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1 **Modelling T Cell Proliferation: Dynamics Heterogeneity Depending on**  
2 **Cell Differentiation, Age, and Genetic Background**

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6 **Short Title: Modelling T Cell Proliferation in aging**

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16 **Abbreviations**

17 BrdU : Bromo-deoxyuridine

18 EdU: 5-ethynyl-2'-deoxyuridine

19 ODE: Ordinary Differential Equation

20 DP: double positive thymocyte CD4<sup>+</sup>CD8<sup>+</sup>

21 TN: triple negative thymocyte CD4<sup>-</sup>CD8<sup>-</sup>CD3<sup>-</sup>

22 Treg: regulatory T cells

23

24 **Key words:** cell proliferation; modelling immune system; aging; lymphocyte dynamics;  
25 cell differentiation; cell death; cell cycle; flow cytometry; mathematical model; state  
26 transition diagram

27

28

29 **Abstract**

30 Cell proliferation is the common characteristic of all biological systems. The  
31 immune system insures the maintenance of body integrity on the basis of a continuous  
32 production of diversified T lymphocytes in the thymus. This involves processes of  
33 proliferation, differentiation, selection, death and migration of lymphocytes to peripheral  
34 tissues, where proliferation also occurs upon antigen recognition. Quantification of cell  
35 proliferation dynamics requires specific experimental methods and mathematical  
36 modelling. Here, we assess the impact of genetics and aging on the immune system by  
37 investigating the dynamics of proliferation of T lymphocytes across their differentiation  
38 through thymus and spleen in mice. Our investigation is based on single-cell multicolour  
39 flow cytometry analysis revealing the active incorporation of a thymidine analogue  
40 during S phase after pulse-chase-pulse experiments *in vivo*, versus cell DNA content. A  
41 generic mathematical model of state transition simulates through ODEs the evolution of  
42 single cell behaviour during various durations of labelling. It allows us to fit our data, to  
43 deduce proliferation rates and estimate cell cycle durations in sub-populations. Our model  
44 is simple and flexible and is validated with other durations of pulse/chase experiments.  
45 Our results reveal that T cell proliferation is highly heterogeneous but with a specific  
46 “signature” that depends upon genetic origins, is specific to cell differentiation stages in  
47 thymus and spleen and is altered with age. In conclusion, our model allows us to infer  
48 proliferation rates and cell cycle phase durations from complex experimental 5-ethynyl-  
49 2'-deoxyuridine (EdU) data, revealing T cell proliferation heterogeneity and specific  
50 signatures.

51

52 **Author summary**

53       We assess the impact of genetics and aging on immune system dynamics by  
54 investigating the dynamics of proliferation of T lymphocytes across their differentiation  
55 through thymus and spleen in mice. Understanding cell proliferation dynamics requires  
56 specific experimental methods and mathematical modelling. Our investigation is based  
57 upon single-cell multicolour flow cytometry analysis thereby revealing the active  
58 incorporation in DNA of a thymidine analogue during S phase after pulse-chase  
59 experiments in vivo, versus cell DNA content. A generic mathematical model that  
60 simulates the evolution of single cell behaviour during the experiment allows us to fit our  
61 data, to deduce proliferation rates and mean cell cycle phase durations in sub-populations.  
62 This reveals that T cell proliferation is constrained by genetic influences, declines with  
63 age, and is specific to cell differentiation stage, revealing a specific “signature” of cell  
64 proliferation. Our model is simple and flexible and can be used with other pulse/chase  
65 experiments.

66

67

68

## 69 **Introduction**

70 Cell division is a characteristic of biological systems, with variability of rates of  
71 proliferation and interphase duration according to the type of organism, organ, and period  
72 of life. In the immune system, cell proliferation is essential for the high turnover of  
73 diversified lymphocytes that insures dynamic cognitive function and maintenance of  
74 body integrity. The production of T cells occurs through processes of proliferation,  
75 differentiation, migration, selection and death in the thymus to finally export less than 6%  
76 of cells to peripheral tissues as naïve T cells (1). T cells circulate through the whole  
77 organism and interact with environmental and body antigens to proliferate and  
78 differentiate into effector/memory T cells upon antigenic recognition. Quantification and  
79 interpretation of lymphocyte dynamics remains a challenge in systems immunology (2,  
80 3). Studies on T cell dynamics at various ages have questioned the mechanisms of thymic  
81 involution (4) beginning at puberty, followed by immuno-senescence and inflammation  
82 with aging (5), and physiological accumulation of effector/memory peripheral T cell  
83 clones, particularly of CD8 phenotype, in both aged humans (6) and mice (7). Although  
84 the number of T cells decreases with aging, promiscuous CD4 T cells accumulate (8).  
85 Thymic hypoplasia or thymectomy in young individuals also affects the repertoire of  
86 peripheral T cells and immunocompetence (9). Thus, thymic production is primordial so  
87 as to maintain a homeostatic and dynamic equilibrium in terms of cell populations and  
88 repertoires, thereby allowing an effective and adaptive immune surveillance. Sequential T  
89 cell development and CD4/CD8 lineage decision models were previously described (10-  
90 13). However, there remain open questions concerning the rates of thymocyte production  
91 that result from various processes, such as differentiation, proliferation, selection, and  
92 death of T cells, and that influence the homeostasis and life-span of naïve and

93 effector/memory T cells in secondary lymphoid organs. Several theoretical approaches  
94 and experimental immunological protocols are available to investigate lymphocyte  
95 dynamics (3) and to model them (14). A number of studies have tried (15-17), often with  
96 the help of modelling (1, 18-21), to quantify thymocyte production, thymic output, and  
97 the processes involved in maintenance of a T lymphocyte dynamic equilibrium in the  
98 periphery in humans or mice (1, 22-26). It was suggested that the naïve T cell pool is  
99 maintained by peripheral division in humans, at variance to thymic output in mice, and  
100 with significant differences in lifespan of CD8 peripheral T cells in young and old  
101 C57BL/6 mice (27). Some findings also suggested different genetic regulations of thymic  
102 output according to strains of mice (28). The flexibility of T cell behaviour has already  
103 been approached, in particular for peripheral cells (29), but references concerning  
104 quantitative proliferation are still lacking.

105         Altogether, these studies were performed with various experimental methods, on  
106 various species, strains, ages, and cell populations. Consequently, no clear consensus has  
107 been reached concerning the influence of genetics and age on T cell dynamics. We have  
108 previously observed that both the genetic background and aging affect T cell dynamics  
109 and repertoires (30). Indeed, C57BL/6 and FVB mice differ in their T cell composition at  
110 steady state but also in their kinetics of T cell reconstitution after a transient  
111 immunosuppression. In FVB mice, there is an acceleration of thymic involution and of  
112 the immunological aging quantified by alterations in the homeostatic control of  
113 lymphocyte numbers and repertoire diversity in the periphery (30).

114         To determine whether or not the differences in the dynamic behaviour of T cells  
115 previously observed between the FVB and B6 mice strains are related to the proliferation  
116 capacities of T cells during their differentiation, we assayed cell proliferation *in vivo* by

117 active DNA labelling of cells in S phase. We designed an original *in vivo* cell labelling  
118 experiment with 5-ethynyl-2'-deoxyuridine (EdU) during a pulse-chase-pulse experiment,  
119 followed by multi-parameter flow cytometry analysis, on the basis of earlier methods that  
120 measured bivariate amounts of incorporated label versus DNA content in single cells (15,  
121 31). To our knowledge, there is currently no model able to analyse the bi-dimensional dot  
122 plot flow cytometry data, in order to infer dynamic parameters of cell cycle phases after  
123 complex pulse-chase experiments. Thus, to interpret the complex cell dynamics during  
124 the experiment, we designed a generic mathematical model that describes the dynamics  
125 of cells that transit from G0/G1 to S and then to G2/M phase of the cell cycle according  
126 to variable periods of pulse-chase. Fitting of the model to the experimental bi-  
127 dimensional flow cytometry data allowed us to infer rates of proliferation, and to give an  
128 estimate of mean duration times of cell cycle phases. Our mathematical model is also able  
129 to fit data from other pulse/chase experiment protocols and by extension can potentially  
130 be used to investigate cell cycle kinetics in any cell type.

131 This study provides a detailed proliferation assessment and cell quantification in  
132 subpopulations of T cells, along their differentiation from thymus to spleen, and through  
133 aging. For the first time, we show that genetic origin and age drive a particular  
134 "signature" of proliferation and cycle phase durations, according to T cell differentiation  
135 stage and T cell lineage. FVB mice have significantly lower rates of T cell proliferation  
136 than C57BL/6 mice in both thymus and spleen. Proliferation decreases with age in both  
137 strains. These results are discussed in the perspective of T cell dynamics and proliferation  
138 of T cells, where aging and genetic peculiarities could be of importance.

139

140 **Results**

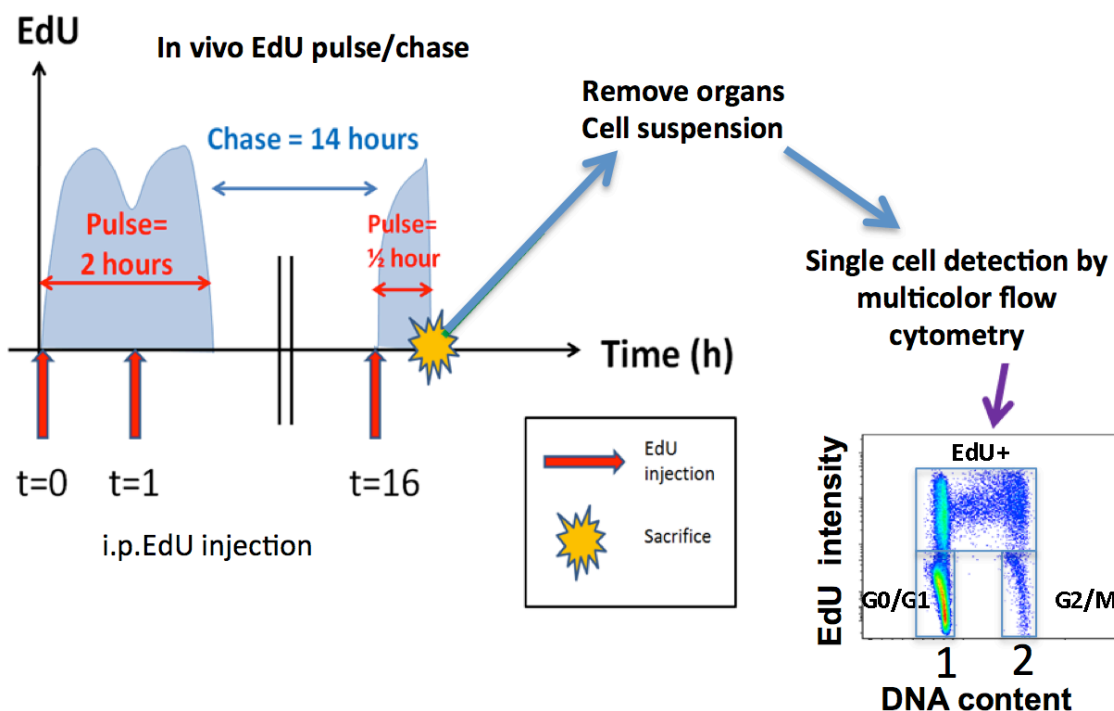
141 **Assessment of *in vivo* cell proliferation by active DNA labelling**

142 To determine proliferation properties of cell populations, active labelling of cells  
143 during S phase of the cell cycle was done with EdU, a thymidine analogue, during  
144 pulse/chase periods. In our specific case, two pulse periods were separated by a fourteen-  
145 hour chase interval (Fig 1). Since the *in vivo* half-life of EdU is about one hour (32), this  
146 was equivalent to a pulse-chase-pulse experiment comprising an initial two-hour pulse  
147 followed by a fourteen-hour chase and then a second thirty-minute pulse. This protocol  
148 was chosen because it allows the labelling of cell populations with low proliferation rates,  
149 which would not be detected with only one EdU injection. Multicolour flow cytometry  
150 then allowed the identification of the cell cycle phases and the quantitative active EdU  
151 labelling status, using bi-dimensional EdU label/DNA content dot plots that represent  
152 single cell analysis. According to DNA content, cells can be classified in G0/G1 phase  
153 (DNA content equal to 1), G2/M phase (DNA content equal to 2), or S phase (DNA  
154 content between 1 and 2). According to the intensity of EdU label, one can distinguish  
155 between labelled cells that have progressed through S phase during the pulse, and non-  
156 labelled cells. Thus, the dot plots represent the end snapshot of the cell population  
157 evolution through periods of pulse/chase. Three groups of cells can be delimited on our  
158 dot plots (Fig 1): G0/G1 unlabelled, G2/M unlabelled, and EdU labelled cells. Since the  
159 experiment ends with a pulse phase, there are no remaining unlabelled cells in S phase.

