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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*Address correspondence to Janko Nikolich-Žugich at nikolich@arizona.edu or Jennifer Uhrlaub at juhrlaub@email.arizona.edu or by mail at P.O. Box 245221, Department of

Immunobiology, University of Arizona College of Medicine-Tucson, 1501 N. Campbell Ave., Tucson, AZ 85724.

ABSTRACT

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 West Nile virus (WNV) in both humans naturally exposed to, and mice experimentally infected with, 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 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.

Keywords: Immune aging/ immune rejuvenation/ IL-7/ CD8 T cells

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 (reviewed in (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) (rev. in (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 (rev, in (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, which transforms 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 (Rev. in (Nikolich-Žugich, 2018). 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

88 apheresis subjects, where 15-20% of PBMC were retained by the apheresis machine,
89 and there was a correlation between the number of donations, lymphopenia, and
90 reactivation of persistent herpesviruses (e.g. Varicella-Zoster virus) (Zhao et al., 2021).
91 Moreover, severity of COVID-19 in older adults has been shown to correlate to reduced
92 numbers of CD4 and CD8 Tn cells (Rydyznski Moderbacher et al., 2020). A limitation of
93 these studies is that they did not independently control for aging, and, in most cases,
94 baseline samples were not available to answer whether this was a preexisting condition,
95 a virus-induced lymphodepletion, or a combination of both. All of these intriguing
96 correlative studies raise the possibility of causal association between the (age-related)
97 Tn lymphopenia and functional immunity decline with aging.

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

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

136

137

138 **RESULTS**

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

140 Previously unencountered and emerging viral infections often cause increased
141 disease severity and mortality in older populations. Indeed, patients who have
142 experienced the most severe symptoms caused by WNV were significantly older than
143 those who had milder disease ((Campbell, Marfin, Lanciotti, & Gubler, 2002) and Figure
144 1A), and similar findings have been made in other emerging infectious diseases
145 including SARS-CoV, Chikungunya virus, and SARS-CoV-2. Consistent with our prior
146 data in general geriatric populations (Wertheimer et al., 2014) our cohort of WNV-
147 exposed participants exhibited significantly lower levels of circulating CD8, but not CD4,
148 Tn cells when compared individuals who experienced asymptomatic infection (Figure
149 1B, C). We determined T cell phenotype frequencies using a combination of CCR7,
150 CD45RA, CD28 and CD95, to define naïve T cells as CCR7hi,CD45RAhi,CD28int/hi
151 and CD95low. Participants who experienced the most severe symptoms, meningitis and
152 encephalitis, exhibited a measurable loss of both Tn CD4 and CD8 T cells as compared
153 to at least one other group. In a retrospective study, where a limited number of patient
154 samples were collected long after asymptomatic infection or disease recovery, it is
155 impossible to determine whether this was a cause, an epiphenomenon, or an effect, of
156 their severe infection. To examine the nature of associations between age, Tn cell
157 deficit, and severe WNV disease, we examined this issue in an established mouse
158 model of age-related susceptibility to WNV (Brien, Uhrlaub, Hirsch, Wiley, & Nikolich-
159 Zugich, 2009).
160

161 *The murine model recapitulates the descriptive findings from human subjects.*

162 It is well established that the naïve T cell compartment contracts with age and is
163 proportionally less well represented than the T cells carrying memory phenotypes
164 (Zhang, Weyand, & Goronzy, 2021). A direct correlation with disease severity, however,
165 has not been conclusively established. To determine if our WNV mouse model is valid
166 to interrogate this association, we measured the number of circulating CD8 Tn cells in
167 WNV-infected adult (3-5 month old) and old (19-22 month old) mice on day 7 p.i.; a time

168 point prior to onset of WNV disease (Figure 2A). Mice that will eventually succumb to
169 WNV exhibited lower numbers of circulating naïve CD8 and CD4 T cells at this early
170 time point when compared to those that will survive (Figure 2B, C). This disparity held
171 regardless of age, despite the fact that old mice exhibit reduced numbers of Tn cells
172 overall (Figure 2B, C). These data support the possibility that a numeric advantage in
173 naïve T cells is directly linked to WNV resistance regardless of age. These results are
174 also consistent with the human data from Figure 1 with the exception that there is also a
175 robust loss of naïve CD4 T cells in susceptible mice. Of note, there was overlap in CD8
176 Tn numbers between humans with severe and asymptomatic WNV disease, as well as
177 between mice that succumbed and survived WNV infection. This suggests that the
178 numbers of CD8 Tn cells are not the only determinant of increased WNV resistance,
179 consistent with the extensive literature on the multipronged nature of immune defense
180 against WNV (Brien et al., 2009; Purtha et al., 2007; Richner et al., 2015). The overlap
181 in the human and mouse data provides confidence that this mouse model can be used
182 to examine whether, and to what extent, reversing age-induced lymphopenia and,
183 specifically, a deficit in Tn cells, may afford improved immune protection against WNV.
184
185 *IL-7 complex treatment increases the number of circulating Tn cells*
186 IL-7 is a pleiotropic cytokine that provides an indispensable homeostatic survival
187 signal to Tn cells, and that drives Tn and other T cell subsets into homeostatic
188 proliferation under the conditions of lymphopenia. Delivered *in vivo* as a cytokine-
189 antibody complex, IL-7C has been shown to drive naïve T cell proliferation and increase
190 thymopoeisis in unmanipulated as well as lymphopenic mice (Boyman, Kovar,
191 Rubinstein, Surh, & Sprent, 2006). Despite its therapeutic impact in adult mice, this
192 model has not been rigorously interrogated for reversal of age-related lymphopenia and
193 reduced susceptibility to infection. We treated old mice with IL-7C (3 i.p. injections,
194 every other day), and sacrificed them on days 7, 10, and 14 post-first IL-7C treatment to
195 assess total spleen and LN content (schema, Figure 3A). Total cellularity of secondary
196 lymphoid tissues (spleen and LNs) remained elevated through day 14 post-first
197 treatment (Figure 3B), an expansion mirrored by the numbers of CD4 and CD8 T cells
198 (Figure 3C, D). Additionally, numbers of CD4 and CD8 Tn cells in treated old mice were

199 no longer statistically significantly reduced relative to those in adult mice (Figure 3E, F).
200 The numerical increase in CD4 T cells was driven by split increases in Tcm and Tvm
201 phenotype cells, neither of which were independently statistically significant (Figure 3G,
202 H). In the CD8 pool, T cells with a CM or Tvm phenotype dominated the increase in
203 numbers (Figure 3I, J). To determine whether the expansion of T cells by IL-7C also
204 included CD8 T cells specific for the immunodominant epitope of WNV, $NS4b_{2488}$, we
205 enumerated the number of these CD8 T cell precursors on day 13 post-treatment in
206 unimmunized mice (Figure 3K). Similar to our prior work with other immunodominant
207 CD8 epitopes (Rudd, Venturi, Davenport, & Nikolich-Zugich, 2011; Smithey, Li, Venturi,
208 Davenport, & Nikolich-Žugich, 2012), we found a marked ~62% loss of NS4b-specific
209 CD8 precursors in old animals compared to adult counterparts (Figure 3F). We found
210 that IL-7C treatment led to a numerical increase in CD8 precursors specific for the WNV
211 NS4b immunodominant epitope that, on average, no longer statistically differed from
212 that in adult animals (Figure 3K). The response between individual animals, however,
213 was heterogenous, with many animals exhibiting no increase, and others exhibiting up
214 to a two-fold increase in precursor numbers. Further, and as would be expected, NS4b-
215 specific precursors were of a similar phenotype as compared to the rest of the CD8 T
216 cell pool (Table 1).

