

# Experimental and mathematical approaches to quantify recirculation kinetics of lymphocytes

Vitaly V. Ganusov<sup>1\*</sup> and Michio Tomura<sup>2</sup>

<sup>1</sup>Department of Microbiology, University of Tennessee, Knoxville, TN 37996, USA

<sup>2</sup>Immunology laboratory, Pharmaceutical Division, Osaka Ohtani University, Osaka, Japan

\*Corresponding author: [vitaly.ganusov@gmail.com](mailto:vitaly.ganusov@gmail.com)

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## Abstract

One of the properties of the immune system that makes it different from nervous and endocrine systems of mammals is the ability of immune cells to migrate between different tissues. Lymphocytes such as T and B cells have the ability to migrate from the blood to secondary lymphoid tissues such as spleen, lymph nodes, and Peyer's patches, and then migrate back to the blood, i.e., they can recirculate. Recirculation of lymphocytes has been a subject of intensive investigation decades ago with wealth of data on the kinetics of lymphocyte recirculation available. However, these data have not been widely used to estimate the kinetics of recirculation of different lymphocyte subsets in naive and immunized animals. In this paper we review pioneering studies addressing the question of lymphocyte recirculation, overview quantitative approaches that have been used to estimate the kinetics of lymphocyte recirculation, and provide currently published estimates of the residence times of resting lymphocytes in secondary lymphoid tissues of mammals.

**Keywords:** lymphocyte migration, recirculation, T cells, B cells, dwell time, residence time, mathematical modeling

**Abbreviations:** LNs: lymph nodes, iLNs: inguinal lymph nodes, mLNs: mesenteric lymph nodes, TDLs: thoracic duct lymphocytes, EEL: efferent lymph lymphocytes, LCMV: lymphocytic choriomeningitis virus, PPs: Peyer's patches.

## 19 Introduction

20 Adaptive immune system of mammals includes two major subsets of lymphocytes, B and T lympho-  
21 cytes [1, 2]. One of the major functions of the adaptive immune system is to protect its host from  
22 invading microorganisms such as viruses and bacteria. Because microbes can enter the host from  
23 multiple areas such as via skin, lung or gut mucosa, there is a need to have lymphocyte being present  
24 near these tissues. In addition, because every lymphocyte is in general specific to only one microbial  
25 determinant (epitope) and there are many microbial determinants, only a small percent of lympho-  
26 cytes would be able to recognize any specific microbe. For example, recent estimates suggest that only  
27 1 in  $10^5 - 10^6$  of T lymphocytes would recognize a given epitope; that is in a mouse that has  $\sim 2 \times 10^8$   
28 lymphocytes only about 200-2000 T cells would be specific to a given epitope [3]. While pathogens in  
29 general have multiple epitopes, only few of those can be recognized by lymphocytes. For example, in  
30 B6 mice lymphocytic choriomeningitis virus (LCMV) has about 30 epitopes that are recognized by  
31 CD8 T cells at all and only a few recognized strongly [4]. It is probably impossible to put a few thou-  
32 sands of T cells in all potential places of entry of a pathogen. Instead, lymphocytes have the ability  
33 to recirculate between different tissues in the body, thus increasing chances of encountering antigen  
34 they are specific for. This ability to migrate from the blood into several specific tissues and then back  
35 to the blood (i.e., to recirculate) makes adaptive immune system different from several other major  
36 systems of mammals such as nervous and endocrine systems. Because lymphocyte recirculation is  
37 a fundamental property of the mammalian immune system, our knowledge of how immune system  
38 works would be greatly incomplete if do not have solid understanding of the kinetics of lymphocytes  
39 recirculation, i.e., how quickly lymphocytes migrate to peripheral tissues, how long they spend in the  
40 tissues and return back to circulation. Understanding lymphocyte recirculation kinetics may be not  
41 just an academic exercise as blocking lymphocyte migration by anti-VLA4 antibodies – VLA4 is an  
42 integrin regulating lymphocyte entry into several tissues – has been shown to be effective in reducing  
43 symptoms of multiple sclerosis [5]. However, such treatment has serious side effects suggesting that  
44 deeper understanding how lymphocyte migration is regulated is needed [6, 7].

45 Ability of lymphocytes to recirculate between blood and tissues depends strongly on the type of  
46 lymphocyte, type of the tissue, and conditions of the host [8–18]. Specifically, naive T cells — cells  
47 that have not yet encountered their cognate antigen — are able to recirculate between blood and  
48 secondary lymphoid organs such as lymph nodes, spleen, and Peyer’s patches [9, 14, 19]. Activated T  
49 cells have the ability to migrate to nonlymphoid tissues [14, 20]; however, whether activated T cells  
50 in nonlymphoid tissues can migrate back to the blood remains poorly understood [21]. Inflammation  
51 may also change the pattern of lymphocyte migration; for example, intravenous (i.v.) infection may  
52 lead to trapping of recirculating lymphocytes in the spleen [22]. In this review we will focus on aspects  
53 of recirculation of resting (naive and memory) T lymphocytes with the major focus on migration of  
54 these cells via secondary lymphoid tissues, and how mathematical modeling has helped so far to  
55 quantify kinetics of this recirculation. Our main focus on recirculation of resting T cells is due to  
56 lack of good quantitative data and mathematical models on recirculation of activated T and B cells.  
57 However, we will provide a novel analysis of older data on recirculation kinetics of activated T cells  
58 in mice.

59 Because this review is about lymphocyte recirculation it is important to outline some basic  
60 anatomical features of the mammalian immune system. Since mice are the smallest mammalian ani-  
61 mal model used to study lymphocyte recirculation, we focus our description specifically on murine  
62 secondary lymphoid organs. The major secondary lymphoid organs of mice are lymph nodes (LNs),  
63 spleen, and Peyer’s patches (PPs). Fluids that leak out of blood vessels are collected by the lymph

64 phatic vessels which bring this interstitial fluid via afferent lymphatics to tissue-draining lymph nodes.  
65 Each lymph node drains fluids from specific tissues and fluids (lymph) exit lymph nodes via efferent  
66 lymphatics often into another lymph node [23, 24]. Lymph from the final lymph nodes is collected  
67 into two big vessels, left and right lymphatic ducts, which are connected to the blood and which  
68 return collected interstitial fluids and cells back to circulation [2]. In mice and humans, the right  
69 lymphatic duct collects lymph from the upper right part of the body (about 1/4 of all interstitial  
70 fluid) and left lymphatic duct (also called thoracic duct) collects lymph from the rest (about 3/4)  
71 of the body. Lymph nodes can be roughly divided into several groups such as skin-draining lymph  
72 nodes, lung-draining lymph nodes, and gut-draining lymph nodes. A typical laboratory mice strain  
73 has about 30 lymph nodes [23] while humans have hundreds (perhaps over a thousand) lymph nodes  
74 [24–26]. Peyer’s patches are lymph node-like structures found in the gut. Peyer’s patches do not  
75 have afferent lymphatics and efferent lymph from Peyer’s patches flows into mesenteric (gut-draining)  
76 lymph nodes. Finally, spleen is probably the largest single secondary lymphoid organ in mice and  
77 humans [25, 27–31]. Spleen is not connected directly to the lymphatic system and lymphocytes enter  
78 the spleen from the blood and exit the spleen into the blood. In contrast, lymphocytes may enter  
79 lymph nodes or Peyer’s patches from the blood via high endothelial venules, and lymphocyte may  
80 also enter lymph nodes by migrating from the blood to peripheral tissues such as skin or gut, and  
81 then enter lymph nodes with afferent lymph. Thus, lymphocytes have several different pathways for  
82 recirculation in the body.