160

161

162

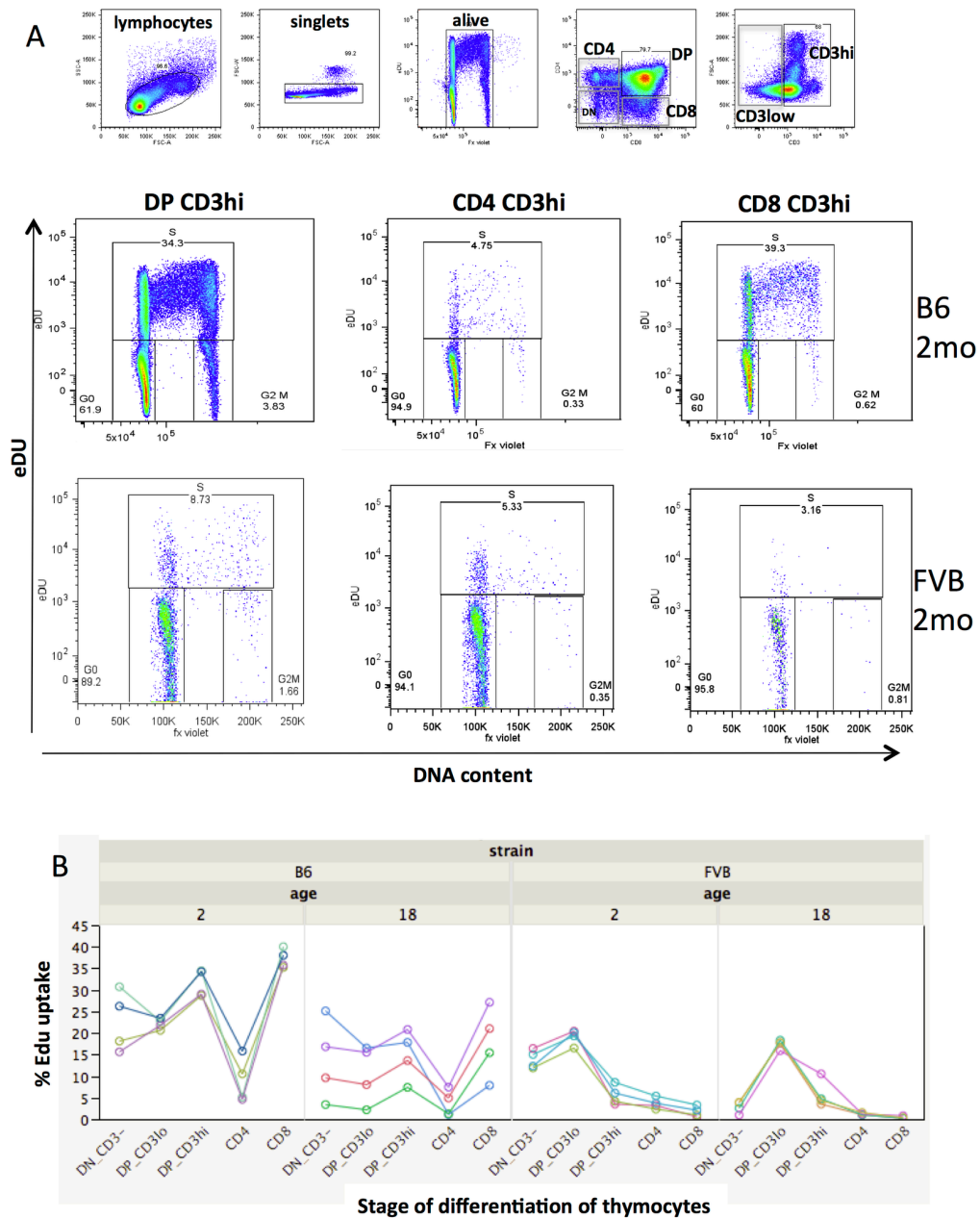


163  
 164 **Fig 1. EdU pulse chase experiment for quantification of cell proliferation.** Mice were  
 165 injected intra-peritoneally with EdU at 0, 1, and 16 hours equivalent to a "pulse-chase-  
 166 pulse" experiment. Thirty minutes after the last pulse, the mice were sacrificed, lymphoid  
 167 organs removed, and cell suspensions were analysed by single-cell multicolour flow  
 168 cytometry. The bi-dimensional dot plot reveals the intensity of EdU label (log scale) and  
 169 DNA content (linear scale arbitrary unit) in lymphocytes. Manual gating allows  
 170 identification of unlabelled cells in either G0/G1 (G) or G2/M (M) that have not transited  
 171 through S phase during pulse, and EdU labelled cells (G'+S'+M') that have transited  
 172 through S phase.  
 173

174 **Differential cell proliferation in T cells according to differentiation stage, age and**  
 175 **strain**

176 Our purpose was to assess the variation of T cell dynamics and heterogeneity in  
 177 individuals, in time and location, by quantification of T cell proliferation in two different  
 178 strains (FVB and B6), through aging (at 2 and 18 months) and during differentiation in  
 179 lymphoid organs (from thymus to spleen). As exemplified in Fig 2A, in the thymus, the  
 180 percentage of EdU labelled cells varies significantly according to strains of mice and the  
 181 stage of thymocyte differentiation. Thus, comparing the FACS profiles between typical 2-  
 182 month-old FVB and B6 mice, one can observe that the proportion of EdU<sup>+</sup> cells is much  
 183 lower in FVB mice, showing lower proliferation capacities in this strain. A given group

184 of mice depicts a particular signature of proliferation according to strain and age (Fig 2B)  
185 revealing the heterogeneity of proliferation across the stages of differentiation. The  
186 percentages of labelled cells after 16 hours are given in S2 Table. With aging, at 18  
187 months, which corresponds to middle life in mice, the proliferation of thymocytes in B6  
188 is reduced by about 1.5 fold in all populations. In 18-month-old FVB mice, the  
189 proliferative capacities are even more reduced in immature CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup> TN cells and  
190 thereafter are quite comparable to the altered proliferation capacities already detected in  
191 young FVB mice. In conclusion, T-cell dynamics is very heterogeneous, depending on  
192 the differentiation stages as detected by the variation in the EdU uptake. Cell proliferation  
193 is altered with age, and genetic origin influences the proliferation capacities, which are  
194 depressed in FVB mice. Thus, a typical biological signature of thymocytes is revealed,  
195 allowing one to distinguish strains of mice and ages, although precise quantification of  
196 dynamic parameters is required.



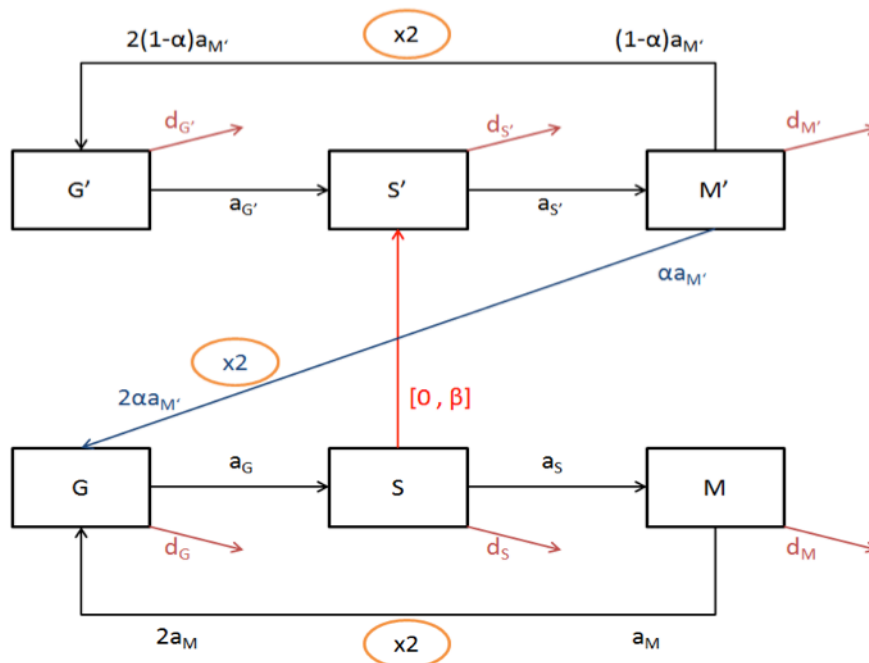
197

198 **Fig. 2. Thymocyte proliferation in mice according to cell differentiation stage, age**  
 199 **and genetic origin.** Cell proliferation was assessed in C57BL/6 and FVB mice of 2 or 18  
 200 months as in Fig 1. (A) Multi-colour flow cytometry and hierarchical gating in

201 thymocytes showing EdU vs DNA content dot plot in 2-month-old mice. (B) Percentage  
202 of EdU+ labelled cells according to differentiation stages through time: from the most  
203 immature CD3-CD4-CD8- triple negative cells (TN), CD3<sup>lo</sup>CD4+CD8+ (DP CD3<sup>lo</sup>),  
204 CD3<sup>hi</sup>CD4+CD8+ (DP CD3<sup>hi</sup>) to single positive mature CD4+CD3+ or  
205 CD8+CD3+stage. Each curve represents the %EdU uptake for one of the 4 mice per  
206 group.

### 207 **Model of labelling experiment and fit to experimental data**

208 Our aim was to quantitatively estimate proliferation parameters and mean life-span  
209 of cells (here restricted to inter-mitotic time), according to age and strain, in a standard  
210 way independent of pulse-chase experiment duration, and to express results as a  
211 proliferation rate/day. Thus we designed a mathematical model to interpret and fit the  
212 data obtained from the experimental cytometry bi-dimensional dot plots. To model  
213 system dynamics and the variation of “stocks” of cells as a function of time, equivalent  
214 representations can be proposed: the hydraulic metaphor, stock-flow/transition diagrams,  
215 integral equations, and differential equations (33). We chose to explain our model with a  
216 state-transition diagram (Fig 3), a visual language that is accessible to biologists and  
217 provides an equivalence to a system of ODEs (34, 35). A state-transition diagram allows  
218 representation of the parallel processes occurring in cells and during the experiment.  
219 Through time, the cells can progress across three stages, G0/G1, S, G2/M or exit the  
220 population (death, differentiation, migration). During the alternative phases of pulse and  
221 chase of EdU, the cells can exist in one of two stages, either EdU unlabelled (Edu<sup>-</sup>) or  
222 EdU labelled (EdU<sup>+</sup>). Thus, by combination, the cells can progress in six different stages  
223 represented by six differential equations. Moreover, the cells progress through the cycle  
224 in the context of EdU bio-availability (pulse or chase). Altogether, the multiplicity of the  
225 states (3x2x2) results in a system of twelve equations (see Materials and Methods section  
226 and Tables 1 to 3 for the description of the mathematical model).



227

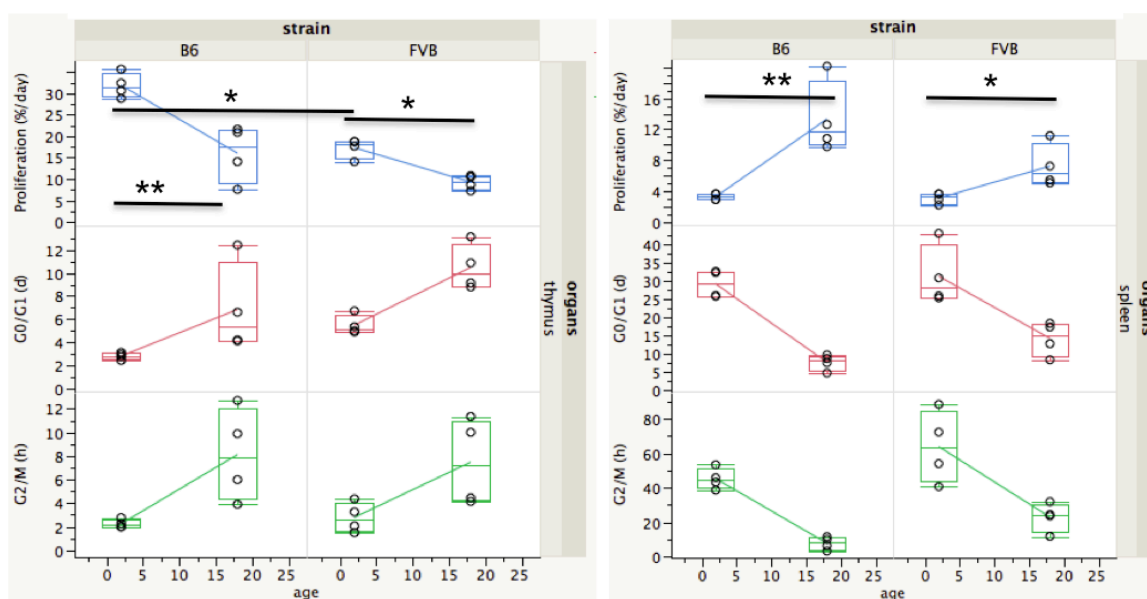
228 **Fig. 3. State transition model of cell cycle and EdU labeling.** According to the FACS  
 229 dot plot, the evolution of cells through the cell cycle and EdU labelling can be  
 230 represented by a transition diagram. Unlabelled cells can transit successively in the three  
 231 phases of the cell cycle: G0/G1 (G), S (S), and G2/M (M). Terms in  $a_X$  correspond to  
 232 rates of entry into the next phase of cells in X phase (X being either G, S, or M). The exit  
 233 of cells, either due to death, differentiation, or migration is represented by terms in  $d_X$ .  
 234 During the pulse-phase the cells in S phase incorporate EdU (red arrow) and enter into S'  
 235 with rate  $\beta$  and continue the cycle to M' and G'. During the chase phase, in the absence  
 236 of EdU, the labelled cells lose labelling upon several cell divisions (blue arrow) (de-  
 237 labelling) with rate  $\alpha$ . Unlabelled cells can enter into S phase but remain unlabelled.  
 238

239 Under our experimental conditions, the EdU labelling protocol is modelled by a  
 240 first pulse phase (between  $t=0$  and  $t=2$  hours), a chase phase (between  $t=2$  and  $t=16$   
 241 hours) and a second pulse phase (between  $t=16$  and  $t=16.5$  hours). We defined an  
 242 experimental result as a two-dimensional vector, representing the three percentages of  
 243 G0/G1 unlabelled, G2/M unlabelled, and labelled cells which are equivalent to the G, M,  
 244 and  $G'+S'+M'$  in the model. Fitting to experimental data was done by simulating the  
 245 progression of cells through the cell cycle and labelling over a bi-dimensional range of

246 parameters. To constrain parameters, we used the biological hypotheses detailed in  
 247 Materials and Methods (Table 3).

248 **Average proliferation rates and cell cycle durations in thymus and spleen from B6**  
 249 **and FVB mice**

250 We first compared proliferation rates for whole thymus and whole spleen,  
 251 abstracting the phenotypic heterogeneity of T cells, in young and old B6 and FVB mice  
 252 (Fig 4, S1 Table). In the thymus, proliferation rates can vary up to three-fold, from 32%  
 253 per day in young B6 mice to 9% per day in old FVB mice. Proliferation rates of  
 254 thymocytes in young B6 mice are in general twice the value of those in young FVB mice  
 255 ( $p < 0.05$ ). The proliferation rates of thymocytes from old B6 mice and young FVB mice  
 256 are similar (around 17%/day). In both strains, there is a two-fold drop of proliferation  
 257 rates in thymus with aging ( $p < 0.05$  in spleen and  $p < 0.01$  in thymus). In the spleen, in  
 258 contrast to the thymus, the proliferation rates increase with age. From about 3% per day  
 259 in both young strains, it doubles in old FVB ( $p < 0.05$ ) and quadruples in old B6 mice  
 260 ( $p < 0.01$ ).



