Enhanced efficacy of vaccination with vaccinia virus in old versus young mice

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Abstract

Immunosenescence is believed to be responsible for poor vaccine efficacy in the elderly. To overcome this difficulty, research into vaccination strategies and the mechanisms of immune responses to vaccination is required. By analyzing the innate and adaptive immune responses to vaccination with vaccinia virus (VACV) in mice of different age groups, we found that immune cell recruitment, production of cytokines/chemokines and control of viral replication at the site of intradermal vaccination were preserved in aged mice and were comparable with younger groups. Analysis of cervical draining lymph nodes (dLN) collected after vaccination showed that numbers of germinal center B cells and follicular T helper cells were similar across different age groups. The number of VACV-specific CD8 T cells in the spleen and the levels of serum neutralizing antibodies 1 month after vaccination were also comparable across all age groups. However, following intranasal challenge of vaccinated mice, body weight loss was lower and virus was cleared more rapidly in aged mice than in younger animals. In conclusion, vaccination with VACV can induce an effective immune response and stronger protection in elderly animals. Thus, the development of recombinant VACV-based vaccines against different infectious diseases should be considered as a strategy for improving vaccine immunogenicity and efficacy in the elderly.

1 Introduction

Old people have increased susceptibility to viral and bacterial infections (1) and in people above 65, about a third of mortality is related to infections (2, 3). Prophylactic vaccination is recommended for the elderly to reduce the burden and severity of infectious diseases (4). However, the elderly respond poorly to the majority of existing vaccines, including vaccines against influenza virus, pneumococcus, hepatitis B, tetanus, pertussis, and diphtheria (5–10). It is important, therefore, to search for ways to overcome this barrier.

The reported decline in the immune system fitness with age, is thought to contribute to reduced vaccine efficacy in humans and mice (5, 6, 11, 12). This decline impacts both innate and adaptive immunity. Impaired recognition of microorganisms and their components, inadequate receptor signaling, and altered cytokine production have all been reported (13). Additionally, dysfunctionality of innate immune cells such as neutrophils, NK cells, monocytes, macrophages and dendritic cells in their ability to migrate, perform phagocytosis, kill bacteria and secrete cytokines have been noted (2, 14–16). Decline in the performance of multiple aspects of the adaptive immune response with age also occurs. This includes decreased numbers of naïve T cells, a reduced TCR repertoire, an impaired clonal expansion and generation of functional effector and memory T cells, a decrease in

immunoglobulin class switch recombination, and restricted B cell diversity and antibody production (7, 11, 17).

Vaccinia virus (VACV), a dsDNA poxvirus (18), is the vaccine used to eradicate smallpox (19). VACV replicates in the cytoplasm of infected cells and has a large genome containing approximately 200 genes (20). Between one third and one half of these genes encode proteins dedicated to immune evasion (21). Although VACV is immunosuppressive, vaccination with VACV in humans and mice results in the generation of robust, long-lasting antibody and T-cell memory that provides protection against re-infection (21–25). The ability of VACV to generate such potent humoral and cellular memory, and its proven ability to protect a population against infectious disease, makes it an excellent model system for studying immune response to vaccination. In this study, we use a mouse model of VACV intradermal vaccination that generates protective immunity against re-infection (26). In this model, both antibody and T cell memory responses are robust and consistent and contribute to protection against subsequent challenge with VACV (27, 28). Although VACV has been studied intensively in multiple models, the influence of aging on the immune and vaccination responses to VACV is unexplored. In this study, we analyzed the innate and adaptive immune response to VACV infection and evaluated subsequent resistance to re-infection in three different age groups of mice.

2 Materials and Methods

2.1 Animals and study design

C57BL/6 female mice were used in the study. All animals were purchased from Charles River and housed in the Cambridge University Biomedical Services facility. All animal experiments were conducted according to the Animals (Scientific Procedures) Act 1986 under PPL 70/8524 issued by UK Home Office.

The animal experiments included intradermal (i.d.) vaccination and intranasal (i.n.) challenge (Fig. 1). Animals of 7, 22 and 54 weeks old (wo) received i.d. injections with 10^4 plaque-forming unit (PFU) of VACV strain Western Reserve (WR) or control vehicle (0.01% BSA/PBS) into both ear pinnae. VACV used for infection of animals was purified from infected cells by sedimentation through a sucrose cushion and subsequently through a sucrose density gradient. Virus infectious titers were determined by plaque assay on BSC-1 cells and frozen at -70 ℃ until use. To evaluate the immune response during the acute stage post vaccination, ear tissues and cervical draining lymph nodes (dLN) were collected at day (d) 7 after i.d. infection for measurement of infectious viral titers (by plaque assay), leukocyte infiltration (by FACS) and levels of cytokines/chemokines (by Luminex assay). Serum and spleens were obtained at 29 d post i.d. injections to measure the titers of anti-VACV neutralizing antibodies and the composition of T cell subpopulations.

To assess the efficacy of vaccination, vaccinated mice (33 d post i.d. VACV infection) and naïve (non-vaccinated) mice were challenged i.n. with ~10^7 PFU of VACV WR. The body weights of animals were monitored daily. Whole lungs were collected at 12, 24 and 48 h post challenge to measure the viral load and the levels of cytokines/chemokines in tissue.

The baseline of immunological parameters was measured in the blood, spleens and lungs of naïve, uninfected animals (n=4).

2.2 Flow cytometry

78 FACS analysis was performed to measure the immune cells present in ear tissue, cervical dLN, blood
79 and spleens of vaccinated and mock-vaccinated animals.

80 Ear pinnae were collected at 7 d post i.d. infection, then separated into dorsal and ventral layers and
81 both leaflets were placed into 1.5 ml of the RPMI-1640 (Gibco, Cat. # 21875034) medium containing
82 750 U/ml of collagenase I (Gibco, Life Technologies, Cat. # 17018-029) and 100 U/ml of DNase I
83 (Invitrogen, Cat. # 18047-019), followed by 1 h incubation at 37 °C on an orbital shaker, at 1100
84 rpm. Suspensions containing digested ear samples were mashed through a 70-µm cell-strainer, mixed
85 with 10 ml of RPMI-1640 medium containing 35% of isotonic Percoll (Sigma, Cat. # P1644-500ML)
86 and centrifugated for 10 min at 940 relative centrifugal force (rcf) without use of brake, at 21 °C.
87 Then the supernatants were removed and the cells were washed with PBS.

88 To obtain cells from spleen or dLN, organs were mashed through 70-µm cell-strainers and washed
89 with PBS.

90 Before antibody staining of prepared cell suspensions, red blood cells (RBC) were lysed with BD
91 Pharm Lyse (BD Biosciences, Cat. # 555899) and washed twice. The suspensions were then passed
92 through 70-µm Pre-Separation Filters (Miltenyi, Cat. # 130-095-823) and cells were counted using a
93 NucleoCounter NC-250 (Chemometec).

94 For the staining of cell surface markers, the samples were incubated with Zombie Fixable Viability
95 dye (Suppl. Table 1) and, after one washing step, purified rat anti-mouse CD16/CD32 antibody
96 (Mouse BD Fc Block) (BD Biosciences, Cat. # 553141) was added to the cell suspension to block
97 non-specific binding. For intracellular Bcl-6 and Ki-67 staining, Foxp3 / Transcription Factor
98 Staining Buffer Set (eBioscience, Cat. # 00-5523-00) was used. Then surface or intracellular markers
99 were stained with monoclonal antibodies (mAbs). The myeloid panel for surface staining of ear
100 tissue included: CD45, Siglec-F, CD11c, CD11b, Ly6C, Ly6G, as well as dump channel markers
101 (CD3, CD5, CD19, NK1.1). The lymphoid cells in ear tissue were identified using mAbs to CD45,
102 NK1.1, CD3, CD4, CD8 and with MHC dextramer H-2Kb/TSYKFESV. For assessment of VACV-
103 specific CD8 T cells in the dLN, the cells were stained with mAbs to CD45, CD19, CD3, CD8 and
104 with MHC dextramer H-2Kb/TSYKFESV. The panel for identification of germinal center B cells and
105 follicular helper T lymphocytes in dLNs included mAbs to CD4, CXCR5, PD-1, B220, Bcl-6 and ki-
106 67. Subpopulations of CD4 and CD8 T cells in spleen were determined by staining with mAbs to
107 CD45, CD3, CD8, CD4, CD62L and CD44 and with MHC dextramer H-2Kb/TSYKFESV. All dyes
108 and mAbs used in the study are listed in Suppl. Table 1. After final washing steps, cells were
109 resuspended with PBS containing 4% paraformaldehyde and were analyzed by FACS on a BD
110 LSRFortessa (BD Biosciences). Gating strategies are shown in Suppl. Figs. 1-5.

111 For the Trucount assay, blood was collected into Micro K3EDTA Tubes (Sarstedt, Cat. #
112 41.1395.005) to prevent clot formation. Then, 50 µl of whole blood was pipetted into the bottoms of
113 BD Trucount Tubes (BD Biosciences, Cat. # 340334), followed by 5 min incubation with Mouse BD
114 Fc Block. The samples were then stained with mAbs to CD45, CD3, CD4, CD8, CD19, NK1.1,
115 CD11b and Ly6G (Suppl. Table 1). After RBC lysis, and without washing steps, the absolute
116 numbers of different leukocyte populations were determined by analysis on a BD LSRFortessa. The
117 gating strategy is shown in Suppl. Fig. 6.

