Quantitative Restoration of Immune Defense in Old Animals Determined by Naïve Antigen-Specific CD8 T cell Numbers

Jennifer L. Uhrlaub[1,2*], Mladen Jergović[1,2], Christine M. Bradshaw[1,2], Sandip Sonar[1,2], Christopher P. Coplen[1,2], Jarrod Dudakov[3,4], Kristy O. Murray[5,6], Marion C. Lanteri[7], Michael P. Busch[7], Marcel R. M. van den Brink[8,9], and Janko Nikolich-Žugich[1,2, 3*]

1- Department of Immunobiology, University of Arizona College of Medicine, Tucson, AZ, USA
2- University of Arizona Center on Aging, University of Arizona, College of Medicine, Tucson, Tucson, AZ, USA
3- Program in Immunology, Clinical Research Division, and Immunotherapy Integrated Research Center, Fred Hutchinson Cancer Research Center, Seattle, WA 98109, USA;
4-Department of Immunology, University of Washington, Seattle, WA 98109, USA;
5- Department of Pediatrics, Section of Pediatric Tropical Medicine and National School of Tropical Medicine, Baylor College of Medicine, Houston, TX 77030, USA
6-William T. Shearer Center for Human Immunobiology, Texas Children's Hospital, Houston, TX 77030, USA
7- Blood Systems Research Institute, Vitalant Research Institute, San Francisco, CA, 94118, USA
8- Department of Medicine and Immunology Program, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA
9-Immunology Program, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA

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30 *Address correspondence to Janko Nikolich-Žugich at nikolich@arizona.edu or Jennifer
31 Uhrlaub at juhrlaub@email.arizona.edu or by mail at P.O. Box 245221, Department of
32 Immunobiology, University of Arizona College of Medicine-Tucson, 1501 N. Campbell
33 Ave., Tucson, AZ 85724.
34

SUMMARY

Older humans and animals often exhibit reduced immune responses to infection and vaccination, and this often directly correlates to the numbers and frequency of naïve T (Tn) cells. We found such a correlation between reduced numbers of blood CD8+ Tn cells and severe clinical outcomes of WNV in both humans naturally exposed to, and mice experimentally infected with, West Nile virus (WNV).

To examine possible causality, we sought to increase the number of CD8 Tn cells by treating C57BL/6 mice with IL-7 complexes (IL-7C, anti-IL-7 mAb bound to IL-7), shown previously to efficiently increase peripheral T cell numbers by homeostatic proliferation. T cells underwent robust expansion following IL-7C administration to old mice increasing the number of total T cells (>four-fold) and NS4b:H-2D^{b}-restricted antigen-specific CD8 T cells (two-fold). This improved the numbers of NS4b-specific CD8 T cells detected at the peak of the response against WNV, but not survival in the face of WNV challenge. IL-7C treated old animals also showed no improvement in WNV-specific effector immunity (neutralizing antibody and *in vivo* T cell cytotoxicity). To test quantitative limits to which CD8 Tn cell restoration could improve protective immunity, we transferred graded doses of Ag-specific precursors into old mice and showed that injection of 5,400 (but not of 1,800 or 600) adult naïve WNV-specific CD8 T cells significantly increased survival after WNV. These results set quantitative limits to the level of Tn reconstitution necessary to improve immune defense in older organisms and are discussed in light of targets of immune reconstitution.

Introduction

Prolonged T cell suppression after myeloablative conditioning is a major clinical problem with considerable clinical implications for therapies such as hematopoietic cell transplantation. Consequently, much work has gone into developing therapies that can boost T cell reconstitution, with varying degrees of success, most of which focus on stimulating thymic function (Granadier, Iovino, Kinsella, & Dudakov, 2021).

Aging is also accompanied by a variable degree of lymphopenia (Groarke & Young, 2019) due to combined effects of early-life thymic involution and late-life defects in lymphocyte maintenance in secondary lymphoid organs (SLO) (Nikolich-Žugich, 2018). This lymphopenia in the last third of life correlates to decreased ability of the immune system to respond to infection and vaccination (Nikolich-Žugich, 2018). Age-related lymphopenia affects all lymphocytes over time, but is drastically evident for naïve T (Tn) cells that reside in SLO such as the spleen and lymph nodes (LN) (Thompson, Smithey, Surh, & Nikolich-Žugich, 2017). Tn cells are the most diverse T cell subset, in charge of defending the organism against new infection, transforming Tn cells (CD62LhiCD44lo in mice) specific for the invading infection into effector (Te, CD62LloCD44hi) and subsequently into memory (Tem – effector memory, also CD62LloCD44hi; and Tcm- central memory T cells, CD62LloCD44hiCD49hi). With aging, disturbances in SLO maintenance of Tn cells leads to the accumulation of antigen-independent memory cells (virtual memory; Tvm, CD62LloCD44hiCD49dlo) impacting their phenotype and function and diminishing their ability to protect the organism against infection. It is well established that both CD4 and CD8 Tn cells diminish in numbers by the last third of life in mice (Thompson et al., 2017), non-human primates (Cicin-Sain et al., 2007; Cicin-Sain et al., 2010), and humans ((Groarke & Young, 2019; Wertheimer et al., 2014), albeit with slightly different kinetics depending on the species and compartment sampled. Associations between Tn lymphopenia and decreased immunity in old age were reported. Studies have shown that the CD8 response to live single-cycle poxvirus vaccination directly correlates to the CD8 Tn numbers in non-human primates (Cicin-Sain et al., 2010). Correlations were also found in a study of long-term repeated apheresis subjects, where 15-20% of PBMC were

89 retained by the apheresis machine, and there was a correlation between the number of
90 donations, lymphopenia and reactivation of persistent herpesviruses (e.g. Varicella-
91 Zoster virus) (Zhao et al., 2021). Moreover, severity of COVID-19 in older adults has
92 been shown to correlate to reduced numbers of CD4 and CD8 Tn cells (Rydyznski
93 Moderbacher et al., 2020). A limitation of these studies is that they did not
94 independently control for aging, and, in most cases, baseline samples were not
95 available to answer whether this was a preexisting condition, a virus-induced
96 lymphodepletion, or a combination of both. All of these intriguing correlative studies
97 raise the possibility of causal association between the (age-related) Tn lymphopenia
98 and functional immunity decline with aging.

99 Reconstitution of T lymphopoiesis and maintenance in older organisms, also
100 known as immune rejuvenation, has been a highly sought goal in regenerative
101 immunotherapy. Success in that regard has been partial, with no clear translation into
102 an accepted medical protocol. Several treatments in humans and mice transiently
103 (usually up to 45-60 days in mice) reawaken thymic production of Tn cells, including
104 surgical (T. S. P. Heng et al., 2005) and various forms of chemical (Sutherland et al.,
105 2005) sex steroid ablation (SSA), and treatment with various growth factors, including
106 the human growth hormone (Taub, Murphy, & Longo, 2010) and the keratinocyte growth
107 factor (KGF) (Min et al., 2007). Functional evidence that immunity was improved
108 following such treatments in *bona fide* old mice was less abundant (T. S. Heng et al.,
109 2012; Min et al., 2007) and did not involve testing of immune protection using lethal
110 infectious challenge. We have recently discovered that KGF- and pharmacological SSA-
111 induced thymic rebound readily led to restoration, followed by a decline over time, of
112 both thymic cellularity and of the influx of recent thymic emigrants (RTE) into the blood
113 (Thompson et al., 2019) in old mice. Such RTE, however, sparsely populated old
114 spleens and lymph nodes (LN), and treated mice showed no improvement in survival
115 following challenge with West Nile virus (Thompson et al., 2019). That led to
116 investigations of LN involution with age, and its role in immune senescence and
117 reduced immune defense with aging.

118 As part of these studies, we sought to conclusively address the role of age-linked
119 lymphopenia in reduced immune defense with aging. Here, we first used IL-7
120 complexes (IL-7C, IL-7 bound to an anti-IL-7 Ab, (Boyman, Ramsey, Kim, Sprent, &
121 Surh, 2008) to increase lymph node cellularity by means of homeostatic proliferation
122 and found a doubling of CD8 Tn precursors specific for the immunodominant NS4b
123 epitope of WNV. However, most of these cells were no longer true naïve CD8 cells, but
124 rather antigen-independent Tvm. Upon challenge with WNV, we found no improvement
125 of immune protection, and no increase in either CD8 effector T cells or their cytotoxic
126 function, consistent with our prior findings that Tvm cells do not proliferate well upon
127 stimulation. These mice also did not produce robust anti-WNV antibodies, suggesting
128 that CD4 and/or B cell defects were not remedied either. To evaluate whether an
129 adequate reconstitution of true naïve cells could protect old mice against lethal
130 challenge, we transferred graded amounts of naïve CD8+ NS4b/Db-specific TCR
131 transgenic cells. We found that numbers of Ag-specific Tn cells needed to confer
132 protection to old mice were slightly above the numbers of Ag-specific Tn precursors
133 present in young adult mice, suggesting that both the numbers and the quality of
134 precursors are important in reconstitution. These results establish the numerical limits
135 of CD8 Tn reconstitution and are discussed in light of strategies for immune
136 reconstitution with aging.