261

262 **Fig. 4. Proliferation rates and cell cycle phase durations in thymus and spleen.**

263 Proliferation rates (%/day), G0/G1 and G2/M phase durations (in days) in B6 and FVB  
264 mice (n=4 per group) according to age (2 and 18 months) in whole thymus (left) and  
265 whole spleen (right). Values are indicated in the box plot, the median is the 50th  
266 percentile, the lower and upper limits of the box are the 25th and 75th percentiles and the  
267 whiskers indicate 1.5x(interquartile range). \*: p<0.05. \*\*: p<0.01. (See Materials and  
268 Methods section for statistical tests used). Statistics are for proliferation rates only. Mean  
269 values and SD are given in S1 Table.  
270

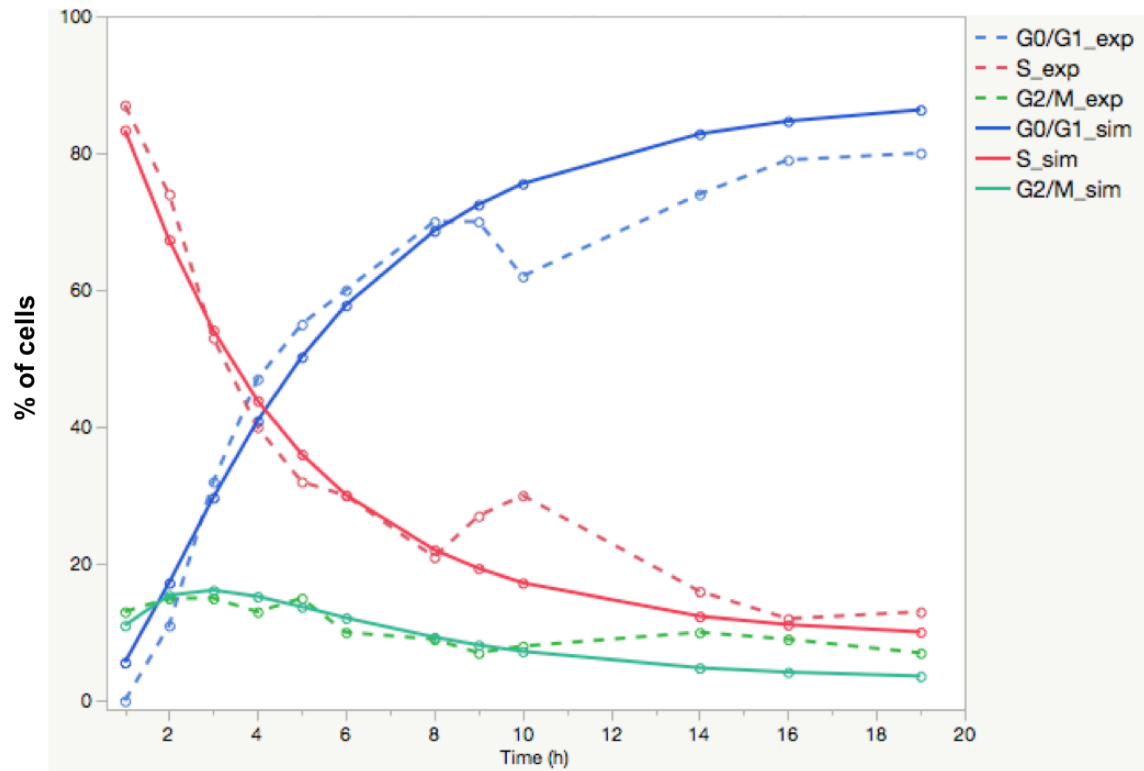
271 The duration of G0/G1 phase, S, and G2/M gives an indication of the mean time  
272 between two cell cycles (inter-mitotic time), which can be calculated as 1/proliferation  
273 rate. Again, these values are heterogeneous; the duration of G0/G1 phase increases with  
274 aging, G2/M duration varies from 2 hours in young B6 thymocytes to up to 63 hours in  
275 young FVB splenocytes.

#### 276 **Model validation: Simulation of another pulse-chase experiment from the literature**

277 In order to consolidate our results and validate our mathematical model, we  
278 simulated a different pulse/chase experiment, as performed in 1990 by Baron and Penit.  
279 This *in vivo* experiment also assesses whole thymocyte kinetics from six- to eight-week-  
280 old B6 mice, using a thymidine analogue Bromo-deoxyuridine (BrdU) uptake, versus  
281 DNA content measurement (15). To simulate this experiment with our model, we set up a  
282 single injection of BrdU at the beginning of the experiment (1-hour pulse), followed by a  
283 nineteen-hour chase phase, during which within the BrdU-labelled cells the percentages  
284 of cells in G0/G1, S, and G2/M cells were quantified according to DNA content. We used  
285 cell cycle parameters retrieved from our own fitting procedure on the whole thymus of  
286 our 2-month old B6 mice (underlined in red in S1 Table), and we simulated the  
287 experiment by setting the initial state to be 100% of cells in S phase. We also set  $\alpha=0$  and  
288  $d_G=a_G$ . The comparison of the experimental results provided by Baron and Penit (15) and  
289 the simulation of their pulse/chase protocol with our parameters obtained from B6 data is  
290 shown in Fig 5. The ability of our simulation to visibly approach Baron and Penit's

291 experimental results confirms the plausibility of the biological parameters obtained in our  
292 young B6 mice and points to the fact that our model can be run with other experimental  
293 conditions of pulse/chase.

294



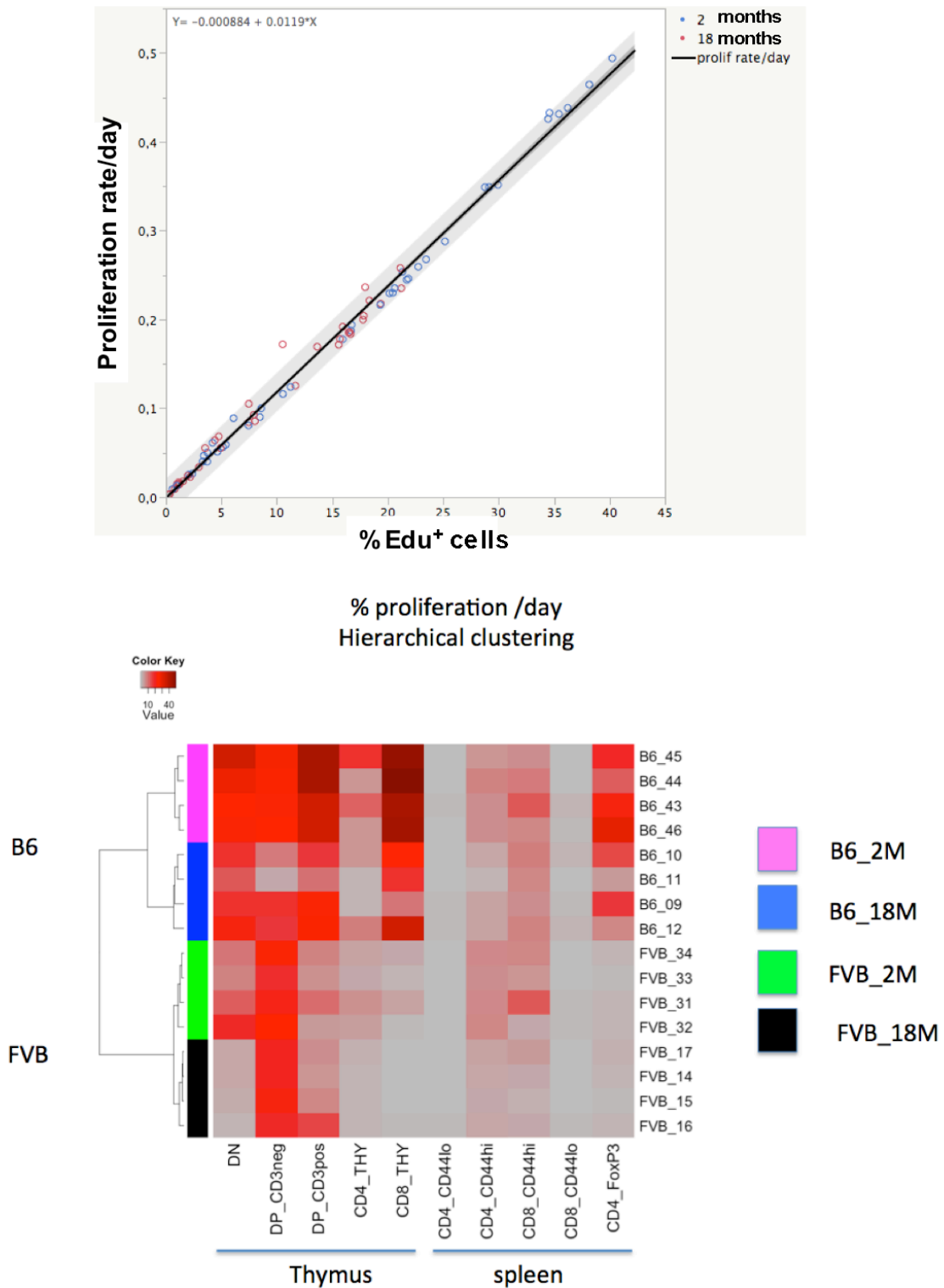
295

296 **Fig. 5. Validation of the mathematical model and parameter values with pulse/chase**  
297 **experimental data from (15).** Young B6 mice received a single BrdU pulse, and within  
298 the BrdU-labelled cells the percentages of cells in G0/G1, S or G2/M is recorded over a  
299 nineteen hour chase period. The points and dashed lines represent Baron experimental  
300 data. Simulation of the same pulse chase conditions using our mathematical model, with  
301 the mean parameter values (S1 Table) obtained from our 2-month-old B6 mice (n=4).  
302 The points and continuous lines represent the results of our simulation.  
303

### 304 **Signature of proliferation according to T cell differentiation stage, age, and genetics**

305 The complexity and heterogeneity of cell populations that compose the thymus led  
306 us to analyse, more deeply, the proliferation rates of cells according to their  
307 differentiation stages, thus across time. Thymocytes were further decomposed in the

308 earliest immature triple negative thymocyte CD4<sup>-</sup>CD8<sup>-</sup>CD3<sup>-</sup>(TN), in DP (double positive  
309 thymocyte CD4<sup>+</sup>CD8<sup>+</sup>, sub-divided into DP CD3<sup>lo</sup> and DP CD3<sup>hi</sup> according to low or  
310 high expression of CD3), CD4, and CD8 mature CD3<sup>+</sup> populations (S1 Figure upper  
311 panel). In the spleen, CD4 and CD8 T cells were separated as CD44<sup>lo</sup> (naïve) and CD44<sup>hi</sup>  
312 (antigen-experienced). CD4<sup>+</sup>Foxp3<sup>+</sup> (regulatory T cells, Treg) cells were also identified  
313 as a sub-population of CD4 T cells (S1 Fig lower panel). From all data collected in the  
314 thymus, we were able to establish a linear correlation between the observed percentages  
315 of EdU<sup>+</sup> cells and the fitted proliferation rates that is similar in the two strains of mice  
316 and is independent of the age (Fig 6). The equation  $y = -0.000884 + 0.0119x$  allows  
317 estimation of the proliferation rates,  $y$ , for further experiments using the same pulse/chase  
318 protocol duration, from  $x = \%EdU^+$  cells ( $r^2 = 0.99$ ,  $p < 0.01$ ). Estimated parameter values  
319 are given in S2 Table.



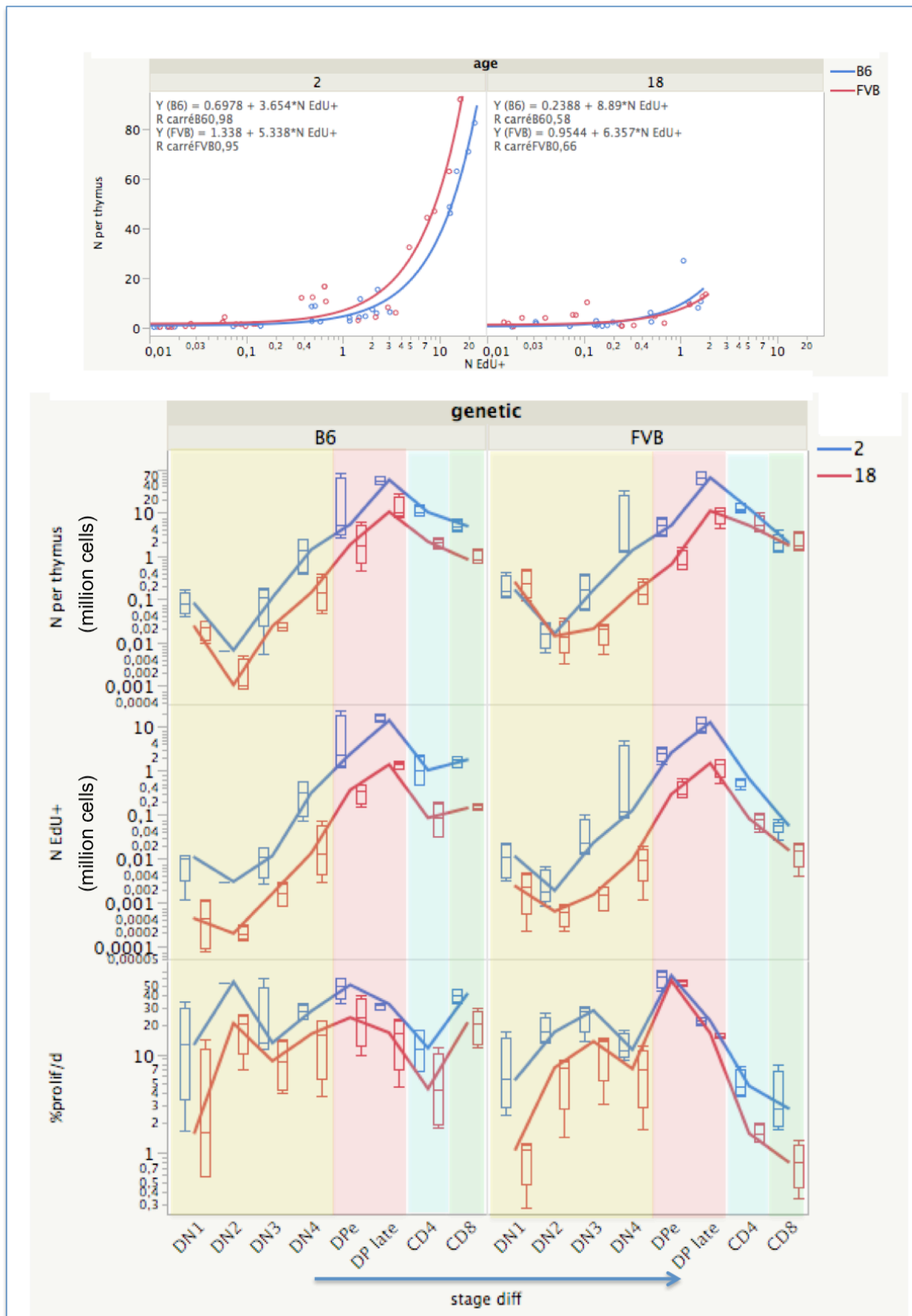
320

321 **Fig. 6. Signatures of lymphocyte proliferation according to strains and ages.** The  
 322 upper panel shows the correlation between the proliferation rate and percentages of Edu<sup>+</sup>  
 323 cells observed 16 h after the pulse/chase/pulse experiment in thymus of B6 and FVB  
 324 mice (80 values) and according to the age of mice (2 months: blue circle, 18 months: red  
 325 circles). Hierarchical unsupervised clustering showing the clusters of mice according to  
 326 the rates of proliferation. Values and statistics are given in Tables S1, S2, S3, S4.

