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Inactivation of the viral interleukin 1β receptor improves CD8$^+$ T-cell memory responses elicited upon immunization with modified vaccinia virus Ankara

Caroline Staib,[1] Sigrid Kisling,[1] Volker Erfle[1] and Gerd Sutter[1,2]

Correspondence
Gerd Sutter
sutge@pei.de

[1]GSF Institute of Molecular Virology and Institute of Virology, Technical University of Munich, Trogerstrasse 4b, 81675 München, Germany

[2]Department of Virology, Paul-Ehrlich-Institute, Paul-Ehrlich-Strasse 51-59, 63225 Langen, Germany

Interleukin 1 (IL1) is an important regulator of inflammatory responses and contributes to host immune defence against infection. Vaccinia virus encodes a viral soluble IL1β receptor (IL1βR), which modulates the acute-phase host response to infection and might influence the induction of immune responses against virus-associated antigens. Here, modified vaccinia virus Ankara (MVA) mutants defective in IL1βR production were produced by insertion of selectable marker gene sequences that precisely deleted the IL1βR coding sequences from the MVA genome (MVA-ΔIL1βR). Analysis of MVA mutants indicated that deletion of the IL1βR gene did not abrogate the formation of MVA progeny upon tissue culture propagation. After high-dose intranasal infection with MVA-ΔIL1βR, mice showed no signs of fever or other illness, suggesting that the avirulent phenotype remained preserved for MVA-ΔIL1βR. Following vaccination of mice, MVA-ΔIL1βR or non-mutated MVA induced similar acute-phase immune responses. Importantly, when monitored at the memory phase, significantly higher vaccinia virus-specific total CD8$^+$ and HLA-A*0201-binding peptide epitope-specific T-cell responses were found after vaccination of HLA-A*0201-transgenic and non-transgenic mice with MVA-ΔIL1βR. Moreover, 4–6 months after vaccination, MVA-ΔIL1βR provided higher levels of protection against lethal respiratory challenge infection with virulent vaccinia virus strain Western Reserve compared with wild-type MVA. These data suggest that deletion of the viral IL1βR gene may be considered a relevant approach to amplify the virus-specific CD8$^+$ memory T-cell response and duration of protective immunity obtained after MVA vaccination.

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INTRODUCTION

Modified vaccinia virus Ankara (MVA) is an attenuated strain of vaccinia virus (Mayr & Munz, 1964). Because of its avirulence found following inoculation of animals and its striking deficiency in producing substantial amounts of new viral progeny in most cells of mammalian origin, MVA can be used under laboratory conditions of biosafety level 1 (Mayr *et al.*, 1975; Meyer *et al.*, 1991; Carroll & Moss, 1997; Drexler *et al.*, 1998). MVA serves as an efficient vector virus for expression of recombinant genes (Sutter & Moss, 1992, 1995) and as a candidate recombinant vaccine (Moss *et al.*, 1996) with a high safety profile, since MVA has been tested for pre-immunization in over 100 000 humans being vaccinated against smallpox without causing notable side effects (Stickl *et al.*, 1974; Mayr *et al.*, 1978). Several MVA vector vaccines have already entered clinical evaluation (Corona Gutierrez *et al.*, 2002; Cosma *et al.*, 2003; McConkey *et al.*, 2003; Rochlitz *et al.*, 2003). Most recently, MVA was reassessed as a candidate second-generation vaccine against smallpox in comparison with immunization with conventional vaccinia virus strains (Belyakov *et al.*, 2003; Drexler *et al.*, 2003; Earl *et al.*, 2004; Wyatt *et al.*, 2004).

MVA was obtained by long-term serial passage in chicken embryo fibroblast (CEF) tissue cultures, which resulted in much loss of genomic information, including many genes regulating virus–host interactions (Meyer *et al.*, 1991; Antoine *et al.*, 1998). The MVA homologues of genes encoding recognized poxvirus immune-evasion molecules (reviewed by Moss & Shisler, 2001; Alcami, 2003) including the viral IFN type I and type II receptors, the interleukin 1β (IL1β) converting enzyme inhibitor SPI-2, the vaccinia complement-binding protein, the vaccinia semaphorin, the 35 kDa chemokine-binding protein and the tumour necrosis factor (TNF)-α receptor are deleted or fragmented. Interestingly, some viral genes with immunomodulatory

function are maintained in the MVA genome and their possible relevance for the use of MVA-based vaccines remains to be determined. One such example is the coding sequence for the viral IL1β receptor (IL1βR), which is highly conserved in the MVA genome (Antoine *et al.*, 1998), and expression of IL1βR in MVA-infected cells has been described (Blanchard *et al.*, 1998). IL1 is a cytokine that plays an important role in the regulation of inflammatory processes and the host innate immune response against infectious agents (reviewed by Sims, 2002). In contrast to its cellular counterpart, soluble viral IL1βR has a specific affinity only for IL1β (Alcami & Smith, 1992), the major endogenous pyrogen (Alcami & Smith, 1996). During vaccinia virus infection of mice, IL1βR was shown to prevent fever by interaction with IL1β. Furthermore, deletion of the IL1βR gene in vaccinia virus accelerated the appearance of symptoms of illness and mortality in intranasally infected mice, suggesting that the blockade of IL1β by vaccinia virus can diminish the systemic acute-phase response to infection and modulate the severity of the disease (Alcami & Smith, 1996). In contrast, virulence was reduced following intracranial infection of mice with IL1βR-deficient vaccinia virus, possibly reflecting a beneficial role of IL1β on brain-tissue infection (Spriggs *et al.*, 1992). Therefore, the viral IL1βR gene represents an interesting target for molecular engineering in the search for optimized vaccinia viruses as candidate second-generation vaccines. Here, we evaluated the effects of deletion of the IL1βR gene from the MVA genome. The construction of an MVA IL1βR deletion mutant allowed us to analyse the significance of IL1βR synthesis following *in vitro* and *in vivo* infection with MVA. Our data suggested that inactivation of the IL1βR gene may be beneficial for the development of future MVA vaccines.

