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Deletion of the rat cytomegalovirus immediate early 1 gene results in a virus capable of establishing latency but with lower levels of acute virus replication and latency that compromise reactivation efficiency

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Summary.

The IE1 and IE2 proteins encoded by the major immediate-early (MIE) transcription unit of cytomegaloviruses are thought to play key roles in the switch between latent and lytic cycle infection. Whilst IE2 is essential for triggering the lytic cycle, the exact roles of IE1 have not been resolved. An MIE-exon 4 deleted rat cytomegalovirus (ΔIE1) failed to synthesize the IE1 protein and did not disperse promyelocytic leukemia bodies (PML) early post-infection, but it was still capable of normal replication in fibroblast cell culture. However, ΔIE1 had diminished

ability to infect salivary glands persistently in vivo and to reactivate from spleen explant cultures ex vivo. Quantitation of viral genomes in spleens of infected animals revealed a reduced amount of ΔIE1 virus produced during acute infection, suggesting a role for IE1 as a regulator in establishing a chronic or persistent infection, rather than in more directly influencing the latency or reactivation processes.

Main Text.

The cytomegaloviruses (CMV) are a large family of β-herpesviruses that are extremely species specific. While the initial infection is usually asymptomatic, the virus can become latent in multiple organs and reactivate during periods of immunosuppression. Like other herpesviruses, the CMV's display an ordered cascade of gene expression during productive infection (Mocarski *et al.*, 2007). The immediate early (IE) genes are expressed shortly after viral entry into the cell and the major immediate early (MIE) mRNA is expressed in the presence of cycloheximide *in vitro*. The IE proteins appear to regulate the expression of many downstream viral genes and are believed to be critical for reactivation processes following the transcriptional activation of the MIE CMV region (Stinski & Isomura, 2008).

The structural organization of the MIE regions of human CMV (HCMV), African green monkey CMV (SCMV), murine CMV (MCMV), and the rat CMVs (RCMV) are very similar to each other (Meier & Stinski, 2006; Sandford *et al.*, 1993). An enhancer region lies upstream of two spliced genes which share an untranslated leader sequence (exon 1), exon-2 which contains the first ATG, and exon-3. Two proteins are expressed from the MIE locus. In HCMV and RCMV, these are IE1 and IE2, and IE1 and IE3 in MCMV. Thus exon-4 is spliced to exon 2/3 to form IE1 while alternate splicing of exon-5 to exon 2/3 forms IE2 (IE3 in MCMV, respectively;

see Fig. 1a) (Keil *et al.*, 1987; Messerle *et al.*, 1992; Sandford *et al.*, 1993; Stinski *et al.*, 1983). No viable IE2/IE3 deletion mutants of any CMV have been isolated, suggesting that IE2/IE3 is absolutely essential for viral replication even in cell culture (Angulo *et al.*, 2000; Marchini *et al.*, 2001; White *et al.*, 2004), but IE1 is generally considered non-essential in cell culture, especially at high MOI.

Prevention of virus latency or reactivation could provide potential attractive targets for therapeutic intervention, but at present very little is known about these processes in CMV. Partly because of the high levels of CpG-suppression of IE1 exon-4 but not of the IE2 exon-5 coding regions compared to the rest of the viral genome (Honess *et al.*, 1989), and because of its association with metaphase chromosomes, which resembles a feature of EBV EBNA1 (Lafemina *et al.*, 1989), the HCMV IE1 protein has at various times been proposed to be involved in the establishment of or reactivation from latency. To study the influence of IE1 in this context, several deletion mutants have been constructed and analyzed. Both partial and complete IE1 exon 4 deletion mutants of HCMV have been described (Ahn *et al.*, 1998; Greaves & Mocarski, 1998; Huh *et al.*, 2008; Lee *et al.*, 2004; Mocarski *et al.*, 1996). They are characterized by defective replication at a low multiplicity of infection (MOI) in primary human fibroblasts, a slower lytic replication cycle especially in PML-over-expressing cells, greatly reduced ability to form or spread as plaques after DNA transfection, increased apoptosis, and a greater susceptibility to inhibition by beta-interferon, but none of these have or could be assessed in vivo in human hosts or animal models.