327 Proliferation rates/day are significantly different ( $p < 0.05$ ) comparing :  
328 **In thymus** : B6 2M vs B6 18M: 2M>18M in DP, CD8 ; FVB 2M vs FVB 18M:  
329 2M>18M in DN and CD4 ; B6 2M vs FVB 2M:B6>FVB in DP CD3<sup>+</sup>, CD8 ; B6 18M vs  
330 FVB 18M:B6>FVB in CD8  
331 **In spleen** : B6 2M vs B6 18M: 2M>18M in CD4 CD44<sup>hi</sup> ; FVB 2M vs FVB 18M:  
332 2M>18M in CD4 CD44<sup>hi</sup> ;B6 2M vs FVB 2M:B6>FVB in CD4, CD4 Foxp3<sup>+</sup> ; B6 18M  
333 vs FVB 18M:B6>FVB in CD4 Foxp3<sup>+</sup>, CD8 CD44<sup>hi</sup>  
334

335       Because the intragroup variability of proliferation rates is low while inter-group  
336 heterogeneity is high (S1 Fig), unsupervised hierarchical clustering allows identification  
337 of clusters of mice according to age, strain, and differentiation stage giving particular  
338 signatures (Fig 6). The heterogeneity of cell dynamics is revealed at the various steps of  
339 differentiation. If decomposition of the population is performed so as to identify rare  
340 populations as DN1 to DN4, then to DP that increase CD3 expression through maturation  
341 while modulating CD4 and CD8 co-expression, then one can observe oscillations in  
342 proliferation (S4 Table and S1 Fig median panel), as previously observed in young B6  
343 mice using other protocols (36). Hence in the thymus, while DN1 cells (the early thymic  
344 precursors) display minimal proliferation, in particular in old FVB mice (1.2%/day), CD8  
345 mature thymocytes are the cells that display the highest proliferation rate (45%/day in  
346 young and a 2-fold decrease to 21%/day in old B6 mice) (S4 Table). Thus, the inter-  
347 mitotic time for CD8 CD3<sup>+</sup> mature thymocytes in young B6 are of about 2 days (S2  
348 Table), suggesting expansion of mature thymocytes before thymic export, while such  
349 proliferation is limited in FVB. The relative durations of G0/G1 and G2M/ phase and thus  
350 inter-mitotic time increase with aging in most thymic populations (S2 Figure). In the  
351 spleen aging perturbations are more irregular. Altogether these durations are again  
352 heterogeneous according to the stage of differentiation and T cell lineage.

353

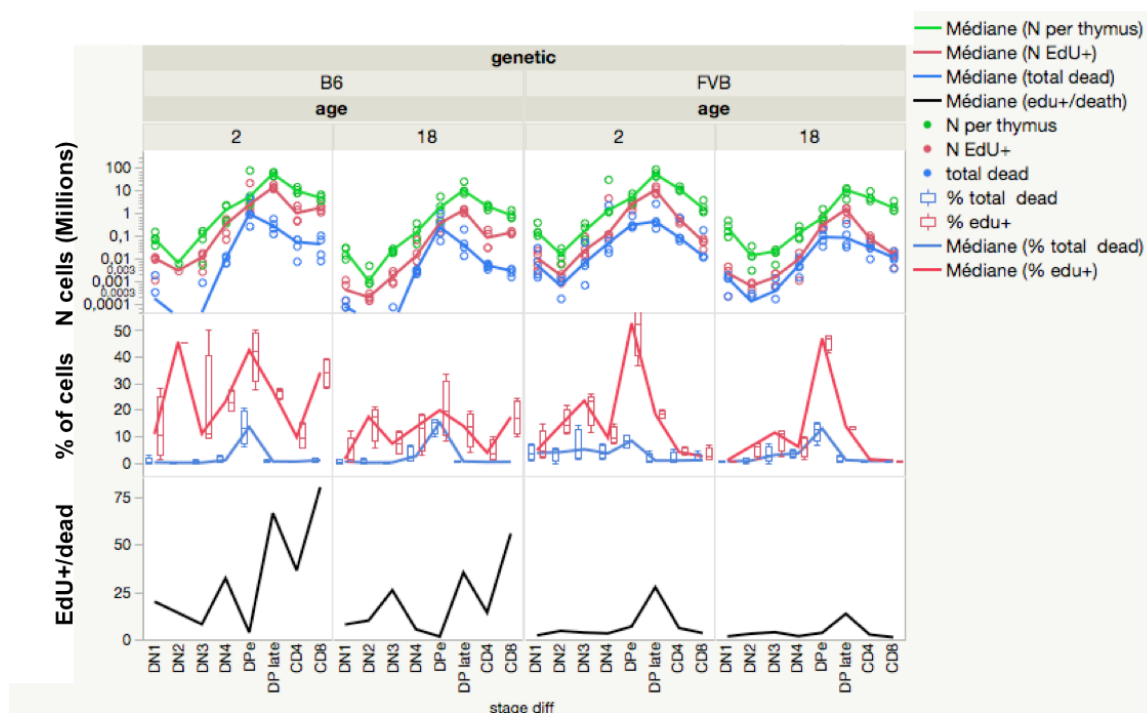


354

355 **Fig. 7. Quantification of dividing cells during thymocyte differentiation.**  
 356 For each stage of thymocyte differentiation, the number of cells and the number of EdU+

357 cells (in millions) is quantified by FACS after pulse/chase. The correlation between these  
358 numbers is shown according to strains and ages of mice (upper panel). Box plots  
359 represent the quartiles and the lines represent the evolution of the median values through  
360 stage of differentiation thus across time, in four mice per group (lower panel). DN CD3<sup>-</sup>  
361 cells are in yellow, DP cells are in red and decomposed into DPearly (CD4<sup>hi</sup>CD8<sup>hi</sup>) and  
362 DP late (CD4<sup>med</sup>CD8<sup>med</sup>). Then DP cells differentiate either into CD4<sup>+</sup>CD3<sup>+</sup> (blue) or into  
363 CD8<sup>+</sup>CD3<sup>+</sup> (green) mature T cells.  
364

365       Numbers of cells per differentiation stage in the thymus are in accordance with the  
366 progression of thymocytes within 4 weeks as previously modelled in young FVB mice  
367 (1). These numbers are correlated to the numbers of EdU<sup>+</sup> cells (Fig 7 upper panel). Thus,  
368 an exponential growth of cells occurs during their residence in the thymus from DN2  
369 where they actively divide up to late DP cells (Fig 7 lower panel). The minimal estimate  
370 of exponential cell divisions in B6 mice is 13 divisions in about 22 days, higher than the  
371 previous 9 divisions estimate (37). This estimation does not take into account the cell  
372 death that occurs essentially at DN4 (1-3% of cells are dying) and at early DP stage for  
373 cells co-expressing the highest levels of CD4 and CD8 (death slightly increases from 13  
374 to 15% in B6 mice and from 8 to 12% in FVB mice with ageing) during the thymic  
375 selection processes (Fig 7, S5 Table). We calculated the ratio of cell number EdU<sup>+</sup>/death  
376 as a performance index of T cell expansion. In FVB, the EdU<sup>+</sup>/death ratio is lower than in  
377 B6 at all stages of differentiation (S5 Table, Fig 8). Thus, although the total number of  
378 cells reaches similar expansion, the balance between division and death appears quite  
379 different in both strains.



380

381 **Fig. 8. Quantification of cell proliferation and death in thymus.** For each stage of  
 382 thymocyte differentiation, the total number of live cells (green), of accumulated EdU<sup>+</sup>  
 383 (red) cells, and of dead cells (blue) after the pulse/chase/pulse are shown (upper panel).  
 384 The percentage of EdU<sup>+</sup> cells (red) and dead cells (blue) among each differentiation stage  
 385 identified is given (lower panel). Quantification is done by FACS and given as the  
 386 median (line) of 4 mice per group (points). In DN2 and DN3 cells in some B6 mice death  
 387 is so rare that quantification is below 10 cells/thymus and sometimes up to 0 and cannot  
 388 be represented on the log scale. The ratio of EdU<sup>+</sup>/dead cells gives a performance of cell  
 389 expansion.

390

391 When cells migrate from the thymus to the spleen they reduce their proliferation.  
 392 Naïve CD44<sup>lo</sup> T cells reduce their proliferation rate to <1%/day in all groups (S1 Fig, S3  
 393 Table, S4 Table). Thus a minimal inter-mitotic time of 226 days (a value similar to 218  
 394 days found in young B6 mice (27)), can be estimated for the more active CD8 naïve T  
 395 cells in 2-month-old mice. In contrast, effector/memory T cells expressing CD44<sup>hi</sup> after  
 396 antigenic stimulation (38), are actively labelled, suggesting inter-mitotic times of 12 to 16  
 397 days in CD4 and CD8 from 2-month-old B6 while it increases about two-fold (32 and 37  
 398 days) in FVB old mice. Regulatory T cells are the T cell population with maximal

399 proliferation rates in young B6 (21%/d), dividing every 5 to 12 days, respectively, in 2-  
400 and 18-month-old mice. In contrast, proliferation rates are as low as 1.4%/day suggesting  
401 that Treg cells divide only every 83 days in FVB mice, independently of the age.

402

## 403 **Discussion**

404 Investigation of cell proliferation and turnover in space and time currently remains  
405 a challenge in immunology, in order to quantify the parameters of cell population  
406 dynamics *in vivo* according to tissue/organ and cell population heterogeneity, as well as  
407 to identify peculiarities related to the state of individuals, such as age and genetic origin  
408 (2, 3, 39-41).

## 409 **Experimental approach and mathematical modelling**

410 The model developed here allows estimation of proliferation rates and inter-mitotic  
411 time (with estimated parameters and confidence intervals for G0/G1 and G2/M cell cycle  
412 phase durations as described in Protocol S1 and Table S6) and allows quantitative  
413 comparison of the effect of age and genetics on lymphocyte dynamics from their early  
414 differentiation in thymus to their final maturation in spleen.

415 **Heterogeneity of experimental approaches can be solved by modelling.** Various  
416 experimental approaches, such as active cell labelling during cell cycle with nucleotide  
417 analogues, deuterated water or glucose, passive DNA content measurement, depletion of  
418 dividing cells, assessment of cyclin activity, and measurement at the level of populations  
419 or single cells, were recently reviewed (2). Mathematical or computer modelling is  
420 necessary to interpret complex data and obtain standardized values for parameters such as  
421 proliferation rates and cell cycle phase durations. This is the only way to compare results  
422 issuing from different experimental approaches (2) (P. Loap et al. in preparation). BrdU,  
423 or more recently EdU labelling pulse-chase experiments, with various durations and  
424 routes of administration were used to investigate lymphocyte turnover and life-span in  
425 mice. Only a few reports concerned bi-dimensional analysis of BrdU/DNA content (15,  
426 42), and these investigations were limited to whole organ cell populations. Indeed,

427 sensitivity of fluorochromes to reveal BrdU labelling, and the small number of parameter  
428 investigations limited studies using old cytometers. In the last few years, the introduction  
429 of Clickit EdU technology and multi-colour flow cytometry has allowed investigation of  
430 cell phenotype together with EdU and DNA content labelling, as set up in this paper.  
431 Since complex experimental pulse chase periods change from one study to another, as in  
432 Baron's paper (15), the only way to interpret the results and to infer quantitative  
433 parameter values of proliferation per day is to develop a mathematical model.

434 Our mathematical model, designed with visual language accessible to biologists, is  
435 easy to manipulate to quantify lymphocyte dynamics parameters and can be used to  
436 simulate all kinds of pulse/chase experimental designs for further investigations.  
437 Moreover, it is flexible and can be easily modified to accommodate other phases of the  
438 cell cycle or different kinetics of label incorporation. This model was successfully applied  
439 to previous results from Penit group obtained with a different experimental pulse/chase  
440 protocol (cf Fig 5).

441 **Cell heterogeneity and granularity of modelling.** In the mathematical model and  
442 the process of fitting, it is assumed that the studied population has uniform cycling  
443 parameters. We make abstraction of certain important transition factors, such as  
444 differentiation, death, and migration between different compartments, as if the population  
445 was a closed system.

446 The estimated parameter values must be considered as averages over the whole  
447 population studied. Values for the total thymus are in fact an average over all sub-  
448 populations in the thymus, weighted by their relative importance in numbers of cells (DP  
449 cells are the most numerous intra-thymic population, and therefore results for the total  
450 thymus are similar to those for DP cells). A correct understanding of kinetic

451 heterogeneity within the whole population therefore requires further identification into  
452 sub-populations so as to increase the resolution. However, precise rates of proliferation  
453 are difficult to calculate for heterogeneous populations during the differentiation process  
454 without integrating transition to cell death and transition between cell stages into a  
455 mathematic model. This is a general problem in the literature since to our knowledge  
456 there is currently no model that integrates such complex dynamical transitions. Indeed  
457 there is cell transition within populations (from rare DN, to DP to SP in thymus and from  
458 naïve to effector/memory in spleen), transition to cell death, and transition to another  
459 organ (from thymus to spleen) or recirculation. Here we can only quantify the numbers of  
460 dead cells at instant time (and not estimate rates/day) and death is not integrated in the  
461 current mathematical model. Inter-mitotic time and cell phase duration are only indicative  
462 but allow to compare the behaviour of various cell populations and conditions. While  
463 CD8 CD3<sup>+</sup> mature thymocytes from young B6 mice have high proliferation rates  
464 (allowing for an inter-mitotic time of about 2.2 days) and possible expansion before  
465 thymic output, the CD8 CD3<sup>+</sup> mature thymocytes from old FVB mice display a 100-fold  
466 lower proliferation rate and thus would be exported before having time to divide.

467         One should consider that between two pulses or within long pulse periods, cells  
468 may transit from differentiation stage or migrate to other tissues. However with a sixteen-  
469 hour long pulse/chase/pulse experiment these transitions are rather limited. The  
470 observation of a linear relationship between the number of cells labelled by EdU and the  
471 proliferation rate (Fig 6) is probably explained by the fact that as the duration of the  
472 experiment is smaller on average than the duration of one full cell cycle, labelled cells  
473 have not had the time to transit to another compartment, so that the number of labelled  
474 cells is globally proportional to the rate of entry into S phase and the proliferation rate, as  
475 mice are in steady-state. Nonetheless if we were to model a longer experimental protocol,

476 it would undoubtedly be necessary to take into account these processes. Moreover, the  
477 parameter values observed with our sixteen-hour pulse/chase/pulse are confirmed by  
478 simulating the context of a single pulse followed by a nineteen-hour chase, as in the  
479 Baron paper that assessed whole thymocyte proliferation.

480       Considering these limitations, we believe that our experimental design and  
481 modelling is able to give, if not exact absolute values, at least a correct understanding of  
482 the relative differences between the heterogeneous populations studied.

483       **Overlap of phases with DNA content analysis.** Since DNA content only allows  
484 one to distinguish three phases in the cell cycle, it is not possible to give separate  
485 estimates of durations for G2 and M phases or, more problematically, for G0 and G1  
486 phases. Thus G0/G1 phase duration is an average between G0 and G1 durations,  
487 weighted by their relative importance in numbers of cells. New protocols and modelling  
488 introducing Ki-67 labelling to separate G0 from G1 cells are under investigation (P. Loap  
489 et al. in preparation).

