presenting cells (APCs) were incubated with CD3+ T cells taken from (A) vaccinated C57BL/6 and A/Ncr mice; and (B) from the F1 progeny of a C57BL/6 X A/Ncr cross (*$P<0.0035$ and ¤$P>0.05$).

In order to directly compare the activity of the APCs from the A/Ncr and C57BL/6 PLNs, we used memory splenic CD3+ T cells taken from F1 vaccinated progeny of C57BL/6 X A/Ncr mice. APCs from the C57BL/6 mice induced significant proliferation in the F1 CD3+ T cells compared to the non-infected controls from 24 hours p.i. (Fig. 6B). APCs from the A/Ncr strain also induced a lower, but statistically significant, rise in cellular proliferation from 12 hours p.i. (Fig. 6B). These data confirm that the PLN cells from the C57BL/6 strain induce better proliferation of responder cells than those of the A/Ncr strain. Thus, a reason for A/Ncr sensitivity to ECTV is likely to be a delayed, less robust immune response at the lymph node draining the site of infection.

CD4+ and CD8+ T cells provide differing levels of IFNγ in both strains

The previous experiments measured early cytokine synthesis and early APC function by the PLN that drains the site of infection. In order to compare the adaptive immune response, A/Ncr and C57BL/6 mice were infected with 3000 PFU of the attenuated EV-138 virus. This virus is non-lethal to the A/Ncr strain following a FP infection, therefore enabling us to measure immunologic changes that cannot be measured in the rapidly lethal wild-type virus (Fig. 1C). We found that following infection the C57BL/6's splenic cell count began to increase as early as 4 days p.i.; however, an increase in the A/Ncr's splenic cell count was not detected until 8 days p.i., indicating a slower immune response in this strain (Fig. 7A). Although the A/Ncr's spleen was slower to respond, we found that its infectivity, as determined by plaque assay, was 3 logs higher than that of the C57BL/6's at day 6 p.i. Moreover, infectious virus was found in the A/Ncr's spleen at 8 days p.i., by which time the C57BL/6 had cleared all splenic virus (Fig. 7B). A similar response was observed in liver samples which consistently had infective titres 2-3 logs higher in the A/Ncr strain compared to that of the C57BL/6 strain at days 6 and 8 p.i. (data not shown). We also measured the number of IFNγ+ CD4+ and CD8+ T cells