Table 1		Adult	Old	Old+IL7-C
Tn	%CD44low CD62Lhigh of Db-NS4b CTLp (+/-SEM)	65.1 (2.6)	56.2 (4.2)	47.6 (7.1)
Tvm	%CD49dlow of CM Db-NS4b+ CTLp (+/-SEM)	93.7 (2.0)	75.7 (6.8)	83.8 (4.5)

217

218 The relative improvement in total T cell numbers, from days 10 to 14, including an
219 overall increase in WNV-specific CD8 precursors on day 13 following IL-7C treatment
220 suggested that functional immunity could be improved within this temporal window,
221 because lymphopenia in old mice was measurably reduced and residual IL-7C (if any)
222 did not cause further T cell proliferation in secondary lymphoid tissues.
223

224 ***IL-7c treated old mice gain numbers, but not function, of WNV-specific CD8 T cell***
225 ***responses***
226
227 To conclusively test whether this increase in T cell numbers is sufficient to overcome
228 infectious disease susceptibility, we challenged IL-7C treated mice with WNV. Based
229 on the above kinetics of IL-7C action, we chose day 13 as an optimal time point for
230 challenge, reasoning that the mice would benefit from an expanded T cell pool at the
231 time when IL-7C is no longer driving further proliferation of T cells, and should not
232 influence the development of a functional immune response to WNV. Adult (3-4
233 months); old (19-22 months); and IL-7C treated old (19-22 months) mice were
234 challenged with 1000pfu of WNV and followed for WNV-specific CD8 T cell responses
235 and survival (Figure 4A). On day 7 post-infection, old mice treated with IL-7C showed a
236 3-fold improvement in the number of WNV NS4b-specific CD8 T cells measured in the
237 blood (Figure 4B). However, this improvement failed to translate to better survival
238 (Figure 4C), despite the fact that we observed roughly equivalent numbers of WNV-
239 specific CD8 T cells in old IL-7C treated mice compared to adult unmanipulated mice.
240 When we segregated each experimental group into animals that died or survived WNV
241 challenge, we found that those that survived again exhibited a numeric advantage in
242 WNV-specific cells over those that succumbed (Figure 4D). The effect, however, was
243 not robust enough to ensure that the group, as a whole, survives better than their
244 untreated counterparts.
245 Multiple effector mechanisms of adaptive immunity must synergize to clear WNV
246 infection. These include potent neutralizing antibodies as well as CD4 and CD8
247 cytotoxic T cell effector function. To mechanistically determine whether any functional
248 improvement in immunity was restored by IL-7C treatment we interrogated the immune
249 response in IL7-C treated old mice in a step-wise manner. IL-7C have the potential to
250 enhance B cell lymphopoiesis, and to thereby increase B cell numbers, although they
251 are not expected to directly affect the mature B cell compartment (Boyman et al., 2008).
252 Previous studies have reported that IL-7C co-delivered with immunization can boost
253 humoral immune responses through enhanced generation of germinal center B cells
254 and Tfh cells (Seo et al., 2014). Therefore, we first analyzed neutralizing antibody titers

255 at day 7 post-WNV infection as a proxy for generalized improvement in B cell
256 responses. We detected no improvement in WNV neutralization by sera of old mice
257 treated with IL7-C relative to untreated controls (Figure 4E). We next examined
258 whether WNV-specific CD8 or CD4 T cells exhibited improved killing function following
259 IL-7C treatment by measuring cytotoxicity in vivo (Figure 4F, G). We included targets
260 specific for one WNV-specific CD4 immunodominant epitope, ENV_{641}, in this assay
261 since CD4 T cells are known to mount cytotoxic responses against WNV in adult mice
262 and data about their specific contribution to viral clearance with age is limited (Brien,
263 Uhrlaub, & Nikolich-Zugich, 2008). We found that, despite numeric increases in
264 circulating NS4b-specific CD8 T cells, IL-7C treated old mice did not have improved
265 cytotoxic capacity to clear $NS4b_{2488}$ peptide-loaded targets as compared to untreated
266 old mice (Figure 4F). There was also no improvement in killing of the ENV_{641}-loaded
267 targets (Figure 4G), consistent with the fact that we saw no increase in CD4 Tn cell
268 numbers (Fig. 3E). These data indicate an overall lack of improvement in cytotoxic
269 function in old mice treated with IL-7C. As these assessments were done prior to the
270 onset of disease, we measured memory antibody responses in WNV survivors as an
271 indication of whether IL7-C treatment impacted overall humoral immunity. We
272 determined that neither total envelope nor DIII lateral ridge specific antibodies were
273 improved by IL7-C treatment prior to WNV infection (Figure H, I). In fact, when
274 comparing the survivors of WNV infection, it appears that old mice treated with IL-7C
275 established an overall lower level of circulating WNV-specific antibodies at this point in
276 memory (Figure H, I). Our interpretation of this data is that the expansion of CD4 T cells
277 by IL7-C treatment does not afford a short- or lasting- impact on antibody responses to
278 WNV infection. At face value, these results suggest that numerical improvement in CD8
279 and CD4 T cells afforded by IL-7C treatment is not sufficient to improve functional
280 immunity.
281 To investigate why a numeric increase in antigen-specific precursors was not
282 able to restore T cell responses we considered the possibility that the expansion
283 induced via IL7-C treatment may push the cells into an exhausted state, impairing their
284 ability to participate in the WNV response. T cells with an ‘exhausted’ phenotype are
285 typically seen in the course of chronic infections and cancer (Collier et al., 2021; Reiner,