490       **Parameter constraint and fit.** The relatively low information content of the  
491 experimental dot plots (three proportions, i.e., a two-dimensional vector) constrains our  
492 search for parameters. We notably restrain ourselves to the steady-state case (Hyp.4)  
493 since we believe that the in vivo conditions of the experiment in normal mice justify this  
494 hypothesis. We also fix loss of cells in S and G2/M to be null (Hyp.5), as confirmed by  
495 observations (15). Finally, we fix S phase duration to 6.5 hours (Hyp.7); this is a value  
496 that has been measured in the same type of cells, as simulated here (15). A stretched cell  
497 cycle duration model (43) was recently proposed for mature T and B cells (44), but there  
498 is no indication this model could also occur in immature cells.

499 **Heterogeneity of lymphocyte dynamics according to cell population, age and genetic**  
500 **origin**

501 The seminal work of Penit has addressed the quantification of proliferation in the  
502 thymus of young B6 mice (36). However, to our knowledge we are the first to give  
503 proliferation rate/day at various granularities (from whole organ to rare populations)  
504 according to the time evolution of lymphocyte differentiation, from thymus to spleen, and  
505 in relation to the age and genetic origin of mice. FVB and B6 mice were chosen, because  
506 these strains belong to different phylogenetic clusters (45, 46) and display dissimilar T-  
507 Cell Receptor (TCR) repertoires as explained by chromosomal deletion of six Beta chain  
508 Variable region (BV) TCR families in FVB (47). B6 and FVB mice differ in their  
509 lymphocyte and T cell compositions (48) and numbers in the various populations at  
510 steady-state (30). Moreover, FVB mice display accelerated thymic involution and aging  
511 of VB repertoire as compared to B6 mice (30). Thus, this suggests that variation in  
512 proliferation rates or cell death might explain these differences.

513 We have previously modelled thymocyte dynamics in young FVB mice and shown  
514 that 83% of the produced thymocytes die, with 35% of DP dying per day (1). Here, we  
515 quantify thymocyte cell death as apoptotic cells in sub G0/G1 and as cells with a DNA  
516 content  $>4$ , corresponding to macrophages engulfing dying cells. While in total thymus  
517 about 2-3% of cells are in death at an instant time, major cell death occurs between DN3  
518 and DN4 stages if VDJ recombination fails (3-5% of instant death) and at the DP stage by  
519 negligence and negative selection processes, with up to 15% instant cell death at early DP  
520 stages before CD4 and CD8 modulation. During death by neglect (49) and negative  
521 selection macrophages remove dying auto-reactive thymocytes, before cell membrane  
522 permeabilisation and chromatin condensation (50). While the thymus of FVB young mice  
523 contains a higher number of cells than B6 mice (90 vs 81 millions of cells), we observe

524 global defective proliferation rates as compared to B6 mice, in particular at early DN1  
525 and DP stages but also for the proliferation of CD8 mature thymocytes (3%/day in FVB  
526 vs 40%/day in B6), and accumulation of cells at late DP stage. Conversely, in FVB mice,  
527 thymocyte death is generally higher. Thus, the death/EdU<sup>+</sup> ratio is higher at all stages  
528 except in DP. (51). Our results show that genetics and age influence T cell dynamics, in  
529 particular increase in the time spent in G0/G1 in thymus with aging, related to the G0  
530 elongation theory of aging. Aging thus increases the inter-mitotic time, and contributes to  
531 the decrease of the total number of thymocytes as the individual ages. Impaired  
532 thymocyte proliferation and higher cell death in FVB mice could be related to the  
533 immaturity of their thymic epithelial cells (52) that normally interact with thymocyte  
534 during differentiation, selection, and migration, as recently modelled in B6 mice (35).  
535 These defects in FVB mice may participate in the accelerated thymic involution and  
536 aging and the high CD4/CD8 ratio (30), also observed here (CD4/CD8= 7 for FVB and 2  
537 for B6). As modelled by Cohen's group in a reactive animation (53), the cell competition  
538 for epithelial cells and decreased ratio of the dissociation rates of CD8<sup>+</sup> and CD4<sup>+</sup>  
539 thymocytes to 0.3, would induce an increase of the CD4/CD8 ratio to 7, thus possibly  
540 limiting the emergence of CD8 and their proliferation in FVB mice. As a consequence it  
541 could explain that while the recovery to steady state values following transient depletion  
542 of dividing cells is effective in young FVB mice (1), this process is defective in old FVB  
543 mice, leading to CD8 clonal expansions in periphery in spleen (30). The defective Treg  
544 proliferation observed here in FVB mice with increased time passed in G0/G1 and G2/M  
545 even in young mice, may explain these TCR repertoire alterations in old mice. Indeed, in  
546 FVB mice, the transfer of Treg from young mice or the stimulation of their proliferation  
547 by low doses IL-2 treatment, initiated at 15 months of age, prevents repertoire alterations  
548 and CD8 clonal expansion at 2 years (30).

549           The global proliferation rate of splenocytes increases with aging from 3 to 13% per  
550 day in B6 and from 3 to 7% per day in FVB. This 2- to 4-fold increase in proliferation  
551 with aging reflects, however, considerable heterogeneity according to naïve or  
552 effector/memory T cells. Our previous modelling using transient depletion of dividing  
553 cells (1) led to the conclusion that “recent thymic emigrants” in the spleen, which  
554 represent around 50% of all naïve T cells in young mice, make 1 to 2 cell divisions  
555 during the 9 days after thymic output, allowing to complete the thymic clonal expansion  
556 (of positively selected T cells) up to obtain at least  $2^4$  to  $2^5$  cells per naïve cell clone. The  
557 mean proliferation rate of effector/memory T cells is 14 times that of naïve T cells. This  
558 high level of proliferation in effector memory antigen-experienced T cells (inter-mitotic  
559 time varies from 12 to 19 days in both strains) is in accordance with our previous results  
560 showing that immunological memory maintenance is dependent on active T cell division  
561 and that specific depletion of dividing T cells induces immunological amnesia while  
562 primary responses were intact (54). However, with aging, the inter-mitotic time of  
563  $CD44^{hi}$  cells increases. This suggests that the accumulation of  $CD44^{hi}$  cell number  
564 observed with aging is related to increased cell longevity rather than to active  
565 proliferation. This decrease in proliferation of effector memory T cells with aging could  
566 be related to hyper-glycosylation of T cell surface macromolecules, leading to altered T  
567 cell signalling (55). Altered signalling pathways occur in T cells with aging, in particular  
568 as alteration of lipid raft polarisation, altering early steps of T cell activation, with  
569 increased activity of SHP-1 acting as a negative feedback on lymphocyte proliferation  
570 (56, 57). This could explain the altered vaccine response in old individuals, and should be  
571 considered in systems biology approaches for vaccine design (58).

572           The  $CD4^+Foxp3^+$  regulatory T cells, which represent less than 10% of CD4 splenic  
573 T cells, display the highest proliferation rate within splenic T cells. This is in accordance

574 with their peculiar selection by medullary thymic epithelial cells (59) presenting  
575 promiscuous antigens because of Auto-Immune REgulator (AIRE) expression (60) of  
576 autoreactive Treg, then triggered in peripheral tissues to division by self-antigens  
577 cognition. We have previously shown that dominant tolerance to allogeneic grafts is  
578 related to antigen specific Treg cells selection in thymus (61). In fact, a delicate balance  
579 between proliferation or suppressive function of Treg is of importance to insure the  
580 negative feedback loop, which controls the proliferation of other T cells. When Treg cells  
581 are under “suppressive mode”, they are anergic, cannot proliferate (62) and thus less  
582 sensitive to specific depletion of dividing T cells than other CD4 T cells. Thus,  
583 specifically targeting cells in proliferation can induce dominant tolerance (51).

584 Our general conclusion is that FVB mice have lower proliferation rates than B6  
585 mice, and that with aging T lymphocytes have lower proliferation rates, although all mice  
586 are kept in the same environment. This certainly contributes to an accelerating aging.  
587 This reveals a specific “signature” of proliferation across differentiation stages in each of  
588 the two strains. This is in favour of the influence of genetic background and age on T cell  
589 dynamics and thus on the homeostatic equilibrium of the immune system. Therefore, the  
590 sensitivity of dividing cells - to depletion treatment by conditional immuno-pharmaco-  
591 genetics that also reveal cell dynamics (1, 30, 51, 54) or to gene therapy where retrovirus  
592 integration requires cell division (63) - could be very different with aging and genetic  
593 origin. Cell proliferation, age and genetics then influence the induction or control  
594 memory (54) and induction of tolerance (30, 51). Thus, knowledge on lymphocyte  
595 dynamics and proliferation of sub-populations is of importance for clinical applications.

596

## 597 **Materials and Methods**

### 598 **Mice**

599 C57BL/6/N (B6) and FVB/N (FVB) mice were obtained from Charles River  
600 Laboratories maintained in SPF conditions and used at 2 and 18 months of age. Mice  
601 were manipulated according to European council directive 86/609/EEC of 24 November  
602 1986 and with the approval of an ethics committee.

### 603 **In vivo EdU treatment and multicolor flow cytometry analysis**

604 The experimental protocol is summarized in Fig 1. Mice received three intra-  
605 peritoneal injections of 1 mg of EdU (5-ethynyl-2'-deoxyuridine, Life technologies) at 0,  
606 1 and 16 hours and were killed half an hour after the last injection to remove thymus and  
607 spleen. Cell suspensions were obtained by mechanical disruption of organs in PBS + 3%  
608 newborn calf serum at 4°C, were then washed and submitted to CD16/CD32 blockage  
609 with 2.4G2 hybridoma cell supernatant for 10 minutes. Cell membrane labeling was then  
610 done for 20 minutes with antibodies coupled with fluorochromes Percp, APC-H7, and  
611 APC. After washing, the cells were fixed and permeabilized with the ClickIT fixative,  
612 and EdU was revealed by Click-IT containing Alexa488-azide. The last step consisted of  
613 staining with antibodies labeled with PE and PE-cy7 for 30 minutes and washing with  
614 Perm/Wash buffer (Becton-Dickinson). 1 µL FxCycle™ violet stain (Life Technologies)  
615 was added per tube, 30 minutes before acquisition. Cell parameters were acquired on  
616 LSR2 equipped with 405, 488, and 633 nm lasers (Becton-Dickinson) at a rate of 2000  
617 cells/sec with DIVA software. Cells were analysed with Flowjo ([www.flowjo.com](http://www.flowjo.com)) on  
618 the basis of structural cell markers FSC-A (Forward Scatter-Area), SSC-A (Side Scatter-  
619 Area), FSC-W (Forward Scatter-Width) to remove debris, apoptotic cells, and doublets.  
620 Multicolour expression of specific markers CD3, CD4, CD8, CD25, CD44 allowed  
621 identification of cell sub-population phenotype by hierarchical gating. Then, DNA

622 content and EdU label enabled construction of bi-dimensional dot plots to determine cell  
623 kinetics in each population. Dead cells were quantified in subG0/G1 apoptotic cells and  
624 cell that display a DNA content >4N, corresponding to macrophages engulfing dying  
625 cells before apoptosis.

## 626 **Mathematical model**

627 We designed an ODE (Ordinary Differential Equation) model describing cell  
628 dynamics during an EdU labelling experiment. Cells cycle through G0/G1, S, G2/M and  
629 are either unlabelled or labelled by EdU, giving six populations of cells represented by  
630 the six following ODEs (system of equations (1)):

631

$$\begin{aligned} \frac{dG}{dt} &= 2a_M M + 2\alpha a_{M'} M' - (a_G + d_G)G \\ \frac{dS}{dt} &= a_G G - (a_S + d_S)S - [0, \beta]S \\ \frac{dM}{dt} &= a_S S - (a_M + d_M)M \\ \frac{dG'}{dt} &= 2(1 - \alpha)a_{M'} M' - (a_{G'} + d_{G'})G' \\ \frac{dS'}{dt} &= a_{G'} G' - (a_{S'} + d_{S'})S' + [0, \beta]S \\ \frac{dM'}{dt} &= a_{S'} S' - (a_{M'} + d_{M'})M' \end{aligned} \quad (1)$$

633

(1)

634 Populations are described in Table 1, parameters in Table 2, the equivalent state transition  
635 diagram is in Fig 2.

636

637

638 **Table 1. Cell populations described in the model and initial values used in the fit**  
 639 **according to our hypotheses.**

<b>Population name</b>	<b>Population description</b>	<b>Initial value used for the fit</b>
$G$	Cells in G0 or G1 phase, unlabelled	$(G + G')_{ss} = \frac{200a_M(a_S+d_S)}{2a_M(a_S+d_S)+2a_Ma_G+(a_G+d_G)(a_S+d_S)}$
$S$	Cells in S phase, unlabelled	$(S + S')_{ss} = \frac{200a_Ma_G}{2a_M(a_S+d_S)+2a_Ma_G+(a_G+d_G)(a_S+d_S)}$
$M$	Cells in G2 or M phase, unlabelled	$(M + M')_{ss} = \frac{100(a_G+d_G)(a_S+d_S)}{2a_M(a_S+d_S)+2a_Ma_G+(a_G+d_G)(a_S+d_S)}$
$G'$	Cells in G0 or G1 phase, labelled	0
$S'$	Cells in S phase, labelled	0
$M'$	Cells in G2 or M phase, labelled	0

640

641

642 **Table 2. Model parameters and values used for the fit in our experimental**  
 643 **conditions.**

Parameter name	Parameter description	Value used for the fit
$a_G, a_{G'}$	Rate of entry into S phase of G1 phase unlabelled, labelled cells	$a_G = a_{G'}$ = parameter to fit
$a_S, a_{S'}$	Rate of entry into G2 phase of S phase unlabelled, labelled cells	$a_S = a_{S'} = 1/6.5$
$a_M, a_{M'}$	Rate of entry into cell division of M phase unlabelled, labelled cells	$a_M = a_{M'}$ = parameter to fit
$d_G, d_{G'}$	Rate of cell death, differentiation or migration of G0/G1 phase unlabelled, labelled cells	$d_G = d_{G'} = a_G$
$d_S, d_{S'}$	Rate of cell death, differentiation or migration of S phase unlabelled, labelled cells	$d_S = d_{S'} = 0$
$d_M, d_{M'}$	Rate of cell death, differentiation or migration of G2/M phase unlabelled, labelled cells	$d_M = d_{M'} = 0$
$\alpha$	Proportion ( $0 \leq \alpha \leq 1$ ) of labelled M phase cells shedding label during cell division	$\alpha = 0$ (no cell loses its label with cell division)
$\beta$	Rate of labelling of S phase cells during the pulse phase	$\beta = \infty$ (instant labelling by EdU)
$p$	Proliferation rate	Result of the fit

644

645 G, S, M are numbers of cells in G0/G1, S, G2/M phase of the cell cycle,  
 646 respectively.  $G', S', M'$  are the corresponding EdU labelled cell numbers.  $a_X$  terms  
 647 correspond to rates of entry into the next phase of cells in X phase (X being either G, S,  
 648 M or  $G', S', M'$ ). The exit of cells, either due to death, differentiation, or migration is  
 649 represented by  $d_X$  terms. EdU labelling intensity is modelled by two terms:  $\beta$  is the rate of  
 650 labelling of S phase cells by EdU, while  $\alpha$  is the rate of loss of labelling by labelled cells  
 651 after division. To distinguish pulse and chase phases of the experiment, we have  
 652 introduced the symbol  $[0, \beta]$ , meaning that during the chase phase (no EdU) we use 0,  
 653 while during the pulse phase we use  $\beta$  in the equation (equivalent to a Dirac function

654 multiplied by  $\beta$ , with the Dirac function equal to 1 during pulse and 0 during chase). The  
655 proliferation rate is defined as  $p = a_M M + a_{M'} M'$ , i.e., it is the number of cells in G2/M  
656 phase, unlabelled (M) and labelled (M'), which divide at rate  $a_M$  (unlabelled) or  $a_{M'}$   
657 (labelled).

