

1 **Mucosal CD8⁺ T cell responses induced by an MCMV based vaccine**
2 **vector confer protection against influenza challenge**

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19 **Abstract**

20 Cytomegalovirus (CMV) is a ubiquitous β -herpesvirus that establishes life-long latent infection
21 in a high percentage of the population worldwide. CMV induces the strongest and most durable
22 CD8⁺ T cell response known in human clinical medicine. Due to its unique properties, the virus
23 represents a promising candidate vaccine vector for the induction of persistent cellular
24 immunity. To take advantage of this, we constructed a recombinant murine CMV (MCMV)

25 expressing an MHC-I restricted epitope from influenza A virus (IAV) H1N1 within the immediate
26 early 2 (*ie2*) gene. Only mice that were immunized intranasally (i.n.) were capable of controlling
27 IAV infection, despite the greater potency of the intraperitoneally (i.p.) vaccination in inducing
28 a systemic IAV-specific CD8⁺ T cell response. The protective capacity of the i.n. immunization
29 was associated with its ability to induce IAV-specific tissue-resident memory CD8⁺ T (CD8T_{RM})
30 cells in the lungs. Our data demonstrate that the protective effect exerted by the i.n.
31 immunization was critically mediated by antigen-specific CD8⁺ T cells. CD8T_{RM} cells promoted
32 the induction of IFN γ and chemokines that facilitate the recruitment of antigen-specific CD8⁺ T
33 cells to the lungs. Overall, our results showed that locally applied MCMV vectors could induce
34 mucosal immunity at sites of entry, providing superior immune protection against respiratory
35 infections.

36 **Author summary**

37 Vaccines against influenza typically induce immune responses based on antibodies, small
38 molecules that recognize the virus particles outside of cells and neutralize them before they
39 infects a cell. However, influenza rapidly evolves, escaping immune recognition, and the
40 fastest evolution is seen in the part of the virus that is recognized by antibodies. Therefore,
41 every year we are confronted with new flu strains that are not recognized by our antibodies
42 against the strains from previous years. The other branch of the immune system is made of
43 killer T cells, which recognize infected cells and target them for killing. Influenza does not
44 rapidly evolve to escape T cell killing; thus, vaccines inducing T-cell responses to influenza
45 might provide long-term protection. We introduced antigen from influenza into the murine
46 cytomegalovirus (MCMV) and used it as a vaccine vector inducing Killer T-cell responses of
47 unparalleled strength. Our vector controlled flu replication and provided relief to infected mice,
48 but only if we administered it through the nose, to activate killer T cells that will persist in the
49 lungs close to the airways. Therefore, our data show that the subset of lung-resident killer T
50 cells is sufficient to protect against influenza.

51 Introduction

52 Respiratory infections caused by influenza viruses usually are associated with mild-to-
53 moderate disease symptoms but are linked with high morbidity and mortality in susceptible
54 populations like the elderly, young children, patients with co-morbidities and
55 immunocompromised patients. Influenza virus causes seasonal epidemics, with typically 3 to
56 5 million cases of severe illness worldwide, according to WHO reports [1], and influenza type
57 A viruses (IAV) cause the more severe disease form. Vaccines against influenza are based on
58 the induction of adaptive immunity that targets the projected yearly epidemics. While most
59 vaccines are based on inactivated IAV formulations inducing anti-IAV IgG responses, live
60 attenuated influenza vaccines (LAIV) are also used as formulations for i.n. administration. This
61 is based on the assumption that the induction of local immunity may provide superior immune
62 protection [2, 3]. However, it remains unclear whether this protection depends on local IgA
63 responses, on cytotoxic T cell responses, or on their combined antiviral activity. Of note,
64 functional T cell responses were shown to substantially contribute to antiviral IAV immunity [4-
65 6]. In particular, cytotoxic influenza-specific CD8⁺ T lymphocytes (CTLs) promote viral
66 clearance indirectly by secretion of pro-inflammatory cytokines such as IFN γ [7] and directly
67 by perforin/Fas-mediated killing of infected epithelial cells in the bronchoalveolar space [8].
68 However, it remained unclear if T cell responses alone may control IAV, or if Ig responses were
69 the crucial contributor to LAIV-mediated immune protection. We considered that this question
70 could be addressed by developing a vaccine formulation that optimizes T cell responses
71 against IAV, while excluding the humoral ones.

72 CMV infection induces sustained functional T cell responses that are stronger in the long-term
73 than the immune response to any other infectious pathogen [9]. Experiments in the mouse
74 model have shown that defined CMV epitope-specific CD8⁺ T cells accumulate in tissues and
75 blood and are maintained at stable high levels during mouse CMV (MCMV) latency [10]. This
76 phenomenon was termed “Memory Inflation” [11]. While some MCMV derived peptides, as the
77 ones derived from the IE3 (₄₁₆RALEYKNL₄₂₃) and M139 proteins (₄₁₉TVYGFCLL₄₂₆) induce

78 inflationary responses, other peptides, such as the M45-derived ($_{985}\text{HGIRNASFI}_{993}$), induce
79 conventional CD8⁺ T cell response [12]. The exceptionally long-lasting cellular immunity to
80 CMV antigens has raised the interest in CMV as a potential new vaccine vector [13]. Antigen-
81 experienced CD8⁺ T cells are subdivided into CD62L⁻ effector memory (CD8T_{EM}) and CD62L⁺
82 central memory CD8⁺ T cells (CD8T_{CM}). The antigen-specific CD8⁺ T cells during latent
83 infection bear predominantly a CD8T_{EM} phenotype and localize in secondary lymphoid or non-
84 lymphoid organs [14]. They may provide immune protection against diverse viral targets [13,
85 15-18], but also against bacterial [19] or tumor antigens [20, 21].

86 Both CD8T_{EM} and CD8T_{CM} subsets recirculate between the blood, the lymphoid organs, and
87 the peripheral tissues. A special subset of memory CD8⁺ T cells (CD8T_{RM}) resides in non-
88 lymphoid tissues such as lungs, the female reproductive tract (FRT), the skin, the brain or the
89 small intestine [22-25]. These cells lose the capacity of recirculating, maintain themselves at
90 the site of infection, and their phenotype and transcriptional profile differ from classical memory
91 T cells [26]. The well-characterized CD8T_{RM} cells express C-type lectin CD69 [22] and the
92 integrin $\alpha\text{E}\beta 7$, also known as CD103 [26]. They provide rapid and superior protection against
93 pathogens at the site of infection [22, 26, 27]. A recent publication argued that a vaccine
94 formulation adjuvanted by IL-1 β enhances the immune control of IAV by improving mucosal T
95 cell responses [28], but IL-1 β improved both humoral and cellular responses in their study.
96 Hence, the contribution of CD8T_{RM} to IAV immune control remains unclear.

97 CD8T_{RM} are found in the salivary glands of MCMV-infected animals [29], but not in their lungs
98 [30]. We showed that i.n. infection with MCMV induces inflationary CD8⁺ T cell responses, but
99 also that memory inflation is stronger upon i.p. infection [31]. The i.n. administration of an
100 MCMV vaccine vector induced CD8T_{RM} responses in the lungs [25] and only i.n. immunization
101 restricted the replication of respiratory syncytial virus (RSV) upon challenge [25], indicating
102 that CD8T_{RM} elicited by i.n. administration of MCMV vectors might provide immune protection
103 against respiratory virus infections, yet this evidence remains correlative. Upon antigen
104 encounter, CD8T_{RM} cells quickly reactivate at the mucosal site and secrete cytokines and

105 chemokines or support the release of inflammatory mediators by other immune cells [24, 32,
106 33]. Lung airway CD8_{RM} cells provide protection against respiratory virus infection through
107 IFN γ and help to recruit circulating memory CD8⁺ T cells to the site of infection in an IFN γ -
108 dependent way [32]. Therefore, to understand if CD8_{RM} cell may provide immune control of
109 respiratory infections may help to refine strategies for tissue-targeted vaccine design.

