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CD8+ T cells induced by adenovirus-vectored vaccine are capable of preventing establishment of latent murine γ-herpesvirus 68 infection

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ABSTRACT

CD8+ T cells are known to control infections, but their role in preventing latent infection from establishing has not been thoroughly investigated.

We hypothesized that a potent CD8+ T cell response patrolling the mucosal viral entry points could kill the first infected cells and thereby abrogate the infection before latency is established.

To investigate this, replication deficient adenovirus serotype 5 vectors encoding murine γ-herpesvirus-68 CD8+ T cell epitopes linked to the T cell adjuvant Invariant chain, were developed. We show that intranasal vaccination of mice reduces the risk of establishment of latent infection from multiple intranasal ID50 challenges with murine γ-herpesvirus-68 by 81% per exposure at 14 days post vaccination. Protection waned over time, but immune responses were extended by heterologous prime-boost vaccination applied simultaneously intramuscularly and intranasally, and animals vaccinated 66 days prior to challenge showed a strong trend of long-term protection.

Our data provides evidence that CD8+ T cells are able to protect against establishment of latent infection. Although the protective efficacy is difficult to maintain over time, this proof-of-concept study suggests a role for a CD8+ T cell arm in future vaccine strategies against latent human viral infections caused by pathogens such as HIV and multiple herpes virus.

1. Introduction

Viral pathogens like Human Immunodeficiency Virus (HIV), Hepatitis B and C Virus and a number of herpes viruses cause persistent chronic or latent infections. Classical MHC class I restricted cytotoxic T cells are known to have potential to control viral replication once the infection is established, but are not generally believed to be capable of preventing acquisition of infection or to eliminate the infection once latency is established [1]. This paradigm might not always be completely true. In a recently published study from our group, a vaccine that induced a strong T cell response and no relevant antibody response against Simian Immunodeficiency Virus (SIV) caused 2 out of 6 vaccinated rhesus macaques to remain free from detectable viral load despite evidence of transient infection [2]. Additionally, unconventional MHC class II and HLA-E restricted CD8+ T cells have been observed capable of abrogating very early SIV infections [3].

Thus, we hypothesized that a vaccine capable of inducing potent and long-lasting CD8+ T cells against conventional MHC class I binding epitopes should be able to kill the first infected cells and thereby prevent the establishment of latent infection.

In natural HIV infections, viral growth only starts declining once a profound T-cell expansion has occurred [4]. The hypothesized CD8+ T cell mediated protection would therefore likely require a

Abbreviations: BAL, Bronchoalveolar Lavage; ChAd63, chimpanzee adenovirus serotype 63; ChAd63-lipoly, lipoly encoded in ChAd63; dpv, days post vaccination; hAd5, human adenovirus serotype 5; hAd5-lipoly, lipoly encoded in hAd5; HIV, Human Immunodeficiency Virus; Ii, MHC class II associated Invariant chain; i.n., intranasal; MHV-68, murine γ-herpesvirus-68; qPCR, Quantitative Polymerase Chain Reaction; SIV, Simian Immunodeficiency Virus; YFP, Yellow Fluorescent Protein.

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very abundant number of virus-specific CD8+ T cells positioned at the site of viral entry, in order to eliminate the infection as early as possible after initial seeding. For HIV, seeding of the latent reservoir occurs already within few days after mucosal virus exposure [5], providing a very narrow window for potential clearing of the infection.

Despite this time-constraint, there is abundant evidence supporting that local CD8+ T cell immunity is key for good vaccine efficacy in the acute phase of infection from latent pathogens like SIV [6] and herpesvirus [7]. It is also well established that CD8+ T cell responses affect the viral load of the latent phase of an SIV infection [8], but there is currently no consensus on the importance of local immunity for protection from establishment of latent viral infection. Masopust has previously hypothesized that a quantitatively robust and mucosally localized cellular immune response could impact the establishment of latent infection [6], but to our knowledge, this has not been adequately tested.

In this proof of concept study, we chose murine γ-herpesvirus 68 (MHV-68) as the infectious model to test if vaccine induced CD8+ T cells could protect against establishment of a model latent infection. MHV-68 is a well characterized as a model for latent viral infection such as Epstein-Barr and Kaposi's sarcoma-associated virus [9], but more importantly, MHV-68 is a highly stringent model, potentially exceeding even HIV in terms of rapid seeding of the latent reservoir [10] and resistance to the vaccine induced CD8+ T cell response once the latent reservoir is established [9]. Within 24 h after intranasal infection in mice, MHV-68 reaches B cells residing in the lung [10], followed by a non-lytic expansion phase in the splenic B-cells, which also makes up the major latent reservoir at 12–14 days after the initial infection [9]. While it has previously been shown that an adenoviral based vaccine can reduce the latent viral load for a MHV-68 infection [7], the possibility of preventing establishment of latent infection has, to our knowledge, not been adequately tested.

To ensure the potent CD8+ T cell response which we hypothesize to be necessary, the experimental vaccine antigen was designed by selecting eight minimal CD8+ T cell epitopes which had each been confirmed to be highly immunogenic for CD8+ T cells in the C57BL/6 mouse in at least two independent studies [12–14]. The epitopes were combined to a polytope in sequence with the genetic adjuvant Invariant chain (Ii) (Iipoly) which enhances adenovirus, gene-gun, and MVA induced CD8+ T cell responses for a wide selection of antigens [7,15–20]. The C57BL/6 mouse was chosen as model, the viral set point is dependent on the mouse-strain specific MHC haplotypes, where the $H2^b$ positive strains, such as C57BL/6, has a relatively high latent viral load, making latent infection easily detectable [11]. Iipoly was encoded in two different serotypes of adenoviral vectors, human serotype 5 (hAd5) and Chimpanzee serotype 63 (ChAd63), which are each known to be potent inducers of CD8+ T cells [2,21]: hAd5-Iipoly and ChAd63-Iipoly.

To evaluate the protective efficacy of the designed vaccine, we applied a repeated-low-dose challenge regimen. This mimics natural infection and facilitates the theoretical opportunity for the immune system to kill the very first of the infected cells before viral spread. Furthermore, unlike a single high-dose challenge, this challenge regimen allows detection of partial protection mediated by the vaccination [22].