118 **2.3 Intravascular staining**

119 To discriminate leukocytes resident in ear tissue from cells located in vasculature, intravascular
120 staining was undertaken as described (29). Briefly, 5 mins before culling, mice were given an

121 intravenous infusion into the tail vein of anti-CD45-BV421 mAb (BioLegend, Cat. # 103134). Ears
122 were then collected, and cells were isolated as described under Flow cytometry above. The cell
123 suspension from ear tissue was stained with anti-CD45-PE (BioLegend, Cat. # 103106). Blood
124 leukocytes were gated as double positive (CD45-BV421^{+} CD45-PE^{+}) cells, while tissue immune cells
125 were positive only for CD45-PE (see Suppl. Fig. 7).

126 **2.4 Identification of cytokines and chemokines in ear, dNL and lung tissues**

127 Whole ears, dLN or lungs were homogenized in 1.5 ml flat-bottom tubes containing 400 µl of 0.5%
128 BSA/PBS using an OMNI Tissue Homogenizer with plastic hard tissue probes (OMNI International).
129 The tissue homogenates were centrifugated at 10,000 rcf for 20 min, at 4 °C and supernatants were
130 obtained and stored at -70 °C. Magnetic Luminex Mouse Premixed Multi-Analyte kits were
131 purchased from R&D Systems, to assess levels of IFNγ, TNFα, IL-1β, IL-4, IL-6, IL-10, IL-33,
132 CCL2, CCL3, CCL4, CCL5, CCL7, CCL20, CXCL1, CXCL2, CXCL5 (LIX) and CXCL10 using a
133 Luminex 200 analyzer (Luminex Corporation).

134 **2.5 Measurement of viral loads in ear and lung tissues**

135 Whole ears and lungs were homogenized as described above. The homogenates underwent 3 cycles
136 of freezing-thawing-sonicating to rupture cells and release the virus. Titers of infectious virus in ear
137 samples were then determined by plaque assay using BSC-1 cell monolayers.

138 The VACV load in the lungs of vaccinated mice was measured by determining the virus genome
139 copy number by qPCR as described (30). Genome copy number correlated well with measurement of
140 virus infectivity by plaque assay (Suppl. Fig. 8). Supernatant samples from lung tissue homogenates
141 were prepared by centrifugation of samples at 1000 rcf for 5 min, followed by 10-fold dilution of
142 supernatants with nuclease-free water (Cat. # AM9930, Ambion). The reaction mix for real-time
143 qPCR included: 2 µl of template, 10 µl of 2x qPCRBIO Probe Mix (Cat. # PB20.21-5,
144 PCRBiosystems), 0.8 µl of 10 µM VACV gene *E9L* forward primer
145 (CGGCTAAGAGTTGCACATCCA), 0.8 µl of 10 µM *E9L* reverse primer
146 (CTCTGCTCCATTTAGTACCGATTCT), 0.4 µl of 10 µM *E9L* probe (TaqMan MGB Probe –
147 AGGACGTAGAATGATCTTGTA, Applied Biosystems). The reaction volume was adjusted to 20
148 µl with nuclease-free water. A plasmid containing the VACV *E9L* gene served as a standard and was
149 a gift from Brian M Ward, University of Rochester Medical Center, USA. qPCR assays were run on
150 a ViiA 7 Real-Time PCR System (Applied Biosystems) with the following protocol: initial
151 denaturation step at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 5 sec,
152 annealing and extension at 60 °C for 30 sec.

153 **2.6 Assessment of VACV neutralizing anybody titer in serum**

154 Blood was collected into Microvette CB 300 µl tubes with clot activator (Sarstedt, Cat.# 16.440.100).
155 Blood samples were left at room temperature for 2 h to allow clot formation. After centrifugation at
156 10,000 rcf for 5 min at room temperature, serum was collected and stored at -70 °C. Titers of
157 neutralizing antibodies were assessed by plaque reduction neutralization test as described (31). Serum
158 samples were incubated at 56 °C for 30 min to inactivate complement, then two-fold serial dilutions
159 were prepared (1:50, 1:100, 1:200, 1:400, 1:800 and 1:1600) using 2.5% fetal bovine serum
160 (FBS)/1% PenStrep/DMEM medium. The diluted serum samples, or reference samples (medium
161 only), were mixed 1:1 with medium containing 3.2×10^2 PFU/ml of VACV WR that had been

purified by sedimentation through a sucrose density gradient. After 1 h incubation at 37 °C, samples were titrated by plaque assay and half maximal inhibitory concentrations (IC50) were calculated.

2.7 Statistical analysis

SPSS v.25 and GraphPad Prism v.8 were used for statistical analysis. The Mann–Whitney U-test was applied for the comparisons of two groups of animals and two-way repeated measures (RM) ANOVA tests were performed for the analysis of time series data. The Spearman's correlation test was used for relation analysis of variables. P values <0.05 were considered significant.

3 Results

Three groups (7, 22 or 54 weeks old [wo]) of female C57BL/6 mice were used representing young adults, middle-aged and old animals. Fifty-four-wo animals were chosen to represent the elderly group based on their general appearance (graying coat, thinning hair) and the death rate in the colony (~10% lethality over a 6-week period not associated with the experiment). One of the features of immunosenescent phenotype is a decline in naïve T cell numbers, which correlates with increased morbidity and mortality (32, 33). Notably, the 54-wo mice had significantly decreased absolute numbers of CD4 and CD8 cells in the blood and spleen in comparison with younger animals (Fig. 2A). This reduction was due to the decline of naïve subpopulations of CD4 and CD8 cells, while effector T cell numbers were increased in 54- and 22-wo mice in comparison with the 7-wo group (Fig. 2B, Suppl. Fig. 5).

3.1 Immune response to intradermal infection with VACV is conserved across different age groups

Intradermal (i.d) infection with VACV leads to the development of skin lesions 5-6 days (d) post infection that usually heal within 21 d (26). Using this infection model, immune cell recruitment, the levels of cytokines/chemokines and the viral load in ear tissues 7 d post i.d. infection was analyzed in three different age groups of mice.

FACS analysis of leukocyte populations in the infected ear showed that ~97% represented cells that had infiltrated into the tissue, whereas immune cells from blood circulation constituted only 3% of the total leukocytes (Suppl. Fig. 7). In comparison with 7- and 22-wo mice, infected ear tissues of 54-wo animals showed significantly less infiltration of different leukocyte populations including NK, CD4 and CD8 T cells, Ly6C^{+} (inflammatory) monocytes and CD45^{+}CD3^{-}CD5^{-}CD19^{-}NK1.1^{-}Siglec-F^{-}CD11c^{+} cells, which represent a mixed population of dendritic cells and macrophages (Fig 3A). The presence of VACV-specific CD8 T cells in infected ear tissue of 54-wo mice was also reduced compared to the other groups (Fig. 3B). Given that amounts of CD4 and CD8 T cells in the elderly mice were diminished before the infection (Fig. 2), the lower numbers of lymphoid populations infiltrating ears are likely reflective of the reduced availability of T cells in the blood circulation.

The levels of cytokines and chemokines detected in ear tissue of the old animals at 7 d after i.d. infection did not differ greatly from the young and middle-age groups (Fig. 3C). Only the levels of IL-10 and CCL5 were reduced, while the concentrations of TNFα and IL-6 were increased in 54-wo mice in comparison with 7- and 22-wo animals. However, the amplitude of these changes did not exceed 2-fold. In addition, viral loads in infected ear tissues were similar in all groups (Fig. 3D). Hence, the infected ear tissue was able to respond to the infection via production of inflammatory mediators and control virus infection to equivalent levels across all age groups. These data provide further evidence that the lower cell recruitment into the ear tissue of 54-wo mice was likely due to the

204 reduced availability of circulating cells rather than due to changes in local responses in the infected
205 tissue. Thus, functionally, the immune response to i.d. vaccination with VACV was preserved in 54-
206 wo mice, and the ability to control VACV replication at the site of infection was equal across 7-, 22-
207 and 54-wo groups.

208 3.2 54-week old mice have enhanced cytokine response to intradermal VACV infection in 209 draining lymph nodes

210 To investigate the effect of age on the adaptive immune response to vaccination with VACV, cervical
211 draining lymph nodes (dLN) were analyzed at 7 d post i.d. infection. This showed a trend in
212 reduction of absolute numbers of VACV-specific CD8 T cells in the 54-wo mice in comparison with
213 22- and 7-wo groups (Fig. 4A). However, the amounts of germinal center (GC) B cells and T
214 follicular helper (Tfh) lymphocytes were not significantly different between all groups (Fig. 4B, C).

215 Next, the levels of cytokines and chemokines in dLN were measured at 7 d post vaccination.
216 Amongst 17 different molecules assessed, IFNγ, IL-33, CCL2, CCL3, CCL4, CCL5, CCL7 and
217 CXCL1 were detectable (Fig. 4D) and the levels of IFNγ, CCL2 and CCL5 were significantly higher
218 in 54-wo animals than in younger mice, while the others were similar in all groups. Thus, the dLNs
219 of old animals responded well to VACV vaccination, expressing high levels of inflammatory
220 mediators and generating appropriate cellular adaptive immune responses.