Rodent CMVs have long provided well-established animal models to study latency and reactivation in vivo (Reddehase *et al.*, 2002; Reddehase *et al.*, 2008). In analogy to HCMV, an MCMV IE1 deletion mutant has been constructed that replicates similar to wild-type (WT) virus at both high and low MOI in vitro, but is attenuated in vivo (Ghazal *et al.*, 2005). Here, we

73 investigated the influence of IE1 on RCMV (English isolate) replication in vitro and in vivo as
74 well as its ability to reactivate ex vivo.

75 Despite the similarity in overall MIE region structural organization, there is little
76 homology (just 15% identity) at the predicted amino acid level between human, old world
77 primate and rodent CMV IE1 proteins. The major conserved feature in exon 4 is a glutamate rich
78 domain found toward the C terminus in all versions. Therefore, they could play very different
79 roles or act through very different mechanisms. To examine the role of IE1 during RCMV
80 replication, we constructed an IE1 mutant virus with a completely deleted exon 4 (ΔIE1ex4).
81 Virion DNA of a previously described recombinant RCMV with the endogenous RCMV
82 enhancer replaced by the MCMV enhancer preceded by a lox-lacZ-lox cassette (Sandford *et al.*,
83 2001) was co-transfected with a transfer vector lacking exon 4 (Fig. 1b) into rat embryo
84 fibroblast (REF) cells and providing IE1 complementation in trans. Homologous recombination
85 in REF cells resulted in the loss of the lacZ expression cassette and thus in white plaques, and
86 also in reconstitution of the endogenous RCMV enhancer (Fig. 1b). A revertant virus (RevIEex4)
87 was also constructed as a control for the absence of other inadvertent genetic defects by co-
88 transfection of virion DNA with the revertant transfer vector as above except that screening was
89 for β-galactosidase-positive virus. RevIE1ex4 was passaged through a REF cell line expressing
90 the Cre protein, which resulted in the loss of the lacZ cassette with one loxP site remaining in the
91 virus. Both recombinant viruses were purified by limiting dilution and analyzed by Southern blot
92 (Fig. 1c) and DNA sequencing of the MIE region. Relative total virion DNA/pfu ratios were
93 measured to show that ΔIE1ex4 does not have many more non-infectious particles than WT and
94 that adjacent exons are unaffected (Fig. S1 and S2).

95 To verify that the lack of exon 4 resulted in the lack of IE1 protein expression in infected
96 cells at low MOI, we performed Western blot analysis using a polyclonal rabbit antiserum that

recognizes a domain within exon 3 of both IE1 and IE2. Thus, the 68-kDa IE2 protein could be detected at normal levels, but as expected ΔIE1ex4 failed to express any of the 92-kDa IE1 protein (Fig. 1d). Next we compared growth characteristics of ΔIE1ex4 with WT RCMV and RevIE1ex4 in fibroblast tissue culture. As has been shown for MCMV (Ghazal *et al.*, 2005), ΔIE1ex4 replicated with almost identical efficiency to WT and RevIE1ex4 viruses at both low and high MOI in this highly permissive cell type (Fig. 1 e and f).