By vaccination of C57BL/6 mice we show that CD8+ T cells induced by intranasal (i.n.) application of the hAd5-Iipoly vaccine can reduce the risk of establishment of infection when challenged 14 days post vaccination (dpv). Immune memory and protection subsides over time, but heterologous prime-boost vaccination through the i.m. and i.n. routes combined provided improved maintenance of local and systemic immunity, and correspondingly showed a very strong trend towards prevention of infection. We conclude that under optimal conditions CD8+ T cells can prevent or abrogate latent infection.

2. Results

To confirm immunogenicity of the eight epitopes included in the hAd5 vector *in vivo,* 5 animals were vaccinated s.c. and the splenic CD8+ T cell response was analyzed 15 dpv. It was found that each animal was able to raise a potent CD8+ T cell response to each of the epitopes included in the vaccine (Fig. 1A).

When looking at the immune response after i.n. or i.m. administration of the hAd5-Iipoly vaccine 14 dpv, we see that i.n. administration induced a markedly higher number of IFN-γ producing CD8+ T cells in the Bronchoalveolar Lavage (BAL) compared to i.m. immunization with hAd5-Iipoly and to the negative control (Ii-GP) i.n. (Fig. 1B). I.m. administration gave rise to the most potent systemic response, but a significant response was also induced by i.n. vaccination compared to i.n. control vaccination (Fig. 1C, $P < 0.01$, Mann-Whitney *U* test). It was also furthermore confirmed that neither i.n. nor i.m. administration of hAd5-Iipoly gave rise to any relevant CD4 + T cell response restricted to the encoded epitopes (Supplementary Fig. 3).

The hypothesised protective capacity of the CD8+ T cell response and certainly their effectiveness against early localized infection relies on the presence of a strong response at the viral entry point [7], and the most potent BAL response was obtained by i.n. immunisation. Therefore, the protective potential of the hAd5-Iipoly vaccine was investigated by i.n. immunization of mice followed by three i.n. ID_{50} challenges of MHV-68 on day 14, 16 and 18 post vaccination. The infection status of the animals was determined by qPCR on splenic DNA at 4 weeks after the last challenge (46 dpv) which is at least two weeks post clearance of acute viral replication [9]. We found that 7 out of 13 vaccinated animals were protected from three i.n. ID_{50} challenges with MHV-68, whereas all animals in the unvaccinated group were infected (Fig. 1D). This is an 81.35% reduction in risk of infection per exposure (calculation, see materials and methods) with a p-value of 0.0051 (Fisher's exact test), which strongly supports that CD8+ T cells can prevent establishment of a latent viral infection. Notably, no difference in viral load was detected between those of the vaccinees that did become infected and the controls (Fig. 1D) meaning that the vaccine-induced CD8+ T cell response had almost no effect on post-viral replication. This is consistent with previous claims that CD8+ T cells in $H2^b$ mice cannot diminish the latent MHV-68 infection once established [11] and supports the use of our qPCR assay for detection of infection in C57BL/6 mice as the latent viral load is unaffected by vaccination.

To investigate long term protection, mice were vaccinated as described for the acute immune response experiment described above, and the immune responses were analyzed 66 dpv. Similar to the observed acute response, the strongest mucosal CD8+ T cell response at 66 dpv was obtained by vaccinating through the i.n. route (Fig. 2A). I.n. vaccination did also give rise to a significant response systemically (Fig. 2B, $P < 0.01$, Mann-Whitney *U* test) compared to negative control i.n. The systemic responses were approximately similar in magnitude to those seen at 14 dpv (Fig. 1B compared to Fig. 2B), but the responses in the BAL after i.n. vaccination were markedly decreased (Fig. 1A compared to Fig. 2A). qPCR on splenic DNA at 4 weeks after the last of three i.n. challenges showed no protection at 64 dpv compared to 14 dpv (Fig. 2C).

As an effector memory phenotype, characterised by CD8 T cells by producing both IFN-γ and TNF-α, has been associated with chronic viral control [29], this was also addressed (Supplementary Fig. 4). All i.n. immunizations led to BAL responses with a reason-