221 3.3 VACV vaccination induces strong adaptive immune response in mice of different ages

222 Next, we compared the cellular and humoral memory responses induced by vaccination with VACV.
223 Spleens and blood samples were obtained from mice 29 d post vaccination of 7-, 22- and 54-wo as
224 well as from mock-vaccinated animals. The total numbers of splenic CD8 T cells were equivalent
225 within the three vaccinated groups, while the absolute numbers of CD4 T cells were slightly reduced
226 in 54-wo mice in comparison with in 7- and 22-wo groups (Fig. 5A). The most pronounced changes
227 in numbers of splenic CD8 and CD4 T subsets were observed for effector T cells. In comparison with
228 baseline parameters before vaccination, effector CD8 and CD4 T lymphocytes increased
229 considerably as a result of vaccination for all groups of mice (Fig. 2B, Fig. 5B). Notably, effector T
230 cells in the 54-wo group expanded proportionally greater than in younger groups. However, analysis
231 of VACV-specific CD8 T cells showed that their absolute counts were comparable within all age
232 groups (Fig. 5C). As for the humoral immune response, the ability of serum to neutralize VACV was
233 identical in all three groups (Fig. 5D). These observations show that vaccination with VACV
234 generates memory immunity in 54-wo mice that is quantitively indistinguishable from that generated
235 in 7- or 22-wo mice.

236 3.4 54-week old mice are better protected against VACV intranasal challenge than those 237 from younger groups

238 To measure the ability of vaccinated groups to respond to re-infection, the three age groups of
239 vaccinated animals and young naïve mice were challenged i.n. with a dose of VACV equivalent to ~
240 300 LD50. All vaccinated groups had mild or moderate weight loss (about 15% maximum) after
241 challenge followed by full recovery. In contrast, naïve mock-vaccinated mice had >25% weight loss
242 and were culled at humane endpoint (Fig. 6A). Notably, following challenge, the 54-wo mice lost
243 less body weight and recovered faster than young and middle-aged groups. Results of viral load
244 measurement in the lungs of challenged mice indicated that the 54-wo mice cleared the virus faster
245 than other groups (Fig. 6B). For the majority of immunized elderly mice, no VACV genome copies
246 were detected in lungs at 24 h post i.n. challenge. Interestingly, the 22-wo mice were slower than 7-

247 and 54- wo groups at clearing the virus, despite the weight loss post challenge being similar between
248 the 7- and 22-wo groups (Fig. 6A, B).

249 Lastly, the levels of inflammatory mediators in the lung tissue of vaccinated and naïve mice were
250 measured (Fig. 7, Suppl. Fig. 9). Baseline levels before i.n. infection did not vary significantly
251 between groups. In comparison with the naïve mice, all vaccinated animals responded very quickly to
252 i.n infection. At just 12 h post-challenge, the levels of IFNγ, CCL7, CXCL1, CXCL2, CXCL10 rose
253 substantially (Suppl. Fig. 9), although there was little variation between the different age groups.
254 Only CXCL1 was increased in the elderly mice, and IFNγ levels tended to be higher in the old and
255 middle-aged mice than in young animals. At 24 h post infection, the majority of lung cytokines and
256 chemokines were reduced in the elderly mice compared with other vaccinated age groups. Moreover,
257 at 48 h after challenge, the levels of inflammatory mediators in the 54-wo group were decreased
258 further and started returning to their initial (baseline) concentrations. This may reflect the faster virus
259 clearance. Therefore, the results of weight loss measurement, viral load and cytokine dynamics in
260 lungs indicate that mice vaccinated at the age of 54-wo had robust protection against reinfection with
261 a lethal dose of VACV, and this protection was even stronger than in the mice vaccinated when 7-
262 and 22-wo.

263 **4 Discussion**

264 In the current study, we show that i.d. vaccination with VACV leads to successful development of
265 immunological memory in old mice bearing an immunosenescent phenotype. Surprisingly, despite a
266 general reduction of naïve CD8 and CD4 cells (Fig. 2), reduced recruitment of immune cells into the
267 site of i.d. infection (Fig. 3A, B) and inflammatory signatures characteristic of a phenomenon
268 sometimes known as inflammaging (Fig. 3C and 4D), 54-week-old mice demonstrated better
269 vaccination efficacy against challenge than the younger animals.

270 Vaccination of humans with VACV results in long lasting immunological memory even after
271 administration of a single dose of vaccine (23, 24, 34), and its high efficacy has been validated by the
272 eradication of smallpox. Little information is available concerning VACV vaccine performance in
273 elderly people or mice. One study has reported that vaccination of aged BALB/c mice with
274 recombinant VACV expressing influenza hemagglutinin was effective in generating anti-
275 hemagglutinin antibodies and influenza-specific cytotoxic T cells (35). The basis of high
276 immunogenicity of VACV is not known. However, local immunosuppression by VACV allows the
277 virus to replicate at the site of infection for at least 12 d post i.d vaccination (26). This extended
278 replication period provides constant antigen exposure to the immune system, probably facilitating the
279 generation of strong immunological memory. This immune suppression may be mediated by the
280 scores of immune modulatory proteins expressed by VACV early after infection (21, 36). Many
281 VACV immunomodulators target pattern recognition receptor and interferon receptor signaling to
282 block anti-viral responses in infected cells. In vaccination models, deletion of two or three such genes
283 leads to enhanced safety but decreased immunogenicity of vaccine and impaired protection against
284 challenge (28). The highly attenuated VACV strain modified vaccinia Ankara (MVA), which is
285 replication deficient in many cell types, results in the generation of significantly lower antibody titers
286 in comparison with WR (37, 38). This may explain the potency of VACV in developing robust
287 immunological memory even in old mice. However, this does not explain why better protection
288 against challenge was observed in older mice than in younger counterparts.

289 One of the features of the immune system in the elderly is the presence of chronic, low grade
290 inflammation, which is sometimes called inflammaging (39). The characteristics of this phenomenon

291 are upregulated activity of NF-κB (40, 41), increased levels of proinflammatory cytokines and
292 chemokines such as TNFα, IL-1, IL-6, IL-8, IL-12, CCL2, CXCL10 (42), accumulation of damage-
293 associated molecular patterns and dysfunctional organelles (43), as well as changes in gut microbiota
294 and metabolism (44). This phenomenon may go some way in explaining the enhanced production of
295 NF-κB-regulated cytokines TNFα and IL-6 in the VACV-infected ear tissue of 54-wo mice (Fig. 3C)
296 as well as CCL2 in dLNs (Fig. 4D). IL-6 and TNF superfamily ligands act as adjuvants and increase
297 immunogenicity of vaccines (45, 46). Therefore, in the case of VACV vaccination, inflammaging
298 might be beneficial by providing additional pro-inflammatory stimulus to drive the cascade of events
299 leading to immune memory development.

300 Increased production of TNFα and IL-6 along with low levels of IL-10 (Fig. 3C) in old animals after
301 i.d. vaccination, might compensate for the reduced recruitment of immune cells into the vaccination
302 site (Fig. 3A). This might contribute to the control of virus infection after i.d. infection (Fig. 3D) and
303 provide adequate conditions for the generation of immunological memory. Notably, the numbers of
304 Tfh and GC B cells in dLN (Fig. 4B, C), as well as VACV-specific CD8 T cells in the spleen and
305 neutralizing antibody levels (Fig. 5C, D), were similar across all age groups. Nonetheless, it is
306 unclear how the old mice achieved faster clearance of VACV and reduced weight loss after challenge
307 (Fig. 6A, B).

308 Our results show that absolute counts of splenic effector CD8 T cells expanded substantially and
309 proportionally higher in the elderly group than in the younger mice (Fig. 2B and 5B). This cannot be
310 explained simply by the increased numbers of splenic VACV-specific CD8 T cells (that have been
311 identified by MHC-I dextramer staining) because their absolute counts are too low and similar across
312 all three age groups (Fig. 5C). This difference might be due to the expansion of VACV-specific CD8
313 T cells against different VACV epitopes and/or the expansion of low-affinity CD8 T cells, which
314 have not been recognized by the type of MHC-I dextramers used in this study. These cells may
315 contribute to the rapid clearance of VACV in the elderly after challenge. Also, despite numerous
316 publications describing functional inefficiency of senescent effector T cells, there are reports that
317 effector T cells from elderly people can have superior immune response to antigen stimulation than
318 younger counterparts (47–49).

319 In conclusion, this study demonstrates that vaccination of elderly mice is very efficient and not
320 inferior to younger animals. Immunescenescence and inflammaging may be more accurately viewed
321 as immunoadaptation and immunoremodeling in old age, rather than just a slow decline in immune
322 system function (50, 51). The majority of vaccines were created for, and are used in, children and
323 young adults (6), and vaccines designed for the elderly population are needed that consider the
324 specific characteristics of immune system in old age. Given the performance of VACV vaccination
325 shown in the current study, further investigation to understand the mechanisms of its high
326 immunogenicity is warranted.

327 5 Ethics Statement

328 This study was carried out in accordance with the regulations of The Animals (Scientific Procedures)
329 Act 1986. All protocols and procedures were approved by the UK Home Office and performed under
330 the project licence PPL 70/8524.

331 6 Conflict of Interest

332 The authors declare that the research was conducted in the absence of any commercial or financial
333 relationships that could be construed as a potential conflict of interest.

