Selective B-cell expression of the MHV-68 latency-associated M2 protein regulates T-dependent antibody response and inhibits apoptosis upon viral infection

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To better understand the role of the M2 protein of the murine herpes virus strain 68 (MHV-68) in vivo, B-lymphocyte-restricted, M2-transgenic mice were constructed. The transgenic mice contained normal B-cell subpopulations in bone marrow, lymph nodes and spleen. After immunization with sheep red blood cells, spleens from M2-transgenic mice had increased germinal centres. Transgenic mice responded to the T-cell-dependent antigen keyhole limpet haemocyanin (KLH) with higher levels of secondary IgM and IgG2a antibodies than WT mice. Normal and M2-transgenic mice were infected with WT and M2 frame-shift mutant (M2FS) MHV-68 viruses. The pathogenesis of M2-transgenic mice infected with the M2-deficient mutant virus did not revert to that observed upon infection of normal mice with WT virus. However, the higher reactivation levels late after M2-transgenic mice were infected with WT virus reflected the importance of M2 as a target for the immune response, and thus with an impact on the establishment of latency. Finally, there was markedly less apoptosis in B-cells from M2-transgenic mice infected with either WT or M2FS mutant than from similarly infected WT mice, consistent with the published inhibitory influence of M2 on apoptosis in vitro. Thus, M2 provides a strategy to increase the pool of germinal centre B-cells through inhibition of apoptosis in the infected cell.

INTRODUCTION

The murine herpes virus strain 68 (MHV-68) is a model for gammaherpesvirus infection, and the availability of genetically modified mice lacking components of the innate and adaptive immune system, together with genetically manipulated MHV-68 viruses, has provided useful tools to explore the pathogenesis and the role of specific viral genes in the virus–host interaction. Intranasal inoculation of mice with MHV-68 results in a productive infection in the lungs, with maximal viral titres observed between days 5 and 10 post-infection (p.i.), and viral clearance on days 9–15 p.i. During the subsequent viraemia, MHV-68 spreads to the spleen where it becomes latent, mainly in germinal centre B-lymphocytes and in the memory B-lymphocyte pool (Flaño et al., 2003; Thorley-Lawson & Babcock, 1999; Thorley-Lawson, 2001; Willer & Speck, 2003). During latent infection, there is no virus replication and the viral genome is present as a nuclear episome (Clambey et al., 2002). Initial establishment of latency in the spleen is associated with a marked splenomegaly and mononucleosis. The splenomegaly is driven by CD4+ cells and is dependent on the presence of MHV-68-infected B-cells in the spleen. The resolution of splenomegaly is achieved by CD8+ cells, which are also important in the long-term control of persistent infection.

This work focused on the role of the M2 gene, which is transcribed during latency, a major target for the host cytotoxic T-lymphocyte response (Husain et al., 1999) and with a pivotal role in the establishment, maintenance and reactivation of latency in B-cells (Herskowitz et al., 2005; Macrae et al., 2003). The M2 ORF is located at the left end of the MHV-68 genome and shares positional homology with latency-associated genes in other gammaherpesviruses. Functional studies performed in vitro have indicated that M2 might have an important role in B-cell activation,
proliferation and survival (Jacoby et al., 2002), although studies with M2-deficient viruses (Jacoby et al., 2002; Macrae et al., 2003) indicate that M2 does not have an impact on the acute phase in the lung. Thus, the main goal of our work was to assess the role of M2 in the host response to virus infection using the infection of a B-cell-restricted M2-transgenic mouse by MHV-68 as a model system.

RESULTS

Construction of transgenic mice, determination of the number of copies and expression of the M2 transgene

Selective B-lymphocyte transgenic M2 expression in FVB/N mice was obtained from a litter of nine mice, after injection of fertilized eggs with the expression cassette of the pπs plasmid containing the M2 transgene. Southern blot analysis revealed two females and one male with a transgenic DNA fragment of the expected size. The two female founders were crossed with WT FVB/N male mice. The resulting F1 mice were further crossed to yield the F2 generation. Southern blot analysis of both founder mice revealed the presence of the injected expression cassette of the plasmid pπs (data not shown). The number of copies of the transgene incorporated in the genome of the two founders, as assessed by quantitative reverse transcription (RT)-PCR, varied between 10 and 30.