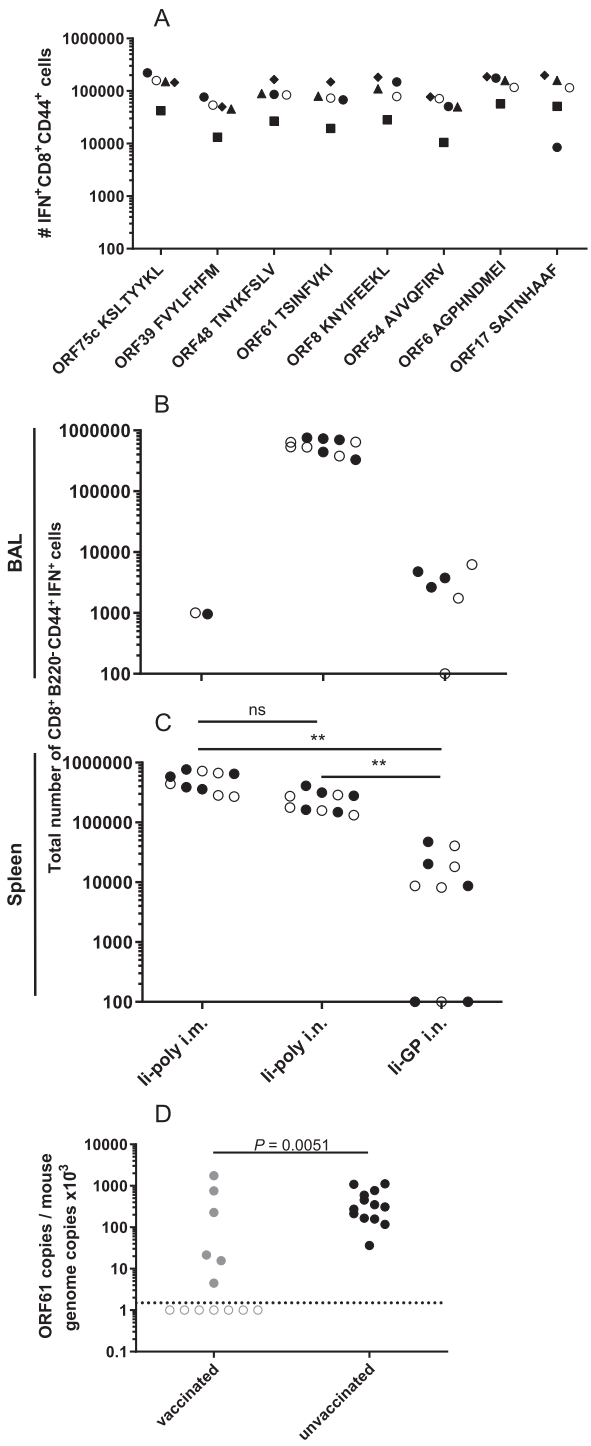

Fig. 1. Early vaccine induced immune responses and protection. A: 5 C57BL/6 mice were vaccinated with hAd5-lipoly and CD8+ T cell responses against the eight lipoly epitopes were measured 15 dpv. Each symbol represents one mouse. B & C: 5 C57BL/6 mice per group were vaccinated i.m. or i.n. with hAd5-lipoly or i.n. with hAd5 encoding an irrelevant antigen (Ii-GP). The immune responses at14 dpv are shown in total numbers of IFN-γ producing CD8+ CD44+ cells in the BAL (B) and the spleen (C). The background responses from non-stimulated cells have been subtracted from all samples of the corresponding animals. Open circles: stimulation with early response epitopes (orf 39, orf 75c and orf 48). Black dots: stimulation with late response epitopes (orf 61, orf8, orf54, orf 6 and orf 17) [12]. BAL cells for the 5 mice immunized i.m. were pooled in (B). ** Statistical significance of $P < 0.01$ (Mann-Whitney *U* test). D: 13 C57BL/6 mice per group were vaccinated i.n. with hAd5-lipoly or not vaccinated and challenged 14, 16 and 18 dpv. 4 weeks after last challenge, viral load was determined by qPCR on spleens. $P = 0.0051$ (Fisher's exact test). Samples below the detection limit (dashed line) of the qPCR were regarded as not infected (open circles). qPCR reactions were performed in triplicates.

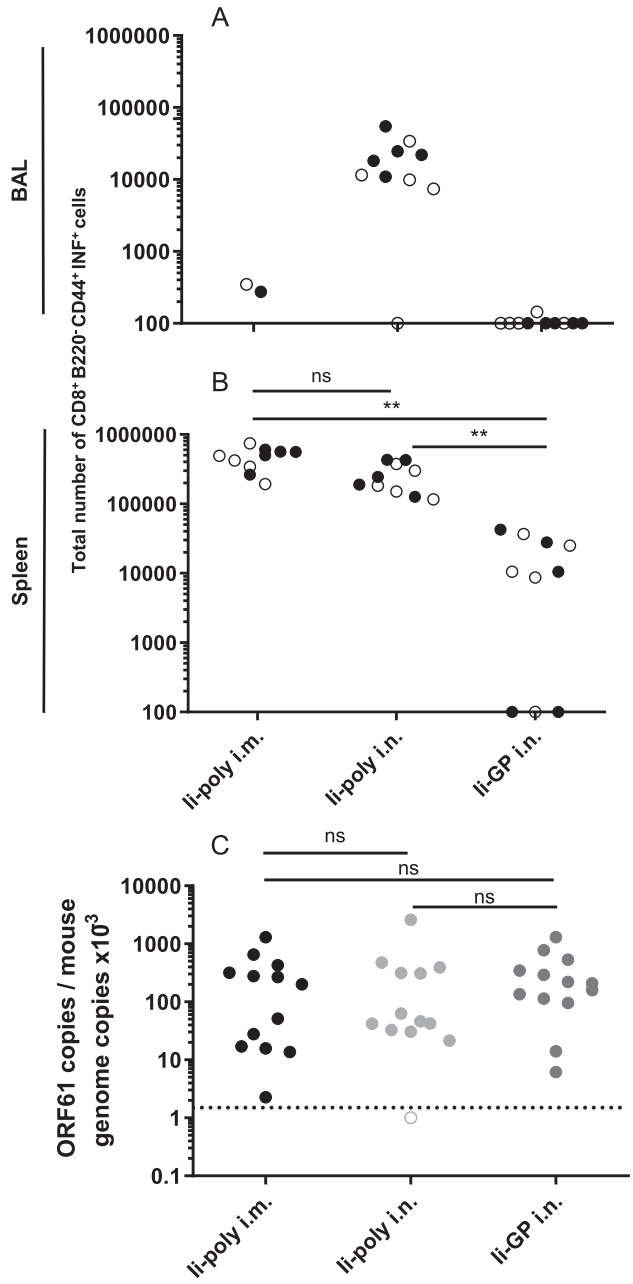

Fig. 2. Long term vaccine induced immune responses and protection. 5 C57BL/6 mice per group were immunized i.m. or i.n. with hAd5-lipoly or i.n. with hAd5 encoding an irrelevant antigen (Ii-GP). A & B: The immune responses 66 dpv are shown as total numbers of IFN-γ producing CD8+ CD44+ cells in the BAL (A) and in the spleen (B). The background responses from non-stimulated cells have been subtracted from all samples of the corresponding animals. BAL cells for the 5 mice immunized i.m. were pooled in (A). ** Statistical significance of $P < 0.01$ (Mann-Whitney *U* test). C: 13 C57BL/6 mice per group were immunized i.m. or i.n. with hAd5-lipoly or i.n. with hAd5-Ii-GP and challenged 64, 70 and 76 dpv. 4 weeks after last challenge (104 dpv), viral load was determined by qPCR on spleens. Open circles: undetectable viral load. Statistical test: Kruskal-Wallis one-way analysis of variance. qPCR reactions were performed in triplicates.

able proportion of double positive cells, confirming that the immunizations induced highly functional CD8 T cell responses, regardless of their protective capacity.

Prime-boost immunisations can enhance T cell mediated immune responses and protection, but priming with viral-vectored vaccines is known to create vector-immunity which reduces, but not completely abrogates, the enhancing effect of a boosting immunisation with the same vectored vaccine [23].