658 A simulation is run by assigning: the twelve parameters of the cell cycle  
659 ( $a_G, a_S, a_M, d_G, d_S, d_M$ ) and ( $a_{G'}, a_{S'}, a_{M'}, d_{G'}, d_{S'}, d_{M'}$ ); the two parameters corresponding to de-  
660 labelling and labelling, respectively ( $\alpha, \beta$ ); the initial state of the cell populations  
661 ( $G_0, S_0, M_0$ ) and ( $G'_0, S'_0, M'_0$ ); and the labelling protocol, i.e., the beginning and end of  
662 pulse and chase phases.

### 663 **Fit of simulations to experimental results**

664 The results of simulations were fitted to experimental results represented by  
665 EdU/DNA flow cytometry dot plots. On the dot plot, three groups of cells can be  
666 individualized and their relative percentages determined according to EdU labelling and  
667 DNA content:  $G_0/G_1 \text{ EdU}^-$ ,  $G_2/M \text{ EdU}^-$ , and  $\text{EdU}^+$ . We therefore defined an  
668 experimental result by three percentages: ( $G_{\text{exp}}, M_{\text{exp}}, (G'+S'+M')_{\text{exp}}$ ). To fit our model to  
669 experimental results and to restrict the search for parameters, we made hypotheses listed  
670 in Table 3:

671 -Hyp.1: Parameters during pulse and chase are equal, reducing to six the number of  
672 parameters of the cell cycle, i.e.,  $(a_G, a_S, a_M, d_G, d_S, d_M) = (a_{G'}, a_{S'}, a_{M'}, d_{G'}, d_{S'}, d_{M'})$ .

673 -Hyp.2: EdU cell labelling is instantaneous, i.e.,  $\beta = \infty$ . While mathematically not  
674 integrable as such, this is very simply implemented in the computer model by transferring  
675 at the start of the pulse phase all unlabelled S cells to the labelled S compartment, and by  
676 allowing the unmarked G1 cells to only go into the labelled S compartment during pulse  
677 phase.

678 -Hyp.3: There is no shedding of EdU label during the time of the experiment, i.e.,  $\alpha=0$ .

679 -Hyp.4: Cells are in a steady-state, and so is the distribution of cells in each cycle phase

680  $((G+G')_{SS}, (S+S')_{SS}, (M+M')_{SS})$  as described by system of equations (2):

$$681 \quad \begin{cases} \frac{d(G+G')}{dt} = 0 \\ \frac{d(S+S')}{dt} = 0 \\ \frac{d(M+M')}{dt} = 0 \end{cases} \Leftrightarrow \begin{cases} (G + G')_{SS} = \frac{200a_M(a_S+d_S)}{2a_M(a_S+d_S)+2a_Ma_G+(a_G+d_G)(a_S+d_S)} \\ (S + S')_{SS} = \frac{200a_Ma_G}{2a_M(a_S+d_S)+2a_Ma_G+(a_G+d_G)(a_S+d_S)} \\ (M + M')_{SS} = \frac{100(a_G+d_G)(a_S+d_S)}{2a_M(a_S+d_S)+2a_Ma_G+(a_G+d_G)(a_S+d_S)} \end{cases} \quad (2)$$

682 with  $(G + G')_{SS} + (S + S')_{SS} + (M + M')_{SS} = 100(\%)$ .

683 As a consequence, the total number of cells during the experiment is constant and

684 percentages can be used. This also gives a conservation equation as follows:

$$685 \quad a_M M + a_{M'} M' = d_G G + d_S S + d_M M + d_{G'} G' + d_{S'} S' + d_{M'} M'$$

686 -Hyp.5: There is no exit during S or G2/M phase (15), i.e.,  $d_S=d_M=d_{S'}=d_{M'}=0$ .

687 -Hyp.6: One injection of EdU is modelled by a one-hour pulse (32).

688 -Hyp.7: S phase lasts 6.5 hours (15), i.e.,  $a_S=1/6.5$ .

689

690 **Table 3. Biological and experimental hypotheses, allowing for constraint of**  
 691 **parameter values.**

Hypothesis number	Hypothesis description	Consequence for parameters
1	EdU labelling does not affect cell-cycle kinetics	$(a_G, a_S, a_M, d_G, d_S, d_M) = (a_G', a_S', a_M', d_G', d_S', d_M')$
2	Labelling of S phase cells is instantaneous during the pulse phase	$\beta = \infty$
3	Labelled cells do not divide enough times during the experiment to shed label	$\alpha = 0$
4	No dynamics perturbation under physiological conditions: steady-state cell number in each phase of cell cycle and in each cell population	$(G_0 = (G+G')_{SS}, S_0 = (S+S')_{SS}, M_0 = (M+M')_{SS})$ $a_M M + a_M' M' = d_G G + d_S S + d_M M + d_G' G' + d_S' S' + d_M' M'$
5	No cell death or differentiation during S phase and G2/M phase	$d_S = d_M = d_S' = d_M' = 0$
6	Bio-disponibility of EdU is one hour after an injection	None
7	S phase lasts 6.5 hours	$a_S = 1/6.5$

692

693 With these hypotheses, ten out of the twelve parameters of the cell cycle are fixed,

694 except for  $a_M$  and  $a_G$ , since:

695  $(a_G, a_S = 1/6.5, a_M, d_G = a_G, d_S = 0, d_M = 0) = (a_G', a_S', a_M', d_G', d_S', d_M')$ . The two parameters of EdU  
 696 labelling intensity are:  $(\alpha = 0, \beta = \infty)$ . The initial state is:  
 697  $(G_0 = (G+G')_{SS}, S_0 = (S+S')_{SS}, M_0 = (M+M')_{SS}, G'_0 = 0, S'_0 = 0, M'_0 = 0)$ .

698

699 To fit our model to experimental results, we run simulations over a two-  
 700 dimensional range of parameters  $(a_G, a_M)$ . The time step chosen with the integration tool  
 701 was 0.01 hour (36 seconds). The best fit is the couple of parameters which minimizes the  
 702 Euclidean distance between  $(G_{exp}, M_{exp}, (G'+S'+M')_{exp})$  and  $(G_{sim}, M_{sim}, (G'+S'+M')_{sim})$ .

703 All results obtained have a Euclidean distance to experiment of less than 0.05. We  
704 deduce from the best fit the mean duration of G0/G1 phase  $1/a_G$ , the mean duration of  
705 G2/M phase  $1/a_M$ , and the proliferation rate  $p$ , which with our hypotheses is equal to  
706  $200a_M a_G / (2a_M + 13a_M a_G + 2a_G)$  (in percentage of cells dividing per hour).

707 The identifiability of parameters obtained during the fitting procedure and some  
708 illustrative results are given in S1 protocol and S6 Table. We also give standard  
709 deviations for our estimates of proliferation rates in total thymus and spleen, calculated  
710 with the Hessian matrix (variance-covariance matrix method).

711

## 712 **Statistics**

713 Statistics were performed using the R software ([www.r-project.org](http://www.r-project.org)). Statistical  
714 significance level was fixed at  $\alpha=0.05$  (Type 1 error). Before applying comparison tests,  
715 all data were assessed for normality by Shapiro-Wilk's test and for equality of variances  
716 by Levene's test. These results led us to the following choice of statistical tests: (i) if the  
717 normality hypothesis did not hold, we used the non-parametric Mann-Whitney test (ii)  
718 otherwise, we used the Student's two-tailed unpaired T-test with a hypothesis of either  
719 equal variances or unequal variances, depending on the result of Levene's test. We  
720 computed arithmetical means and standard deviations (variance square root) for each  
721 group ( $n=4$ ).

## 722 **Software**

723 The mathematical model of cell proliferation and its fitting to experimental data  
724 were developed and simulated using Python (<http://www.python.org>). To allow high-  
725 throughput analyses, we developed a program in Python to implement the fitting  
726 procedure on tables of values extracted from the dot plot manual gates with FlowJo

727 software or from published results recovered from 2-dimensional descriptions of cell

728 kinetics. Graphs were produced with JMPpro11.

729

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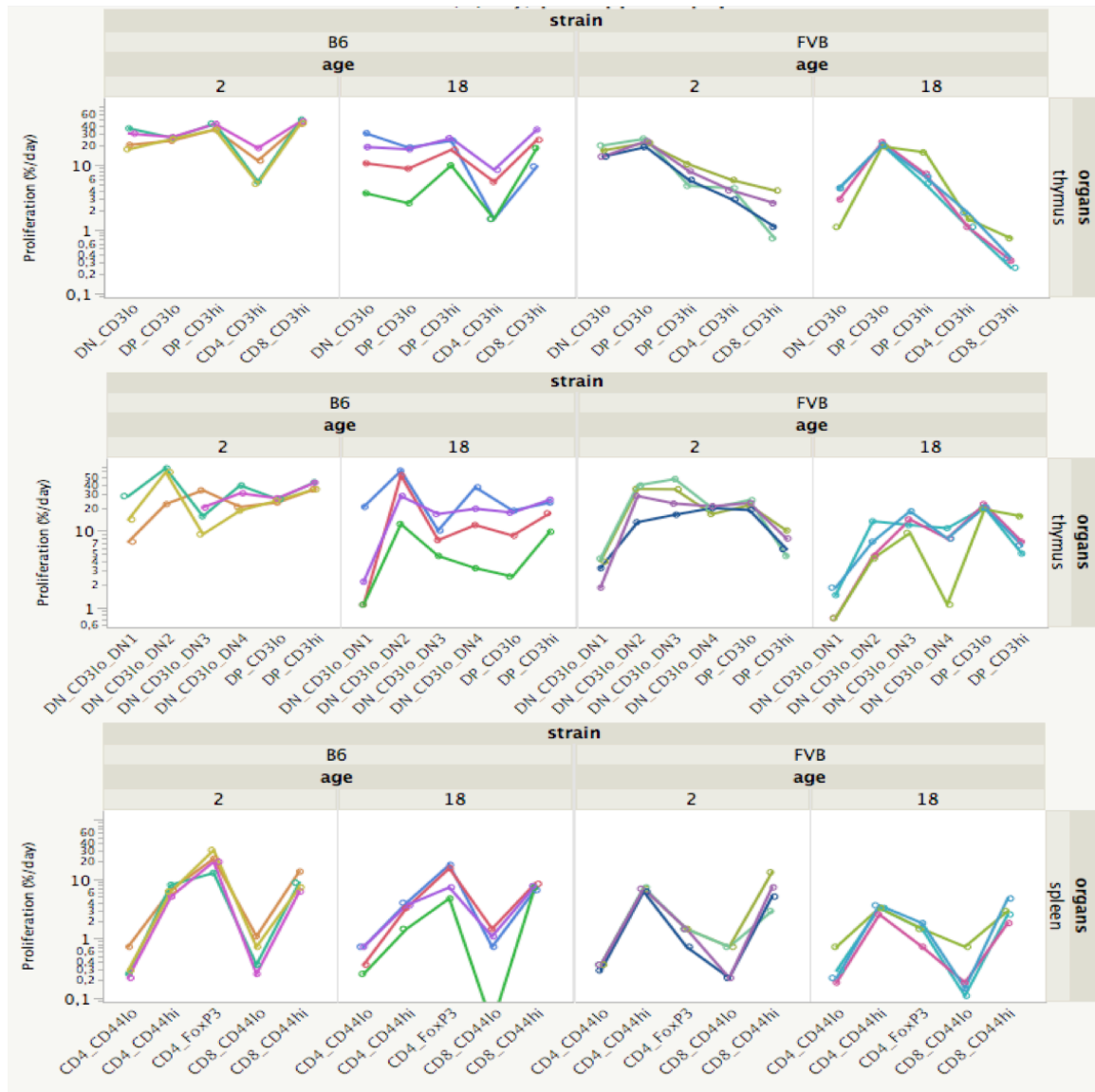
936 **Supporting information**  
 937 **(text only, figures are submittend as Tiff)**

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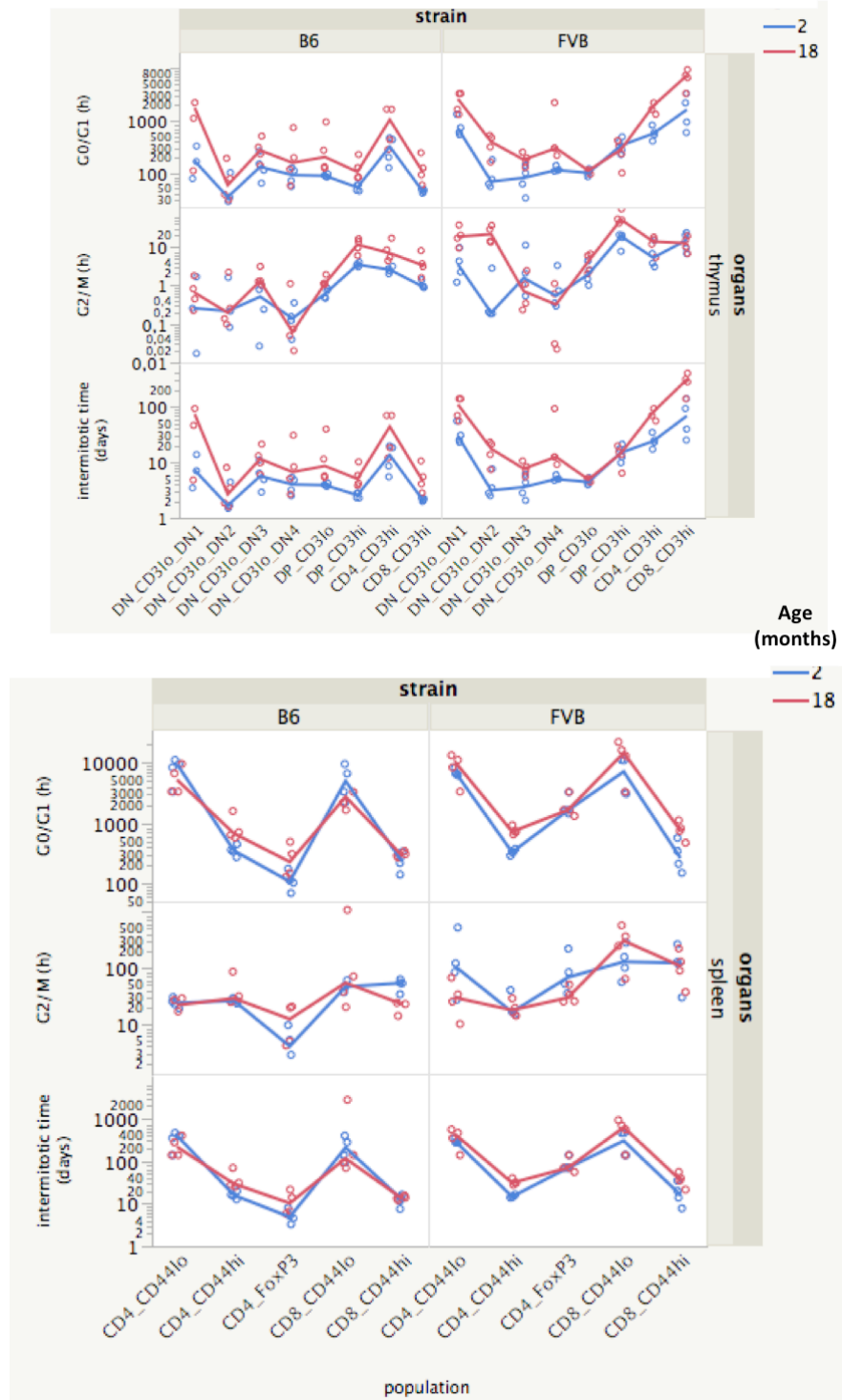
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**S1 Fig. Heterogeneity of proliferation according to strain, age, organ and T cell population.** These values are only indicative, since transition of cells from one stage to another and transition to death are not modelled. Each line represents the values obtained for one mouse. The means of these values are given in tables S1 to S4.