The dispersion of promyelocytic leukemia (PML) nuclear bodies is thought to increase viral transcription efficiency. Both the HCMV and MCMV IE1 proteins are responsible for the disruption of PML-associated nuclear bodies very early after infection (Ahn *et al.*, 1998; Ahn & Hayward, 1997; 2000; Ghazal *et al.*, 2005; Nevels *et al.*, 2004; Tang & Maul, 2003; Tavalai *et al.*, 2006; Wilhelmi *et al.*, 2008; Wilkinson *et al.*, 1998; Xu *et al.*, 2001). To reveal a possible role for RCMV IE1 in PML disruption, REF cells were either mock-infected or infected with WT RCMV, ΔIE1ex4, or RevIE1ex4. PML bodies were detected by a polyclonal antiserum (Ahn *et al.*, 1998) 8 hrs post-infection and visualized by fluorescent microscopy (Fig. 2a). Whereas both WT and RevIE1ex4 dispersed PML bodies normally, ΔIE1ex4 did not. Both IFA and Western blotting confirmed that there was no change in the pattern or levels of expression of the IE2 protein in typical punctate pre-RC structures from the mutant virus. Therefore, since ΔIE1ex4 replicated efficiently in fibroblast cell culture, it seems that the dispersion of PML is not a prerequisite for RCMV to replicate in vitro.

In the rat animal model, WT virus becomes detectable in the salivary gland (by direct virus culture) by two weeks post-infection where it persists for at least 12 weeks thereafter, but is cleared from the spleen by one week post-infection before latency is established (not directly culturable). To investigate IE1 biological function in vivo, we compared infections of rats with

121 WT, ΔIE1ex4, and RevIE1ex4, respectively, using 1 x 10^7 PFU administered intraperitoneally.
122 We monitored acute infection by harvesting spleens at 3 days and salivary glands at 16 days post-
123 infection. In spleens, all viruses could be detected in the infected animals by a direct plaque assay
124 (Table 1). However, virus could be detected in all salivary glands of animals infected with either
125 WT or RevIE1ex4, whereas only 5 out of 8 animals infected with ΔIE1ex4 were positive with a
126 ten-fold decrease in viral plaques. Thus, ΔIE1ex4 can replicate normally in the spleen during
127 acute infection and can spread to the salivary gland but appears to have reduced replication
128 efficiency. Next, we investigated virus persistence in spleen and salivary gland explants. As
129 expected, neither virus could be detected by direct plaque assay at 120 days post-infection in the
130 spleen. In salivary glands, both infectious WT and RevIE1ex4 virus were detectable in all 12
131 animals, but directly infectious ΔIE1ex4 virus was only detected in 4 out of 12 animals.

132 In our infected adult rat model, we define latency as resolution of productive infection on
133 the organismal level, such that virus cannot be detected by direct plaque assay culture, but can
134 still be detected as stable low levels of DNA and by explant co-cultivation; however, latency in
135 any organ can coexist with productive infection in the salivary glands, as shown in the mouse
136 model (Simon *et al.*, 2006). To examine whether IE1 plays a role in reactivation from latency in
137 explants at 120 days post-infection, spleens were excised and small pieces were co-cultured on
138 REF cells. Whereas spleens from all 12 WT and RevIE1ex4 virus infected animals consistently
139 reactivated after two weeks of culture, only one of 12 spleens from ΔIE1ex4-infected animals
140 reactivated three weeks after explant. Nevertheless, 6/6 of these spleens tested contained mutant
141 viral DNA as detected by PCR (Fig S3). Therefore, ΔIE1ex4 can become latent in the spleen, but
142 demonstrates a reduced ability to reactivate. This points to a substantial, nevertheless non-
143 essential role for IE1 in reactivation (since virus could be recovered from one spleen) from

144 latency ex vivo, as has recently been reported for an MCMV IE1 deletion mutant (Busche *et al.*,
145 2009).