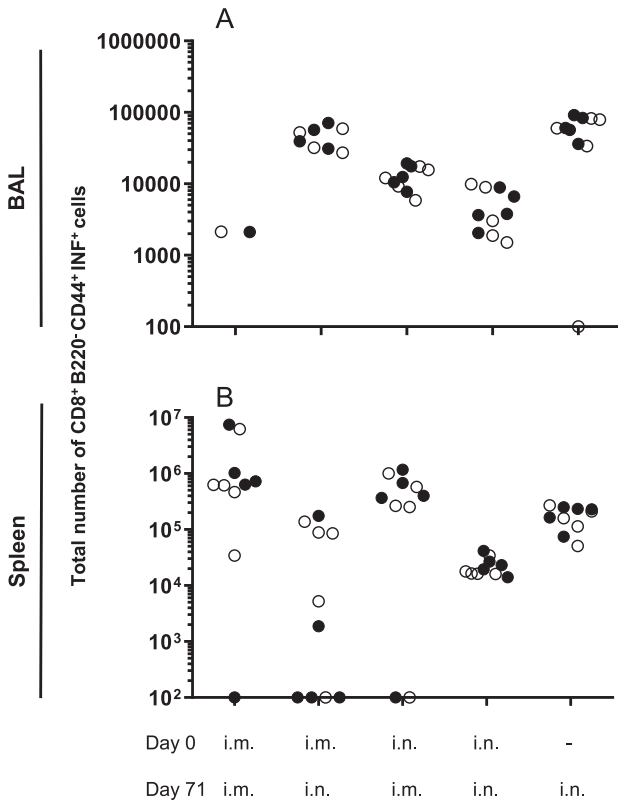

Fig. 3. Immune responses after homologous prime-boost. 5 C57BL/6 mice per group were primed either i.n. or i.m. with hAd5-lipoly followed by boosting 71 days later either through same or through the opposite route. One group was immunized once i.n. on day 71 for comparison. Immune responses 14 days post last immunization are shown as total numbers of IFN-γ producing CD8+ CD44+ cells in the BAL (A) and the spleen (B). The background responses from non-stimulated cells have been subtracted from all samples of the corresponding animals. Please notice that the y-axis differs for (B) compared to all other ICS results.

Additionally, it has been reported that pre-existing hAd5 immunity can be bypassed by mucosal administration of the vaccine [24]. To investigate the possibilities of prolonging the protective effect of the hAd5-lipoly vaccine, we primed mice either i.n. or i.m. with hAd5-lipoly followed by boosting 71 days later either through same or through the opposite route. A group of mice were immunized once i.n. for comparison.

The CD8+ T cell responses in the BAL at 14 days after the last immunization (Fig. 3A) were pronounced for the mice receiving i.m. priming and i.n. boost or a single i.n. immunization, however without any notable effect of the priming. Mice receiving i.n. priming and i.m. boost had low responses, and mice receiving only i.m. immunizations had almost undetectable lung-mucosa CD8+ T-cell responses. These results suggested that suboptimal memory responses from i.n. vaccination could not be enhanced by i.n. vaccination with a homologous vector, and we again observed that BAL responses were primarily dependent on a mucosal administration. In contrast, splenic responses after homologous i.m. prime and i.m. boost were larger than the responses seen after i.m. prime and i.n. boost, suggesting that i.n. boosting does not have any relevant enhancing effect on a systemic response, but also that vector-immunity from i.m. priming does completely block the effect of i.m. boosting (Fig. 3B).

Heterologous prime-boost regimens can bypass vector specific immunity and give rise to drastically improved mucosal CD8+ T cells compared to single immunization [19]. To investigate if this could improve mucosal responses for our antigen, we produced

