Medical Microbiology and Immunology (2019) 208:439–446
https://doi.org/10.1007/s00430-019-00615-8

ORIGINAL INVESTIGATION

Transcripts expressed in cytomegalovirus latency coding for an antigenic IE/E phase peptide that drives "memory inflation"

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Received: 3 April 2019 / Accepted: 11 April 2019 / Published online: 19 April 2019

Abstract

Roizman's definition of herpesviral latency, which applies also to cytomegaloviruses (CMVs), demands maintenance of reactivation-competent viral genomes after clearance of productive infection. It is more recent understanding that failure to complete the productive viral cycle for virus assembly and release does not imply viral gene silencing at all genetic loci and all the time. It rather appears that CMV latency is transcriptionally "noisy" in that silenced viral genes get desilenced from time to time in a stochastic manner, leading to "transcripts expressed in latency" (TELs). If a TEL happens to code for a protein that contains a CD8 T cell epitope, protein processing can lead to the presentation of the antigenic peptide and restimulation of cognate CD8 T cells during latency. This mechanism is discussed as a potential driver of epitope-selective accumulation of CD8 T cells over time, a phenomenon linked to CMV latency and known as "memory inflation" (MI). So far, expression of an epitope-encoding TEL was shown only for the major immediate-early (MIE) gene *m123/ie1* of murine cytomegalovirus (mCMV), which codes for the prototypic MI-driving antigenic peptide *YPHFMPTNL* that is presented by the MHC class-I molecule L^d. The only known second MI-driving antigenic peptide of mCMV in the murine MHC haplotype H-2^d is *AGPPRYSRI* presented by the MHC-I molecule D^d. This peptide is very special in that it is encoded by the early (E) phase gene *m164* and by an overlapping immediate-early (IE) transcript governed by a promoter upstream of *m164*. If MI is driven by presentation of TEL-derived antigenic peptides, as the hypothesis says, one should find corresponding TELs. We show here that E-phase and IE-phase transcripts that code for the MI-driving antigenic peptide *AGPPRYSRI* are independently and stochastically expressed in latently infected lungs.

Keywords Antigen presentation · Antigenic peptide(s) · CD8 T cells · Gene m164 · IE1 peptide · Latency · Latent infection · Memory inflation (MI) · RT-qPCR · Stochastic gene expression · Transcripts expressed in latency (TEL) · Viral gene expression

Edited by: Stipan Jonjic.

This article is part of the Special Issue on Immunological Imprinting during Chronic Viral Infection.

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Introduction

The establishment of viral latency [1–4] and "memory inflation" (MI) of virus-specific CD8 T cells are hallmarks of cytomegalovirus (CMV) infection and are proposed to be linked mechanistically [5–7]. In the BALB/c mouse model of CMV infection [8], the major immediate-early (MIE) gene *m123/ie1* of murine CMV (mCMV) [9–12] codes for the prototype of an antigenic peptide of CMVs, namely IE1 peptide *YPHFMPTNL* that is presented to CD8 T cells by the MHC class-I (MHC-I) molecule L^d [13, 14]. In latent mCMV infection, MIE genes *m123/ie1* and *m128/ie2* [15] were found to be expressed in the lungs in a stochastic fashion and independently of each other [16, 17]. A first link to relate transcripts expressed in latency (TELs) to immune

surveillance of the latent state by IE1 epitope-specific tissue-patrolling CD8 T cells was revealed by work of Simon and colleagues [18]. Specifically, absence of the functional IE1 peptide *YPHFMPTNL* after infection with a recombinant mCMV expressing the non-functional IE1 peptide analogue *YPHFMPTNA* (for a strategy review, see [19]) was found to be associated with a higher IE1 TEL activity in latently infected lungs [18]. By implication, this indicated for the first time that a viral peptide is presented and recognized during latency.

