

1 **Tolerance induction in memory CD4 T cells is partial and reversible**

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3 Joshua I Gray^{1#}, Shaima Al-Khabouri¹, Fraser Morton¹, Eric T Clambey², Laurent Gapin³,

4 Jennifer L Matsuda³, John W Kappler³, Philippa Marrack³, Paul Garside¹, Thomas D Otto¹,

5 Megan KL MacLeod¹

6

7 ¹Institute of Infection, Immunity and Inflammation, University of Glasgow, Glasgow, UK

8 ²Department of Anesthesiology, University of Colorado Denver, Anschutz Medical Campus,

9 Aurora, CO USA 80045

10 ³National Jewish Health, Denver CO, USA

11 [#]Current address: Columbia Center for Translational Immunology, Columbia University, New

12 York NY, USA 10032

13

14 **Keywords**

15 Memory CD4 T cells, Tolerance, Proliferation, Mitotic catastrophe

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17

18 **Abstract**

19

20 Memory T cells respond rapidly in part because they are less reliant on heightened levels of
21 costimulatory molecules. This presents challenges to silencing memory T cells in tolerance
22 strategies for autoimmunity or allergy. We find that memory CD4 T cells generated by infection
23 or immunisation survive secondary activation with antigen delivered without adjuvant,
24 regardless of their location in secondary lymphoid organs or peripheral tissues. These cells
25 were, however, functionally altered following a tertiary immunisation with antigen and
26 adjuvant, proliferating poorly but maintaining their ability to produce inflammatory cytokines.
27 Transcriptional and cell cycle analysis of these memory CD4 T cells suggest they are unable
28 to commit fully to cell division potentially because of low expression of DNA repair enzymes.
29 In contrast, these memory CD4 T cells could proliferate following tertiary reactivation by viral
30 re-infection. These data suggest that tolerance induction in memory CD4 T cells is partial and
31 can be reversed.

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36 **Introduction**

37

38 Memory CD4 T cells play central roles in enhancing immune protection against pathogens the
39 host has previously encountered(Jaigirdar and MacLeod, 2015). However, activated and
40 memory CD4 T cells also contribute to disease processes in chronic inflammatory conditions,
41 including rheumatoid arthritis and multiple sclerosis(Cope et al., 2007, McGinley et al., 2018,
42 Raphael et al., 2020). Most current treatments for these conditions require continued use of
43 drugs that dampen or deplete immune mediators or cells. A cure for these diseases will require
44 deletion or retraining of the CD4 T cells that contribute to pathology.

45

46 Antigen-specific tolerance strategies have been used for many years to treat allergies and
47 there are ongoing trials in autoimmune patients(Gunawardana and Durham, 2018, Pearson
48 et al., 2017, Rayner and Isaacs, 2018, Serra and Santamaria, 2019) The underlying rationale
49 for these strategies is based on our knowledge of tolerance induction in T cells, mainly
50 developed from experiments examining TCR-activation of naïve CD4 T cells in the absence
51 of costimulatory and inflammatory signals(Greenwald et al., 2005, Miller et al., 2007, Nurieva
52 et al., 2011). Much less is known about the consequences of activating memory CD4 T cells
53 through their TCR alone. Memory CD4 T cells can respond more quickly to a secondary
54 challenge because, in part, they are less reliant on heightened level of costimulatory
55 signals(Holzer et al., 2003, London et al., 2000, MacLeod et al., 2006). While this contributes
56 to rapid pathogen control, this presents significant hurdles for treatments that aim to induce
57 antigen-specific tolerance in autoimmunity, allergy or transplantation(Hartigan et al., 2019,
58 MacLeod and Anderton, 2015).

59

60 A deeper understanding of the functional and molecular consequences of activating memory
61 CD4 T cells with TCR signals alone is required to surmount these hurdles. We recently
62 demonstrated that memory CD4 T cells reactivated with antigen delivered in the absence of
63 adjuvant return to the memory pool and survive longterm(David et al., 2014). However, tertiary

64 reactivation led to a curtailed response. Here we address two outstanding questions: 1.
65 whether the consequences of reactivating memory CD4 T cells with antigen alone are similar
66 in lymphoid organs and peripheral tissues and 2. what the underlying cellular changes
67 responsible for the curtailed tertiary response are. These are important questions as the
68 pathology for many autoimmune and allergic conditions is present in peripheral tissues, and
69 understanding the mechanisms of memory CD4 T cell tolerance is essential to improve
70 treatments and monitor therapeutic success. Our data show that while memory CD4 T cell
71 responses are altered following exposure to tolerogenic signals, their ability to respond and
72 produce inflammatory cytokines is not permanently restrained.

73

74 **Results**

75

76 **Antigen-specific memory CD4 T cells in lymphoid organs and in peripheral tissues 77 respond to tolerogenic signals but fail to increase in number**

78 To investigate the consequence of reactivating memory CD4 T cells with tolerogenic signals
79 (antigen delivered without adjuvant), we needed to track antigen-specific CD4 T cells following
80 secondary and tertiary reactivation. To achieve this, we generated memory CD4 T cells in
81 lymphoid organs and peripheral tissues by infecting C57BL/6 mice with WSN Influenza A virus
82 (IAV) intranasally (i.n.). We used MHC class II tetramers containing the immunodominant IAV
83 peptide nucleoprotein (NP)₃₁₁₋₃₂₅ to identify NP₃₁₁₋₃₂₅-specific memory CD4 T cells in the
84 spleen, lung draining mediastinal lymph node (MedLN) and lung (Supplementary Figure 1).

85

86 Previously(David et al., 2014), we delivered antigen intravenously (i.v.) as it is a well-
87 established tolerogenic injection route(David et al., 2014, Jenkins and Schwartz, 1987, Liblau
88 et al., 1996). As expected, i.v. injection of NP₃₁₁₋₃₂₅ and the adjuvant PolyIC led to an increase
89 of T cells within the spleen in mice previously infected with IAV. However, there was no
90 increase in the numbers of antigen-specific CD4 T cells in the MedLN or lung (Supplementary

91 Figure 2). These data suggested that the antigen failed to access the MedLN and lung and
92 that this delivery route could not be used to address our questions.

93

94 In contrast, i.n. instillation of peptide in the presence (immunogenic) or absence (tolerogenic)
95 of PolyIC led to reactivation of the memory CD4 T cells in all three organs (Figure 1A-B).
96 Importantly, delivery of peptide in the absence of adjuvant i.n. to naïve animals led to functional
97 tolerance of the antigen-specific CD4 T cell population, validating this injection route for
98 assessment of memory CD4 T cell tolerance induction (Supplementary Figure 3).

99

100 We first examined the immediate consequences of reactivating memory CD4 T cells with
101 immunogenic or tolerogenic signals. Five days following instillation of NP₃₁₁₋₃₂₅ peptide
102 delivered with or without PolyIC, antigen-specific CD4 T cells showed evidence of activation
103 via increased expression of the proliferation marker, Ki67, in all three organs (Figure 1B-C).
104 Memory CD4 T cells reactivated in the presence of adjuvant increased in number as expected
105 (Figure 1D). In contrast, there was no accumulation of memory CD4 T cells reactivated
106 following the tolerogenic instillation of peptide alone. This suggests that while these cells
107 entered the cell cycle, they either failed to complete mitosis or rapidly underwent cell death
108 following proliferation.

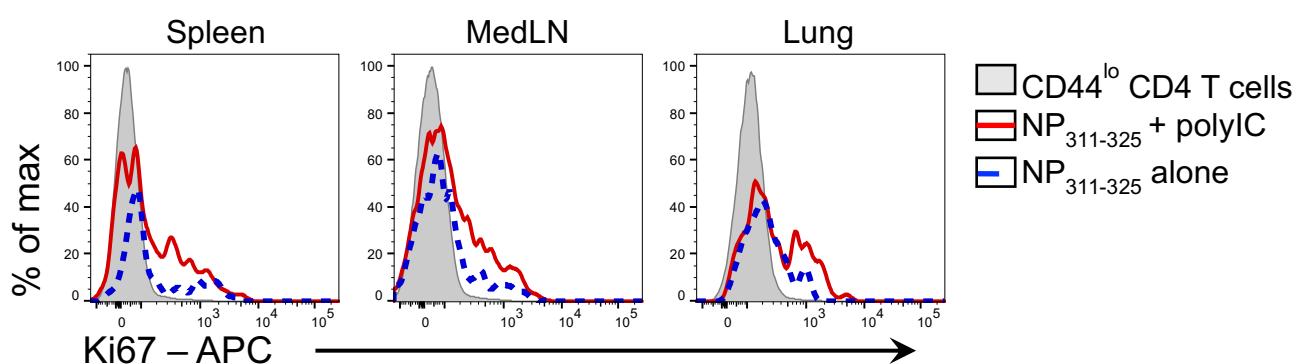
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110 Gray, Figure 1

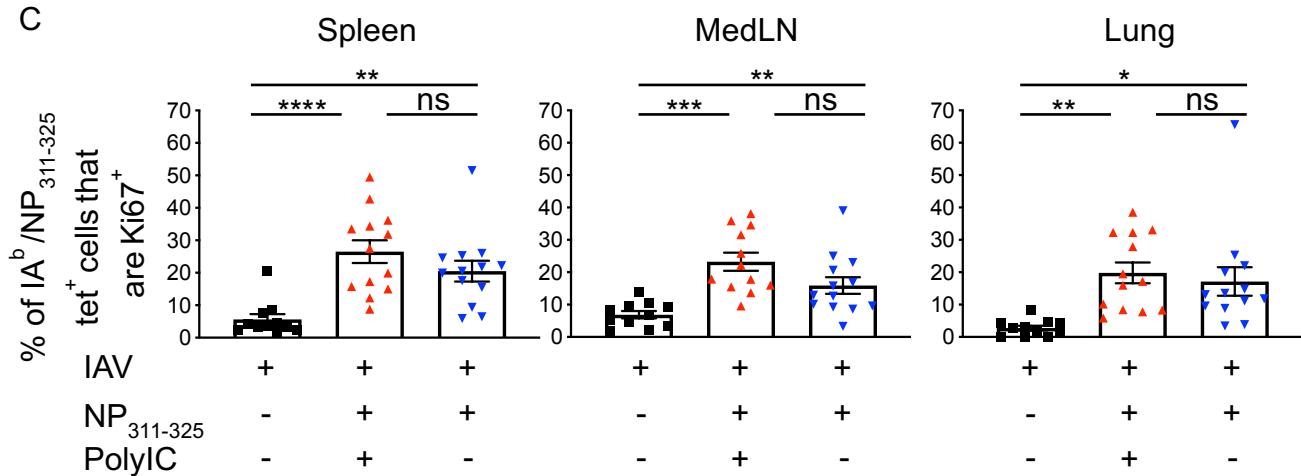
111 A

112 d-30: IAV d0: NP₃₁₁₋₃₂₅ +/-
113 PolyIC i.n. d5: analysis.