## 83 **Mathematical modeling of lymphocyte recirculation**

84 By definition recirculation of lymphocytes is a dynamic process and therefore mathematical modeling  
85 is likely to be a useful tool for understanding of lymphocyte recirculation. Mathematical modeling  
86 is required to accurately quantify the kinetics of lymphocyte recirculation and to estimate the rates  
87 of lymphocyte migration from the blood to tissues and lymphocyte residence times in tissues. There  
88 have been many uses of mathematical models to understand cell migration. For example, mathemat-  
89 ical models have been used to gain insights of how lymphocytes move in lymphoid and nonlymphoid  
90 tissues and how tissue composition impacts movement patterns of T cells [32, 33]. Here our main  
91 focus will be on experimental studies providing quantitative data on lymphocyte migration between  
92 tissues, and on mathematical modeling attempts to quantify lymphocyte recirculation kinetics (Table  
93 1).

94 Kinetic aspects of lymphocyte recirculation has been studied since 1950<sup>th</sup> using multiple mam-  
95 malian species such as mice, rats, sheep, and pigs (see more below). Prior to pioneering experiments  
96 by Gowans the role of small lymphocytes found in the blood was unknown. Collecting lymphocytes  
97 from the thoracic duct lymph of rats over several days led to a decline in the number of lympho-  
98 cytes found in the lymph. However, return of cells collected by thoracic duct cannulation back into  
99 circulation prevented loss of lymphocytes from the lymph [34]. These key experiments thus estab-  
100 lished that lymphocytes are able to recirculate between blood and thoracic duct lymph. By labeling  
101 lymphocytes collected from the blood or lymph (e.g., by thoracic duct (rats) or individual lymph  
102 node (sheep, pigs) cannulation) with radioactive labels further experiments demonstrated that indeed  
103 lymphocytes migrate from the blood to the efferent lymphatics of lymph nodes [8, 35–40].

## 104 Spleen

105 Spleen is a large secondary lymphoid organ and it was previously estimated that about 20% of all  
106 lymphocytes in a human are found in the spleen [25, 31]. In mice, around 50% of all lymphocytes  
107 from secondary lymphoid tissues are found in the spleen [41, 42]. While it has been estimated that  
108 many lymphocytes travel via the spleen of mice, rats, or pigs [28, 30, 43] the exact time lymphocytes  
109 spend in the spleen has not been accurately quantified. Previous studies documented accumulation  
110 of radioactively labeled lymphocytes in the spleen after i.v. infusion of such cells [43–45] or dynamics  
111 of labeled cells in the blood in normal or splenectomized pigs [29]. However, data from these studies  
112 have not been analyzed using mathematical models, and thus, these previous data did not lead to  
113 estimates of lymphocyte residence (or dwell) times in the spleen.

114 An interesting approach was taken by Ford who designed an apparatus allowing maintenance of  
115 viable rat spleens for an extended period of time (up to 10 days, [46]). By creating an artificial  
116 circulation system connecting the spleen's blood vessels, the author could monitor concentration of  
117 lymphocytes that exit the spleen during the perfusion of labeled thoracic duct lymphocytes that  
118 had been injected into circulation. Similar experiments with isolated pig spleens was done later by  
119 another group [28]. Verbal analysis of the data on migration thoracic duct lymphocytes via isolated  
120 perfused spleen led to estimate of lymphocyte residence time in the rat spleen of 4-5 hours [46, 47] and  
121 in the pig spleen of 2-4 hours [28]. A relatively complex mathematical model was proposed and fitted  
122 to the data of Ford [46]. This mathematical model-based analysis predicted that only about 10-25%  
123 of lymphocytes migrating via spleen pass via the marginal zone of the spleen (i.e., enter the spleen  
124 parenchyma). Cells entering the marginal zone of the spleen had a residency time of 50 minutes in the  
125 tissue. About 10% of cells existing the marginal zone migrated to the white pulp where the residency  
126 time was 4.6 hours. In contrast, remaining 90% of lymphocytes exited marginal zone into the red  
127 pulp with the average residency time of 5 minutes in that subcompartment of the spleen [48]. While  
128 Hammond [48] did not calculate the average time lymphocytes spend in the spleen, given the estimates  
129 provided, the average residency time of TDLs in the spleen is  $50 + 0.1 \times 4.6 \times 60 + 0.9 \times 5 = 82$  min  
130 or 1.4 hours. This is a significantly shorter residency time than concluded by Ford [46] by visual  
131 analysis of the data.

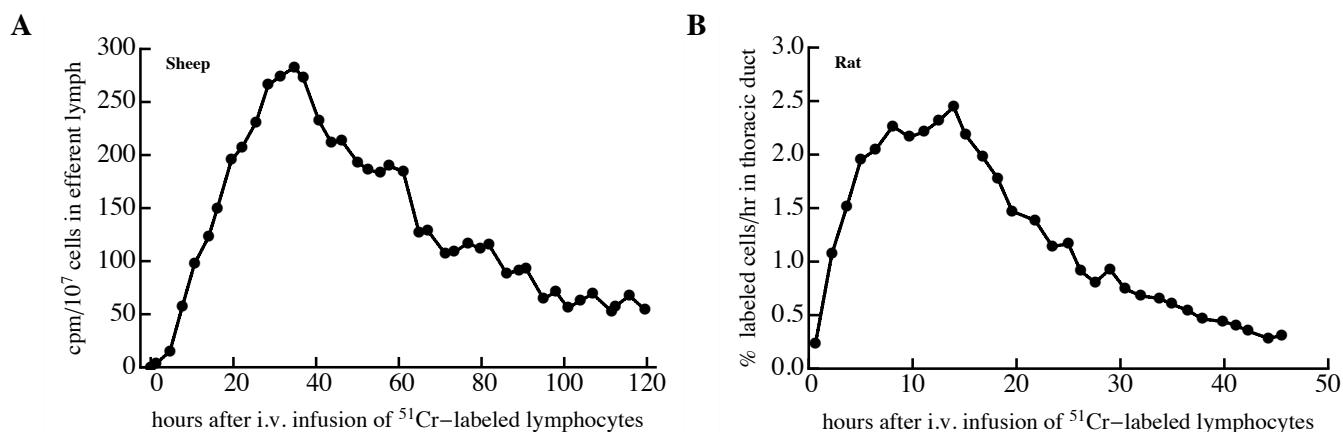
132 Ganusov and Auerbach [49] used another set of data on migration kinetics of thoracic duct  
133 lymphocytes (TDLs) in rats. In these experiments (see also below), lymphocytes collected via thoracic  
134 duct cannulation were transferred into syngenic rats and the accumulation and loss of transferred  
135 cells (labeled with a radioactive label) were measured in multiple tissues of rats [50]. By fitting a  
136 mathematical model to these experimental data, Ganusov and Auerbach [49] estimated recirculation  
137 kinetics of TDLs including residence of these cells in major secondary lymphoid tissues of rats. The  
138 model fits predicted that average residence time of TDLs in the spleen is 2.4 hours which is also  
139 shorter than a previous estimate given in Ford [46] but longer than the estimate obtained from  
140 parameters of Hammond [48].