In all of the F1 and F2 mice bred from the two selected founders that were analysed, expression was confirmed by RT-PCR (Fig. 1), and confirmed by fluorescent staining of spleen sections from sheep red blood cell (SRBC)-immunized M2-transgenic mice (Fig. S1 in JGV Online). In addition, the higher yield of virus from M2-transgenic mice (see below) is also consistent with expression of the transgene and the resulting establishment of tolerance to M2.

Normal B-cell populations in bone marrow and spleen of M2-transgenic mice

To assess the role of M2 during B-cell development, flow cytometric analysis of lymphocyte suspensions prepared from bone marrow, spleen and lymph nodes was performed. The frequency of bone marrow B-cell subpopulations that define successive stages of B-cell development was determined on the basis of IgM and IgD surface markers on B220+ cells [pro- or pre-B-cells B220low IgM-, immature B-cells, B220high IgM+ and mature B-cells B220hi IgM+ (Fig. 2a)]. The IgM/IgD ratios were similar in both control and transgenic mice [immature B-cells, IgM+ IgD-, transitional B-cells IgM+ IgDlow and mature recirculating B-cells, IgM+ IgD+ (Fig. 2b)]. Similarly, there was no difference between control and M2-transgenic mice in marginal zone (B220+ CD23− CD21bright) and follicular B-cell (B220+ CD23+ CD21+) subsets in the spleen (Fig. 2c). Collectively, these findings indicated that expression of M2 did not affect B-cell bone marrow development and splenic maturation. Similar results were observed in the lymph nodes (results not shown).

B-cell-restricted M2-transgenic mice have higher levels of secondary IgM and IgG2a antibody responses to T-dependent antigen and increased numbers of germinal centres

The impact of M2 expression on the immune function in vivo was tested by immunizing M2-transgenic and control WT mice with T-independent [2,4-dinitrophenyl–lipo polysaccharide (DNP–LPS) and DNP–Ficoll] and T-dependent [DNP–keyhole limpet haemocyanin (DNP–KLH)] antigens. Similar anti-DNP antibody responses to DNP–LPS and DNP–Ficoll were observed in control and transgenic mice 10 and 21 days p.i. (data not shown). Regarding DNP–KLH, levels of DNP-specific IgG1, IgG2a, IgG2b, IgG3 and IgM antibodies were measured 10 days after primary immunization and 7 days after the secondary immunization on day 21 with DNP–KLH. As indicated, higher levels of IgM and IgG2a were observed in the secondary humoral responses of M2-transgenic mice (day 28) (Fig. 3a). The higher levels of these antibodies to the T-dependent antigen and the increased numbers of germinal centres determined by the number of peanut agglutinin (PNA)-positive areas in the spleen sections (Fig. 3b) reinforce the idea that an increased expansion of B-cells during the germinal centre reaction in the M2-transgenic mice reflects a viral strategy to increase the size of germinal centres and the memory B-cell pool, the main reservoir for virus in vivo.

Expression of the M2 protein protects against apoptosis induced by infection with MHV-68

To study the impact of the M2 transgene in the context of infection, groups of 8-week-old WT and M2-transgenic mice were infected intranasally with WT (MHV-68) and
M2-deficient recombinant (M2FS) viruses. Spleen cell suspensions prepared 21 days p.i. were stained with PI and apoptosis was assessed by the magnitude of the hypodiploid sub-G1 peak. In contrast to the abundant apoptosis in the normal mice infected with either of the two viruses, there was little or no apoptosis with either virus in the B-cell transgenic mice (Fig. 4), a finding consistent with observations in vitro (Madureira et al., 2005).