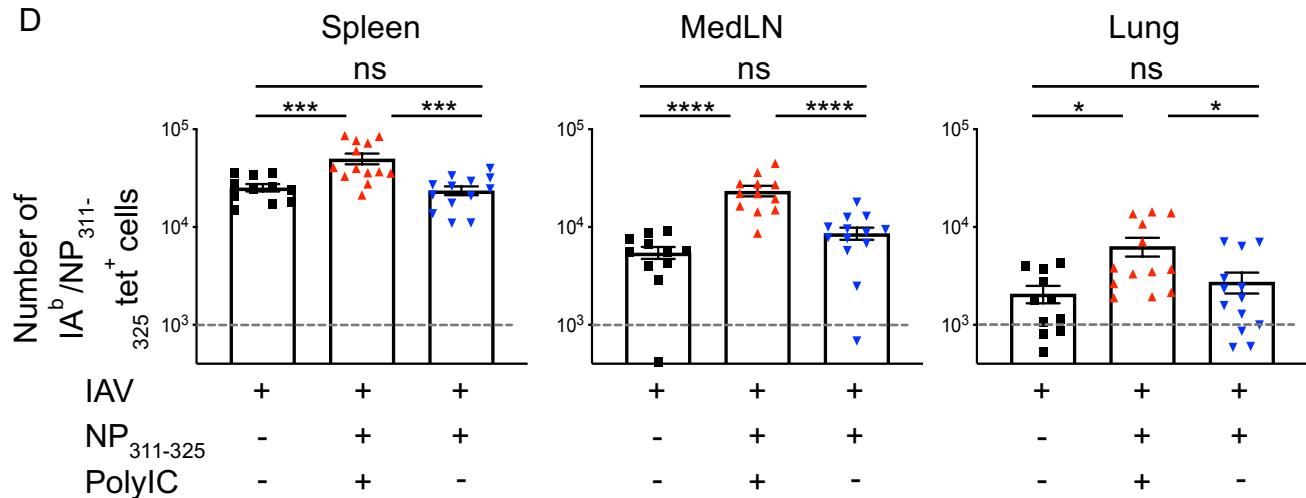
114 B



C



D



115 **Figure 1: NP₃₁₁₋₃₂₅-specific memory CD4 T cells reactivated with peptide delivered in**
116 **the absence of adjuvant respond but fail to accumulate**
117 *C57BL/6 mice were infected with IAV on day -30. On day 0, some of these mice were*
118 *immunised with NP₃₁₁₋₃₂₅, +/-, PolyIC i.n. IA^b/NP₃₁₁₋₃₂₅ CD44^{hi} CD4 T cells were examined 5*
119 *days later in the spleen, MedLN, and lung (A) and their Ki67 expression (B, C) or their numbers*
120 *determined (D). In C and D, each symbol represents one mouse and error bars are SEM. In*
121 *D the grey dashed line represents the background staining in naïve animals. Data are*
122 *combined from 3 experiments (3-5 mice/experiment). Cells are gated as shown in*
123 *Supplementary Figure 1. All statistics calculated using a one-way ANOVA with multiple*
124 *comparisons; ns = not significant, * = <0.05, ** = <0.01, *** = <0.001, **** = <0.0001.*

125
126
127 **Memory CD4 T cells previously exposed to tolerogenic signals fail to expand upon**
128 **subsequent reactivation despite entry into the cell cycle**

129
130 To examine the longer term consequences of reactivating memory CD4 T cells with
131 tolerogenic signals, we set up the experiment displayed in Figure 2A. Thirty days after infection
132 with IAV, animals were given NP₃₁₁₋₃₂₅ i.n. delivered with (immunogenic) or without
133 (tolerogenic) PolyIC. After a further thirty days, we either examined the memory cells or
134 performed a tertiary immunisation with NP₃₁₁₋₃₂₅ conjugated to ovalbumin (OVA) protein
135 delivered with the adjuvant alum.

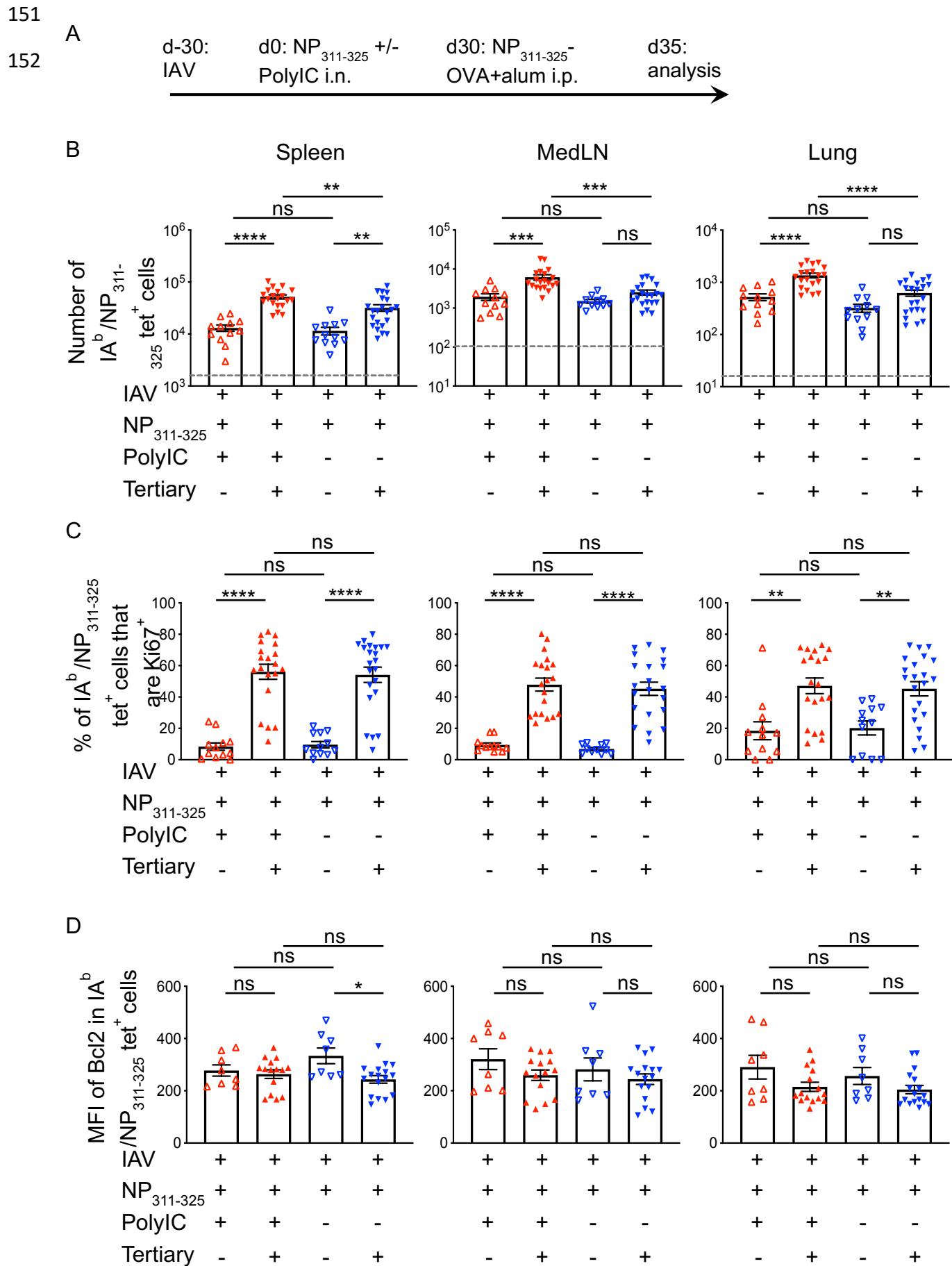
136
137 Prior to tertiary reactivation, there were similar numbers of memory antigen-specific CD4 T
138 cells in the two groups in each organ (Figure 2B). The antigen-specific CD4 T cells reactivated
139 with an immunogenic secondary injection were able to mount a robust response in all organs
140 upon tertiary reactivation. In contrast, the CD4 T cells previously exposed to tolerogenic
141 signals expanded only slightly in the spleen and not at all in the MedLN and lung. There was,
142 however, no difference in expression of Ki67 in the reactivated CD4 T cells (Figure 2C).

143

144 We also examined the expression of the pro-survival molecule Bcl2 to determine whether the
145 memory CD4 T cells were more prone to apoptosis following tolerogenic activation (Figure
146 2D). However, there were no differences in the expression of Bcl2 between the two groups
147 regardless of whether we examined the memory or the recalled cells in any of the three
148 organs. This suggests increased apoptosis could not account for the poor accumulation of the
149 tertiary reactivated memory CD4 T cells exposed to tolerogenic signals.

150

Gray, Figure 2



153 **Figure 2: NP₃₁₁₋₃₂₅-specific memory CD4 T cells previously reactivated with peptide**
154 **without adjuvant fail to accumulate in the lung after tertiary reactivation**

155 C57BL/6 mice were infected with IAV on day -30. On day 0, mice received NP₃₁₁₋₃₂₅ +/- PolyIC
156 and some of these mice were immunised i.p with NP-OVA with alum on day 30 (A). The
157 numbers of IA^b/NP₃₁₁₋₃₂₅ CD44^{hi} CD4 T cells were examined 5 days later in the spleen, MedLN,
158 and lung (B) and their expression of Ki67 (C) and Bcl2 (D) determined. Each symbol
159 represents one mouse and error bars are SEM. In B, the grey dashed line represents the
160 background staining in naïve animals. Data are combined from 4 experiments (4-
161 8 mice/experiment). All statistics calculated using a one-way ANOVA with multiple
162 comparisons; ns = not significant, * = <0.05, ** = <0.01, *** = <0.001, **** = <0.0001.

163

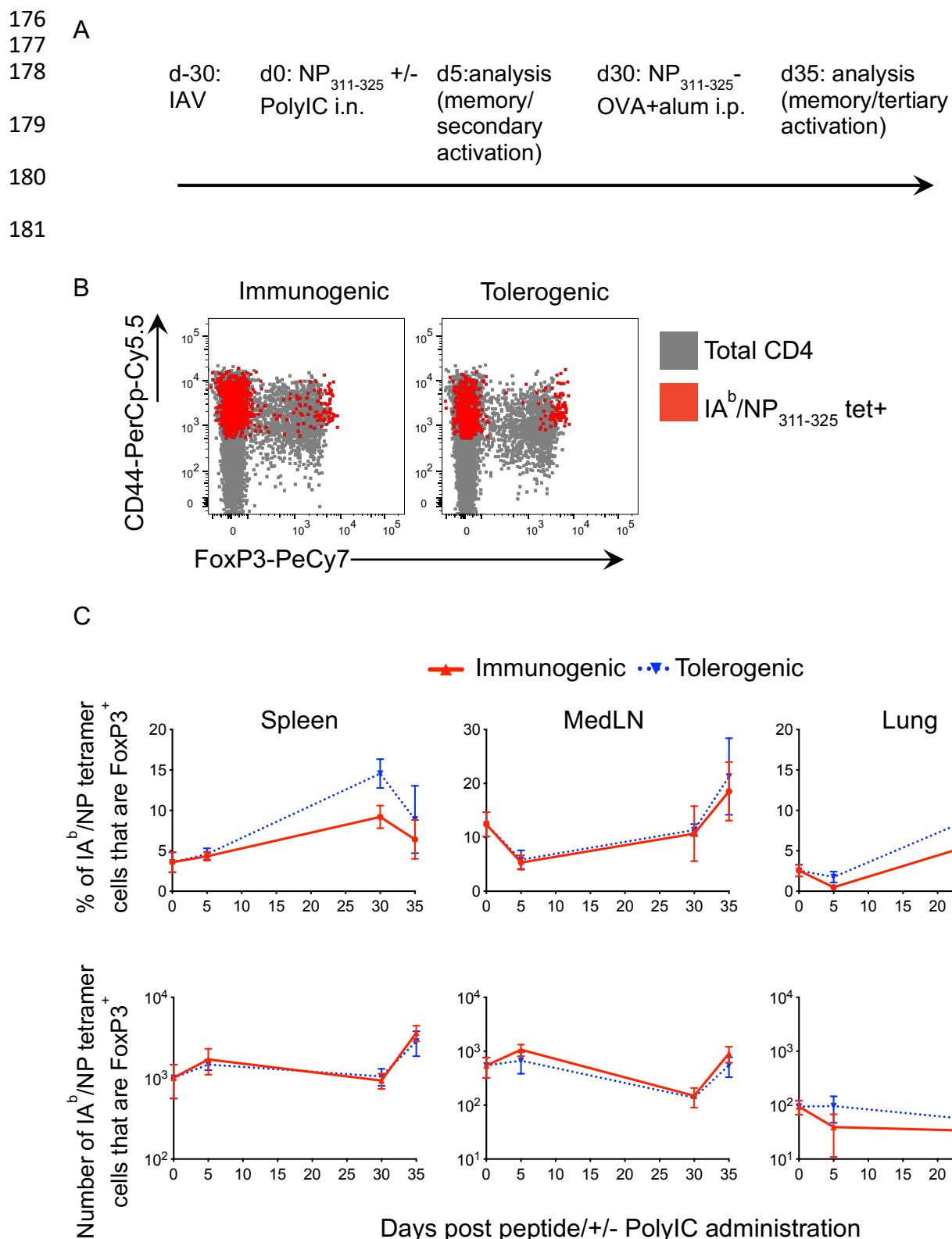
164 **Memory CD4 T cells reactivated with tolerogenic signals are not converted to regulatory**
165 **T cells**

166 Previous studies have shown that activation with antigen alone can lead to tolerance via the
167 induction of FoxP3-expressing regulatory T cells (Zhang et al., 2013). We found few NP₃₁₁₋₃₂₅-
168 specific FoxP3⁺ cells at any timepoint examined (Figure 3). While there was a slightly
169 increased percentage of antigen-specific FoxP3⁺ cells in the mice exposed to tolerogenic
170 signals at one time point in the spleen, there were no differences in the number of FoxP3⁺
171 NP₃₁₁₋₃₂₅-specific cells between the groups. This suggests that Treg conversion does not
172 explain the poor accumulation of antigen-specific memory CD4 T cells previously exposed to
173 tolerogenic signals.