110 In this study, we constructed an MCMV vector expressing the MHC-I restricted peptide
111 ⁵³³IYSTVASSL₅₄₁ (IVL₅₃₃₋₅₄₁) [34] from IAV H1N1 hemagglutinin (HA)-MCMV^{IVL} under the
112 transcriptional control of the *ie2* promoter. We investigated the potential of this recombinant
113 virus to induce HA-specific CD8⁺ T cells that confer protection against a lethal IAV challenge.
114 We showed that i.n., but not i.p. immunization with MCMV^{IVL} resulted in a robust protection
115 against an IAV challenge. Protection following i.n. MCMV^{IVL} immunization was associated with
116 high levels of antigen-specific CD8_{RM} cells in the lungs, and targeted depletion of lung-
117 CD8_{RM} cells revealed that the control of the IAV in the lungs depended on these cells.

118

119 Results

120 Generation of recombinant MCMV and its replication *in vitro* and *in vivo*.

121 We showed recently that MCMV vector expressing a single, optimally positioned MHC-I
122 restricted antigenic epitope provides a more efficient immune protection than vectors
123 expressing the full-length protein [21]. Therefore, we constructed an MCMV influenza vaccine
124 vector by inserting the coding sequence for the H-2K^d MHC-I restricted peptide IYSTVASSL
125 from the hemagglutinin (HA) of the H1N1 (PR8) IAV [34] into the C-terminus of the MCMV *ie2*
126 gene. The resulting recombinant virus was called MCMV^{IVL} (Fig 1A). To test if the recombinant
127 virus retained its capacity to replicate in host cells, virus replication was assessed by multi-
128 step growth kinetics assays in NIH-3T3 cells *in vitro* and by *ex vivo* quantification of virus titers
129 in livers, lungs and spleens 5 days post-infection (dpi) and in salivary glands 21 dpi. MCMV^{IVL}
130 showed identical replication properties as the MCMV^{WT}, both *in vitro* (Fig 1B) and *in vivo* (Fig

131 1C), indicating that the insertion of the IVL₅₃₃₋₅₄₁ epitope does not impair virus replication and
132 spread.

133 **Intranasal immunization with MCMV^{IVL} induces a lower magnitude of CD8⁺ T cell**
134 **response than intraperitoneal immunization**

135 We have shown that mucosal infection with MCMV by the i.n. route induces memory inflation,
136 although to a lower extent than upon the i.p. infection route [31]. To define if this pattern would
137 hold true for the artificially incorporated influenza epitope as well, we compared the magnitude
138 of the CD8⁺ T cell responses to MCMV^{IVL} and MCMV^{WT} induced via the i.n. and i.p. route,
139 respectively. The kinetics of antigen-specific CD8⁺ T cell responses in peripheral blood was
140 determined by IVL-tetramer staining. While we did not observe any difference at early times
141 post immunization, i.p. immunization induced an overall stronger inflationary CD8⁺ T cell
142 response during latency (Figs 2A and 2B). This pattern was observed both in relative terms
143 (Fig 2A) and in absolute cell counts (Fig 2B). We next analyzed the IVL-specific CD8⁺ T cell
144 responses in spleens, lungs and mediastinal lymph nodes (mLNs) at times of latency
145 (>3months post infection (p.i)). Similarly, i.p. immunization induced higher levels of IVL-specific
146 CD8⁺ T cells than the i.n. immunization in the spleen and lungs, both in relative (Figs 2C and
147 2D) and/or in absolute terms (Figs 2F and 2G). There were no significant differences in the
148 mLNs (Figs 2E and 2H). We also analyzed the primed (CD11a^{hi}CD44⁺) and IVL-specific CD8⁺
149 T cells during latency post-immunization for KLRG1 and CD62L expression in order to assess
150 the fractions of central memory (T_{CM}: KLRG1⁻CD62L⁺); effector (T_{EFF}: KLRG1⁺CD62L⁻) and
151 effector memory (T_{EM}: KLRG1⁻CD62L⁻) subsets in blood, spleen and lung cells of i.p. and i.n
152 immunized mice (S1 Fig). While the fraction of CD8T_{CM} cells remained relatively low in all
153 compartments irrespectively of the route of administration, i.p. infection resulted in a response
154 polarized towards effector cells, whereas i.n. immunization induced a higher fraction of EM
155 cells in all analyzed organs. In sum, mucosal (i.n.) immunization with MCMV^{IVL} induces a
156 systemic inflationary IVL-specific CD8⁺ T cell response, whereas the overall magnitude of the
157 IAV-specific CD8⁺ T cell response is less pronounced compared to that induced in i.p.

158 immunized mice. Moreover, CD8⁺ T cell responses induced via the mucosal route skew
159 towards an effector memory phenotype.

160

161 **Intranasal immunization with MCMV^{IVL} facilitates the elimination of IAV in a CD8⁺**
162 **T cell-dependent manner**

163 To test whether immunization with MCMV^{IVL} protects against IAV infection, latently immunized
164 BALB/c mice were i.n. challenged with IAV. IAV titers in the lungs were quantified 5 dpi. Viral
165 loads in mice that were immunized with MCMV^{WT} via either the i.p. or the i.n. route were
166 comparable to those detected in mock-immunized mice (Fig 3A). In contrast, mice immunized
167 with MCMV^{IVL} via the i.n. route showed significantly lower IAV titers than in any other group.
168 Interestingly, i.p. MCMV^{IVL} immunization also resulted in reduced IAV loads, but to a lower
169 extent than the i.n. immunization (Fig 3A). Similarly, animals immunized with MCMV^{WT} suffered
170 the most severe weight loss whilst i.n. immunization of MCMV^{IVL} led to the least pronounced
171 body weight loss. I.p. immunization with MCMV^{IVL} displayed an intermediate level (Fig 3B).
172 Numerous studies have reported that CD8⁺ T cells play an important role in protecting against
173 influenza infection [35, 36] and it was reasonable to assume that our vector provided immune
174 protection by eliciting CD8⁺ T cell responses. To formally prove that efficient immune control
175 of IAV observed in the MCMV^{IVL} (i.n.) immunized group depends on CD8⁺ T cells, we depleted
176 these cells by systemic treatment of mice with a depleting anti-CD8 α antibody (depletion
177 efficiency is shown in S2A Fig) one day prior to IAV challenge and quantified viral titers in lungs
178 6 dpi. While the virus titer was below the detection limit in mice that were i.n. immunized with
179 MCMV^{IVL} and received isotype control antibodies, CD8⁺ T cell depletion indeed resulted in a
180 significant increase of the IAV titer to levels comparable to the groups that were i.p. immunized
181 with MCMV^{IVL} and to control mice immunized with MCMV^{WT} by i.n. route (Fig 3C). CD8⁺ T cell
182 depletion also slightly increased virus titers in both control groups - MCMV^{IVL} (i.p.) and
183 MCMV^{WT} (i.n.), but not as pronounced as in the MCMV^{IVL} i.n. immunized group (Fig 3C). Similar
184 as Fig. 3B, animals of all experimental groups showed a comparable body weight loss post-

185 challenge, whereas i.n. MCMV^{IVL} immunized mice showed a faster recovery than the i.p.
186 immunized mice (Fig 3D). Of note, this difference disappeared in the groups lacking CD8⁺ T
187 cells (Fig 3E). Together, these data demonstrate that IAV-specific CD8⁺ T cells induced by the
188 mucosal (i.n.) administration of MCMV^{IVL} confer protection against IAV in the lungs of
189 vaccinated mice.

190 We further compared the lung pathology upon IAV challenge by histology. A moderate
191 perivascular inflammation was observed in the lungs of most mice (stars), and to a lesser
192 degree surrounding the bronchioles (arrows) (Fig 3F). The CD8⁺ T cell depleted group showed
193 more severe pathology than isotype-treated controls, but the difference was not very
194 pronounced (Fig 3G). Taken together, these data imply that intranasal immunization with the
195 MCMV^{IVL} vector can limit IAV growth in the lungs by inducing IAV-specific CD8⁺ T cell
196 responses, whereas the clinical outcome is only moderately improved.