286 2021). The number of divisions and other factors required to induce this state are not
287 fully understood. To investigate whether homeostatic proliferation driven by IL-7C
288 treatment increases the proportion of responding WNV-specific T cells with an
289 exhausted phenotype we measured exhaustion markers in both adult and old mice
290 treated with IL7-C on day 7 of WNV. We included an adult cohort in these experiments
291 to dissociate aging from treatment-associated phenotypes. Our data confirm that a
292 higher percentage of WNV-specific CD8 T cells in aged mice express PD-1, LAG-3, or
293 2B4 regardless of whether they have been treated with IL7-C (Figure 5A, B, C). The
294 expression of these molecules could contribute to their poor cytotoxic function, and
295 overall lack of improvement in survival, experienced by the old animals. These data
296 suggest that the homeostatic proliferation induced by IL7-C treatment is not *per se*
297 pushing T cells into an exhausted state. Rather, consistent with some reports (Decman
298 et al., 2012) we hypothesize that the age of the animal may have already determined
299 the phenotype of the available T cell pool and this state is maintained through the
300 expansion initiated by IL7-C treatment to deleterious effect.
301 However, the fact that a similar increase in precursor numbers induced by IL-7C
302 treatment did not lead to either improved immunity or improved cytotoxicity, suggested
303 that some other property of reconstituted cells may be of critical importance. To directly
304 address whether an increase in CD8 Tn precursors specific for an invading pathogen
305 can overcome the age-related defects in immune defense and afford improved survival
306 to old mice, we performed adoptive transfer experiments. We transferred graded levels
307 of WNV-specific CD8 T cells ($NS4b_{2488}$ specific, referred to as WNV-I (Aguilar-
308 Valenzuela et al., 2018)) the day before challenge into old mice to boost the number of
309 precursors available to respond. These transgenic T cells have a naïve phenotype at
310 time of transfer and are not yet primed or cytotoxic. After challenge, the transferred
311 cells quantitatively restored the WNV-specific CD8 T cell numbers measured in the
312 blood on day 7 post-infection (Figure 6A). The differences between old mice treated
313 with IL-7C and those receiving adoptively transferred cells was that the former mounted
314 a response from endogenous, old precursors, and these precursors were driven into
315 homeostatic proliferation by IL-7C; the latter received naïve, unperturbed NS4b-specific
316 precursors from adult donors. Both the age and the immediate prior proliferation

317 induced by IL-7C could have impacted their response. More importantly, in the transfer
318 experiments, we were able to significantly improve survival in old mice following WNV
319 challenge with 5,400 transferred WNV-specific CD8+ Tn cells; but not with the two lower
320 transfer boluses of 1,800 and 600 cells (Figure 6B).

321 To put these numbers in the context of physiological precursor numbers available
322 to old and adult animals, we determined the efficacy of engraftment of transferred cells.
323 Some estimates of transfer efficiency posit that only about 10% of the transferred T cells
324 engraft in i.v. injected mice (Blattman et al., 2002; Moon et al., 2009). We enumerated
325 the total number of NS4b-specific CD8 T cells that were either endogenous (CD45.2) or
326 transgenic (CD45.1). Indeed, our experiments confirmed that 24h after transfer, we
327 could recover between 12-15% of transgenic cells (Supp. Fig 1A). Note here that the
328 numbers of engrafted cells were quantified in direct comparison to the number of
329 endogenous naïve precursors recovered from adult or old mice as they were
330 enumerated alongside each other (Supp. Fig 2). This means that a dose of 5,400
331 transferred cells reconstituted about 650-810 NS4b precursors, that were effectively
332 capable to significantly protect the old animal against WNV with survival not significantly
333 different from untreated adult mice. Of interest, an adult mouse contains 500-700 NS4b-
334 specific CD8+ precursors. Importantly, old mice that received 5400 WNV-I Tg CD8 T
335 cells efficiently recruited them to the WNV response (Figure 6C) and exhibited improved
336 cytotoxic function measured by increased killing (Figure 6D). This offers a plausible
337 mechanism for improved survival of this group (Figure 6B). We conclude that restoring
338 the precursor numbers of an immunodominant CD8 Tn specificity to adult levels, and
339 with cells that have a Tn phenotype, may be enough to confer a significant degree of
340 protection to an old mouse against WNV, despite concurrent defects in many other
341 arms of the immune response.
342

343 **DISCUSSION**

344

345 Immune therapies that seek to overcome age-induced lymphopenia are an
346 attractive strategy to reduce susceptibility to infectious diseases in older adults.
347 Conceptually, increasing the total cellular resources of the immune system, regardless

348 of antigen specificity, would provide the best solution for broad-based protection against
349 diverse challenges. IL-7C treatment has been shown to increase peripheral T cell
350 numbers even in the absence of thymic output (Boyman et al., 2008). In this work, we
351 formally demonstrated that IL-7C treatment can transiently increase T cell numbers in
352 old mice that have suffered substantial thymic involution, predominantly due to an
353 expansion of virtual central memory Tvm phenotype cells. We found significant, but
354 variable, expansion of the numbers of CD8 precursors specific for WNV, and an overall
355 increase in both CD8 and CD4 T cell numbers. However, these expanded cells did not
356 protect mice from WNV infection, and we could find no evidence that the cytotoxic
357 function of either CD8 or CD4 T cells was improved in IL-7C treated old mice. While we
358 did not enumerate B cells, their protective responses were similarly flat, with no
359 improvement in neutralization capacity, despite an increase in total numbers of CD4 T
360 cells. Therefore, under our experimental conditions, IL-7C treatment showed no
361 appreciable immune protection benefit to the old mice, similar to our prior studies with
362 unconjugated IL-7 in non-human primates (Okoye et al., 2015).
363 Given that protection against WNV normally requires a coordinated and multi-
364 faceted response, it may not be surprising that the increase in T cells alone cannot
365 augment protection. It was more puzzling, however, that we could find no evidence of
366 improved responses from the numerically increased T cell pool. To further explore this
367 question and test whether we could improve protection against WNV by boosting a
368 single arm of protective immunity, we performed adoptive transfer of graded numbers of
369 transgenic CD8 T cells. We demonstrated that sufficient numbers of transferred cells
370 (approximately equivalent to the precursor content of a single adult mouse) were able to
371 significantly improve survival, albeit not to restore it to the levels of adult mice. These
372 results begin to provide quantitative targets of CD8 T cell reconstitution, and also
373 provide a proof-of-principle that the aged immune system can coordinate an effective
374 immune response if the proper T cell resources are provided.
375 One key unanswered question remained regarding the relationship between
376 precursor T cell numbers and their quality. Specifically, adoptively transferred old mice
377 contained similar numbers of overall precursors relative to the IL-7C treated old mice,
378 suggesting that somehow the quality of the two precursor populations was different. To

379 address the potential differences, we considered prior work from ours and other groups
380 on age-related changes in Tn cell maintenance. Specifically, with aging, an increasing
381 number of Tn cells change their maintenance pattern and become Tvm (Chiu, Martin,
382 Stolberg, & Chensue, 2013; Decman et al., 2012; Rudd et al., 2011) to the point that
383 most of the central memory phenotype cells in SPF mice are of Tvm origin. While work
384 from other groups (Lee, Hamilton, Akue, Hogquist, & Jameson, 2013) and us
385 (Renkema, Li, Wu, Smithey, & Nikolich-Žugich, 2014) demonstrated that Tvm cells in
386 young adult mice exhibit strong proliferation and enhanced effector function relative to
387 Tn cells, we have also shown that with age Tvm cells exhibit reduced proliferative
388 response to cognate antigen (Renkema et al., 2014). By contrast, the response to
389 homeostatic cytokines remains preserved in Tvm cells. Additional studies will be
390 necessary to directly compare in vivo antiviral function and protective capacity of Tvm
391 and Tn cells of different ages with and without prior expansion by IL-7C and similar
392 treatments. Moreover, as these studies were focused on improving immune protection
393 against acute lethal challenge, it remains to be investigated whether and to what extent
394 the interventions used here, including IL-7C, could potentially improve responses to
395 vaccination, including memory responses. That notwithstanding, the work presented
396 here places quantitative limits on desirable levels of immune reconstitution and opens
397 provocative questions about their quality, that remain to be answered in the future.
398