137

138

139 **RESULTS**

140 *Older age and reduced naïve T cell numbers are associated with WNV disease severity.*

141 Previously unencountered and emerging viral infections often cause increased
142 disease severity and mortality in older populations. Indeed, patients who have
143 experienced the most severe symptoms caused by WNV are significantly older than
144 those who had milder disease (Campbell, Marfin, Lanciotti, & Gubler, 2002) and Figure
145 1A, and similar findings have been made in other emerging infectious diseases
146 including SARS-CoV, Chikungunya virus, and SARS-CoV-2. Consistent with our prior
147 data in general geriatric populations (Wertheimer et al., 2014) our cohort of WNV-
148 exposed participants also exhibited significantly lower levels of circulating CD8, but not
149 CD4, Tn cells (Figure 1B, C). In a retrospective study, where patient samples were
150 collected after disease recovery, it is impossible to determine whether this is a cause,
151 an epiphenomenon, or an effect, of their severe infection. To examine the nature of
152 associations between age, Tn cell deficit, and severe WNV disease, we examined this
153 issue in an established mouse model of age-related susceptibility to WNV (Brien,
154 Uhrlaub, Hirsch, Wiley, & Nikolich-Zugich, 2009).
155

156 *The murine model recapitulates the findings in humans.*

157 It is well established that the naïve T cell compartment contracts with age and
158 becomes dominated by T cells with a memory phenotype (Zhang, Weyand, & Goronzy,
159 2021). A direct correlation with disease severity, however, has not been conclusively
160 established. To determine if our WNV mouse model is valid to interrogate this
161 association, we measured the number of circulating CD8 Tn cells in WNV-infected adult
162 (3-5 month old) and old (19-22 month old) mice on day 7 p.i. (Figure 2A). We found that
163 mice succumbing to WNV had lower numbers of circulating naïve CD8 and CD4 T cells
164 as compared to those that survived (Figure 2B, C). This disparity held regardless of
165 age, despite the fact that old mice exhibit reduced numbers of Tn cells overall (Figure
166 2B, C). These data support the possibility that a numeric advantage in naïve T cells is
167 directly linked to WNV resistance regardless of age. These results are also consistent
168 with the human data from Figure 1 with the exception that there is also a reduced

169 number of naïve CD4 T cells in susceptible mice. Of note, there was overlap in CD8 Tn
170 numbers between humans with severe and asymptomatic WNV disease, as well as
171 between mice that succumbed and survived WNV infection. This suggests that the
172 numbers of CD8 Tn cells are not the only determinant of increased WNV resistance,
173 consistent with the extensive literature on the multipronged nature of immune defense
174 against WNV (Brien et al., 2009; Purtha et al., 2007; Richner et al., 2015). The overlap
175 in the human and mouse data provided confidence that the mouse model can be used
176 to examine whether, and to what extent, reversing age-induced lymphopenia and,
177 specifically, a deficit in Tn cells, may afford improved immune protection against WNV.
178
179 *IL-7 complex treatment increases the number of circulating Tn cells*
180 IL-7 is a pleiotropic cytokine that provides an indispensable homeostatic survival
181 signal to Tn cells, and that drives Tn and other T cell subsets into homeostatic
182 proliferation under the conditions of lymphopenia. Delivered *in vivo* as a cytokine-
183 antibody complex, IL-7C has been shown to drive naïve T cell proliferation and increase
184 thymopoeisis in unmanipulated as well as lymphopenic mice (Tan et al., 2002). Despite
185 its therapeutic impact in adult mice, this model has not been rigorously interrogated for
186 reversal of age-related lymphopenia and reduced susceptibility to infection. We treated
187 old mice with IL-7C (3 i.p. injections, every other day), and sacrificed them on days 7,
188 10, and 14 post-first IL-7C treatment to assess total spleen and LN content (Figure 3A).
189 Total cellularity of secondary lymphoid tissues (spleen and LNs) remained elevated
190 through day 14 post-first treatment (Figure 3B), an expansion mirrored by the numbers
191 of CD4 and CD8 T cells (Figure 3C, D). Additionally, numbers of CD4 and CD8 Tn cells
192 in treated old mice were no longer statistically significantly reduced relative to those in
193 adult mice (Figure 3E, F). We also discovered an expansion of CD8, but not CD4, T
194 cells exhibiting a Tvm phenotype, whereas in the CD4 compartment the increase was
195 driven mostly by Tcm cells (Figure 3G, H). To determine whether the expansion of T
196 cells by IL-7C also included CD8 T cells specific for the immunodominant epitope of
197 WNV, $NS4b_{2488}$, we enumerated the number of these CD8 T cell precursors on day 13
198 post-treatment in unimmunized mice (Figure 3I). Similar to our prior work with other
199 immunodominant CD8 epitopes (Rudd, Venturi, Davenport, & Nikolich-Zugich, 2011;

200 Smithey, Li, Venturi, Davenport, & Nikolich-Žugich, 2012), we found a marked ~62%
201 loss of NS4b-specific CD8 precursors in old animals compared to adult counterparts
202 (Figure 3F). We found that IL-7C treatment led to a numerical increase in CD8
203 precursors specific for the WNV NS4b immunodominant epitope that, on average, no
204 longer statistically differed from that in adult animals (Figure 3I). The response between
205 individual animals, however, was heterogenous, with many animals exhibiting no
206 increase, and others exhibiting up to a two-fold increase in precursor numbers. The
207 relative improvement in total T cell numbers, from days 10 to 14, including an overall
208 increase in WNV-specific CD8 precursors on day 13 following IL-7C treatment
209 suggested that functional immunity could be improved within this temporal window,
210 because lymphopenia in old mice was measurably reduced and residual IL-7C (if any)
211 did not cause further T cell proliferation in secondary lymphoid tissues.
212
213 ***IL-7c treated old mice gain numbers, but not function, of WNV-specific CD8 T cell***
214 ***responses***
215
216 To conclusively test whether this increase in T cell numbers is sufficient to overcome
217 infectious disease susceptibility, we challenged IL-7C treated mice with WNV. Based
218 on the above kinetics of IL-7C action, we chose day 13 as an optimal time point for
219 challenge, reasoning that the mice would benefit from an expanded T cell pool at the
220 time when IL-7C is no longer driving further proliferation of T cells, and should not
221 influence the development of a functional immune response to WNV. Adult (3-4
222 months); old (19-22 months); and IL-7C treated old (19-22 months) mice were
223 challenged with 1000pfu of WNV and followed for WNV-specific CD8 T cell responses
224 and survival (Figure 4A). On day 7 post-infection, old mice treated with IL-7C showed a
225 3-fold improvement in the number of WNV NS4b-specific CD8 T cells measured in the
226 blood (Figure 4B). However, this improvement failed to translate to better survival
227 (Figure 4C), despite the fact that we observed roughly equivalent numbers of WNV-
228 specific CD8 T cells in old IL-7C treated mice compared to adult unmanipulated mice.
229 When we segregated each experimental group into animals that died or survived WNV
230 challenge, we found that those that survived again exhibited a numeric advantage in

231 WNV-specific cells over those that succumbed (Figure 4D). The effect, however, was
232 not robust enough to ensure that the group, as a whole, survives better than their
233 untreated counterparts.
234
235 Multiple effector mechanisms of adaptive immunity must synergize to clear WNV
236 infection. These include potent neutralizing antibodies as well as CD4 and CD8
237 cytotoxic T cell effector function. To mechanistically determine whether any functional
238 improvement in immunity was restored by IL-7C treatment we interrogated the immune
239 response in IL7-C treated old mice in a step-wise manner. IL-7C have the potential to
240 enhance B cell lymphopoiesis, and to thereby increase B cell numbers, although they
241 are not expected to directly affect the mature B cell compartment (Boyman et al., 2008).
242 Previous studies have reported that IL-7C co-delivered with immunization can boost
243 humoral immune responses through enhanced generation of germinal center B cells
244 and Tfh cells (Seo et al., 2014). Therefore, we first analyzed neutralizing antibody titers
245 at day 7 post-WNV infection as a proxy for generalized improvement in B cell
246 responses. We detected no improvement in WNV neutralization by sera of old mice
247 treated with IL7-C relative to untreated controls (Figure 4E). We next examined
248 whether WNV-specific CD8 or CD4 T cells exhibited improved killing function following
249 IL-7C treatment by measuring cytotoxicity in vivo (Figure 4G, H). We included targets
250 specific for one WNV-specific CD4 immunodominant epitope, ENV_{641}, in this assay
251 since CD4 T cells are known to mount cytotoxic responses against WNV in adult mice
252 and data about their specific contribution to viral clearance with age is limited (Brien,
253 Uhrlaub, & Nikolich-Zugich, 2008). We found that, despite numeric increases in
254 circulating NS4b-specific CD8 T cells, IL-7C treated old mice did not have improved
255 cytotoxic capacity to clear $NS4b_{2488}$ peptide-loaded targets as compared to untreated
256 old mice (Figure 4G). There was also no improvement in killing of the ENV_{641}-loaded
257 targets (Figure 4H), consistent with the fact that we saw no increase in CD4 Tn cell
258 numbers (Fig. 3E). These data indicate an overall lack of improvement in cytotoxic
259 function in old mice treated with IL-7C. At face value, these results suggest that
260 numerical improvement in CD8 and CD4 T cells afforded by IL-7C treatment is not
261 sufficient to improve functional immunity.