Pathogenesis of normal and B-cell-restricted M2-transgenic mice to infection with MHV-68 and M2-deficient (M2FS) virus

To study the pathogenesis of MHV-68 infection in the M2-transgenic mice, groups of M2-transgenic and WT mice were infected intranasally with 10^4 p.f.u. MHV-68 or M2FS viruses and monitored by infectious centre assays over a period of 90 days p.i. to determine levels of latent viruses in the spleen. First, comparing WT FVB/N mice infected with MHV-68 or M2FS virus, at day 14 p.i. there was an approximately tenfold reduction in the number of infectious centres in WT mice infected with M2FS virus, as compared with mice infected with MHV-68, which is in agreement with similar work in BALB/c mice (Simas et al., 2004) (Fig. 5a). At later time points, days 43 (Fig. 5c) and 71 (Fig. 5d) p.i., the number of infectious centres was consistently higher in the M2FS virus-infected mice than in mice infected with WT MHV-68, as previously described in BALB/c mice (Marques et al., 2008; Simas et al., 2004). Importantly, even at 90 days p.i. (Fig. 5e), the long-term latency of MHV-68 in FVB/N mice was not significantly affected by the disruption of the M2 protein, also as previously described for BALB/c mice. Thus, we confirmed that the acute latency deficit and long-term latency of M2-deficient MHV-68 virus observed in BALB/c mice (Simas et al., 2004) also occurs in FVB/N mice. Secondly, comparing infection of WT and M2-transgenic mice infected with MHV-68 virus, reactivation levels were significantly higher on day 43 p.i. (Fig. 5c) in the transgenic mice. Although the same trend was detected on day 71 p.i. (Fig. 5d), this difference was not statistically significant (P=0.1473), and on day 90 p.i. (Fig. 5e) there was no difference between the two mice groups infected with MHV-68. Thirdly, comparing infection of WT and M2-transgenic mice with M2FS there were no major differences between the FVB/N WT and the M2-transgenic mice, apart from at day 71 p.i., on which a higher number of latent reactivation-competent virus was observed in the WT mice infected with M2FS mutant virus (Fig. 5d). Finally, the latency deficiency observed upon infection of normal mice with the M2-deficient virus was not reversed when M2 was provided by infection of M2-transgenic mice with the M2FS virus. One possibility could be the impact of the M2 transgene on the physiology of the B-cell. For example, it is known that M2 drives B-cell proliferation and differentiation in an IL-10-dependent manner (Siegel et al., 2008). For this reason, we measured the IL-10 levels in sera from naïve and SRBC-stimulated normal and M2-transgenic mice. Although the levels of IL-10 were low, they were nevertheless significantly above background (*P<0.05), and there was no significant difference between the WT and M2-transgenic mice (P>0.05, not significant) (Fig. S2).

DISCUSSION

Virus host evasion strategies provide ready-made tools for genetic manipulation of cell biology and immune responses. The M2 gene of MHV-68 is known to be involved in the establishment, maintenance and reactivation of latency in B-cells (Herskowitz et al., 2005; Macrae et al., 2003; Rodrigues et al., 2006). Here, as an approach to better understand the biological role of M2, we constructed M2-transgenic mice with expression restricted to B-cells. The construction of transgenic mice is a powerful alternative strategy to study mechanisms of immunity in vivo, and the use of specific promoters to restrict cellular expression is an additional refinement, which may overcome the undesirable effects of whole-body transgenesis. Our findings not only confirm the importance of the M2 protein as an immunodominant determinant of cellular immunity, but also provide a strategy for greatly reducing apoptosis in the germinal centre. These data are consistent with previous work suggesting that M2 may manipulate the activation, proliferation and survival of B-cells. The validity of the model was justified by the generation of two founder lines with similar characteristics and with RT-PCR assays demonstrating expression of M2 in splenocytes but not in thymocytes (Fig. 1).