## 141 Lymph nodes

142 Migration of lymphocytes from blood to lymph nodes and then back to the blood has been the main  
143 focus of many studies on lymphocyte recirculation. In part, this stems from the fact that in small  
144 animals such as rats cells that had migrated via lymph nodes can be easily collected via the thoracic  
145 duct cannulation, and, thus, the tempo of lymphocyte movement from the blood to thoracic duct

146 can be easily recorded. In larger animals an efferent lymphatic vessel exiting a given lymph is wide  
147 enough to allow cannulation and collection of cells exiting that specific lymph node.

148 As described above, following the classical studies by Gowans illustrating ability of lymphocytes  
149 to recirculate between blood and thoracic duct lymph, there have been multiple studies investigating  
150 details of lymphocyte migration from blood to efferent lymph (reviewed in [30, 51–53]). Transit times  
151 via a particular lymph node (e.g., inguinal or cervical) have been inferred by transferring labeled  
152 lymphocytes into the animal and measuring rate of exit of labeled lymphocytes via a cannulated  
153 node in large animals such as sheep and pigs (e.g., [8, 9, 16, 54–57, Figure 1A]). Alternatively,  
154 kinetics of lymphocyte migration from the blood to the thoracic duct (i.e., via the whole lymphatic  
155 system) have been measured in smaller animals such as mice and rats (e.g., [36, 50, 58–65, Figure  
156 1B]).



**Figure 1:** Typical examples of experimental data on lymphocyte migration from blood to lymph. In such studies, lymphocytes were isolated from a tissue (e.g., efferent lymph, lymph nodes, blood, spleen) and labeled with a label. In older studies labels were radioactive while newer studies involved fluorescent labels. Then cells were transferred intravenously into the same (e.g., sheep, panel A) or syngenic (e.g., rat, panel B) host. The accumulation of labeled cells in the efferent lymph of a given lymph node (panel A) or in the thoracic duct (panel B) were then followed. Different measures of the number of labeled cells have been used such as the number of labeled cells per fixed number of total isolated cells (e.g., estimated by measuring counts-per-minute (cpm) from the sample, panel A) or as the number or percent of injected cells (measured by total radioactivity) per unit of time (panel B). Data were digitized from previous publications (panel A: Frost et al. [54], panel B: Smith and Ford [50]).

157 While the data on the migration kinetics of lymphocytes via individual lymph nodes or from the  
158 blood to thoracic duct have been collected (e.g., Figure 1) very few studies attempted to estimate  
159 the lymphocyte residence (or dwell) time in lymph nodes from these cannulation experiments. For  
160 example, by simply looking at the data it is unclear which characteristic of the distribution observed  
161 in Figure 1 represents the average residence time. Mode, median, and average could all potentially be  
162 good estimates of the average residency time, and intuitively the average of the distribution has been  
163 treated in experimental studies as an estimate for residence time of lymphocytes in lymph nodes,  
164 e.g., about 48 hours in sheep or 24 hours in rats [30, 58]. However, to accurately estimate the average  
165 residence time one need to use mathematical modeling that takes physiology of the recirculatory and  
166 lymphatic system of mammals into account.

167 As far as we know the first mathematical modeling-based attempt to quantify lymphocyte mi-  
168 gration via lymph nodes was in a series of papers by Stekel et al. [66–68]. The main idea of the  
169 mathematical model considered in these papers was the ability of lymphocytes to attach to and

170 deattach from the lymphoid tissues while in lymph nodes or the spleen [66]. Deattached cells move  
171 through the tissue and this “movement” was described by a transport equation. Attached cells,  
172 however, would not move, and thus the process of “attachment – detachment” generated a skewed  
173 distribution and matched data on thoracic duct cannulation in rats [66, 67]. This work suggested 20h  
174 residency time of lymphocytes in the lymph nodes and 6 h residency time in the spleen of rats [66].  
175 The model was further used to explain different kinetics of lymphocyte migration in irradiated or  
176 thymectomized rats [67, 68]. It is now well understood that the basic assumption of the model that  
177 cell attachment to structures in lymph nodes prevent lymphocyte exit is incorrect; rather, lymphocyte  
178 use structures including fibroblastic reticular cells to move around and to exit lymph nodes although  
179 precise mechanisms regulating lymphocyte exit from lymph nodes remain to be fully defined [69].  
180 By using intravital imaging of T lymphocytes moving in murine lymph nodes and by mathematical  
181 modeling of T cell movement in the nodes Grigorova et al. [69] estimated the half-life time of T  
182 cells in the lymph nodes of mice to be 4-5 hours. Another study analyzed importance of directional  
183 movement of T cells in lymph nodes using digital reconstruction of a rat lymph node but the actual  
184 residency times of lymphocytes in the nodes was not estimated [70].

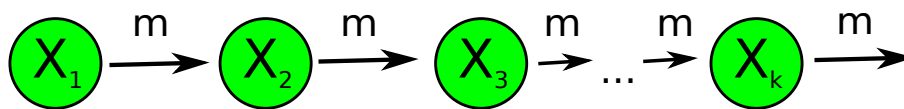
185 An important study quantified the residence time of antigen-specific naive and memory CD8 T  
186 cells using a novel “transfer-and-block” technique [71]. In this approach, naive or memory CD8 T  
187 cells, specific to the GP33 epitope of LCMV were transferred into congenic hosts and 24 hours after  
188 cell transfer further entry of lymphocytes into LNs was blocked by using anti-CD62L antibodies.  
189 CD62L is expressed on T cells and is generally required for cell entry into LNs [72]. The declining  
190 number of LCMV-specific T cells remaining in the LNs at different times after the blockade was used  
191 to infer the lymphocyte residence time. Interestingly, the authors found a non-monotonic rate of loss  
192 of T cells from LNs; naive and memory CD8 T cell populations had initial residency times of 5-6 h in  
193 LNs which increased significantly at later times for both naive and memory T cell populations to 15-  
194 16 hours [71]. Re-analysis of these data in another study revealed that the data could be accurately  
195 explained by a model in which residency time of lymphocytes is density-dependent and declines with  
196 time since blockade (if blockade was 100% efficient) [49]. In this reanalysis, naive and memory CD8  
197 T cells had different residency times (16 and 9 hours for naive and memory T cells, respectively)  
198 [49]. Thus, the use of the same data but different assumptions on lymphocyte migration may result  
199 in different estimates of lymphocyte recirculation kinetics.