In parallel to these virological approaches focussing on TELs, protective CD8 T cells were found to accumulate in latently infected lungs long after resolution of productive infection [20]. When compared to lung infiltrate CD8 T cells present during acute infection, activated $CD62L^{lo}$ CD8 T cells specific for the IE1 epitope and capable of secreting effector cytokine IFN-γ upon peptide stimulation were found to be enriched in latently infected lungs both in relative terms and also in absolute numbers [21]. Notably, CD8 T cells specific for a panel of other antigenic peptides known at the time (m04, M83, M84) were not enriched [21]. Subsequent work exploited these findings and provided a more complete time course of the enrichment phenomenon, coining the now popular term "memory inflation" (MI) [22], which from today's point of view is a misnomer, as the accumulating cells are effector rather than memory cells (for more details and recent developments, see reviews in this issue of *MMIM* [23–27]).

Shortly after the first description of epitope-selective accumulation of CD8 T cells in latently infected lungs [21], an antigenic peptide *AGPPRYSRI,* derived from the early (E) phase protein m164/gp36.5 [28] and presented by the MHC-I molecule D^d, was identified and found to behave like the IE1 peptide in terms of driving the accumulation of cognate CD8 T cells during viral latency [29]. So, to maintain the hypothesis that MI during latency is driven by epitope-encoding TEL activity, m164 transcripts should be present in latently infected tissues.

Materials and methods

Viruses and mice

Bacterial artificial chromosome (BAC)-cloned virus MW97.01 derived from BAC plasmid pSM3fr [30, 31] is herein referred to as mCMV-WT.BAC. Cell culture-derived high titer virus stocks were generated by a standard protocol [32].

8- to 9-week-old female BALB/c mice were purchased from Harlan Laboratories and housed in the Translational Animal Research Center (TARC) of the University Medical Center of the Johannes Gutenberg-University Mainz for at least 1 week under specified pathogen-free (SPF) conditions.

Experimental HCT and establishment of latent mCMV infection

Sex-matched syngeneic hematopoietic cell transplantation (HCT) with 8- to 9-week-old female BALB/c mice as bone marrow cell donors and recipients was performed with modifications as described previously [32]. In brief, after hematoablative conditioning by total-body γ-irradiation with a single dose of 6.5 Gy, 5×10^6 syngeneic bone marrow cells were infused into the tail vein of the recipients. Subsequently, the recipients were footpad-infected with 10^5 PFU of mCMV-WT.BAC. Establishment of latency was controlled at 6 months post-transplantation as described previously [33].

Quantitation of latent viral genomes in lung tissue

DNA from the postcaval lobe of latently infected lungs was extracted with the DNeasy blood and tissue kit (catalog no. 69504; Qiagen, Hilden) according to the manufacturer's instructions. Briefly, DNA was isolated, and viral and cellular genomes were quantitated in absolute numbers by *M55*-specific and *pthrp*-specific qPCRs normalized to a $\log_{10}$-titration of standard plasmid pDrive_gB_PTHrP_Tdy [33, 34].

In vitro transcripts

Plasmid pDrive-m164_IE, which contains sequences encompassing open reading frames (ORFs) *m164* to *m166*, was constructed as follows: a PCR fragment was amplified by using oligonucleotides m164_IE_BamHI_for (ATATGGATCCGTCGGGTTGTTCGCTTATGG) and m164-IE_HindIII_rev (ATACAAGCTTACCGGCCTATGAGATACTCG) from viral DNA (strain Smith ATCC VR-1399) as template. The 2214-bp product was digested with *Bam*HI and *Hin*dIII (ThermoFisher Scientific, Dreieich) and inserted into the *Bam*HI- and *Hin*dIII-digested pDrive vector (Qiagen) to generate pDrive-m164_IE that was linearized with *Eco*RI (ThermoFisher Scientific) and used as template for in vitro transcription with the MAXIscript SP6/T7 Transcription Kit (catalog no. AM1320, ThermoFisher Scientific). In vitro transcripts IE1 and m164_E_IE were described previously [16, 17, 35].