334 7 Author Contributions

335 GS and BF provided the funding. ES, GS and BF designed the study. ES performed all experiments
336 and statistical analysis. GS and BF supervised the work. ES, BF and GS wrote the manuscript.

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344 10 Data Availability Statement

345 Raw data supporting the conclusions of this manuscript will be made available by the authors,
346 without undue reservation, to any qualified researcher.

347 11 References

348 1. Hepper, H. J., Sieber, C., Cornel, S., Walger, P., Peter, W., Bahrmann, P. et al. Infections in the
349 elderly. *Crit Care Clin* (2013) *29*(3):757-774. doi:10.1016/j.ccc.2013.03.016.
350 2. Haq, K., and McElhaney, J. E. Ageing and respiratory infections: the airway of ageing. *Immunol*
351 *Lett* (2014) *162*(1 Pt B):323-328. doi:10.1016/j.imlet.2014.06.009.
352 3. Kline, K. A., and Bowdish, D. M. Infection in an aging population. *Curr Opin Microbiol* (2016)
353 *29*63-67. doi:10.1016/j.mib.2015.11.003.
354 4. Weinberger, B. Vaccines for the elderly: current use and future challenges. *Immun Ageing*
355 (2018) *15*3. doi:10.1186/s12979-017-0107-2.
356 5. Chen, W. H., Kozlovsky, B. F., Effros, R. B., Grubeck-Loebenstein, B., Edelman, R., and Sztein,
357 M. B. Vaccination in the elderly: an immunological perspective. *Trends Immunol* (2009)
358 *30*(7):351-359. doi:10.1016/j.it.2009.05.002.
359 6. Ciabattini, A., Nardini, C., Santoro, F., Garagnani, P., Franceschi, C., and Medaglini, D.
360 Vaccination in the elderly: The challenge of immune changes with aging. *Semin Immunol* (2018)
361 *40*83-94. doi:10.1016/j.smim.2018.10.010.
362 7. Goodwin, K., Viboud, C., and Simonsen, L. Antibody response to influenza vaccination in the
363 elderly: a quantitative review. *Vaccine* (2006) *24*(8):1159-1169.
364 doi:10.1016/j.vaccine.2005.08.105.
365 8. Tin Tin Htar, M., Stuurman, A. L., Ferreira, G., Alicino, C., Bollaerts, K., Paganino, C. et al.
366 Effectiveness of pneumococcal vaccines in preventing pneumonia in adults, a systematic review
367 and meta-analyses of observational studies. *PLoS One* (2017) *12*(5):e0177985.
368 doi:10.1371/journal.pone.0177985.
369 9. Weston, W. M., Friedland, L. R., Wu, X., and Howe, B. Vaccination of adults 65 years of age
370 and older with tetanus toxoid, reduced diphtheria toxoid and acellular pertussis vaccine

371 (Boostrix(®)): results of two randomized trials. *Vaccine* (2012) *30*(9):1721-1728.
372 doi:10.1016/j.vaccine.2011.12.055.
373 10. Yang, S., Tian, G., Cui, Y., Ding, C., Deng, M., Yu, C. et al. Factors influencing immunologic
374 response to hepatitis B vaccine in adults. *Sci Rep* (2016) *6*27251. doi:10.1038/srep27251.
375 11. Goronzy, J. J., and Weyand, C. M. Understanding immunosenescence to improve responses to
376 vaccines. *Nat Immunol* (2013) *14*(5):428-436. doi:10.1038/ni.2588.
377 12. Solana, R., Pawelec, G., and Tarazona, R. Aging and innate immunity. *Immunity* (2006)
378 *24*(5):491-494. doi:10.1016/j.immuni.2006.05.003.
379 13. Molony, R. D., Malawista, A., and Montgomery, R. R. Reduced dynamic range of antiviral
380 innate immune responses in aging. *Exp Gerontol* (2018) *107*130-135.
381 doi:10.1016/j.exger.2017.08.019.
382 14. Gomez, C. R., Nomellini, V., Faunce, D. E., and Kovacs, E. J. Innate immunity and aging. *Exp*
383 *Gerontol* (2008) *43*(8):718-728. doi:10.1016/j.exger.2008.05.016.
384 15. Metcalf, T. U., Cubas, R. A., Ghneim, K., Cartwright, M. J., Grevenynghe, J. V., Richner, J. M.
385 et al. Global analyses revealed age-related alterations in innate immune responses after
386 stimulation of pathogen recognition receptors. *Aging Cell* (2015) *14*(3):421-432.
387 doi:10.1111/acel.12320.
388 16. Shaw, A. C., Goldstein, D. R., and Montgomery, R. R. Age-dependent dysregulation of innate
389 immunity. *Nat Rev Immunol* (2013) *13*(12):875-887. doi:10.1038/nri3547.
390 17. Henry, C., Zheng, N. Y., Huang, M., Cabanov, A., Rojas, K. T., Kaur, K. et al. Influenza Virus
391 Vaccination Elicits Poorly Adapted B Cell Responses in Elderly Individuals. *Cell Host Microbe*
392 (2019) *25*(3):357-366.e6. doi:10.1016/j.chom.2019.01.002.
393 18. Moss, B. (2013), Poxviridae, in *Fields Virology*, edited by B. N. Fields, D. M. Knipe, and P. M.
394 Howley, pp. 2129-2159, Philadelphia : Wolters Kluwer Health/Lippincott Williams & Wilkins,
395 19. Fenner, F., D. A. Henderson, I. Arita, Z. Jezek, and I. D. Ladnyi (1988), *Smallpox and its*
396 *eradication*, 1460 pp., World Health Organization,
397 20. Goebel, S. J., Johnson, G. P., Perkus, M. E., Davis, S. W., Winslow, J. P., and Paoletti, E. The
398 complete DNA sequence of vaccinia virus. *Virology* (1990) *179*(1):247-66, 517.
399 21. Smith, G. L., Benfield, C. T., Maluquer de Motes, C., Mazzon, M., Ember, S. W., Ferguson, B.
400 J. et al. Vaccinia virus immune evasion: mechanisms, virulence and immunogenicity. *J Gen*
401 *Virol* (2013) *94*(Pt 11):2367-2392. doi:10.1099/vir.0.055921-0.
402 22. Belyakov, I. M., Earl, P., Dzutsev, A., Kuznetsov, V. A., Lemon, M., Wyatt, L. S. et al. Shared
403 modes of protection against poxvirus infection by attenuated and conventional smallpox vaccine
404 viruses. *Proc Natl Acad Sci U S A* (2003) *100*(16):9458-9463. doi:10.1073/pnas.1233578100.
405 23. Crotty, S., Felgner, P., Davies, H., Glidewell, J., Villarreal, L., and Ahmed, R. Cutting edge:
406 long-term B cell memory in humans after smallpox vaccination. *J Immunol* (2003)
407 *171*(10):4969-4973.
408 24. Taub, D. D., Ershler, W. B., Janowski, M., Artz, A., Key, M. L., McKelvey, J. et al. Immunity
409 from smallpox vaccine persists for decades: a longitudinal study. *Am J Med* (2008)
410 *121*(12):1058-1064. doi:10.1016/j.amjmed.2008.08.019.
411 25. Xu, R., Johnson, A. J., Liggitt, D., and Bevan, M. J. Cellular and humoral immunity against
412 vaccinia virus infection of mice. *J Immunol* (2004) *172*(10):6265-6271.
413 26. Tscharke, D. C., and Smith, G. L. A model for vaccinia virus pathogenesis and immunity based
414 on intradermal injection of mouse ear pinnae. *J Gen Virol* (1999) *80*(Pt 10):2751-2755.
415 doi:10.1099/0022-1317-80-10-2751.
416 27. Sumner, R. P., Ren, H., and Smith, G. L. Deletion of immunomodulator C6 from vaccinia virus
417 strain Western Reserve enhances virus immunogenicity and vaccine efficacy. *J Gen Virol* (2013)
418 *94*(Pt 5):1121-1126. doi:10.1099/vir.0.049700-0.