METHODS

Viruses and cells. Vaccinia virus strain Western Reserve was originally provided by Bernard Moss (NIH, Bethesda, USA), vaccinia virus strains CVA and MVA were originally obtained from Anton Mayr (University of Munich, Germany), and CVA from the second and MVA from the 582nd passage on CEFs (cloned isolate F6) were used for this study. All viruses were propagated and titrated following a standard methodology (Moss & Earl, 1991). To generate vaccine preparations, viruses were routinely purified by ultracentrifugation through sucrose and reconstituted in 1 mM Tris/HCl, pH 9·0. Primary CEFs and rabbit kidney RK-13 (ATCC CCL-37) cells were grown in minimal essential medium (MEM) supplemented with 10 % fetal bovine serum (FBS) and maintained at 37 °C and 5 % CO_2.

Plasmids. The transfer plasmid pΔK1L-184R carries two DNA fragments that represent flanking sequences of MVA ORF 184R (nt 162021–163001, GenBank accession no. U94848) that have been inserted into multiple cloning sites 1 and 2 of plasmid pΔK1L (Staib *et al.*, 2000). One fragment, designated flank 184R-I, consists of a 486 bp MVA DNA sequence starting in the 5′-intergenic region of ORF 184R and ending at the start codon for translation of ORF 184R; the other fragment, flank 184R-II, is a 544 bp PCR fragment of MVA DNA extending from the codon for 184R translation termination into the 3′-intergenic region of the 184R gene.

Genetic modification of MVA. Mutant MVAs were obtained following the transient K1L-based host-range selection protocol as described previously (Staib *et al.*, 2000; Staib & Sutter, 2003). Briefly, for the generation of deletion mutant viruses, monolayers of 1×10^6 confluent CEFs were infected with MVA at an m.o.i. of 0·01 infectious units (IU) per cell. At 90 min post-infection (p.i.), cells were transfected with 1·5 μg plasmid pΔK1L-184R DNA using FuGENE (Roche) as recommended by the manufacturer. At 48 h p.i., transfected cells were harvested and plated on RK-13 cell monolayers for growth selection. Mutant viruses were isolated by plaque cloning on RK-13 cells and then passaged on CEF cells to remove the selectable marker gene K1L. For control purposes, we generated revertant virus MVA-IL1βRev using a standard methodology (Staib & Sutter, 2003), reinserting a DNA fragment comprising the complete IL1βR gene sequence under the transcriptional control of its authentic promoter at the site of deletion VI (depicted in Fig. 1) into the genome of MVA-ΔIL1βR.

Analysis of viral DNA by PCR and Southern blotting. Genomic viral DNA was isolated from infected CEFs as described previously and analysed by PCR using oligonucleotides annealing within the flanking regions flank 184R-I and -II, respectively

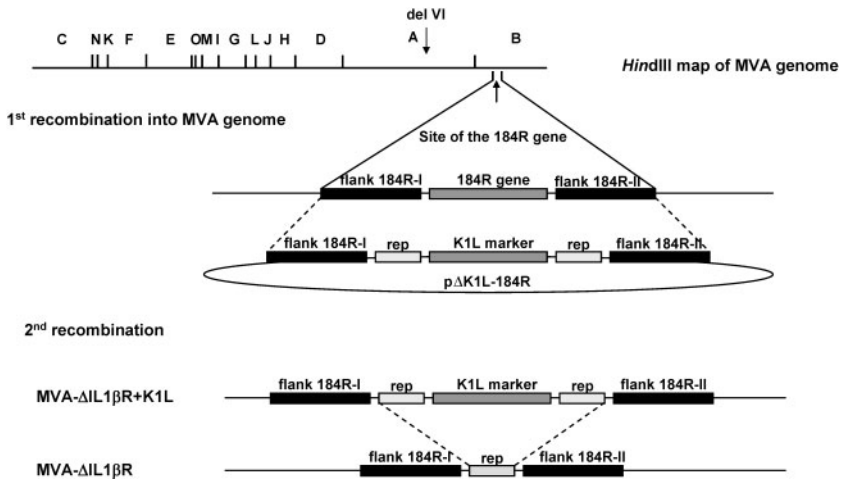

Fig. 1. Construction of IL1βR-deficient MVA. Upper panel, schematic map of the MVA genome. Sites of the restriction endonuclease *Hin*dIII within the genome of MVA are indicated. The position of the 184R ORF (IL1βR gene) is indicated. MVA DNA sequences adjacent to the IL1βR coding sequence (flank 184R-I and -II) were cloned into plasmid pΔK1L to allow transient insertion of the K1L gene by homologous recombination at the site of ORF 184R, resulting in deletion of this gene sequence. The final mutant virus, MVA-ΔIL1βR, was obtained after deletion of the K1L marker gene during a second step of homologous recombination involving synthetic repetitive sequences (rep). The site of deletion VI in the MVA genome (used for construction of revertant virus) is indicated (del VI).

(primer pair 1) (Staib *et al.*, 2000) or within flanking sequences of deletion site VI in the MVA genome (primer pair 2: Del-VI-5′, 5′-CCTGGACATTTAGTTTGAGTGTTCCTGAAT-3′, and Del-VI-3′, 5′-CTCAGCATCTAGTTGATATTCCAACCTCTT-3′). Specific DNA fragments were amplified by 30 cycles of PCR at an annealing temperature of 52 °C (pair 1) or 55 °C (pair 2).

Alternatively, total DNA isolated from virus-infected cells was digested with *Eco*RI, separated by gel electrophoresis in 0·8 % agarose, transferred to a Hybond-N membrane (Amersham) and hybridized to a DNA probe consisting of a PCR fragment from flank 184R-I sequences labelled with [α-^{32}P]dCTP. Prehybridization and hybridization were performed according to Sambrook *et al.* (1989). Blots were exposed to a Kodak BioMax film.

Radioimmunoprecipitation of virus-infected cell lysates. CEF cells grown in six-well tissue culture plates were infected with MVA at an m.o.i. of 20 IU per cell. At 6 h p.i., the virus inoculum was replaced with methionine-free MEM containing 5 % FBS and 50 μCi (1·85 MBq) [^{35}S]methionine ml^{-1} and incubated for a further 2 h at 37 °C. Cells were lysed in RIPA buffer containing 0·15 M NaCl, 0·01 M Tris/HCl pH 7·4, 1 % NP-40, 0·1 % SDS, 1 % sodium deoxycholate, 1 mM PMSF and incubated for 16 h at 4 °C with 50 % protein A–Sepharose coupled to rabbit polyclonal anti-B15R antibody (Alcami & Smith, 1992). Immune complexes were washed in RIPA buffer, dissolved in Laemmli buffer and proteins were separated by 10 % SDS-PAGE.

