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1 # Neuropilin-1 regulates the secondary CD8 T cell response to virus infection

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14 <u>Running title:</u> Neuropilin-1 regulates secondary CD8 T cell responses

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15 **Abstract**

16 Neuropilin-1 plays important roles in axonal guidance in neurons, and in the growth of new blood vessels.
17 There is also a growing appreciation for roles played by neuropilin-1 in the immune response. This molecule is
18 important for the function of regulatory T cells, however roles in other T cell populations have not been
19 identified. Here we show that neuropilin-1 is expressed during the peak of the antiviral CD8 T cell response
20 during murine gammaherpesvirus infection. Using a conditional knockout model, we deleted Nrp1 either before
21 infection, or after CD8 T cell memory had been established. We found deletion of Nrp1 skewed the acute CD8
22 T cell response toward a memory precursor-like phenotype, however the ensuing resting memory response
23 was similar regardless of Nrp1 expression. Interestingly Nrp1 deletion had differing effects on the recall
24 response depending on the timing of deletion. When deleted before infection, Nrp1 deficiency inhibited the
25 secondary response. Deletion just prior to re-exposure to virus lead to an enhanced secondary response.
26 Interestingly these effects were observed only in mice infected with a persistent strain of murine
27 gammaherpesvirus, and not a non-persistent mutant strain. These data highlight a multifaceted role for
28 neuropilin-1 in memory CD8 T cell differentiation, dependent upon the stage of the T cell response and
29 characteristics of the infectious agent. Several therapeutic anti-cancer therapies focus on inhibition of Nrp1 to
30 restrict tumor growth, so knowledge of how Nrp1 blockade may affect the CD8 T cell response will provide a
31 better understanding of treatment consequences.

32

33 **Importance**

34 CD8 T cell responses are critical to control both virus infections and tumors. The ability of these cells to persist
35 for long periods of time can result in lifelong immunity, as relatively small populations of cell can expand rapidly
36 to counter re-exposure to the same insult. Understanding the molecules necessary for this rapid secondary
37 expansion is critical if we are to develop therapies that can provide lifelong protection. This report shows an
38 important and complex role for the molecule neuropilin-1 in the secondary response. Several cancer therapies
39 targeting neuropilin-1 are in development, and this work will lead to better understanding of the effect these
40 therapies could have upon the protective CD8 T cell response.

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41 # INTRODUCTION

42

43 Neuropilin-1 (Nrp1) is a type I transmembrane protein with multiple domains that functions as a co-receptor for
44 several ligands, such as semaphorins (SEMA), vascular endothelial growth factor (VEGF), and transforming
45 growth factor beta (TGFβ). The molecule itself lacks kinase activity, but it associates with other receptors such
46 as integrins, plexins and VEGF receptor, that mediate transmembrane signaling[1]. It was first studied in the
47 nervous system, where neuropilin-1 is known to participate in neuronal development and provide cues for
48 axonal guidance[2]. Later the interaction between VEGF and Nrp1 was found to play an important role in
49 angiogenesis[3,4]. The involvement of Nrp1 in the growth of new blood vessels in tumor vasculature promotes
50 tumor progression, and its blockade can restrict tumor growth[5,6]. Nrp1 can also be expressed by tumor cells
51 themselves, and a peptide which inhibits VEGF-Nrp1 interactions has been shown to induce a apoptosis of
52 Nrp1-expressing breast tumor cells[7].

53

54 Tumors often elaborate an immunosuppressive microenvironment, and neuropilin-1 has been shown to play
55 important roles in suppression mediated by regulatory T cells (T_{reg}). A recent study showed Nrp1 on T_{reg} was
56 required for the suppression of the anti-tumor T cell response, and to cure inflammatory colitis[8]. Engagement
57 of Nrp1 promoted T_{reg} quiescence and limited differentiation, resulting in enhanced T_{reg} stability in the tumor[8].
58 Expression of Nrp1 differentiates natural from inducible regulatory T cells in some physiological settings[9,10],
59 and also identifies $CD4^+C25^-$ T cells with inhibitory function and the ability to recruit conventional T_{reg}[11].
60 Interestingly a recent report showed Nrp1 expression on group 3 innate lymphoid cells (ILC3s) in the lung with
61 lymphoid tissue inducer activity, and suggested functions in the early development of tertiary lymphoid
62 aggregates in the lung and/or pulmonary angiogenesis[12]. Collectively these studies imply Nrp1 not only has a
63 major impact in modulating responses to tumors, but also plays a role in immune regulation and tissue
64 remodeling other physiological settings.

65

66 Nrp1 clearly plays important roles in immune regulatory cell populations, however its role on conventional T
67 cell populations has not been determined. CD8 T cells are important in controlling virus infections and
68 restricting growth of tumors, providing lifelong immunity by developing into memory cells that can respond

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69 rapidly to re-infection. Memory cells develop from memory precursors present early in the T cell response[13],
70 and signals from cytokines, costimulatory molecules and CD4 T cells are necessary for them to develop
71 optimal recall responses[14]. In this study, we investigated the role of Nrp1 on the CD8 T cell response to murine
72 gammaherpesvirus (MHV-68) infection using a conditional knockout model. By means of this strain, we could
73 restrict the deletion only to CD8 T cells, and regulate the timing of the deletion by tamoxifen administration.
74
75 We show that Nrp1 is highly upregulated on CD8 T cells during the acute phase of viral infection, and deletion
76 of Nrp1 during this window skewed the T cells more toward memory precursors than terminally differentiated
77 effector cells. Interestingly, 'early' Nrp1 deletion resulted in weaker CD8 T cell expansion following virus
78 rechallenge, suggesting Nrp1 signaling during priming promotes optimal 'programming' of memory CD8 T cells.
79 Interestingly when deletion of Nrp1 occurred just before the recall response, the magnitude of the response
80 was higher, indicating Nrp1 signals restrain the recall response. Interestingly these effects were only observed
81 with a persistent strain of MHV-68, but Nrp1 did not appear to affect recall responses in infection with a non-
82 persistent strain of the virus. What emerges is a complex role for Nrp-1 in the CD8 T cell response, which is
83 dependent both upon the timing of Nrp-1 expression during the primary vs secondary response, and the nature
84 of the infection.
85
86
87
88

89 # MATERIALS AND METHODS

90

91 **Mice and MHV-68 infection**

92 C57BL/6NCrl (B6) mice were originally obtained from Charles River Laboratory. Nrp1 E8i-CreERT2 R26-YFP
93 mice were kindly provided by Dr. Dario A. Vignali, from The University of Pittsburgh. Primers used to genotype
94 the strain were as follows:
95