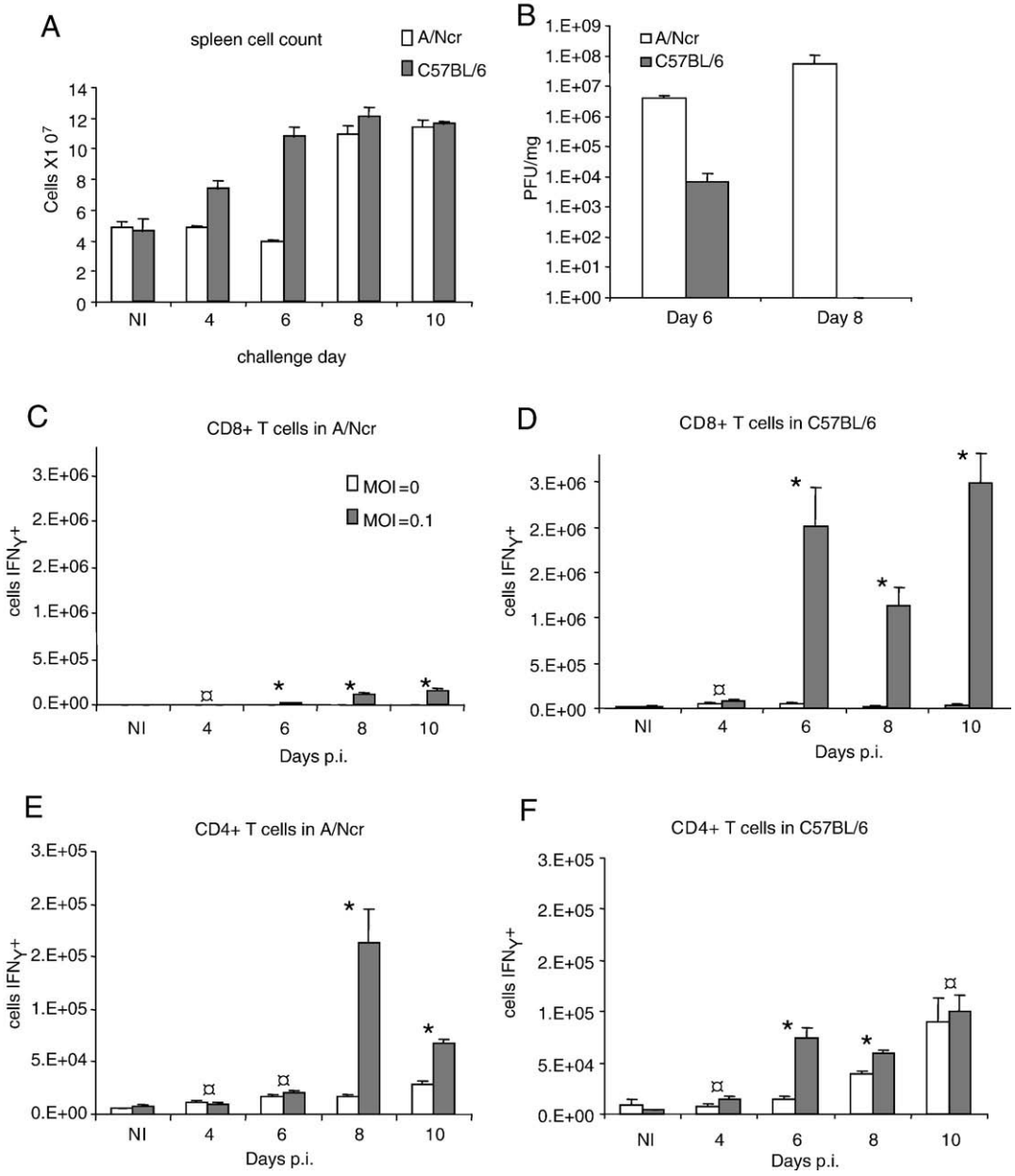

Fig. 7. CD4+ and CD8+ splenocytes release different levels of IFNγ. (A) Splenic cell counts in C57BL/6 mice infected with 3000 PFU of an attenuated (EV138) virus increased from 4 days p.i. A similar increase was not observed in the A/Ncr strain until 8 days p.i. (B) Infectivity titres in the spleens of A/Ncr and C57BL/6 mice. Infected (MOI = 0.1) and non-infected (MOI = 0) CD8+ splenocytes from A/Ncr (C) and C57BL/6 mice (D) were harvested and tested for IFNγ levels at days 4, 6, 8 and 10 p.i. CD4+ splenocytes were also harvested at the same time points and tested for IFNγ production (E and F) (*$P < 0.0037$ and ¤$P > 0.1$).

in the spleen. By 6 days p.i. we found a significantly large increase in the number IFNγ+/CD8+ cells in the C57BL/6's spleen; conversely, we found that the A/Ncr strain only had a small (2 logs lower than that of the C57BL/6) but significant increase in cell numbers from day 6 p.i. (Figs. 7C and D). With regard to IFNγ+/CD4+ cells, we found that the A/Ncr strain had a large increase in cells at day 8 and day 10. The C57BL/6 strain had small but significant elevations compared to the A/Ncr at days 6 and 8 (Figs. 7E and F); however, on a per cell basis the IFNγ being generated by the C57BL/6's T cells is at least 10 fold in excess of the IFNγ produced by the A/Ncr's T cells.

A delayed footpad infection therapeutically protects against an intranasal challenge

The protective immune response induced in C57BL/6 mice following subcutaneous infections with ECTV suggest a prophylaxis/therapeutic utility first demonstrated in humans with variola. Skin inoculation with VARV (variolation) was the first attempt of prophylaxis through vaccination and yielded mortality rates of 0.5-1% versus 20-40% for respiratory infections (Fenner et al., 1988). Anecdotal evidence suggests that vaccinia vaccination against smallpox is efficacious up to 4 days p.i. (Mortimer, 2003b). Human MPXV infections also appear to be milder following intra-dermal infection, although this may to be due to the virus strain or other factors (Cunha, 2004; Reed et al., 2004; Sejvar et al., 2004). To test if the C57BL/6 strain could be used to model the protective therapeutic effect of vaccination we evaluated an ECTV FP infection for efficacy against an ECTV IN infection. We found that 60% of mice infected IN with a 600 PFU inoculum died by day 13 p.i. and experienced a maximum of 28±0.8% body weight-loss and scruffy coats – both of which are indicative of disease (Figs. 8A and B). Mice infected via the FP route (also 800 PFU) survived and had a maximum weight-loss of 3.3±1.8%. Furthermore, we found that complete protection was conferred when mice were IN infected 24 hours post FP infection. Indeed, all mice survived even when infected via the FP and IN routes simultaneously, although these mice lost the most weight.