Analysis of virus growth. To determine low- or high-multiplicity growth profiles, confluent CEF monolayers (grown on six-well plates) were infected with 0·05 or 10 IU MVA or mutant MVA per cell, respectively. After virus adsorption for 60 min at 37 °C, the inoculum was removed. Cells were washed twice with RPMI 1640 and incubated with fresh RPMI 1640 containing 10 % FBS at 37 °C and 5 % CO_2. At multiple time points p.i., infected cells were harvested and virus was released by freeze–thaw and brief sonication. Virus titres were determined following standard procedures, as described previously (Hornemann *et al.*, 2003).

Cellular vaccinia virus responses. For *ex vivo* monitoring of peptide-specific acute- and memory-phase CD8$^+$ T-cell responses, splenocytes from vaccinia virus-immunized HHD mice were prepared and incubated for 5 h with HLA-A*0201-binding peptide (VP35#1; Drexler *et al.*, 2003) at 10^{-6} M. After 2 h, brefeldin A was added at a final concentration of 1 μg ml^{-1} (GolgiPlug; BD Biosciences Pharmingen). Splenocytes were live/dead stained with PBS containing 1 % BSA and 1 μg ethidium monoazide bromide (EMA; Molecular Probes) ml^{-1} and blocked for non-specific Fcγ III and -II receptor-mediated binding with 5 μg purified anti-CD16/CD32 (Fc Block; BD Biosciences Pharmingen) ml^{-1} for 20 min at 4 °C. Cell-surface staining was performed with phycoerythrin (PE)-conjugated anti-CD8 (clone 53-6.7) and allophycocyanin (APC)-conjugated anti-CD62L (clone Mel-14) for 30 min at 4 °C. After permeabilization of cells (Cytofix/Cytoperm kit; BD Biosciences Pharmingen), intracellular cytokine staining was performed for 30 min at 4 °C using FITC-conjugated anti-IFN-γ (clone XMG1.2) or FITC-conjugated anti-TNF-α (clone MP6-XT22) or the respective FITC-labelled IgG1 isotype control (clone R3-34) (all from BD Biosciences Pharmingen). Splenocytes were analysed by four-colour flow cytometry (FACSCalibur) using CellQest software (both from Becton Dickinson). For detection of *ex vivo* vaccinia virus-specific total CD8$^+$ T-cell responses, a fraction of the splenocytes, isolated from HHD or C57BL/6 mice 6 months after vaccination, was infected for 7 h with MVA prior to the addition of brefeldin A. After a further incubation for 16 h, cells were stained and analysed as described above. Statistical analysis (*f*- or *t*-test) was performed using GraphPad Prism 4 software. Vaccination experiments were performed at least three times.

Animal models. Female 6–8-week-old transgenic HHD$^{+/+}$ β_2m$^{-/-}$ D$^{b-/-}$ mice (HHD) (Pascolo *et al.*, 1997) or female 6–8-week-old BALB/c or C57BL/6 mice were used for vaccination experiments. HHD mice were inoculated with 0·25 ml virus vaccine by the intraperitoneal route and monitored for HLA-A*0201-restricted T-cell responses at days 10 and 180 post-immunization. For protection assays, animals were vaccinated once with 0·03–0·25 ml virus vaccine given by the intranasal or intraperitoneal route. At 3–6 months post-immunization, animals were anaesthetized, infected intranasally with vaccinia virus Western Reserve (10^7 p.f.u. diluted in 30 μl PBS) and monitored for at least a further 3 weeks for morbidity and mortality, with daily measurement of individual body weights and scoring of signs of illness as described previously (Alcami & Smith, 1996). Animals suffering from severe systemic infection and having lost >30 % body weight were sacrificed. The mean change in body weight was calculated as the percentage of the mean weight for each group on the day of challenge. Body temperature was determined with an Electronic Laboratory Animal Monitoring System (BioMedic Data Systems) using subcutaneously implanted microchip battery-free transponders and a DAS-5004 Pocket Scanner for data collection. Mean changes in body temperature were calculated by subtracting the pre-challenge (days −3 to 0) baseline temperature of each group from each subsequent time point. Experiments in animal models were performed at least twice.

RESULTS

Deletion of IL1βR coding sequences from the MVA genome

In order to analyse the possible role of IL1βR gene expression during MVA infection, we constructed MVA knockout mutants lacking ORF 184R (IL1βR). The coding sequences of viral IL1βR together with its presumed promoter sequence are well conserved within the MVA genome. As with the previously characterized IL1βR of vaccinia virus strain Western Reserve, the predicted MVA polypeptide consists of 326 aa with an identity level of 99 % (Alcami & Smith, 1992; Spriggs *et al.*, 1992; Antoine *et al.*, 1998). Using PCR, we amplified DNA segments located up- and downstream of the 184R coding sequence and inserted these fragments into the deletion vector pΔK1L (Fig. 1), which contains the vaccinia virus K1L gene as a selectable marker. Following transfection of MVA-infected cells with pΔK1L-184R, the 184R flanking regions allowed introduction of the K1L marker gene and simultaneous deletion of the IL1βR gene sequence in the MVA genome by homologous recombination. The resulting viruses were selected on RK-13 cells, where K1L function is essential for MVA growth. After isolation of clonally pure mutant viruses, the K1L marker cassette was removed following passage on CEF cells, yielding the final mutant virus MVA-ΔIL1βR (Fig. 1).

Molecular characterization and unimpaired *in vitro* replication of mutant virus MVA-ΔIL1βR

After isolation of the MVA deletion mutants, we first wished to confirm the correct removal of IL1βR coding sequences at the genetic level. We analysed viral DNA extracted from CEFs infected with wild-type, mutant or IL1β-revertant

MVA (MVA-IL1β-Rev) by PCR using oligonucleotide primers specific for MVA genomic sequences adjacent to the IL1βR gene locus or specific for the site of deletion VI within the MVA genome, the insertion site of the reintroduced IL1βR gene in MVA-IL1β-Rev (Fig. 2a). The IL1βR gene-specific PCR specifically amplified a 2·1 kb DNA fragment from wild-type templates, whereas the use of DNA from MVA-ΔIL1βR and revertant MVA-infected cells generated 1·1 kb PCR products corresponding to the expected reduction in size after deletion of ORF 184R. PCR analysis specific for MVA sequences at the site of deletion VI revealed the expected 306 bp DNA fragments for MVA-ΔIL1βR and MVA DNA, whereas the use of genomic DNA from revertant virus resulted in amplification of a 1·6 kb DNA fragment, suggesting the correct insertion of the complete IL1βR gene expression cassette. Furthermore, we digested viral DNAs with restriction endonuclease *Eco*RI and revealed DNA fragments containing the IL1βR gene locus by Southern blot analysis. Confirming the PCR data, we detected a 6500 bp *Eco*RI fragment in the genomic DNA of deletion mutant MVA-ΔIL1βR compared with a 7800 bp MVA DNA fragment (Fig. 2b), corresponding to the expected loss of 1·3 kb and again verifying correct deletion of the targeted ORF 184R sequences.