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Nrp1-Forward	AGGTTAGGCTTCAGGCCAAT
Nrp1-Reverse	GCAGATCTCTTCCCTGCAAC
Rosa26-YFP-1	GCGAAGAGTTTGTCCTCAACC
Rosa26-YFP-2	GGAGCGGGAGAAATGGATATG
Rosa26-YFP-3	AAAGTCGCTCTGAGTTGTTAT
E8i-Cre-IC-1	CTAGGCCACAGAATTGAAAGATCT
E8i-Cre-IC-2	GTAGGTGGAAATTCTAGCATCATCC
E8i-Cre-ER7-For	CCACCGAGTCCTGGACAAGATCAC
E8i-Cre-IRES-Rev	CCTCGACTAAACACATGTAAAGCATG

96

97 Mice were maintained under specific pathogen–free conditions in the Dartmouth Center for Comparative
98 Medicine and Research. The Animal Care and Use Committee of Dartmouth College approved all animal
99 experiments. MHV-68 containing a frameshift mutation in ORF73 (FS73) and the revertant virus (FS73R) were
100 originally obtained from Stacey Efstathiou at The University of Cambridge, UK. 7-week-old mice were primarily
101 infected with $4x10^3$ PFU by the intranasal route in 30µl Hank's balanced salt solution (HBSS), and
102 rechallenged with $1x10^6$ PFU WT MHV-68 by the intraperitoneal route.

103

104 **Tamoxifen treatment, T cell purification, and adoptive transfer**

105 Tamoxifen (VWR) was suspended in 5% (v/v) EtOH-corn oil (Ward's science), warmed at 37°C for at least 30
106 minutes before treatment, and 1 mg/100µl/mouse was given intraperitoneally for 5 consecutive days; starting
107 at day -6 relative to infection (d-6) for early Nrp1 deletion and d28 for late Nrp1 deletion. CD8 T cells were
108 purified from the spleens of early Nrp1 deleted (d28) or late Nrp1 deleted (d34) Nrp1 E8i-CreERT2 R26-mice
109 using EasySep™ Mouse CD8 T Cell Isolation Kits (Stemcell Technologies) according to the manufacturer's
110 instructions. Single T cell preparations were >95% pure as determined by flow cytometry. CD8 T cell
111 populations containing $2x10^4$ ORF61 tetramer$^+$ memory CD8 T cells were injected by the retro-orbital route into
112 B6-Ly5.1 recipients. The recipients were rechallenged one day after the adoptive T cell transfer, and
113 splenocytes were collected on day 6 post-infection.

114

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115 **Cell preparation, flow cytometry, and proliferation assay**

116 Single-cell suspensions from spleen were prepared by passing them through cell strainers, and resuspended
117 in Gey's solution (150 mM NH_4Cl, 10 mM $KHCO_3$, and 0.05% phenol red) for 5 min to lyze red cells. Cell
118 suspensions were then filtered through a 70 µm nylon cell strainer (BD Biosciences), washed, and
119 resuspended in PBS with 2% bovine growth serum (BGS) and APC-conjugated tetramer specific for the MHV-
120 68 dominant epitope (ORF61, NIH tetramer core facility) at room temperature for 1 hour, followed by 10 µg/ml
121 Fc Block (2.4G2; Dartab) on ice for 10 min before staining with the following fluorochrome-conjugated
122 antibodies (Abs): anti-cluster of differentiation (CD) 8-BV510 (CD8; 53-6.7), anti-CD45.2-BV421 (104), anti-
123 CD45.2-BV650 (104), anti-CD304-BV421 (Neuropilin-1; 3E12), and anti-KLRG1-PE-Cy7 (2F1/KLRG1; all from
124 BioLegend), and anti-CD4-APC (GK.15) and anti-CD127-APC 780 (A7R34; all from eBioscience).
125 LIVE/DEAD^{TM} Fixable Near-IR Dead Cell Stain Kit (Thermo Fisher) was used of cell viability, and Click-iT^{TM}
126 Plus EdU Pacific Blue^{TM} Flow Cytometry Assay kit (Thermo Fisher) was used to assess cell proliferation. Cells
127 were analyzed with MACSQuant (Miltenyi) FACS Aria (Becton Dickinson) or CytoFLEX S (Beckman Coulter)
128 flow cytometers at the Dartlab flow cytometry core facility.

129

130 **Statistical Analysis**

131 Two way ANOVA-Sidak's or Dunnett's multiple comparison test was used (GraphPad Prism Version 7.0). P
132 values of less than 0.05 were considered statistically significant.

133

134

135 # RESULTS

136 **Nrp1 is upregulated with both persistent and non-persistent MHV-68 infection**

137 Our previous research has shown the CD8 T cell response differs between infection with a mutant MHV-68
138 with a deletion in ORF73 (FS73)[15,16], which is essential for latent infection, when compared with a revertant
139 virus that retains the ability to persist in the host. In order to understand the role of Nrp1 on CD8 T cells upon
140 MHV-68 infection, we initially measured the kinetics of Nrp1 expression on CD8 T cells after either persistent
141 (FS73R) or non-persistent (FS73) MHV-68 infection. Mice were infected with the relevant virus, then at various
142 times post infection spleens cells were stained with MHC/peptide tetramers and anti-CD8 antibody to measure

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143 the frequency of CD8 T cells recognizing the dominant epitope[17] (Fig. 1A and B). Consistent with our previous
144 studies[16], the magnitude of the CD8 T cell response was greater in the FS73R infected mice during the first
145 four weeks of infection, however memory populations were of similar size in both strains (Figs 1A and B). Nrp1
146 expression was low in both cases during the early stages of infection (d7), but were significantly upregulated
147 on d14, when CD8 T cell responses peak in MHV-68 infection[16,17] (Fig. 1C). Nrp-1 expression slowly declined
148 after 14 days, and had reduced to baseline expression levels by 60 days post-infection. While Nrp-1 was
149 induced with these kinetics in both FS73 and FS73R infection, the induction was significantly greater after
150 FS73 infection from days 14-21 post infection, but not significantly different thereafter (Fig. 1C and D). This
151 lead to the T cell response to FS73 being dominated by Nrp1^{hi} cells during the acute infection (Fig. 1E),
152 whereas there were more similar proportions of Nrp1^{hi} and Nrp1^{lo} cells at most times during the response to
153 FS73R (Fig. 1F). In both cases the majority of memory CD8 T cells at d100 were Nrp1^{hi} (Figs 1E and 1F).
154 These data indicate the absence of persistent infection leads to a greater induction of Nrp1 in the responding
155 CD8 T cell population.
156