Because simultaneous FP and IN infections were sub lethal, we next investigated if a FP infection could confer resistance to an IN infection in a post-exposure therapeutic manor. To address this, we infected mice with 800 PFU of ECTV IN followed by 800 PFU FP inoculations on days 0, 1, 2 and 3 p.i. We found that the FP inoculations could protect all mice up to the last measured point of 3 days p.i. and that 80% of control animals were dead by day 12 p.i. ($p=0.02$) (Figs. 8C and D). These data suggest that post-exposure vaccination alone, as supported by other studies, or coupled with post-exposure antiviral therapy, should be considered as a therapeutic treatment for smallpox and/or human monkeypox in immuno-competent individuals (Mortimer, 2003b; Samuelsson et al., 2008; Paran et al, in press).

Conclusions

Taken together, our data reveal that the C57BL/6 strain has the potential to more accurately model smallpox and human-monkeypox

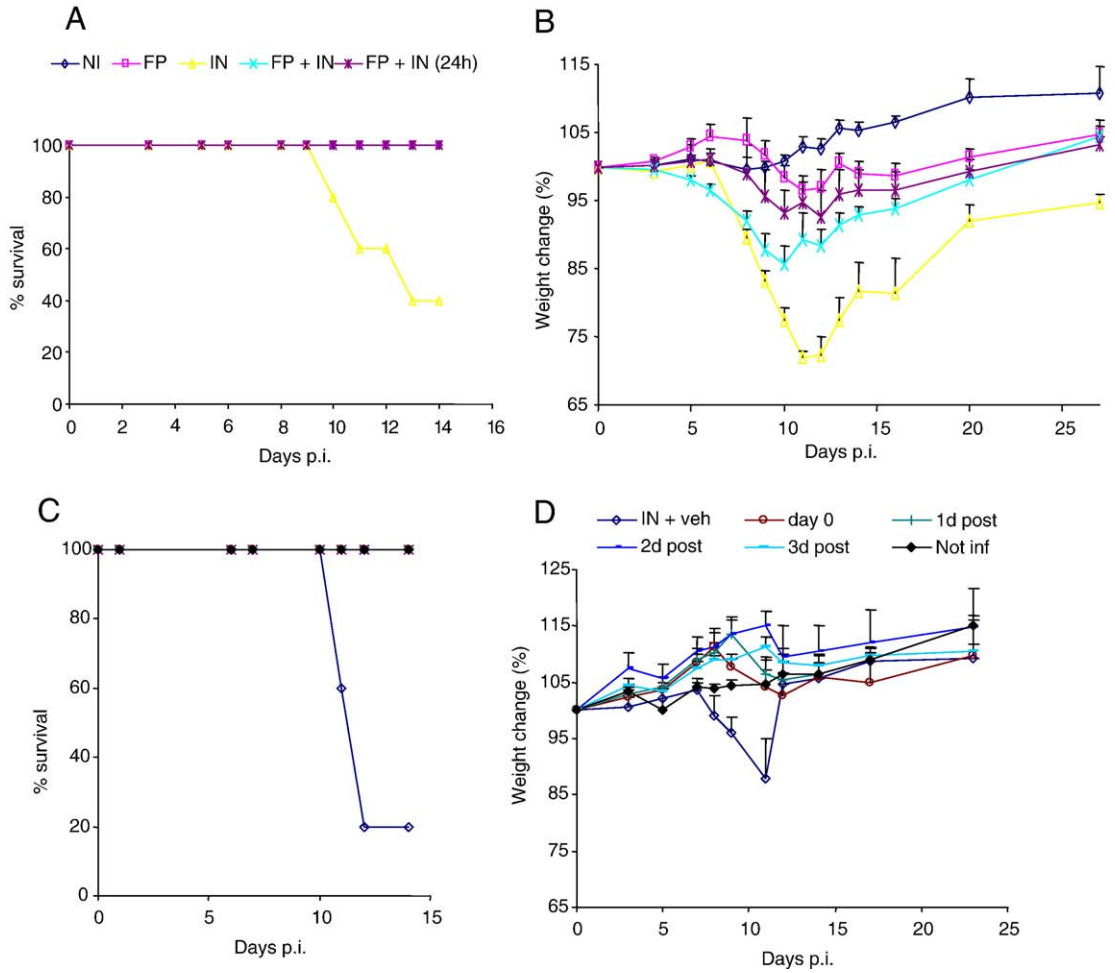

Fig. 8. A footpad infection protects the C57BL/6 strain against a lethal IN challenge. Survival of infected C57BL/6 mice: (NI, not infected; FP, footpad; IN, intranasal; FP+IN, footpad and intranasal together; and FP+IN (24 h), footpad infection administered 24 hours before IN infection; are shown). (B) Weight-loss of indicated groups. (C) The survival of mice infected IN followed by FP infections on days 0, 1, 2 and 3 p.i. are shown. (D) Weight changes.