174

175

Gray, Figure 3



182 **Figure 3: NP₃₁₁₋₃₂₅-specific memory CD4 T cells exposed to soluble antigen in the**
183 **absence of adjuvant are not converted to Tregs**

184 *C57BL/6 mice were infected with IAV on day -30. On day 0, mice received NP₃₁₁₋₃₂₅ +/- PolyIC*
185 *i.n. and some of these mice were immunised i.p with NP-OVA and alum on day 30 (A). The*
186 *expression of FoxP3 by IA^b/NP₃₁₁₋₃₂₅ CD44^{hi} CD4 T cells was determined and shown in red*
187 *and total CD4 T cells in grey in representative FACS plots (B) and the percentages and*
188 *numbers of FoxP3 by IA^b/NP₃₁₁₋₃₂₅ CD44^{hi} CD4 T cells days 0, 5 and 35 shown in (C). The data*
189 *are combined from 1 experiment/timepoint with 4-5 mice/group/timepoint. Each symbol*
190 *represents the mean of between 4-5 mice and the error bars are SEM, *** = <0.001.*

191

192 **Memory CD4 T cells exposed to tolerogenic signals display evidence of mitotic**
193 **catastrophe following reactivation**

194 Thus far our experiments indicated that memory CD4 T cells activated with tolerogenic signals
195 can survive in the memory pool but accumulate poorly upon tertiary reactivation. To take an
196 unbiased approach to investigate this failure, we performed transcriptomics analysis. For this
197 we required a significant number of memory CD4 T cells that could be easily isolated for
198 analysis. As identification of antigen-specific CD4 T cells by MHC tetramers requires ligation
199 of the TCR by MHC molecules and the number of epitope specific cells are limited, we
200 developed a novel triple transgenic reporter mouse TRACE (T cell Reporter of Activation and
201 Cell Enumeration). We generated a transgenic animal in which the IL-2 promoter drives
202 expression of rtTA. These animals were crossed to B6.Cg-Tg(tetO-cre)1Jaw/J mice and
203 B6.129X1-Gt(ROSA)26SorTm(EYFP+)Cos mice. In these animals, T cells activated through
204 the TCR when the animals are given doxycycline (Dox) become permanently EYFP+
205 (Supplementary Figure 4A).

206

207 Feeding of the Dox+ diet for one week was sufficient to induce a small population of EYFP+
208 cells, even in the absence of immunisation. However, delivery of OVA conjugated to 20 μ m
209 polyethylene carboxylate beads in combination with the strong adjuvant combination of anti-

210 CD40 and PolyIC(Kurche et al., 2010) drove an increased population of EYFP+ CD4 T cells
211 above background (Supplementary Figure 4B,C). OVA was used for these studies as we
212 required a protein containing multiple CD4 T cell epitopes which could be obtained free of
213 contaminating microbial products, which would be present in recombinant IAV proteins.
214 Moreover, we found that recombinant nucleoprotein had intrinsic adjuvant properties(Macleod
215 et al., 2013).

216

217 To reactivate the memory CD4 T cells, we returned to systemic i.v. delivery of OVA protein as
218 this is widely accepted as a consistent tolerogenic route(David et al., 2014, Jenkins and
219 Schwartz, 1987, Liblau et al., 1996). We found that secondary immunisation with OVA in the
220 TRACE mice led to anaphylactic shock, likely a consequence of anti-OVA antibodies. To avoid
221 this, we sorted EYFP+ CD4 T cells at day 8 after immunisation and transferred these cells into
222 naïve C57BL/6 animals that were injected with OVA or OVA and LPS i.v. after 22 days, 30
223 days since the cells were first primed. Thirty days following this, the recipient animals were
224 immunised with OVA+alum and CD4+ EYFP+ cells isolated 5 days later for RNA-seq analysis
225 (Figure 4A).

226

227 Gene expression from 5 individual mice in each experimental condition were analysed. One
228 sample from the tolerogenic group was excluded as the number of EYFP+ CD4 T cells
229 collected was 2.5-10fold higher than any of the other samples, suggesting an abnormal
230 response or potential contamination during sorting. Of the differently expressed genes
231 (DEGs), 898 were expressed at lower levels and 667 at higher levels in the tolerogenic group
232 compared to the immunogenic group (Figure 4B). Analysis of DEGs expressed at higher levels
233 in the tolerised samples failed to find consistent changes across all four samples. We,
234 therefore, concentrated on DEGs that were expressed at lower levels in the tolerised samples.
235 Gene ontogeny (Panther(Mi et al., 2019)) analysis of the biological processes associated with
236 these DEGs indicated overrepresentation of gene products involved in 'DNA-dependent DNA

237 replication', 'spindle organisation' and 'cell cycle checkpoints' (Table 1 and Supplementary
238 Excel File 1).

239

240 We performed gene set enrichment analysis and found that the DEGs were enriched for genes
241 within the GO term, Cell Cycle Checkpoints (Figure 4C); DEGs within this GO term that were
242 expressed at lower levels in the tolerogenic samples are displayed as a heatmap (Figure 4D).
243 A number of these molecules play key roles at various stages of the cell cycle and in spindle
244 formation and function. These genes include: the essential cyclin, Cdk1(Santamaria et al.,
245 2007); Aurkb, a key component of the Chromosome Passenger Complex required for normal
246 spindle assemble(Joukov and De Nicolo, 2018); Mad1l1 (also known as MAD1), a component
247 of the spindle-assembly checkpoint(Hardwick and Murray, 1995, Musacchio, 2015); and
248 Cdk5rap2 which plays a number of roles in spindle checkpoints(Lizarraga et al., 2010, Zhang
249 et al., 2009).

250

251 Table 1: Gene over-representation analysis of DEGs expressed at lower levels in EYFP+ CD4
252 T cells in tolerogenic groups (top 3)

	Number of genes	Number of genes within DEGs	Fold enrichment	Raw value	p	Adjusted value (FDR)	p
DNA-dependent DNA replication (GO:0006261)	103	13	3.71	1.16E-04	3.98E-02		
Spindle organization (GO:0007051)	134	15	3.29	1.22E-04	4.10E-02		
Cell cycle checkpoint (GO:0000075)	152	16	3.1	1.41E-04	4.56E-02		

253

254 Dysfunction of the spindle checkpoint is linked to death by mitotic catastrophe, a form of cell
255 death that occurs when cells are unable to complete mitosis(Nitta et al., 2004, Shang et al.,
256 2010). As the percentages of CD4 T cells that were Ki67+ after tertiary activation were
257 equivalent regardless of whether or not they had been activated with immunogenic or
258 tolerogenic signals (Figure 2C), these data suggests that memory CD4 T cells activated with
259 tolerogenic signals can enter the cell cycle but fail to complete cell division following tertiary

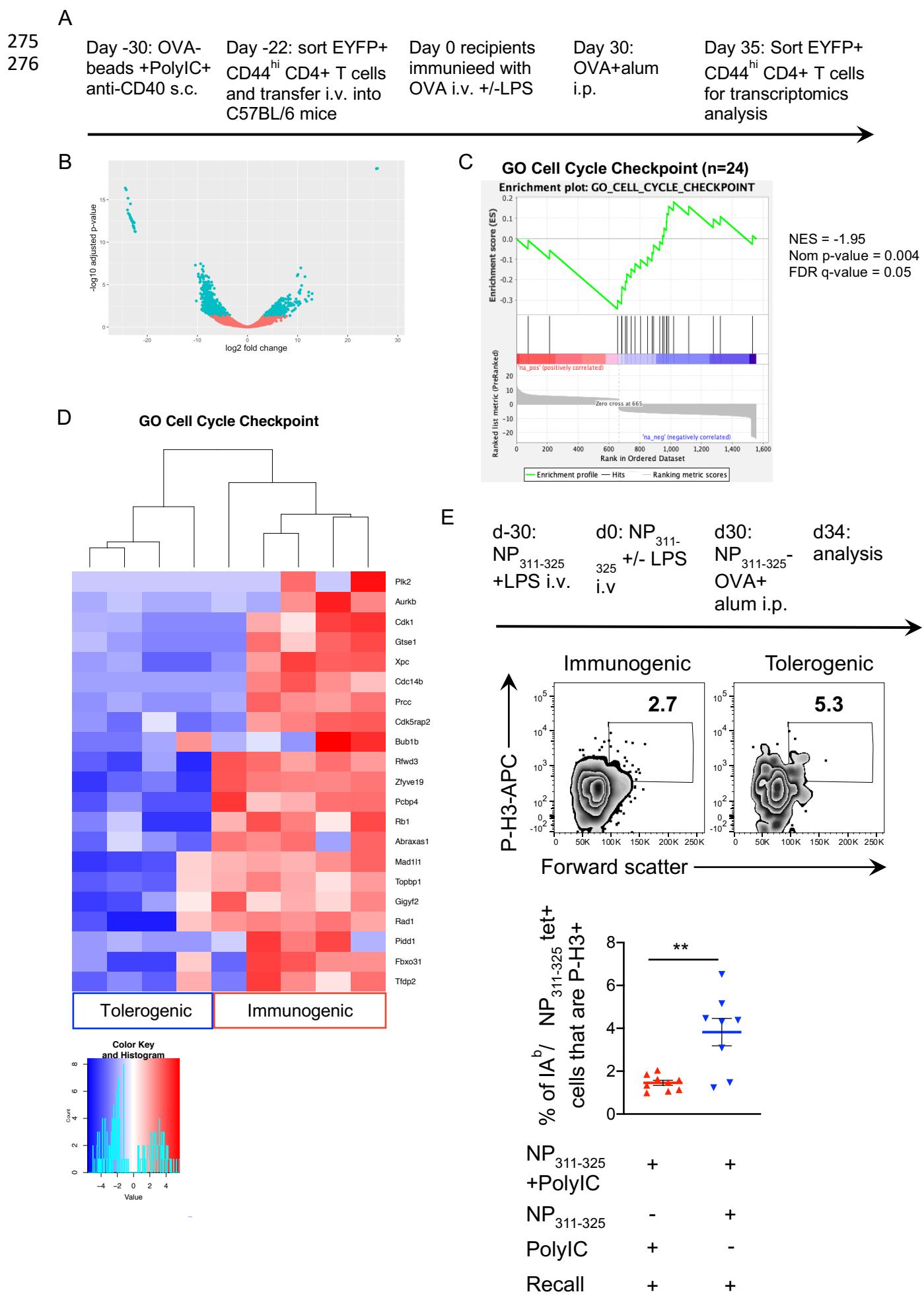
260 reactivation. To investigate this, we examined the proportion of reactivated memory antigen-
261 specific T cells in mitosis reasoning that more CD4 T cells would be found in mitosis in
262 reactivated cells previously exposed to tolerogenic signals as they would be 'stuck' in mitosis.