399 **EXPERIMENTAL PROCEDURES**

400

401 Study Subjects and Blood Samples

402 This study was approved by the Institutional Review Boards at the University of Arizona
403 (Tucson, AZ; # 080000673; currently #2102460536), the Oregon Health and Science
404 University (Portland, OR; # IRB00003007); the University of Texas Health Science
405 Center at Houston, TX; and the Blood Systems Research Institute, San Francisco, CA.
406 – presently Vitalant Research Institute. Exclusion criteria included known
407 immunosuppressive pathology, stroke, cancer, or use of steroids within the last 5 years.
408 WNV-infected donors were enrolled by the University of Texas at Houston, 2006–2009
409 or Blood Systems Research Institute (BSRI), 2009 – 2011. Samples, demographics
410 (Supp. Table I), and symptoms data were collected and analyzed as previously
411 described (Lanteri et al., 2014) after the subjects provided an informed consent
412 approved by the UCSF Committee on Human Research (protocol #10-01255) or the
413 Committee for the Protection of Human Subjects at the University of Texas Health
414 Science Center at Houston (HSC-SPH-03-039), respectively. Blood was drawn into
415 heparinized Vacutainer CPT tubes (BD Bioscience) and processed fresh at respective
416 sites per manufacturer's recommendations to isolate peripheral blood mononuclear cells
417 (PBMC) and plasma; K2-EDTA tubes were used to determine complete blood counts.
418 PBMCs were frozen in 90% fetal bovine serum (FBS) and 10% DMSO.

419

420 Flow Cytometry – human samples

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

429 min at 4°C. Flow cytometry acquisition was performed on a BD Bioscience Fortessa and
430 analyzed using FlowJo software (Tree Star).
431
432 Mice
433 Mouse experiments were carried out in strict accordance with the recommendations in
434 the Guide for the Care and Use of Laboratory Animals of the National Institutes of
435 Health. Protocols were approved by the Institutional Animal Care and Use Committee at
436 the University of Arizona (IACUC 19-580, PHS Assurance No. A-3248-01. Old (19-22
437 months) and adult (3-5 months) were obtained from the National Institute on Aging
438 Rodent Resource or Jax laboratories. Mice in each experiment were age matched
439 within 4 weeks. WNV-I mice were obtained from Drs Jason Netland and. Michael J.
440 Bevan (Aguilar-Valenzuela et al., 2018). Footpad (f.p.) injections and retro-orbital
441 intravenous cell transfers were performed under isoflurane anesthesia. IL-7 cytokine
442 was obtained from the Biological Resources Branch (BRB), Developmental
443 Therapeutics Program (DTP), Division of Cancer Treatment and Diagnosis (DCTD),
444 National Cancer Institute (NCI). IL-7R antibody (clone M25) was purchased from
445 BioXcell. 0.59ug of IL-7 was complexed with 2.9ug of M25 antibody for 30 min at RT
446 then administered via intraperitoneal injection in 200uL USP saline on days 1, 3, and 5
447 of the experiment. Euthanasia was performed by isoflurane overdose or cervical
448 dislocation.
449
450 Virus
451 West Nile virus NY 385–99, isolated from the liver of a snowy owl, and in its second
452 passage, was a kind gift from Dr. Robert Tesh (University of Texas Medical Branch at
453 Galveston, Galveston, TX). Virus was prepared as previously described (Brien et al.,
454 2009). Mice were infected by footpad (f.p.) route with 1,000 pfu WNV/50 µl/mouse and
455 monitored for survival until 45 days p.i.
456
457 Tissue harvest, cell counts, flow cytometry, neutralization titers, antibody ELISA
458 Blood was collected via retro-orbital route and subjected to hypotonic lysis of red blood
459 cells. Spleen and peripheral lymph nodes (inguinal, brachial, axillary, and cervical) were

460 collected and disassociated over 40uM mesh. Cells were resuspended and stained
461 overnight at 4°C in a saturating concentration of antibodies against CD49d (9C10,
462 MFR4.B), CD44 (IM7), CD62L (MEL-14), CD3 (17A2), CD4 (RM4–5), CD8a (53–6.7),
463 CD45.1 (A20), CD244.2 (m2B4 (B6) 458.1), CD223 (LAG-3, C9B7W), PD-1 (29F.1A12)
464 (eBioscience and BioLegend) and H2-Db/NS4b tetramers (NIH Tetramer Facility,
465 Atlanta, GA) followed by Live/Dead viability dye (ThermoFisher Sci). Cytotoxic
466 precursor enumeration was accomplished as described in (Uhrlaub, Smithey, &
467 Nikolich-Žugich, 2017). NS4b-Tg CD45.1 CD8 T cells were purified with Miltenyi Biotec
468 reagents and columns using negative magnetic selection. Cell counts and purity (>95%)
469 were confirmed by FCM analysis and count beads before adoptive transfer.
470 Optimization procedures for flow cytometry are based on current standards and
471 included full-minus-one (FMO) gating controls. Samples were acquired on a BD
472 LSRFortessa cytometer (BD Biosciences) or Cytek Aurora (Cytek Biosciences) and
473 analyzed with FlowJo software (Tree Star). WNV neutralization titers were performed as
474 described in (Wong et al., 2020) and scored as a 90% reduction in viral inoculum. WNV
475 ELISA's for both total ENV and DIII were also performed as described in (Wong et al.,
476 2020) and directly reported as O.D.
477
478 In Vivo Killing Assay
479 Splenocytes from C57BL/6 mice were disassociated into a single cell suspension and
480 labeled with 1μM CellTrace Far Red (ThermoFisher Sci). Targets were then divided
481 into two populations: $NS4b_{2488}$ targets were labeled with Cell Trace Violet
482 (ThermoFisher Sci) 5mM for 10^{-6}M peptide loaded targets and 0.5mM for non-loaded
483 negative control) and ENV_{641} targets were labeled with CFSE (ThermoFisher Sci) 5mM
484 for 10^{-6}M peptide loaded targets and 0.5mM for non-loaded negative control. All
485 labeling was done as described in manufacturer instructions. Peptide loading was 1
486 hour at 37°C. After thorough washing, targets were pooled and injected intravenously
487 (i.v.) into infected and naïve mice. After 4 hours mice were sacrificed spleen
488 disassociated to a single cell suspension. Splenocytes were gated on total targets
489 (CellTrace Far Red positive) then CellTrace Violet high and low or CFSE high and low.
490 Calculation of killing: 100% - [(% peptide bearing targets IMMUNE recipients / % no

491 peptide targets IMMUNE recipients) / (% peptide bearing targets NAIVE recipients / %
492 no peptide targets NAIVE recipients) x 100].
493

494 **STATISTICS**:

495 GraphPad Prism® version 9.2.0 (GraphPad Software) was used for data analysis. All
496 experiments were performed at least twice. Significance was determined as described
497 in figure legends.

498

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506

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510

511 **AUTHOR CONTRIBUTIONS:**

512 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.,
513 and C.P.C. performed experiments and analyzed the data; K.O.M., M.C.L., M.P.B.
514 contributed human samples; J.L.U. prepared the figures; J.L.U. and J.N-Ž. wrote the
515 manuscript; all authors edited the manuscript.