197 **Intranasal immunization with MCMV^{IVL} induces antigen-specific tissue-resident** 198 **memory CD8⁺ T (CD8T_{RM}) cells in the lungs.**

199 We assumed that intranasal MCMV^{IVL} immunization may control the IAV replication by inducing
200 CD8T_{RM} cell responses in the lungs. In order to test this hypothesis we identified the CD8T_{RM}
201 cell compartment by staining cells with the CD69 [22] and the CD103 [26] marker at times of
202 MCMV latency (> 3 months p.i.), as shown in Fig 4A. Only a few CD8T_{RM} (CD69⁺CD103⁺) cells
203 could be detected in the spleen and blood regardless of the route of immunization (Fig 4B).
204 CD8T_{RM} cells frequencies and counts were significantly higher in the lungs of mice that were
205 i.n. immunized with MCMV^{IVL} compared to the i.p. route (Figs 4B and 4C). Resident memory
206 T cells reside in the mucosal tissue layer and are non-migratory [27]. To confirm that CD8T_{RM}
207 cells induced in the lungs of mice i.n. immunized with MCMV^{IVL} are indeed located within the
208 airway mucosa, *in vivo* cell labeling experiments were performed [37, 38]. Here, intravenous
209 (i.v.) injection of a fluorescent anti-CD45 antibody prior sampling allows for the discrimination
210 of circulating leukocytes (fluorescence-positive) from emigrated or tissue-resident leukocytes

211 (fluorescence-negative). The CD69⁺CD103⁺ cells from lungs were virtually absent from the
212 CD45-labelled fraction (S3 Fig). IVL-specific CD8T_{RM} fractions were present solely in the lungs
213 but not in the spleen and the blood (Fig 4D) and few IVL-tetramer⁺ CD8T_{RM} cells were induced
214 by MCMV^{IVL} i.p. immunization (Figs 4D-4F). In contrast, in mice immunized with MCMV^{IVL} via
215 the i.n. route, approximately half of the CD8T_{RM} cells were IVL-tetramer⁺ (Fig 4F). CD8T_{RM} cells
216 were also induced in the group that was i.n. immunized with MCMV^{WT}, but not IVL-specific
217 (S4A Fig). In addition, there was an overall higher percentage of the CD69⁺CD103⁺CD8⁺ T cells
218 in the lungs of i.n. immunized mice (S4B Fig), although the absolute cell numbers did not show
219 a significant difference (S4C Fig). CD8T_{RM} cells showed low expression of Eomes whereas
220 CD69⁺CD103⁺CD8⁺ T cells showed high expression of Eomes which is consistent with primed
221 CD8⁺ T cells (S4D Fig). In summary, i.n. immunization with MCMV^{IVL} induces an accumulation
222 of IAV-specific CD8T_{RM} response in the lungs.

223 **Pulmonary CD8T_{RM} cells improve viral clearance and the production of CD8⁺ T** 224 **cell-recruiting chemokines during IAV infection**

225 Resident memory T cells reside in the epithelial barrier of mucosal tissue [27] that is in close
226 proximity to the airways. Hence, they can respond rapidly to a virus challenge at the site of
227 infection [25, 27]. To define the relevance of lung CD8T_{RM} cells in protection against IAV
228 challenge, we specifically depleted the airways CD8⁺ T cells by i.n. administration of αCD8
229 antibodies one day before challenge (Fig 5A, S2B and S2C Figs). To assess the impact of
230 pulmonary CD8⁺ T-cell depletion on antiviral immunity, on day 4 post-challenge, we quantified
231 the IAV titers in the lungs of i.n. MCMV^{IVL} immunized mice. Strikingly, targeted depletion of
232 pulmonary CD8T_{RM} cells was associated with a significantly higher viral burden during IAV
233 infection (Fig 5B). The data indicate that CD8T_{RM} cells induced by i.n. immunization with
234 MCMV^{IVL} promote the clearance of IAV.

235 Influenza virus infection can induce a vigorous cytokine storm in airways and lungs, which
236 promotes the recruitment of inflammatory cell. IFN γ as a pivotal antiviral cytokine is expressed

237 early after influenza virus infection [39]. It has been demonstrated that CD8_{RM} cells activate
238 rapidly when they re-encounter the cognate antigen and provide protection by secreting
239 cytokines such as IFN γ and granzyme B [40, 41]. *Morabito et al.* showed that intranasal
240 immunization with an MCMV-based vaccine vector induced CD8_{RM} cells and IFN γ was
241 secreted at the very early time upon challenge during RSV infection [25].

242 Therefore, we measured the production of IFN γ in the bronchoalveolar lavage fluid (BALF)
243 early upon challenge. IFN γ levels were generally low at day 2 post-challenge and no difference
244 could be observed between groups regardless of airway CD8⁺ T cells depletion (Fig 5C). On
245 day 4, the IFN γ level was significantly increased compared to the level on day 2, but more
246 IFN γ was induced in the control group than in the one lacking airway CD8⁺ T cell in the lungs
247 (Fig 5C). IFN γ was also induced in the MCMV^{IVL} i.p. immunization group and extremely low
248 level of IFN γ could be detected in the MCMV^{WT} control group (S5A Fig). Thereby suggesting
249 that primary cognate antigen immunization is needed for the rapid IFN γ secretion and that
250 resident CD8⁺ T cells may not be the major IFN γ producer. In contrast to these effects,
251 depletion of lung airway CD8⁺ T cells increased the concentration of IL-6 as compared to the
252 group that was intranasally immunized with MCMV^{IVL} and treated with isotype control
253 antibodies (Fig 5D). Similarly, a higher concentration of IL-6 was also detected in the i.p.
254 immunization group, whereas the MCMV^{WT} control group displayed the highest IL-6 levels
255 (S5B Fig). Finally, very low levels of other cytokines could be detected in all groups, both on
256 day 2 and 4 post-challenge and regardless of the depletion of the airway CD8⁺ T cell (S5C Fig),
257 suggesting that the presence of pulmonary CD8_{RM} cells does not affect the Th1, Th2 and
258 Th17 immune profile during early IAV infection.

259 It has been demonstrated that T_{RM} cells help to recruit immune cells to the infection site through
260 the induction of chemokines such as CCL3 and CXCL9 in the female reproductive tract (FRT),
261 and CCL4 in the lungs, either by direct chemokine expression or by their induction in nearby
262 cells, such as epithelial cells [24, 25].

263 To determine whether i.n. immunization with MCMV^{IVL} induced inflammatory chemokines
264 expression upon IAV challenge, a series of inflammatory chemokines were measured in the
265 BALF on day 2 and day 4 upon IAV challenge (Figs 5E and 5F). As shown in Fig 5E, airway
266 depletion of CD8⁺ T cells reduced CCL3, CCL4, CCL5 levels on day 2. On day 4, CCL3 and
267 CCL4 levels were significantly higher in the MCMV^{IVL} i.n. group than in the MCMV^{IVL} i.p. and
268 in the MCMV^{WT} i.n. immunization groups. Airway CD8⁺ T cell depletion reduced the level of
269 CCL3 and CCL4 to values in the i.p. MCMV^{IVL} immunization group (Fig 5F). CXCL9 levels were
270 comparable between the MCMV^{IVL} i.n. and i.p. immunization groups, but dramatically lower in
271 the MCMV^{WT} immunization group (Fig 5F), which was consistent with the low IFN γ level in the
272 BALF, as IFN γ is known as an inducer of CXCL9, which then acts as a T cell-attracting
273 chemokine. Together, these data indicate that CD8T_{RM} cells induced by i.n. immunization with
274 MCMV^{IVL} promote the induction of the pro-inflammatory chemokines CCL3, CCL4, CCL5 and
275 CXCL9, along with a reduction of IL-6 in the lungs.

276 **CD8T_{RM} cells facilitate the expansion of CD8⁺ T cells in the lungs.**

277 Since i.n. immunization induced stronger chemokine responses in comparison to the i.p.
278 immunization route, we decided to define whether CD8T_{RM} cells induced by MCMV^{IVL}
279 promoted the accumulation of CD8⁺ T cells in the lungs. CD8⁺ T cell numbers on day 2 and 4
280 post IAV challenge were quantified in presence or absence of airway CD8⁺ T cells. In the
281 MCMV^{IVL} i.n. immunization group, IVL-specific and total CD8⁺ T cell numbers increased from
282 day 2 to day 4 post-challenge, but only in mice that were not depleted for airway CD8⁺ T cells
283 (Figs 6A and 6B). IVL-specific CD8⁺ T cell counts in the lung tissue and BAL were slightly
284 higher in the MCMV^{IVL} i.n. group than in the i.p. immunized group (S6A and S6B Figs).
285 Interestingly, this differed from results prior to IAV challenge, where significantly larger
286 amounts of IVL-specific CD8⁺ T cells were detected in the i.p. group (Fig 2G). IVL-specific and
287 total CD8⁺ T cell counts increased significantly in the BALF of i.n. immunized mice by day 4
288 post IAV challenge, indicating that CD8⁺ T cells accumulate in the lungs and migrate to the
289 bronchoalveolar space (Figs 6C and 6D). In mice where CD8⁺ T cells were depleted prior to

290 challenge, very few IVL-tetramer⁺ CD8⁺ T cells (Fig 6C) and CD8⁺ T cells (Fig 6D) could be
291 detected, both on day 2 and at day 4 post-challenge.