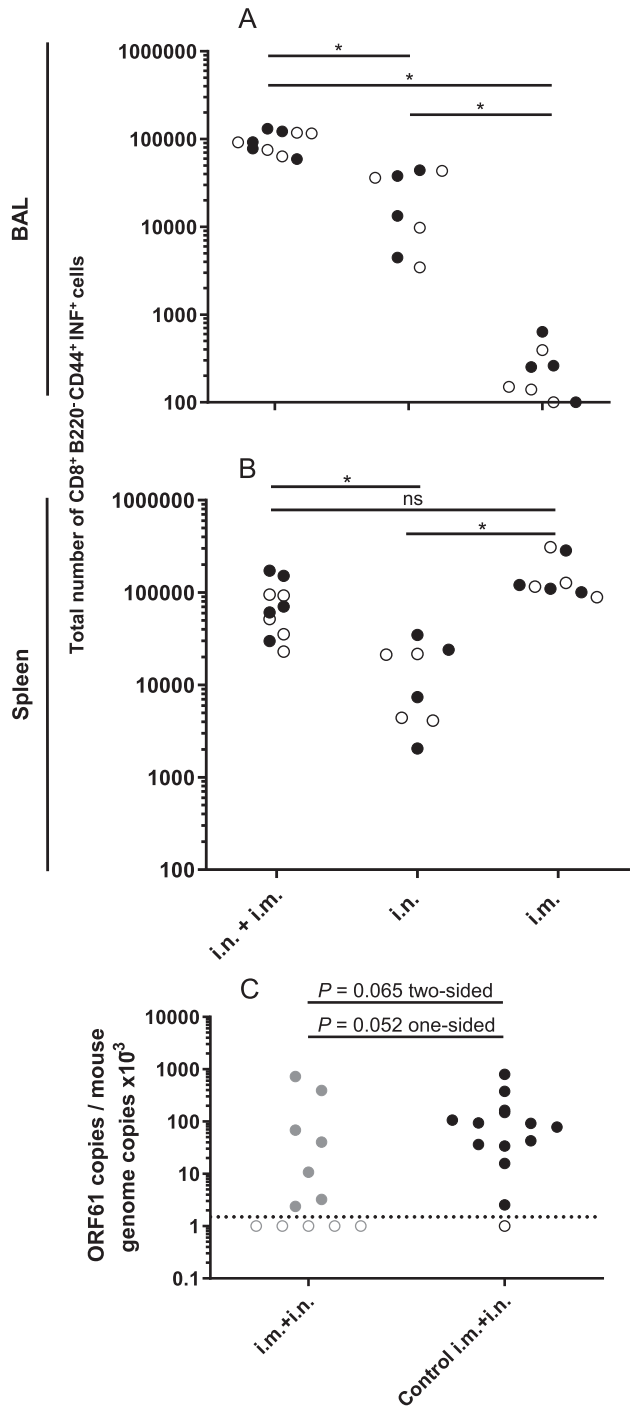

Fig. 4. Long term immune responses and protection after heterologous prime-boost immunization. A & B: 5 C57BL/6 mice per group were immunized i.n., i.m. or simultaneous i.n. and i.m. with hAd5-lipoly vaccine on day 0 and with ChAd63-lipoly on day 60. The immune responses 60 days post last immunization (day 120) are shown in total numbers of IFN-γ producing CD8+ CD44+ cells in the BAL (A) (dotted line indicates 0) and in the spleen (B). The background responses from non-stimulated cells have been subtracted from all samples of the corresponding animals. * Statistical significance of $P < 0.05$ (Mann-Whitney *U* test). C: C57BL/6 mice were immunized both i.m. and i.n. with hAd5-lipoly vaccine (12 mice) or hAd5 containing an irrelevant antigen (control i.m. + i.n., 13 mice) at day 0 and with ChAd63-lipoly or control on day 60. The mice were challenged at 60 and 67 days post last immunization (day 120 and 127). 62 days after last challenge (day 189), viral load was determined by qPCR on spleens. Open circles: undetectable viral load. $P = 0.0652$ (Fisher's exact test, two-sided) or $P = 0.0522$ (one-sided). qPCR reactions were performed in triplicates.

the ChAd63-Iipoly vaccine to use in a heterologous prime-boost regimen (23,24) together with the hAd5-Iipoly vaccine [2,21].

It was recently found that the synergy of intranasal and systemic immunity using adenovirus vectors enhances long-term vaccine-induced control of acute Influenza A infection. Uddbäck *et al.* showed that combined local and systemic immunization elicited long-term (8 months) CD8+ T-cell mediated protection as compared to either i.n. (3 months protection) or s.c. immunization (1 month protection) [25]. A similar synergy of the combined route was shown earlier against MHV-68 in BALB/c mice [7], and we therefore decided to vaccinate with combined i.m. and i.n. immunization in the heterologous prime-boost regimen in an attempt to extend protective efficacy. The systemic and mucosal immune responses were determined 60 days after the last immunization, and as anticipated, we saw a significantly higher number of mucosal IFN-γ producing CD8+ T cells after combined immunization than after i.n. or i.m. administration alone (Fig. 4A). Furthermore, the systemic CD8+ T cell response induced by double-route immunization was as strong as for i.m. administration alone and significantly stronger than the response after i.n. administration alone (Fig. 4B). Mice immunized by the heterologous prime-boost regimen were challenged with two ID_{50} doses of MHV-68 at day 120 and 127 (day 60 and day 67 after the last immunization), and the viral load was assessed at day 189 (62 days post last challenge). Only 7 out of 12 vaccinees had detectable virus load and are regarded as infected, compared to 13 out of 14 in the control group (Fig. 4C). The results are not formally significant, but a strong tendency of protection was observed in the vaccinees ($P = 0.065$, two-way Fisher's exact test), and again there was no difference in virus load between infected animals in the vaccinated and control group. In combination with the conclusive results obtained at 14 dpv, these results lend support to the notion that a vaccine induced CD8+ T-cell response can reduce the risk of latent viral infection, when effective antiviral CD8+ T cells are present in sufficient numbers.

3. Discussion

In terms of generating high magnitude mucosal immune responses, we show that a mucosal or a mixed mucosal/systemic vaccination strategy is superior to systemic vaccination alone, both for the acute response (Fig. 1B) and for the memory responses (Figs. 2A and 4A). This resembles previous findings by Uddbäck et al., Belyakov et al. and others [7,25,26]. For long term maintenance of this high magnitude CD8+ response, we show the benefit of vaccinating in a heterologous prime boost regimen and notably the use of simultaneous mucosal and intramuscular immunization (see Figs. 2A and 4A). Most importantly, we have shown that under optimal conditions CD8+ T-cells are capable of preventing acquisition of latent viral infection, presumably by eliminating the acute infection before latency is established as latent viral load is unaffected by vaccination in animals with detectable infection.

Our results share strong similarities with the results of the previously mentioned SIV study [2], where an adenoviral vectored vaccine inducing CD8+ T cells, and no relevant CD4 + T cell or antibody responses, gave rise to a very similar protective effect against repeated low dose challenges. Those findings strongly support the results presented in this work, and vice versa, and ultimately adds evidence to the hypothesis that CD8+ T cells can prevent establishment of a latent infection. It would be relevant to further investigate the effect of the vaccine-induced mucosal CD8+ T cells on the progression of the pathogen at time points very early after the initial infection, in order to better understand this mechanism. However, such studies would be challenging to conduct when using a low-dose challenge regimen, as it would require a very large numbers of animals and a method to detect the extremely low viral loads at early time-points of infection.

The protective effect was difficult to maintain long term. Prime boost immunization regimens are widely used to enhance the longevity of immune responses. However, in the context of viral vectored vaccines, vector immunity from the priming immunization makes it questionable if homologous boosting regimens can enhance responses. When looking at the mucosal responses after homologous prime-boost vaccination, we observed that priming through the airways efficiently blocked the effect of boosting through the same route. Interestingly, and in alignment with previously published studies [24] we saw that mucosal boosting seemed to circumvent the vector immunity from i.m. priming and gave rise to a marked mucosal CD8+ T cell response which was comparable to that of a single i.n. immunization. This feeds in to the discussion of the potential for hAd5 vectored vaccines in populations with pre-existing Ad5 immunity, as it supports the potential use of hAd5 viral vectored vaccines if administered mucosally rather than systemically. In addition to what was shown by Croyle et al. [24] our data suggests that the route of antigen stimulation that gave rise to the pre-existing immunity is crucial for the effect of the subsequent mucosal vaccination, but also that, in contrast to the mucosal vaccination and responses, the effect of i.m. boosting on the splenic response did not seem to be affected by whether the priming were i.m. or i.n..

However, changing the site of homologous prime-boost immunization could not be used to extend or augment immune responses in the mucosal compartment. Instead, and in alignment with the general observations in the field, using a heterologous prime-boost regimen rather than a homologous was a more efficient strategy for boosting and further extending an antigen-specific immune response.