516

DATA AVAILABILITY:

All data available upon request.

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636

637 **FIGURE LEGENDS:**

638

639 Figure 1

640 **Older adults experience more severe WNV disease and have a dearth of naïve**
641 **CD8 T cells.**

642 A cohort of 52 patients with WNV disease severity clinically classified as Asymptomatic,
643 WN Fever (WNF), WN Meningitis (WNM), and WN meningitis and encephalitis (WNME)
644 were retrospectively recruited to participate in our study. The age of the patients
645 experiencing the most severe disease is significantly older than asymptomatic (A).
646 Naïve ($CD95^{low}$ and $^{CD28int/high}$, also confirmed additionally to be CCR7hi and CD45RA+)
647 CD4 and CD8 T cells were phenotyped by FCM and counts were extrapolated from
648 clinical cell blood count (CBC) data collected on the fresh blood sample. Statistical
649 significance evaluated by Kruskal-Wallis with Dunn's multiple comparisons test between
650 all groups, +SEM. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

651

652 Figure 2

653 **Decreased numbers of naïve CD8 T cells in both young and old mice correlate**
654 **with increased susceptibility to WNV.**

655 Adult (3-5 months) and old (19-22 months) C57BL/6 mice were infected with WNV,
656 1000pfu, f.p.; bled on day 7 for FCM; and followed for susceptibility (A, schema).
657 Absolute numbers of naïve CD8 or CD4 T cells were enumerated per mL of blood on
658 day 7 post-infection by FCM (B, C). Data compiled from two independent experiments.
659 Statistical significance evaluated by t-test, median. *$P \leq 0.05$; **$P \leq 0.01$; ****$P \leq$
660 0.0001.

661

662 Figure 3

663 **IL-7C treatment expands the total number of T cells available in secondary**
664 **lymphoid tissues.**

665 Old (19-22 months) C57BL/6 mice were treated with IL-7C on days 1, 3, and 5 of the
666 experiment and sacrificed on days 7, 10, and 14 post-first treatment alongside untreated
667 adult (3-5 months) and old (19-22 months) (schema, A). The total number of
668 lymphocytes (B), CD4 T cells (C), and CD8 T cells (D) are increased at indicated day(s)
669 post-first treatment. The total number of naïve, CD44 low and CD62L high, CD4 and
670 CD8 T cells are not significantly increased at any day post-treatment (E, F). The
671 expansion of CD4 T cells is not dominated by either CM (CD44 high, CD62L high, and
672 CD49d high) or virtual memory (CD44 high, CD62L high, and CD49d low) phenotyped
673 cells (G,H). Both CM and VM phenotype CD8 T cells are expanded at day 10 post-first
674 treatment (I,J). WNV-specific CD8 CTLp (Db-NS4b$_{2488}$) are not reliably increased by IL-
675 7C treatment (K). (B-J) Data shown is representative of two experiments with similar
676 results and statistical significance evaluated by one-way Anova with Dunn's multiple
677 comparisons test. All comparisons are to untreated old mice. *$P \leq 0.05$; **$P \leq 0.01$. (K)
678 IL-7C treated group is day 13 post-first treatment. Data compiled from three
679 independent experiments and statistical significance evaluated by t-test comparison to
680 O, median. *$P \leq 0.05$.

681

682 Figure 4

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

685 Adult (3-5 months), Old (19-22 months), and Old (19-22 months) mice treated with IL-
686 7C 13 days before challenge are infected with WNV (1000pfu, f.p.; n=45-52 per group).
687 On day 7 post-WNV infection mice are bled and monitored for survival (schema, A).
688 Number of WNV-specific CD8 T cells per mL of blood on day 7 post-WNV (B). WNV
689 survival at day 45 post-infection (C). Number of WNV-specific CD8 T cells from (B)
690 broken by those that survived and those that died from WNV in each group (C).
691 Neutralizing antibody titers on day 7 post-WNV broken by those that survived and those
692 that from died from WNV in each group (E). *In vivo* killing assay on day 7 post-WNV for
693 $NS4b_{2488}$ (F) and ENV_{641} (G). Total WNV-ENV and DIII-specific memory antibody levels
694 >45 days post-infection (Figure H, I). Data compiled from two experiments. Statistical
695 significance evaluated by anova in (B, F, G) and t-test in (D) and (E) as represented on
696 graphs. Significance of survival evaluated by Log-rank (Mantel-Cox) test. $^{*}P \leq 0.05$; $^{**}P$
697 ≤ 0.01; $^{***}P \leq 0.001$; $^{****}P \leq 0.0001$.

698

699 Figure 5

700 **IL7-C treatment does not induce T cell exhaustion.**

701 Adult (3-5 months) and Old (19-22 months) mice were treated with IL-7C 13 days before
702 infection with WNV (1000pfu, f.p.). On day 7 post-WNV infection mice were bled and
703 circulating PBMC's were analyzed by FCM for expression of PD1, CD244 (2B4), and
704 CD223 (LAG-3) on $NS4b_{2488}$+ CD8 T cells (A-C). Data compiled from two experiments.
705 Statistical significance evaluated by anova in (all groups) and t-test in (by age) as
706 represented on graphs. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