157 **Tamoxifen-induced Nrp1 excision and YFP expression on CD8 T cells**

158 In order to determine the role of Nrp1 in the antiviral CD8 T cell response, we wished to use an inducible
159 knockout system that deletes Nrp1 selectively in CD8 T cells, and only after induction by tamoxifen, as Nrp1 is
160 important in the development of embryonic blood vessels and a systemic knockout is lethal[4]. We therefore
161 exploited conditional Nrp1 knockout transgenic mice (Nrp1 E8i-CreERT2 R26-YFP; Nrp1cKO mice, Fig. 2)
162 where the E8i-creERT2 cassette confers CD8 specificity[18], but cre is translocated to the nucleus only after
163 tamoxifen treatment. These mice also contain floxed Nrp-1 allele and a Rosa26-flox-stop-flox-YFP sequences
164 resulting in deletion of Nrp-1 and expression of YFP in cells where cre is active. Then we characterized these
165 mice to verify inducible deletion by treating Nrp1cKO mice or B6 with tamoxifen (1 mg/mouse) for 5
166 consecutive days (Fig. 3A), and measured Nrp1 expression on CD8 T cells 48 hours after the last treatment.
167 We observed that YFP expression was induced on CD8 T cells in Nrp1cKO (Fig. 3B, third panel) but not B6
168 mice (Fig. 3B, second panel). While there was only a very small population of CD8 T cells expressing YFP
169 after vehicle treatment (Fig. 3B, first panel), this rose to 66% following tamoxifen treatment (Fig. 3B, third
170 panel). Tamoxifen-mediated induction of YFP was not observed in CD4 T cells in these mice (Fig. 3B, forth

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171 panel), confirming that cre-mediated deletion was limited to CD8 T cells. To confirm YFP expression correlated
172 with Nrp1 cell surface expression, we stained for Nrp1 on CD8 T cells from mice treated as described, and
173 found this molecule was absent from the YFP^{+} population, but present on a proportion of YFP^{-} cells (Fig. 3B,
174 lower panels, Fig. 3C). After tamoxifen treatment there was still an easily detectable population of Nrp1^{+} CD4 T
175 cells (Figs. 3B and 3C), demonstrating the absence of Nrp1 deletion in this population. These data confirmed
176 that tamoxifen treatment effectively abrogated Nrp1 expression on CD8 T cells and cells lacking Nrp1 were
177 marked by YFP fluorescence.
178

179 **Effect on the CD8 T cell responses of Nrp1 deletion**

180 To test the effect of Nrp1 on CD8 T cell differentiation during murine gammaherpesvirus infection, we treated
181 Nrp1 mice with tamoxifen as described above, and infected them with FS73 or FS73R two days after the last
182 tamoxifen treatment (Fig. 4A). Vehicle treated mice exhibited a very small YFP+ population, but this was
183 greatly enlarged after tamoxifen treatment (Fig. 4B). To measure the effect of Nrp1 on CD8 T cell expansion
184 and memory formation, we compared the proportion of YFP^{+} and YFP^{-} cells that stained with a tetramer
185 identifying CD8 T cells recognizing the dominant ORF61 epitope. In this way we had an internal control in
186 each mouse, normalizing for variations in virus titers or other variables from mouse to mouse, and enabling the
187 use of paired statistical tests for significance. The frequency of tetramer positive cells were not significantly
188 different in YFP^{+} and YFP^{-} cells at 14 days post-infection, regardless of virus strain (Fig. 4C), indicating the
189 magnitude of the effector response was not altered by absence of Nrp1. However we did detect differences in
190 the differentiation status of the CD8 T cells. On day 14 in blood, we found that most tetramer^{+}YFP^{+}CD8 T cells
191 (Nrp1 deleted) had the phenotype of precursors of memory cells (KLRG-1^{-}CD127^{+}), whereas the majority of
192 tetramer^{+}YFP^{-}CD8 T cells (expressing Nrp1) were terminally differentiated effector T cells (KLRG-1^{+}CD127^{-};
193 Fig. 4D and 4E). These results were comparable between persistent and non-persistent infections, although in
194 FS73R infection the KLRG-1^{+}CD127^{+} population was as prominent as the KLRG-1^{-}CD127^{+} population (Fig.
195 4E). We have previously shown that most memory CD8 T cells after infection with either the FS73 or FS73R
196 viruses do not upregulate CD127, unlike that seen in other infection models[16]. Here we observed most YFP^{+}
197 and YFP^{-} CD8 T cells in the spleen became KLRG-1^{+}CD127^{-} by day 28 (Fig. 4F and 4G), indicating the
198 absence of Nrp-1 does not affect this phenotype. These data showed Nrp1 favored the differentiation of

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199 effector CD8 T cells during the peak response, and in it's absence T cells differentiated preferentially toward
200 the memory precursor phenotype. However these changes did not endure to the memory phase, where both
201 Nrp1 sufficient and deficient CD8 T cells had similar phenotypes.
202
203 **Effect of Nrp1 on the recall response**
204 Having observed a role for Nrp1 in the formation of memory CD8 T cells during the effector response, we next
205 tested whether the quality of the resulting memory population was altered by re-challenging with virus. As in
206 previous experiments, tamoxifen was administered before infection to delete Nrp1, then splenic CD8 T cells
207 were purified on d28 post-infection, and adoptively transferred into congenic recipient mice (Fig. 5A). YFP
208 expression was then used to identify the response from CD8 T cells with intact Nrp1 (YFP^-) or deleted Nrp1
209 (YFP^+; Fig. 5B). Recipients were rechallenged with virus, then six days later spleens were removed and the
210 expansion of adoptively transferred cells measured by flow cytometry. Memory CD8 T cells from mice infected
211 with FS73 expanded comparably regardless of Nrp1 expression status (Fig. 5C), whereas the expansion of
212 memory cells from FS73R donors was significantly reduced when they lacked Nrp1 (YFP^+, Fig. 5D).
213 In the previous experiment Nrp1 was absent both during the primary CD8 T cell response, where
214 memory 'programming' occurs, and also after CD8 T cells differentiated into memory cells. Next we tested
215 whether the absence of Nrp1 during the secondary response alone affected T cell expansion. To examine this,
216 we performed a similar experiment, but this time tamoxifen treatment started at d28 post-infection, then
217 spleens were harvested two days after the cessation of treatment (Fig. 6A). As before, purified CD8 T cells
218 were transferred into congenic recipient mice that were then infected with MHV-68. Six days later both YFP^+
219 and YFP^- CD8 T cells from FS73 infected donors expanded to a similar extent (Fig. 6B). However YFP^+ CD8 T
220 cells from FS73R donor mice expanded to a greater extent than YFP^- cells (Fig. 6C), indicating Nrp1
221 expression during the secondary response limits the extent of T cell expansion. This greater expansion was
222 attributed to better cell survival (lower frequencies of dead cells; Fig. 7A) and more actively proliferating cells
223 (EdU incorporation; Fig. 7B) among the YFP^+ population. These data indicated that Nrp1 restrains cellular
224 proliferation and survival during the recall response, but it also plays a different role during the priming or post-
225 priming phase of the response, promoting the development of memory cells capable of making an optimal
226 recall response.