262 To investigate why a numeric increase in antigen-specific precursors was not
263 able to restore T cell responses we considered the possibility that the expansion
264 induced via IL7-C treatment may push the cells into an exhausted state, impairing their
265 ability to participate in the WNV response. T cells with an 'exhausted' phenotype are
266 typically seen in the course of chronic infections and cancer (Collier et al., 2021; Reiner,
267 2021). The number of divisions and other factors required to induce this state are not
268 fully understood. To investigate whether homeostatic proliferation driven by IL-7C
269 treatment increases the proportion of responding WNV-specific T cells with an
270 exhausted phenotype we measured exhaustion markers in both adult and old mice
271 treated with IL7-C on day 7 of WNV. We included an adult cohort in these experiments
272 to dissociate aging from treatment-associated phenotypes. Our data confirm that a
273 higher percentage of WNV-specific CD8 T cells express PD-1, LAG-3, or 2B4
274 regardless of whether they have been treated with IL7-C (Figure 5A, B, C). The
275 expression of these molecules could contribute to their poor cytotoxic function, and
276 overall lack of improvement in survival, experienced by the old animals. These data
277 suggest that the homeostatic proliferation induced by IL7-C treatment is not *per se*
278 pushing T cells into an exhausted state. Rather, consistent with some reports (Decman
279 et al., 2012) we hypothesize that the age of the animal may have already determined
280 the phenotype of the available T cell pool and this state is maintained through the
281 expansion initiated by IL7-C treatment to deleterious effect.
282 However, the fact that a similar increase in precursor numbers induced by IL-7C
283 treatment did not lead to either improved immunity or improved cytotoxicity, suggested
284 that some other property of reconstituted cells may be of critical importance. The
285 differences between old mice treated with IL-7C and those receiving adoptively
286 transferred cells was that the former mounted a response from endogenous, old
287 precursors, and that these precursors were driven into homeostatic proliferation by IL-
288 7C; the latter received naïve, unperturbed NS4b-specific precursors from adult donors.
289 Both the age and the immediate prior proliferation could have made a difference in their
290 responses. To directly address whether an increase in CD8 Tn precursors specific for
291 an invading pathogen can overcome the age-related defects in immune defense and
292 afford improved survival to old mice, we performed adoptive transfer experiments. We

293 transferred graded levels of WNV-specific CD8 T cells (NS4b$_{2488}$ specific, referred to as
294 WNV-I (Aguilar-Valenzuela et al., 2018) the day before challenge into old mice to boost
295 the number of precursors available to respond. These transgenic T cells have a naïve
296 phenotype at time of transfer and are not yet primed or cytotoxic. After challenge, the
297 transferred cells quantitatively restored the WNV-specific CD8 T cell numbers measured
298 in the blood on day 7 post-infection (Figure 6A).
299 More importantly, in these experiments, we were able to significantly improve
300 survival in old mice following WNV challenge with 5,400 transferred WNV-specific CD8+
301 Tn cells; but not with the two lower transfer boluses of 1,800 and 600 cells (Figure 6A).
302 To put these numbers in the context of physiological precursor numbers available
303 to old and adult animals, we determined the efficacy of engraftment of transferred cells.
304 Some estimates of transfer efficiency posit that only about 10% of the transferred T cells
305 engraft in i.v. injected mice (Blattman et al., 2002; Moon et al., 2009). We enumerated
306 the total number of NS4b-specific CD8 T cells that were either endogenous (CD45.2) or
307 transgenic (CD45.1). Indeed, our experiments confirmed that 24h after transfer, we
308 could recover between 12-15% of transgenic cells (Supp. Fig 1). This meant that a dose
309 of 5,400 transferred cells reconstituted about 650-810 NS4b precursors, that were
310 effectively capable to significantly protect the old animal against WNV with survival not
311 significantly different from untreated adult mice. Of interest, an adult mouse contains
312 500-700 NS4b-specific CD8+ precursors. Importantly, old mice that received 5400
313 WNV-I Tg CD8 T cells exhibited improved cytotoxic function as measured by increased
314 killing (Figure 5C). This offers a plausible mechanism for improved survival of this group
315 (Figure 6B). We conclude that restoring the precursor numbers of an immunodominant
316 CD8 Tn specificity to adult levels, and with cells that have a Tn phenotype, may be
317 enough to confer a significant degree of protection to an old mouse against WNV,
318 despite concurrent defects in many other arms of the immune response.
319
320 **DISCUSSION**
321
322 Immune therapies that seek to overcome age-induced lymphopenia are an
323 attractive strategy to reduce susceptibility to infectious diseases in older adults.

324 Conceptually, increasing the total cellular resources of the immune system, regardless
325 of antigen specificity, would provide the best solution for broad-based protection against
326 diverse challenges. IL-7C treatment has been shown to increase peripheral T cell
327 numbers even in the absence of thymic output (Boyman et al., 2008). In this work, we
328 formally demonstrated that IL-7C treatment can transiently increase T cell numbers in
329 old mice that have suffered substantial thymic involution, predominantly due to an
330 expansion of virtual central memory Tvm phenotype cells. We found significant, but
331 variable, expansion of the numbers of CD8 precursors specific for WNV, and an overall
332 increase in both CD8 and CD4 T cell numbers. However, these expanded cells did not
333 protect mice from WNV infection, and we could find no evidence that the cytotoxic
334 function of either CD8 or CD4 T cells was improved in IL-7C treated old mice. While we
335 did not enumerate B cells, their protective responses were similarly flat, with no
336 improvement in neutralization capacity, despite an increase in total numbers of CD4 T
337 cells. Therefore, under our experimental conditions, IL-7C treatment showed no
338 appreciable immune protection benefit to the old mice, similar to our prior studies with
339 unconjugated IL-7 in non-human primates (Okoye et al., 2015).
340 Given that protection against WNV normally requires a coordinated and multi-
341 faceted response, it may not be surprising that the increase in T cells alone cannot
342 augment protection. It was more puzzling, however, that we could find no evidence of
343 improved responses (or any responses) from the numerically increased T cell pool. To
344 further explore this question and test whether we could improve protection against WNV
345 by boosting a single arm of protective immunity, we performed adoptive transfer of
346 graded numbers of transgenic CD8 T cells. We demonstrated that sufficient numbers of
347 transferred cells (approximately equivalent to the precursor content of a single adult
348 mouse) were able to significantly improve survival, albeit not to restore it to the levels of
349 adult mice. These results begin to provide quantitative targets of CD8 T cell
350 reconstitution, and also provide a proof-of-principle that the aged immune system can
351 coordinate an effective immune response if the proper T cell resources are provided.
352 One key unanswered question remained regarding the relationship between
353 precursor T cell numbers and their quality. Specifically, adoptively transferred old mice
354 contained similar numbers of overall precursors relative to the IL-7C treated old mice,

355 suggesting that somehow the quality of the two precursor populations was different. To
356 address the potential differences, we considered prior work from ours and other groups
357 on age-related changes in Tn cell maintenance. Specifically, with aging, an increasing
358 number of Tn cells changes their maintenance pattern and become Tvm (Chiu, Martin,
359 Stolberg, & Chensue, 2013; Decman et al., 2012; Rudd et al., 2011) to the point that
360 most of the central memory phenotype cells in SPF mice are of Tvm origin. While work
361 from other groups (Lee, Hamilton, Akue, Hogquist, & Jameson, 2013) and us
362 (Renkema, Li, Wu, Smithey, & Nikolich-Žugich, 2014) demonstrated that Tvm cells in
363 young adult mice exhibit strong proliferation and enhanced effector function relative to
364 Tn cells, we have also shown that with age Tvm cells exhibit reduced proliferative
365 response to cognate antigen (Renkema et al., 2014). By contrast, the response to
366 homeostatic cytokines remains preserved in Tvm cells. Additional studies will be
367 necessary to directly compare in vivo antiviral function and protective capacity of Tvm
368 and Tn cells of different ages with and without prior expansion by IL-7C and similar
369 treatments. That notwithstanding, the work presented here places quantitative limits on
370 desirable levels of immune reconstitution and opens provocative questions about their
371 quality, that remain to be answered in the future.
372