263

264 The percentages of re-activated antigen-specific T cells in mitosis were determined by
265 expression of phosphorylated (p)Histone 3, present only during mitosis(Hans and Dimitrov,
266 2001). We focused on cells with increased forward scatter as cells increase in size during cell
267 division(Bohmer et al., 2011). CD4 T cells were examined 4 days after tertiary reactivation of
268 mice first immunised with NP₃₁₁₋₃₂₅ and LPS, then reactivated with NP₃₁₁₋₃₂₅ delivered with or
269 without LPS and finally reactivated with NP-OVA+alum (Figure 4E). Very few antigen-specific
270 T cells were positive for p-H3, but consistently more CD4 T cells were p-H3 positive in mice
271 previously immunised with NP₃₁₁₋₃₂₅ delivered without, than with, LPS (Figure 4E). This
272 indicates that the cells in mice that received tolerogenic signals were more likely to be mitosis,
273 suggestive of a failure to complete cell division.

274

Gray, Figure 4



277 **Figure 4: Transcriptomics and cell cycle analysis indicates that memory CD4 T cells**
278 **exposed to tolerising signals undergo mitotic catastrophe following further**
279 **reactivation in vivo**

280 FACS sorted EYFP+ CD4 T cells from TRACE mice immunized with OVA+anti-CD40 and
281 PolyIC were transferred into naïve C57BL/6 mice that were then immunised with OVA +/- LPS
282 then re-immunised with OVA+alum i.p. 30 days later (A). EYFP+ CD4 T cells were FACS
283 sorted after a further 5 days and RNA isolated for transcriptomic analysis. The DEGs are
284 displayed in a volcano plot (B). GESA and heatmap show expression of DEGs contained
285 within the GO term 'Cell Cycle Checkpoints' (GO: 0000075) (C). DEGs within the GO term
286 and expressed at lower levels in the tolerogenic samples are displayed in a heatmap (D).
287 C57BL/6 mice immunised with NP₃₁₁₋₃₂₅ and LPS were injected with NP₃₁₁₋₃₂₅ +/- LPS 30 days
288 later and finally immunised after a further 30 days with NP-OVA+alum. 5 days later the
289 percentages of forward scatter high IA^b/NP₃₁₁₋₃₂₅ tetramer+ cells that expressed
290 phosphorylated Histone3 were examined (E). In (E) cells are gated as in supplementary Figure
291 1 and plots are concatenated from 4 mice per group. Data are combined from 2 experiment
292 with 4-5 mice/group, error bars are SEM. Statistical analysis in B calculated by a T-test, ** =
293 <0.01.

294

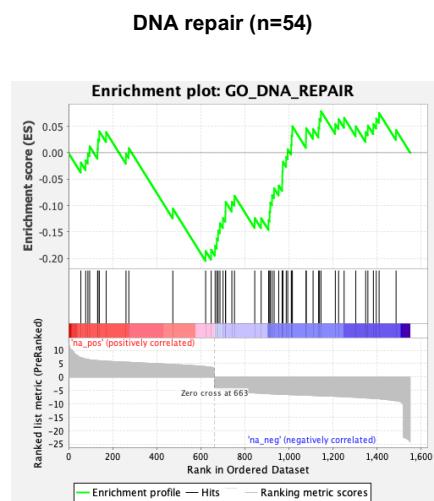
295 Mitotic catastrophe often occurs in cells with DNA damage(Vakifahmetoglu et al., 2008). We,
296 therefore, examined whether any DEGs were enriched in genes involved in GO term DNA
297 repair. This was indeed the case (Figure 5A); the DEGs expressed at lower levels in the
298 tolerised samples are shown as a heatmap (Figure 5B). The GSEA of the DEGs expressed at
299 lower levels in the tolerogenic samples indicates that multiple genes contribute to this
300 enrichment. These data suggest that tolerised memory CD4 T cells display poor repair of their
301 DNA during reactivation-induced DNA synthesis and that this is a consequence of reduced
302 expression of a number of different genes. This, coupled with the low expression of cell cycle
303 checkpoint proteins, likely compromises their ability to commit to cell division.

304

305Gray, Figure 5

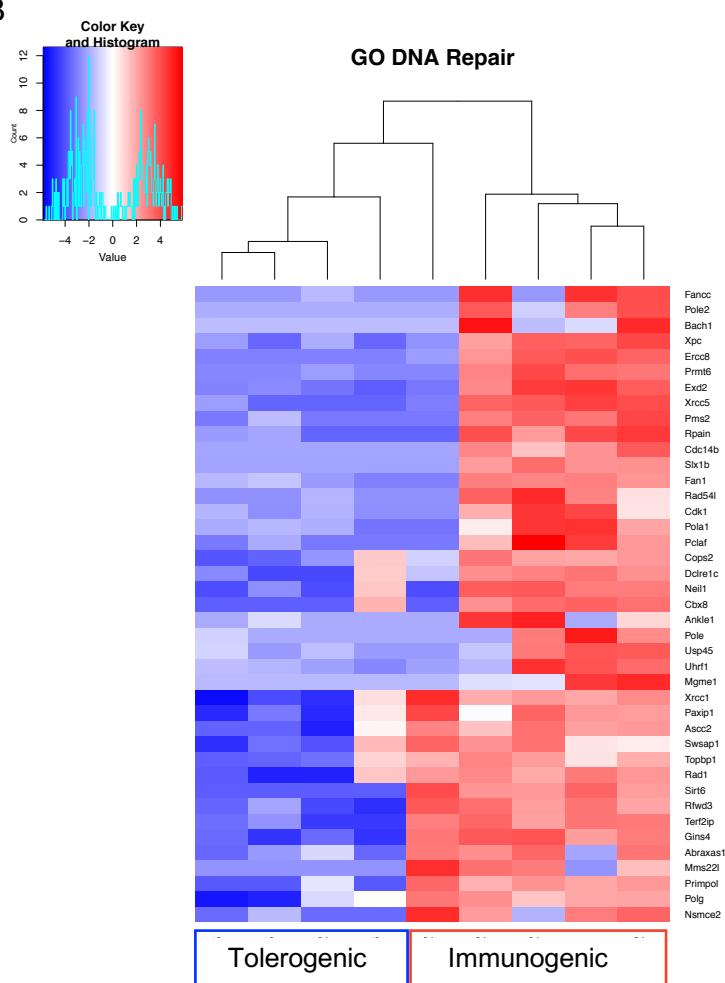
306

A



NES = -1.78
 Nom p-value = 0.012
 FDR q-value = 0.09

B



307 **Figure 5: Transcriptomics analysis indicates that memory CD4 T cells exposed to**
308 **tolerising signal have reduced expression of DNA repair enzymes**
309 FACS sorted EYFP+ CD4 T cells from TRACE mice immunized with OVA+anti-CD40 and
310 PolyIC were transferred into naïve C57BL/6 mice that were then immunised with OVA +/- LPS
311 then re-immunised with OVA+alum i.p. 30 days later as in Figure 4. EYFP+ CD4 T cells were
312 isolated by FACS after a further 5 days and RNA isolated for transcriptomic analysis. GSEA
313 shows significant enrichment of the genes involved within the GO term 'DNA repair' (GO:
314 0006281) (A) and the DEGs expressed at lower levels in the tolerogenic samples are
315 displayed in a heatmap (B).

316

317 **Memory CD4 T cells exposed to tolerogenic signals continue to produce cytokine but**
318 **fail to provide accelerated help to primary responding B cells**

319 Our data indicate that memory CD4 T cells reactivated with tolerogenic signals have impaired
320 proliferative responses. We also wanted to determine whether these cells were impaired in
321 other ways. To investigate this, we used the IAV infection model to generate sufficient cells in
322 multiple organs to examine *ex vivo* cytokine production; cytokine responses are limited in
323 antigen/adjuvant models(MacLeod et al., 2008).

324

325 Thirty days after mice were infected with IAV, they were injected with immunogenic or
326 tolerogenic NP₃₁₁₋₃₂₅ i.n. and then rested for 30 days (Figure 6A). Bone marrow dendritic cells
327 loaded with NP₃₁₁₋₃₂₅ were used to examine the *ex vivo* cytokine potential of the memory CD4
328 T cells and activated CD4 T cells from mice given a tertiary immunisation with NP₃₁₁₋₃₂₅-OVA
329 and alum delivered i.p. (Supplementary Figure 5).

330

331 The numbers of IFN- γ , TNF or IL-2 producing antigen-specific memory CD4 T cells were
332 equivalent in mice exposed to immunogenic or tolerogenic NP₃₁₁₋₃₂₅ peptide 35 days
333 previously (Figure 6B). Five days after reactivation with NP-OVA+alum, there was an increase
334 of TNF and IL-2 producing cells in the spleen and the MedLN in mice previously exposed to

335 NP₃₁₁₋₃₂₅ and PolyIC. In contrast, there was no increase in the number of cytokine producing
336 cells in mice previously exposed to tolerogenic NP₃₁₁₋₃₂₅. In neither group did we see an
337 increase in IFN- γ producing CD4 T cells. Together, these data suggest that, while exposure
338 to tolerogenic signals affected accumulation of T cells, it did not prevent their ability to produce
339 cytokines.

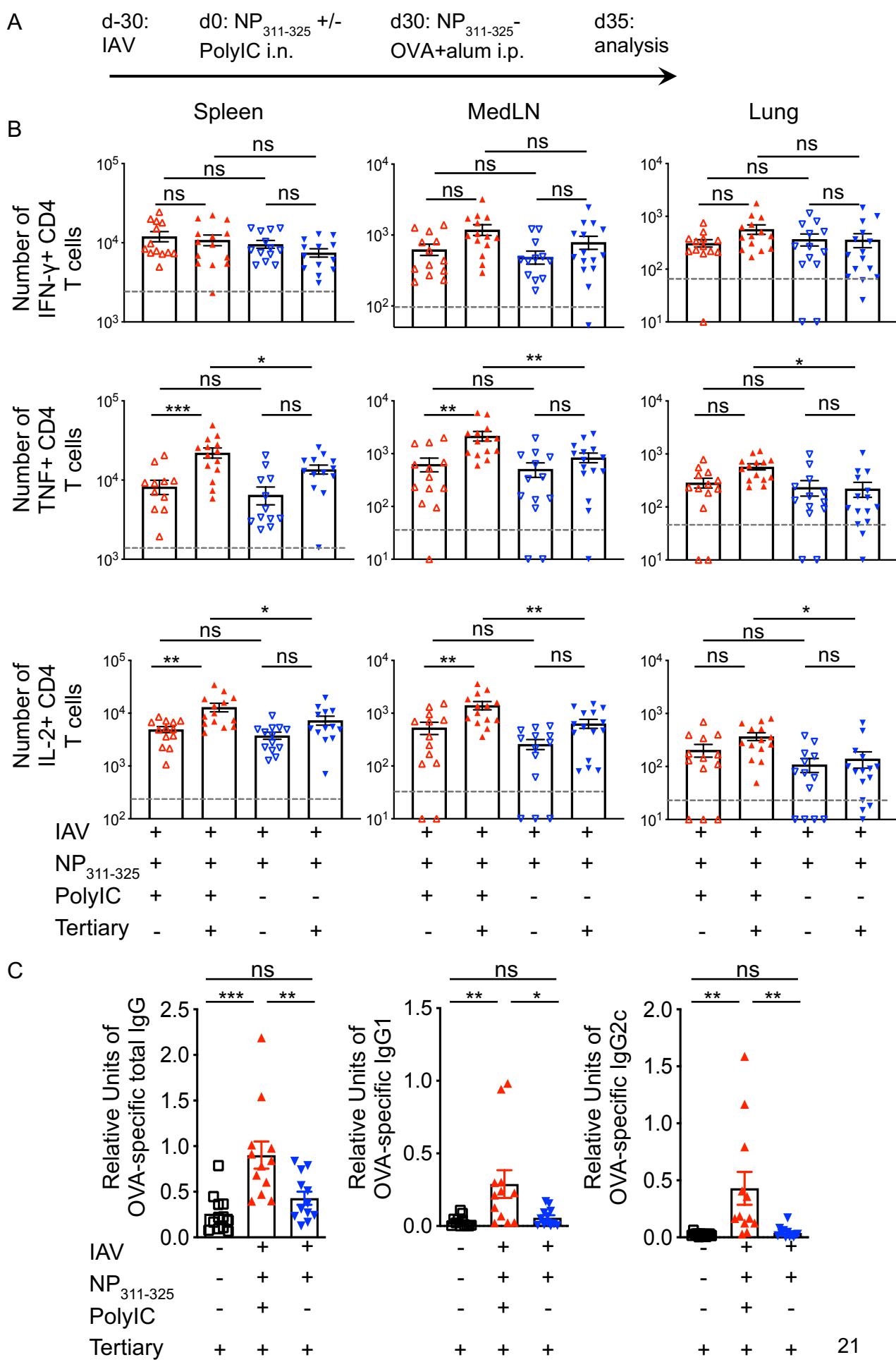
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341 To investigate the functional responses of the T cells further, we examined their ability to
342 provide accelerated help to primary responding OVA-specific B cells(David et al., 2014,
343 MacLeod et al., 2011). We measured the levels of class-switched, OVA-specific antibody 5
344 days after the tertiary reactivation. As expected, primary responding mice had very little class-
345 switched OVA-specific antibody and IAV-infected mice previously exposed to immunogenic
346 signals had clearly detectable levels of OVA-specific immunoglobulin(MacLeod et al., 2011).
347 In contrast, IAV-infected mice that had previously received tolerogenic signals failed to
348 produce these antibodies at levels above primary immunised animals, demonstrating an
349 impaired functional response (Figure 6C).