419 28. Sumner, R. P., Ren, H., Ferguson, B. J., and Smith, G. L. Increased attenuation but decreased
420 immunogenicity by deletion of multiple vaccinia virus immunomodulators. *Vaccine* (2016)
421 *34*(40):4827-4834. doi:10.1016/j.vaccine.2016.08.002.
422 29. Anderson, K. G., Mayer-Barber, K., Sung, H., Beura, L., James, B. R., Taylor, J. J. et al.
423 Intravascular staining for discrimination of vascular and tissue leukocytes. *Nat Protoc* (2014)
424 *9*(1):209-222. doi:10.1038/nprot.2014.005.
425 30. Baker, J. L., and Ward, B. M. Development and comparison of a quantitative TaqMan-MGB
426 real-time PCR assay to three other methods of quantifying vaccinia virions. *J Virol Methods*
427 (2014) *196*126-132. doi:10.1016/j.jviromet.2013.10.026.
428 31. Ren, H., Ferguson, B. J., Maluquer de Motes, C., Sumner, R. P., Harman, L. E., and Smith, G. L.
429 Enhancement of CD8(+) T-cell memory by removal of a vaccinia virus nuclear factor-κB
430 inhibitor. *Immunology* (2015) *145*(1):34-49. doi:10.1111/imm.12422.
431 32. Lynch, H. E., Goldberg, G. L., Chidgey, A., Van den Brink, M. R., Boyd, R., and Sempowski,
432 G. D. Thymic involution and immune reconstitution. *Trends Immunol* (2009) *30*(7):366-373.
433 doi:10.1016/j.it.2009.04.003.
434 33. Youm, Y. H., Horvath, T. L., Mangelsdorf, D. J., Kliewer, S. A., and Dixit, V. D. Prolongevity
435 hormone FGF21 protects against immune senescence by delaying age-related thymic involution.
436 *Proc Natl Acad Sci U S A* (2016) *113*(4):1026-1031. doi:10.1073/pnas.1514511113.
437 34. Pütz, M. M., Alberini, I., Midgley, C. M., Manini, I., Montomoli, E., and Smith, G. L.
438 Prevalence of antibodies to Vaccinia virus after smallpox vaccination in Italy. *J Gen Virol* (2005)
439 *86*(Pt 11):2955-2960. doi:10.1099/vir.0.81265-0.
440 35. Ben-Yehuda, A., Ehleiter, D., Hu, A. R., and Weksler, M. E. Recombinant vaccinia virus
441 expressing the PR/8 influenza hemagglutinin gene overcomes the impaired immune response and
442 increased susceptibility of old mice to influenza infection. *J Infect Dis* (1993) *168*(2):352-357.
443 36. Albarnaz, J. D., Torres, A. A., and Smith, G. L. Modulating Vaccinia Virus Immunomodulators
444 to Improve Immunological Memory. *Viruses* (2018) *10*(310.3390/v10030101.
445 37. de Freitas, L. F. D., Oliveira, R. P., Miranda, M. C. G., Rocha, R. P., Barbosa-Stancioli, E. F.,
446 Faria, A. M. C. et al. The Virulence of Different Vaccinia Virus Strains Is Directly Proportional
447 to Their Ability To Downmodulate Specific Cell-Mediated Immune Compartments. *J Virol*
448 (2019) *93*(610.1128/JVI.02191-18.
449 38. Ramírez, J. C., Gherardi, M. M., and Esteban, M. Biology of attenuated modified vaccinia virus
450 Ankara recombinant vector in mice: virus fate and activation of B- and T-cell immune responses
451 in comparison with the Western Reserve strain and advantages as a vaccine. *J Virol* (2000)
452 *74*(2):923-933.
453 39. Frasca, D., and Blomberg, B. B. Inflammaging decreases adaptive and innate immune responses
454 in mice and humans. *Biogerontology* (2016) *17*(1):7-19. doi:10.1007/s10522-015-9578-8.
455 40. Bektas, A., Schurman, S. H., Sen, R., and Ferrucci, L. Human T cell immunosenescence and
456 inflammation in aging. *J Leukoc Biol* (2017) *102*(4):977-988. doi:10.1189/jlb.3RI0716-335R.
457 41. Tilstra, J. S., Clauson, C. L., Niedernhofer, L. J., and Robbins, P. D. NF-κB in Aging and
458 Disease. *Aging Dis* (2011) *2*(6):449-465.
459 42. Minciullo, P. L., Catalano, A., Mandraffino, G., Casciaro, M., Crucitti, A., Maltese, G. et al.
460 Inflammaging and Anti-Inflammaging: The Role of Cytokines in Extreme Longevity. *Arch*
461 *Immunol Ther Exp (Warsz)* (2016) *64*(2):111-126. doi:10.1007/s00005-015-0377-3.
462 43. Franceschi, C., Garagnani, P., Vitale, G., Capri, M., and Salvioli, S. Inflammaging and 'Garb-
463 aging'. *Trends Endocrinol Metab* (2017) *28*(3):199-212. doi:10.1016/j.tem.2016.09.005.
464 44. Franceschi, C., Garagnani, P., Parini, P., Giuliani, C., and Santoro, A. Inflammaging: a new
465 immune-metabolic viewpoint for age-related diseases. *Nat Rev Endocrinol* (2018) *14*(10):576-
466 590. doi:10.1038/s41574-018-0059-4.

467 45. Gupta, S., Termini, J. M., Kanagavelu, S., and Stone, G. W. Design of vaccine adjuvants
468 incorporating TNF superfamily ligands and TNF superfamily molecular mimics. *Immunol Res*
469 (2013) *57*(1-3):303-310. doi:10.1007/s12026-013-8443-6.
470 46. Luo, J., Zhang, B., Wu, Y., Tian, Q., Zhao, J., Lyu, Z. et al. Expression of interleukin-6 by a
471 recombinant rabies virus enhances its immunogenicity as a potential vaccine. *Vaccine* (2017)
472 *35*(6):938-944. doi:10.1016/j.vaccine.2016.12.069.
473 47. Chen, G., Lustig, A., and Weng, N. P. T cell aging: a review of the transcriptional changes
474 determined from genome-wide analysis. *Front Immunol* (2013) *4*121.
475 doi:10.3389/fimmu.2013.00121.
476 48. Vescovini, R., Biasini, C., Fagnoni, F. F., Telera, A. R., Zanlari, L., Pedrazzoni, M. et al.
477 Massive load of functional effector CD4+ and CD8+ T cells against cytomegalovirus in very old
478 subjects. *J Immunol* (2007) *179*(6):4283-4291.
479 49. Weng, N. P., Akbar, A. N., and Goronzy, J. CD28(-) T cells: their role in the age-associated
480 decline of immune function. *Trends Immunol* (2009) *30*(7):306-312.
481 doi:10.1016/j.it.2009.03.013.
482 50. Franceschi, C., Salvioli, S., Garagnani, P., de Eguileor, M., Monti, D., and Capri, M.
483 Immunobiography and the Heterogeneity of Immune Responses in the Elderly: A Focus on
484 Inflammaging and Trained Immunity. *Front Immunol* (2017) *8*982.
485 doi:10.3389/fimmu.2017.00982.
486 51. Fulop, T., Larbi, A., Dupuis, G., Le Page, A., Frost, E. H., Cohen, A. A. et al.
487 Immunosenescence and Inflamm-Aging As Two Sides of the Same Coin: Friends or Foes. *Front*
488 *Immunol* (2017) *8*1960. doi:10.3389/fimmu.2017.01960.
489

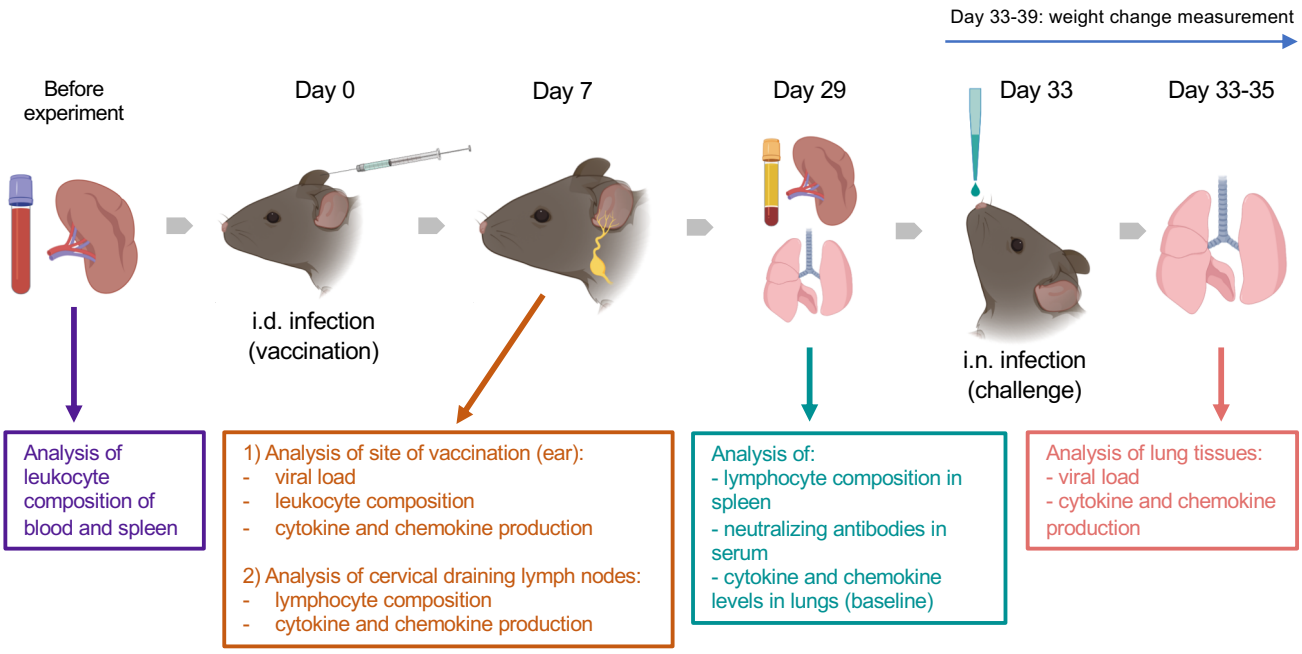

Figure 1. Experimental design. Groups (*n*=4-5) of 7-, 22- and 54-week old C57BL/6 mice were used in the study. Various parameters were measured before and at 7 and 29 d after intradermal (i.d.) infection with 10^4 PFU of VACV WR, as well as following intranasal (i.n.) challenge of immunised or naïve mice with ~10^7 PFU of VACV WR.