373 **EXPERIMENTAL PROCEDURES**

374

375 Study Subjects and Blood Samples

376 This study was approved by the Institutional Review Boards at the University of Arizona
377 (Tucson, AZ; # 080000673; currently #2102460536), the Oregon Health and Science
378 University (Portland, OR; # IRB00003007); the University of Texas Health Science
379 Center at Houston, TX; and the Blood Systems Research Institute, San Francisco, CA.
380 – presently Vitalant Research Institute. Exclusion criteria included known
381 immunosuppressive pathology, stroke, cancer, or use of steroids within the last 5 years.
382 WNV-infected donors were enrolled by the University of Texas at Houston, 2006–2009
383 or Blood Systems Research Institute (BSRI), 2009 – 2011. Samples, demographics and
384 symptoms data were collected and analyzed as previously described (Lanteri et al.,
385 2014) after the subjects provided an informed consent approved by the UCSF
386 Committee on Human Research (protocol #10-01255) or the Committee for the
387 Protection of Human Subjects at the University of Texas Health Science Center at
388 Houston (HSC-SPH-03-039), respectively. Blood was drawn into heparinized
389 Vacutainer CPT tubes (BD Bioscience) and processed fresh at respective sites per
390 manufacturer's recommendations to isolate peripheral blood mononuclear cells (PBMC)
391 and plasma; K2-EDTA tubes were used to determine complete blood counts. PBMCs
392 were frozen in 90% fetal bovine serum (FBS) and 10% DMSO.

393

394 Flow Cytometry – human samples

395 Frozen PBMCs were thawed in RPMI medium supplemented with 10% FBS, penicillin
396 and streptomycin in the presence of DNAse (Millipore Sigma) then rested overnight in
397 X-Vivo medium (Lonza) supplemented with 5% human male AB serum. 1–3 $\times 10^6$
398 PBMC were stained with a saturating concentration of the following antibodies: CD3
399 AF700 (clone SP34-2, BD Biosciences), CD4 eFluor450 (clone OKT4, eBioscience),
400 CD8β ECD (2ST8.5H7, Beckman Coulter), CD95 APC (clone DX2, BD Biosciences),
401 and CD28 PercpCy5.5 (clone CD28.2, BioLegend) in 1XPBS, 2% FBS and next
402 incubated with LIVE/DEAD® Fixable Dead Cell Stain (ThermoFisher Sci) , both for 30

403 min at 4°C. Flow cytometry acquisition was performed on a Biosciences Fortessa and
404 analyzed using FlowJo software (Tree Star).
405
406 Mice
407 Mouse experiments were carried out in strict accordance with the recommendations in
408 the Guide for the Care and Use of Laboratory Animals of the National Institutes of
409 Health. Protocols were approved by the Institutional Animal Care and Use Committee at
410 the University of Arizona (IACUC 19-580, PHS Assurance No. A-3248-01. Old (19-22
411 months) and adult (3-5 months) were obtained from the National Institute on Aging
412 Rodent Resource or Jax laboratories. Mice in each experiment were age matched
413 within 4 weeks. WNV-I mice were obtained from Drs Jason Netland and. Michael J.
414 Bevan (Aguilar-Valenzuela et al., 2018). Footpad (f.p.) injections and retro-orbital
415 intravenous cell transfers were performed under isoflurane anesthesia. IL-7 cytokine
416 was obtained from the Biological Resources Branch (BRB), Developmental
417 Therapeutics Program (DTP), Division of Cancer Treatment and Diagnosis (DCTD),
418 National Cancer Institute (NCI). IL-7R antibody (clone M25) was purchased from
419 BioXcell. 0.59ug of IL-7 was complexed with 2.9ug of M25 antibody for 30 min at RT
420 then administered via intraperitoneal injection in 200uL USP saline on days 1, 3, and 5
421 of the experiment. Euthanasia was performed by isoflurane overdose or cervical
422 dislocation.
423
424 Virus
425 West Nile virus NY 385–99, isolated from the liver of a snowy owl, and in its second
426 passage, was a kind gift from Dr. Robert Tesh (University of Texas Medical Branch at
427 Galveston, Galveston, TX). Virus was prepared as previously described [REF: Uhrlaub
428 et al., 2011]. Mice were infected by footpad (f.p.) route with 1,000 pfu WNV/50 µl/mouse
429 and monitored for survival until 45 days p.i.
430
431 Tissue harvest, cell counts, flow cytometry, neutralization titers - mice
432 Blood was collected via retro-orbital route and subjected to hypotonic lysis of red blood
433 cells. Spleen and peripheral lymph nodes (inguinal, brachial, axillary, and cervical) were

434 collected and disassociated over 40uM mesh. Cells were resuspended and stained
435 overnight at 4°C in a saturating concentration of antibodies against CD49d (9C10,
436 MFR4.B), CD44 (IM7), CD62L (MEL-14), CD3 (17A2), CD4 (RM4–5), CD8a (53–6.7),
437 CD45.1 (A20), CD244.2 (m2B4 (B6) 458.1), CD223 (LAG-3, C9B7W), PD-1 (29F.1A12)
438 (eBioscience and BioLegend) and H2-Db/NS4b tetramers (NIH Tetramer Facility,
439 Atlanta, GA) followed by Live/Dead viability dye (ThermoFisher Sci). Cytotoxic
440 precursor enumeration was accomplished as described in (Uhrlaub, Brien, Widman,
441 Mason, & Nikolich-Zugich, 2011) NS4b-Tg CD45.1 CD8 T cells were purified with
442 Miltenyi Biotec reagents and columns using negative magnetic selection. Cell counts
443 and purity (>95%) were confirmed by FCM analysis and count beads before adoptive
444 transfer. Optimization procedures for flow cytometry are based on current standards
445 and included full-minus-one (FMO) gating controls. Samples were acquired on a BD
446 LSRFortessa cytometer (BD Biosciences) or Cytek Aurora (Cytek Biosciences) and
447 analyzed with FlowJo software (Tree Star). WNV neutralization titers were performed as
448 described in (Wong et al., 2020) and scored as a 90% reduction in viral inoculum.
449
450 In Vivo Killing Assay
451 Splenocytes from C57BL/6 mice were disassociated into a single cell suspension and
452 labeled with 1μM CellTrace Far Red (ThermoFisher Sci). Targets were then divided
453 into two populations: $NS4b_{2488}$ targets were labeled with Cell Trace Violet
454 (ThermoFisher Sci) 5mM for 10^{-6}M peptide loaded targets and 0.5mM for non-loaded
455 negative control) and ENV_{641} targets were labeled with CFSE (ThermoFisher Sci) 5mM
456 for 10^{-6}M peptide loaded targets and 0.5mM for non-loaded negative control. All
457 labeling was done as described in manufacturer instructions. Peptide loading was 1
458 hour at 37°C. After thorough washing, targets were pooled and injected intravenously
459 (i.v.) into infected and naïve mice. After 4 hours mice were sacrificed spleen
460 disassociated to a single cell suspension. Splenocytes were gated on total targets
461 (CellTrace Far Red positive) then CellTrace Violet high and low or CFSE high and low.
462 Calculation of killing: 100% - [(% peptide bearing targets IMMUNE recipients / % no
463 peptide targets IMMUNE recipients) / (% peptide bearing targets NAIVE recipients / %
464 no peptide targets NAIVE recipients) x 100].

465 **STATISTICS**:

466 GraphPad Prism® version 9.0.2 (GraphPad Software) was used for data analysis. All
467 experiments were performed at least twice. Significance was determined as described
468 in figure legends.

469

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477

478 **CONFLICT OF INTEREST:**

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481

482 **AUTHOR CONTRIBUTIONS:**

483 J.L.U., J.N-Ž., J.D. and M.R.M.vdB. designed the research; J.L.U., M.J., C.M.B., S.S.,
484 and C.P.C. performed experiments and analyzed the data; K.O.M., M.C.L., M.P.B.
485 contributed human samples; J.L.U. prepared the figures; J.L.U. and J.N-Ž. wrote the
486 manuscript; all authors edited the manuscript.