948

949 **S2 Fig. Heterogeneity of duration of cycle phase and intermitotic time in thymic and**  
 950 **splenic sub-populations, and variability with strains of mice and aging.** The G0/G1  
 951 and G2/M estimated durations are in hours. The inter-mitotic time durations are given in  
 952 days. The lines represent median values of 4 mice per group.

953

954

955 **S1 Table. Parameter values obtained by fitting the mathematical model to whole**  
956 **spleen and whole thymus.**

**Population = total**

organs	strain	age	Proliferation (%/day)		G0/G1 (d)		G2/M (h)	
			Mean	Std Dev	Mean	Std Dev	Mean	Std Dev
spleen	B6	2	3,2	0,4	29,1	3,8	45,3	6,1
		18	13,3	4,7	7,5	2,2	7,9	3,7
	FVB	2	3,1	0,7	31,2	8,2	63,8	20,8
		18	7,2	2,8	14,1	4,6	23,0	8,3
thymus	B6	2	31,9	2,9	2,8	0,3	2,2	0,4
		18	16,0	6,6	6,8	3,9	8,1	3,9
	FVB	2	17,3	2,2	5,5	0,8	2,8	1,3
		18	9,3	1,7	10,5	2,0	7,5	3,7

957

958 Mean and standard deviation with n=4 mice per group

959

960

961 **S2 Table. Thymocyte dynamics parameter values in B6 and FVB mice aged 2 and 18**  
 962 **months.**

strain	age	diff stage	n cells	labelled / 16 h	prolif/d	G0/G1 (d)	G2M (d)	total time (d)
B6	2	TN	1,98	24,32	27,84	3,39	0,03	3,69
		DP CD3 <sup>-</sup>	30,52	22,18	25,19	3,68	0,03	3,98
		DP CD3 <sup>+</sup>	46,33	31,75	38,89	2,18	0,15	2,60
		DP	78,16	27,97	33,26	2,65	0,11	3,03
		CD4 CD3 <sup>+</sup>	5,64	9,06	10,03	12,52	0,10	12,89
	18	CD8 CD3 <sup>+</sup>	5,16	37,53	45,68	1,86	0,07	2,20
		TN	0,29	16,04	17,80	5,52	0,06	5,85
		DP CD3 <sup>-</sup>	4,29	10,59	11,63	16,44	0,06	16,77
		DP CD3 <sup>+</sup>	10,30	15,05	19,21	5,19	0,43	5,89
		DP	14,95	13,54	16,83	6,43	0,35	7,06
FVB	2	CD4 CD3 <sup>+</sup>	1,43	3,67	4,24	40,35	0,31	40,93
		CD8 CD3 <sup>+</sup>	0,86	18,40	21,36	5,42	0,19	5,88
		TN	1,48	10,97	12,21	8,85	0,06	9,18
		DP CD3 <sup>-</sup>	51,42	19,37	22,17	4,20	0,09	4,57
		DP CD3 <sup>+</sup>	5,11	5,65	7,50	13,56	0,56	14,39
	18	DP	56,92	18,09	20,88	4,47	0,11	4,85
		CD4 CD3 <sup>+</sup>	6,50	3,72	4,30	24,88	0,22	25,38
		CD8 CD3 <sup>+</sup>	1,33	1,73	2,18	62,81	0,38	63,46
		TN	0,95	2,25	2,72	39,75	0,23	40,25
		DP CD3 <sup>-</sup>	6,98	17,47	20,42	4,47	0,18	4,91
		DP CD3 <sup>+</sup>	0,57	5,80	8,98	10,89	2,39	13,55
		DP	7,61	16,62	20,23	4,43	0,26	4,96
		CD4 CD3 <sup>+</sup>	5,17	1,19	1,51	66,04	0,33	66,64
		CD8 CD3 <sup>+</sup>	1,42	0,39	0,46	273,10	0,39	273,77
		total	DN_CD3 <sup>-</sup>	DP_CD3 <sup>lo</sup>	DP_CD3 <sup>hi</sup>	CD4	CD8	
B6_2M	>>		>	>>		>>	B6_18M	
FVB_2M	>	>>>			>>		FVB_18M	
B6_2M	>			>>>		>>>	FVB_2M	
B6_18M						>	FVB_18M	

963

964 For each differentiation stage, the mean number of cell/thymus (n=4) and standard  
 965 deviation is given. TN corresponds to immature cells CD4<sup>+</sup>CD8<sup>-</sup>CD3<sup>-</sup> cells. DP CD3<sup>-</sup> and  
 966 DP CD3<sup>+</sup> stages are the decomposition of DP cells. The percentage of labelled cells/16h  
 967 and the percentage of proliferation/day are correlated, as shown in Fig 7. Estimated  
 968 duration of G0/G1 and G2/M are given in days. The duration of S phase is fixed to 6.5  
 969 hours. Total time indicates hypothetical inter-mitotic time (1/proliferation rate). These  
 970 values are only indicative, since transition of cells from one stage to another and to death  
 971 are not modelled. **Statistical analysis obtained by fitting the procedure for the**  
 972 **proliferation rate (%/day) in thymus.** Statistics are given for the populations of total  
 973 thymus, DN CD3<sup>-</sup> (TN); DP CD3<sup>lo</sup>, DP CD3<sup>hi</sup>, CD4<sup>+</sup>CD3<sup>+</sup> and CD8<sup>+</sup>CD3<sup>+</sup> thymocytes.  
 974 B6\_2M: 2 month-old B6 mice, B6\_18M: 18 month-old B6 mice, FVB\_2M: 2 month-old  
 975 FVB mice, FVB\_18M: 18 month-old FVB mice. Level of significance of statistical tests:  
 976 > (resp. <) indicates that in population p, group a (on the left) has a mean which is  
 977 superior (resp. inferior) to group b (on the right) with a level of significance of p<0.05;  
 978 >> (resp. <<) is for p<0.01; >>> (resp. <<<) is for p<0.001.

979

980

981 **S3 Table. Splenocyte dynamics parameter values in B6 and FVB mice aged 2 and 18**  
 982 **months.**

strain	age	Population	Proliferation (%/day)		G0/G1 (d)		G2/M(d)		intermitotic time (d)	
			Mean	St Dev	Mean	St Dev	Mean	St Dev	Mean	St Dev
B6	2	CD4_CD3hi	2,3	0,2	43,2	3,1	1,1	0,2	44,6	3,3
		CD4_CD44hi	6,4	1,2	14,7	3,0	1,1	0,1	16,1	3,0
		CD4_CD44lo	0,4	0,2	335,2	140,2	1,0	0,2	336,5	140,0
		CD4_FoxP3	21,2	7,4	4,8	1,8	0,2	0,2	5,2	2,0
		CD8_CD3hi	3,9	2,0	27,6	10,4	2,1	0,4	30,0	10,9
		CD8_CD44hi	8,8	3,2	9,9	3,4	2,1	0,5	12,3	3,8
		CD8_CD44lo	0,6	0,4	224,3	137,9	2,0	0,4	226,5	138,1
	18	total spleen	3,2	0,4	29,1	3,8	1,9	0,3	31,2	4,0
		CD4_CD3hi	2,3	1,1	60,2	49,8	1,8	1,5	62,3	51,2
		CD4_CD44hi	3,1	1,1	36,3	19,7	1,7	1,2	38,3	20,9
		CD4_CD44lo	0,5	0,2	237,1	124,0	0,8	0,5	238,1	124,4
		CD4_FoxP3	11,1	6,1	11,1	7,0	0,5	0,4	11,9	7,3
		CD8_CD3hi	5,6	1,3	17,4	4,1	1,0	0,2	18,6	4,3
		CD8_CD44hi	7,4	0,7	12,5	1,3	0,9	0,2	13,7	1,4
FVB	2	CD8_CD44lo	0,8	0,6	757,0	1317,7	12,4	21,4	769,7	1339,0
		total spleen	13,3	4,7	7,5	2,2	0,3	0,2	8,1	2,3
		CD4_CD3hi	1,4	0,2	72,3	12,6	2,6	1,9	75,2	11,6
		CD4_CD44hi	6,8	0,5	13,7	1,4	0,9	0,5	14,9	1,0
		CD4_CD44lo	0,3	0,0	287,0	38,7	7,8	9,4	295,1	34,7
		CD4_FoxP3	1,3	0,4	82,5	35,3	4,0	3,5	86,8	34,7
		CD8_CD3hi	2,9	2,0	41,7	23,2	5,8	3,3	47,7	26,0
	18	CD8_CD44hi	7,0	4,3	13,2	7,7	5,6	4,0	19,0	11,6
		CD8_CD44lo	0,5	0,3	294,5	189,2	6,2	4,0	300,9	187,1
		total spleen	3,1	0,7	31,2	8,2	2,7	0,9	34,1	8,7
		CD4_CD3hi	1,4	0,3	70,6	15,2	0,9	0,2	71,8	15,4
		CD4_CD44hi	3,2	0,5	31,2	4,9	0,8	0,3	32,3	5,1
		CD4_CD44lo	0,4	0,3	374,5	178,8	1,4	1,0	376,2	179,7
		CD4_FoxP3	1,4	0,5	81,7	37,1	1,4	0,5	83,3	37,6
18	CD8_CD3hi	2,7	1,3	40,1	18,8	4,4	2,7	44,8	21,5	
	CD8_CD44hi	3,0	1,2	32,6	10,9	4,9	3,2	37,8	14,1	
	CD8_CD44lo	0,3	0,3	565,6	325,9	12,9	8,8	578,7	330,6	
	total spleen	7,2	2,8	14,1	4,6	1,0	0,3	15,3	4,9	
	<b>total</b>	<b>CD4</b>	<b>CD4_CD44lo</b>	<b>CD4_CD44hi</b>	<b>CD4_FoxP3</b>	<b>CD8</b>	<b>CD8_CD44lo</b>	<b>CD8_CD44hi</b>		
	B6_2M	<<		>>					B6_18M	
	FVB_2M	<		>>>					FVB_18M	
B6_2M		>>>		>>				FVB_2M		
B6_18M				>	>		>>	FVB_18M		

983

984 Estimated percentage of proliferation/day from the model, allowing estimation of  
 985 duration of G0/G1, G2/M phase, and the potential inter-mitotic time for various cell  
 986 populations. Whole CD4-CD3<sup>hi</sup> and CD8-CD3<sup>hi</sup> cells are decomposed into CD44<sup>hi</sup>  
 987 (effector/memory) and CD44<sup>lo</sup> (naïve) cells showing the heterogeneity of dynamics  
 988 according to the granularity of populations. CD4-Foxp3 are regulatory T cells Foxp3<sup>hi</sup>.  
 989 These values are only indicative, since transition of cells from one stage to another and to  
 990 death are not modelled. **Statistical analysis obtained by fitting the procedure for the**  
 991 **proliferation rate (%/day), in spleen.** Statistics f are given for the populations of total  
 992 spleen; CD4, CD4 CD44<sup>lo</sup>, CD4 CD44<sup>hi</sup>, CD4 FoxP3+; CD8, CD8 CD44<sup>lo</sup> and CD8  
 993 CD44<sup>hi</sup> splenocytes. B6\_2M: 2 month-old B6 mice, B6\_18M: 18 month-old B6 mice,  
 994 FVB\_2M: 2 month-old FVB mice, FVB\_18M: 18 month-old FVB mice. **Level of**  
 995 **significance of statistical tests:** > (resp. <) indicates that in population p, group a (on the  
 996 left) has a mean which is superior (resp. inferior) to group b (on the right) with a level of  
 997 significance of p<0.05; >> (resp. <<) is for p<0.01; >>> (resp. <<<) is for p<0.001.