292 CD8⁺ T cells in the lung tissue were further analyzed by *in vivo* labeling of circulating cells.
293 Anti-CD45 antibodies were injected i.v. 3-5 min prior to euthanasia and organ collection. The
294 IVL-specific CD8⁺ T cell population failed to expand upon airway CD8⁺ T cell depletion, with
295 significantly lower numbers in CD45⁻ subset on day 4. IAV-specific CD8⁺ T cell counts showed
296 an increased trend both in the CD45⁺ and in the CD45⁻ subsets on day 4 post-challenge (Fig
297 6E). Airway CD8⁺ T cell depletion prevented also the expansion of total CD8⁺ T cells counts on
298 day 4 (Fig 6F). Interestingly, only the CD45 unlabeled fraction of the total CD8 pool expanded
299 on day 4 (Fig 6F), in contrast to IVL-specific CD8⁺ T cells, where CD45⁺ cells also expanded
300 (Fig 6E). It is important to note that the number of total CD45⁻ CD8⁺ T cells increased in the i.n.
301 immunization group, arguing that the recruitment by CD8T_{RM} cells was antigen-independent.
302 Thus, non-cognate antigen-specific cells were also attracted from the circulation to the lungs
303 and this accumulation of cells was abrogated by mucosal CD8⁺ T cell depletion (Fig 6F).

304 We surmised that the accumulation of CD8⁺ T cells in the lungs and in the BALF might be due
305 to an expansion of CD8T_{RM} upon IAV challenge. Surprisingly, the number of CD8T_{RM} cells in
306 the lungs did not expand from day 2 to day 4; if anything, their frequency decreased (Figs 7A
307 and 7B). Likewise, IVL-Tetramer⁺ CD8T_{RM} cell counts were also slightly reduced from day 2 to
308 day 4 post-challenge (Fig 7C), although IVL-Tetramer⁺ CD8 counts in the BALF increased at
309 the same time (Fig 6A). It is important to note that the effect of the i.n. depletion was local,
310 since the frequencies and counts of IVL-specific CD8⁺ T cells in the blood (S6C and S6D Figs)
311 and spleen (S6E and S6F Figs) did not significantly differ upon i.n. αCD8 or upon isotype-
312 control antibody administration. Therefore, our data indicated that CD8T_{RM} cells may confer
313 protection by recruiting circulating CD8⁺ T cells upon IAV challenge.

314 Discussion

315 Influenza-specific CD8⁺ T cells are known to contribute to virus elimination, because the
316 clearance of influenza virus is delayed in T cell-deficient mice [5, 42]. However, previous
317 evidence did not clarify whether vaccines solely inducing influenza-specific CD8⁺ T cell
318 responses improve immune protection. To avoid confounding humoral immune responses and
319 focus on the potential of optimally primed CD8⁺ T cells in protecting against influenza, we
320 generated a new MCMV based vaccine vector. CMV vaccine vectors expressing exogenous
321 antigenic peptides fused to a CMV gene induce CD8⁺ T cell responses of unparalleled strength
322 [13, 15, 21, 25]. We show here that robust CD8⁺ T cell responses against a single MHC-I
323 restricted epitope derived from the HA protein of IAV, promote the clearance of IAV from lungs,
324 but only upon i.n. immunization. While some pathology was observed even in immunized mice,
325 arguing that the protection was not complete, depletion assays confirmed that CD8⁺ T cells are
326 crucial for the immune protection observed in our model. Remarkably, immunization by the i.p.
327 route induced even stronger systemic CD8⁺ T cell responses, but very poor protection. This
328 conundrum was resolved once we noticed that only i.n. immunization induces T_{RM} responses
329 in the lung.

330 CD8T_{RM} cells act as sentinels in the host and form the first line of defense, providing rapid and
331 effective protection to fight against pathogens invasion [23, 25, 27, 43]. Prior studies have
332 revealed that direct delivery of vaccines to the target tissue is necessary for generation of T_{RM}
333 cells [25, 44] and that sustained lung CD8T_{RM} responses in MCMV-infected mice are generated
334 by immunoproteasome-independent antigenic stimulation [45], akin to the CD8 expansions in
335 memory inflation [21], arguing that they are induced by similar and overlapping mechanisms.
336 This was consistent with our observation that approximately half of CD8T_{RM} cells induced by
337 MCMV^{IVL} i.n. immunization were IVL-specific, arguing that site-specific immunization with
338 MCMV is necessary for the generation of memory-inflation-like antigen-specific CD8T_{RM} cells.
339 Some prior studies have claimed that skin-resident CD8T_{RM} cells may confer protection in an
340 antigen-unspecific manner [46], whereas others argued that only the antigen-specific CD8T_{RM}
341 cells respond to cognate antigens [47]. MCMV^{WT} induced robust CD8T_{RM} responses in our

342 model, but these were not specific for IAV, and did not provide any protection against IAV in
343 our study. Site-specific anti-CD8 α antibody administration depleted CD8 T_{RM}, and increased
344 IAV titers in immunized mice, indicating that CD8T_{RM} cells facilitated IAV elimination. Thus, the
345 protection against IAV challenge required antigen-specific CD8T_{RM} cells in our model.

346 Early upon IAV challenge, the IVL-specific CD8⁺ T cells expanded strongly and rapidly in the
347 lungs of i.n. immunized mice. The non-specific CD8⁺ T cell population also expanded
348 dramatically, suggesting that the expansion was not antigen driven. However, total and IVL-
349 specific CD8⁺ T cells expanded poorly in the lungs when airway CD8⁺ T cells were depleted.
350 Finally, IAV challenge expanded IVL-specific CD8⁺ T cell counts in the blood and spleen of i.n.
351 immunized mice to levels observed in the i.p. immunized controls, although the levels were
352 significantly lower in the i.n. immunization group before challenge. Overall, these results
353 indicated that i.n. immunization facilitated CD8⁺ T cell responses upon challenge, both locally
354 in the lungs and systemically in the blood and spleen.

355 It has long been assumed that CD8T_{RM} cells have poor proliferative capacity upon challenge.
356 Previous work has demonstrated that airway CD8⁺ T cells fail to expand *in vivo* upon
357 intratracheal transfer [32] and that CD8T_{RM} cells induced by MCMV infection display a limited
358 proliferative capacity in salivary glands [48]. However, this is in contrast to two recent studies
359 demonstrating that CD8T_{RM} cells in the skin [47] and FRT [49] maintain the capacity of *in situ*
360 proliferation upon cognate antigen stimulation. Such stimulation differentiates circulating
361 effector memory CD8⁺ T cells into CD8T_{RM} cells without displacing the pre-existing CD8T_{RM}
362 population [47]. In our study, CD8⁺ T cells accumulated in the lungs upon IAV challenge, but
363 the CD8T_{RM} population did not expand and the number of antigen-specific CD8T_{RM} cells even
364 displayed a reduction trend. Therefore, our data argued that either lung CD8T_{RM} in general or
365 CD8T_{RM} induced by MCMV i.n. immunization in particular may behave differently from CD8T_{RM}
366 in other organs. This distinction, however, goes beyond the scope of our current work and
367 remains to be addressed in future studies.

368 We have shown in this study that concentrations of CCL3, CCL4 and CXCL9 in the BALF of
369 the MCMV^{IVL} i.n. immunization group are significantly higher than in MCMV^{IVL} i.p. or MCMV^{WT}
370 i.n. immunized mice. Intravital CD45 labeling showed that CD8⁺ T cells accumulating in the
371 lungs are sequestered from the bloodstream, but not CD8T_{RM}, arguing that circulating antigen-
372 specific cells were attracted into the lungs under the presence of mucosa-resident CD8⁺ T cells.
373 This is in line with the work of *Schenkel* et al. showing a rapid local induction of chemokines
374 CXCL9 and CCL3/4 in the FRT upon re-infection, and recruitment of memory CD8⁺ T cells from
375 the periphery [24]. Depletion of mucosal CD8⁺ T cells depressed chemokine levels in the BALF
376 to levels seen in the i.p. immunization group. This, together with the high levels of IFN γ in the
377 MCMV^{IVL} i.n. immunization group and extremely low IFN γ in MCMV^{WT} i.n. immunization points
378 to a putative model where antigen-specific re-stimulation induces IFN γ , which drives
379 chemokine responses that recruit CD8⁺ T cells from the bloodstream to the lungs.