The first step in the characterization of the M2-transgenic mice was the analysis of surface markers. There were no differences between the WT and the M2-transgenic mice in their B-cell subpopulations in the bone marrow and spleen (Fig. 2). Therefore, the B-cell-restricted expression of the MHV-68 M2 protein in vivo did not impact on B-cell development, a finding consistent with the absence of a strong in vivo phenotype for the VAV1, VAV2 and VAV3 triple-knockout mice (Pearce et al., 2004). As the downstream target for M2 is VAV1/2, we concluded that VAV proteins are redundant and overlapping in their function.

We then performed functional assays to assess the impact of the transgene on the serological immune response. Transgenic mice immunized with the T-dependent antigen (DNP–KLM) produced statistically higher levels of IgM and IgG2a anti-DNP antibodies than normal mice (Fig. 3a). The predominant subtype observed following MHV-68 infection, as well as in other viral infections, is IgG2a, suggesting a preferential class switching (Coutelier et al., 1987). Similarly, when M2-transgenic mice were challenged with a T-dependent antigen, there was a significant increase of IgG2a, especially in the secondary challenge.

Particularly significant was the considerably diminished B-cell apoptosis in MHV-68-infected M2-transgenic mice.
Based on these results, we suggest that M2 competes with T-cells for activation of B-cells to provide a viral replication deficit, either in tissue culture or during the acute phase, in the lungs of infected mice (Jacoby et al., 2001). Thus, before investigating the pattern of establishment and maintenance of latency in the FVB/N M2-transgenic mice infected with MHV-68 and M2FS-deficient viruses, we analysed infection in the WT FVB/N background, and observed that FVB/N WT mice infected with M2FS showed the same phenotype as BALB/c, namely an acute latency deficit and an elevated long-term latency. Therefore, control of infection through recognition of M2 peptides by CD8+ T-cells might not be restricted to the H-2k+ haplotype, as previously suggested (Marques et al., 2008).

To explore the role of M2 in the establishment of latency, M2-transgenic and WT mice were infected with WT MHV-68 and M2 deletion mutant (M2FS) viruses, and the numbers of reactivation-competent virus plaques in the spleen were then measured as an indication of latently infected B-cells (Fig. 5). The yield of virus recovered in normal mice infected with the M2-deficient virus increased at the late but not at earlier phase of infection. This phenotype was not observed in the M2-transgenic mouse, where the response to WT and M2-deficient viruses was similar. The observation of higher reactivation centres in the late phase of latency in the M2-transgenic mice infected with WT viruses is consistent with the establishment of tolerance to M2 on the one hand, and the proven importance of M2 as a determinant of protective cellular immunity on the other, and thus with an impact on the establishment of persistence (Husain et al., 1999). Therefore, the higher virus yield in M2-transgenic mice is consistent with the establishment of tolerance to M2 (and the consequent loss of a major immunodominant CD8+ T-cell epitope), although no formal evidence for this has been presented.

In conclusion, the B-cell-restricted transgenic expression of the MHV-68 ORF M2 provides a novel approach to explore the mechanism and possible exploitation of the M2 gene. It has the merit of being relevant in the context of an MHV-68 infection, providing a good platform to study the impact in vivo of a single virus protein during acute and latent phases of virus infection. Although we have not found an effect on the distribution of B-cell subsets in the bone marrow and spleens of the M2-transgenic mice, we have found an interesting phenotype when these mice were challenged with a thymus-dependent protein antigen, or when infected with MHV-68 or an M2-deficient recombinant virus. Infection of normal mice with M2-deficient MHV-68 resulted in an increased reactivation in the late but not the early phase of latency, an observation not seen when the M2-transgenic mice were similarly infected. More importantly, we have demonstrated in vivo that M2 protein not only contains an immunodominant CD8+ T-cell response, but also protects B-cells from apoptosis in vivo, thereby providing a viral strategy for increasing the population of potential host cells for the establishment of latency. In contrast to our expectation, the latency deficiency phenotype associated with the M2-deficient virus was not reversed by similar infection of
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(a) 