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227

228

229 ## DISCUSSION

230

231 This work shows clearly that neuropilin 1 plays an important role both in the differentiation of memory precursor
232 cells and their capacity to mount a recall response. Cell surface Nrp1 expression was induced at the peak of
233 the virus-specific CD8 T cell response, then declined slowly as these cells differentiated to memory cells.
234 Interestingly we detected higher level induction in mice infected with the non-persistent FS73 strain. Our
235 previous research showed a potent CD8 T cell response is induced after infection with either FS73 or FS73R
236 strains of MHV-68[16]. However the capacity of the persistent strain to establish a latent infection in the spleen
237 leads to splenomegaly, which increases the total number of virus-specific CD8 T cells, and also likely creates a
238 more pro-inflammatory environment in the spleen. While the regulation of Nrp1 in T cells is not known, it is
239 possible Nrp1 expression is restrained by these pro-inflammatory signals. It is particularly interesting that Nrp1
240 deletion only affects recall responses in FS73R infected mice, despite observing lower Nrp1 expression after
241 infection with this persistent strain. The reasons for this effect are currently unclear, but may be due to the fact
242 that during persistent infection there is sporadic reactivation, re-exposing the T cell response to viral antigens.
243 This would lead to antigen presentation by infected B cells, which express the Nrp1 ligand semaphorin 4A[19],
244 potentially signaling to virus-specific memory CD8 T cells. In the acute infection with the non-persistent FS73
245 strain, these interactions with semaphorin 4A expressing antigen presenting cells would be limited to lung-
246 draining dendritic cells and infected lung epithelial cells, but few B cells. Sporadic re-exposure to viral antigen
247 during persistent infection endows the memory CD8 T cells with the ability to elaborate antiviral effector
248 functions more quickly[16], indicating a heightened state of readiness to counter the virus when it reactivates.
249 Other evidence for these cells being in a different state of differentiation relative to memory cells in non-
250 persistently infected animals includes lower levels of Bcl-2, lower IL-2 production and a faster turnover[16].
251 Maintaining memory CD8 T cells in this differentiation state may be more dependent on Nrp1, which may also
252 have an impact on their ability to mount a recall response.

253

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254 Our data show that, regardless of virus strain, the absence of Nrp1 on CD8 T cells at the time of infection
255 resulted in a bias toward KLRG1^{-}CD127^{+} memory precursor phenotype cells at the expense of KLRG1^{+}CD127^{-}
256 effector cells at the peak of the response. However this reduction in the proportion of effector cells did not
257 reduce the overall magnitude of the response, indicating T cell proliferation is unaffected. Nrp1 therefore may
258 have a role in restraining the differentiation of memory precursors, while favoring differentiation of effector T
259 cells. This is counter to what may be expected based on a previous report that found Nrp1 at the
260 immunological synapse acts through the phosphatase PTEN to restrain Akt phosphorylation in T_{reg}[19]. In CD8 T
261 cells Akt promotes effector cell differentiation and glycolytic metabolism acting through mTOR and Tbet[20],
262 whereas inhibition of Akt promotes memory differentiation[21,22]. Therefore Nrp1 may be expected to restrict Akt
263 phosphorylation and promote memory differentiation, counter to that which we observed. This is likely due
264 differences in the signal transduction pathways present in T_{reg} and CD8 T cells. Further work is necessary to
265 uncover the underlying mechanism, and to determine why this apparent skewing to memory precursors does
266 not result in a larger long-term memory population.
267
268 Our use of a conditional deletion model where Nrp1 expression is abrogated on approximately half the CD8 T
269 cells, and the other half retain Nrp-1 expression, allowed us to perform very precise internally controlled
270 experiments measuring the secondary CD8 T cell response. A complex picture of the roles of Nrp1 during the
271 recall response emerges, with contrasting functions during different stages of the response. When deleted prior
272 to infection, the absence of Nrp1 reduced the size of the recall response, indicating Nrp1 promotes the ability
273 of memory cells to expand upon antigen re-exposure. While Nrp1 expression was most prominent on effector
274 cells, it was still expressed at a low level on memory CD8 T cells at d28 post infection. Therefore Nrp1 could
275 be acting in this context either by 'programming' appropriate differentiation of memory cells during the effector
276 response, signaling during the contraction and early memory phases, or potentially both.
277
278 To interrogate the function of Nrp-1 during the recall response itself, we allowed the effector and memory
279 response to develop in the presence of Nrp1 then deleted it just prior to memory cell harvest and cell transfer
280 to secondary recipients. In this context deletion of Nrp1 enhanced the recall response, by promoting T cell
281 viability and proliferation. This indicates during the differentiation of memory cells to effector cells Nrp1 serves

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282 to repress CD8 T cell proliferation and limit cell survival, presumably to limit the size of the T cell response and
283 prevent immunopathology during the recall response.
284
285 A previous report detailed a role for Nrp1 in the initiation a primary T cell response from human T cells[23].
286 Antibody blockade of Nrp1 reduced the proliferation of naïve T cells 50-60% when stimulated with allogeneic
287 dendritic cells, and Nrp1 was shown to cluster to dendritic cell:T cell contact areas. This contrasts with our
288 finding that the primary response was not affected by the presence or absence of Nrp1. There are many
289 differences between this report and our study, including the use of human cells vs mice, studying whole T cell
290 populations vs CD8 T cells, and studying allogeneic responses vs antigen-specific responses. One additional
291 potential reason for the difference in our findings is that antibody blockade of Nrp1 at the cellular interface may
292 result in steric hindrance of other important interactions, thereby reducing signaling through the immunologic
293 synapse. This is not a concern in our studies, as we used genetic deletion to ablate Nrp1 expression.
294
295 Our finding that Nrp1 expressed during the secondary response represses the proliferative response can be
296 seen as consistent with previous studies reporting inhibition of T cell responses by Nrp1. Semaphorin 3A
297 (Sema3A), a physiological ligand of Nrp1[24,25,26], inhibits *in vitro* DC-T cell interaction[27] and tumor-T cell
298 interaction[28], and by blocking Sema3A, hence suppressing the downstream signaling involving Nrp1, T cell
299 activation and proliferation were restored. However these studies used whole T cell populations, which include
300 T_{reg}, so the inhibition observed may have been due to T_{reg}-mediated suppression, in which Nrp1 plays a key
301 role[8,29,30], rather than direct interactions with CD8 T cells.
302
303 A previous report identified Nrp1 upregulation on anergic mouse CD8 T cells, but Nrp-1 did not appear to play
304 any role in the tolerant phenotype[31]. However to our knowledge our study is the first to interrogate Nrp1
305 function directly on an antigen-specific antiviral CD8 T cell response *in vivo*. It highlights complex roles for this
306 molecule in memory CD8 T cell differentiation and the secondary immune response, which depend upon the
307 stage of the response and the nature of the infection. While Nrp1-semaphorin interactions are known to have
308 an important impact on anti-tumor immunity, this study shows additional roles in antiviral immunity, and the
309 memory CD8 T cell response.