350

Gray, Figure 6

351



352 **Figure 6: Activation of CD4 T cells with peptide in the absence of adjuvant does not**
353 **affect CD4 T cell cytokine production but does prevent them providing accelerated help**
354 **to B cells**

355 *C57BL/6 mice were infected with IAV on day -30. On day 0, mice received NP₃₁₁₋₃₂₅ +/- PolyIC*
356 *and some of these mice were immunised i.p with NP-OVA with alum on day 30 (A). On day*
357 *35, cells from the spleen, MedLN and lung were co-cultured with bmDCs loaded with NP₃₁₁₋*
358 *₃₂₅ for 6 hours in the presence of Golgi Plug and the number of IFN- γ , TNF and IL-2 producing*
359 *CD44^{hi} CD4+ T cells examined (B). The levels of IgG, IgG1 and IgG2c anti-OVA antibodies in*
360 *the serum was determined on day 5 (C). Each symbol represents one mouse and error bars*
361 *are SEM. In B the grey dashed line represents the background staining in naïve animals. Data*
362 *in B are combined from 2-3 experiments (3-5 mice/experiment). Data in C are combined from*
363 *3 experiments with 4 mice/experiment. All statistics calculated using a one-way ANOVA with*
364 *multiple comparisons; ns = not significant, * = <0.05, ** = <0.01, *** = <0.001, **** = <0.0001.*

365

366 **Memory CD4 T cells exposed to tolerogenic signals expand following reactivation with**
367 **influenza virus**

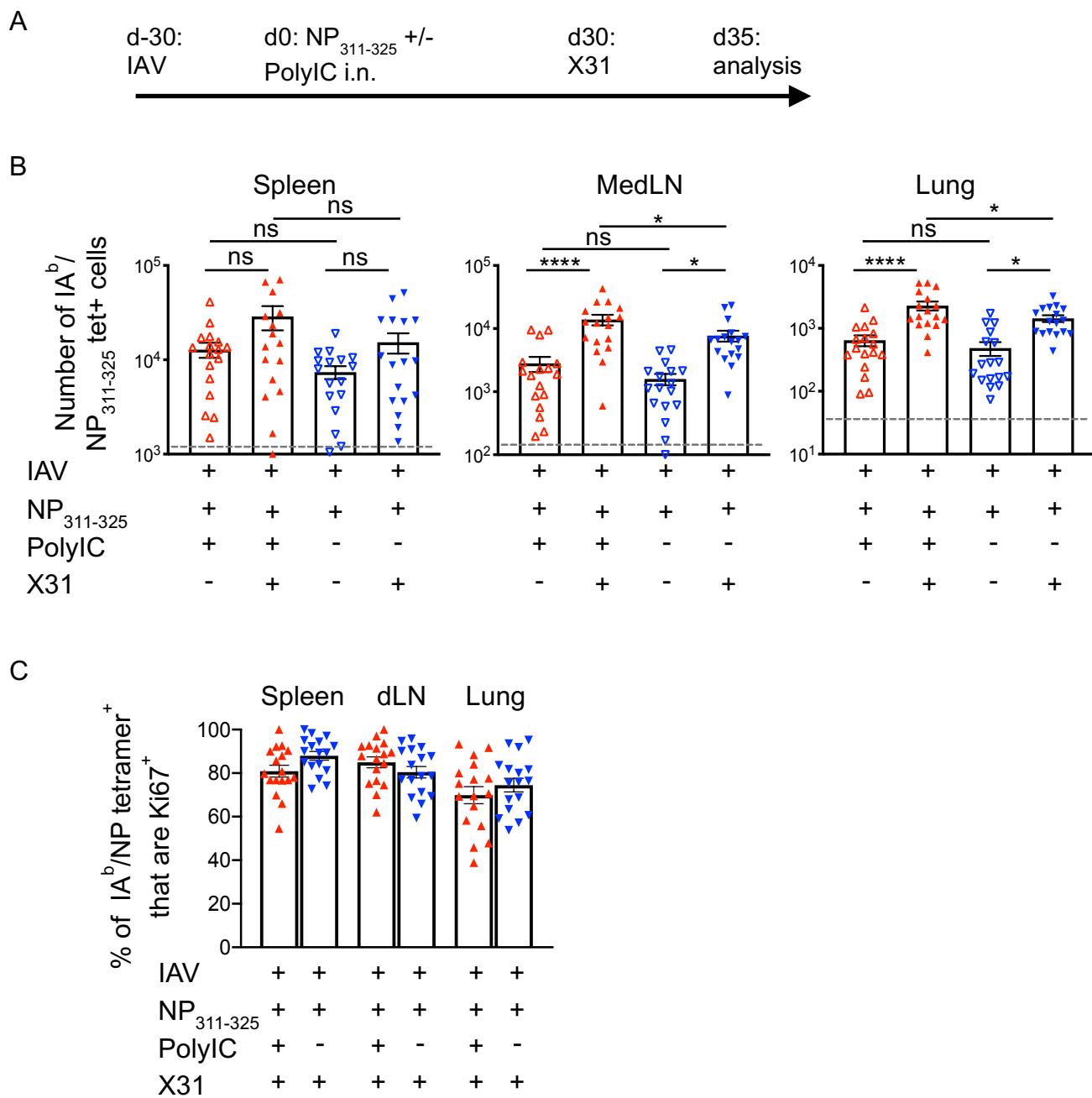
368 Finally, we wanted to test whether the failure of CD4 T cells exposed to tolerogenic signals to
369 accumulate could be rescued by reactivation with a more inflammatory stimulus. We therefore
370 challenged IAV infected mice given immunogenic or tolerogenic signals with an heterosubtypic
371 form of IAV, X31 (Figure 7A).

372

373 Five days following re-infection, we found significant increases in the numbers of antigen-
374 specific CD4 T cells in the lungs and MedLN of mice regardless of their immunisation history
375 (Figure 7B). This expansion was less clear in the spleen regardless of previous immunisation.
376 In all organs, the majority of the antigen-specific CD4 T cells were Ki67 positive indicating a
377 more robust response following IAV infection compared to immunisation (Figure 7C versus
378 Figure 2C).

379

Gray, Figure 7



382 **Figure 7: CD4 T cells exposed to peptide in the absence of adjuvant expand following
383 re-infection with IAV**

384 *C57BL/6 mice were infected with IAV on day -30. On day 0, mice received NP₃₁₁₋₃₂₅ +/- PolyIC
385 i.n. and some of these mice were infected with 100PFU of X31 i.n. 30 days after this (A). The
386 numbers of IA^b/NP₃₁₁₋₃₂₅ CD44^{hi} CD4 T cells were examined 5 days later in the spleen, MedLN,
387 and lung (B) and their expression of Ki67 determined (C). In B and C, each symbol represents
388 one mouse and error bars are SEM. In A, the grey dashed line represents the background
389 staining in naïve animals. Data are combined from 3 experiments (5-6 mice/experiment). All
390 statistics calculated using a one-way ANOVA with multiple comparisons; ns = not significant,
391 * = <0.05, ** = <0.01, *** = <0.001, **** = <0.0001.*

392

393 **Discussion**

394

395 Memory CD4 T cells respond to low doses of antigen and costimulatory signals suggesting
396 they will be refractory to tolerance induction(Blair et al., 2011, Holzer et al., 2003, London et
397 al., 2000, MacLeod et al., 2006). This presents significant hurdles for therapies that aim to
398 induce antigen-specific T cell tolerance(MacLeod and Anderton, 2015, Pearson et al., 2017,
399 Ten Brinke et al., 2019).

400

401 Our previous(David et al., 2014) and current data demonstrate that some but not all functions
402 of memory CD4 T cells are altered following the exposure of memory CD4 T cells to antigen
403 delivered in the absence of adjuvant. Our findings demonstrate that tolerance induction in
404 these cells is both subtle and complex. This contrasts with investigations of tolerance induction
405 in naïve CD4 T cells that consistently show silencing of multiple T cell functions(Greenwald et
406 al., 2005, Liu et al., 2019, Miller et al., 2007, Nurieva et al., 2006, Nurieva et al., 2011).

407

408 Consistently in our own and others research, memory CD4 T cells reactivated with antigen
409 alone fail to accumulate following tertiary activation with antigen and adjuvant(David et al.,

410 2014, Mackenzie et al., 2014). Our analysis of cell proliferation and survival signals suggest
411 that this is not a consequence of reduced entry into the cell cycle nor low expression of anti-
412 apoptosis molecules. Instead, our data indicate that memory CD4 T cells reactivated following
413 exposure to antigen fail to complete mitosis, a characteristic associated with the phenomenon
414 of mitotic catastrophe.

415

416 Mitotic catastrophe has mainly been studied in tumour cells treated with ionising radiation or
417 drugs that cause DNA damage(Kimura et al., 2013, Maskey et al., 2013, Mc Gee, 2015,
418 Vakifahmetoglu et al., 2008). Our transcriptomic data indicate that reactivated memory CD4 T
419 cells previously exposed to tolerogenic signals have reduced expression of molecules
420 involved in sensing and repairing DNA damage and in the control of various stages of the cell
421 cycle. Our data suggest, therefore, a novel form of cell death for memory CD4 T cells.

422

423 In contrast to the poor accumulation of memory CD4 T cells exposed to tolerogenic signals,
424 this did not shut down cytokine production. We found only small increases in cytokine-
425 producing CD4 T cells following reactivation regardless of T cell activation history. If these
426 cells are not undergoing cell division, they would not be at risk of death via mitotic catastrophe.
427 These data suggest that non-cytokine producing memory CD4 T cells are more likely to
428 proliferate than those committed to cytokine production. This agrees with the general concepts
429 within the Tcentral/Teffector memory cell classification and with findings from ourselves and
430 others that cells either making IFN- γ , or that are CD62L^{lo}, proliferate poorly on
431 reactivation(Dutta et al., 2013, MacLeod et al., 2008, Thomas et al., 2010).

432

433 We did find that memory CD4 T cells previously exposed to tolerogenic signals were unable
434 to provide accelerated help for primary responding B cells, suggesting cell proliferation may
435 be required for this functional response. This contrasts with our previous study in which
436 memory cells exposed to tolerogenic signals could help primary responding B cells produce
437 class switched antibody(David et al., 2014). There are multiple differences in experimental

438 procedure between our previous study and the experiments here including the antigen (3K
439 peptide versus NP₃₁₁₋₃₂₅) form of priming (antigen versus infection) and route of tolerance
440 induction (intravenous versus intranasal) that could explain this difference. Regardless, in our
441 studies and similar research from others, memory CD4 T cells reactivated with antigen
442 delivered without adjuvant accumulate poorly following a subsequent immunisation(David et
443 al., 2014, Mackenzie et al., 2014). This suggests that consistently and, regardless of
444 specificity, priming, memory cell location, and route of injection of tolerogenic signals, memory
445 CD4 T cells reactivated with antigen in the absence of adjuvant proliferate poorly following
446 reactivation with antigen and adjuvant.