(b) 

(c) 

Day of serum collection
Fig. 3. The response of M2 B-cell transgenic mice to T-dependent antigen DNP–KLH and the formation of germinal centres in M2 B-cell transgenic mice are increased. (a) Control and M2 B-cell-restricted transgenic mice were immunized with the TD antigen DNP–KLH. Relative amounts of DNP-specific IgM, IgG1, IgG2a and IgG2b were determined by ELISA. Serum was collected before immunization and 7 and 14 days after primary immunization (day 0). Mice were then re-immunized at 21 days, bled at day 28 and the secondary immune response measured. Arrows represent the days at which mice were immunized (days 0 and 21). Open and filled circles represent mean ± s.o for control and transgenic mice, respectively. Each circle represents one mouse. Results of the ELISA are shown as absorbance at 492 nm (x-axis) versus day of serum collection (y-axis). There was no significant difference between control and transgenic titres of DNP-specific IgG1 and IgG2b antibodies, whereas titres of DNP-specific IgM and IgG2a were significantly elevated (\(P<0.05\)) in the secondary response of the M2-transgenic mice. (b) Cryostat sections of spleens from SRBC-immunized control and transgenic mice were stained with rat anti-mouse Ig coupled to Alexa Fluor 488 (left) and counterstained with biotinylated PNA followed by streptavidin–TexasRed (right). (c) Graphical representation of the number of splenic germinal centres assessed by immunofluorescence. The numbers of B220\(^+\) follicular areas and PNA\(^+\) germinal centres were counted in fields from histological cryostat sections from control and M2-transgenic mice. Data are mean ± SEM per microscopic field (18–20 fields were analysed for each genotype). \(P<0.05\); comparison indicated by horizontal lines.

M2-transgenic mice. The possibility that this might be related to the known impact of M2 on IL-10 expression (Siegel et al., 2008) was not supported as there was no significant difference between levels of serum IL-10 in control and SRBC-immunized normal and M2-transgenic mice. Another possibility is that the impact of expression of the M2 transgene on the physiology of B-cells might in some, as yet unexplained, way have been responsible for the failure to reverse the latency deficiency phenotype.

METHODS

Mice. FVB/N mice were bred and kept at the Instituto Gulbenkian de Ciência (IGC). The M2-transgenic founder mice were produced by Dr Moises Mallo and the Transgenic Facility of IGC. All the animal work was conducted in compliance with Portuguese and European laws and was performed at the IGC animal house, licensed by the Direcção Geral de Veterinária (Laboratory permission 520/000/000/2518/99 and Portaria 1005/92 and Directive 86/609/EEC, respectively) following Federation of European Laboratory Animal Science Associations recommendations.

Gene amplification and plasmid construction. The M2 gene was amplified by PCR using Pfu DNA polymerase, from template DNA of MHV-68. The primers used were: Up, 5’-ATGAGGTTCTTGGTTCTCAGG-3’; Low, 5’-TTACTCTCTGGCCCCACTCCAC-3’. PCR was conducted on a PTC-100 Peltier-Effect Cycling apparatus with 200 μM dNTPs, 1 μM primers and 3 mM MgSO\(_4\) at 95 °C for 2 min, 30 cycles of 95 °C for 1 min, 44 °C for 1 min and 74 °C for 2 min, and finally 74 °C for 5 min. The gene was cloned into pMSCVneo, subcloned as a 5’ EcoRI-3’ XhoI fragment into the pCIS2+ vector, and then subcloned with a downstream SV40 poly(A) tail as a 5’ Clad-3’ Apal fragment into the pEUS vector. This vector, constructed by Joanna B. Wilson, and was a gift from Dr Pedro Simas (Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa) (who cloned the M2 gene in the pUS plasmid), is based on the plasmid backbone of pBluescript (SK-) and incorporates the promoter (150 bp) and enhancer (700 bp) sequences of the mouse Ig\(_\mu\) heavy chain, upstream of the mouse APRT intron (150 bp). The recombinant vector was digested with KpnI and SstI to release the expression cassette from the bacterial backbone vector. After purification from an agarose gel, the expression cassette was microinjected into FVB/N fertilized eggs. These were then transferred into pseudopregnant foster females, producing the founder mice. These founders were crossed with WT FVB/N mice to obtain hemizygous mice. Subsequent crossing of hemizygous mice yielded homozygous animals. Control animals were littermates without the transgene.