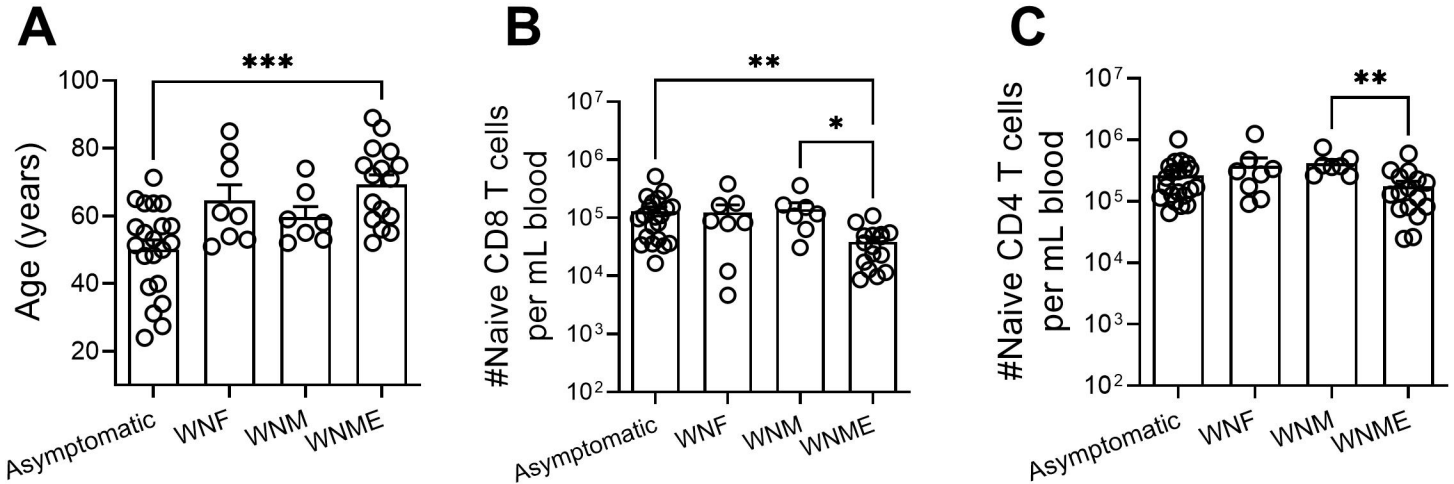
707

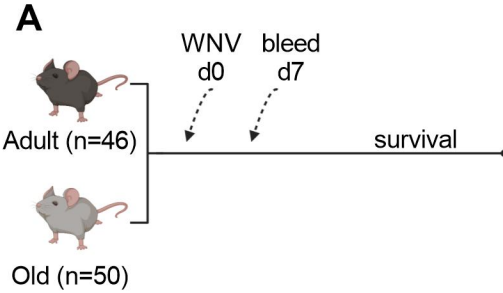
708

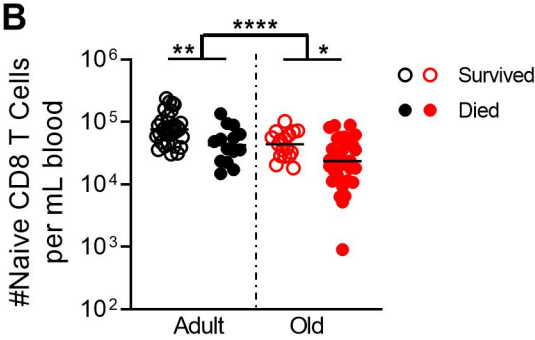
709 Figure 6

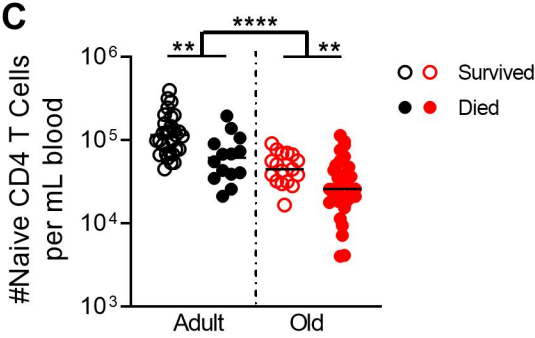
710 **Adoptive transfer of WNV-I Tg CD8 T cells rescues old mice from WNV**
711 **susceptibility.**

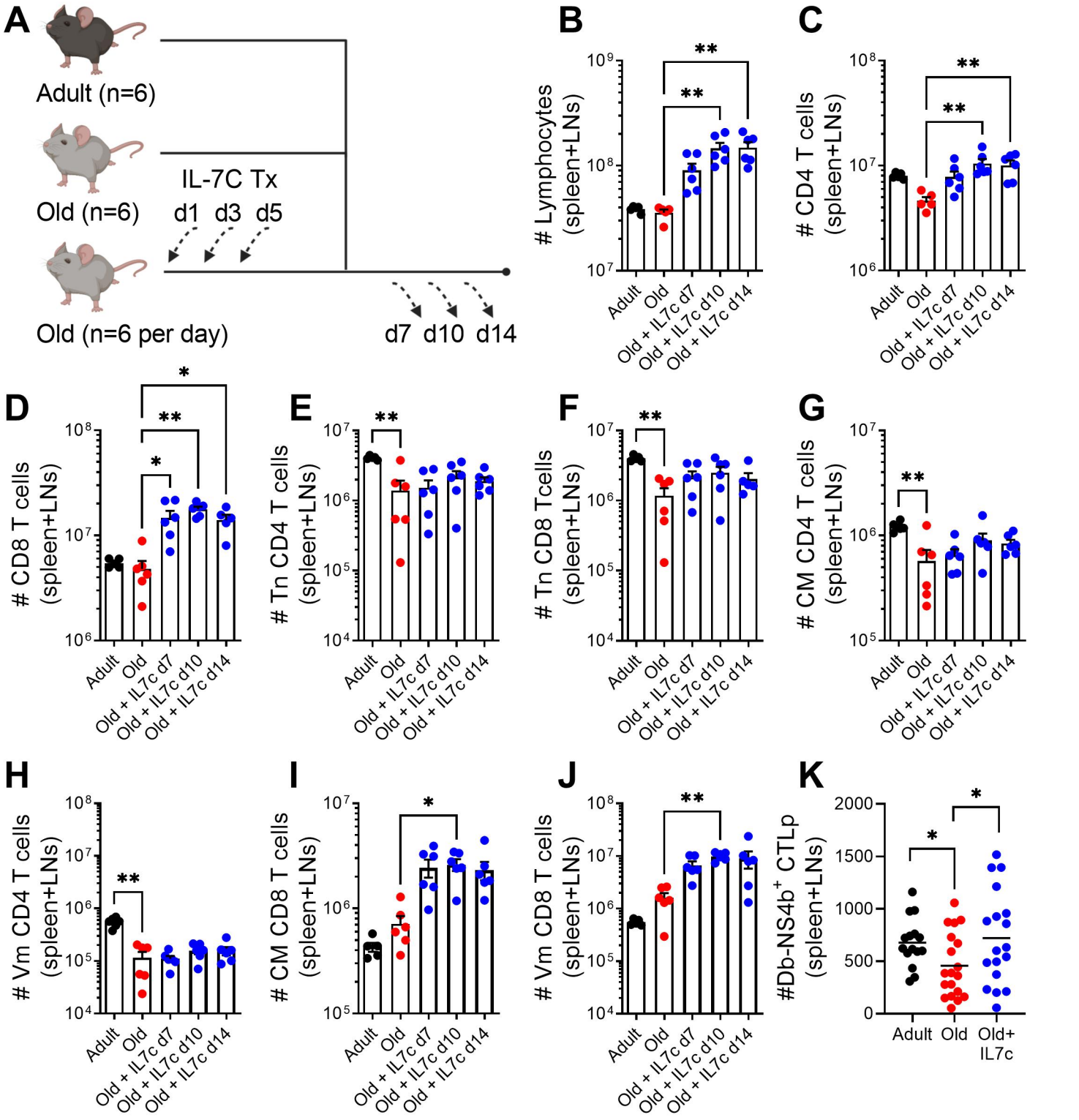
712 WNV-I CD8 T cells (5400, 1600, or 600) were transferred to groups of old mice (19-22
713 months) alongside adult (3-5 months) and Old (19-22months) mice the day before WNV
714 challenge (1000pfu, f.p.). On day 7 post-infection mice were bled and total numbers of
715 circulating WNV-specific CD8 T cells were enumerated per mL of blood (A). Mice were
716 monitored for survival to day 45 post-infection (B). The frequency of donor WNV-I CD8
717 T cells (CD45.1) contributing to the response in (A) is shown in (C). *In vivo* killing assay
718 on day 7 post-WNV for $NS4b_{2488}$ (D). Data compiled (A-C) or representative (D) of two
719 experiments. Statistical significance evaluated by anova (A, C, D) with multiple
720 comparisons test. Significance of survival evaluated by Log-rank (Mantel-Cox) test. *P
721 ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001.