The IL1βR protein is produced during MVA infection (Blanchard *et al.*, 1998) and we wished to demonstrate that the generated mutants failed to synthesize this polypeptide and that expression was restored following infection with MVA-IL1β-Rev. Therefore, we performed immunoprecipitation experiments with polyclonal IL1βR-specific antibodies using lysates of metabolically labelled CEFs infected with MVA, MVA-ΔIL1βR or MVA-IL1β-Rev (Fig. 2c). The antiserum precipitated a specific protein of approximately 45 kDa from cell lysates obtained after infection with wild-type or revertant MVA corresponding in size to the glycosylated product of the IL-1βR polypeptide found in vaccinia virus Western Reserve-infected cells (Alcami & Smith, 1992). In contrast, this protein was not detected in lysates from mock-infected or MVA-ΔIL1βR-infected cells,

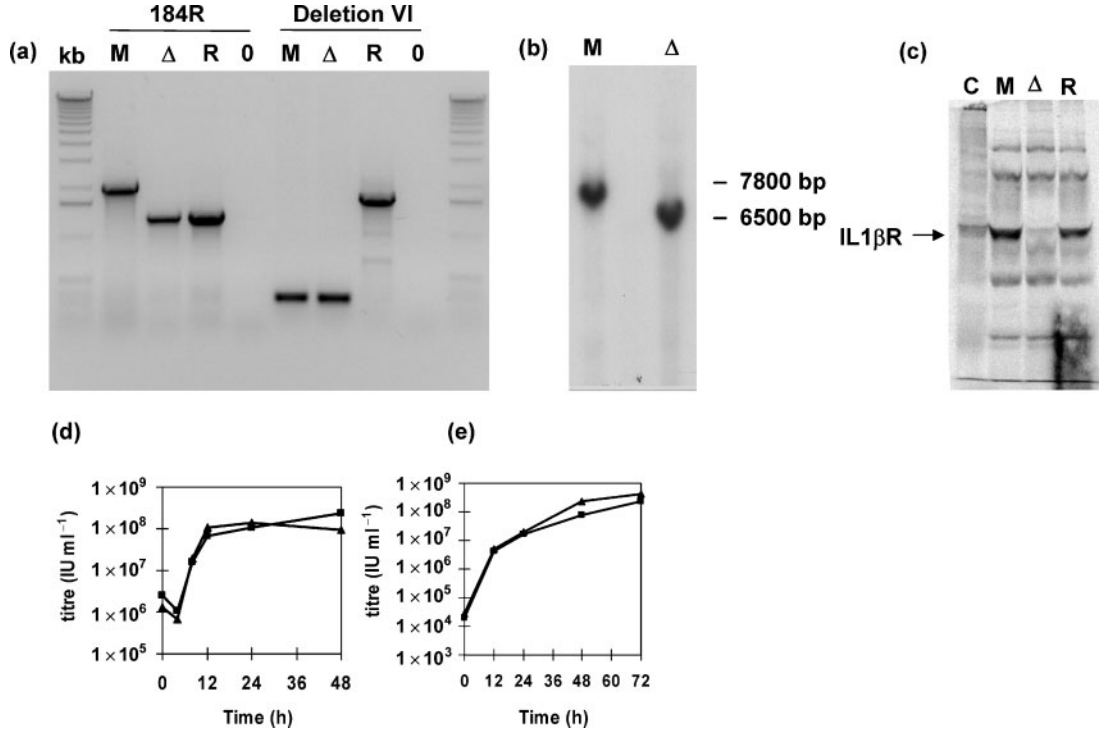

Fig. 2. *In vitro* characterization of MVA-ΔIL1βR. (a) PCR analysis of viral DNA. Genomic template DNA was prepared from cells infected with MVA (M), MVA-ΔIL1βR (Δ) or MVA-ΔIL1β-Rev (R) and incubated with oligonucleotide primers adjacent to the 184R gene locus or deletion VI to amplify specific DNA fragments. PCR products were separated by agarose gel electrophoresis. 0, PCR without template; kb, 1 kb ladder molecular mass marker (Invitrogen). (b) Southern blot analysis of viral DNA. Genomic DNA was prepared from MVA (M)- or MVA-ΔIL1βR (Δ)-infected cells, digested with *Eco*RI, separated by agarose gel electrophoresis and transferred to a nylon membrane. DNA fragments specific for the IL1βR gene locus were detected using a [^{32}P]dCTP-labelled specific probe. (c) Radioimmunoprecipitation of IL1βR protein from lysates of CEFs infected with MVA (M), MVA-ΔIL1βR (Δ) or MVA-ΔIL1β-Rev (R) or mock infected (C), and radiolabelled. Immunoprecipitation was performed using polyclonal anti-B15R antibody coupled to protein A–Sepharose. The arrow indicates the IL1βR protein. (d, e) Analysis of virus growth in CEFs after high (d)- or a low (e)-multiplicity infection with MVA-ΔIL1βR (■) or MVA (▲).

demonstrating that the generated deletion mutant virus failed to produce viral IL1βR. In addition, the data suggested that the revertant virus MVA-IL1β-Rev allowed synthesis of similar amounts of IL-1βR compared with wild-type MVA, despite the fact that the IL-1βR gene had been reintroduced at a heterologous site in the viral genome.

We also wanted to assess the replicative capacity of mutant MVA-ΔIL1βR in comparison with wild-type MVA. After infection of CEFs, we found comparable amounts of new viral progeny being formed with almost identical kinetics during one-step (Fig. 2d) and multiple-step (Fig. 2e) virus growth. These data clearly suggested that inactivation of MVA ORF 184R did not affect the *in vitro* multiplication of the virus.