200 Mandl et al. [73] extended the study of Harp et al. [71] by transferring polyclonal naive CD4 and  
201 CD8 T cells into congenic mice and by blocking entry of new cells 2 hours after cell transfer by using a  
202 combination of antibodies to CD62L and VLA4. The authors found that a residence time of naive T  
203 cells was dependent on the type of the cell (CD4 vs. CD8 T cell). In contrast with previous work [71]  
204 the authors observed that after the blockade the percent of transferred cells declined exponentially  
205 over time. By fitting a line to log-transformed cell frequencies the authors estimated that naive CD4  
206 and CD8 T cells spend on average 12 and 21 hours, respectively, in lymph nodes in mice [73].

207 It is interesting to note that we only know of one study that estimated residence time of lym-  
208 phocytes from data generated by cannulating individual lymph nodes in sheep [74]. The authors  
209 proposed that lymphocyte migration within a lymph node can be described as a Markov process  
210 with  $n$  states and probability of jump from one state to another forward ( $i \rightarrow i + 1$ ) or backward  
211 ( $i \rightarrow i - 1$ ). Reaching the  $n^{th}$  state implied exit of a lymphocyte from the lymph node. The model was  
212 fitted to the cannulation data similar to that in Figure 1A and predicted the average residence time  
213 of lymphocytes in sheep lymph nodes of 31 hour [74]. Using a different mathematical model, which  
214 incorporated lymphocyte recirculation kinetics in the whole body, we have analyzed similar data on  
215 lymphocyte migration via individual lymph nodes in sheep (McDaniel and Ganusov (in preparation)).

216 Depending on the dataset we found the average residence time of blood-derived lymphocytes in ovine  
 217 lymph nodes to be 18-22 hours. This is another demonstration that the estimate of the lymphocyte  
 218 residence time may not be robust to the choice of a model [75].

219 We recently performed mathematical modeling-assisted analysis of experimental data on recir-  
 220 culation of thoracic duct lymphocytes (TDLs) in rats [49]. In these experiments [50], lymphocytes  
 221 collected in rats via thoracic duct cannulation were transferred into a series of syngenic hosts and  
 222 accumulation and loss of the transferred cells in multiple lymphoid tissues was followed over time.  
 223 By fitting a series of mathematical models to experimental data we estimated the TDL residence  
 224 time in multiple tissues including lymph nodes. Interestingly, in contrast with previous studies that  
 225 found differences in T lymphocyte residence times in different lymph nodes (e.g., mesenteric vs. sub-  
 226 cutaneous LNs) [71, 73] we found the average residence time in subcutaneous or mesenteric LNs or  
 227 PPs to be 10 hours [49, see more below].



**Figure 2:** Schematic of the mathematical model describing loss of photoconverted cells from a LN of Kaede mice. We assume that cells entering a LN must undergo  $k$  exponentially distributed transitions before they can exit the node. In essence, this assumes that residency time of lymphocytes in a lymph node follows a gamma distribution. In the model  $X_i$  is the number of lymphocytes residing in the  $i^{th}$  compartment and  $m$  is the rate of transition of cells between subcompartments (see also eqns. (1)–(2)). Cells exiting the last subcompartment exit the lymph node into efferent lymph.

228 A novel experimental technique, the Kaede mice, allows to accurately track exit of lymphocytes  
 229 from a given tissue such skin or a lymph node [17, 76]. Kaede mice express a photoconvertible  
 230 protein which upon exposure to violet light changes color from green to red [76]. This unique system  
 231 allows to label cells in one location, for example, an inguinal lymph node (iLN) or skin, and track the  
 232 movement of labeled cells to other tissues in the body [17, 77]. We developed a simple mathematical  
 233 model to track the dynamics of photoconverted (red) lymphocytes in the iLNs of Kaede (or other  
 234 photoconvertible mice, e.g., KikGR [78, 79]) mice. The model assumes that cells exit the LN and are  
 235 not able to re-enter the same LN. Experiments have shown that labeling of cells in the iLN distribute  
 236 between all LNs and the spleen with approximately of 2-3% of cells in LNs being red [76]. Therefore,  
 237 until the percent of photoconverted cells in the iLN is above 8-10%, re-entry of such cells into the  
 238 node can likely be neglected if we assume that photoconversion and surgery associated with it do not  
 239 induce strong inflammation impacting cell migration. In experiments, the lack of inflammation was  
 240 recorded by similar size of iLNs prior and after the photoconversion.

241 Because our previous study suggested that distribution of residence times of TDLs in LNs was  
 242 best described by a gamma distribution (and not by exponential distribution) [49], for experiments  
 243 with Kaede we describe exit of lymphocytes from a LN as cell “migration” via multiple ( $k$ ) subcom-  
 244 partments in the LN:

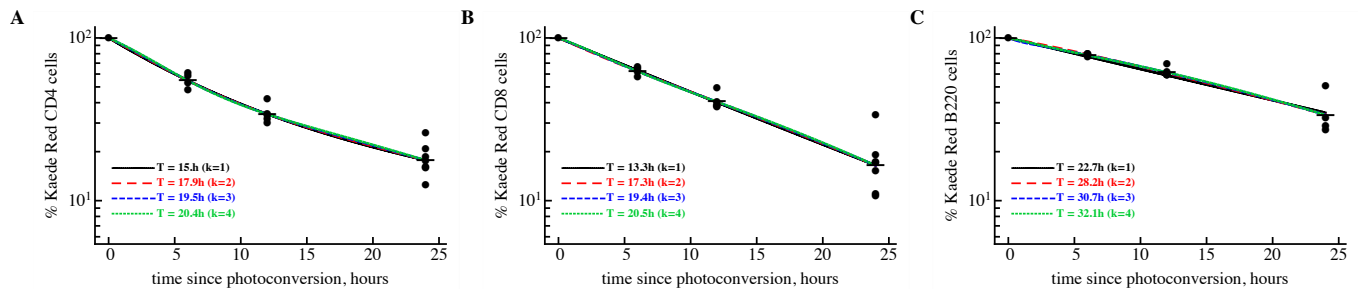
$$\frac{dx_1(t)}{dt} = -mx_1(t), \quad (1)$$

$$\frac{dx_i(t)}{dt} = m(x_{i-1}(t) - x_i(t)), \quad i = 2 \dots k, \quad (2)$$

245 where  $m$  is the migration rate via a given subcompartment. The average residence time of lympho-  
 246 cytes in the lymph nodes is then given by  $T = k/m$ . Given that following photoconversion cells in  
 247 all subcompartments at the steady state have equal densities,  $x_i(0) = 1/k$ , the mathematical model  
 248 (eqns. (1)–(2)) has a unique solution for the total number of cells in the LN  $x(t) = \sum_{i=1}^k x_i(t)$ :