Even under the artificially optimized vaccination circumstances; using a heterologous prime-boost regimen, simultaneously administered through both a systemic and mucosal route, and a vaccine design incorporating strong CD8 epitopes specific for the HLA-alleles of the C75BL/6 mouse, we still see declining protection over time. This underlines that the necessary CD8+ T cell immunity may be difficult to induce in a real-life settings, with variety in HLA haplotypes and maintain for clinically relevant time-frames which may be several years. The prime-boost experiments in this study investigates how boosting affects the magnitude and duration of the host response and thereby the protective effect. Yet, other studies show how continuous antigen stimulation can mature the immune response in terms of phenotype and functional qualities [27,28]. Selinger et al. report how multiple antigen stimulations lead to an evolving host response that affects the protective outcome [28]. Masopust et al. describes how every immunization altered the T cell phenotype and that a higher number of immunizations lead to retained effector-memory phenotype and interestingly also to increased accumulation in mucosal tissues [27]. While the data presented here suggests that the time since the last antigen stimulation is a key determinant for the protective capacity of the CD8+ T cell mediated immune response, it is also worthwhile to consider how the complex changes in functional qualities of the CD8+ T cell response following repeated antigen stimulation can enhance the protective efficacy and longevity of the immune response for example by altering the functional phenotype of the response. In addition to looking at IFN-γ producing CD8 T cells, we also evaluated the functionality of the CD8 responses by the total number of double functional CD8 T cells capable of producing both IFN-γ and TNF-α (Supplementary Fig. 4), as T cells producing both IFN-γ and TNF-α have been shown to be superior in clearing pathogens compared to cells producing either cytokine alone [29]. Although our results were not conclusive, we did observe a tendency towards a higher

number of double-functional CD8+ T cells in the BAL after the two immunization regimens leading to reduced per-exposure risk of infection (14 dpv after single i.n. immunization and following heterologous prime boost i.n. and i.m. combined immunizations, Figs. 1D and 4C) compared to the immunization regimen incapable of conveying protection (66 dpv after single i.n. immunization, Fig. 2C). No difference in the total number double functional CD8 T cells was seen in the spleen.

In conclusion, while we show evidence of short term CD8+ T cell mediated prevention or abrogation of latent infection, our data fail to show significant long term protection. However, MHV-68 is a highly stringent model of latent viral infection in terms of rapid seeding of latent reservoir and resistance to the vaccine induced CD8+ T cell response once the latent reservoir is established. Potentially, other latent infection may have less stringent requirements.

Thus, while our results suggest that CD8+ T cells can only prevent a latent MHV-68 infection under the best of circumstances, we also interpret our results to strongly advocate for incorporation of a mucosal CD8+ T cell arm in future experimental vaccine-designs against pathogens causing latent infections.

4. Materials and methods

4.1. Cell cultures

All cell lines were cultured in DMEM-1965 supplemented with fetal bovine serum (FBS) (Sigma) (10%), penicillin/streptomycin, L-glutamine (not NIH/3T3 cells) and sodium pyruvate (not NIH/3T3 and vero-cre cells).

4.2. Animals

All animals were C57BL/6 females obtained from Taconic M&B (Ry, Denmark) and housed at the Panum Institute, University of Copenhagen. All experimental procedures were approved by the national animal ethics committee (Dyreforsøgstilsynet, permit number 2011/561-120) and performed according to national guidelines.

4.3. Murine γ-herpesvirus 68

MHV-68 encoding yellow fluorescent protein (YFP) was a gift from Kristine Niss Arfelt (INF, UCPH, DK). MHV-68 encoding YFP coupled to histone 2b and wildtype MHV-68 was kindly shared by Christopher Collins (Emory University, Atlanta, US). The three MHV-68 variants are reported to be clinically and pathologically similar [30]. The MHV-68 virus stock was propagated and titered using NIH/3T3 cells layered with methylcellulose.

4.4. Antigen design

The polytope was designed of eight epitopes as described in the introduction. Linkers between epitopes were designed to ensure correct processing of the polytope [31,32]. The cleavage sites were evaluated using the NetChop 3.1 server on http://www.cbs.dtu.dk/. The amino acid sequence of the polytope is LKFKSLTYYKLFVYLFH FMESLTNYKFSLVALNTSINFVKIYYYKNYIFEEKLPGLAVVQFIRVTGIAG PHNDMEITRKSAITNHAAF. The polytope was produced by GenScript and was cloned into a hAd5 shuttle vector encoding CMV promoter cassette, the MHC class II chaperone protein Ii, the porcine teschovirus self-cleaving peptide 2A (P2A) [33] and EGFP for vector tracking (Iipoly). See Supplementary Fig. 1 for a schematic overview of the antigen design.

4.5. Adenoviral vectors

Adenoviruses were produced as previously described using homologous recombination, plaque cloning, amplification and CsCl banding [34]. Infectivity was determined with the Adeno-X Rapid Titer Kit (Clontech) on HEK 293T cells. Negative control virus, Ii-GP, was produced following the same protocol [16].

4.6. Mouse immunization and challenges

Mice, 7–10 weeks old, were immunized with 2×10^7 IFU hAd5 or 10^9 particles of ChAd63 vaccine diluted in PBS. Mice were anesthetized with isofluorane before s.c. immunizations, with isofluorane or Ketamine/Xylazine before i.m. immunizations, and with Ketamine/Xylazine before any i.n. injections.

Challenges were performed i.n. with ID_{50} MHV-68. ID_{50} was experimentally found to be 3.55 plaque forming units for MHV-68 in the C57BL/6 mouse (data not shown).

For acute protection experiments, mice were challenged with MHV-68 encoding YFP on day 14, wt MHV-68 on day 16 and MHV-68 encoding YFP coupled to histone 2b on day 18 after vaccination. For long-term protection experiments after single vaccination, mice were challenged with the same MHV-68 variants as above at 64, 70 and 76 dpv respectively. For the heterologous prime-boost experiment, mice were challenged with MHV-68 encoding YFP only.

4.7. DNA extraction

Mice were sacrificed by bleeding under anesthesia. Spleens and lungs were removed and immediately frozen on dry ice or at −80 °C. Organs were purified using GenElute™ Mammalian Genomic Purification Kit (Sigma).

4.8. Intracellular cytokine-staining (ICS) and flow cytometry

Splenocyte and BAL cell suspensions were obtained as described [7]. BAL cells from animals receiving only i.m. immunizations were pooled due to very low cell counts. The i.m. groups induced inferior BAL immunity even if when comparing the pooled i.m. BAL samples to single i.n. BAL samples.