Avirulence of MVA-ΔIL1βR following high-dose respiratory infection of mice

An important question was whether the inability to produce the viral IL1βR protein would influence the outcome of MVA infection *in vivo*. Previous work in mice with vaccinia virus Western Reserve deletion mutants revealed either enhancement of respiratory disease after intranasal infection (Alcami & Smith, 1992) or reduced virulence after intracranial infection (Spriggs *et al.*, 1992). The more severe respiratory infection appeared to be linked to induction of fever response and the functional activity of the viral IL1βR neutralizing IL1β as the major endogenous pyrogen (Alcami & Smith, 1996). Therefore, we tested mutant virus MVA-ΔIL1βR following intranasal infection of mice. Severity of disease in this mouse model is well reflected by changes in body weight and the appearance of characteristic signs of illness (Williamson *et al.*, 1990; Alcami & Smith, 1996; Drexler *et al.*, 2003; Reading *et al.*, 2003; Reading & Smith, 2003b). Additionally, we wished to monitor changes in body temperature because of the possible onset of febrile reactions. We transplanted BALB/c mice with subcutaneous microchip transponders to allow computable readings, and 1 week later infected the animals with 10^8 IU MVA or MVA-ΔIL1βR, or with 5×10^5 p.f.u replication-competent vaccinia virus CVA or 3×10^4 p.f.u Western Reserve as a control. Animals were monitored daily over a period of 3 weeks (Fig. 3). Infection of mice with MVA or mutant MVA-ΔIL1βR did not result in any obvious disease. In contrast, infection with the replication-competent viruses CVA and WR caused a drastic loss of body weight (Fig. 3a) and severe signs of illness, also reflected by a reduced body temperature (Fig. 3b). In MVA-infected animals, body temperature remained stable over the observation period. Taken together, these data suggested preservation of the attenuated phenotype of MVA after deletion of the IL1βR gene from its genome.

Early immune response induced by vaccination with MVA-ΔIL1βR

In the following experiments, we wished to assess the possible influence of deletion of the immunomodulatory IL1βR gene on MVA immunogenicity. First, we vaccinated HLA-A*0201-transgenic mice (HHD) with a single dose of MVA, deletion mutant or revertant virus and monitored the induction of vaccinia virus-specific $CD8^+$ T-cell responses directly *ex vivo* in the acute phase of the immune response using the orthopoxvirus-specific HLA-A*0201-restricted peptide epitope VP35#1 (Drexler *et al.*, 2003). By FACS analysis of freshly prepared splenocytes from vaccinated animals, we were able to detect 0·4–2·45 % activated $CD8^+$ T cells after immunization with MVA-ΔIL1βR, whereas in animals inoculated with MVA or revertant virus, levels of IFN-γ-releasing $CD8^+$ T cells ranged from 0·16 to 0·82 %. We analysed 12 individual mice per group for VP35#1-specific T-cell induction. However, the difference between the groups of vaccinees with regard to higher levels of T-cell immunogenicity elicited by MVA-ΔIL1βR (Fig. 4) was not statistically significant ($P = 0{\cdot}07$).

MVA-ΔIL1βR vaccination improves the T-cell memory response and long-term protective capacity

The mature form of the inflammatory cytokine IL1β has multiple effects *in vivo*, as revealed by the study of mice deficient for different components of the IL1 system (Sims, 2002). An active area of ongoing research on IL1 function is to elucidate the likely importance of this cytokine in protective T-cell immunity, including activation of professional antigen-presenting cells and memory T cells (Khayyamian *et al.*, 2002; Iwasaki, 2003).

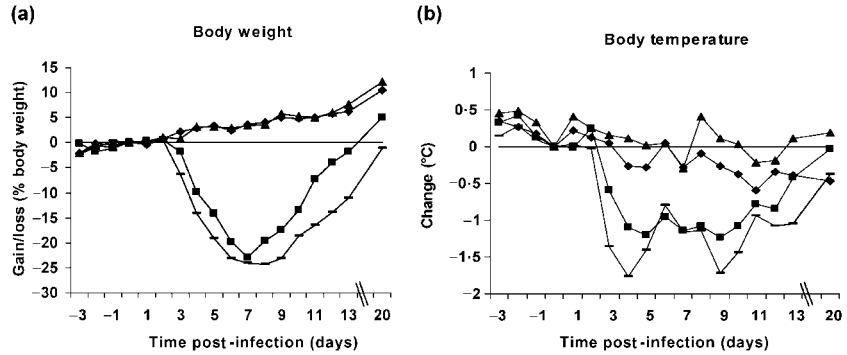

Fig. 3. Analysis of virus virulence in a mouse model for respiratory poxvirus infection. Characterization of infection with MVA, MVA-ΔIL1βR, CVA and strain Western Reserve. BALB/c mice ($n = 10$) were inoculated by the intranasal route with 1×10^8 IU MVA (◆) and MVA-ΔIL1βR (▲) or 5×10^5 p.f.u CVA (■) and 3×10^4 p.f.u Western Reserve (–). Body weight (a) and body temperature (b) were monitored daily and expressed as the mean for each group.

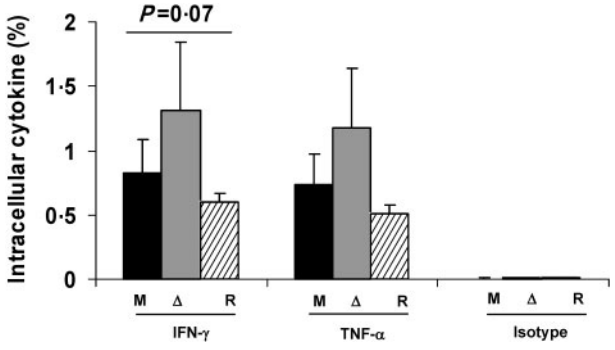

Fig. 4. *Ex vivo* analysis of vaccine-induced CD8$^+$ T cells. HHD mice were vaccinated with a single dose of MVA-ΔIL1βR, MVA or revertant virus. After 10 days, splenocytes were stimulated with the HLA-A*0201-restricted vaccinia-specific VP35#1 or influenza M1 58–66 epitope (irrelevant control) peptide, then stained with EMA, PE–anti-CD8, APC–anti-CD62L and FITC–anti-IFN-γ or -anti-TNF-α, or the respective FITC-labelled isotype control. Cells were analysed by flow cytometry for the presence of VP35#1 peptide-specific, activated (CD62L^{low}) CD8$^+$ T cells. The magnitude of the induced T-cell response is depicted as the percentage of cytokine-secreting CD8$^+$ T cells within the live (EMA$^-$) and CD8$^+$ cell population. Results are shown as the mean of 12 mice per group vaccinated with either MVA-ΔIL1βR (shaded bars), MVA (filled bars) or MVA-IL1βR-Rev (hatched bars). Error bars indicate SE $\times$ 1·96.