487

488 **DATA AVAILABILITY:**

489 All data available upon request.

490

491 **REFERENCES**

492 Aguilar-Valenzuela, R., Netland, J., Seo, Y. J., Bevan, M. J., Grakoui, A., & Suthar, M. S. (2018). Dynamics
493 of Tissue-Specific CD8(+) T Cell Responses during West Nile Virus Infection. *J Virol, 92*(10).
494 doi:10.1128/jvi.00014-18
495 Blattman, J. N., Antia, R., Sourdive, D. J., Wang, X., Kaech, S. M., Murali-Krishna, K., . . . Ahmed, R. (2002).
496 Estimating the precursor frequency of naive antigen-specific CD8 T cells. *J Exp Med, 195*(5), 657-
497 664. doi:10.1084/jem.20001021
498 Boyman, O., Ramsey, C., Kim, D. M., Sprent, J., & Surh, C. D. (2008). IL-7/anti-IL-7 mAb complexes
499 restore T cell development and induce homeostatic T Cell expansion without lymphopenia. *J*
500 *Immunol, 180*(11), 7265-7275. doi:10.4049/jimmunol.180.11.7265
501 Brien, J. D., Uhrlaub, J. L., Hirsch, A., Wiley, C. A., & Nikolich-Zugich, J. (2009). Key role of T cell defects in
502 age-related vulnerability to West Nile virus. *J Exp Med, 206*(12), 2735-2745.
503 doi:10.1084/jem.20090222
504 Brien, J. D., Uhrlaub, J. L., & Nikolich-Zugich, J. (2008). West Nile virus-specific CD4 T cells exhibit direct
505 antiviral cytokine secretion and cytotoxicity and are sufficient for antiviral protection. *J*
506 *Immunol, 181*(12), 8568-8575. doi:10.4049/jimmunol.181.12.8568
507 Campbell, G. L., Marfin, A. A., Lanciotti, R. S., & Gubler, D. J. (2002). West Nile virus. *The Lancet*
508 *Infectious Diseases, 2*(9), 519-529. doi:https://doi.org/10.1016/S1473-3099(02)00368-7
509 Chiu, B. C., Martin, B. E., Stolberg, V. R., & Chensue, S. W. (2013). Cutting edge: Central memory CD8 T
510 cells in aged mice are virtual memory cells. *J Immunol, 191*(12), 5793-5796.
511 doi:10.4049/jimmunol.1302509
512 Cicin-Sain, L., Messaoudi, I., Park, B., Currier, N., Planer, S., Fischer, M., . . . Nikolich-Zugich, J. (2007).
513 Dramatic increase in naive T cell turnover is linked to loss of naive T cells from old primates. *Proc*
514 *Natl Acad Sci U S A, 104*(50), 19960-19965. doi:10.1073/pnas.0705905104
515 Cicin-Sain, L., Smyk-Pearson, S., Currier, N., Byrd, L., Koudelka, C., Robinson, T., . . . Nikolich-Zugich, J.
516 (2010). Loss of naive T cells and repertoire constriction predict poor response to vaccination in
517 old primates. *J Immunol, 184*(12), 6739-6745. doi:10.4049/jimmunol.0904193
518 Collier, D. A., Ferreira, I., Kotagiri, P., Datir, R. P., Lim, E. Y., Touizer, E., . . . Gupta, R. K. (2021). Age-
519 related immune response heterogeneity to SARS-CoV-2 vaccine BNT162b2. *Nature*.
520 doi:10.1038/s41586-021-03739-1
521 Decman, V., Laidlaw, B. J., Doering, T. A., Leng, J., Ertl, H. C., Goldstein, D. R., & Wherry, E. J. (2012).
522 Defective CD8 T cell responses in aged mice are due to quantitative and qualitative changes in
523 virus-specific precursors. *J Immunol, 188*(4), 1933-1941. doi:10.4049/jimmunol.1101098
524 Granadier, D., Iovino, L., Kinsella, S., & Dudakov, J. A. (2021). Dynamics of thymus function and T cell
525 receptor repertoire breadth in health and disease. *Semin Immunopathol, 43*(1), 119-134.
526 doi:10.1007/s00281-021-00840-5
527 Groarke, E. M., & Young, N. S. (2019). Aging and Hematopoiesis. *Clin Geriatr Med, 35*(3), 285-293.
528 doi:10.1016/j.cger.2019.03.001
529 Heng, T. S., Reiseger, J. J., Fletcher, A. L., Leggatt, G. R., White, O. J., Vlahos, K., . . . Boyd, R. L. (2012).
530 Impact of sex steroid ablation on viral, tumour and vaccine responses in aged mice. *PLoS One,*
531 *7*(8), e42677. doi:10.1371/journal.pone.0042677
532 Heng, T. S. P., Goldberg, G. L., Gray, D. H. D., Sutherland, J. S., Chidgey, A. P., & Boyd, R. L. (2005). Effects
533 of Castration on Thymocyte Development in Two Different Models of Thymic Involution. *The*
534 *Journal of Immunology, 175*(5), 2982-2993. doi:10.4049/jimmunol.175.5.2982
535 Lanteri, M. C., Lee, T. H., Wen, L., Kaidarova, Z., Bravo, M. D., Kiely, N. E., . . . Busch, M. P. (2014). West
536 Nile virus nucleic acid persistence in whole blood months after clearance in plasma: implication

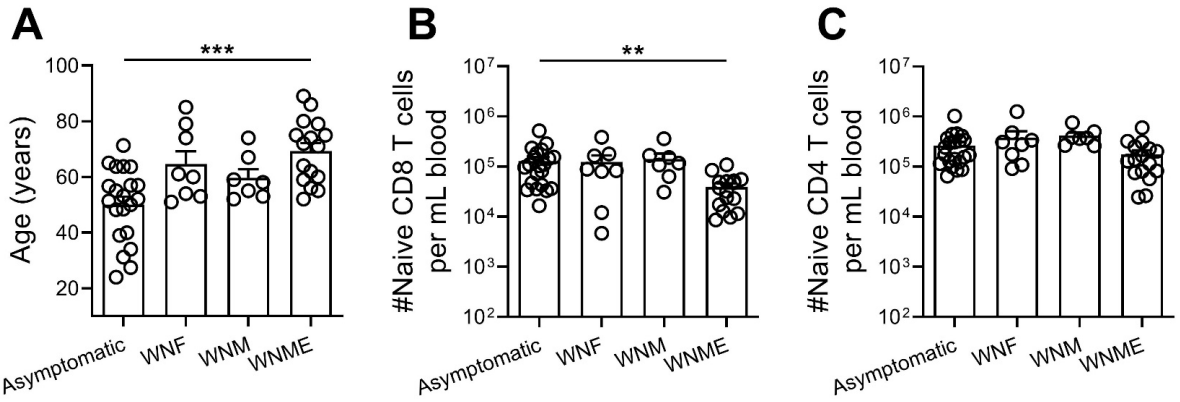
537 for transfusion and transplantation safety. *Transfusion, 54*(12), 3232-3241.
538 doi:10.1111/trf.12764
539 Lee, J. Y., Hamilton, S. E., Akue, A. D., Hogquist, K. A., & Jameson, S. C. (2013). Virtual memory CD8 T
540 cells display unique functional properties. *Proc Natl Acad Sci U S A, 110*(33), 13498-13503.
541 doi:10.1073/pnas.1307572110
542 Min, D., Panoskaltsis-Mortari, A., Kuro, O. M., Holländer, G. A., Blazar, B. R., & Weinberg, K. I. (2007).
543 Sustained thymopoiesis and improvement in functional immunity induced by exogenous KGF
544 administration in murine models of aging. *Blood, 109*(6), 2529-2537. doi:10.1182/blood-2006-
545 08-043794
546 Moon, J. J., Chu, H. H., Hataye, J., Pagán, A. J., Pepper, M., McLachlan, J. B., . . . Jenkins, M. K. (2009).
547 Tracking epitope-specific T cells. *Nat Protoc, 4*(4), 565-581. doi:10.1038/nprot.2009.9
548 Nikolich-Žugich, J. (2018). The twilight of immunity: emerging concepts in aging of the immune system.
549 *Nat Immunol, 19*(1), 10-19. doi:10.1038/s41590-017-0006-x
550 Okoye, A. A., Rohankhedkar, M., Konfe, A. L., Abana, C. O., Reyes, M. D., Clock, J. A., . . . Picker, L. J.
551 (2015). Effect of IL-7 Therapy on Naive and Memory T Cell Homeostasis in Aged Rhesus
552 Macaques. *J Immunol, 195*(9), 4292-4305. doi:10.4049/jimmunol.1500609
553 Purtha, W. E., Myers, N., Mitaksov, V., Sitati, E., Connolly, J., Fremont, D. H., . . . Diamond, M. S. (2007).
554 Antigen-specific cytotoxic T lymphocytes protect against lethal West Nile virus encephalitis. *Eur J*
555 *Immunol, 37*(7), 1845-1854. doi:10.1002/eji.200737192
556 Reiner, S. L. (2021). A Regenerative Perspective on Successful and Failed T-Cell Immunity. *Cold Spring*
557 *Harb Perspect Biol, 13*(7). doi:10.1101/cshperspect.a037937
558 Renkema, K. R., Li, G., Wu, A., Smithey, M. J., & Nikolich-Žugich, J. (2014). Two separate defects affecting
559 true naive or virtual memory T cell precursors combine to reduce naive T cell responses with
560 aging. *J Immunol, 192*(1), 151-159. doi:10.4049/jimmunol.1301453
561 Richner, J. M., Gmyrek, G. B., Govero, J., Tu, Y., van der Windt, G. J., Metcalf, T. U., . . . Diamond, M. S.
562 (2015). Age-Dependent Cell Trafficking Defects in Draining Lymph Nodes Impair Adaptive
563 Immunity and Control of West Nile Virus Infection. *PLoS pathogens, 11*(7), e1005027.
564 doi:10.1371/journal.ppat.1005027
565 Rudd, B. D., Venturi, V., Davenport, M. P., & Nikolich-Zugich, J. (2011). Evolution of the antigen-specific
566 CD8+ TCR repertoire across the life span: evidence for clonal homogenization of the old TCR
567 repertoire. *J Immunol, 186*(4), 2056-2064. doi:10.4049/jimmunol.1003013
568 Rydyznski Moderbacher, C., Ramirez, S. I., Dan, J. M., Grifoni, A., Hastie, K. M., Weiskopf, D., . . . Crotty,
569 S. (2020). Antigen-Specific Adaptive Immunity to SARS-CoV-2 in Acute COVID-19 and
570 Associations with Age and Disease Severity. *Cell, 183*(4), 996-1012.e1019.
571 doi:10.1016/j.cell.2020.09.038
572 Seo, Y. B., Im, S. J., Namkoong, H., Kim, S. W., Choi, Y. W., Kang, M. C., . . . Sung, Y. C. (2014). Crucial roles
573 of interleukin-7 in the development of T follicular helper cells and in the induction of humoral
574 immunity. *J Virol, 88*(16), 8998-9009. doi:10.1128/jvi.00534-14
575 Smithey, M. J., Li, G., Venturi, V., Davenport, M. P., & Nikolich-Žugich, J. (2012). Lifelong persistent viral
576 infection alters the naive T cell pool, impairing CD8 T cell immunity in late life. *J Immunol,*
577 *189*(11), 5356-5366. doi:10.4049/jimmunol.1201867
578 Sutherland, J. S., Goldberg, G. L., Hammett, M. V., Uldrich, A. P., Berzins, S. P., Heng, T. S., . . . Boyd, R. L.
579 (2005). Activation of Thymic Regeneration in Mice and Humans following Androgen Blockade.
580 *The Journal of Immunology, 175*(4), 2741-2753. doi:10.4049/jimmunol.175.4.2741
581 Tan, J. T., Ernst, B., Kieper, W. C., LeRoy, E., Sprent, J., & Surh, C. D. (2002). Interleukin (IL)-15 and IL-7
582 jointly regulate homeostatic proliferation of memory phenotype CD8+ cells but are not required
583 for memory phenotype CD4+ cells. *J Exp Med, 195*(12), 1523-1532. doi:10.1084/jem.20020066