Cells were incubated for 5 h in 3 μM monensin with or without 1 μg/mL of relevant peptides. The cells were stained against surface markers (PerCP/Cy5.5 anti CD8, FITC anti CD4, APC/cy7 anti CD44 (all from Biolegend), and Pacific Blue anti B220 (Life technologies)), fixed, permeabilized, and stained intracellularly against IFN-γ.

The peptides used were the eight MHV-68 CD8+ T-cell epitopes included in the polytope, either individually or pooled in an early response type group (ORF 39, ORF75c and ORF48) and a late response type group (ORF61, ORF8, ORF 54, ORF 6, ORF17) as defined by Gredmark-Russ et al. [12]. Peptides were obtained from Schafer-N ApS, DK.

Flow cytometry was performed on the LSR-II (BD Biosciences) flow cytometer and data analysis was performed using FlowJo software. Epitope specific CD8+ T-cell responses were measured as B220-, CD8+, CD44+, IFN-γ+ cells and presented in total number of cells per organ. Gating strategy, see Supplementary Fig. 2. Non-stimulated samples were used as background controls, and their response values have been subtracted from the peptide-stimulated samples of the corresponding animal before statistical analyses and graphical presentation.

For all figures with logarithmic y-axis, the values ≤ 0 have been manually adjusted to 100 to allow them to be visible in the plot. All statistical tests are performed on non-adjusted data.

4.9. Quantitative polymerase chain reaction (qPCR)

Viral loads were determined on 25 ng purified DNA by qPCR using Brilliant II QPCR Master Mix (Agilent). Primers and FAM/BHQ-1 probes were directed towards the MHV-68 ORF61 [7] and against the murine housekeeping gene GAPDH [35]. Cycling conditions were 10 min at 95 °C, followed by 50 cycles of 30 sec at 95 °C, 1 min at 58 °C and 30 sec at 72 °C. All samples were run in triplicates. Mouse genomic DNA for the standard curve for GAPDH was purified from spleen tissue from naïve BALB/C mice. The MHV-68 standard curve was made on bacterial artificial chromosome DNA diluted in 10 ng/μL mouse genomic DNA. Non-template controls included ultra-purified water and virus-free mouse DNA. Samples were run on CFX384 Touch™ Real-Time PCR Detection System. The data analysis was based on the mean of triplicates and viral loads are presented as ORF61 copies per 1000 GAPDH copies.

The detection limit of the qPCR of MHV-68 was 5 copies per reaction, corresponding to a sensitivity of approximately 5 copies of ORF61 per 10,000 copies of mouse genome.

In spite of persistent efforts, it was unfortunately not possible to create MHV-68 strain specific primer/probe sets with sufficient sensitivity.

4.10. Calculation of vaccine efficacy when the first challenge is given 14 dpv

The calculations were performed as described by Hudgens and Gilbert [36] for repeated low dose challenges.

Vaccine efficacy (VE) is defined as $VE = 1 - \frac{P_v}{P_c}$, where P_v is the risk of infection per challenge in the vaccinated group and P_c is the risk of infection per challenge in the control group.

After three exposures, 7 out of 13 animals were not infected. The risk of being infected per exposure can be written as 1 – risk of escaping infection:

Risk of escaping infection per exposure = $1 - P_v$

For three repeated challenges this gives

Risk of escaping infection = $(1 - P_v)^3 = \frac{7}{13}$

From this equation, the risk of infection in the vaccinated group can be estimated as $P_v = 1 - \sqrt[3]{\frac{7}{13}}$

The vaccine efficacy per exposure at 14 dpv against a challenge with ID_{50} is calculated to:

$$VE = 1 - \frac{1 - \sqrt[3]{\frac{7}{13}}}{1} = 0.81355 \approx 81\%$$

The 95% confidence interval for the proportion 7/13 can be constructed using minus two times the loglikelihood function for the binomial distribution to locate all values within 3.841 of the minimum value. This results in a 95% confidence interval for $1 - P_V$ ranging from 27.94 to 78.34% which may be back-transformed to the scale of VE:

Lower limit: 0.6537784 = 65%
Upper limit: 0.9218645 = 92%

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Conflict of interest disclosure

PJH is an inventor on a patent describing the MHC class II associated Invariant chain as a vaccine adjuvant, but commercial rights for exploitation within herpesvirus vaccines resides with GlaxoSmithKline Biologicals SA. The authors declare that no other conflict of interest exists. All authors have approved the final article.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.04.034.