146 Mock-infected animals were negative for infectious virus at all time points. In addition,
147 DNA was isolated from infected spleens harvested 120 days post-infection and measured for
148 latent DNA levels with an RCMV IE1-specific probe by quantitative PCR methods described
149 previously (Voigt *et al.*, 2007). Each sample was measured in duplicate and absolute quantities
150 were calculated using a calibration curve of serial dilutions of subcloned plasmids containing IE1
151 or c-myc DNA fragments. Total viral DNA levels were normalized by calculating the IE1/100
152 copies c-myc-ratio. For both the WT and RevIE1ex4 virus, ratios ranged between 0.03-6.2 for
153 wt-infected and 0.08-6.7 for RevIE1ex4-infected animals. In contrast, ratios for animals infected
154 with ΔIE1ex4 ranged from 0.005-0.04 (Fig. 2b). Therefore, ΔIE1ex4 evidently can become latent
155 in the spleen, but its ability to reactivate is severely compromised. Whereas, the overall effect of
156 restricted reactivation must be caused by the deletion in IE1, it is not necessarily related to any
157 direct qualitative deficiency of the mutant viral genomes present to become latent or to reactivate,
158 but may rather simply reflect the lower quantity of mutant virus present. Less mutant virus was
159 detectable in the spleen, and therefore the chance of it being reactivated is likely to be small
160 (Reddehase *et al.*, 1994). The IE1 deletion also results in an acute replication deficit which is in
161 accordance with a lower titer in the salivary gland at day 16 post-infection. But because ΔIE1ex4
162 is attenuated in vivo, the infection conditions likely did not provide equal levels of multiplication
163 of the viruses, and thus do not produce an equivalent viral genome load during latency.
164
165 To exclude a contamination with WT or RevIE1ex4 virus in this one reactivated animal
166 and to see if the reduced acute replication in vivo plus reduced reactivation of ex vivo ΔIE1ex4
167 was related to the lack of IE1 protein expression, viruses were isolated from salivary glands that

had been persistently infected, as well as from spleens from which virus had been reactivated ex vivo. Recovered viruses were grown on REF cells and lysates were analyzed by Western blot assays. Again only 68-kDa IE2 but not 92-kDa IE1 protein was detected (Fig. S4).

Overall, our results indicate greatly reduced levels of acute replication by RCMV lacking the IE1 gene in the in vivo model in the salivary gland, which evidently leads to much lower levels of stably maintained latency and of reactivation in the spleen. The results are consistent with the findings of reduced virulence and lower virus titers for MCMV lacking IE1 in adult BALB/c and SCID mice (Ghazal *et al.*, 2005), although they did not address latency or reactivation. Similar findings were also recently described in a newborn mouse model (Busche *et al.*, 2009). However, using 10 to 100-fold higher compensatory levels of MCMV lacking IE1 compared to wild-type controls to generate equal levels of established latency, the latter authors measured stable latency and used three distinct criteria for reactivation (including lung explants), but found no evidence for a role of IE1 in these two latter aspects of MCMV biology. Therefore, we can only conclude that there is a strong positive effect of IE1 on acute infection levels in vivo, whereas it is quite plausible that RCMV ΔIE1ex4 (like MCMV) has no defect in the ability to maintain or reactivate from latency.

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Figure and Table legends.

Fig. 1. Construction and characterization of recombinant viruses. (A) Structural organization of the MIE region of RCMV. The MIE enhancer (Enh) drives the two main IE transcripts, IE1 and IE2, by alternative splicing. (B) Isolation of mutant viruses following homologous recombination in REF/IE1 cells. Recombinant viruses were analyzed by *Hin*dIII digest, southern blot (using a probe directed against exon 4 (C)) and sequencing. The deletion of the IE1 protein was confirmed in Western blot analysis using a polyclonal antiserum directed against a peptide within exon 3 of the RCMV MIE region, and therefore detects both the IE1 and IE2 proteins (D). For comparison of growth capacities, one-step (MOI 0.01; E) and multi-step (MOI 5; F) growth curves were done with WT RCMV, ΔIE1ex4, and RevIE1ex4.

Fig. 2. Analysis of IE1 function in fibroblast cell culture and in vivo. (A) IE1 is needed for disruption of PML bodies at 8-h after infection of REF cells. REF cells were either mock-infected or infected with WT RCMV, ΔIE1ex4, or RevIE1ex4 with an MOI of 3 and subjected to double-label IFA with polyclonal antisera against IE1/IE2 exon3 (FITC, green) and PML (rhodamine, red). Nuclei were stained with DAPI (blue). IE2 was detected as punctate pre-RC domains in all three viruses. (B) Quantitative PCR of DNA extracted from latently infected rat spleens 120 days after infection with WT RCMV, ΔIE1ex4, and RevIE1ex4.