380 In summary, our data demonstrate that CD8T_{RM} cells promote the induction of chemokines,
381 which help to drive the recruitment of IVL-specific CD8⁺ T cells and facilitates the elimination
382 of IAV. Furthermore, optimal induction of CD8T_{RM} cells in the lungs by the MCMV vector can
383 be only achieved after i.n. vaccination. Therefore, immunization with an MCMV vector at the
384 local site provided CD8⁺ T cell-based protection against IAV infection. Our results therefore
385 demonstrate that CD8⁺ T cell induction, and CD8T_{RM} in particular, contribute to vaccination
386 outcomes in influenza infection independently of humoral immune responses, and the
387 selection of the adequate immunization route plays a critical role in terms for promoting
388 superior protective efficacy.

389 **Materials and Methods**

390 **Ethics statement**

391 Mice were housed and handled in agreement with good animal practice as defined by EU
392 directive EU 2010/63 and ETS 123 and the national animal welfare body “Die Gesellschaft für
393 Versuchstierkunde /Society of Laboratory Animals (GV-SOLAS)”. Animal experiments were

394 performed in accordance with the German animal protection law and were approved by the
395 responsible state office (Lower Saxony State Office of Consumer Protection and Food Safety)
396 under permit number: 33.9-42502-04-14/1709.

397 **Mice**

398 BALB/c mice were purchased from Janvier (Le Genest Saint Isle, France) and housed in the
399 animal facility of the HZI Braunschweig under SPF conditions according to FELASA
400 recommendations [50].

401 **Cells**

402 Bone marrow stromal cell line M2-10B4 (CRL-1972) and NIH-3T3 fibroblasts (CRL-1658) were
403 purchased from American Type Culture Collection (ATCC). The cells were maintained in
404 DMEM supplemented with 10% FCS, 1% L-glutamine, and 1% penicillin/streptomycin.
405 C57BL/6 murine embryonic fibroblasts (MEFs) were prepared in-house and maintained as
406 described previously [51].

407 **Viruses**

408 BAC-derived wild-type murine cytomegalovirus (MCMV^{WT} clone: pSM3fr 3.3) [52] was
409 propagated on M2-10B4 lysates and purified on a sucrose cushion as described previously
410 [53]. Virus titers were determined on MEFs by plaque assay as shown elsewhere [54].

411 Recombinant MCMV was generated by the “en passant mutagenesis”, essentially as
412 described previously [55, 56]. In brief, we generated a construct containing an antibiotic
413 resistance cassette coupled with the insertion sequence and the restriction site *Sce-I*. This
414 construct was flanked by sequences homologous to the target region of insertion within the
415 MCMV BAC genome. Then, the fragment containing the insertion sequences was integrated
416 into the MCMV BAC genome by homologous recombination. In a second step, *Sce-I* was
417 induced to linearize the BAC followed by a second round of induced homologous

418 recombination to re-circularize it and select for clones that discarded the antibiotic selection
419 marker but retained the inserted sequence.

420 The PR8M variant of Influenza A/PR/8/34 was obtained from the strain collection at the Institute
421 of Molecular Virology, Muenster, Germany. Virus stocks from chorioallantoic fluid of
422 embryonated chicken eggs were generated as previously described [57].

423 **Tetramers and Antibodies**

424 ⁵³³IYSTVASSL₅₄₁ (IVL₅₃₃₋₅₄₁)-tetramer was bought from MBL (cat. NO.TS-M520-1), anti-CD8 α
425 depletion antibody (clone: YTS 169.4). Rat IgG2b isotype antibody (clone: LTF-2) was
426 purchased from Bio X Cell. Antibodies for flow cytometry included anti-CD3-APC-eFluor780
427 (clone: 17A2; eBioscience), anti-CD4-Pacific Blue (clone: GK1.5; BioLegend), anti-CD8 α -
428 PerCP/Cy5.5 (clone: 53-6.7; BioLegend), anti-CD11a-PE/Cy7 (clone: 2D7; BD Bioscience),
429 anti-CD44-Alexa Fluor 700 (clone: IM7; BioLegend), anti-CD45-APC-eFluor780 (clone:30-
430 F11;Biolegend), anti-CD62L-eVolve 605 (clone: MEL-14; eBioscience), anti-CD127-PE &
431 PE/Cy7 (clone: A7R34; BioLegend), anti-KLRG1-FITC & BV510 (clone: 2F1/KLRG1;
432 BioLegend), anti-CD103-APC (clone: 2E7; BioLegend), anti-CD69-FITC (clone: H1.2F3;
433 BioLegend) and anti-IFN γ -APC (clone: XMG1.2; BioLegend), anti-Eomes-PE & PE/Cy7 (clone:
434 Dan11mag; eBioscience).

435 **Virus *in vitro* infection**

436 NIH-3T3 cell monolayers were infected with MCMV^{WT} and MCMV^{IVL} at an MOI of 0.1, incubated
437 at 37°C for 1h, upon which the inoculum was removed, cells were washed with PBS, and
438 supplied with fresh medium. Cells were incubated for 6 days; the supernatant was harvested
439 every day and stored at -80°C until titration.

440 **Virus *in vivo* infection**

441 6 to 8 weeks old BALB/c female mice were infected with 2 x 10⁵ PFU MCMV^{WT} and MCMV^{IVL}
442 diluted in PBS. For i.p. infection, 100 μ l virus dilution was injected. For i.n. infection, mice were
443 first anesthetized with ketamine (10 mg/ml) and xylazine (1 mg/ml) in 0.9% NaCl (100 μ l/10 g

444 body weight), then administered with 20 μ l of virus suspension onto nostrils [31]. For IAV
445 challenge, BALB/c mice that were latently (> 3 months) immunized with MCMV were i.n.
446 inoculated with 220 focus forming units (FFU) or with 1100 FFU of PR8M influenza virus as
447 described previously [31].

448 **Infectious virus quantification (MCMV)**

449 MCMV virus from organ homogenates was titrated on MEFs with centrifugal enhancement as
450 described previously [17].

451 **Infectious virus quantification (IAV)**

452 Mice were sacrificed by CO₂ inhalation, whole lungs were excised and mechanically
453 homogenized using a tissue homogenizer. Tissue homogenates were spun down and
454 supernatants were stored at -70°C. Lung virus titers were determined by using the focus-
455 forming assay (FFA), as described before [57] with minor modifications. Briefly, MDCK cells
456 were cultured in MEM, supplemented with 10% FCS, 1% penicillin/streptomycin. Supernatants
457 of lung tissue homogenates were serially diluted in DMEM, supplemented with 0.1% BSA and
458 N-acetylated trypsin (NAT; 2.5 μ g/ml) and added to the MDCK cell monolayers. After 1h, cells
459 were overlaid with DMEM supplemented with 1% Avicel, 0.1% BSA and NAT (2.5 μ g/ml). After
460 24h cells were fixed with 4% PFA and incubated with quenching solution (PBS, 0.5% Triton X-
461 100, 20 mM Glycin). Cells were then treated with blocking buffer (PBS, 1% BSA, 0.5%
462 Tween®20). Focus forming spots were identified using primary polyclonal goat anti-H1N1 IgG
463 (Virostat), secondary polyclonal rabbit anti-goat IgG conjugated with horseradish peroxidase
464 (KPL) and TrueBlue™ peroxidase substrate (KPL). Viral titers were calculated as FFU per ml
465 of lung tissue homogenate.

466 **Isolation of lymphocytes from blood and organs**

467 Blood, spleen and mLNs were prepared as described previously [31]. Lungs were perfused by
468 injecting 5-10 ml PBS into the right heart ventricle. The lungs were cut into small pieces,
469 resuspended in 1 ml RPMI1640 (0.5% FCS), and digested with 1 ml of RPMI1640 with DNase

470 I (Sigma-Aldrich Chemie) and Collagenase I (ROCKLAND™) in a shaker at 37°C for 30 min.
471 Digested tissue was passed through cell strainers and single cell suspensions were washed
472 with RPMI1640, centrifuged at 500x g for 10 min. Subsequently, the cells were resuspended
473 in 7 ml of 40% Easycoll solution (Biochrom), overlaid onto 6 ml of 70% Easycoll solution in a
474 15 ml Falcon and centrifuged at 25 min at 1000x g at room temperature. The interface layer
475 was transferred to a 5 ml tube, washed, and resuspended in RPMI1640 (10% FCS).