Analysis and quantitation of transcripts

Viral transcripts in latently infected lungs were detected by specific quantitative reverse transcriptase PCR (RT-qPCR) as described in principle previously [34]. Briefly, lungs of latently infected HCT recipients were cut into pieces, each representing a 1/11 portion of the lungs, followed by shock-freezing in liquid N_2. Total RNA was isolated with the RNeasy

Mini Kit (catalog no. 7410, Qiagen) according to the manufacturer's instructions. This included an on-column DNAaseI (catalog no. 79254, Qiagen) digestion. For synthesis of cDNA and quantification of transcripts, 100 ng of RNA was used per sample and RT-qPCR was performed using the OneStep RT-PCR Kit (catalog no. 210212, Qiagen). For absolute quantitation of IE1, m164_E_IE and m164_IE transcript numbers, respective dilution series of specific in vitro transcripts were used as standards [34]. Cellular β-actin transcripts were quantified in parallel for normalization. PCR primers and probe [5′ 6-FAM labeled, 3′ black hole quencher (BHQ) labeled] sequences were as follows: (β-actin) β-actin_for: GACGGC CAGGTCATCACTATTG, β-actin_rev: CACAGGATTCCA TACCCAAGAAGG, β-actin_probe: AACGAGCGGTTC CGATGCCC. (mCMV IE1) IE1_for: TGGCTGATTGAT AGTTCTGTTTTATCA, IE1_rev: CTCATGGACCGCATC GCT, IE1_probe: AACGCTCCTCACTGCAGCATGCTT G. (mCMV m164_E) m164_E_for: CAACTGACAGTC GCAGCTCTTC, m164_E_rev: CGGCGGTAACCTGCT ATCC, m164_E_probe: TCGGCCGTGTCCACCAGTTTG ATCT. (mCMV m164_IE) m164_IE_for: CTACCCCCGTCA CCCAGATA, m164_IE_rev: AGCGAATCGCGGTTCTGA, m164_IE_probe: TGCTCACACCGCACGCGAGAAA.

Limiting dilution analysis

The detection limits [reciprocals of the respective most probable numbers (MPN)] for viral transcripts and the corresponding 95% confidence intervals (CI) of the MPN were estimated by limiting dilution analysis [36] using the maximum-likelihood method for calculation [37]. The calculation is based on the Poisson distribution equation $\lambda = -\ln f(0)$, where λ is the Poisson distribution parameter lambda and $f(0)$ is the experimentally determined fraction of negative samples/replicates, that is, in this specific case, samples negative for transcripts in the specific RT-qPCRs performed with graded numbers of the respective in vitro transcripts. By definition, $\lambda = 1$ for an $f(0) = 1/e$. Accordingly, in a semilogarithmic plot of graded in vitro transcript numbers (abscissa) and $-\ln f(0)$ (ordinate), the MPN is revealed as the abscissa coordinate of the point of intersection between $1/e$ and the calculated regression line.