Sequencing. The cloned M2 gene was sequenced using primers derived from the sequence of the pUS plasmid upstream and downstream of the Clad and XhoI sites, respectively: Up, 5’-CTGCGCGGCCACGCGGTCACCTCTC-3’; and Low, 5’-CGATATCGATCCATACCGTGCAGCC-3’. The kit used was a BigDye terminator v1.1, Part No. 436776 (Applied Biosystems) and the cycling conditions were 96 °C for 1 min and 25 cycles of 96 °C for 10 min, 50 °C for 5 min and 60 °C for 4 min. The PCR products were analysed on a 377 DNA Sequencer and 3130xl Genetic Analyzer (Applied Biosystems).

Mouse genotyping by Southern blot and PCR. Founder mice were analysed by Southern blotting to assess integrity of the expression cassette injected into the fertilized eggs and discriminate between positive and negative mice. Genomic DNA (10 μg) isolated from tail biopsies was digested with KpnI and SstI and electrophoresed on a 0.8 % agarose gel. Tails were digested overnight with proteinase K (100 μg ml\(^{-1}\)) at 56 °C and then DNA was precipitated with 2-propanol (0.7 vols) at room temperature. Treatment of the gel, transfer to the membrane Hybridon N+ and hybridization were done according to the instructions with Hybridon N+ (Amersham). The M2 probe was labelled with \(^32\)P-dCTP using the Random Primers DNA Labelling System (Gibco) according to the manufacturer's instructions. Unincorporated probe was removed using G-50 Sephadex Quick Spin columns (Gibco).

Mice were then genotyped by PCR using DNA from tail biopsies obtained at the time of weaning (3 weeks).

Detection of the M2 gene in the DNA by PCR was performed using Taq DNA polymerase and the previously described primers. PCR was conducted on a PTC-100 Peltier-Effect Cycling apparatus and with 200 μM dNTPs, 1 μM primers and 2 mM MgCl\(_2\) at 95 °C for 2 min, 30 cycles of 95 °C for 1 min, 54 °C for 1 min and 72 °C for 2 min, and finally 72 °C for 5 min.

Determination of transgene copy number by RT-PCR. The amount of amplified M2 transgene estimated by LightCycler Fast Start DNA Master SYBR Green I Roche (primers: Up, 5’-ATGAGGTTCTTGGTTCTCAGG-3’; Low, 5’-TTACTCTCTGGCCCCACTCCAC-3’) was normalized against the amount of amplified mouse β-globin (primers: Up, 5’-CAAATCTGCTCACACAGGATAGG-3’; Low, 5’-CCATGACCTGTCACACATCC-3’). PCR used 4 mM MgCl\(_2\) and 200 nM primers. The program for M2 was pre-incubation at 95 °C for 10 min, 45 cycles of amplification (95 °C for 10 min, 70 °C for 5 min, 72 °C for 30 min) and melting curve analysis (65 °C for 15 min); the program for β-globin was pre-incubation at 95 °C for 10 min, 45 cycles of amplification (95 °C for 10 min, 70 °C for 5 min, 72 °C for 5 min).
20 min) and melting curve analysis (65 °C for 15 min). Analysis with the Roche software and calculations of the amount of amplified product were based on the determination of the second derivative maximum and on an arithmetic adjustment of the baseline.