476 **Peptide stimulation**

477 T cells were stimulated with peptides (1 µg/ml) in 85 µl RPMI 1640 for 1h at 37°C,
478 supplemented with brefeldin A (10 µg/ml in 15 µl RPMI 1640) and incubated for additional 5h
479 at 37°C. Cells incubated without any peptide in the same condition were used as negative
480 controls. Cytokine responses were detected by intracellular cytokine staining.

481 **Cell surface staining, intracellular cytokine staining and flow cytometry**

482 Blood cells and lymphocytes from spleen, lung and mLNs were stained with IVL₅₃₃₋₅₄₁-tetramer-
483 PE and surface antibodies for 30 min, washed with FACS buffer and analyzed. For intracellular
484 cytokine stainings, the cells were first stained with cell surface antibodies for 30 min, washed
485 and fixed with 100 µl IC fixation buffer (eBioscience) for 5 min at 4°C. Following this, cells were
486 permeabilized for 3 min with 100 µl permeabilization buffer (eBioscience) and incubated with
487 anti-IFN γ antibody for 30 min. Afterwards, cells were washed with FACS buffer and acquired
488 using an LSR-Fortessa flow cytometer (BD Bioscience).

489 ***In vivo* cell labeling**

490 Mice were intravenously (i.v.) injected with 3 µg anti-CD45-APC/eFluor780 (clone: 30-F11;
491 BioLegend). Mice were euthanatized 3-5 min after injection, and blood, spleen and lungs were
492 collected. Following their isolation from the respective compartment, lymphocytes were stained
493 and analyzed as described above.

494 ***In vivo* CD8⁺ T cell depletion**

495 For systemic *in vivo* CD8⁺ T cell depletion, published protocols [58, 59] were adapted as
496 follows. BALB/c mice were i.p. injected with 200 µg anti-CD8α (αCD8: clone: YTS 169.4) or
497 isotype antibody (Rat IgG2b: clone: LTF-2; Bio X Cell) one day before IAV challenge. To
498 deplete mucosal CD8⁺ T cells in the lungs, BALB/c mice were i.n. administered 10 µg αCD8 or
499 IgG2b in 20 µl of PBS one day before IAV challenge [36].

500 **Collection of bronchoalveolar lavage fluid (BALF)**

501 Mice were sacrificed by CO₂ inhalation, chest cavity was opened and skin and muscle around
502 the neck were gently removed to expose the trachea. A catheter was inserted and lungs were
503 carefully flushed with 1 mL PBS via the trachea. The BALF was transferred into a 1.5 ml tube
504 and stored on ice. The BALF was centrifuged at 500x g at 4°C for 10 min. Supernatant was
505 aliquoted and stored at -80°C until further analysis.

506 **Cytokine and chemokine quantification**

507 Mouse IFNγ enzyme-linked immunosorbent assay (ELISA) MAX™ kits (BioLegend) and the
508 bead-based immunoassay LEGENDplex™ Mouse Inflammation Panel (13-plex, BioLegend)
509 were used to quantify IFNγ and other cytokine levels in the BALF according to the
510 manufacturer's instructions. The bead-based immunoassay LEGENDplex™ Mouse Pro-
511 inflammation Chemokine Panel (13-plex, BioLegend) was used to quantify multiple chemokine
512 levels in the BALF.

513 **Histopathology**

514 Lungs were harvested from BALB/c mice that were latently infected with MCMV^{WT} and
515 MCMV^{IVL} and challenged with IAV during latency. Lungs were fixed in 4% formalin, paraffin
516 embedded, sliced and hematoxylin and eosin (H&E) stained according to standard laboratory
517 procedures.

518 **Statistics**

519 One-way ANOVA analysis was used to compare multiple groups at single time points. Two-
520 way ANOVA analysis was used to compare different groups at multiple time points.
521 Comparisons between two groups were performed using Mann-Whitney U test (two-tailed).
522 Statistical analysis was performed using GraphPad Prism 7.

523

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530

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668

669 **Figure legends**

670 **Fig 1. Generation of the recombinant MCMV expressing the $_{533}$ IYSTVASSL $_{541}$ epitope.**

671 The sequence of IAV epitope $_{533}$ AAIYSTVASSL $_{541}$ (IVL $_{533-541}$) was introduced at the C-terminus
672 of the MCMV *ie2* gene, and the growth of the recombinant virus MCMV^{IVL} was tested *in vitro*
673 and *in vivo*. (A) The location of the *ie2* gene within the MCMV genome at ~186-187kb position
674 is shown; the insertion site of the peptide IVL $_{533-541}$ and the corresponding nucleotide
675 sequences are magnified. (B) MCMV^{IVL} and wild-type MCMV (MCMV^{WT}) growth at a multiplicity
676 of infection of 0.1 was compared in NIH-3T3 cells. Virus titers in supernatants expressed as
677 plaque-forming units (PFU) were established at indicated time points. Group means +/-
678 standard error of the mean (SEM) are shown. The dashed line indicates the limit of detection.
679 (C) BALB/c mice were i.p. infected with 2×10^5 PFU of MCMV^{IVL} or MCMV^{WT} virus. Spleen,
680 lung and liver homogenates were assayed for virus titers 5 days post-infection (dpi). Salivary-
681 gland (SG) homogenates were assayed 21 dpi. Each symbol represents one mouse. Group
682 means +/- standard error of the mean (SEM) are shown. The dashed line indicates the limit of
683 detection.

684 **Fig 2. Intranasal immunization of MCMV^{IVL} induces weaker CD8⁺ T cell response than** 685 **intraperitoneal immunization.**

686 BALB/c mice were infected with 2×10^5 PFU MCMV^{IVL} via the i.n. or i.p. route or with MCMV^{WT}
687 via the i.n. route. IVL-specific CD8⁺ T cell responses were analyzed by IVL-tetramers staining
688 and flow-cytometry. (A, B) Blood leukocytes were analyzed at indicated time points upon
689 infection to define the (A) percentage and (B) cell counts of IVL-specific (Tet⁺) CD8⁺ T cells in
690 peripheral blood. Two independent experiments were performed and results were pooled and
691 shown as group means \pm SEM (n=8-10). (E-H) IVL-specific CD8⁺ T cells in spleen, lungs and
692 mLNns were quantified by IVL-tetramer staining 120 dpi as relative cell percentages (C, D, E)
693 or absolute cell counts (F, G, H) per organ. Two independent experiments were performed and
694 pooled data are shown. Each symbol represents one mouse, n=7-10. Group means \pm SEM
695 are shown. Significance was assessed by Two-way ANOVA test (panels A, B) or one-way
696 ANOVA test (Panels C-H). *P <0.05, **P<0.01, ***P<0.001.

697 **Fig 3. Intranasal immunization with MCMV^{IVL} facilitates the elimination of IAV.**

698 BALB/c mice were immunized with 2×10^5 PFU MCMV^{IVL} or MCMV^{WT} via the i.n. or i.p. route.
699 Mock controls received 100 μ l PBS by i.p. route. Once latency was established (> 3 months
700 p.i), mice were challenged with IAV (PR8). (A) IAV titers in the lungs on day 5 post-challenge
701 (i.n., 220 FFU) by focus-forming assay (FFA). Two independent experiments were performed
702 and pooled data are shown, n=10 (B) Body weight loss upon IAV challenge (i.n., 1100 FFU),
703 n=3-7. (C) CD8⁺ T cells were depleted systemically by i.p. injection of 200 μ g anti-CD8 α
704 antibody (α CD8) or isotype control antibody (IgG2b) one day before PR8 challenge (i.n., 1100
705 FFU). Virus loads in the lung homogenates were quantified on day 6 post-challenge by FFA.
706 Each symbol represents one mouse, n=5. Group means \pm SEM are shown. (D, E) Body
707 weight loss upon IAV challenge (i.n., 1100 FFU) without (D) or with (E) systemic CD8⁺ T cell
708 depletion. Two independent experiments were performed and pooled data are shown, n=10.
709 Group means \pm SEM are shown. (F) H&E staining of the lung tissue on day 5 post-challenge
710 (i.n., 1100 FFU) with or without systemic depletion of CD8⁺ T cell. (G) The score of inflammation
711 in the lungs upon IAV challenge (i.n., 1100 FFU). Bars indicate means, error bars are SEM.