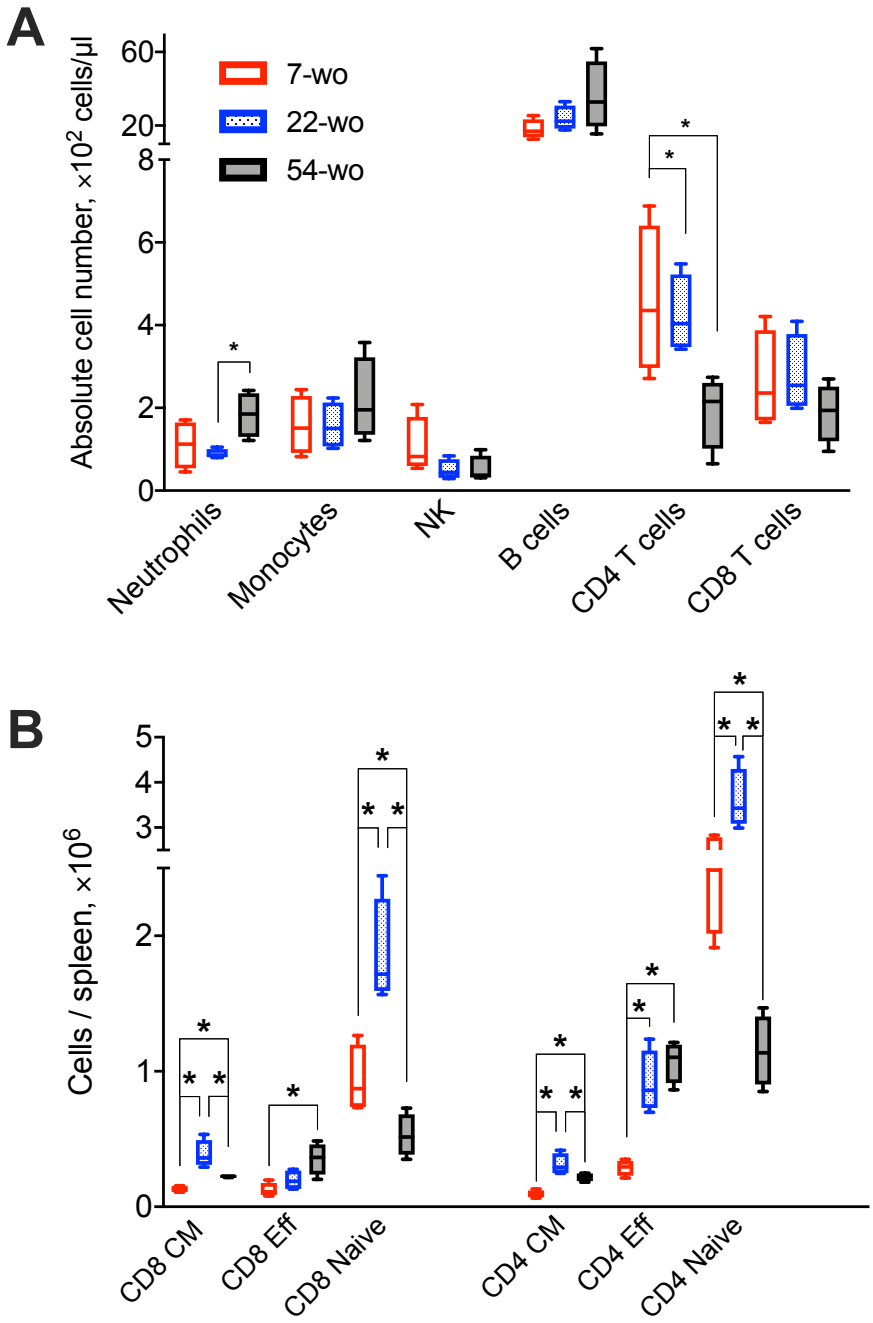

Figure 2. Old mice have decreased numbers of naïve CD8 and CD4 T cells. The absolute numbers of different subpopulation of leukocytes in blood (**A**) and T cells isolated from the spleen (**B**) of 7-, 22- and 54-week old mice (without VACV infection) (4 animals per group). CM, central memory; Eff, effector. P values determined by Mann-Whitney test, * = $p<0.05$.

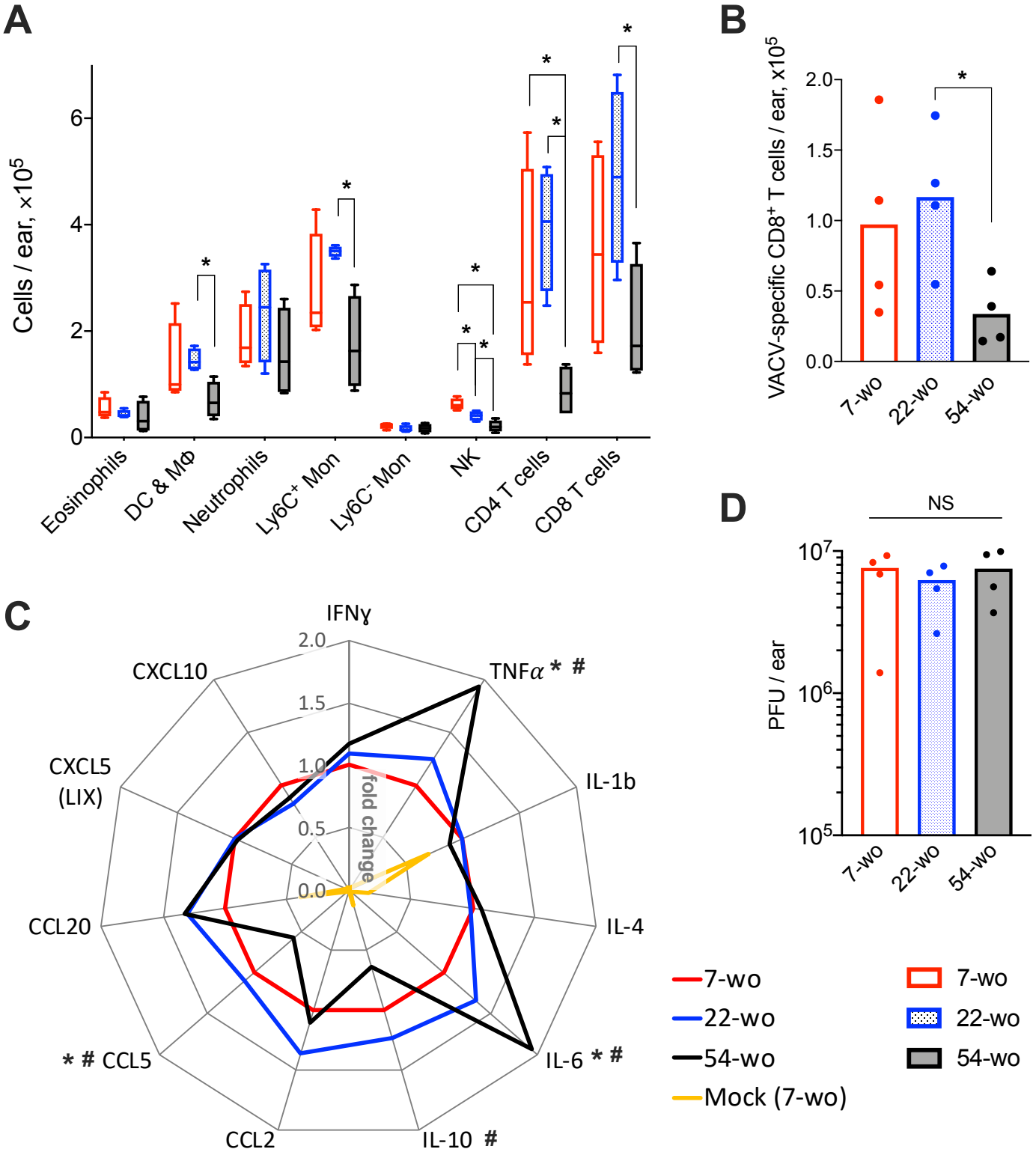

Figure 3. Conservation of the immune response to intradermal infection with VACV across age groups. Ear tissues were collected at 7 d post i.d. infection with VACV or PBS (mock-control) from groups of 7-, 22- and 54-wo mice (*n*=4-5 per group). The absolute numbers of (**A**) different subpopulations of leukocytes and (**B**) VACV-specific CD8 T cells infiltrating ear tissues are shown. DC & MΦ, dendritic cells and macrophages; Mon, monocytes; p values were determined by the Mann-Whitney test, * = p<0.05. (**C**) The levels of cytokines/chemokines detected by multiplex assay (Luminex) in ear tissues are presented as fold change compared with the 7-wo VACV-infected group, which is assigned a value of 1. The means are shown; p values were determined by the Mann-Whitney test, * = p<0.05 between 7- and 54-wo animals. # = p<0.05 between 22- and 54-wo mice. (**D**) Titers of VACV in ear tissues at 7 d post i.d. infection with VACV. PFU, plaque-forming units; NS – non-significant by Mann-Whitney test. The experiment was performed twice and representative data from one experiment are shown.