$$x(t, m) = e^{-mt} \sum_{i=1}^k \frac{(mt)^{i-1}}{(i-1)!}. \quad (3)$$

249 By fitting this solution (eqn. (3)) to experimental data from photoconversion experiments (Figure  
 250 3) we found that this simple model describes well the data for the loss of photoconverted CD8 T cells  
 251 and B cells from the iLN (Figure 3B&C). However, the model was inadequate at describing the data  
 252 for CD4 T cells as judged by the lack of fit test [80, results not shown]. Visually, this is likely because  
 253 the loss of photoconverted CD4 T cells from the iLN is not exponential (compare Figure 3A & 3B).  
 254 These results were independent of the number of subcompartments tested. While it is clear that CD4  
 255 and CD8 T cell populations most likely consist of subpopulations with perhaps different rates of exit  
 256 from the iLNs, e.g., naive and memory T cells, why we were not able to detect such heterogeneity  
 257 for CD8 T cells was unclear. It is possible that heterogeneity in CD4 T cells may come from a more  
 258 diverse sets of cell types present in this group, for example, naive, memory, and regulatory T cells.  
 259 Previously it was noted that naive and memory phenotype CD4 T cells have different exit kinetics  
 260 from LNs [17]. In contrast, LCMV-specific naive and memory CD8 T cells appear to exit iLNs with  
 261 similar kinetics [71].



**Figure 3:** Estimated residence time of lymphocytes depends on the shape of residence time distribution. Lymphocytes in inguinal lymph nodes (iLNs) of Kaede mice were photoconverted [76] and the percent of CD4 T cells (panel A), CD8 T cells (panel B), or B cells (panel C) remaining in the iLNs at different times after photoconversion was recorded; the data from individual mice are shown by dots and horizontal lines denote average percent per time point. We fit a series of mathematical models assuming one (eqn. (3)) or two (eqn. (4)) subpopulations of cells with a different number of subcompartments ( $k = 1 \dots 4$ ) to these data. Fits of the models with 2 subpopulations are shown by lines. To normalize residuals we used  $\log_{10}$  transformation. The model with two cell subpopulations only improved the fit of the data for CD4 T cells (F-test,  $p < 0.005$ ). Data for CD8 T and B cells were well described by a model with one, homogenous population (F-test,  $p > 0.05$ ). Parameters of the model with 2 subpopulations and  $k = 2$  compartments and their 95% confidence intervals (found by bootstrapping residuals with 1000 simulations) for different lymphocyte types are: CD4 T cells:  $m_1 = 0.30$  (0.30, 0.31)/h,  $m_2 = 0.066$  (0.065, 0.067)/h,  $f = 0.53$  (0.52, 0.53),  $T = 17.9$  (17.9, 18.0) h; for CD8 T cells:  $m_1 = 0.46$  (0.43, 0.48)/h,  $m_2 = 0.10$  (0.10, 0.10)/h,  $f = 0.16$  (0.15, 0.15),  $T = 17.9$  (17.9, 18.0) h; B cells:  $m_1 = 0.071$  (0.071, 0.071)/h,  $m_2 = 0.07$  (0.07, 0.07)/h,  $f = 0.37$  (0.37, 0.37),  $T = 28.2$  (28.2, 28.2) h.

262 To more accurately describe the kinetics of loss of photoconverted CD4 T cells from iLNs we  
 263 extended the simple model (eqn. (3)) by allowing 2 subpopulations with relative frequencies  $f$  and



264  $1 - f$  and with different exit kinetics determined by the rates  $m_1$  and  $m_2$ , respectively. In this model,  
265 the total number of photoconverted (red) cells in the iLN is then given by

$$X(t) = fx(t, m_1) + (1 - f)x(t, m_2), \quad (4)$$

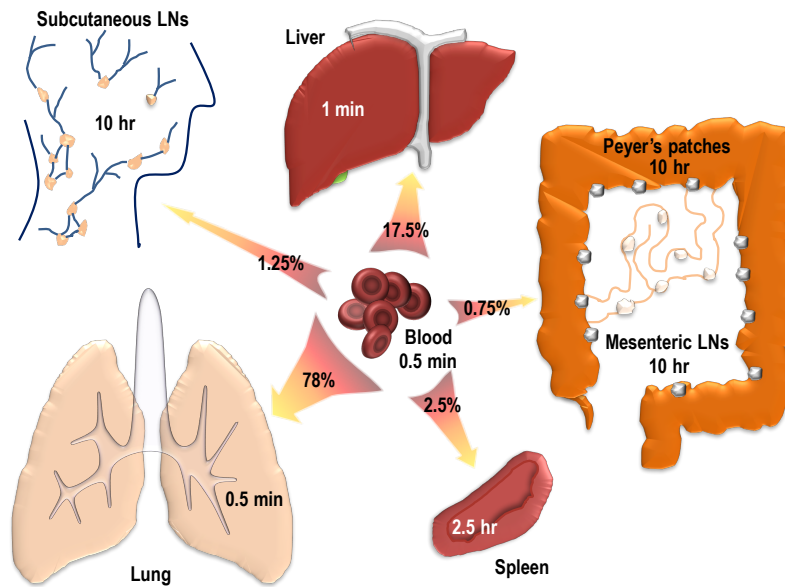
266 where  $x(t, m_i)$  is given in eqn. (3). It is straightforward to extend this model to  $n$  subpopulations.  
267 The average residence time in this model is defined as  $T = fk/m_1 + (1 - f)k/m_2$  where  $k$  is the number  
268 of subcompartments in each of the subpopulations. This 2 subpopulation model can well describe  
269 experimental data on the loss of photoconverted CD4 T cells from iLNs (Figure 3A). Interestingly,  
270 for models that fit the data well (e.g., data for CD8 T cells or B cells) the estimated average residence  
271 time was not strongly dependent on the number of subpopulations assumed (1 or 2 subpopulations,  
272 results not shown). However, the estimate of the average residence time was strongly dependent on  
273 the number of subcompartments assumed, and, as the consequences, on the shape of the distribution  
274 of residence times. For example, the model fits predicted average residence time for CD8 T cells to  
275 be  $T = 13.3$  h for  $k = 1$  or  $T = 22.9$  for  $k = 5$  (eqn. (3)) with moderate reduction in the quality of  
276 the model fit to data at higher  $k$  as judged by AIC (results not shown). Therefore, it appears that  
277 the estimate of the average residence time from photoconversion data is not fully robust. Given our  
278 previous observation that best description of TDL recirculation kinetics via LNs in rats is given by  
279 a gamma distribution with shape parameter  $k = 2$  our results suggest that average residence times  
280 in mouse iLNs are 18 h for CD4 and CD8 T cells, and 28 h for B cells.

## 281 **Whole body recirculation kinetics**

282 An important limitation of many of the previously listed studies is that they considered migration of  
283 lymphocytes only via individual secondary lymphoid tissues such as spleen or individual lymph nodes.  
284 To study lymphocyte migration in the whole organism, Smith and Ford [50] adoptively transferred  
285  $^{51}\text{Cr}$ -labeled TDLs and measured the percent of transferred lymphocytes in different organs of the  
286 recipient rats including the blood, lung, liver, spleen, skin-draining (subcutaneous) and gut-draining  
287 (mesenteric) lymph nodes. These data were initially analyzed with the use of a mathematical model  
288 [81] but TDL residence times in different tissues were not estimated. We developed a simple yet  
289 large mathematical model describing TDL dynamics, and by fitting the model to Smith and Ford  
290 [50] data, for the first time estimated the kinetics of TDL recirculation in the whole body [49, Figure  
291 4].