**Demonstration of transgene expression by RT-PCR.** Total RNA was extracted from tissue homogenates of thymus and spleen from transgenic and control littermates using Trizol Reagent (Sigma). Samples of RNA were digested with DNase I (Invitrogen) and cDNA
synthesis was performed with Moloney murine leukemia virus reverse transcriptase (Invitrogen), according to the manufacturer’s instructions. Detection of the M2 gene in the cDNA was done with PCR and sequenced by RT-PCR using LightCycler Fast Start DNA Master SYBR Green I (Roche) as described above.

Flow cytometry analysis of lymphocyte surface proteins. Cell suspensions of bone marrow and spleen were incubated with titrated concentrations of the antibodies used in the staining combinations given in the text, washed in PBS–2 % FCS–0.02 % sodium azide and subsequently analysed in a FACScalibur (Becton Dickinson). The following rat anti-mouse monoclonal antibodies (BD Pharmingen) were used: anti-IgM–Alexa Fluor 488, anti-IgG2b–APC, CD19–APC, anti-CD23–PE, anti-CD21–FITC and anti-IgD–PE. Except for the four-colour staining, PI was always used to exclude dead cells from the analysis. Data were processed using CellQuest software (Becton Dickinson Immunocytometry Systems). Live lymphocyte counts were deduced from the acquisition of a fixed number of 10 μm latex beads (Coulter) mixed with a known volume of unstained cell suspension.

Stimulation and measurement of germinal centre formation. Mice were injected intraperitoneally with 200 μl 2 % SRBCs, and 7–10 days later spleens were collected, frozen in tissue-Tek OCT Compound (Sakura Finetechinal) and 8 μm cryostat sections were fixed (absolute ethanol, 5 min at room temperature; followed by acetone, 5 min at room temperature) and air dried. Quantification of the germinal centres was performed by immunofluorescence using a titrated concentration of rat anti-mouse Ig coupled to Alexa Fluor 488 (BD Pharmingen) counterstained with biotinylated PNA (Vector Laboratories) followed by streptavidin–TexasRed (BD Pharmingen).

Immunizations with T-dependent antigens and Ig isotype-specific ELISA. Mice aged 16–20 weeks were injected intraperitoneally with DNP–KLH (100 μg) (Biosearch Technologies) in PBS on day 0, rechallenged on day 21 and killed on day 28. Blood was collected for serum on days 0, 7, 14, 21 and 28. Determination of DNP-specific serum antibodies was done by Ig isotype-specific ELISA. Titration of the antibody concentration of rat anti-mouse Ig coupled to Alexa Fluor 488 (Coulter) mixed with a known volume of unstained cell suspension.

The initial dilution of the serum varied according to the Ig isotype specific antibodies. Single dilution factor that fell in the linear part of the curve was presented for all time points (1:6400 for IgM, 1:3200 for IgG1, 1:1600 for IgG2a and IgG2b and 1:400 for IgG3).

Cell culture. BHK-21 cells were cultured in 5 % CO2 at 37 °C in complete GMEM (cGMEM) consisting of Glasgow’s modified Eagle’s medium (GMEM) supplemented with 10 % FCS, 10 % (w/v) tryptose phosphate broth, 100 U penicillin G ml⁻¹, 100 μg streptomycin ml⁻¹ and 2 mM l-glutamine.

Virus production and titration. The MHV-68 clone G2.4 (Efstathiou et al., 1990) used in this work was provided by Dr Pedro Simas. The MHV-68 recombinant virus (M2FS) (provided by Dr Heiko Adler (Institute of Molecular Immunology, Clinical Cooperation Group Hemato poetic Cell Transplantation, GSF, National Research Center for Environment and Health, Munich, Germany) and Dr Stacey Efstathiou (Division of Virology, Department of Pathology, University of Cambridge, Cambridge, UK)) contains a frameshift in the ORF M2 created by insertion of a single guanine between nt 4603 and 4604, immediately downstream of the translational start codon (Husain et al., 1999), creating a novel Apal site and premature stop codon 78 nt downstream.