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313 ## ACKNOWLEDGEMENTS

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381 **Figure Legends**

382

383 **Figure 1. Nrp1 expression on CD8 T cells after persistent (FS73R) and non-persistent (FS73) MHV-68**
384 **infection.** (A) The proportion of ORF61-specific T cells among total splenic CD8 T cells after infection with
385 either the FS73 or FS73R strain of MHV-68. (B) Numbers of ORF61-specific CD8 T cells in spleens of mice
386 infected with either the FS73 or FS73R strain of MHV-68. (C) Histograms showing Nrp1 expression gated on
387 $CD8^+$ORF61 $tetramer^+$ splenocytes at the times post infection shown. Y axes in bottom plots are normalized to
388 the mode. (D) Nrp1 MFI of tetramer positive CD8 T cells compared over time for FS73 and FS73R infection. (E
389 and F) Frequency of ORF61 $tetramer^+$ CD8 T cells that were $Nrp1^-$ or $Nrp1^+$ after FS73 infection (E, panels on
390 right show representative plots showing gating strategy to distinguish $Nrp1^{hi}$ and $Nrp1^{lo}$ cells) or FS73R
391 infection (F). All data show mean ± SD of 4-5 mice per group; ***P*<0.01. Representative data from at least two
392 experiments are shown.

393

394 **Figure 2. Schematic representation of Nrp1 E8i-CreERT2 R26-YFP mice.** In the presence of tamoxifen,
395 regions flanked by loxP sequences are excised, which deletes Nrp1 in CD8 T cells, and removes the stop
396 sequence downstream of the ROSA26 promoter, resulting in YFP expression on CD8 T cells.

397

398 **Figure 3. Tamoxifen administration effectively deltes Nrp1 from CD8 T cells in E8i-CreERT2 R26-YFP**
399 **mice.** (A) Protocol for tamoxifen treatment. (B) YFP expression was measured on CD8 or CD4 T cells after
400 either tamoxifen or vehicle treatment in B6 or E8i-CreERT2 R26-YFP mice. Lower panels show Nrp1 staining
401 on the indicated populations. (C) Histograms showing Nrp1 staining on CD8 (left) and CD4 (right) T cells from
402 tamoxifen treated E8i-CreERT2 R26-YFP mice. Representative data from at least two experiments are shown.
403 Percentages shown are among the total CD8 or CD4 population, as appropriate.

404

405 **Figure 4. Effect of Nrp1 deletion on CD8 T cell responses.** (A) Experimental design. (B) YFP expression in
406 tamoxifen treated B6 and tamoxifen or vehicle treated E8i-CreERT2 R26-YFP mice. (C) Evaluation of the size
407 of the ORF61-specific population in the blood within either the YFP^+ or YFP^- CD8 T cell populations in FS73 or
408 FS73R infected mice. (D-E) At d14 post-infection blood was stained to identify $CD8^+$ORF61 $tetramer^+$ cells,

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409 and the frequencies among this population are shown with respect to KLRG-1 and CD127 expression. (F-G)
410 Spleen cells at d28 post-infection were stained as in (D-E). All data show mean ± SD of 4-5 mice per group;
411 ***P*<0.01, *****P*<0.0001. Representative data from at least two experiments.
412
413 **Figure 5. Effect on the recall response of Nrp1 deletion before infection.** (A) Experimental design showing
414 Nrp1 deletion before infection, then adoptive transfer of memory CD8 T cells followed by infection of congenic
415 secondary hosts. (B) Flow cytometry plots of adoptively transferred CD8 T cell populations. Plots show ORF61
416 tetramer positive CD8 T cell populations (left) and YFP positive populations within the tetramer positive
417 population (right) from FS73 (top) or FS73R (bottom) infected mice. (C-D) Graphs showing expansion of YFP^+
418 and YFP^- $CD8^+$ORF61 $tetramer^+$ populations from mice infected originally with either (C) FS73 or (D) FS73R
419 after secondary exposure to WT MHV-68. Joined lines represented paired samples (YFP^+ and YFP^- cells from
420 the same mouse). Data combined from 2 experiments are presented in C and D. ***P*<0.01.
421
422 **Figure 6. Effect of Nrp1 deletion just prior to the recall response.** (A) Experimental design showing
423 deletion of Nrp1 in memory cells, just before adoptive transfer to secondary congenic hosts. (B-C) Graphs
424 showing expansion of YFP^+ and YFP^- $CD8^+$ORF61 $tetramer^+$ populations from mice infected originally with
425 either (B) FS73 or (C) FS73R after secondary exposure to WT MHV-68. Joined lines represented paired
426 samples (YFP^+ and YFP^- cells from the same mouse). Data combined from 2 experiments are presented.
427 ***P*<0.01.
428
429 **Figure 7. Cell viability and proliferation during recall responses where Nrp1 was deleted in memory**
430 **cells.** Experimental design was the same as that shown in Fig.6A. (A) Graph of the percentage of dead cells
431 after the recall response, determined by gating on $CD8^+$ORF61 $tetramer^+$ cells and a LIVE/DEAD™ stain. (B)
432 Mice were injected with 100□l of 10□M EdU i.p. 16 hour before euthanasia. EdU incorporating proliferating
433 cells were determined by gating on $CD8^+$ORF61 $tetramer^+$ EdU^+ cells after the recall response. All data show
434 mean ± SD of 5-6 mice per group; **P*<0.05, ***P*<0.01. Representative data from at least two experiments.