292 The model fits predicted that TDLs spend very short time in the main blood vessels (about 30  
293 sec) after which the vast majority of lymphocytes (about 95%) gets trapped in vasculature of the lung  
294 or the liver. This trapping is short-lived, however, and within 1 min trapped lymphocytes re-enter  
295 circulation (Figure 4). Only 5% of lymphocyte enter secondary lymphoid tissues per one passage of  
296 lymphocytes via circulatory system, with half of these entering the spleen, and half entering lymph  
297 nodes and Peyer's patches (PPs). Lymphocytes reside for 10 h in LNs/PPs but only for 2.5 h in the  
298 spleen (Figure 4).

299 Because there is a good understanding of the kinetics of blood recirculation in rats, we would  
300 like to provide another interpretation of the kinetics at which TDLs pass via major tissues in rats.  
301 Previous studies found that the total blood volume in rats is proportional to the rat weight [82],  
302 and for 300 g rats, blood volume is  $V_b \approx 20$  mL. Heart volume is dependent on the animal size and  
303 age, and for 6-10 week old rats,  $V_h = 0.5$  mL [83]. Given the high heart rate in rats (462/min)



**Figure 4:** Estimated residence times of thoracic duct lymphocytes (TDLs) in rats [49]. We developed a mathematical model describing migration of lymphocytes in the whole organisms and by fitting the model to experimental data [50], estimated TDL residence times in major nonlymphoid and all major secondary lymphoid tissues of rats. Predicted residence times were short in the blood, lung and liver ( $T \leq 1\text{min}$ ) with 95% of lymphocytes recirculating between these tissues. Residence time in the spleen was 2.5 h with about 2.5% of lymphocytes entering the tissue. The remaining  $\sim 2\%$  of cells were entering lymph nodes and Peyer's patches (PPs), with the average residence time of TDLs in these tissues was 10 hours [49].

304 and stroke volume 0.3 mL per heart beat, the total cardiac output in rats is approximately  $c = 140$   
305 mL/min [84]. This in turn suggests the rate of blood recirculation in rats of  $m_0 = c/V_b = 7/\text{min}$   
306 or residency time of 9 seconds (i.e., on average in 9 seconds all blood passes through the heart).  
307 Given previously estimated rates at while TDLs enter various tissues [49], we predict that per one  
308 circulation of whole rat blood 27% and 6% of TDLs are attaching to the lung and liver vasculature,  
309 respectively. This suggests that the majority of TDLs pass via lung and liver vasculatures without  
310 attachment! Importantly, only 0.8% of TDLs in the blood will migrate to the spleen and 0.6% will  
311 migrate to LNs and PPs in one blood recirculation cycle lasting 9 seconds suggesting that the process  
312 of entering secondary lymphoid tissues by recirculating TDLs is not very efficient.

313 Differential probability of lymphocyte migration via lung and liver vs. secondary lymphoid organs  
314 is interesting but perhaps not unexpected given that these two organs are large and are expected  
315 to collect large volumes of blood. Amount of blood going to any specific tissue (cardiac output)  
316 has been measured in multiple species, for example, by measuring accumulation of labeled small  
317 microspheres after injection into the blood [40, 56, 84]. While we did not find a single study in rats  
318 measuring cardiac output to the same tissues as in our analysis (Figure 4), we found the cardiac  
319 output does not accurately predict the hierarchy of lymphocyte entry into lung, liver, and spleen. In  
320 rats, 0.7%, 3.3%, and 0.6% of cardiac output goes to lung, liver, and spleen, respectively [84], which  
321 is in contrast to 78%, 17%, and 2.5% of lymphocyte entry probability for these tissues [49]. Thus,  
322 migration and retention of lymphocytes in the whole body, while likely is dependent on the blood  
323 flow, is not strictly determined by the amount of blood going to any specific tissue.

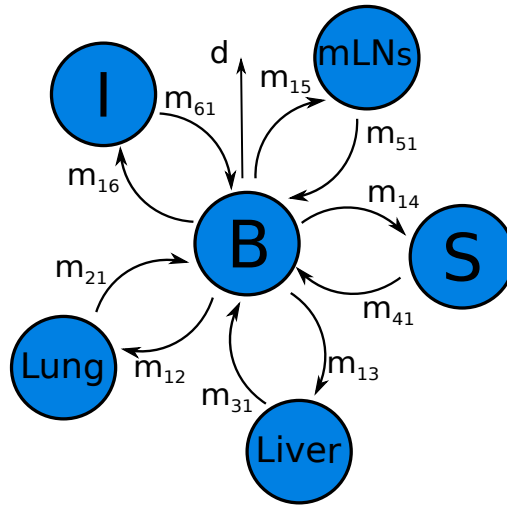
| Lymphocyte type    | Tissue/organ    | Animal species | Residency time $T$ , $h$ | Reference |
|--------------------|-----------------|----------------|--------------------------|-----------|
| TDL                | Spleen          | Rats           | 4.5                      | [46]      |
| TDL                | Spleen          | Rats           | 6.0                      | [66]      |
| TDL                | Spleen          | Rats           | 1.4                      | [48]      |
| TDL                | Spleen          | Rats           | 2.4                      | [49]      |
| Blood lymphocytes  | Spleen          | Pigs           | 3.0                      | [28]      |
| Naive T cells      | Lymph nodes     | Mice           | 4.5                      | [69]      |
| naive CD8 T cells  | Lymph nodes     | Mice           | 6.0                      | [71]      |
| memory CD8 T cells | Lymph nodes     | Mice           | 6.0                      | [71]      |
| naive CD8 T cells  | Lymph nodes     | Mice           | 16.0                     | [49]      |
| memory CD8 T cells | Lymph nodes     | Mice           | 9.0                      | [49]      |
| naive CD4 T cells  | Lymph nodes     | Mice           | 12.0                     | [73]      |
| naive CD8 T cells  | Lymph nodes     | Mice           | 21.0                     | [73]      |
| CD4 T cells        | Lymph nodes     | Mice           | 18.0                     | this work |
| CD8 T cells        | Lymph nodes     | Mice           | 18.0                     | this work |
| B cells            | Lymph nodes     | Mice           | 28.0                     | this work |
| TDL                | Lymph nodes     | Rats           | 20.0                     | [66]      |
| TDL                | Lymph nodes     | Rats           | 10.0                     | [49]      |
| ELL                | Lymph nodes     | Sheep          | 31.0                     | [74]      |
| TDL                | Peyer's patches | Rats           | 10.0                     | [49]      |

**Table 1:** Summary of published estimates of residency times of resting lymphocytes in secondary lymphoid tissues such as spleen, lymph nodes, and Peyer's patches. Here ELL are efferent lymph lymphocytes (lymphocytes isolated by cannulation of individual lymph nodes, e.g., in sheep), TDL are thoracic duct lymphocytes (lymphocytes isolated by thoracic duct cannulation). In most other cases lymphocytes were isolated from lymph nodes and/spleen. Listed values for the lymphocyte residency time are as has been reported by authors and in some cases, half-life time of lymphocytes in the tissue ( $T_{1/2}$ ) was converted to the residency time using formula  $T = \ln 2 \times T_{1/2}$ . In some studies, estimates for the residence time of lymphocytes in lymph nodes were dependent on the lymph node type (e.g., [73]), therefore, the presented estimates are for the pooled data. For time-dependent residency times the initial value was used (e.g., [71]).