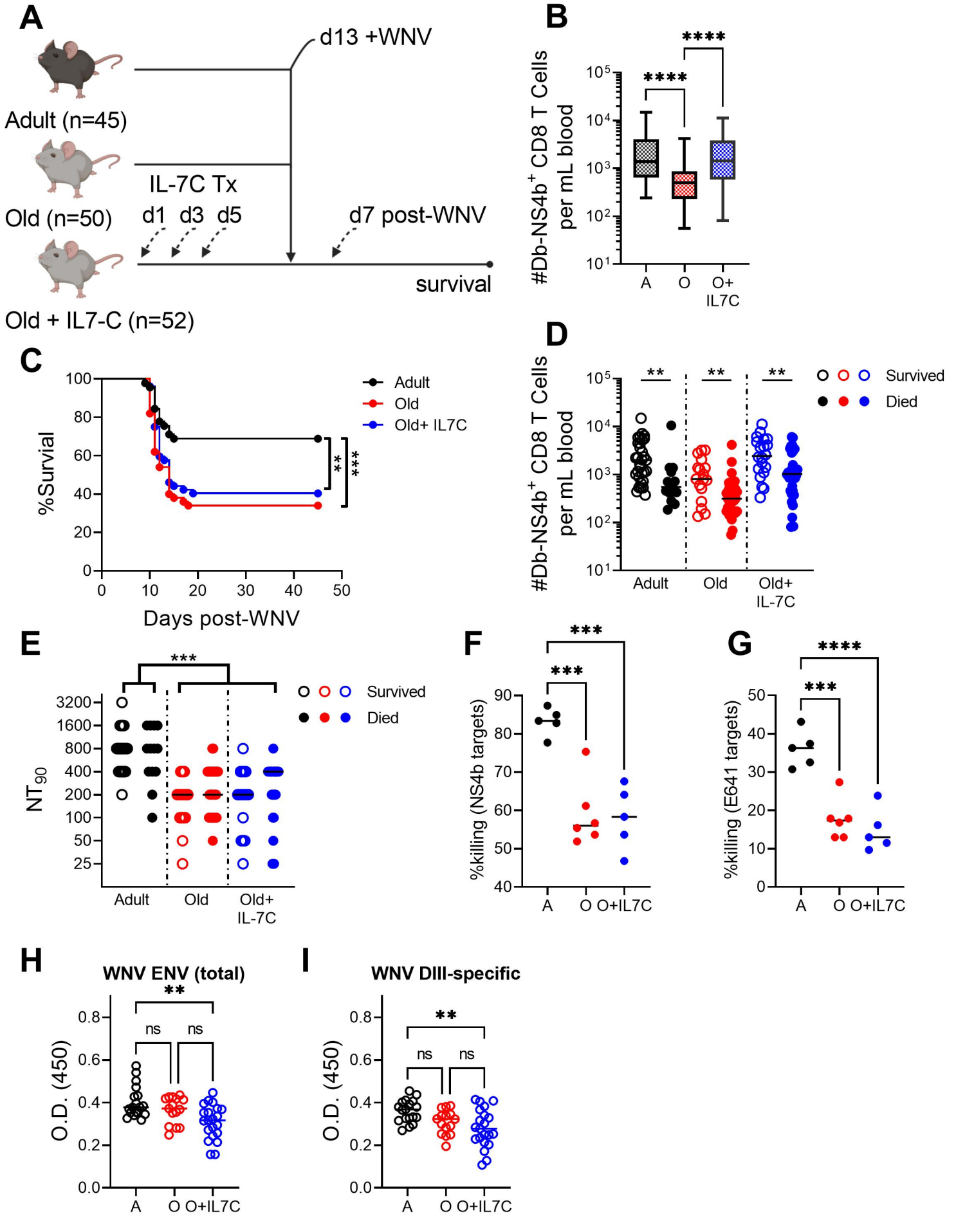

A
***
Age (years)
100
80
60
40
20
Asymptomatic
WNF
WNM
WNME
B
**
*
#Naive CD8 T cells per mL blood
10^7
10^6
10^5
10^4
10^3
10^2
Asymptomatic
WNF
WNM
WNME
C
**
#Naive CD4 T cells per mL blood
10^7
10^6
10^5
10^4
10^3
10^2
Asymptomatic
WNF
WNM
WNME

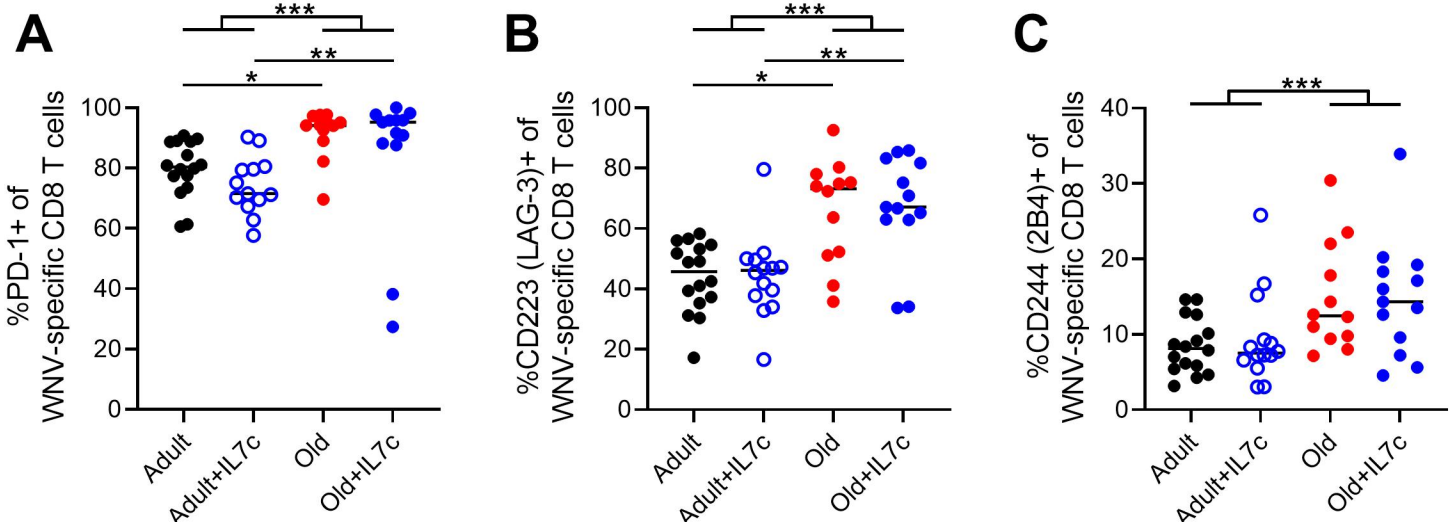

A
WNV
d0
bleed
d7
Adult (n=46)
survival
Old (n=50)