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Figure 1

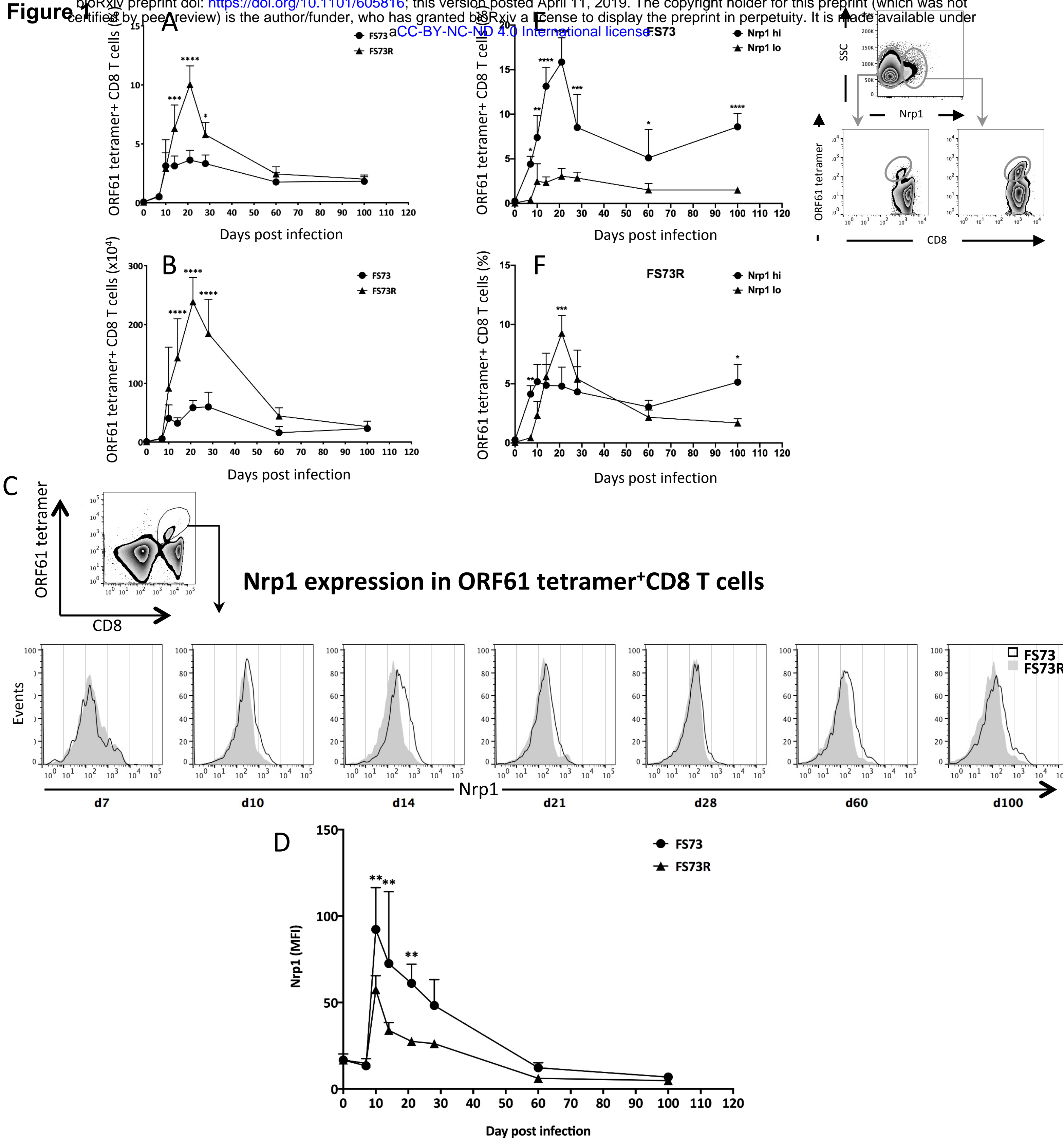

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Figure 2

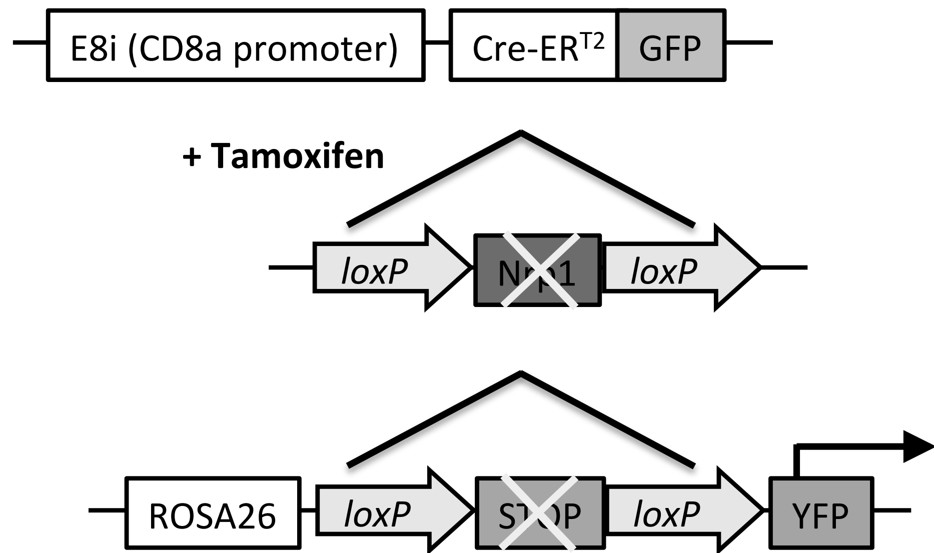

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Figure 3