## 324 Recirculation of activated lymphocytes in mice

325 The vast majority of previous studies focused on quantifying migration of naive and memory lympho-  
326 cytes via secondary lymphoid organs. While such lymphocytes are likely to represent the majority  
327 of cells in an organism in the absence of infection, infections will result in activation of lymphocytes.  
328 Yet, pattern and kinetics of migration of activated lymphocytes remain poorly defined. Cancer im-  
329 munotherapy, involving *in vitro* expansion of populations of cancer-specific CD8 T cells and transfer  
330 of these cells into patients, is one of the novel ways to treat patients [85–87]. Therefore, deeper un-  
331 derstanding of migration kinetics of activated T cells may help to improve the efficacy of T cell-based  
332 cancer therapies.

333 To determine the pattern and kinetics of recirculation of activated T lymphocytes we analyzed  
334 data from an old set of experiments [61]. In these experiments, Sprent [61] injected thymocytes (cells  
335 from the thymus) from CBA (H-2<sup>k</sup>) mice into irradiated CBA  $\times$  C57Bl/6 (H-2<sup>k</sup> $\times$ H-2<sup>b</sup>) F<sub>1</sub> mice and  
336 isolated activated T cells via the thoracic duct cannulation [60]. Activated T cells were specific to the  
337 H-2<sup>b</sup> antigen of the donor. Collected T cells were labeled with <sup>125</sup>IUdR *in vitro* after 1 hour incubation  
338 and then injected intravenously into a series of syngenic CBA mice [61]. <sup>125</sup>IUdR is incorporated into



**Figure 5:** Schematic of assumed recirculation pathways of activated T lymphocytes [61]. In these experiments, lymphocytes were injected into the blood ( $B$ ) and from the blood lymphocytes may enter lung ( $i = 2$ ), liver ( $i = 3$ ), spleen ( $S$ ,  $i = 4$ ), mesenteric LNs (mLNs,  $i = 5$ ), intestine ( $I$ ,  $i = 6$ ) at rates  $m_{1i}$  with  $i = 2, \dots, 6$ , respectively. Lymphocytes in these tissues may return to circulation at rates  $m_{i1}$  with  $i = 2, \dots, 6$ . Lymphocytes may also leave the blood to other unsampled compartments and/or die at rate  $d$  (see eqns. (5)–(8)).

339 newly synthesized DNA, and therefore, only lymphocytes that were actively dividing *in vitro* became  
 340 labeled.

341 Following adoptive transfer recipient mice were sacrificed at different times after cell transfer  
 342 and the percent of labeled lymphocytes was measured in several major organs of mice including  
 343 blood, lung, liver, spleen, mesenteric lymph nodes (mLNs), thymus, kidney, and intestine [61, Figure  
 344 6]. Because very few cells migrated to thymus and kidney, we ignored these tissues in our following  
 345 analysis; inclusion of these tissues did not influence significantly estimates of other parameters (results  
 346 not shown).

347 To estimate the rates of activated T cell migration to major tissues of mice we adopted a mathe-  
 348 matical model from our previous study [49]. In this model we assume that lymphocytes in the blood  
 349 can migrate to multiple tissues such as lung, liver, spleen, , and intestine, and following passage via  
 350 the tissue, the cells would return back to the blood. The rate of lymphocyte entry into  $i^{th}$  tissue  
 351 from the blood is denoted as  $m_{1i}$  and the rate of exit from the  $i^{th}$  tissue into the blood is then  $m_{i1}$   
 352 where  $i = 2, \dots, 6$ . Following our previous work and some initial analyses in the model we assume  
 353 that T cell migration via the lung and liver follows 1st order kinetics (i.e., is described by an expo-  
 354 nential distribution), but residence in the spleen, mLNs and intestine is gamma-distributed. Gamma  
 355 distribution of T cell residence in these tissues was modelled by assuming  $k$  subcompartments with  
 356 migration rate  $m_{i1}$  between subcompartments. With these assumptions the mathematical model is  
 357 given by a set of differential equations:

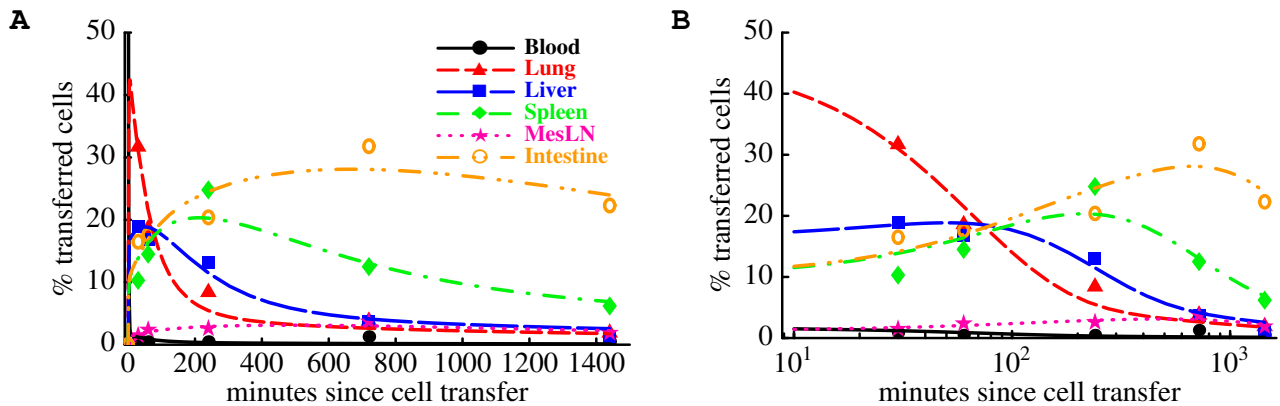
$$\frac{dx_1}{dt} = -x_1 \left( d + \sum_{i=2}^6 m_{1i} \right) + \sum_{i=2}^3 m_{i1}x_i + \sum_{i=4}^6 m_{i1}x_{ik}, \quad (5)$$

$$\frac{dx_i}{dt} = m_{1i}x_1 - m_{i1}x_i, \quad i = 2, 3, \quad (6)$$

$$\frac{dx_{i1}}{dt} = m_{1i}x_1 - m_{i1}x_{i1}, \quad i = 4, 5, 6, \quad (7)$$

$$\frac{dx_{ij}}{dt} = m_{i1}x_{ij-1} - m_{i1}x_{ij}, \quad i = 4, 5, 6, \quad j = 2, \dots, k, \quad (8)$$

358 where  $x_i$  is the percent of labeled cells found in the blood ( $i = 1$ ), lung ( $i = 2$ ), liver ( $i = 3$ ), and  
 359  $x_{ij}$  is the percent of labeled cells found in the  $j^{\text{th}}$  sub-compartment of spleen ( $i = 4$ ), mesenteric LNs  
 360 ( $i = 5$ ), or intestine ( $i = 6$ ), and  $j = 1 \dots k$ ,  $d$  as the rate of removal of lymphocytes from circulation  
 361 (due to death or migration to unsampled tissues such as other lymph nodes). Note that in this model  
 362 we assume that cells migrating to the intestine return directly back to circulation without migrating  
 363 via afferent lymph to mLNs. This assumption was justified by the lack of accumulation of labeled  
 364 cells in the mLNs (Figure 6).