compared to the A/Ncr strain. We conclude that the C57BL/6 mouse strain could be beneficial for testing CMX001 and other anti-orthopoxvirus drugs, and for therapeutic evaluation of vaccines. Comparing the kinetics and magnitude of various measures of the immune response between the C57BL/6 and A/Ncr strain suggests a basis for severe disease and may provide insights into the mechanisms of susceptibility of humans to orthopoxvirus infections.

Our studies further support the hypothesis that Th1 biased immune responses are associated with recovery from orthopoxvirus infected mice. It has also been demonstrated that humans vaccinated with vaccinia virus generate a Th1 biased response (Zaunders et al., 2006). Thus, it is likely that a Th1 biased response is necessary for recovery from human monkeypox or smallpox.

Materials and methods

Cells and virus

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). 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 and 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. A replication deficient virus (EV-138) and a non-attenuated virus encoding GFP (EV-GFP) were also used and purified as above.

Animals

Four to six week old female A/Ncr and C57BL/6 mice were obtained from the National Cancer Institute, Frederick MD., 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. Animal husbandry and experimental procedures were in accordance with PHS policy, and approved by the Institutional Animal Care and Use Committee. Each group consists of 5 animals. Each experiment was repeated thrice in various combinations. Mice were monitored everyday until 21 days p.i. and then on days 28, 35 and 42.

Antiviral compound

The CMX001 analogue of CDV was a gift from Chimerix Inc., (Durham, NC). CMX001 was freshly prepared prior to each experiment by dissolving the compound in sterile, distilled water. Drug was stored at 4 °C.

Viral challenge and tissue assays

Mice were anesthetised with 0.1 ml/10 g body weight of ketamine HCl (9 mg/ml) and xylazine (1 mg/ml) by intraperitoneal injections. Anesthetised mice were laid on their dorsal side with their bodies angled so that the anterior end was raised 45° from the surface; a plastic mouse holder was used to ensure conformity. ECTV was diluted in PBS without Ca^{2+} and Mg^{2+} to the required concentration and slowly loaded into each nare (5 μl/nare) or the FP (10 μl total). Mice were subsequently left in situ for 2-3 mins before being returned to their cages.

At indicated times following exposure to ECTV, groups of mice were treated by gavage with 0.1 ml sterile, distilled water (placebo) or water containing the desired concentration of CMX001. To determine infectious viral titres, mice were sacrificed post challenge, and lung, spleen, and liver tissues and nasal-wash were isolated. Tissue was ground in PBS (10% w/v), frozen and thawed three times, and sonicated for 20 seconds. Virus infectivity (PFU/ml) in tissue homogenates was estimated by titration on BSC-1 monolayers. Arithmetic means were calculated for PFU/ml values above the limit of detection (1×10^2 PFU/ml). Remaining mice were observed for clinical signs of disease (morbidity) and mortality. Moribund mice were euthanized. LD_{50} values were calculated as described (Reed and Muench, 1938).

Clinical chemistry assay

AST (aspartate aminotransferase) and ALT (alanine aminotransferase) analysis was executed on a Cobas Mira Plus Chemistry Analyser (Roche Diagnostics) using a modified International Federation of Clinical Chemistry method. The samples were diluted as needed to provide adequate sample volume for analysis or to bring the results within the linear range of the analyser.

Cell proliferation assay

Antigen presenting cells (APCs) from PLNs were removed from infected or control animals and a single-cell preparation was made in RMPI+0.1% β-mercaptoethanol (Gibco), hepes (cellgro), sodium pyruvate (cellgro) and 10% FCS (Hyclone III). 1×10^4 irradiated cells were plated in 96-well round-bottom plates (costar). Responder cells were isolated from 5 week post-vaccination animals that had been intradermally vaccinated in the flank with a replication deficient virus (ECTV-M138). Single-cell preparations were prepared in pharm-lyses (BD Pharmingen) as per manufacturer's instructions. CD3+ T cells were isolated by a pan T cell isolation (Miltenyibiotec) as per the manufacturer's instructions. 1×10^5 CD3+ T cells were incubated with APCs for 48 hours. Cultures were then pulsed with 1 μCi of ^{3}H-thymidine (MP Biomedicals,) for 12-16 hrs. The following day the cells were harvested onto a filter mat using a cell harvester (TomTec) and counts determined by a liquid scintillation counter.