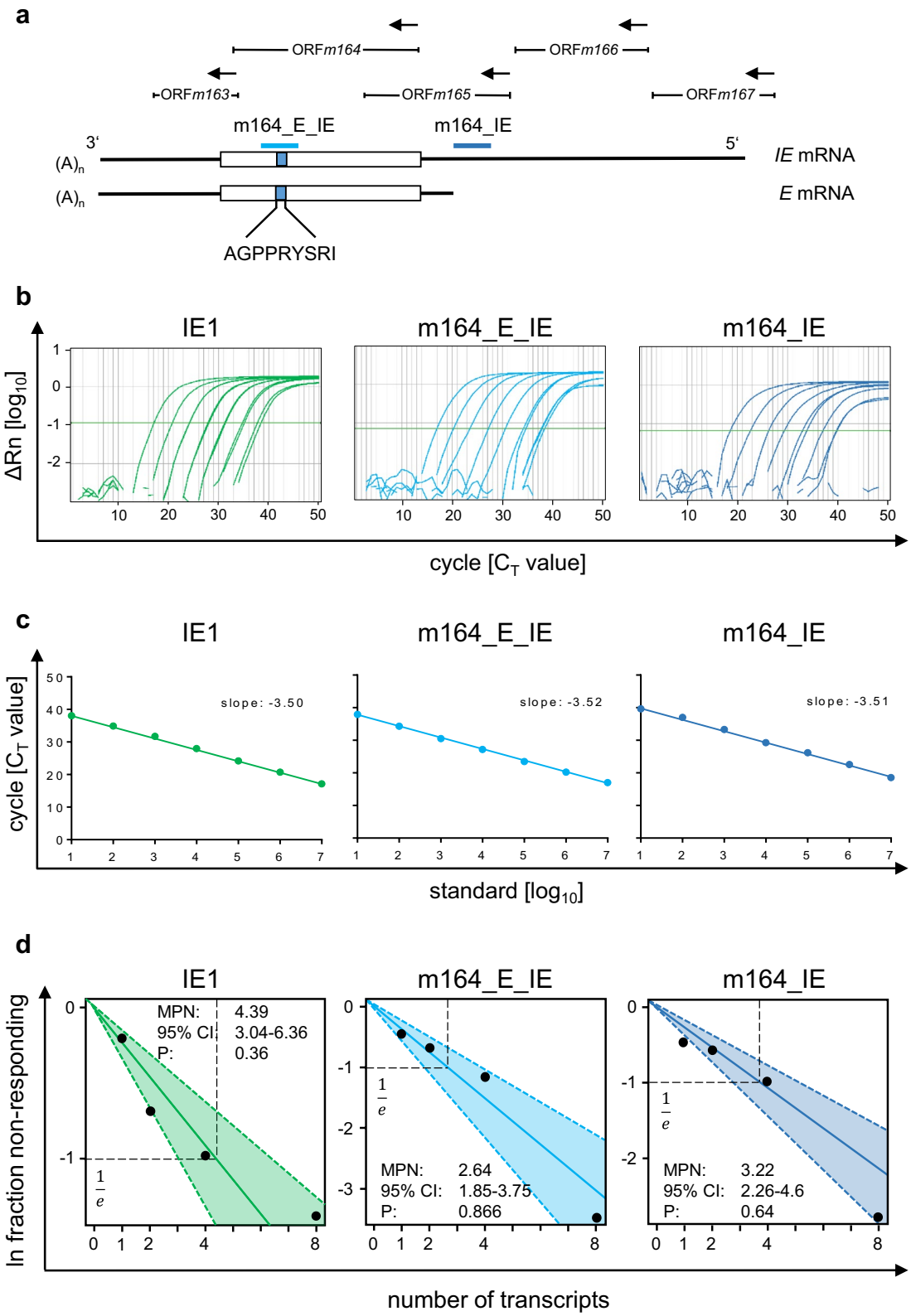
Results

Equivalence in the sensitivity of detecting IE/E phase transcripts coding for the antigenic m164 peptide

The MI-driving antigenic peptide *AGPPRYSRI* was identified by a 'reverse immunology' approach, running prediction algorithms over the deduced amino acid sequences of known mCMV ORFs, followed by testing the candidate peptides in functional assays [29]. Subsequently, two additional antigenic peptides attributed to ORF*m164* were identified in the C57BL/6 mouse model, one presented by MHC-I molecule K^b and the other one by D^b, though neither one is driving MI [38]. As these peptides localize to the ORF*m164* gene product, namely the confirmed E-phase protein gp36.5 [28], it was taken for granted that the three peptides are derived by processing of this protein and that they are presented in the E-phase of the viral gene expression cascade. This was general thinking at the time and still is. Importantly, therefore, more recent work by Fink et al. [39] has revealed a noncanonical expression of antigenic peptide *AGPPRYSRI* from an upstream IE-phase mRNA starting within ORF*m167* and encompassing ORF*m164*. Thus, regarding latency and associated MI, this new insight leads to the question if m164 TELs, provided they exist at all, are the IE-phase or the E-phase TEL or both.