To investigate whether inactivation of viral IL1βR influenced the formation of memory T-cell responses, we vaccinated groups of HHD mice once with 10^8 IU MVA-ΔIL1βR, MVA or MVA-IL1β-Rev and monitored the presence of virus-specific T cells for more than 6 months after this primary immunization. We detected clearly higher levels of approximately 1·7 % VP35#1-reactive and IFN-γ-releasing splenic CD8$^+$ memory T cells in MVA-ΔIL1βR-immunized animals compared with vaccination with non-recombinant MVA or MVA-IL1β-Rev, which resulted in approximately 0·4 and 0·5 % epitope-specific IFN-γ-secreting CD8$^+$ T cells, respectively (Fig. 5a). This difference in favour of MVA-ΔIL1βR vaccination was statistically significant ($P<0·005$) and, interestingly, splenocytes from vaccinees of this group also contained significantly higher amounts of total vaccinia-specific CD8$^+$ memory T cells ($P<0·05$; Fig. 5a).

To monitor whether the different levels of memory T-cell responses coincided with alterations in protection, we challenged HHD mice by intranasal inoculation of 10^7 p.f.u vaccinia virus Western Reserve following vaccination with a single intraperitoneal inoculation of 10^8 IU MVA-ΔIL1βR or MVA more than 6 months previously (Fig. 5b). In mock-vaccinated control animals, infection resulted in the onset of respiratory disease, weight loss and death within 8 days of challenge. Mice inoculated with wild-type MVA were also affected by respiratory illness and substantial loss of body

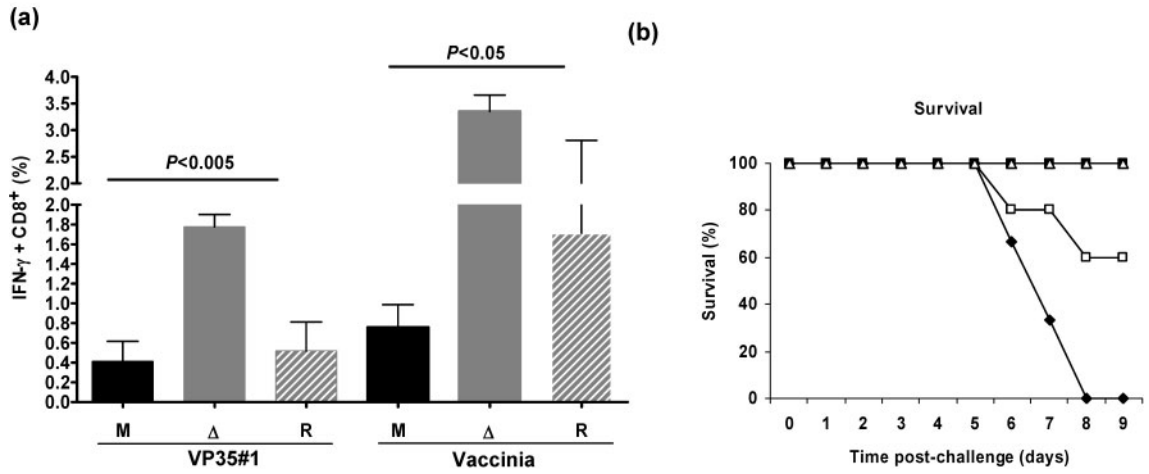

Fig. 5. *Ex vivo* analysis of vaccine-induced memory CD8$^+$ T cells and analysis of vaccine-induced long-term protection in HHD mice. (a) Mice were vaccinated intraperitoneally with a single dose of 10^8 IU MVA-ΔIL1βR, MVA or MVA-IL1β-Rev. After 6 months, splenocytes were either stimulated with the HLA-A*0201-restricted vaccinia-specific VP35#1 peptide or infected with MVA for detection of vaccinia-specific total CD8$^+$ T-cell responses. Cells were stained with EMA, PE–anti-CD8, APC–anti-CD62L and FITC–anti-IFN-γ or the respective FITC-labelled isotype control. Cells were analysed by flow cytometry for the presence of VP35#1 peptide or vaccinia-specific, activated (CD62L^{low}) CD8$^+$ T cells. The mean results from six mice are depicted following vaccination with MVA-ΔIL1βR (shaded columns), MVA (filled columns) or MVA-IL1β-Rev (hatched columns). (b) HHD mice ($n=5$) were immunized intraperitoneally with 10^8 IU MVA-ΔIL1βR (△) or MVA (□). At 6 months after vaccination, mice were challenged with 1×10^7 p.f.u vaccinia virus Western Reserve. Survival was monitored daily and expressed as the percentage of surviving animals per group. Mock-vaccinated (◆) and mock-challenged (■) mice served as control groups.

weight, although these animals were partially protected, since three of five mice in this group survived the challenge infection. Notably, all five animals receiving the MVA-ΔIL1βR vaccine were protected from challenge infection during a 3-week observation period, an outcome that was also reflected in a much lower level of weight loss and illness observed in this group (data not shown). This result implied that vaccination with MVA-ΔIL1βR could indeed have an influence on the durability of protective immunity.

The HHD mouse model allows convenient analysis of epitope-specific HLA-A*0201-restricted $CD8^+$ T-cell responses; however, possibly because of their knockout phenotype for mouse MHC class I, these mice develop unusually low numbers of total $CD8^+$ T cells (Pascolo *et al.*, 1997). As this phenotype might influence the analysis of total vaccinia-specific T-cell responses, we also assessed the number of total $CD8^+$ memory T cells induced by MVA-ΔIL1βR or MVA after vaccination of normal C57BL/6 mice (Fig. 6a). Again, in comparison with conventional MVA vaccination, we found significantly ($P=0{\cdot}003$) higher numbers of vaccinia-specific $CD8^+$ T cells in animals immunized with MVA-ΔIL1βR. These data strongly suggested an improved capacity of MVA-ΔIL1βR to elicit or maintain vaccinia virus-specific $CD8^+$ T-cell memory.