584 Taub, D. D., Murphy, W. J., & Longo, D. L. (2010). Rejuvenation of the aging thymus: growth hormone-
585 mediated and ghrelin-mediated signaling pathways. *Curr Opin Pharmacol, 10*(4), 408-424.
586 doi:10.1016/j.coph.2010.04.015
587 Thompson, H. L., Smithey, M. J., Surh, C. D., & Nikolich-Žugich, J. (2017). Functional and Homeostatic
588 Impact of Age-Related Changes in Lymph Node Stroma. *Front Immunol, 8*, 706.
589 doi:10.3389/fimmu.2017.00706
590 Thompson, H. L., Smithey, M. J., Uhrlaub, J. L., Jeftić, I., Jergović, M., White, S. E., . . . Nikolich-Žugich, J.
591 (2019). Lymph nodes as barriers to T-cell rejuvenation in aging mice and nonhuman primates.
592 *Aging Cell, 18*(1), e12865. doi:10.1111/acel.12865
593 Uhrlaub, J. L., Brien, J. D., Widman, D. G., Mason, P. W., & Nikolich-Zugich, J. (2011). Repeated in vivo
594 stimulation of T and B cell responses in old mice generates protective immunity against lethal
595 West Nile virus encephalitis. *J Immunol, 186*(7), 3882-3891. doi:10.4049/jimmunol.1002799
596 Wertheimer, A. M., Bennett, M. S., Park, B., Uhrlaub, J. L., Martinez, C., Pulko, V., . . . Nikolich-Žugich, J.
597 (2014). Aging and cytomegalovirus infection differentially and jointly affect distinct circulating T
598 cell subsets in humans. *J Immunol, 192*(5), 2143-2155. doi:10.4049/jimmunol.1301721
599 Wong, R., Belk, J. A., Govero, J., Uhrlaub, J. L., Reinartz, D., Zhao, H., . . . Bhattacharya, D. (2020). Affinity-
600 Restricted Memory B Cells Dominate Recall Responses to Heterologous Flaviviruses. *Immunity,*
601 *53*(5), 1078-1094.e1077. doi:10.1016/j.immuni.2020.09.001
602 Zhang, H., Weyand, C. M., & Goronzy, J. J. (2021). Hallmarks of the aging T-cell system. *Febs j*.
603 doi:10.1111/febs.15770
604 Zhao, J., Gabriel, E., Norda, R., Höglund, P., Baden, L., Diedrich, B. A., . . . Edgren, G. (2021). Frequent
605 platelet donation is associated with lymphopenia and risk of infections: A nationwide cohort
606 study. *Transfusion, 61*(2), 464-473. doi:10.1111/trf.16175

607

608 **FIGURES:**

Figure 1



609

610 Figure 1

611 **Older adults experience more severe WNV disease and have a dearth of naïve**

612 **CD8 T cells.**

613 A cohort of 52 patients with WNV disease severity clinically classified as Asymptomatic,
614 WN Fever (WNF), WN Meningitis (WNM), and WN meningitis and encephalitis (WNME)
615 were retrospectively recruited to participate in our study. The age of the patients
616 experiencing the most severe disease is significantly older than asymptomatic (A).
617 These patients also had a reduced number of circulating naive CD8, but not CD4, T
618 cells as compared to asymptomatic (B, C). Statistical significance evaluated by Kruskal-
619 Wallis with Dunn's multiple comparisons test, +SEM. $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