Flow cytometry

Red blood cells were lysed with BD Pharm Lyse (BD Biosciences Pharmingen) and resuspended in RPMI-1640 (BioWhiticker) with 10% FCS (vida supra). 1×10^6 cells were blocked for 10 min with Fc Block (BD Biosciences Pharmingen) and stained with the following antibodies: Gr-1-FITC, CD11b-PerCP Cy5.5, CD3-APC 660, CD4-Pacific Blue 445, CD11c-PE-Cy7, CD8-AF700, CD55-PE 575, CD19-APC and CD45-Pacific Orange (all from BD Biosciences Pharmingen). Cells were fixed with 10% ultrapure formaldehyde (Polysciences Inc.) and analysed using a BD LSR II flowcytometer (BD Biosciences Pharmingen).

Cytokine staining

Antigen presenting cells were generated by infecting naïve splenocytes overnight at an MOI of 0 or 0.1. The cells were then irradiated at 2500 rads, labelled with CFSE (BD Biosciences) and co-cultured with responder populations overnight. Samples were incubated for 4-6 hours with Golgi Plug (BD Biosciences), washed with PBS-1% Fetal Clone II and stained for surface markers. The cells were fixed using Cytofix (BD Biosciences). For intracellular staining, the cells were washed twice in Permwash buffer (BD Biosciences) and incubated with anti-IFNγ antibody. The cells were washed twice in Permwash buffer and analyzed on the BD LSRII. Serum levels of IFNγ

were detected using a mouse IFNγ Flex Set (BD Biosciences) and assayed on the BD LSR II. Serum levels of cytokines were detected using mouse Flex Sets (BD Biosciences) as per manufacturer's instructions. The serum was diluted 1:4 in assay buffer prior to the addition of beads and detection reagent then analyzed on the BD LSR II.

A Bio-plex cytokine assay kit (Bio-rad) was used, as per manufacturer's instructions, for tissue supernatant cytokine staining. 23 cytokines were analysed: IL-1α and β; IL-2, -3, -4, -5, -6, -9, -10; IL-12p40 and IL-12p70; IL-13 and -17; G-CSF; GM-CSF; IFNγ; Eotaxin; KC; MCP-1; MIP-1a and-1b, Rantes and TNFα.

Microarrays

Total RNA was extracted from both PLNs using the RNeasy Mini Kit (Qiagen Inc,) according to the manufacturer's guidelines. The total RNA was qualified by running micro-capillary gels on RNA LabChips using the Bioanalyzer 2100 (Agilent Technologies). The quality was assessed by identifying the 18 s and 28 s ribosomal RNA bands to ensure RNA integrity. The total RNA was then quantified using the NanoDrop® ND-1000 Spectrophotometer (NanoDrop).

Global gene expression profiling of PLNs was performed using GeneChip® Mouse Genome 430 2.0 Arrays (Affymetrix, Inc). PLNs from the C57BL/6 and A/Ncr strains were extracted at naïve, 6, 12, and 24 hours p.i. (n=3). mRNA amplification/labelling and chip washing/hybridization was conducted according to the manufacturer's guidelines.

Data were normalized using accuracy/precision-optimizing gc-RMA algorithms, accounting for probe GC content (Wu and Irizarry, 2004, 2005). A balanced 2-way ANOVA was used to assess gene expression variability ($FDR \leq 0.01$) (Pavlidis, 2003; Reiner et al., 2003). Differentially expressed genes were statistically analyzed for gene functional category enrichment (i.e. Gene Ontology and Pathway) using the 2×2 Fisher's Exact Test implemented in the web-based DAVID platform (Hosack et al., 2003; Huang et al., 2007; Sherman et al., 2007).

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.

Acknowledgments

This work was supported by a subcontract from Chimerix Inc., and NIAID grant NOI-AI-15436, subcontract CN01-A1-30063 to Southern Research and U54-AI-057169 from the NIAID to the Midwestern Regional Center of Excellence for Biodefense and Emerging Infectious Diseases (MRCE).

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