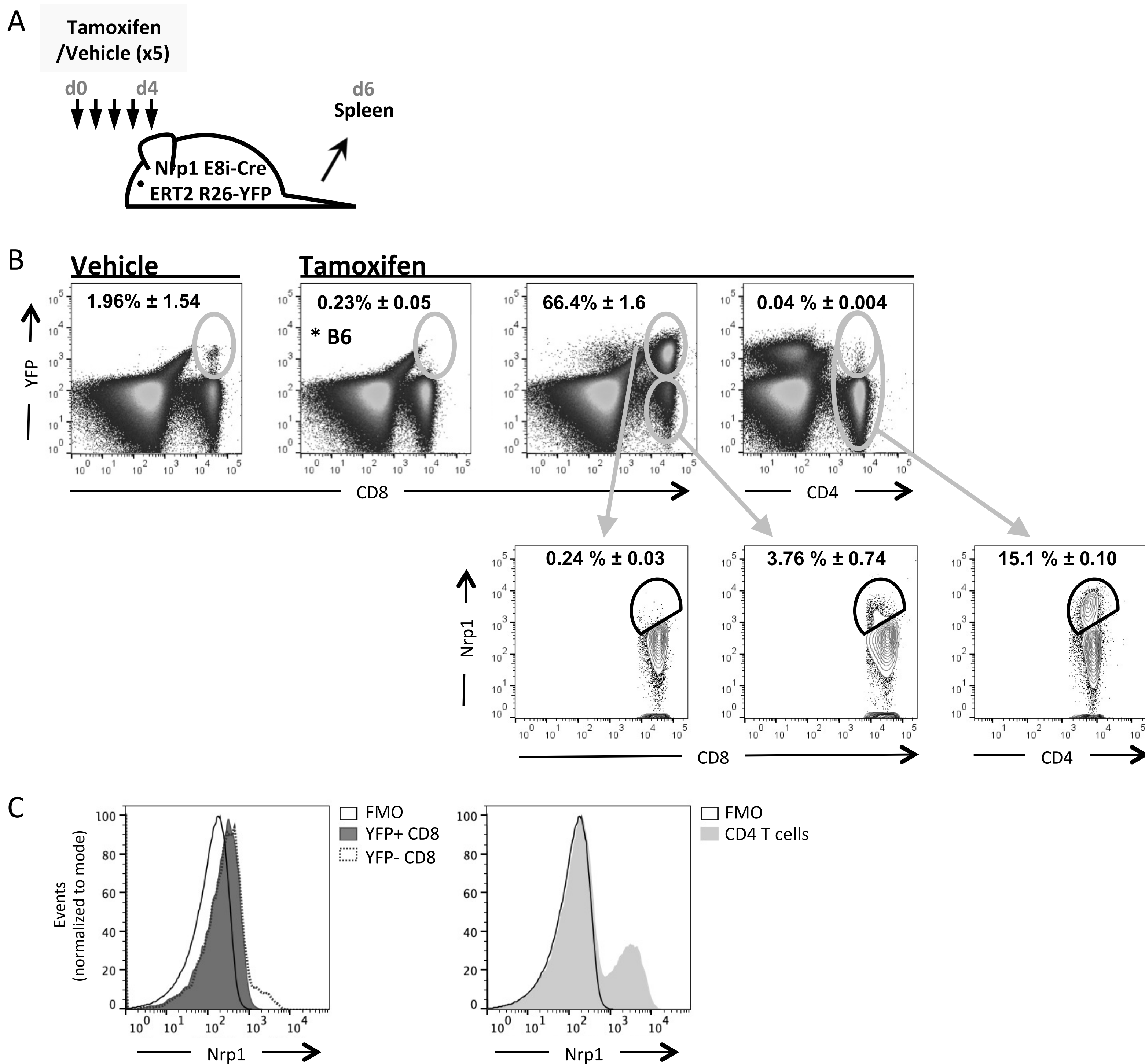

Figure 4

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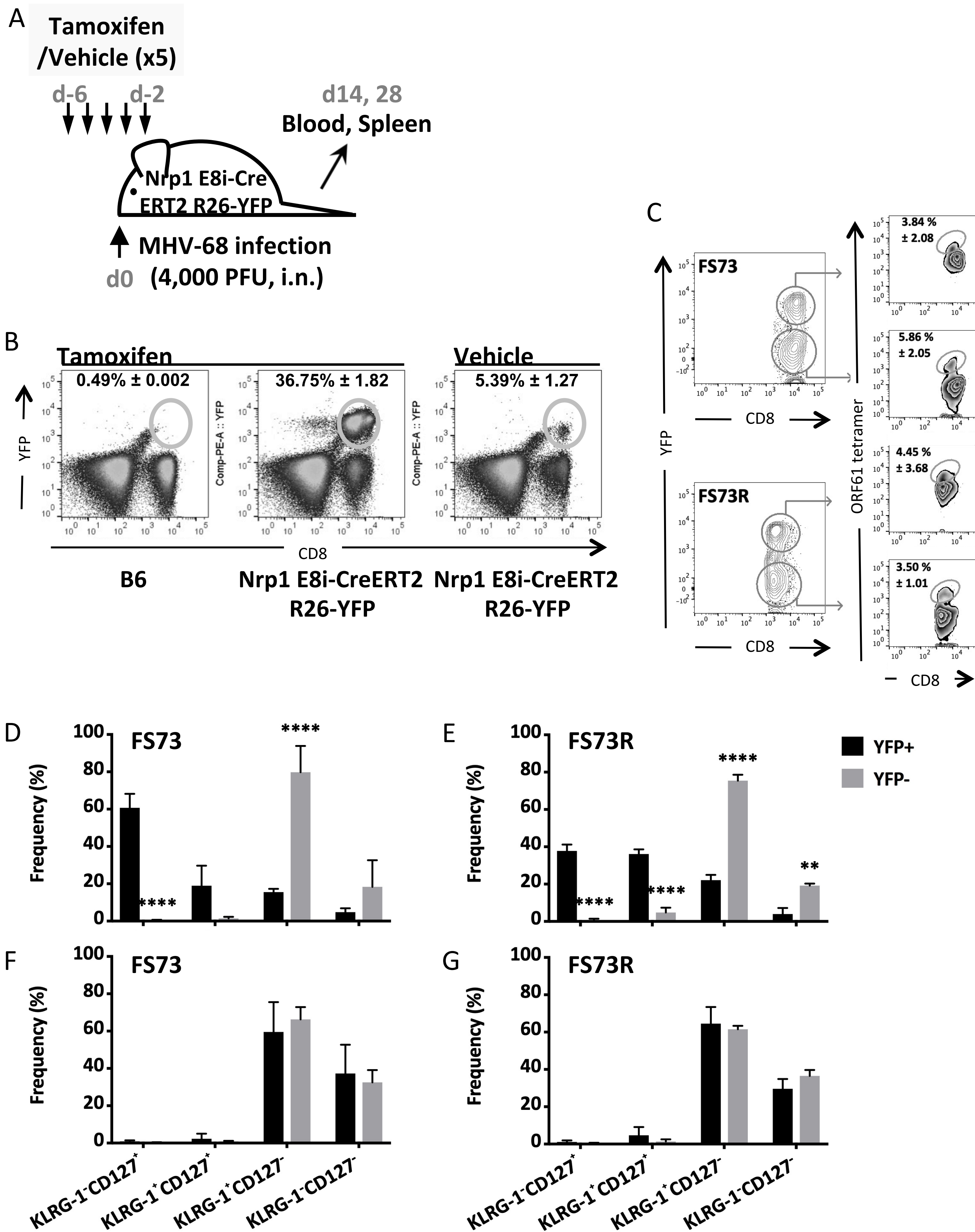

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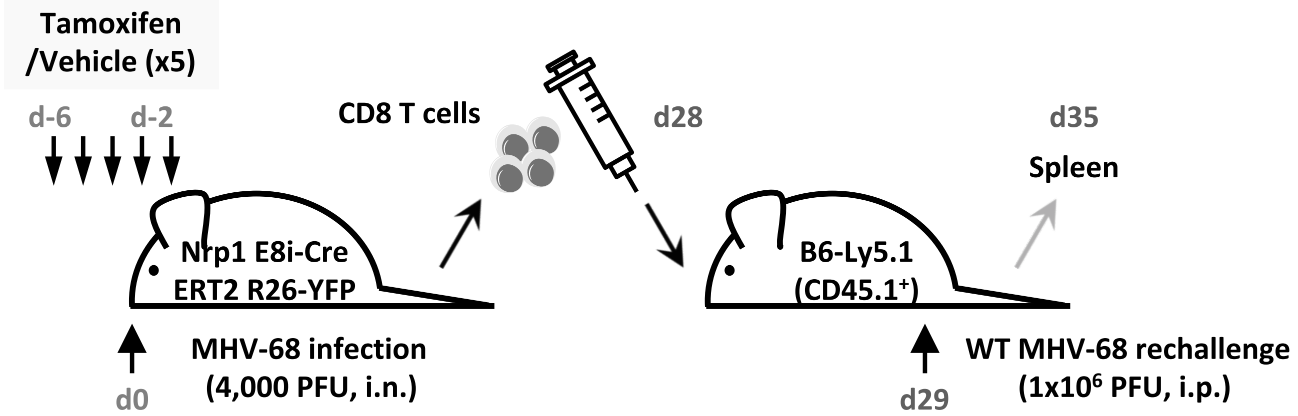
Figure 5