To investigate the long-term efficacy of MVA-ΔIL1βR immunization in more detail, we decided to test the vaccines in the well-established challenge model using non-transgenic BALB/c mice (Alcami & Smith, 1992). We chose intranasal vaccination, as this route of immunization of mice results in lower levels of circulating virus-specific antibodies compared with intramuscular or intraperitoneal vaccination (C. Staib & G. Sutter, unpublished data; Belyakov *et al.*, 2003), which might be an advantage when assessing the potential protective capacity of T-cell immunity. We inoculated groups of BALB/c mice with 10^6 and 10^7 IU MVA-ΔIL1βR, MVA or MVA-IL1β-Rev. Three and a half months after immunization, we subjected animals again to a respiratory infection with 10^7 p.f.u vaccinia virus Western Reserve (Fig. 6b, c). Importantly, all mice that had received the MVA-ΔIL1βR vaccine survived the challenge and animals in the group vaccinated with 10^7 IU MVA-ΔIL1βR demonstrated on average $<13\,\%$

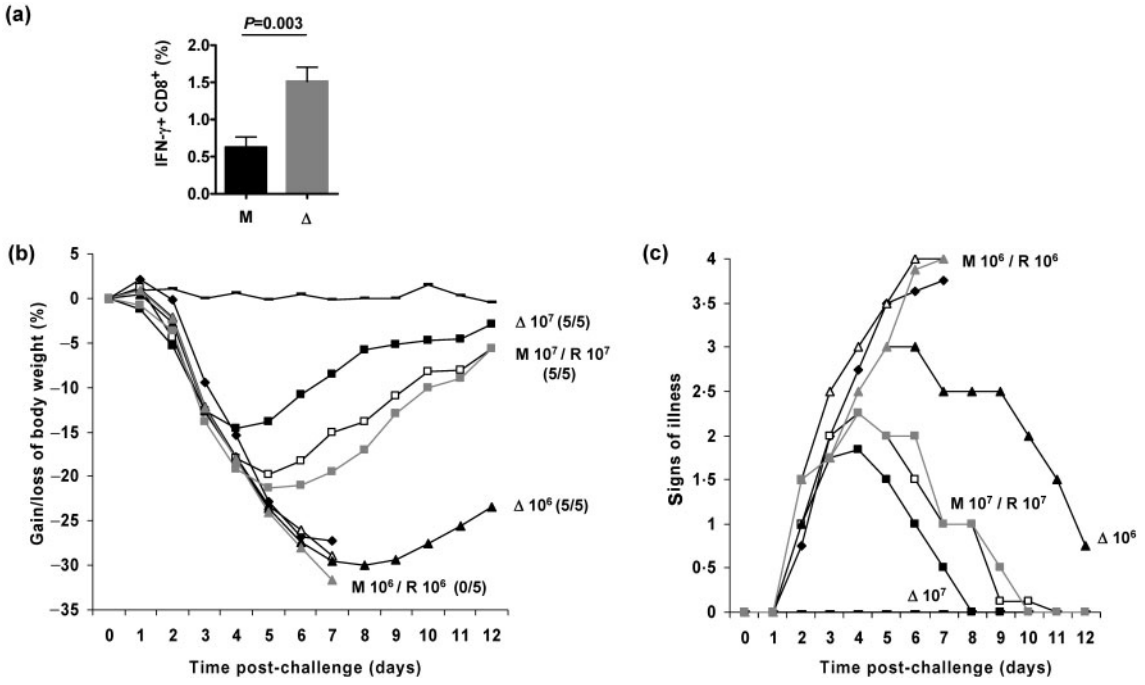

Fig. 6. *Ex vivo* analysis of vaccine-induced memory $CD8^+$ T cells and analysis of vaccine-induced long-term protection in non-transgenic mice. (a) C57BL/6 mice were vaccinated intraperitoneally with a single dose of 10^8 IU MVA-ΔIL1βR or MVA. After 6 months, splenocytes were infected with MVA for detection of vaccinia-specific total $CD8^+$ responses. Cells were stained with EMA, PE–anti-CD8, APC–anti-CD62L and FITC–anti-IFN-γ or the respective FITC-labelled isotype control. Cells were analysed by flow cytometry for the presence of vaccinia-specific, activated ($CD62L^{low}$) $CD8^+$ T cells. The mean results from six mice are depicted following vaccination with either MVA-ΔIL1βR (shaded column) or MVA (filled column). (b, c) BALB/c mice ($n=5$) were immunized intranasally with 10^6 (▲) or 10^7 (■) IU MVA-ΔIL1βR, 10^6 (△) or 10^7 (□) IU MVA, or 10^6 (▲) or 10^7 (■) IU MVA-IL1β-Rev. Three and a half months after vaccination, animals were challenged intranasally with 1×10^7 p.f.u Western Reserve. Weight (b) and signs of illness (c) were monitored daily in individual animals and are expressed as the mean for each group. Mock-vaccinated (◆) and mock-challenged (–) mice served as control groups. Numbers in parentheses indicate surviving animals per group.

reduction in body weight (Fig. 6b) and only mild signs of illness (Fig. 6c). In contrast, there was no protection from severe disease, resulting in death of all the animals, after inoculation with 10^6 IU MVA or MVA-IL1β-Rev vaccine (Fisher's exact test, $P=0{\cdot}029$). While vaccination with a higher dose (10^7 IU) of MVA or MVA-IL1β-Rev prevented death, animals in these groups showed a $\geqslant 20\,\%$ mean weight loss (Fig. 6b) and enhanced signs of disease (Fig. 6c).

DISCUSSION

The MVA ORF 184R encodes a vaccinia virus soluble receptor for IL1β with the proposed function of blocking inflammatory and febrile host responses to infection. The removal of putative immune evasion genes from viral genomes is a promising approach to elucidate further the roles of these regulatory virus proteins in the *in vivo* virus life cycle (Alcami, 2003). Moreover, application of this research to enable a virus such as MVA to be suitable for use as a (recombinant) live viral vaccine may lead directly to second-generation vaccines with rationally improved properties.