712 Significance was assessed by One-way ANOVA test or Two-way ANOVA test. *P<0.05,
713 **P<0.01, ****P<0.0001, ns: no significant difference.

714 **Fig 4. Intranasal immunization with the MCMV^{IVL} induces antigen-specific tissue**
715 **resident CD8⁺ T cells in the lungs.**

716 BALB/c mice were i.n. (○) or i.p. (●) infected with 2 x 10⁵ PFU of MCMV^{IVL} virus. Leukocytes
717 were isolated from peripheral blood, spleen and lungs at latency time (> 3 months p.i), stained
718 with antibodies against CD3, CD4, CD8 α , CD11a, CD44, CD103, CD69, IVL-tetramer and
719 measured by flow cytometry. (A) Representative gating strategy to identify CD8T_{RM} cells
720 (CD69⁺CD103⁺). (B) Percentage of CD8T_{RM} cells in the peripheral blood, spleen and lungs.
721 (C) Absolute CD8T_{RM} cell counts in the lungs of i.n. and i.p. immunized mice. (D) Percentage
722 and (E) absolute counts of IVL-specific CD8T_{RM} cells in the peripheral blood, spleen and lungs
723 of the i.n. and i.p. immunization groups. (F) Representative gating strategy and percentage of
724 IVL-specific cells within the CD8T_{RM} cell subset in blood, spleen and lungs of i.n. and i.p.
725 immunized mice. Two independent experiments were performed and pooled data are shown.
726 Each symbol represents one mouse, n=8-10. Group means +/- SEM are shown. Significance
727 was assessed by One-way ANOVA (B, D, E, F) test or Mann-Whitney U test (C). **P<0.01,
728 ****P<0.0001.

729 **Fig 5. CD8T_{RM} cells facilitate the elimination of IAV.**

730 BALB/c mice were i.n. or i.p. immunized with 2 x 10⁵ PFU MCMV^{IVL} or i.n. with MCMV^{WT}. During
731 latency (> 3 months p.i), mice were treated with α CD8 or IgG2b antibodies and challenged
732 with IAV (PR8) (i.n., 1100 FFU). Leukocytes were isolated from lungs on day 4 post-challenge
733 for flow cytometric analysis. (A) Graphic representation of the mucosal CD8⁺ T cell depletion
734 protocol. (B) IAV titers in the lungs on day 4 post-challenge of MCMV^{IVL} i.n. immunized mice.
735 Two independent experiments were performed and pooled data are shown. Each symbol
736 represents one mouse, n=10. Group means +/- SEM are shown. (C-F) The concentration of
737 inflammatory cytokines and chemokines were measured in the BALF on day 2 and day 4 post-

738 IAV challenge. (C) The concentrations of IFN γ and (D) IL-6 in the BALF of each MCMV^{IVL} i.n.
739 immunized mice are shown as symbols. Group means +/- SEM are shown. (E) The
740 concentration of CCL3, CCL4, CCL5 and CXCL9 in the BALF on day 2 post-challenge. (F) The
741 concentration of CCL3, CCL4 and CXCL9 in the BALF on day 4 post-challenge. Experiments
742 were performed twice with 5-7 mice per group and representative data are shown. Bars
743 indicate means, error bars are SEM. Significance was assessed by Mann-Whitney U test, One-
744 way ANOVA test, or Two-way ANOVA test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

745 **Fig 6. CD8T_{RM} cells facilitate the accumulation of CD8⁺ T cells in the lungs.**

746 BALB/c mice were immunized with 2 x 10⁵ PFU MCMV^{IVL} via the i.n. route. During latency (>
747 3 months p.i), mice were i.n. treated with α CD8 (●;●) or IgG2b (○;○) antibodies and challenged
748 with IAV (PR8) (i.n., 1100 FFU) one day after. Leukocytes were isolated from lung tissue to
749 analyze the CD8⁺ T cell response on day 2 and day 4 post-challenge. (A) Cell counts of IVL-
750 specific CD8⁺ T cells in the lung tissue. (B) Cell counts of total CD8⁺ T cells in the lung tissue.
751 (C) Cell counts of IVL-specific CD8⁺ T cells in the BAL. (D) Cell counts of total CD8⁺ T cells in
752 the BAL. (E, F) anti-CD45 antibodies were injected intravenously 3-5 min before mice
753 euthanasia and (E) IVL-specific CD8⁺ T cells or (F) Total CD8⁺ T cells that were intravitally
754 labeled or remained unlabeled in the lung tissue were counted at 2 or 4 days post IAV
755 challenge. Experiments were performed twice with 5-7 mice per group and representative data
756 are shown. Each symbol represents one mouse, Group means +/- SEM are shown.
757 Significance was assessed by One-way ANOVA test. *P <0.05, **P <0.01, ***P <0.001, ****P
758 <0.0001.

759 **Fig 7. CD8T_{RM} cells do not expand upon IAV challenge.**

760 BALB/c mice were immunized with 2 x 10⁵ PFU MCMV^{IVL} via the i.n. route. During latency (>
761 3 months p.i), mice were i.n. treated with α CD8 (●) or IgG2b (○) antibodies and challenged with
762 IAV (PR8) (i.n., 1100 FFU) one day after. Leukocytes were isolated from lung tissue to analyze
763 the CD8⁺ T cell response on day 2 and day 4 post-challenge. (A) Percentage and (B) Counts

764 of CD8T_{RM} cells in the lungs. (C) Counts of IVL-specific CD8T_{RM} cells in the lungs. Experiments
765 were performed twice with 5-7 mice per group in total and representative data are shown. Each
766 symbol represents one mouse. Group means +/- SEM are shown. Significance was assessed
767 by One-way ANOVA test. *P <0.05, **P <0.01, ns: no significant difference.

768

769 **Supporting information**

770 **S1 Fig. Phenotype of primed and IVL-specific CD8⁺ T cells.**

771 BALB/c mice were immunized with 2 x 10⁵ PFU MCMV^{IVL} via the i.p. or i.n. route. During latency
772 (> 3 months p.i), leukocytes from blood, spleen and lungs were stained with cell surface
773 markers CD3, CD4, CD8, CD11a, CD44, KLRG1, CD62L, IVL-tetramer and analyzed by flow
774 cytometry. Primed cells are defined as CD11a^{hi}CD44⁺, T_{EFF} as KLRG1⁺CD62L⁻, T_{EM} as
775 KLRG1⁻CD62L⁻ and T_{CM} as KLRG1⁻CD62L⁺. (A-B) The percentages of primed and IVL-
776 tetramer⁺ CD8⁺ T cell subsets with different phenotypes in the blood (A & B), spleen (C & D)
777 and lungs (E & F) are shown. Each symbol represents one mouse, pooled data from two
778 independent experiments are shown (n=8-10). Group means +/- SEM are shown. Significance
779 was assessed by Mann-Whitney U test (two-tailed). *P <0.05, **P <0.01, ***P <0.001, ****P
780 <0.0001.

781 **S2 Fig. Efficiency of CD8⁺ T cell depletion.**

782 BALB/c mice were immunized with 2 x 10⁵ PFU MCMV^{IVL} by the i.n. route. (A) During latency
783 (> 3 months p.i), mice were injected 200 µg αCD8 antibody (i.p.) to deplete total CD8⁺ T cells.
784 Same amount of IgG2b antibody was given as isotype control. Leukocytes from blood, spleen
785 and lung were analyzed by flow cytometry and representative flow cytometric panels in blood,
786 spleen and lungs on day 1 post-depletion are shown. (B-C) Mice were administered with 10
787 µg αCD8 antibody (i.n.) to deplete airway CD8⁺ T cells in the lungs or IgG2b as a control. (B)
788 The efficiency of mucosal CD8T_{RM} cell depletion in the lungs of MCMV^{IVL} i.n. immunized mice

789 on day 1 post-depletion. (C) Total and IVL-specific CD8⁺ T cell counts in the blood of MCMV^{IVL}
790 i.n. immunized mice on day 1 post airway CD8⁺ T cell depletion. Bars indicate means, error
791 bars are SEM.