Previous experience with IE1-specific TEL activity in lungs of latently infected mice has revealed transcript numbers that are orders of magnitude lower than during acute infection. As latent viral DNA load and TEL activity vary depending on the experimental conditions, it is difficult to define a rule. To just give an idea of orders of magnitude: in the work by Simon and colleagues [18], lungs latently infected with revertant viruses expressing the IE1 peptide contained ~ 10^4 copies (median value) of latent viral DNA per 10^6 lung cells. In samples with TEL activity above the detection limit (TEL^+), the numbers of transcripts ranged from 9 to 210 IE1 transcripts per 10^4 latent genomes in the published experiments [18], that is 10^1–10^2 transcripts when we think in orders of magnitude. Thus, in this kind of experiments sensitivity of the RT-qPCRs is an issue.

RT-qPCRs were designed to distinguish between m164_IE and m164_E_IE transcripts (Fig. 1a). Note that the sequence overlap makes it impossible to measure m164_E transcripts directly, but quantitation allows it to conclude on existence and quantity of m164_E transcripts from the difference between m164_E_IE and m164_IE transcripts, provided that both RT-qPCRs detect the respective transcripts with comparable efficacy and sensitivity (Fig. 1). Amplification profiles for graded numbers of synthetic transcripts, with synthetic IE1 transcripts included as a reference [17] (Fig. 1b) and slopes of standard curves deduced thereof (Fig. 1c) revealed almost identical amplification efficacies for the three RT-qPCRs. Finally, limiting dilution analysis revealed that both types of m164 RT-qPCRs detect the respective transcripts with sensitivities identical within the 95% confidence intervals, namely being able to detect a most probable number of ~ 3 transcripts (Fig. 1d).


a
ORFm164
ORFm166
ORFm163
ORFm165
ORFm167
m164_E_IE
m164_IE
3'
5'
(A)n
(A)n
IE mRNA
E mRNA
AGPPRYSRI
b
IE1
m164_E_IE
m164_IE
ΔRn [log10]
cycle [CT value]
c
IE1
m164_E_IE
m164_IE
slope: -3.50
slope: -3.52
slope: -3.51
cycle [CT value]
standard [log10]
d
IE1
m164_E_IE
m164_IE
MPN: 4.39
95% CI: 3.04-6.36
P: 0.36
MPN: 2.64
95% CI: 1.85-3.75
P: 0.866
MPN: 3.22
95% CI: 2.26-4.6
P: 0.64
ln fraction non-responding
number of transcripts

◂**Fig. 1** Establishment of RT-qPCRs to detect m164 transcripts. **a** Map of the genomic region from where IE and E mRNAs are transcribed that encompass the sequence coding for the m164 peptide *AGPPRYSRI*. Positions of amplificates of the m164_E_IE (light blue dash) and m164_IE (dark blue dash) RT-qPCRs as well as the position of the antigenic peptide are indicated. **b** RT-qPCR amplification profiles with synthetic in vitro transcripts as templates. RT-qPCRs were run for replicates of $\log_{10}$ dilutions (10^7–10^1). The normalized relative fluorescence intensity (ΔRn) was plotted against the corresponding C_T value for each amplification. **c** Slopes of the standard curves deduced from the amplification profiles define the amplification efficiency of each RT-qPCR. **d** Limiting dilution (Poisson distribution) analysis based on the experimentally determined fractions of negative replicates $f(0)$ for $\log_2$ dilutions of synthetic in vitro transcripts. For each number of transcripts, 16 replicates were subjected to the indicated RT-qPCRs. The plots of $-\ln f(0)$ on the ordinate and the number of transcripts on the abscissa show the Poisson distribution regression lines calculated with the maximum-likelihood method. Shaded areas show the 95% confidence intervals (CI). The MPN (most probable number), which is the reciprocal of the Poisson distribution parameter λ, is revealed as the abscissa coordinate (dashed line) of the point of intersection between $\ln f(0) = \ln 1/e = -1$, and the regression line. By convention, the conformity to the null hypothesis, here the conformity to the Poisson distribution, is accepted for $P > 0.05$