620

Figure 2

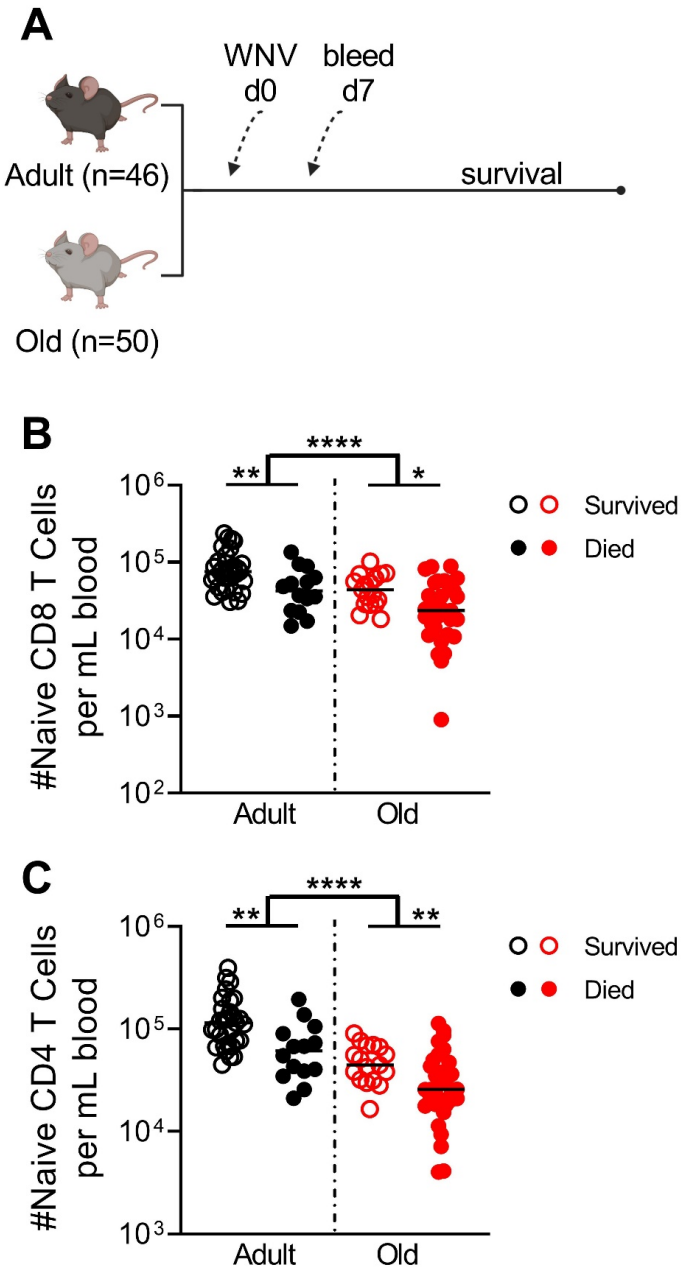
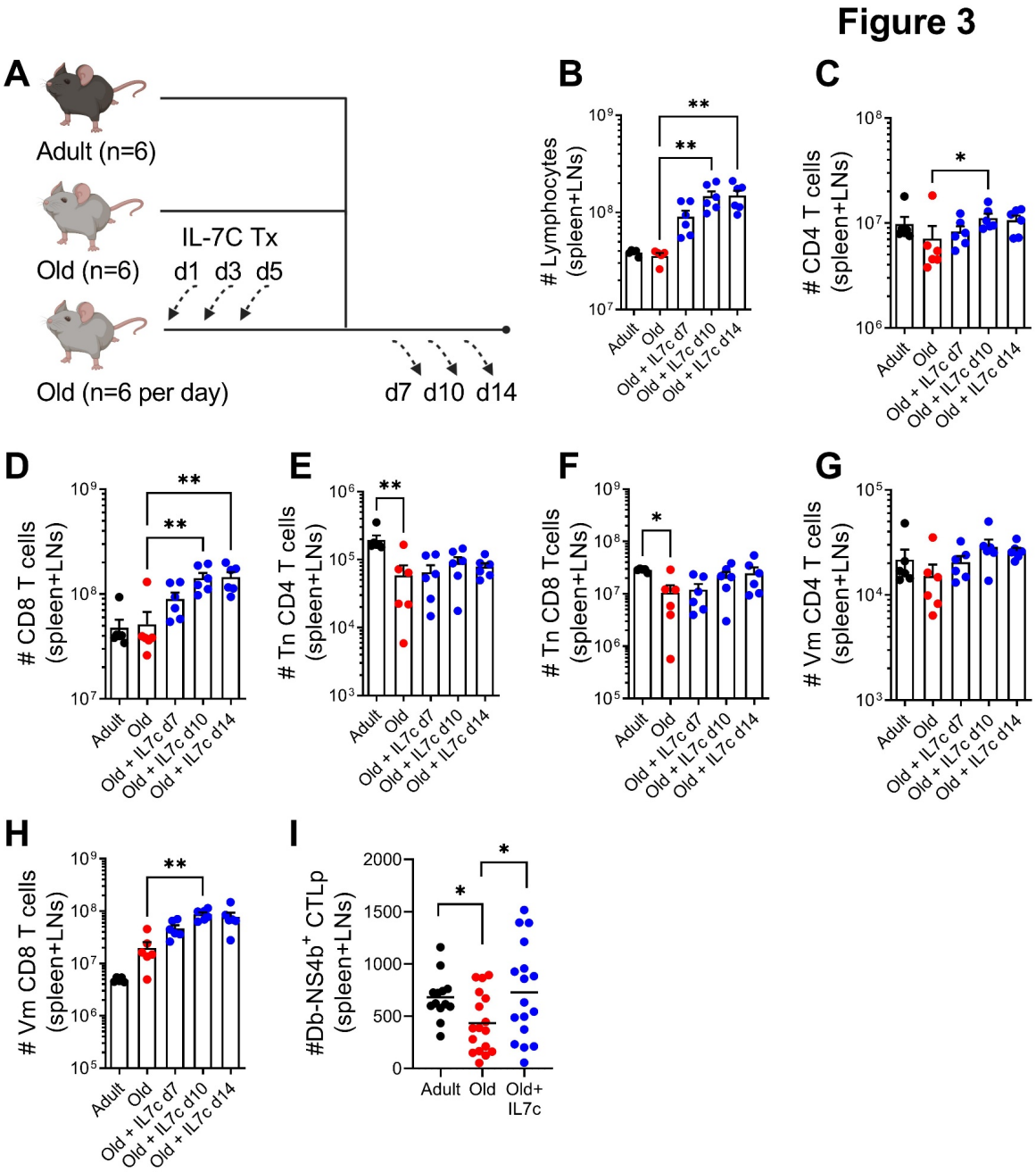


Figure 2

Decreased numbers of naïve CD8 T cells in both young and aged mice convey increased susceptibility to WNV.

Adult (3-5 months) and old (19-22 months) C57BL/6 mice were infected with WNV, 1000pfu, f.p.; bled on day 7 for FCM; and followed for susceptibility (A, schema).

627 Absolute numbers of naïve CD8 T cells were enumerated per mL of blood on day 7
628 post-infection (B). Data compiled from two independent experiments. Statistical
629 significance evaluated by t-test, median. *$P \leq 0.05$; **$P \leq 0.01$; ****$P \leq 0.0001$.
630



631

632 Figure 3

633 **IL-7C treatment expands the total number of T cells available in secondary**
634 **lymphoid tissues.**
635 Old (19-22 months) C57BL/6 mice were treated with IL-7C on days 1, 3, and 5 of the
636 experiment and sacrificed on days 7, 10, and 14 post-first treatment alongside untreated
637 adult (3-5 months) and old (19-22 months) (schema, A). The total number of
638 lymphocytes (B), CD4 T cells (C), and CD8 T cells (D) are increased at indicated day(s)
639 post-first treatment. The total number of naïve, CD44 low and CD62L high, CD8 T cells
640 are not significantly increased at any day post-treatment (E) but those with a virtual
641 memory phenotype (CD44 high, CD62L high, and CD49d low) are elevated at day 10
642 post-first treatment (F). CD8 CTLp ($NS4b_{2488}$) are not reliably increased by IL-7C
643 treatment (G). (B-F) Data shown is representative of two experiments with similar
644 results and statistical significance evaluated by one-way Anova with Dunn's multiple
645 comparisons test. All comparisons are to untreated old mice. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$.
646 (G) IL-7C treated group is day 13 post-first treatment. Data compiled from three
647 experiments and statistical significance evaluated by t-test comparison to O, median. $^{*}P$
648 ≤ 0.05.
649