792 **S3 Fig. Efficiency of *in vivo* CD8⁺ T cell labeling.**

793 BALB/c mice were i.n. immunized with 2 x 10⁵ PFU MCMV^{IVL}. During latency (> 3 months p.i),
794 mice were i.v. injected 3 µg anti-CD45 antibody in 100 µl PBS 3-5min before euthanasia.
795 Leukocytes were isolated from blood, spleen and lungs, stained with cell surface markers
796 against CD4, CD8, CD69, CD103 before flow cytometry. (A) Representative dot plots of the
797 cell surface expression of *ex vivo* labeled CD8 and *in vivo* labeled CD45. (B) Representative
798 backgating showing that lung CD8T_{RM} (CD69⁺CD103⁺) cells are located exclusively within the
799 CD45 unlabeled cells.

800 **S4 Fig. Mucosal immunization induces CD69⁺ and CD8T_{RM} cells, but MCMV^{WT} induces**
801 **only IVL-unspecific CD8T_{RM}.**

802 (A) BALB/c mice were immunized with 2 x 10⁵ PFU MCMV^{WT} via the i.n. route. During latency
803 (> 3 months p.i), leukocytes were isolated from lungs, stained with cell surface markers against
804 CD4, CD8, CD69, CD103 before flow cytometry. Representative dot plots of CD8T_{RM} and IVL-
805 specific CD8T_{RM} cells. (B, C) BALB/c mice were immunized with 2 x 10⁵ PFU MCMV^{IVL} via the
806 i.n. or i.p. route. The (B) percentage and (C) counts of CD69⁺CD103⁺CD8⁺ T cells immunized
807 via the i.n. and i.p. route in the lungs. Pooled data from two independent experiments are
808 shown. Each symbol represents one mouse, n=7-9. Group means +/- SEM are shown. (D)
809 Eomes expression on different subsets of CD8⁺ T cells in the lungs. Significance was assessed
810 by Mann-Whitney U test. ***P < 0.001, ns: no significance.

811 **S5 Fig. Inflammatory cytokines in the BALF upon IAV challenge.**

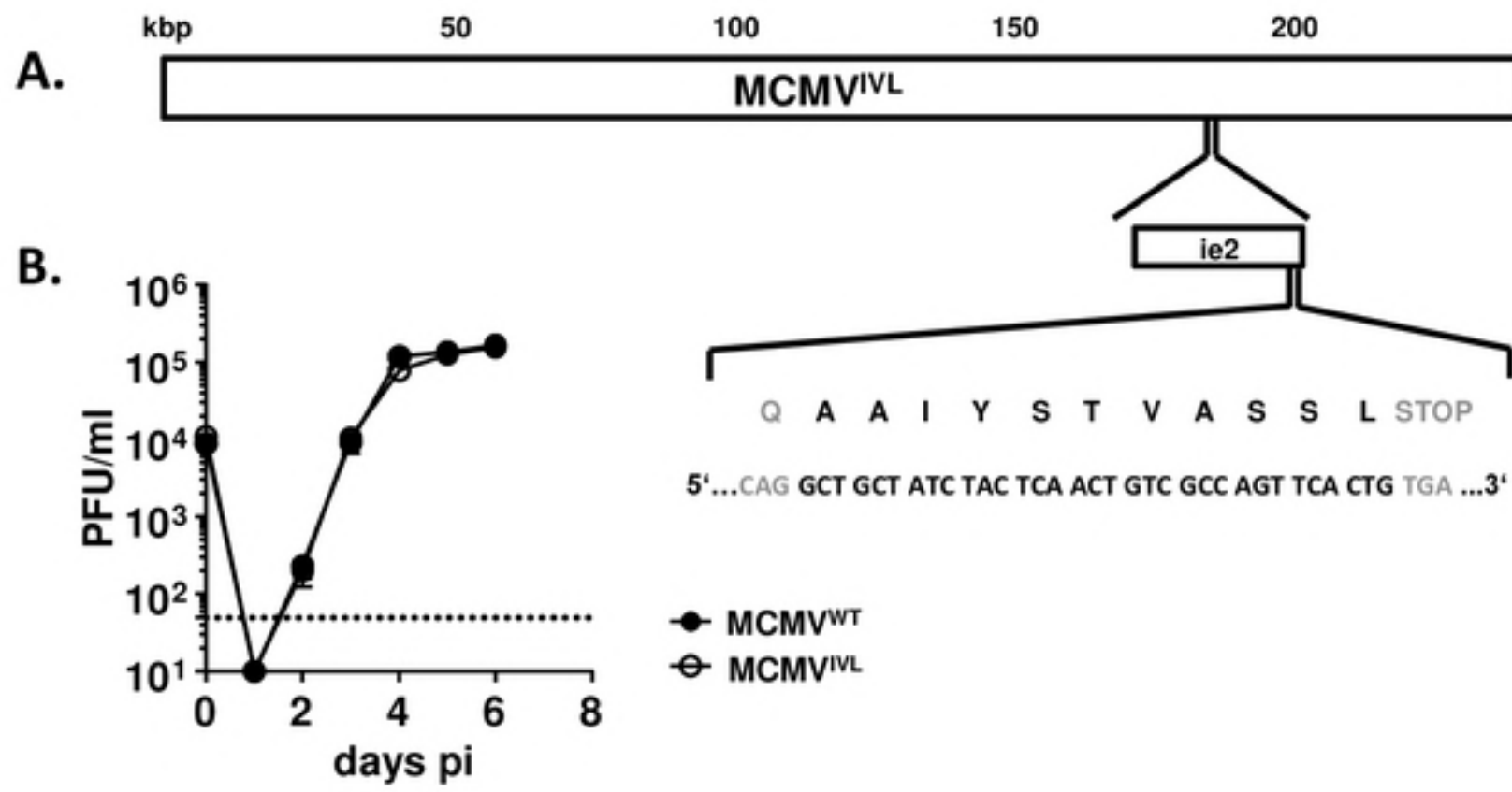
812 BALB/c mice were immunized with 2 x 10⁵ PFU MCMV^{IVL} via the i.n. or i.p. route or with
813 MCMV^{WT} via the i.n. route. During latency (> 3 months p.i), MCMV^{IVL} (i.n.) immunized mice

814 were administered with 10 μ g α CD8 or 10 μ g IgG2b antibody (i.n.). MCMV^{IVL} (i.p.) and MCMV^{WT}
815 (i.n.) immunized mice were administered with 10 μ g IgG2b antibody (i.n.). One day later,
816 animals were challenged with IAV (PR8) (i.n., 1100 FFU). On day 2 and day 4 post-challenge,
817 BALF was harvested and measured cytokines production by bio-plexing. The concentration of
818 (A) IFN γ and (B) IL-6 in the BALF on day 4 post-challenge. Two independent experiments were
819 performed and pooled data are shown. Each symbol represents one mouse, n=5-7. Group
820 means +/- SEM are shown. (C) Cytokine concentrations in the BALF in different immunization
821 group on day 2 and day 4 post-challenge. Bars indicate means, error bars are SEM.
822 Experiments were performed twice with 5-7 mice each group. Significance was assessed by
823 One-way ANOVA test. ****P <0.001.

824 **S6 Fig. Mucosal immunization with MCMV^{IVL} induced vigorous CD8⁺ T cell responses in**
825 **blood, spleen and lungs.**

826 BALB/c mice were immunized with 2 x 10⁵ PFU MCMV^{IVL} by the i.n. or i.p. route or with
827 MCMV^{WT} by the i.n. route. During latency (> 3 months p.i), leukocytes were isolated from lungs.
828 Mice were challenged with IAV (PR8) (i.n., 1100 FFU) one day after airway CD8⁺ T cell
829 depletion. Leukocytes were isolated from lung, BAL, blood and spleen on day 4 post-challenge.
830 (A) Count of IVL-specific CD8⁺ T cells in the lungs and (B) BAL. (C) Percentage of IVL-specific
831 CD8⁺ T cells in the blood and (E) spleen. (D) Count of IVL-specific CD8⁺ T cell in the blood and
832 (F) spleen. Two independent experiments were performed and pooled data are shown. Each
833 symbol represents one mouse, n=5-7. Group means +/- SEM are shown. Significance was
834 assessed by One-way ANOVA test. *P <0.05, **P <0.01.

Fig 1



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