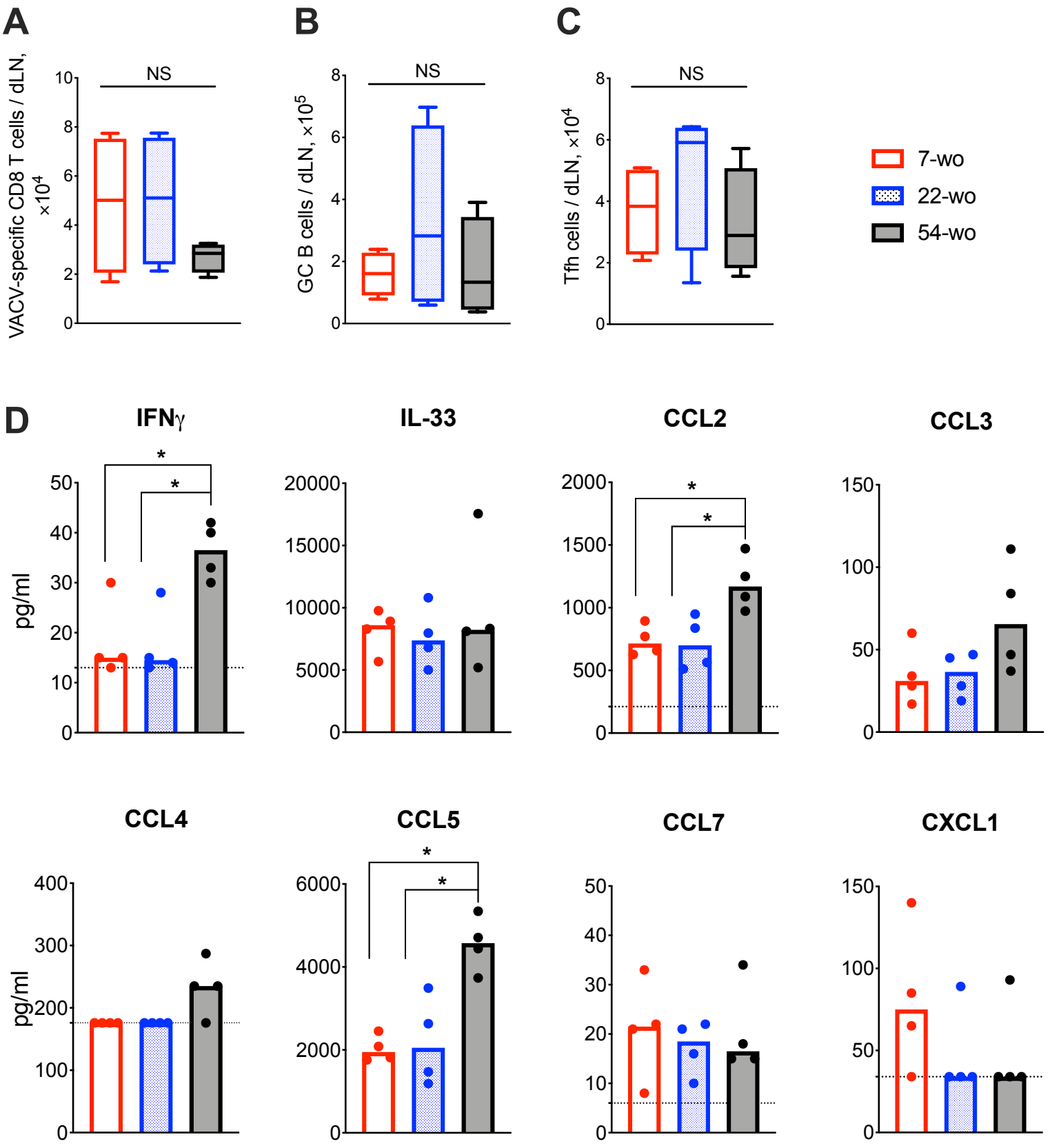

Figure 4. Enhanced cytokine production in the draining lymph nodes (dLN) of 54-wo mice following intradermal infection with VACV. Groups of 7-, 22- and 54-wo mice (*n*=4-5 per group) were infected i.d. with VACV and at 7 d post infection the dLN were collected. The absolute number of (**A**) VACV-specific CD8 T cells, (**B**) germinal center B cells and (**C**) T follicular helper cells were determined by FACS. GC, germinal center; Tfh, T follicular helper; NS, non-significant by Mann-Whitney test. (**D**) The levels of cytokines and chemokines were detected by multiplex assay (Luminex) in cervical dLN from mice treated as above. Medians are shown; dashed lines indicate limit of sensitivity; p values were determined by the Mann-Whitney test, * = $p<0.05$. The experiment was performed twice and representative data from one experiment are shown.

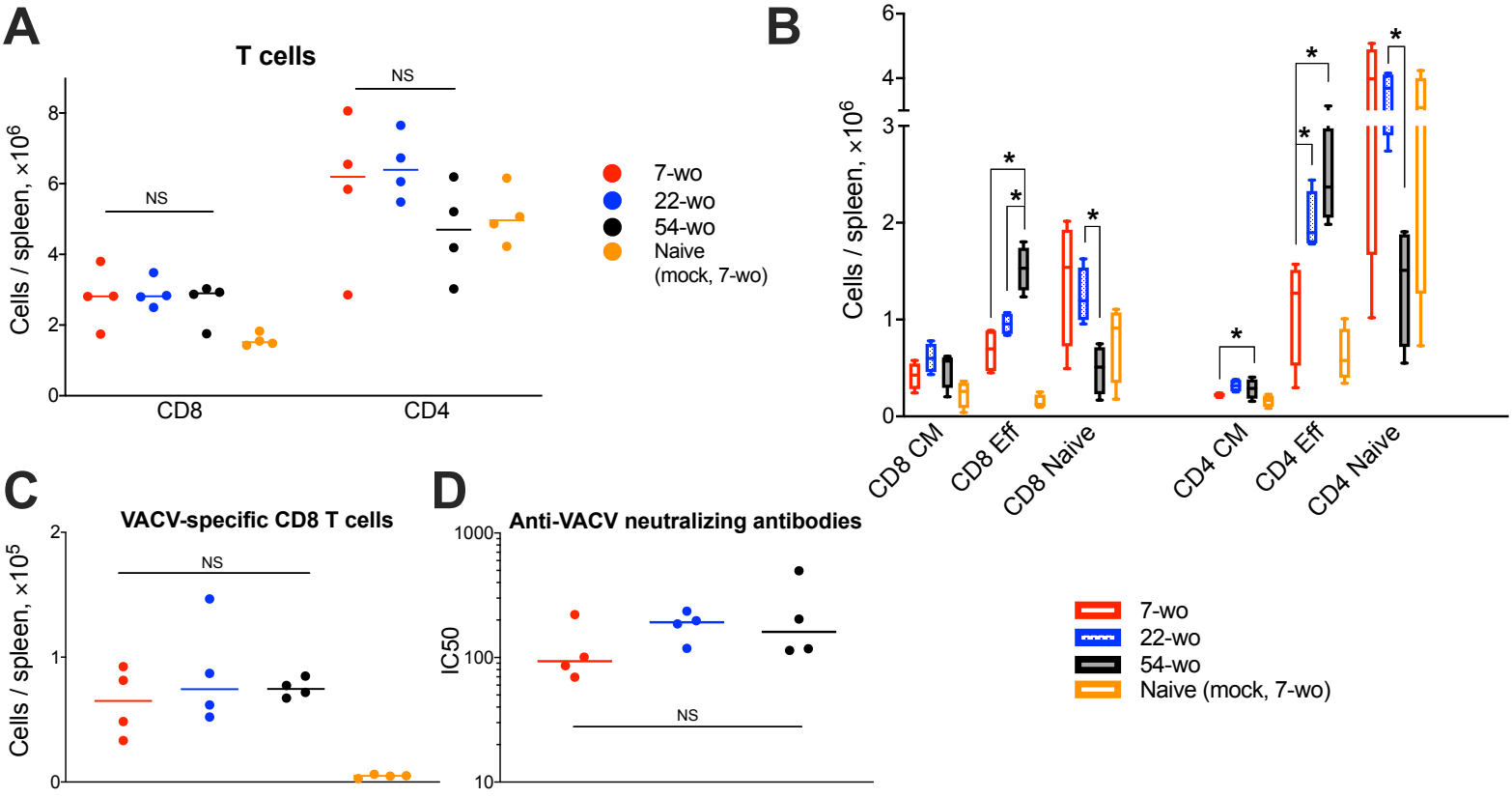

Figure 5. Vaccination with VACV induces a robust adaptive immune response in mice of different ages. Spleens and serum samples were obtained from vaccinated and naïve (mock-vaccinated) mice of different ages at 29 d post i.d. injection (*n*=4-5 animals per group). (**A**) The absolute numbers of total splenic CD8 and CD4 T cells, and (**B**) their subpopulations are shown. Naive, central memory (CM) and effector (Eff). (**C**) Shows VACV-specific CD8 T cells. p values were determined by the Mann-Whitney test, * = $p<0.05$. NS, non-significant. (**D**) Neutralizing antibody responses determined by plaque-reduction neutralization test. IC50, half maximal inhibitory concentration; NS, non-significant by Mann-Whitney test. All experiments were performed twice and representative data from one experiment are shown.