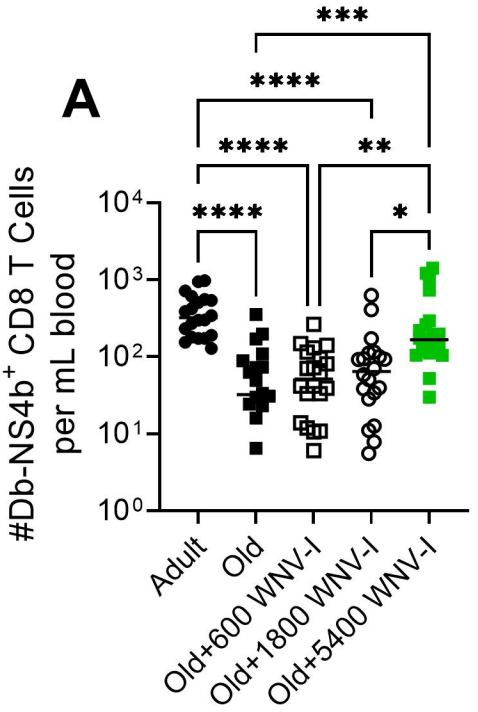

B
****
**
*
#Naive CD8 T Cells
per mL blood
10^6
10^5
10^4
10^3
10^2
Adult
Old
Survived
Died

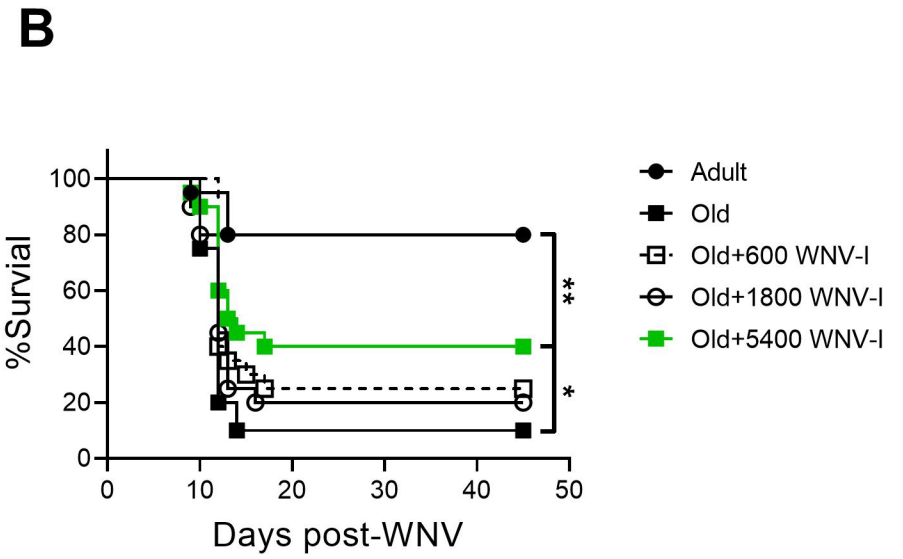

C
****
**
**
#Naive CD4 T Cells
per mL blood
10^6
10^5
10^4
10^3
Adult
Old
Survived
Died

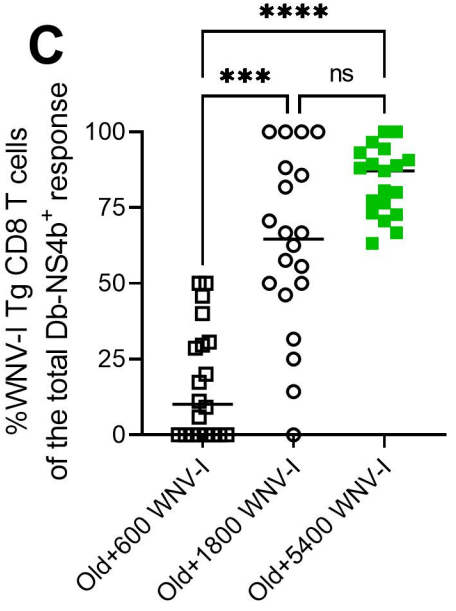

A
Adult (n=6)
Old (n=6)
IL-7C Tx
d1 d3 d5
Old (n=6 per day)
d7 d10 d14
B
Lymphocytes (spleen+LNs)
C
CD4 T cells (spleen+LNs)
D
CD8 T cells (spleen+LNs)
E
Tn CD4 T cells (spleen+LNs)
F
Tn CD8 Tcells (spleen+LNs)
G
CM CD4 T cells (spleen+LNs)
H
Vm CD4 T cells (spleen+LNs)
I
CM CD8 T cells (spleen+LNs)
J
Vm CD8 T cells (spleen+LNs)
K
#Db-NS4b+ CTLp (spleen+LNs)
Adult
Old
Old + IL7c d7
Old + IL7c d10
Old + IL7c d14
Old+ IL7c

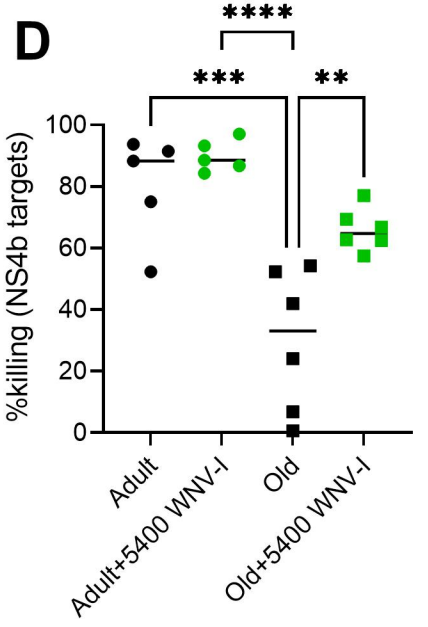

A
d13 +WNV
Adult (n=45)
Old (n=50)
IL-7C Tx
d1 d3 d5
d7 post-WNV
survival
Old + IL7-C (n=52)
B
#Db-NS4b+ CD8 T Cells per mL blood
A
O
O+ IL7C
C
%Survial
Days post-WNV
Adult
Old
Old+ IL7C
D
#Db-NS4b+ CD8 T Cells per mL blood
Survived
Died
Adult
Old
Old+ IL-7C
E
NT90
Survived
Died
Adult
Old
Old+ IL-7C
F
%killing (NS4b targets)
A
O
O+IL7C
G
%killing (E641 targets)
A
O
O+IL7C
H
WNV ENV (total)
O.D. (450)
ns
ns
A
O
O+IL7C
I
WNV DIII-specific
O.D. (450)
ns
ns
A
O
O+IL7C


A
B
C
***
**
*
%PD-1+ of WNV-specific CD8 T cells
%CD223 (LAG-3)+ of WNV-specific CD8 T cells
%CD244 (2B4)+ of WNV-specific CD8 T cells
0
20
40
60
80
100
10
30
Adult
Adult+IL7c
Old
Old+IL7c


A
#Db-NS4b+ CD8 T Cells per mL blood
Adult
Old
Old+600 WNV-I
Old+1800 WNV-I
Old+5400 WNV-I


B
%Survial
Days post-WNV
Adult
Old
Old+600 WNV-I
Old+1800 WNV-I
Old+5400 WNV-I


C
%WNV-I Tg CD8 T cells of the total Db-NS4b+ response
ns
Old+600 WNV-I
Old+1800 WNV-I
Old+5400 WNV-I


D
%killing (NS4b targets)
Adult
Adult+5400 WNV-I
Old
Old+5400 WNV-I