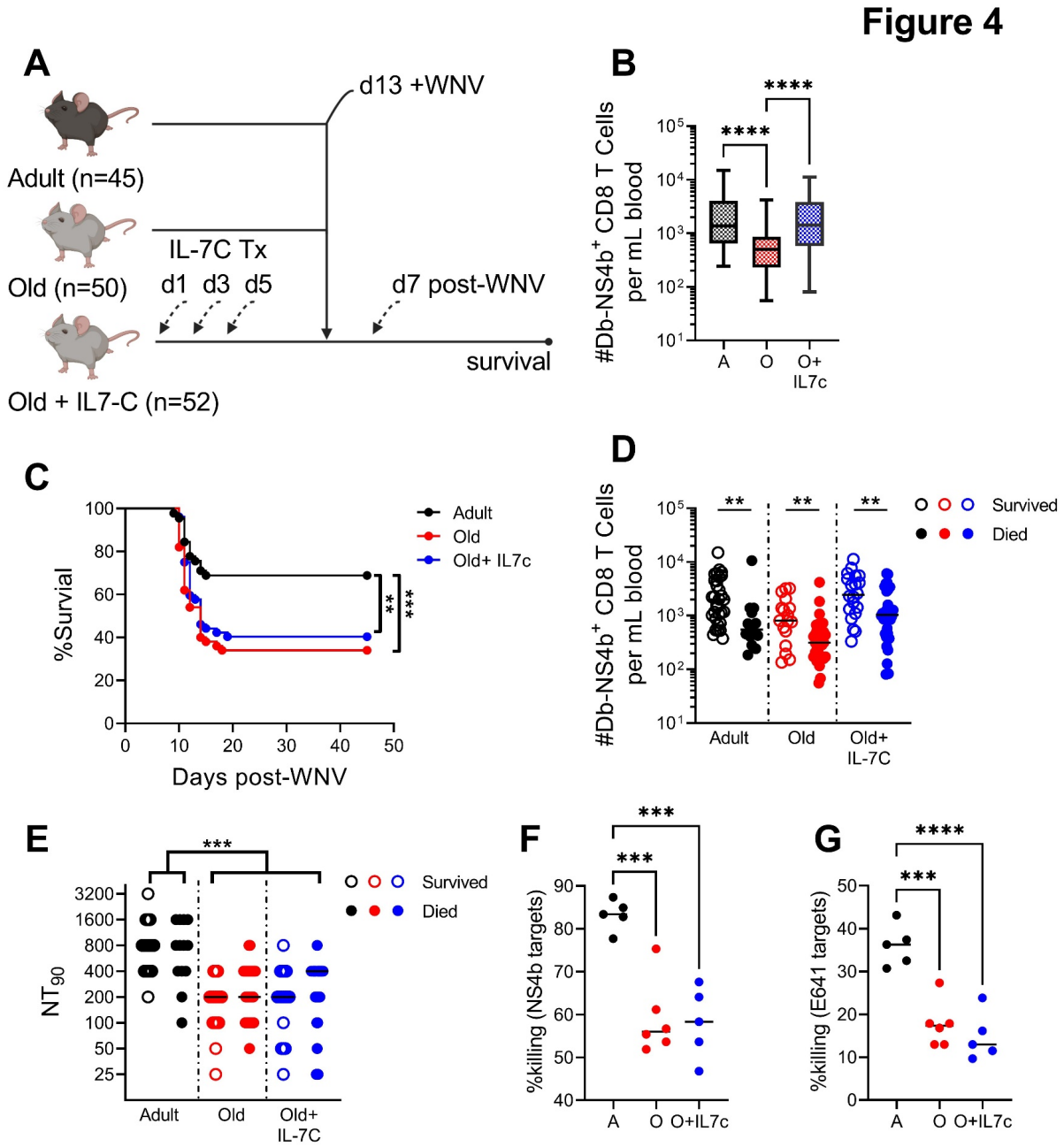


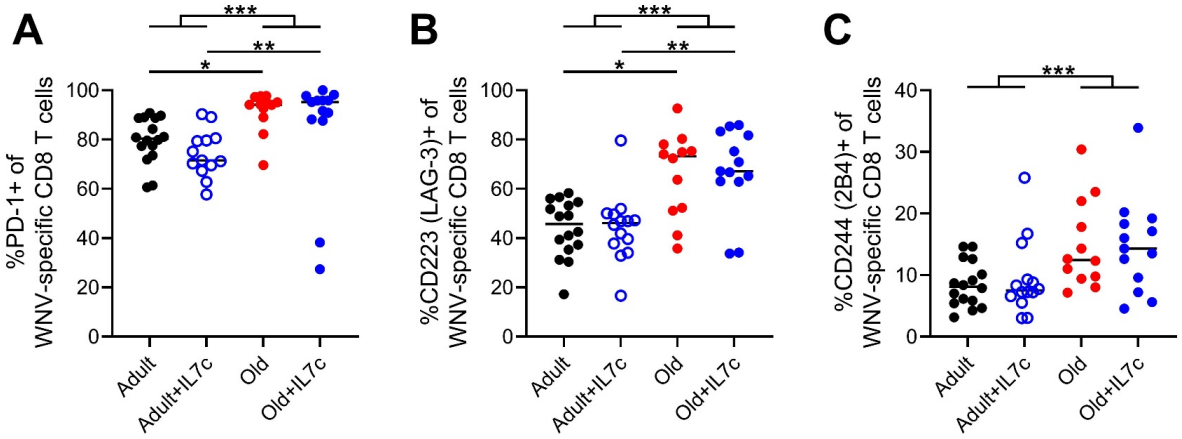
Figure 4

IL-7C treatment of old mice improves WNV-specific CD8 T cell responses without impacting survival.

Adult (3-5 months), Old (19-22 months), and Old (19-22 months) mice treated with IL-7C 13 days before challenge are infected with WNV (1000pfu, f.p.). On day 7 post-WNV infection mice are bled and monitored for survival (schema, A). Number of WNV-

657 specific CD8 T cells per mL of blood on day 7 post-WNV (B). WNV survival at day 45
658 post-infection (C). Number of WNV-specific CD8 T cells from (B) broken by those that
659 survived and those that died from WNV in each group (C). Neutralizing antibody titers
660 on day 7 post-WNV broken by those that survived and those that from died from WNV
661 in each group (E). *In vivo* killing assay on day 7 post-WNV for $NS4b_{2488}$ (F) and ENV_{641}
662 (G). Data compiled from two experiments. Statistical significance evaluated by anova in
663 (B, F, G) and t-test in (D) and (E) as represented on graphs. Significance of survival
664 evaluated by Log-rank (Mantel-Cox) test. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq$
665 0.0001.
666

Figure 5



667
668 Figure 5
669 **IL7-C treatment does not induce T cell exhaustion.**
670 Adult (3-5 months) and Old (19-22 months) mice were treated with IL-7C 13 days before
671 infection with WNV (1000pfu, f.p.). On day 7 post-WNV infection mice were bled and
672 circulating PBMC's were analyzed by FCM for expression of PD1, CD244 (2B4), and
673 CD223 (LAG-3) on $NS4b_{2488}$+ CD8 T cells (A-C). Data compiled from two experiments.
674 Statistical significance evaluated by anova in (all groups) and t-test in (by age) as
675 represented on graphs. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.
676

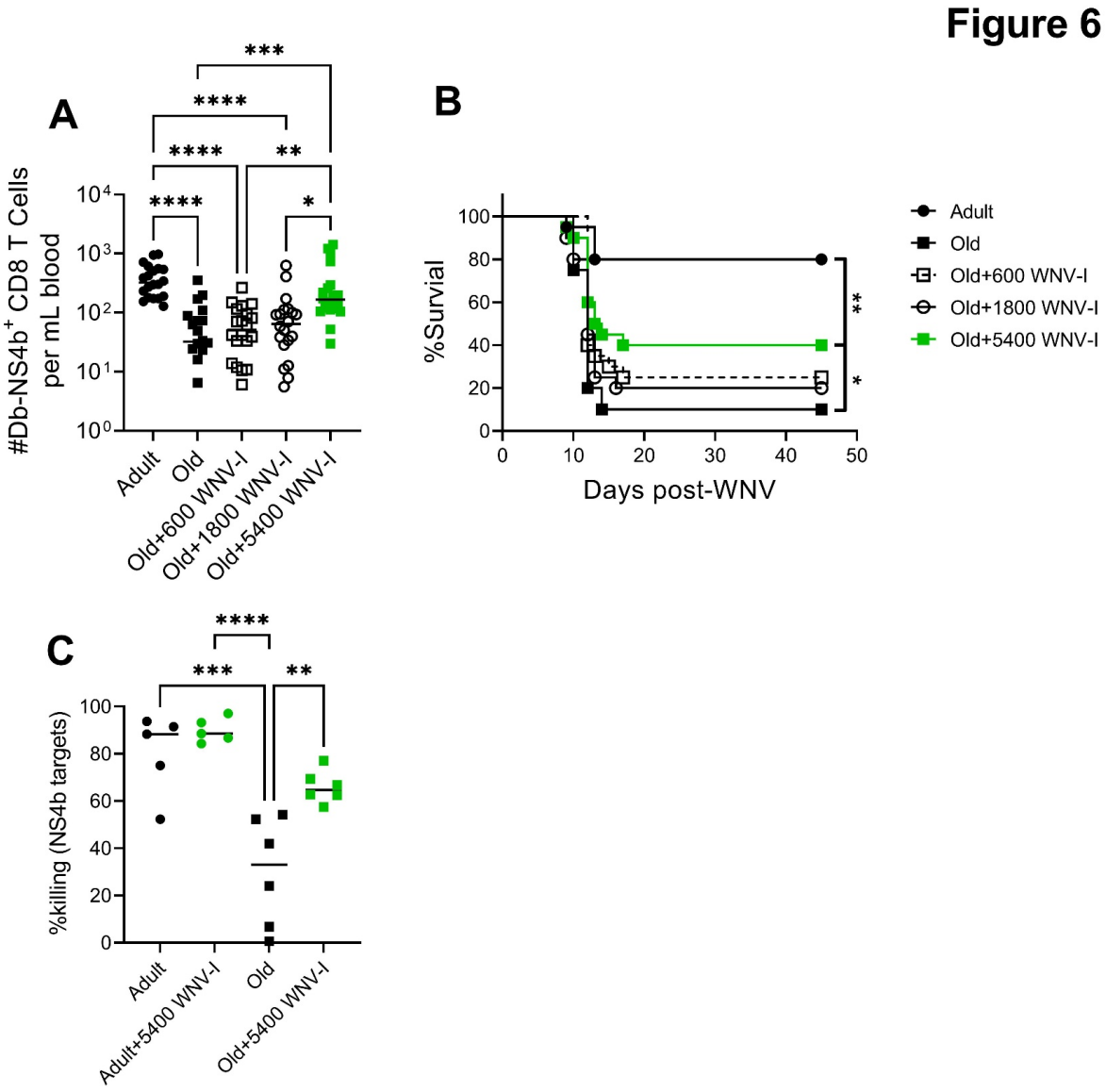


Figure 6

Adoptive transfer of WNV-I Tg CD8 T cells rescues old mice from WNV susceptibility.

WNV-I CD8 T cells (5400, 1600, or 600) were transferred to groups of old mice (19-22 months) alongside adult (3-5 months) and Old (19-22months) mice the day before WNV challenge (1000pfu, f.p.). On day 7 post-infection mice were bled and total numbers of circulating WNV-specific CD8 T cells were enumerated per mL of blood (A). Mice were monitored for survival to day 45 post-infection (B). *In vivo* killing assay on day 7 post-WNV for $NS4b_{2488}$ (C). Data compiled (A-B) or representative (C) of two experiments.

687 Statistical significance evaluated by anova (A, C) with multiple comparisons test.
688 Significance of survival evaluated by Log-rank (Mantel-Cox) test. *$P \leq 0.05$; **$P \leq 0.01$;
689 ***$P \leq 0.001$; ****$P \leq 0.0001$.
690