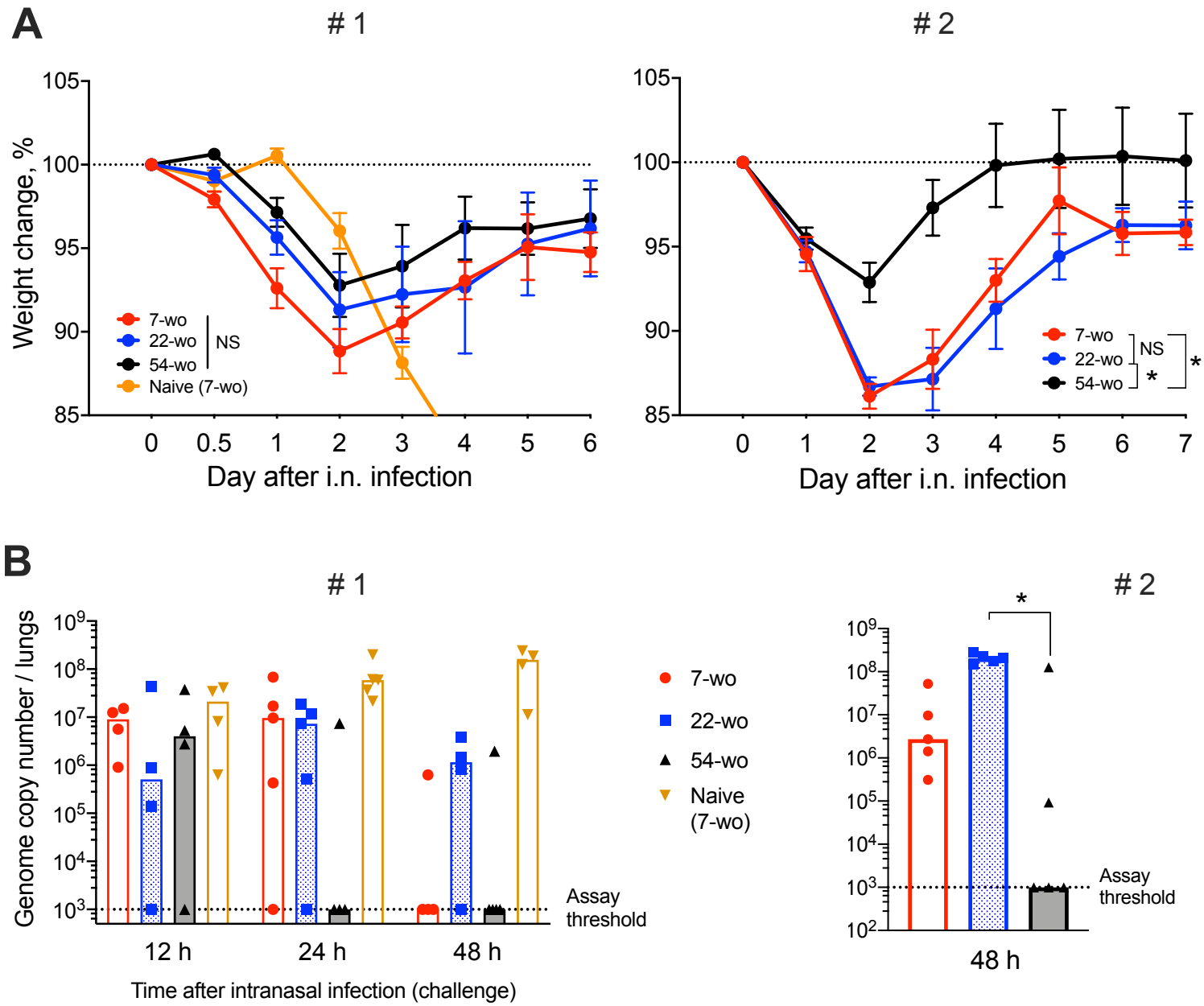

Figure 6. 54-week old mice are better protected against VACV intranasal challenge than mice from younger groups. Groups of 7-, 22- and 54-wo C57BL/6 mice (*n*=4-5 per group) were injected intradermal with 10^4 PFU (per ear, into both ears) of VACV or PBS (mock). These groups were then challenged i.n. with 0.7×10^7 PFU of VACV WR at day 33 post vaccination in experiment #1 (left) and with 1.3×10^7 PFU of VACV WR in experiment #2 (right). (**A**) Body weight changes of mice after intranasal challenge with VACV; within each group, data show a comparison of the weight or each mouse with the weight of the same animal on day zero. The percentages for each group are means with SEM. Statistical analysis by RM ANOVA test. NS, non-significant; * = $p<0.05$. (**B**) Viral genome copy number in both lungs from mice at 12, 24 and 48 h post i.n. challenge were determined by qPCR. Medians are shown.

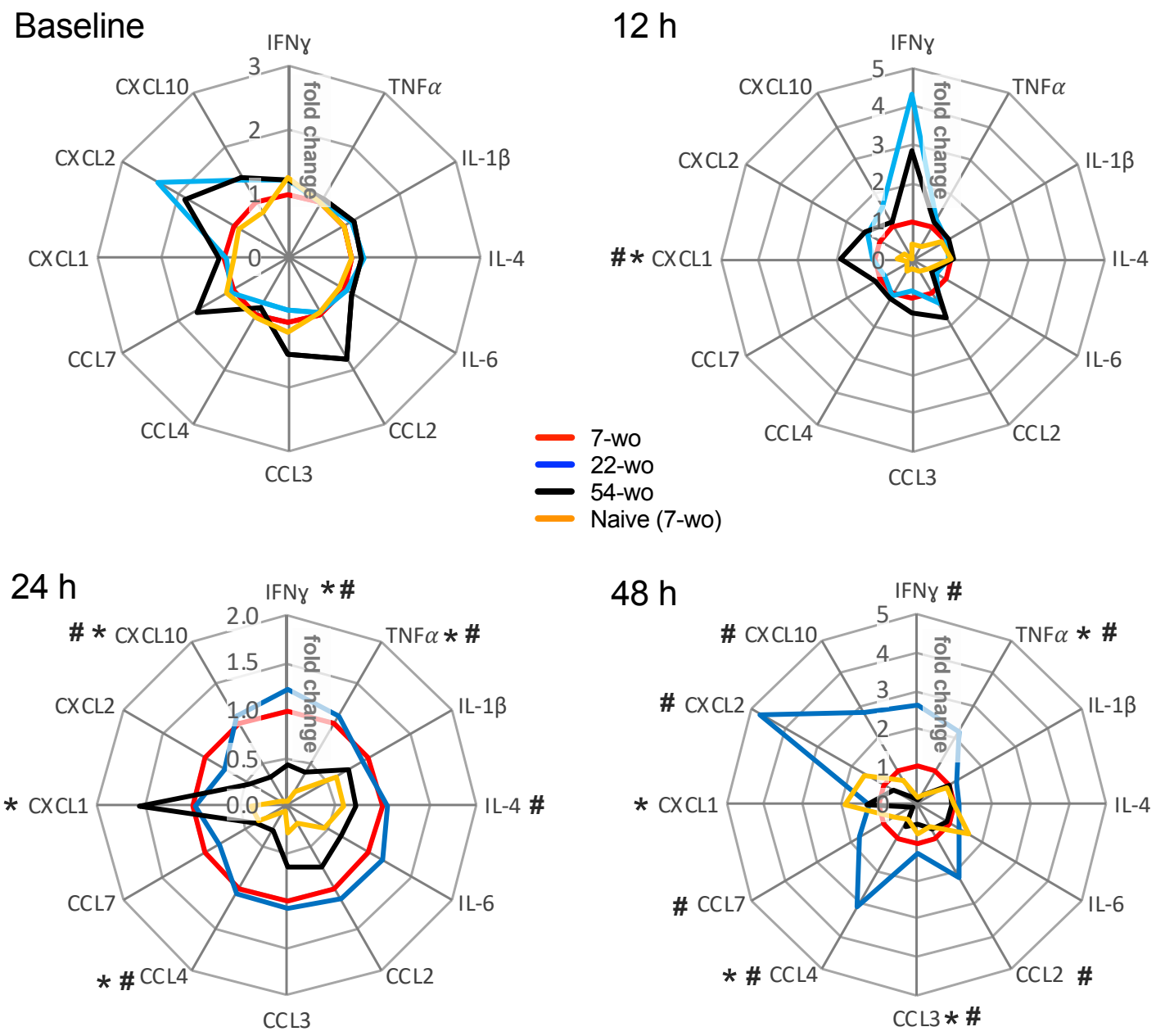

Figure 7. Kinetics of cytokine and chemokine production in lungs after intranasal challenge with VACV correlates with the kinetics of virus clearance. Groups (*n*=4-5) of 7-, 22- and 54-wo C57BL/6 mice were vaccinated i.d. with 10^4 PFU (per ear, into both ears) of VACV or PBS (mock-control). Then 33 d post vaccination animals were challenged i.n. with VACV WR. Lungs of vaccinated and mock-vaccinated mice were collected at 12, 24 and 48 h after challenge. The levels of cytokines and chemokines were measured by multiplex assay (Luminex). Data are shown as the fold change from the vaccinated 7-wo group, which is assigned a value of 1. Means are shown; p values were determined by the Mann-Whitney test. * = $p<0.05$ between 7- and 54-wo animals, # = $p<0.05$ between 22- and 54-wo mice. The experiment was performed twice and data from one representative experiment are shown.