A



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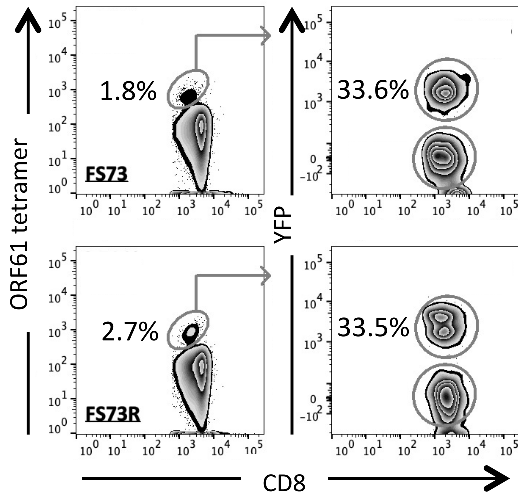

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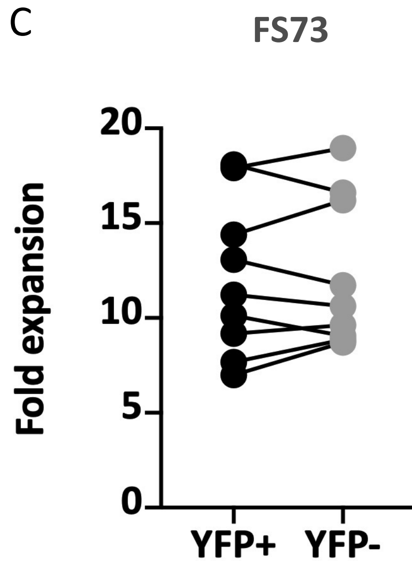

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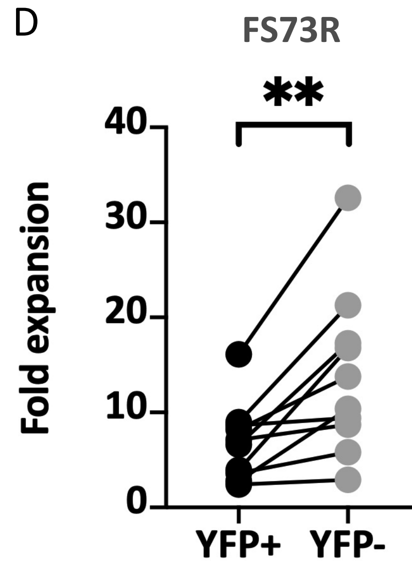

Figure 6

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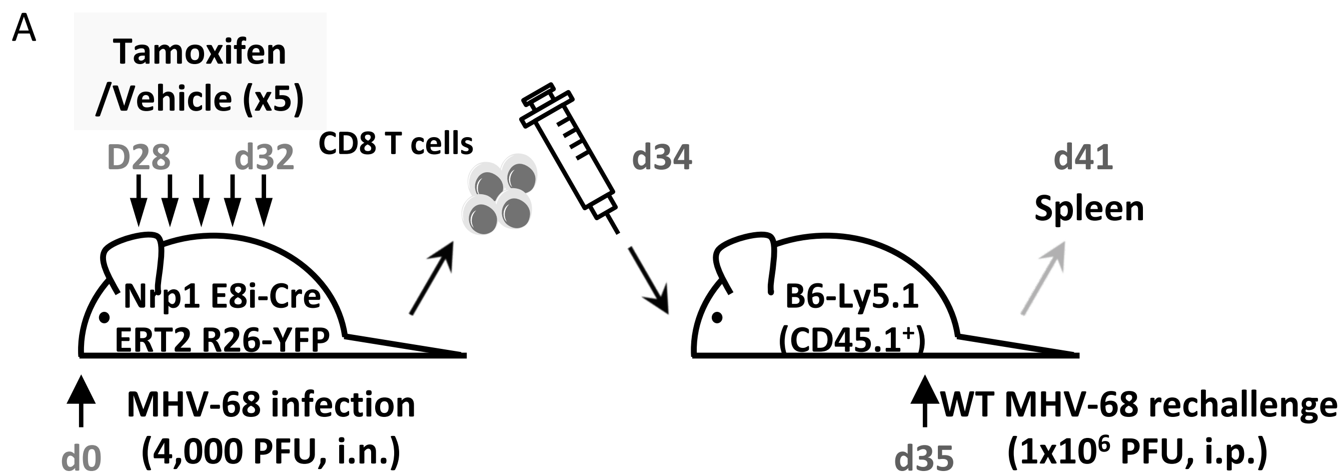

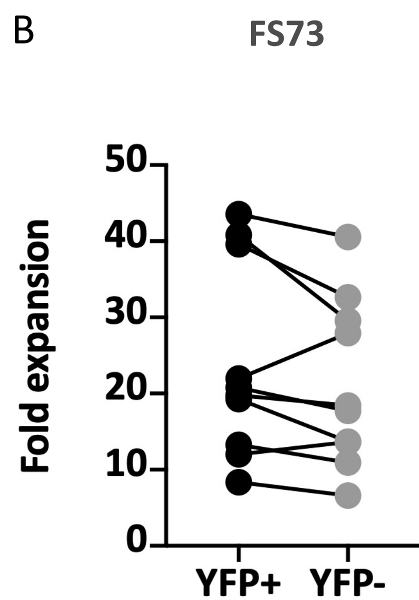

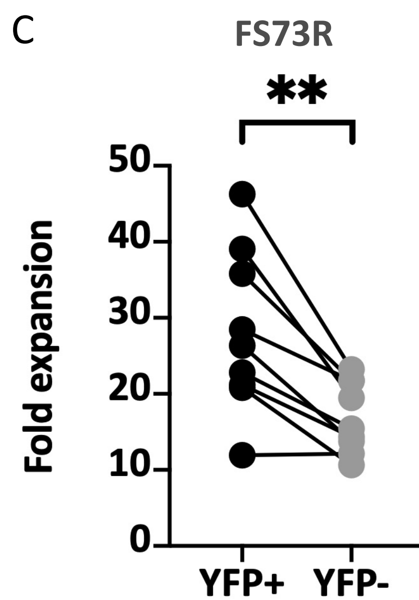

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Figure 7