Detection of m164 transcripts in latently infected lungs

Viral latency in the lungs was studied in an established murine model of experimental syngeneic HCT with BALB/c mice as donors and recipients with concomitant mCMV infection of the recipients ([16–18, 40], for reviews see [3, 6, 7]) For a contextual analysis of TEL activity in the lungs, four lung lobes of each latently infected BALB/c HCT recipient were subdivided into nine pieces, and transcripts IE1, m164_IE, and m164_E_IE were quantitated by the respective RT-qPCRs (Fig. 2a). The fifth lung lobe of each mouse, the postcaval lobe, was subdivided into two pieces and used to determine the latent viral DNA load by qPCR, which was found to be 10^4 viral genomes per 10^6 lung cells in the order of magnitude (not shown), in accordance with the previous work by Simon and colleagues [18]. At a glance, in the lungs of mouse #1 all three TELs were present in numbers clearly above the technical detection limit (DL) of three transcripts

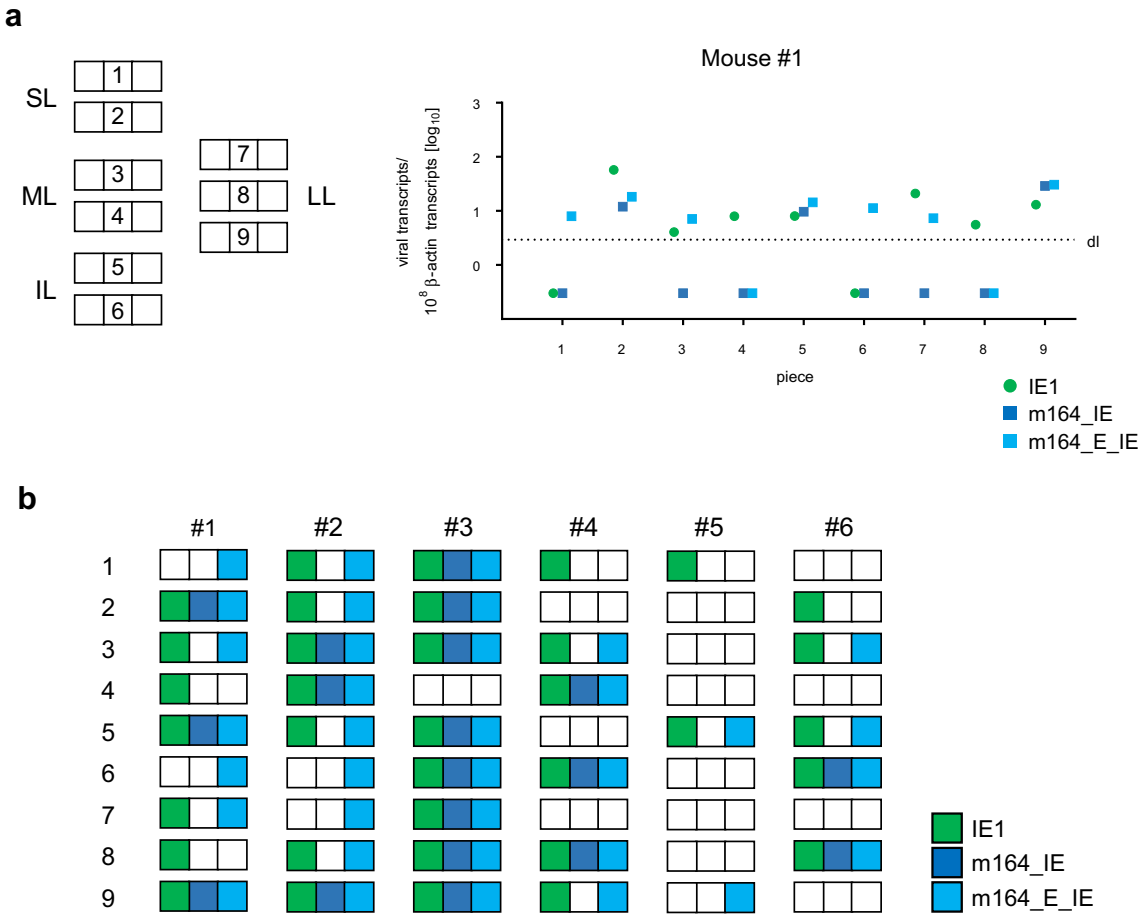


Fig. 2 Stochastic patterns of IE and m164 TEL activity in latently infected lungs. **a** *Left* schematic representation of the lungs localizing the analyzed nine lung tissue pieces to lobes of the lungs: *SL* superior lobe, *ML* middle lobe, *IL* inferior lobe, *LL* left lung. *Right* plot of viral transcript numbers, normalized to 10^8 transcripts of housekeeping gene β-actin, for nine lung tissue pieces shown exemplarily for latently infected mouse #1. *dl* detection limit. **b** Pattern overview of IE1 and m164 TEL activity for six latently infected mice tested, that is for 54 lung tissue pieces altogether. For symbols and color codes, see the internal legends