998

999 **S4 Table. Comparison of proliferation rates according to strain, age, organ, and T**  
 1000 **cell populations.**

			Proliferation (%/day)														
			Population														
strain	age	organs	total	DN1	DN2	DN3	DN4	DP_CD3-	DP_CD3+	CD4	CD8	CD4_CD44lo	CD4_CD44hi	CD4_FoxP3	CD8_CD44lo	CD8_CD44hi	
B6	2	thymus	31,9	12,4	36,7	19,6	27,3	25,2	38,9	10,0	45,9	.	.	.	.	.	.
		spleen	3,2	.	.	.	.	.	.	2,3	3,9	0,4	6,4	21,2	0,6	8,8	
	18	thymus	16,0	6,2	38,7	9,7	17,9	11,7	18,9	4,1	21,6	.	.	.	.	.	.
		spleen	13,3	.	.	.	.	.	.	2,3	5,6	0,5	3,1	11,1	0,8	7,4	
FVB	2	thymus	17,3	3,3	29,0	30,3	19,3	22,1	7,1	4,2	2,1	.	.	.	.	.	.
		spleen	3,1	.	.	.	.	.	.	1,4	2,9	0,3	6,8	1,3	0,5	7,0	
	18	thymus	9,3	1,2	7,4	13,3	6,9	20,4	8,6	1,4	0,4	.	.	.	.	.	.
		spleen	7,2	.	.	.	.	.	.	1,4	2,7	0,4	3,2	1,4	0,3	3,0	

1001 Total represents the whole organ. CD4 and CD8 mature T cells are observed in the  
 1002 thymus and spleen. Mean proliferation rates per day are given during the transition of  
 1003 cells from DN1 to DP\_CD3<sup>+</sup> then CD4 or CD8 in thymus and from naïve (CD44<sup>lo</sup>) to  
 1004 effector/memory (CD44<sup>hi</sup>) differentiation in spleen; Foxp3 cells are a subpopulation of  
 1005 CD4 that are CD44<sup>hi</sup>. These values are only indicative, since the transition of cells from  
 1006 one stage to another and to death are not modelled.  
 1007  
 1008

1009 **S5 Table. Evolution of thymocyte numbers according to strains, ages, and**  
 1010 **differentiation stages.**

genetic	age	stage diff	N per thymus	N EdU+	edu+/death
			Median	Median	Median
B6	2	DN1	0,08	0,01	20,00
		DN2	0,01	0,00	.
		DN3	0,11	0,01	8,00
		DN4	1,37	0,31	32,34
		DPe	5,23	2,38	3,69
		DP late	55,64	13,87	66,74
		DPtotal	63,92	18,68	14,37
		CD4	9,93	1,01	36,44
	18	CD8	4,82	1,74	80,37
		DN1	0,02	0,00	7,86
		DN2	0,00	0,00	10,00
		DN3	0,02	0,00	26,00
		DN4	0,14	0,01	5,30
		DPe	1,79	0,36	1,52
		DP late	10,21	1,36	35,28
		DPtotal	12,00	1,62	6,68
FVB	2	CD4	2,07	0,08	14,17
		CD8	0,83	0,14	55,92
		DN1	0,15	0,01	2,10
		DN2	0,02	0,00	4,50
		DN3	0,16	0,02	3,60
		DN4	1,32	0,12	3,18
		DPe	4,94	2,55	6,83
		DP late	54,75	10,67	27,59
	18	DPtotal	60,76	13,21	17,43
		CD4	11,93	0,57	6,01
		CD8	1,71	0,06	3,34
		DN1	0,23	0,00	1,60
		DN2	0,01	0,00	3,00
		DN3	0,02	0,00	3,83
		DN4	0,13	0,01	1,71
		DPe	0,62	0,29	3,50
	DP late	10,70	1,47	13,54	
	DPtotal	11,32	1,75	7,36	
	CD4	4,93	0,08	2,56	
	CD8	1,75	0,02	1,14	

1011  
 1012 Numbers are given as millions of cells in the thymus (see Fig 7 and 8). The ratio between  
 1013 EdU<sup>+</sup> and dead cells gives a performance of cell expansion. DP<sup>total</sup> represents the sum of  
 1014 the DPe (gated on CD4<sup>hi</sup>CD8<sup>hi</sup>) and DP<sup>late</sup> (gated on CD4<sup>med</sup>CD8<sup>med</sup>).  
 1015  
 1016

1017

1018 **S6 Table. Proliferation rates with confidence intervals and standard deviations of**  
 1019 **single mice, for total thymus and total spleen.**

Sample	Age (months)	Thymus				Spleen			
		Proliferation (%/day)	IC_min	IC_max	sd	Proliferation (%/day)	IC_min	IC_max	sd
B6_43	2	<b>29.4</b>	28.1	30.6	0.18	<b>3.6</b>	1.8	5.2	0.08
B6_44	2	<b>36.3</b>	35.1	37.7	0.14	<b>3.1</b>	1.1	4.8	0.19
B6_45	2	<b>33.2</b>	31.8	34.7	0.09	<b>3</b>	1.1	4.4	0.05
B6_46	2	<b>31</b>	29.5	32.5	0.07	<b>3.5</b>	1.8	5.2	0.18
B6_09	18	<b>21.2</b>	19.9	22.5	0.16	<b>10.8</b>	9.6	11.8	0.08
B6_10	18	<b>14.1</b>	12.9	15.1	0.06	<b>20.6</b>	19.2	21.8	0.12
B6_11	18	<b>7.8</b>	6.3	8.9	0.05	<b>12.8</b>	11.8	14	0.09
B6_12	18	<b>22</b>	20.7	23.3	0.12	<b>9.8</b>	8.5	11.1	0.19
FVB_31	2	<b>18.1</b>	16.6	19.2	0.06	<b>3.5</b>	1.8	5.2	0.18
FVB_32	2	<b>18.9</b>	17.7	20.3	0.11	<b>2.8</b>	0.7	4.8	0.10
FVB_33	2	<b>14.2</b>	13.3	15.5	0.19	<b>2.3</b>	0.4	4.1	0.05
FVB_34	2	<b>18.9</b>	17.7	20.3	0.12	<b>3.7</b>	1.8	5.2	0.07
FVB_14	18	<b>10.6</b>	9.6	11.8	0.13	<b>5.4</b>	3.7	7	0.16
FVB_15	18	<b>8.8</b>	7.4	10	0.12	<b>5.1</b>	3.3	6.6	0.04
FVB_16	18	<b>7.3</b>	5.9	8.5	0.06	<b>7.2</b>	5.5	8.9	0.16
FVB_17	18	<b>11.1</b>	10	12.2	0.02	<b>11.3</b>	10	12.6	0.14

1020

1021 Proliferation rates (%/day) with confidence intervals (CI= IC\_min - IC\_max) and  
 1022 standard deviations (sd) calculated with the use of the Hessian matrix of all sixteen mice  
 1023 in whole thymus and whole spleen. Confidence intervals and standard deviations are  
 1024 calculated as described in the S1 Protocol.

1025

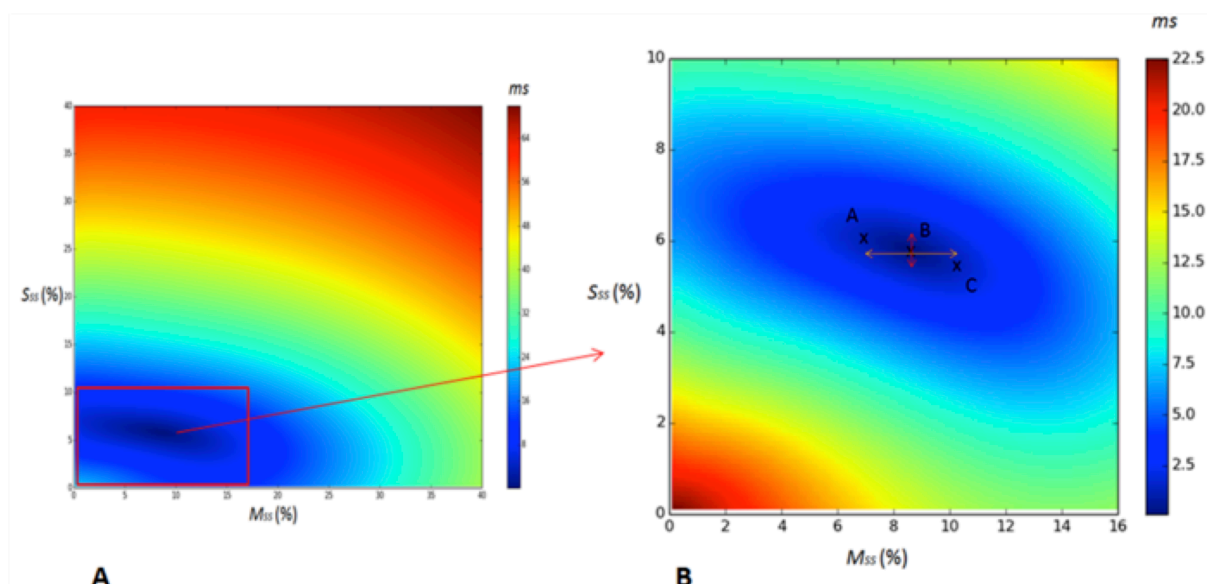
1026

1027 **S1 Protocol: Identifiability of parameters and calculation of confidence intervals**  
1028 **and standard deviations in individual mice allowing for mathematical proliferation**  
1029 **rate and cell cycle phase duration estimation**

1030 To illustrate the fitting procedure which allows estimating proliferation rates and cycle  
1031 phase durations from experimental data, we choose the free parameters to be  $(S+S')_{SS}$  and  
1032  $(M+M')_{SS}$ , which we will call  $S_{SS}$  and  $M_{SS}$  for convenience, i.e. proportions of cells in S  
1033 phase and G2/M phase at steady-state (Hyp.4), since  $S_{SS}$  is directly proportional to  
1034 proliferation rate. In this case, proliferation rate is equal to  $p=a_M M_{SS}=a_S S_{SS}=S_{SS}/6.5$   
1035 (Hyp.7). This choice of parameters is arbitrary and has no incidence on results, since all  
1036 results are fixed by two free parameters. Mean duration of G0/G1 phase is  $1/a_G$  with  
1037  $a_G=a_S S_{SS}/G_{SS}=a_S S_{SS}/(100-S_{SS}-M_{SS})$ , and mean duration of G2/M phase is  $1/a_M$  with  
1038  $a_M=a_S S_{SS}/M_{SS}$ . After having run 160,000 simulations ( $S_{SS}$  and  $M_{SS}$  each ranging from  
1039 0.1% to 40% every 0.1%), the best fit is the pair of parameters, which minimizes the  
1040 Euclidean distance between experiment and simulation.

1041 To take into account the variability of parameters giving a good fit, we define  
1042 "confidence intervals" for our fitting estimates of proliferation rates, corresponding to the  
1043 variability of proliferation rates with a distance to experiment of less than 1. This limit of  
1044 1 is based on the fact that our flow cytometry data (and also our fitting grid) is precise to  
1045 the hundredth of a percent (0.01%), and we therefore judge it appropriate to define 1 as a  
1046 limit distance between experiment and simulation for calculating our confidence  
1047 intervals. It is however of course arbitrary, and the size of confidence intervals are indeed  
1048 directly related to the value of this limit.

1049 An illustrative example of the confidence interval is show below. Confidence intervals  
1050 for proliferation rates of all sixteen mice in whole thymus and whole spleen are shown in  
1051 **S6 Table**.



1052

1053 **Identifiability of parameters obtained during the fitting procedure, with a graphical**  
1054 **representation.** The example is for total thymocytes of a B6 18-month old mouse. After  
1055 having run 160,000 simulations ( $S_{SS}$  and  $M_{SS}$  each ranging from 0.1% to 40% every  
1056 0.1%), the best fit is the pair of parameters which minimizes the Euclidean distance  
1057 between experiment and simulation. Euclidean distance (ms) between experiment and  
1058 simulation is plotted here. Right panel (B) corresponds to zooming in on the red square  
1059 on the left (A). Point B is the best fit (Euclidean distance = 0.11), it corresponds to the  
1060 pair  $S_{SS}=5.7\%$  and  $M_{SS}=8.6\%$ , i.e. proliferation rate is 21.0 % (/day), G0/G1 phase is 97.7  
1061 hours and G2/M phase is 9.8 hours. (In our fitting procedure, we use a resolution of  
1062 0.01% so that the best fit is actually  $S_{SS}=5.74\%$  and  $M_{SS}=8.55\%$  with Euclidean distance  
1063 of 0.01, but for graphical purposes we do not use this resolution in the example). In order  
1064 to quantify the variability of the fit according to parameters, we take all fits with a  
1065 maximum Euclidean distance with experience of 1. These are contained inside a zone  
1066 centered by point B and bordered by points A and C. In this case,  $S_{SS}$  varies between  
1067 5.4% and 6.1% (red double arrow), i.e. between proliferation rates of 19.9% and 22.5%.  
1068 On the other hand,  $M_{SS}$  varies between 7.3 and 9.8% (yellow double arrow). Thus G0/G1  
1069 phase varies between 93.9 and 100.1 hours, and G2/M phase between 7.9 and 11.6 hours,  
1070 leading to an inter-mitotic time varying from 4.5 to 4.9 days in this mouse. "Confidence  
1071 intervals" for our fitting estimates of proliferation rates correspond to the variability of  
1072 proliferation rates with a distance to experiment of less than 1 (red double arrow).

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1081 **Calculation of standard deviations for estimates of proliferation rates.**

1082 To compute standard deviations for our estimated parameters, we use the Hessian matrix  
 1083 defined as  $\nabla^2 f(x^*)$  where  $x^*$  is the point in parameter space which corresponds to the  
 1084 best fit, i.e.  $(S_{SS}^*, M_{SS}^*)$ .

1085 We compute the Hessian matrix in the following way:

$$\nabla^2 f(x^*) = J(x^*)^T J(x^*) + Q(x^*)$$

1086 where we use the Jacobian matrix :

$$J(x^*) = \begin{pmatrix} \frac{\partial r_G(x^*)}{\partial S_{SS}} & \frac{\partial r_G(x^*)}{\partial M_{SS}} \\ \frac{\partial r_M(x^*)}{\partial S_{SS}} & \frac{\partial r_M(x^*)}{\partial M_{SS}} \end{pmatrix}$$

1087 with  $r_G(x^*) = G_{sim}(x^*) - G_{exp}$  and  $r_M(x^*) = M_{sim}(x^*) - M_{exp}$  (and we neglect the  
 1088 term  $Q(x^*)$  with second-order derivatives which approximates to zero close to  $x^*$ ).

1089 We compute numerically the Jacobian in the following way:

$$J(x^*) = \begin{pmatrix} \frac{r_G(S_{SS}^* + \delta, M_{SS}^*) - r_G(S_{SS}^*, M_{SS}^*)}{\delta} & \frac{r_G(S_{SS}^*, M_{SS}^* + \delta) - r_G(S_{SS}^*, M_{SS}^*)}{\delta} \\ \frac{r_M(S_{SS}^* + \delta, M_{SS}^*) - r_M(S_{SS}^*, M_{SS}^*)}{\delta} & \frac{r_M(S_{SS}^*, M_{SS}^* + \delta) - r_M(S_{SS}^*, M_{SS}^*)}{\delta} \end{pmatrix}$$

1090 with  $\delta = 0.1$

1091 We can then calculate the variance-covariance matrix D:

$$D = \sigma^2 (\nabla^2 f(x^*))^{-1}$$

1092 with  $\sigma^2 = r_G(x^*)^2 + r_M(x^*)^2$

1093 The diagonal elements of  $D$  correspond to the variances of the parameters, so finally we  
 1094 can compute standard deviations for each parameter:

$$1095 \sigma_{S_{SS}^*} = \sqrt{D_{11}} \text{ and } \sigma_{M_{SS}^*} = \sqrt{D_{22}}$$

1096 The standard deviation for the proliferation rate is equal to  $a_S \sigma_{S_{SS}^*}$ , since  $p = a_S S_{SS}$ .

1097 We present standard deviations for our proliferation rate estimates in **S6 Table**.

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