447

448 Interestingly, we found that this poor accumulation could be rescued by re-infection with IAV,
449 a much more potent challenge to the host than immunisation. These data suggest that while
450 the responses of memory CD4 T cells can be moderated by exposure to tolerogenic signals,
451 these cells may not be permanently silenced. An alternative explanation is that a portion of
452 the memory CD4 T cells are not reactivated by antigen immunisations and therefore remain
453 blind to the tolerogenic signals and free to respond to the infection. Teasing apart these two
454 possibilities will require detailed understanding of the micro-location of memory CD4 T cells
455 within peripheral and lymphoid organs and which antigen presenting cells reactivate memory
456 CD4 T cells following immunisation and infection. A deeper understanding of these factors will
457 be critical to address the most effective methods of antigen-specific tolerance strategies. Our
458 data, moreover, demonstrate the importance of analysing multiple phenotypic and functional
459 parameters in trials of antigen-specific therapy(Pearson et al., 2017, Ten Brinke et al., 2019).

460

461 **Materials and Methods**

462

463 **Animals**

464 To generate mice in which rtTA reports IL-2 expression we used recombineering to extract the
465 upstream 8.389kb section of the IL-2 promoter from a Bacterial Artificial Chromosome (BAC)

466 RP24208L3 (BAC resource at Children's Hospital Oakland Research Institute, Buffalo, New
467 York). This was subcloned into a plasmid containing the human CD2 locus control region and
468 linked to the rtTA sequence. The transgene, cut and purified from the construct backbone,
469 was used to create transgenic mice at the Transgenic mouse facility at National Jewish Health
470 in Jackson Lab C57BL/6 animals. Two founder pups were identified by PCR but only one was
471 fertile. Progeny of this animal were bred with B6.Cg- Tg(tetO-cre)1Jaw/J (006234) and
472 B6.129X1-Gt(ROSA)26Sor^{tm1(EYFP+)Cos} (006148) both from Jackson Laboratories. 10
473 week old female C57BL/6 mice were purchased from Envigo (UK). TRACE and C57BL/6 mice
474 were maintained at the University of Glasgow under standard animal husbandry conditions in
475 accordance with UK home office regulations (Project License P2F28B003) and approved by
476 the local ethics committee.

477

478 **Immunisations and infections**

479 TRACE mice were given Dox+ chow (Envigo) for a total of 7 days starting two days prior to
480 immunisation with 40µg of ovalbumin (OVA) protein (Worthington) conjugated to 20µm
481 polyethylene carboxylate beads (Polysciences Inc.) with 20µg of polyinosinic:polycytidinic acid
482 (InvivoGen) and 20µg of anti-CD40 (BioXcell) s.c in the scruff. Recipients of TRACE EYFP+
483 T cells were given 40µg of OVA with/out 10µg lipopolysaccharide i.v. in 100µl of PBS. NP₃₁₁₋
484 ₃₂₅ was conjugated to OVA using Imject Maleimide-activated OVA according to the
485 manufacturer's instruction (ThermoFisher) and mice immunised with 1µg NP₃₁₁₋₃₂₅-OVA
486 delivered i.p with 0.1mg alum. For mitosis analysis, C57BL/6 mice were immunised with 20µg
487 NP₃₁₁₋₃₂₅ peptide (IDT) with 10µg of LPS i.v. After 30 days, they were re-immunised with 20µg
488 NP₃₁₁₋₃₂₅ peptide with/out 10µg LPS followed 30 days later by i.p. immunisation with 5µg NP-
489 OVA with 0.1mg of alum i.p. For IAV studies, C57BL/6 mice were briefly anesthetised using
490 inhaled isoflurane and infected with 200-300 plaque forming units of IAV strain WSN in 20µl
491 of PBS intranasally (i.n.). IAV was prepared and titered in MDCK cells. Infected mice were
492 rechallenged with 100PFU of X31 (kindly provided by Prof James Stewart, University of

493 Liverpool). Infected mice were weighed daily. Any animals that lost more than 20% of their
494 starting weight were humanely euthanised.

495

496 **FACS sorting and cell transfers**

497 TRACE mice were euthanised 8 days post-immunisation and single cell suspensions were
498 prepared. Lymphoid organs, including spleen, mediastinal, axillary, brachial and mesenteric
499 lymph nodes, from individual mice were pooled and pre-enriched for CD4 T cells using
500 EasySep™ Mouse T Cell Isolation Kit (Stemcell Technologies). Live, single, EYFP+ CD4 T
501 cells negative for MHCII, B220, CD8 and F4/80 were sorted on a BD FACS Aria. Sorted cells
502 were washed in PBS and 100,000 cells transferred i.v. into naïve C57BL/6 mice.

503 **Tissue preparation**

504 Mice were euthanized either by cervical dislocation or with a rising concentration of carbon
505 dioxide and perfused with PBS-5mM EDTA in experiments examining lungs. Spleen and
506 mediastinal lymph nodes were processed by mechanical disruption. Single cell suspensions
507 of lungs were prepared by digestion with 1mg/ml collagenase and DNase (Sigma) for 40
508 minutes at 37°C. Red blood cells were lysed from spleen and lungs using lysis buffer
509 (ThermoFisher).

510

511 **Flow cytometry**

512 Single cell suspension were stained with PE-labeled IA^b/NP₃₁₁₋₃₂₅ (NIH tetramer core) at 37°C,
513 5% CO₂ for 2 hours in complete RMPI (RPMI with 10% foetal calf serum, 100µg/ml penicillin-
514 streptomycin and 2mM L-glutamine) containing Fc block (24G2). Surface antibodies were
515 added and the cells incubated for a further 20minutes at 4°C. Antibodies used were: anti-CD4
516 BUV805 (BD Biosciences; clone: RM4-5) or CD4 APC-Alexa Fluor 780 (eBioscience; RM4-
517 5), anti-CD44 BUV395 (BD Biosciences; clone: IM7), anti-CXCR5 BV785 (BioLegend;
518 clone:L138D7), anti-PD-1 BV711 (BioLegend: 29F.1A12) and 'dump' antibodies: B220 (RA3-

519 6B2), anti-CD8 (53-6.7) and MHC II (M5114) all on eFluor-450 (eBioscience). Cells were
520 stained with a fixable viability dye eFluor 506 (eBioscience). In some cases, cells were then
521 fixed with FoxP3 Transcription Factor Fixative kit (Thermofisher UK) and stained with anti-
522 FoxP3 PeCy7 (eBioscience; FJK-16S), anti-Bcl2 FITC (Biolegend; Blc/10C4), anti-Ki67
523 BV605 (Biolegend; 16A8). Phosphorylated H3 was detected in cells fixed with 2%PFA/0.5%
524 saponin using Alexa647-labelled anti-Histone H3 (pS28) (HTA28). Cells were acquired on a
525 BD LSR or Fortessa and analysed using FlowJo (version 10 Treestar).

526

527 **T cell cytokine analysis**

528 Bone marrow derived dendritic cells were cultured as described(Inaba et al., 1992) in complete
529 RPMI supplemented with X-63 supernatant for 7 days. A single cell suspension was incubated
530 with 10 μ g/ml NP₃₁₁₋₃₂₅ peptide for 2 hours prior to co-culture with lungs, spleen or lymph node
531 cells in complete RMPI at a ratio of approximately 10 T cells to 1 DC in the presence of Golgi
532 Plug (BD Bioscience). Co-cultures were incubated at 37°C, 5% CO₂ for 6 hours. Cells were
533 incubated with Fc block and surface stained with anti-CD4 BUV805 (BD Biosciences; clone:
534 RM4-5) or CD4 APC-Alexa Fluor 780 (eBioscience; RM4-5), anti-CD44 BUV395 (BD
535 Biosciences; clone: IM7) and 'dump' antibodies: B220 (clone: RA3-6B2), CD8 (53-6.7) and
536 MHC II (clone: M5114) all on eFluor-450 (eBioscience). Cells were fixed with cytofix/cytoperm
537 (BD Bioscience) for 20 minutes at 4°C and stained in permwash buffer with anti-cytokine
538 antibodies for one hour at room temperature (anti-IFN- γ PE (XMG1.2;), anti-TNF Alexa-Fluor-
539 488 (MP6-XT22) anti-IL-2 APC (JES6-5H4) all from eBioscience.

540

541 **RNA isolation for RNA-seq**

542 CD4+ EYFP+ cells from the spleen, mediastinal, axillary, brachial and mesenteric lymph
543 nodes of TRACE cell recipients were FACS sorted as above and cell pellets were stored at -
544 20°C prior to RNA extraction. RNA was extracted and purified from single cell suspensions
545 using RNeasy Micro Kit (Qiagen) according to manufacturer's instructions.

546 **RNA analysis**

547 Sequencing and library prep were conducted by LCSciences Ltd. Total RNA was extracted
548 using Trizol reagent (Invitrogen, CA, USA). Total RNA quantity and purity were analysed using
549 a Bioanalyzer 2100 and RNA 6000 Nano LabChip Kit (Agilent, CA, USA), all samples had RIN
550 numbers >7.0. Approximately 10 μ g of total RNA was subjected to isolate Poly (A) mRNA with
551 poly-T oligoattached magnetic beads (Invitrogen). Following purification, the poly(A)- or
552 poly(A)+ RNA fractions were fragmented using divalent cations under elevated temperature.
553 The cleaved RNA fragments were reverse-transcribed to create the final cDNA library in
554 accordance with the protocol for the mRNA-Seq sample preparation kit (Illumina, San Diego,
555 USA). The average insert size for the paired-end libraries was 300 bp (\pm 50 bp). Paired-end
556 sequencing was done on an Illumina Hiseq 4000 (Ic-bio, China). Cutadapt software(Martin,
557 2011) was used to remove low quality reads and adaptor sequences. High quality reads were
558 then mapped to a C57BL/6 mouse reference genome to which the EYFP sequence was added
559 using HISAT2(Kim et al., 2015). Readcounts were obtained from bam files with FeatureCounts
560 using the default parameters. Differential expressed genes (DEGs) were obtained with
561 DESeq2, using RStudio (RStudio Inc). DEGs were visualised with a volcano plot using the
562 'Enhancedvolcano' package within R. DEGs with a fold change of at least 3 and q-values less
563 than p=0.05 were classed as statistically significant. Heatmaps were generated using the
564 heatmap2 function, using the DESeq2 normalised counts and Panther(Mi et al., 2019) used
565 to determine GO biological processes. GSEA analysis was conducted using UC San
566 Diego/Broad Institute's GSEA software(Mootha et al., 2003, Subramanian et al., 2005).

567

568 **ELISA**

569 OVA specific antibody ELISAs were carried out as desctried(David et al., 2014). Serum from
570 immunised and control mice was titrated in 2-fold serial dilution on plates coated with OVA
571 protein. Anti-mouse IgG, IgG1 or IgG2c biotin detection antibodies (Thermofisher, UK) were
572 used with Extravidin-peroxidase (Sigma Aldrich) and SureBlueTMB substrate (KPL).
573 Absorbance was measured at 450nm using a Sunrise Absorbance Reader (Tecan). The

574 absorbance of each sample was normalised to a positive control on each plate after the
575 background absorbance from a blank well had been removed.

576 **Statistical analysis**

577 Data were analysed using Prism version 7 software (GraphPad). Differences between groups
578 were analysed by unpaired ANOVAs or T-tests as indicated in figure legends. In all figures *
579 represents a p value of <0.05; **: p>0.01, ***: p>0.001, ****: p>0.0001.

580

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591 been deposited on GEO, accession number GSE145310.