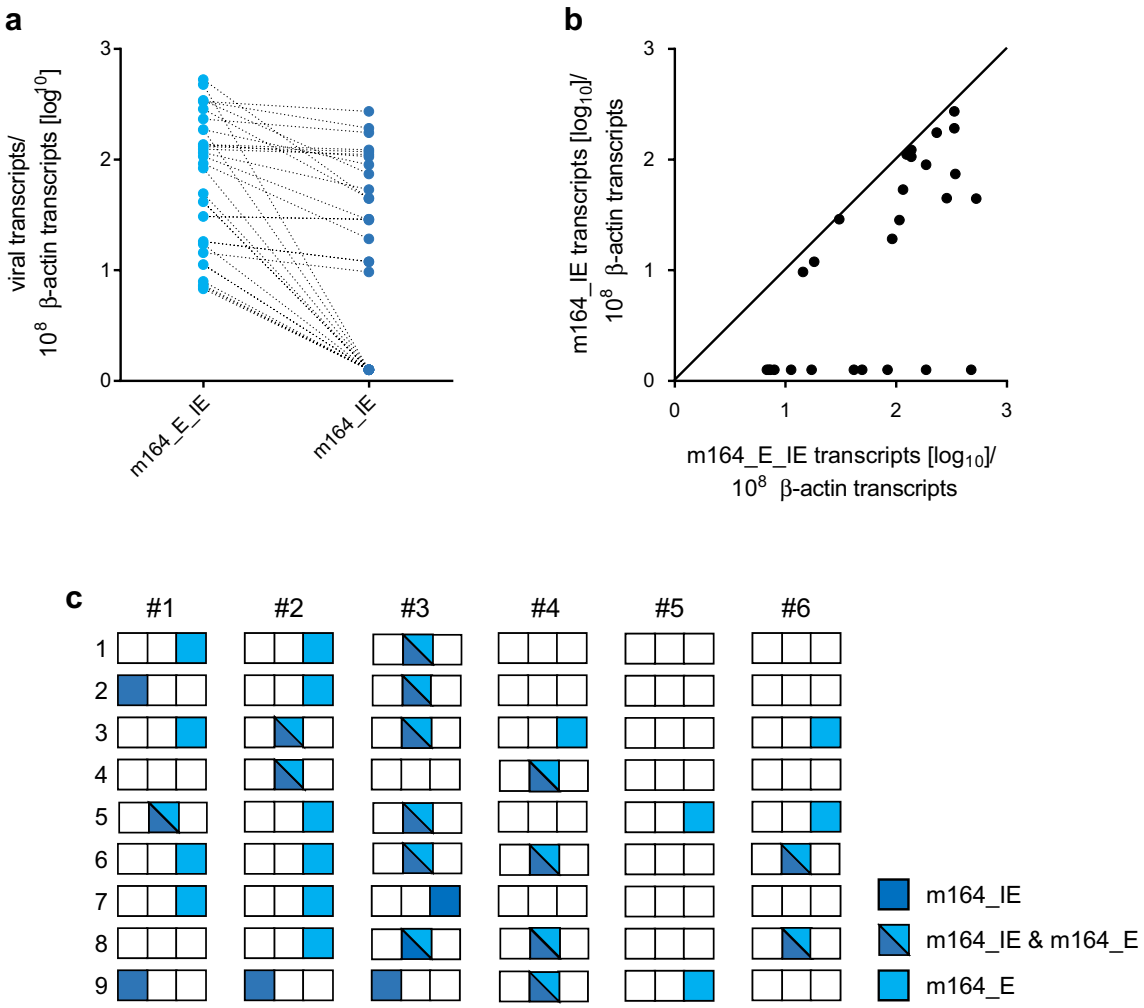


Fig. 3 Stochastic patterns of m164 IE and m164 E TEL activity in latently infected lungs. **a** Correspondence plots of the numbers of m164_E_IE and m164_IE transcripts, each normalized to 10^8 transcripts of housekeeping gene β actin. Data are from m164 TEL-positive pieces compiled from the six latently infected mice tested. **b** Correlation plot of m164_IE transcripts (ordinate) and m164_E_IE transcripts (abscissa) normalized as above. **c** Pattern overview of m164 IE and m164 E TEL activity for six latently infected mice tested, that is for 54 lung tissue pieces altogether. For symbols and color codes, see the internal legend

(recall Fig. 1d), although not every of the nine lung tissue pieces contained all three TELs. For a comprehensive overview, yes–no expression is shown for 54 lung tissue pieces from six mice tested (Fig. 2b), revealing stochastic patterns of expression. Tissue pieces positive in the m164_IE RT-qPCR proved the presence of IE transcripts in 19 out of 54 pieces, and pieces negative for m164_IE but positive for m164_E_IE proved the presence of E transcripts in 16 out of 54 pieces.

This already allowed us to conclude that m164 TELs exist from IE- and E-phase promoters, and stochasticity reflects independent activity of these promoters.

Quantitation of m164 TEL activity in latently infected lungs