Table 1. Detection of WT RCMV, ΔIE1ex4, and RevIE1ex4 in Sprague Dawley rats at different time points after infection. Acute infection was monitored in spleens 3 days post-

infection (6 animals each) and in salivary glands 16 days post-infection (8 animals each). WT virus and RevIE1ex4 could be detected in all spleens and salivary glands, whereas ΔIE1ex4 was only detectable in 5 out of 8 salivary glands with an approx. 10-fold lower amount of virus present. ΔIE1ex4 was also found in only 4 out of 12 animals in salivary glands 120 days post-infection In contrast to WT virus and RevIE1ex4, ΔIE1ex4 could only be reactivated from 1 out of 12 spleens ex vivo.

Supplementary Figure legends.

Fig. S1. Southern blot analysis (using the DIG system, Roche) to measure the equivalency of pfu/particle ratios. Equal pfu input from sucrose banded viruses were analyzed by *Hin*dIII digest and hybridized using a 550-bp probe directed against the e128 gene that is located in the adjacent 7.2 kb RCMV Hind J fragment.

Fig. S2. PCR analysis to verify the integrity of exons 3 and 5 in ΔIE1ex4. Primers located in exons 3 and 5, spanning a region of 1.9 kb in the wild-type and revertant viruses and 390-bp in the mutant virus, were used for virion DNA amplification and sequencing. H_2O was used as a negative control.

Fig. S3. PCR analysis to detect viral DNA in animals infected with ΔIE1ex4. Splenic DNA was extracted from six rats and amplified with primers described in Fig. S2. As a positive control, recombinant (ΔIE1ex4) virion DNA was amplified. DNA from a mock (m) infected spleen could not be amplified, and splenic DNA from a WT-infected animal revealed a 1.9 kb band (not shown).

Fig. S4. Representative Western blot of virus isolated from salivary gland or reactivated from spleen from persistently (120 days) infected animals. Salivary glands (SG) and spleens (S) were harvested from rats infected with WT, ΔIE1ex4 or RevIE1ex4 viruses 120 days post-infection. REF: mock infected REF cells. M: mock infected salivary gland. WT: wild-type RCMV infected spleen.

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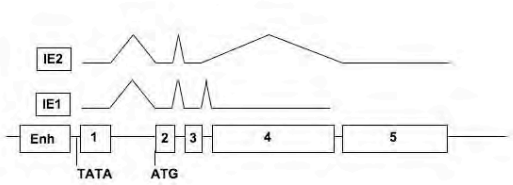
Figure 1

A



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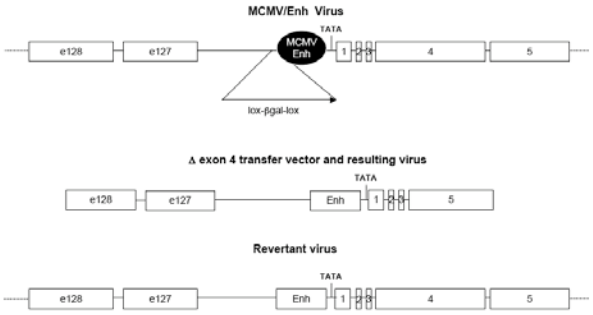

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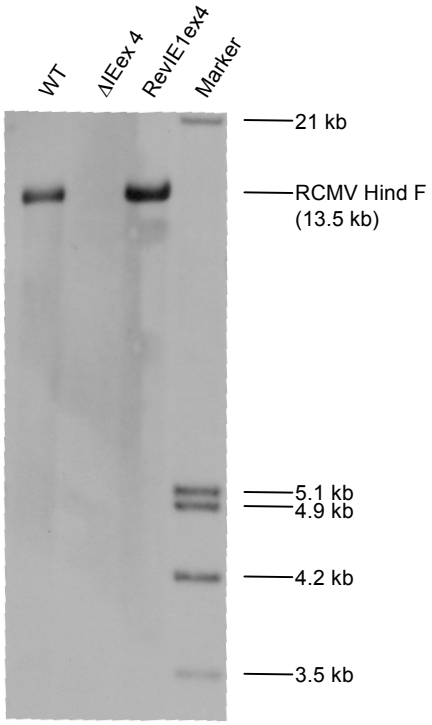