Virus working stocks were grown by low-multiplicity infection of semi-confluent BHK-21 cells (0.001 p.f.u. per cell) in 150 cm² culture flasks, with MHV-68 or with M2FS. After 4 days, supernatants were centrifuged (30 000 g, 2 h, 4 °C) and pelleted virus was resuspended in 2 ml GMEM and ultrasonically disrupted for 30 s. Aliquots were stored at −80 °C. To determine the virus titre, subconfluent BHK-21 cells were incubated with tenfold serial dilutions of virus in GMEM. Following a 1 h adsorption, the medium was replaced with cGMEM containing 0.32 % carboxymethylcellulose and incubated at 37 °C for 4 days. After removal of this medium, cells were fixed with 10 % formaldehyde in PBS and then stained with 0.1 % toluidine blue in formal saline. Viral plaques were counted under a Stemi SV6 microscope (Zeiss) and virus titres were calculated from numbers in duplicate dishes.

Infection of mice with MHV-68 or M2FS. After genotyping, 6- to 8-week-old mice were transferred from the standard animal house to quarantine. Animals were inoculated intranasally under the effect of light halothane anaesthesia with 10⁶ p.f.u. MHV-68 or M2FS virus in 20 μl PBS. At different time points after infection, mice were killed by inhalation of CO₂. The entire experiment was performed twice, with comparable results.

Infectious centre assay. Spleen cell suspensions were tenfold serially diluted, starting with 10⁷ cells ml⁻¹. BHK-21 cells (5 × 10⁴) were added to each sample and the final volume was adjusted to 5 ml with cGMEM. The mixture was incubated for 1 h at 37 °C with gentle shaking, plated out in 60 mm cell culture Petri dishes and then incubated at 37 °C in a humidified incubator for 5 days. Monolayers were fixed with 10 % formaldehyde in PBS and stained with 0.1 % (w/v) toluidine blue in formal saline. Plaques were counted under a Stemi SV6 microscope (Zeiss) and the number of infectious centres per 10⁶ splenocytes was calculated.

Estimation of apoptotic cells. Splenic cell suspensions were stained with a titrated concentration of a monoclonal FITC-labelled rat

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**Fig. 5.** Numbers of latent reactivation-competent virus in control and M2-transgenic mice infected with MHV-68 or M2FS virus. Control and M2-transgenic mice were infected intranasally with 10^4 p.f.u. of either MHV-68 or M2FS and the spleens were assayed for latent virus on days 14–90 post-infection. Wild-type mouse infected with MHV-68 virus (○), WT mouse infected with M2FS virus (●), M2-transgenic infected with MHV-68 virus (□), or M2-transgenic infected with M2FS virus (■). Results were compiled from two independent experiments, and the results for these four groups are presented as the numbers of latent reactivation-competent virus measured on days 14, 21, 43, 71 and 90 p.i. (a–e, respectively). The continuous horizontal dashed line indicates the limit of detection of the assay and the short horizontal line in each group of circles represents the arithmetic mean (ND, denotes that virus was not detectable). Each circle represents an individual mouse. *P<0.05, n.s. not significant; comparison indicated by horizontal lines.
samples were resuspended in PBS with PI, at a final concentration of 100 and 50 U of RNaseA by a two-tailed Mann–Whitney t-test and SPSS v17.0 statistical software. Statistical significance was determined based on cells in the hypodiploid sub-G1 peak after gating on the B220+ splenic B-cell population.

**Statistics.** Statistical significance between the groups was determined by a two-tailed Mann–Whitney t-test and no correction was made for multiple comparisons. Data were analysed using GraphPad Prism v5.0 and SPSS v17.0 statistical software. Statistical significance was accepted at a two-tailed probability of \( P<0.05 \) (*) and \( P<0.01 \) (**).

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