Despite using molecular engineering techniques allowing precise mutagenesis (Staib *et al.*, 2000, 2003), the resulting phenotypes of mutant viruses are unpredictable, and inactivation of the MVA IL1βR gene may serve as further example. Our finding that inactivation of the 184R ORF had no impact on the *in vitro* replicative capacity of the MVA mutant virus was not surprising, since growth deficiencies have not been reported with corresponding mutants derived from vaccinia virus Western Reserve (Alcami & Smith, 1992). Yet the capacity for high-level amplification is of the utmost importance for a virus to be used in vaccine production. Additionally, it should be noted that, with another MVA mutant defective in expression of the viral IFN response gene E3L, we recently found a very unexpected host range phenotype in CEFs, the preferred cell type for MVA vaccine production (Hornemann *et al.*, 2003).

In further experiments, we characterized the *in vivo* properties of MVA-ΔIL1βR. *In vivo*, the removal of an immunomodulator such as the viral IL1βR could have different consequences. On the one hand, it was possible that an unhampered IL1β activity elicited by infection with MVA-ΔIL1βR would have triggered strong inflammation reactions and febrile responses that resulted in adverse effects of vaccination. On the other hand, we speculated that IL1β action could be locally restricted and might influence the potency of MVA immunization in an adjuvant-like manner. Following intranasal infection of BALB/c mice with MVA-ΔIL1βR, we did not detect signs of respiratory illness, despite using high-dose inoculations and the fact that this mouse model system appears to be particularly suitable to assess potential pathogenic consequences of inflammatory responses to viral infection (Alcami & Smith, 1996; Reading *et al.*, 2003; Reading & Smith, 2003a). These findings confirmed that the avirulent phenotype of MVA had been conserved, even after the removal of IL1βR as a vaccinia viral factor having the potential to attenuate the pathogenicity of infection. Maintenance of attenuation is of utmost importance, especially with regard to MVA vaccine development, and may be a consequence of the particular MVA genotype, with other vaccinia virus regulatory or immunomodulatory genes being fragmented or deleted (Antoine *et al.*, 1998). Alternatively, it could be that the disease enhancement observed with vaccinia virus IL1βR deletion mutants requires active *in vivo* replication of the virus after intranasal infection. As there is good recent evidence confirming that MVA is unable to replicate *in vivo* in mice (Ramirez *et al.*, 2000) or macaques (Stittelaar *et al.*, 2001), our data might suggest that transient one-step infection with MVA-ΔIL1βR is simply not sufficient to result in IL1β activities inducing adverse systemic fever or inflammation reactions.

In first-vaccination experiments, we found a slight benefit of MVA-ΔIL1βR immunization when monitoring for acute vaccinia virus epitope-specific T-cell responses. The H3L gene product-derived epitope VP35#1 is the target of an immunodominant HLA-A*0201-restricted T-cell specificity and can be used to examine the induction of virus-specific $CD8^+$ T cells in an epitope-specific manner (Di Nicola *et al.*, 2003; Drexler *et al.*, 2003). In contrast, bulk analysis of total vaccinia virus-specific responses can provide a representative picture based on a multitude of different T-cell specificities, but might not allow us to assess subtle changes in the activation of single T-cell populations. Suspecting a possible effect on T-cell activation, we opted also to assess VP35#1-specific T-cell memory responses, which we had previously found detectable for more than 6 months after vaccination of HLA-A*0201-transgenic mice (Drexler *et al.*, 2003). Indeed, we observed a significant enhancement of VP35#1-specific T-cell responses after vaccination with MVA-ΔIL1βR ($P<0{\cdot}005$). We noticed for the first time a significantly increased total $CD8^+$ T-cell response in transgenic HHD mice ($P<0{\cdot}05$) and, more importantly, we further corroborated this result following vaccination of non-transgenic mice ($P=0{\cdot}003$) harbouring a bona fide pool of $CD8^+$ T cells and allowing for higher variety of MHC class I-presented peptide epitopes in comparison with HHD mice. These additional findings appeared to confirm the long-term beneficial effect of MVA-ΔIL1βR immunization. In addition, we found higher protective capacities against lethal respiratory challenge with vaccinia virus Western Reserve in both transgenic HHD and normal mice.

How can the enhanced vaccine efficacy in the context of IL1β function be explained? Interestingly, results from two recent studies investigating *Leishmania major* infection of susceptible and resistant mice suggested that the ability of dendritic cells (DCs) to secrete IL1α or IL1β is specifically associated with the induction of protective Th1 immunity (Filippi *et al.*, 2003; Von Stebut *et al.*, 2003). In addition, there is recent evidence for IL1β being an essential mediator

of Fas-ligation-induced maturation of murine DCs (Guo *et al.*, 2003). The same work demonstrated that maturation of murine DCs could be completely abrogated by the use of IL1β-neutralizing antibodies, which may function in a similar manner as would be expected with the soluble vaccinia virus IL1βR molecule. Therefore, the lack of IL1β neutralization following vaccination with MVA-ΔIL1βR may lead to improved functionality of DCs to serve as antigen-presenting cells, which might result in better T-cell memory responses.

Similarly, it has been shown that stimulation of endothelial cells with IL1β resulted in human inducible co-stimulator-ligand-mediated activation of memory T cells (Khayyamian *et al.*, 2002). Such activity of IL1β might be the functional basis for our finding that vaccination with MVA-ΔIL1βR appeared predominantly to improve memory T-cell responses, suggesting that the viral IL1βR could have a specific role in abrogating anti-viral memory T-cell responses. This appears alluring in view of recent data either demonstrating an increased persistence of $CD4^+$ compared with $CD8^+$ T cells upon analysis of vaccinia virus-specific memory T cells in humans (Amara *et al.*, 2004) or suggesting that recombinant poxvirus vaccines may potently amplify peak but not necessarily memory T-cell responses following vaccination experiments in the human simian immunodeficiency virus/rhesus macaque model (Santra *et al.*, 2004).

In summary, our analysis recommends deletion of the viral IL1βR gene as a first step towards the development of a new generation of MVA-based vaccines. High virus titres were obtained following *in vitro* propagation of the deletion mutant MVA-ΔIL1βR, and the avirulent phenotype of MVA was well conserved in MVA-ΔIL1βR, even after high-dose *in vivo* infection. Our finding of improved vaccine properties of MVA-ΔIL1βR is particularly promising, since it demonstrates for the first time the possibility of obtaining more efficacious MVA vaccines through rational genetic engineering.

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