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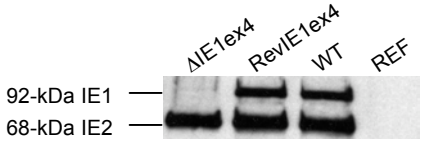

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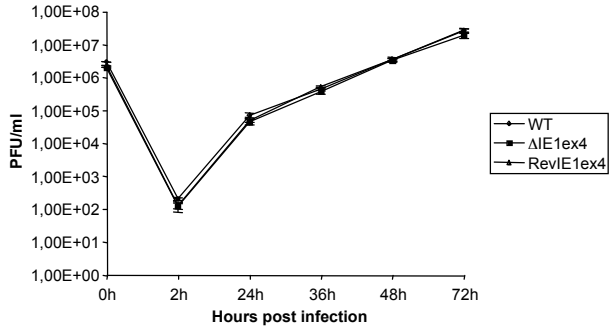
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0h
2h
24h
36h
48h
72h
Hours post infection
WT
ΔIE1ex4
RevIE1ex4

F

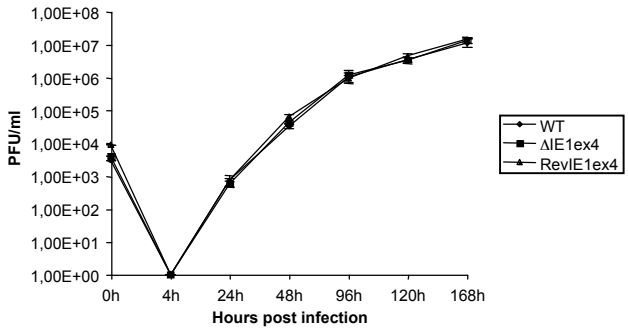
1,00E+08
1,00E+07
1,00E+06
1,00E+05
1,00E+04
1,00E+03
1,00E+02
1,00E+01
1,00E+00
PFU/ml
0h
4h
24h
48h
96h
120h
168h
Hours post infection
WT
ΔIE1ex4
RevIE1ex4