References

[1] Amanna IJ, Slifka MK. Contributions of humoral and cellular immunity to vaccine-induced protection in humans. Virology 2011 Mar 15;411(2):206–15.
[2] Xu H, Andersson AM, Ragonnaud E, et al. Mucosal vaccination with heterologous viral vectored vaccine targeting subdominant SIV accessory antigens strongly inhibits early viral replication. EBioMedicine 2017.
[3] Hansen SG, Sacha JB, Hughes CM, et al. Cytomegalovirus vectors violate CD8+ T cell epitope recognition paradigms. Science 2013 May 24;340(6135):1237874.
[4] Ndhlovu ZM, Kamya P, Mewalal N, et al. Magnitude and kinetics of CD8+ T cell activation during hyperacute HIV infection impact viral set point. Immunity 2015 Sep 15;43(3):591–604.
[5] Whitney JB, Hill AL, Sanisetty S, et al. Rapid seeding of the viral reservoir prior to SIV viraemia in rhesus monkeys. Nature 2014 Aug 7;512(7512):74–7.
[6] Masopust D. Developing an HIV cytotoxic T-lymphocyte vaccine: issues of CD8 T-cell quantity, quality and location. J Intern Med 2009 Jan;265(1):125–37.
[7] Hoegh-Petersen M, Thomsen AR, Christensen JP, Holst PJ. Mucosal immunization with recombinant adenoviral vectors expressing murine gammaherpesvirus-68 genes M2 and M3 can reduce latent viral load. Vaccine 2009 Nov 12;27(48):6723–30.
[8] Schmitz JE, Kuroda MJ, Santra S, et al. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. Science 1999 Feb 5;283(5403):857–60.
[9] Barton E, Mandal P, Speck SH. Pathogenesis and host control of gammaherpesviruses: lessons from the mouse. Annu Rev Immunol 2011;29:351–97.
[10] Flano E, Jia Q, Moore J, Woodland DL, Sun R, Blackman MA. Early establishment of gamma-herpesvirus latency: implications for immune control. J Immunol 2005 Apr 15;174(8):4972–8.
[11] Marques S, Alenquer M, Stevenson PG, Simas JP. A single CD8+ T cell epitope sets the long-term latent load of a murid herpesvirus. PLoS Pathog 2008 Oct;4(10):e1000177.
[12] Gredmark-Russ S, Cheung EJ, Isaacson MK, Ploegh HL, Grotenbreg GM. The CD8 T-cell response against murine gammaherpesvirus 68 is directed toward a broad repertoire of epitopes from both early and late antigens. J Virol 2008 Dec;82(24):12205–12.
[13] Freeman ML, Lanzer KG, Cookenham T, et al. Two kinetic patterns of epitope-specific CD8 T-cell responses following murine gammaherpesvirus 68 infection. J Virol 2010 Mar;84(6):2881–92.
[14] Sehrawat S, Kirak O, Koenig PA, et al. CD8(+) T cells from mice transnuclear for a TCR that recognizes a single H-2K(b)-restricted MHV68 epitope derived from gB-ORF8 help control infection. Cell Rep 2012 May 31;1(5):461–71.
[15] Mikkelsen M, Holst PJ, Bukh J, Thomsen AR, Christensen JP. Enhanced and sustained CD8+ T cell responses with an adenoviral vector-based hepatitis C virus vaccine encoding NS3 linked to the MHC class II chaperone protein invariant chain. J Immunol 2011 Feb 15;186(4):2355–64.
[16] Holst PJ, Sorensen MR, Mandrup Jensen CM, Orskov C, Thomsen AR, Christensen JP. MHC class II-associated invariant chain linkage of antigen dramatically improves cell-mediated immunity induced by adenovirus vaccines. J Immunol 2008 Mar 1;180(5):3339–46.
[17] Ragonnaud E, Andersson AC, Mariya S, et al. Therapeutic vaccine against primate papillomavirus infections of the cervix. J Immunother 2017 Feb;40(2):51–61.
[18] Spencer AJ, Cottingham MG, Jenks JA, et al. Enhanced vaccine-induced CD8+ T cell responses to malaria antigen ME-TRAP by fusion to MHC class II invariant chain. PLoS One 2014;9(6):e100538.
[19] Capone S, Naddeo M, D'Alise AM, et al. Fusion of HCV nonstructural antigen to MHC class II-associated invariant chain enhances T-cell responses induced by vectored vaccines in nonhuman primates. Mol Ther 2014 May;22(5):1039–47.

[20] Grujic M, Holst PJ, Christensen JP, Thomsen AR. Fusion of a viral antigen to invariant chain leads to augmented T-cell immunity and improved protection in gene-gun DNA-vaccinated mice. J Gen Virol 2009 Feb;90(Pt 2):414–22.
[21] Colloca S, Barnes E, Folgori A, et al. Vaccine vectors derived from a large collection of simian adenoviruses induce potent cellular immunity across multiple species. Sci Transl Med 2012;4(115):115ra2.
[22] Nolen TL, Hudgens MG, Senb PK, Koch GG. Analysis of repeated low-dose challenge studies. Stat Med 2015 May 30;34(12):1981–92.
[23] Steffensen MA, Jensen BA, Holst PJ, Bassi MR, Christensen JP, Thomsen AR. Pre-existing vector immunity does not prevent replication deficient adenovirus from inducing efficient CD8 T-cell memory and recall responses. PLoS One 2012;7(4):e34884.
[24] Croyle MA, Patel A, Tran KN, et al. Nasal delivery of an adenovirus-based vaccine bypasses pre-existing immunity to the vaccine carrier and improves the immune response in mice. PLoS One 2008;3(10):e3548.
[25] Uddback IE, Pedersen LM, Pedersen SR, et al. Combined local and systemic immunization is essential for durable T-cell mediated heterosubtypic immunity against influenza A virus. Sci Rep 2016;6:20137.
[26] Belyakov IM, Ahlers JD, Nabel GJ, Moss B, Berzofsky JA. Generation of functionally active HIV-1 specific CD8+ CTL in intestinal mucosa following mucosal, systemic or mixed prime-boost immunization. Virology 2008 Nov 10;381(1):106–15.
[27] Masopust D, Ha SJ, Vezys V, Ahmed R. Stimulation history dictates memory CD8 T cell phenotype: implications for prime-boost vaccination. J Immunol 2006 Jul 15;177(2):831–9.
[28] Selinger C, Strbo N, Gonzalez L, et al. Multiple low-dose challenges in a rhesus macaque AIDS vaccine trial result in an evolving host response that affects protective outcome. Clin Vaccine Immunol 2014 Dec;21(12):1650–60.
[29] Seder RA, Darrah PA, Roederer M. T-cell quality in memory and protection: implications for vaccine design. Nat Rev Immunol 2008 Apr;8(4):247–58.
[30] Collins CM, Speck SH. Tracking murine gammaherpesvirus 68 infection of germinal center B cells in vivo. PLoS One 2012;7(3):e33230.
[31] Le GS, Stamegna P, Walker BD. Portable flanking sequences modulate CTL epitope processing. J Clin Invest 2007 Nov;117(11):3563–75.
[32] Kulkarni V, Rosati M, Valentin A, et al. HIV-1 p24(gag) derived conserved element DNA vaccine increases the breadth of immune response in mice. PLoS One 2013;8(3):e60245.
[33] Kim JH, Lee SR, Li LH, et al. High cleavage efficiency of a 2A peptide derived from porcine teschovirus-1 in human cell lines, zebrafish and mice. PLoS One 2011;6(4):e18556.
[34] Holst PJ, Bartholdy C, Stryhn A, Thomsen AR, Christensen JP. Rapid and sustained CD4+ T-cell-independent immunity from adenovirus-encoded vaccine antigens. J Gen Virol 2007 Jun;88(Pt 6):1708–16.
[35] Sorensen MR, Pedersen SR, Lindkvist A, Christensen JP, Thomsen AR. Quantification of B16 melanoma cells in lungs using triplex Q-PCR – a new approach to evaluate melanoma cell metastasis and tumor control. PLoS One 2014;9(1):e87831.
[36] Hudgens MG, Gilbert PB. Assessing vaccine effects in repeated low-dose challenge experiments. Biometrics 2009 Dec;65(4):1223–32.