592

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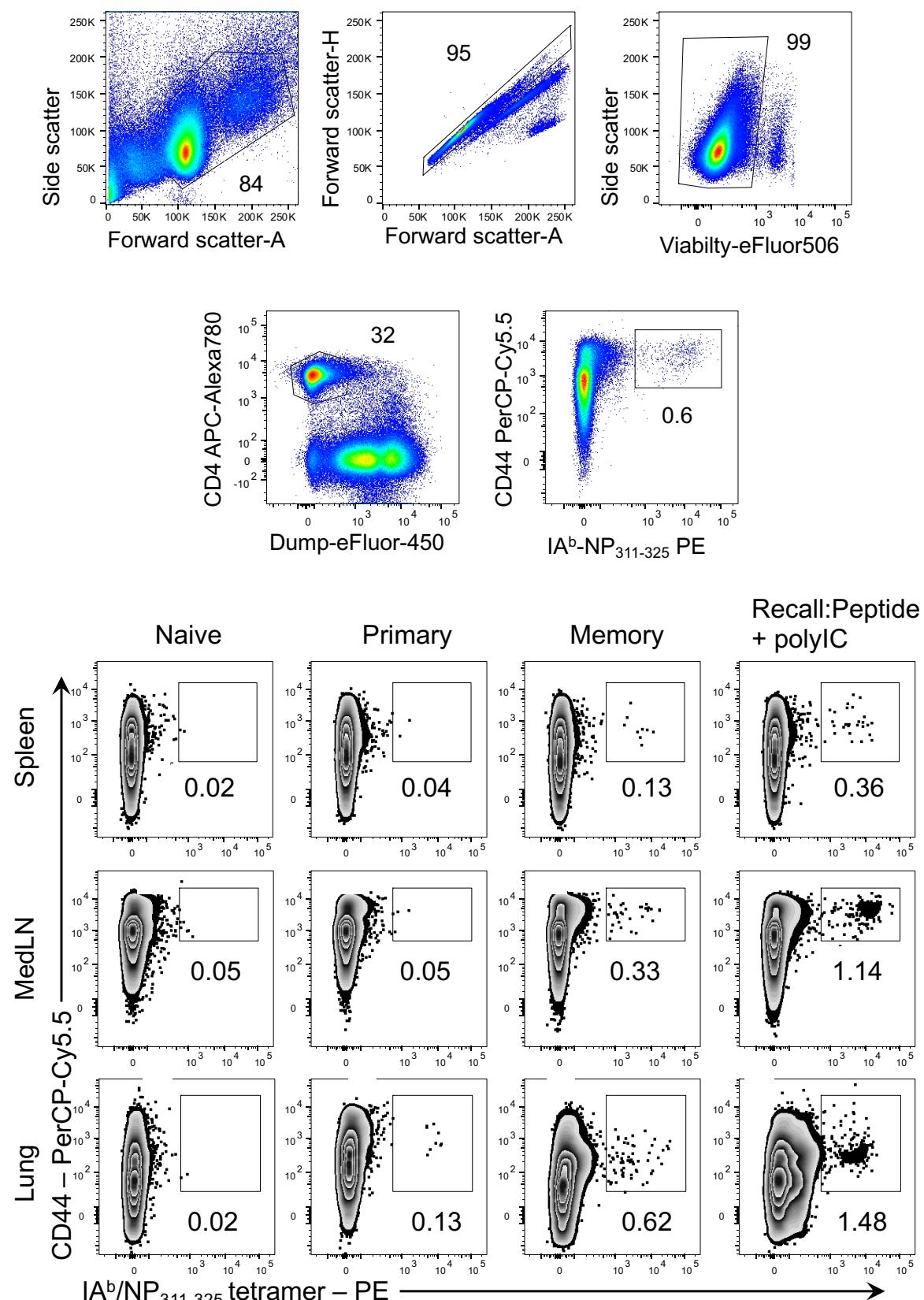
594 **Competing interest statement:** The authors have no competing interests to declare.

595

596 **Author Contribution:** JIG designed and performed experiments, analysed data and wrote
597 the manuscript; TO and S-AK analysed data; FM, ETC, LG, JLM, JWK and PM designed and
598 produced essential tools; PG designed experiments; MKLM designed and performed the
599 research, analysed data, and wrote the manuscript. All authors approved the manuscript.

600

Supplementary Figure 1



604 **Supplementary Figure 1: Gating strategy for IA^b/NP₃₁₁₋₃₂₅ tetramer+ CD44^{hi} cells**

605 C57BL/6 mice were infected with IAV i.n. and some were given NP₃₁₁₋₃₂₅ peptide+PolyIC i.n.
606 on day 30. The percentages of IA^b/NP₃₁₁₋₃₂₅ tetramer+ CD44^{hi} CD4 T cells examined 35 days
607 later in spleen, mediastinal LN, and lung. Cells are gated on live CD4+ lymphocytes that are
608 negative for B220, F4/80, CD8, and MHCII+ as shown in the gating strategy. The numbers on
609 the graph show the percentages of the cells present in the gate within each plot.

610

611

Supplementary Figure 2

612

613

614

615

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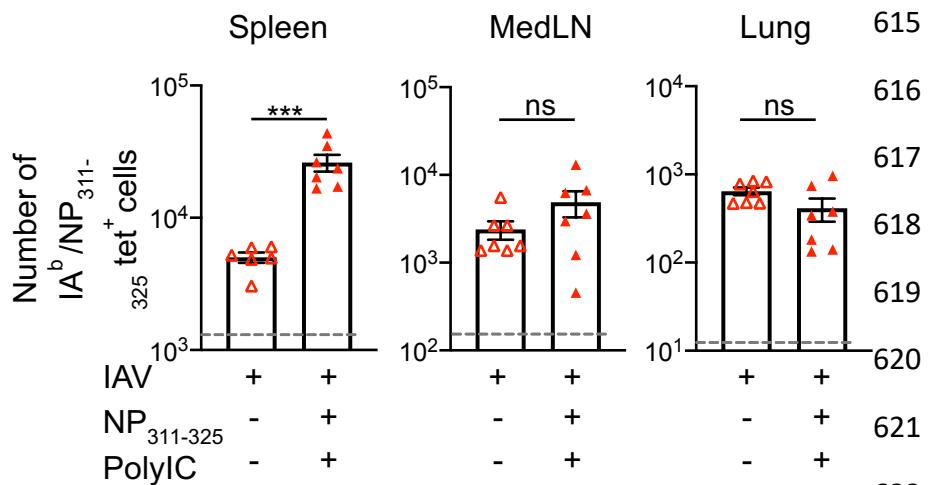
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623

624 **Supplementary Figure 2: Lung memory NP₃₁₁₋₃₂₅-antigen-specific CD4 T cells are not**
625 **reactivated by antigen and adjuvant delivered i.v.**

626 C57BL/6 mice were infected with IAV i.n. on day -30. On day 0, some of these mice were
627 immunised with NP₃₁₁₋₃₂₅, + PolyIC intravenously. The numbers of IA^b/NP₃₁₁₋₃₂₅ CD44^{hi} CD4 T
628 cells were examined 5 days later in the spleen, MedLN, and lung. Each symbol represents
629 one mouse and error bars are SEM. The grey dashed line represents the background staining
630 in naïve animals. Data are combined from two experiments (3-4 mice/experiment). Statistics
631 calculated using a one-way ANOVA with multiple comparisons; ns = not significant, * = <0.05,
632 ** = <0.01, *** = <0.001, **** = <0.0001.

633

634

Supplementary Figure 3

635

636

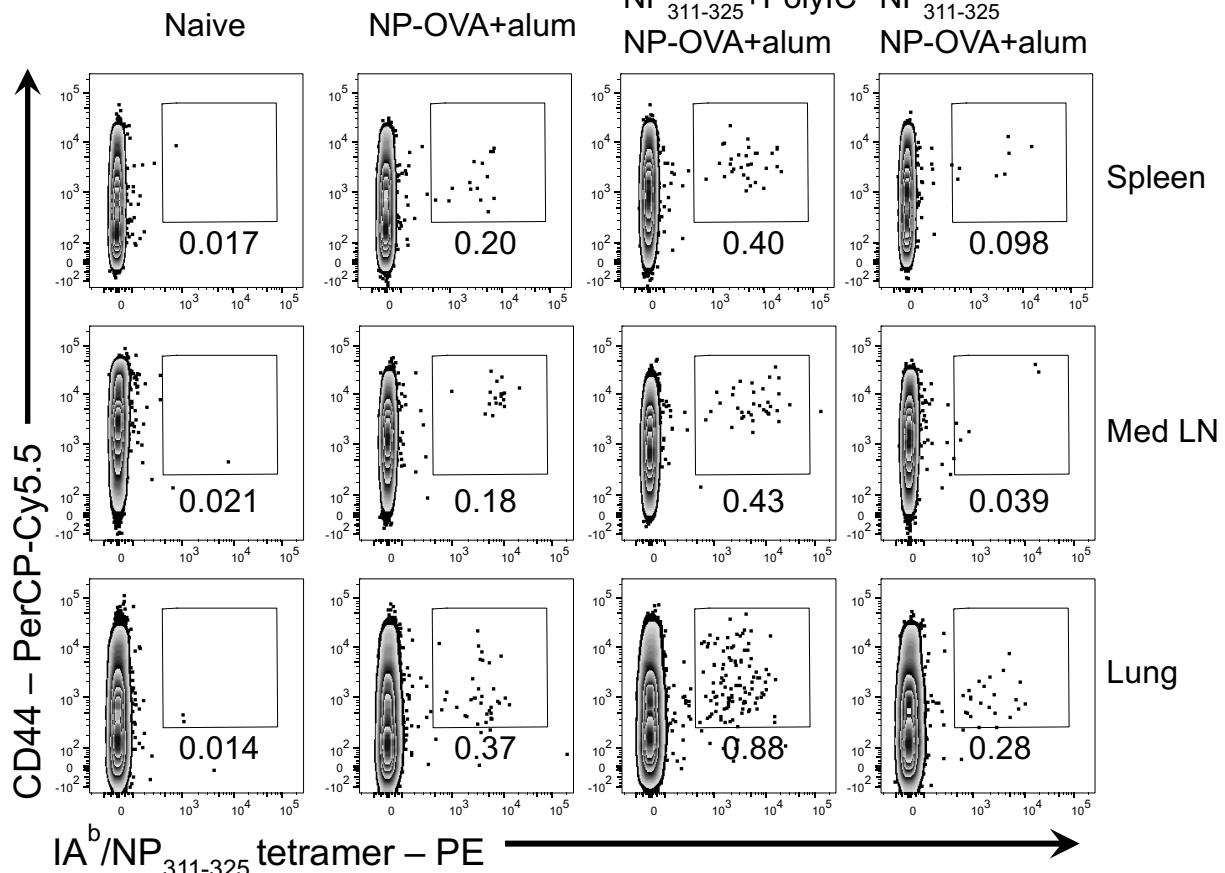
A

d0: NP₃₁₁₋₃₂₅
+/- PolyIC i.n.