Figure 2A

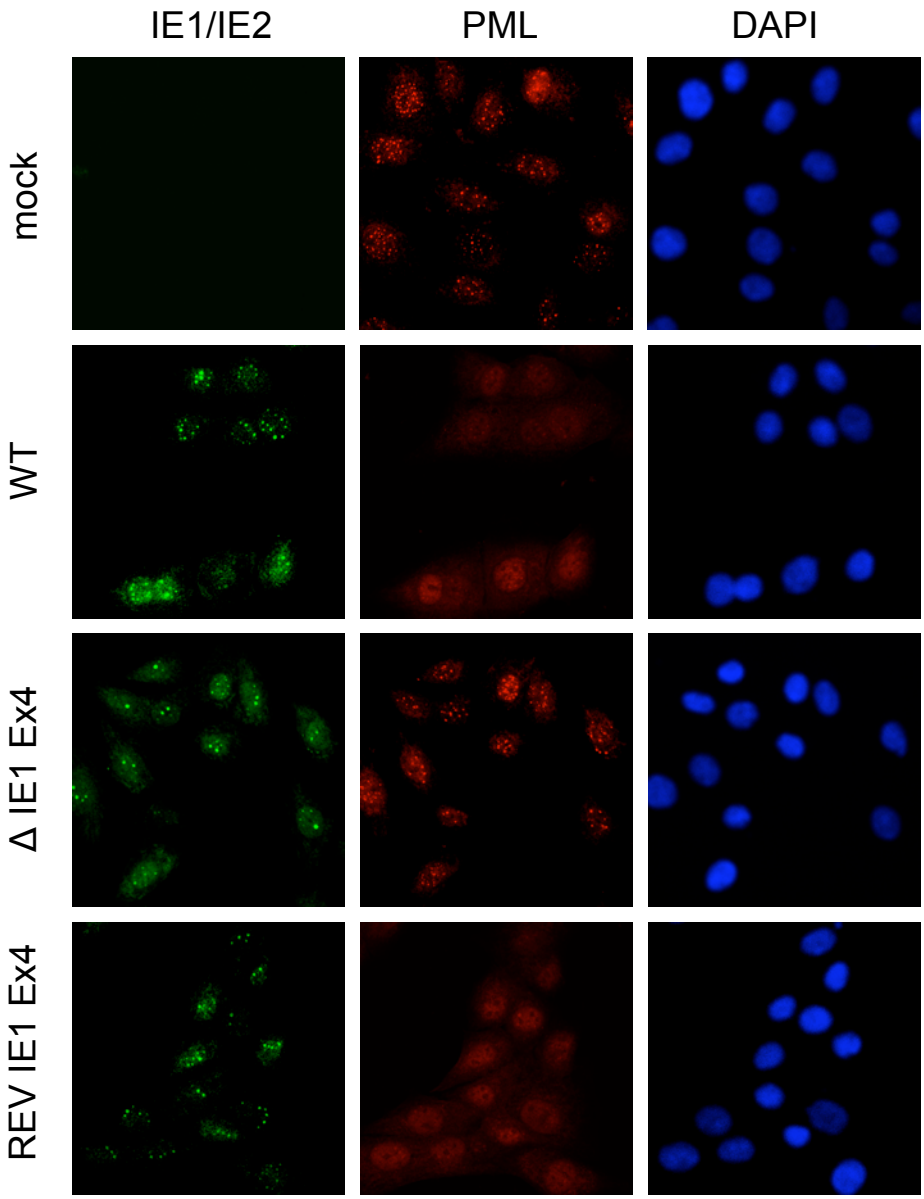

Figure 2B

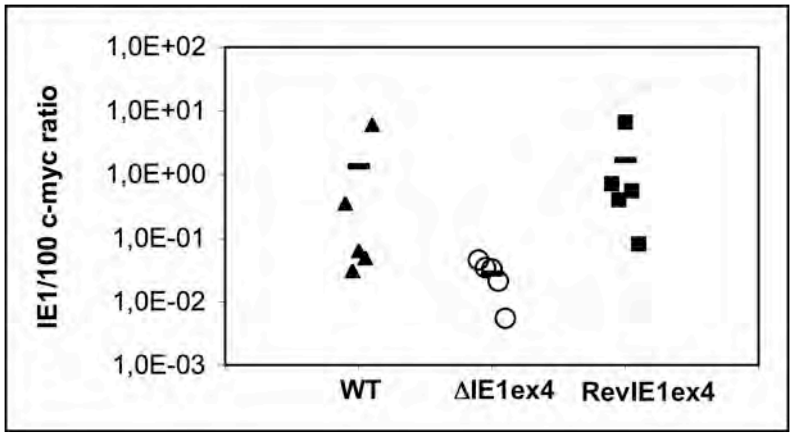
IE1/100 c-myc ratio
1,0E+02
1,0E+01
1,0E+00
1,0E-01
1,0E-02
1,0E-03
WT
ΔIE1ex4
RevIE1ex4

Table 1

Acute infection	**WT**	**RevIE1ex4**	**ΔIE1ex4**
Spleen day 3	6/6	6/6	6/6
Salivary gland day 16	8/8	8/8	5/8*
Persistent virus 120 days p.i.			
Spleen	0/12	0/12	0/12
Salivary gland	12/12	12/12	4/12
Reactivated virus 120 days p.i.			
Spleen	12/12	12/12	1/12

* approx. 10-fold less virus than WT