So far the data revealed the numbers of pieces selectively containing either the TEL m164_IE or the TEL m164_E. To get the full picture of m164 TEL activity, the quantitative data were used to determine mixtures of the two transcripts within tissue pieces (Fig. 3). Figure 3a, b relates transcript numbers determined by RT-qPCRs m164_E_IE and m164_IE for all tissue pieces positive in the dual-detection m164_E_IE RT-qPCR. In Fig. 3b, pieces containing varying mixtures of TELs m164_IE and m164_E cloud below the angle bisector. Finally, Fig. 3c summarizes the stochastic expression patterns showing pieces that contain only IE or only E transcripts, or mixtures of both.

Bottom line message

The current hypothesis on drivers of MI demands transcriptional activity of viral epitope-encoding genes during latency. The data presented here show that, in accordance with this hypothesis, genes coding for the MI-driving antigenic m164 peptide *AGPPRYSRI* are indeed expressed in latency in a stochastic manner. As a specialty, the antigenic peptide can be derived from IE- as well as E-phase transcripts.

Acknowledgements This work was supported by the Deutsche Forschungsgemeinschaft (DFG), SFB490, individual project E4 "Antigen presentation under the influence of murine cytomegalovirus immune evasion genes" (M.G., B.K, and C.K.S.), and SFB1292, individual project TP11 "Viral evasion of innate and adaptive immune cells and inbetweeners" (A.R, J.K.S., and N.A.W.L.).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standard Animal experiments were approved according to German federal law §8 Abs. 1 TierSchG by the ethics committee of the Landesuntersuchungsamt Rheinland-Pfalz, permission number 177-07/G 10-1-052.

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