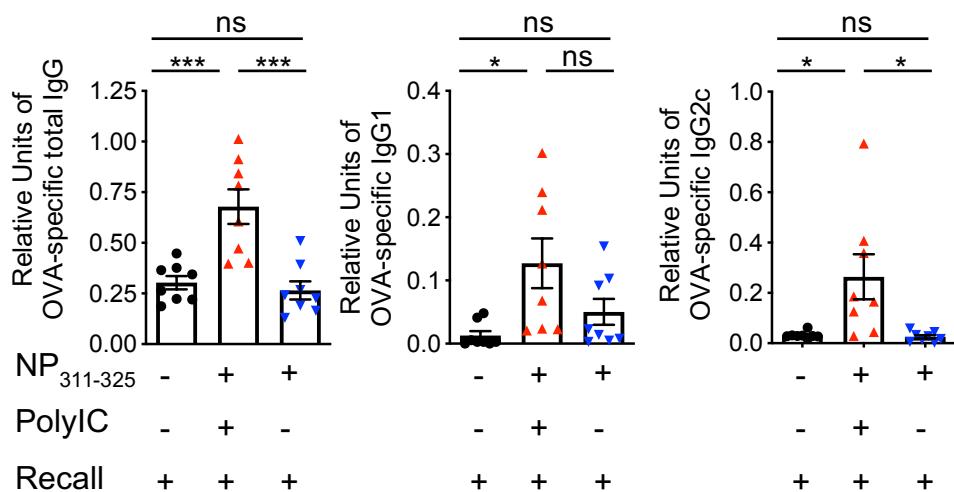
d30: NP₃₁₁₋₃₂₅
- OVA+alum i.p.

d35:
analysis

B



C



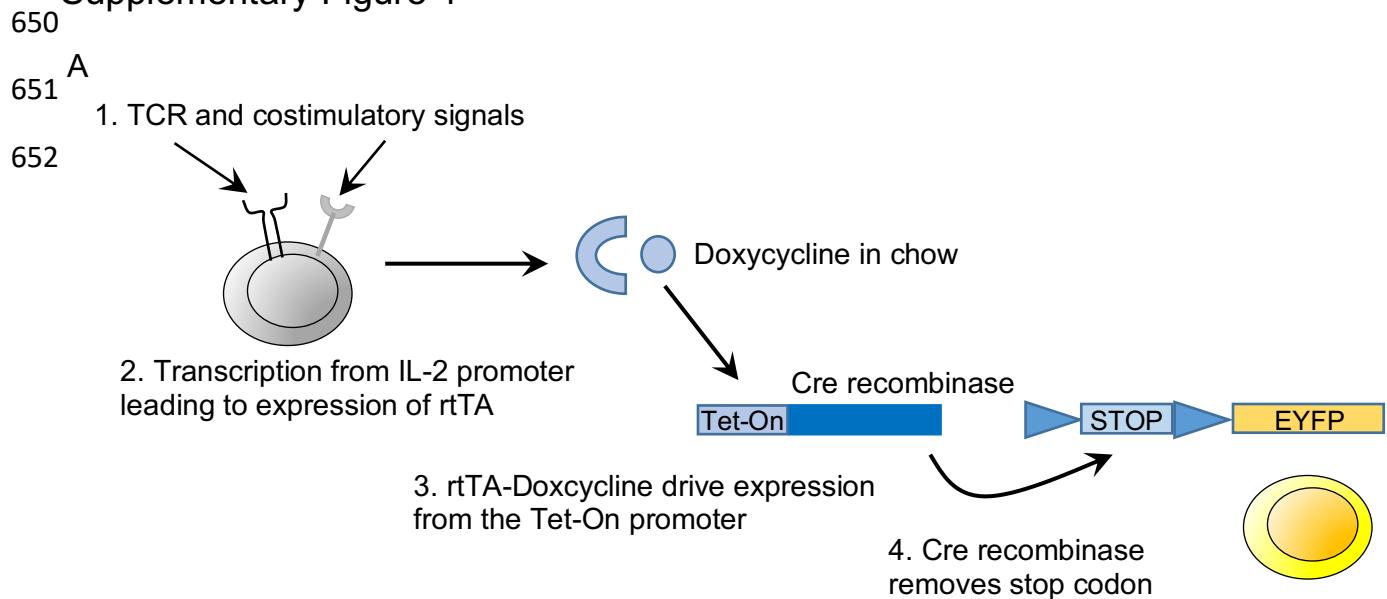
637 **Supplementary Figure 3: Instillation of NP₃₁₁₋₃₂₅ peptide induces functional tolerance in**
638 **naive animals**

639 C57BL/6 mice were instilled with NP₃₁₁₋₃₂₅ peptide +/- PolyIC on day 0 and immunised with
640 NP₃₁₁₋₃₂₅-OVA and alum i.p. 30 days later (A). The percentages of IA^b/NP₃₁₁₋₃₂₅ CD44^{hi} CD4 T
641 cells were examined 5 days after the recall immunisation (B). Cells are gated on live CD4+
642 lymphocytes that are negative for B220, CD8, F4/80, and MHCII+. The numbers on the graph
643 show the percentages of the cells present in the gate within each plot. The levels of IgG, IgG1
644 and IgG2c anti-OVA antibodies was determined on day 5 in the serum (C). Data are from 2
645 experiments with 4mice/group. In C each point represents one mouse and the error bars are
646 SEM). All statistics calculated using a one-way ANOVA with multiple comparisons; ns = not
647 significant, * = <0.05, ** = <0.01, *** = <0.001.

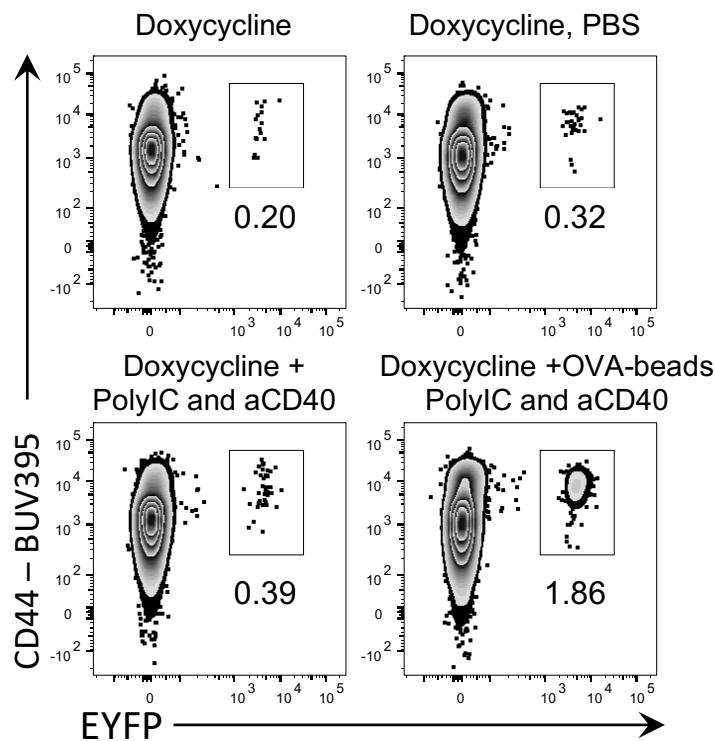
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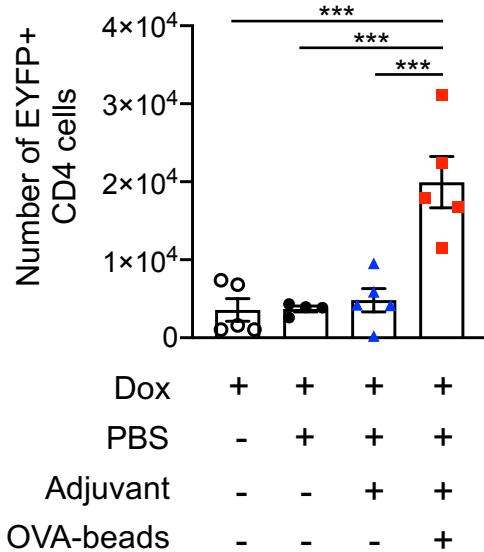
Supplementary Figure 4



B



C



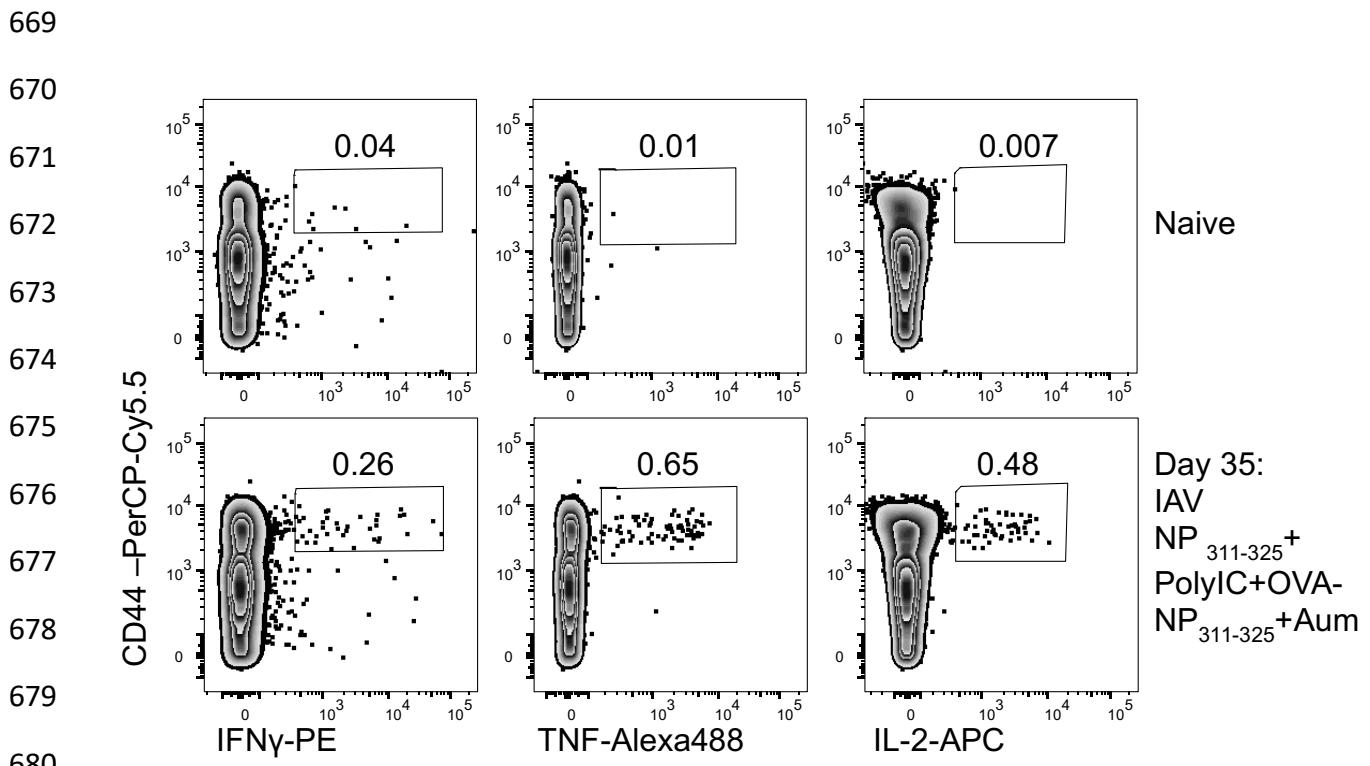
653 **Supplementary Figure 4: TRACE mice enable identification of antigen-reactive CD4 T
654 cells**

655 In TRACE mice, activation through the TCR in the presence of costimulatory signals leads to
656 the expression of rtTA driven by the interleukin 2 promoter. Only in the presence of doxycycline
657 will rtTA be able to bind to the tet-ON promoter leading to the expression of Cre recombinase.
658 Cre recombinase removes the stop codon at the Rosa locus allowing permanent expression
659 of EYFP (A). In B, TRACE mice were given doxycycline chow from day minus 2 until day 5
660 and injected in the s.c. in the scruff on day 0 with nothing, PBS, anti-CD40 and PolyIC, or OVA
661 protein conjugated to 20 μ m beads delivered with anti-CD40 and PolyIC. On day 9, the brachial
662 and axillary lymph nodes were examined by flow cytometry. Cells are gated on live
663 lymphocytes that were CD4+, and negative for B220, F4/80, MHCII and CD8. The numbers
664 show the percentage of CD4+ cells that are in the indicated gates (A). In B, the numbers of
665 EYFP+ CD4 T cells in the lymph nodes are shown with each symbol representing one mouse.
666 Statistics calculated using a one-way ANOVA with multiple comparisons; *** = <0.001.

667

668

Supplementary Figure 5



Supplementary Figure 5: Identification of NP₃₁₁₋₃₂₅ specific cytokine producing CD4 T cells

C57BL/6 mice were infected with IAV on day -30. On day 0, mice received NP₃₁₁₋₃₂₅ in the +/- PolyIC and some of these mice immunised i.p with NP-OVA with alum on day 30. On day 35, cells from the spleen were co-cultured with bmDCs loaded with NP₃₁₁₋₃₂₅ for 6 hours in the presence of Golgi Plug and the percentages of IFN- γ , TNF and IL-2 producing CD44^{hi} CD4+ T cells examined. Cells are gated on live CD4+ lymphocytes as in gating strategy in SF1. Data are representative of 3 experiments (3-5 mice/experiment).

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692

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