**Figure 6:** Experimental data and predictions of a mathematical model on the migration kinetics of *in vivo* activated T lymphocytes in mice. Thymocytes from CBA mice were transferred i.v. into CBA×C57BL/6 mice and activated TDLs were collected 4 to 5 days after cell transfer from the recipient mice. Replicating T cells were labeled with  $^{125}\text{IUdR}$ , adoptively transferred into CBA mice, and the distribution of transferred cells in different murine organs was followed over time (see Sprent [61] for more experimental detail). The percent of labeled lymphocytes recovered from different organs of the recipient mice are shown by markers. We fit the mathematical model of lymphocyte recirculation (eqns. (5)–(8)) to these experimental data; model fits are shown as lines. Plots are shown on the linear (panel A) or a log-scale (panel B). Parameter estimates are given in Table 2.

365 The model was fit to experimental data using least squares. A number of interesting observations  
 366 emerged. First, the model predicted a very short average residence time of activated lymphocytes  
 367 in the blood,  $T \sim 1.2$  min (Table 2). Nearly 65% of activated lymphocytes in the blood migrated to  
 368 the lung and liver where they spent on average 35 min and 2.2 hours, respectively. These residence  
 369 times are substantially higher than those for resting TDLs [49, Figure 4]. It is interesting to note that  
 370 trapping of activated lymphocytes in the liver after the i.v. injection has been observed previously in  
 371 other experiments [22]. The spleen and intestine took another 20% of activated lymphocytes in the  
 372 blood, and the average residence times were 8 and 22 hours in the spleen and intestine, respectively

373 (Table 2). In these experiments very few cells migrated to the mesenteric lymph nodes and we could  
 374 not reliably estimate the residence times of these *in vivo* activated lymphocytes in the mLNs (e.g.,  
 375 note large confidence intervals in Table 2 for this tissue compartment). We also found that about  
 376 15% of transferred cells in the blood per circulation cycle migrated to tissues/organs which have not  
 377 been sampled in these experiments, e.g., skin or other lymph nodes, or these cells have been dying  
 378 at a high rate. Indeed, it is expected that activated lymphocytes undergo programmed cell death  
 379 following clearance of the antigen which has been mimicked by the adoptive transfer experiments.  
 380 In 24 hours post-transfer, only 34% of injected radioactivity could be recovered from recipient mice  
 381 [61, Figure 6 and results not shown]. Nevertheless, this analysis highlights a similar hierarchy of  
 382 migration of naive and activated lymphocytes *in vivo* with the majority of cells entering lung and  
 383 liver vasculature but residing there for a relatively short period of time in these organs as compared  
 384 to other tissues.

| Organ     | Rate of entrance from blood $m_{1i}$ , $\text{h}^{-1}$ | Percent cells going to organ from blood | Rate of exit from organ to blood $m_{i1}$ , $\text{h}^{-1}$ | Residence time in organ, h |
|-----------|--|---|---|----------------------------|
| Lung      | 23.64 (13.3–44.3)                                      | 46.1 (37.1–61.6)                        | 1.7 (1.0–3.6)   | 0.6 (0.3–1.)               |
| Liver     | 8.61 (5.0–12.8)  | 16.8 (12.1–20.6)                        | 0.4 (0.3–0.7)   | 2.2 (1.3–3.3)              |
| Spleen    | 5.4 (2.9–7.7)  | 10.5 (7.3–12.8)                         | 0.2 (0.2–0.4)   | 8.0 (5.7–11.)              |
| MesLN     | 0.7 (0.3–1.6)  | 1.3 (0.6–2.9)                           | 0.1 (0.0–0.8)   | 16.8 (2.5–> 50)            |
| Intestine | 5.5 (2.9–7.3)  | 10.7 (7.2–12.8)                         | 0.1 (0.1–0.1)   | 22.0 (15.4–31.8)           |

**Table 2:** Parameter estimates of the mathematical model that was fitted to the data on migration of *in vivo* activated TDLs in mice [61]. We list i) the rate of TDL entrance into a particular organ from the blood  $m_{1i}$  (second column), ii) the percent of cells leaving the blood into a particular organ ( $m_{1i}/(d + \sum_{i=1}^5 m_{1i})$ , third column), iii) the rate of exit of TDLs from an organ to the blood  $m_{i1}$  (fourth column), and iv) the average residence time of TDLs in the organ (fifth column). The rate of migration of TDLs from the blood to all organs,  $d + \sum_{i=1}^5 m_{1i}$ , is  $51.2 \text{ h}^{-1}$  or the average residence time of cells in the blood is 1.2 min. The average residence time of cells in a particular organ is calculated as  $1/m_{i1}$  (for blood, lung, and liver) and  $2/m_{i1}$  for spleen, mLNs, and intestine. We assume  $k = 2$  subcompartments in these latter organs since this allowed for the best description of the data based on AIC (results not shown). The rate of cell migration from the blood to other organs in the body  $d = 7.50$  ( $3.68 - 10.02$ )  $\text{h}^{-1}$ . No dying cells were assumed to migrate to the liver as including this process did not improve the quality of the model fit to data (not shown). In brackets we show 95% confidence intervals calculated by bootstrapping the residuals with 1000 simulations [88].

## 385 Recirculating and non-recirculating lymphocytes

386 Data and analyses presented so far may create an impression that all (or nearly all) lymphocytes of  
 387 the immune system recirculate. This is not likely to be the case in general. Multiple factors are likely  
 388 to influence ability of lymphocyte to recirculate following adoptive transfer [50, 89]. For example, it  
 389 was noted that handling of lymphocyte *in vitro* at low temperatures dramatically impedes lymphocyte  
 390 recirculation kinetics; passaging of lymphocytes via an intermediate host before final transfer into  
 391 definite hosts restores the ability of lymphocytes to recalculate [89]. Accurate counting of lymphocytes  
 392 entering isolated perfused spleens allowed to conclude that a large fraction of spleen lymphocytes  
 393 never exit the tissue during 7-10 days of perfusion [46]. More recently, experiments involving surgical  
 394 joining of syngenic mice (parabiosis) allowed accurate tracking recirculation kinetics of memory CD8  
 395 T cells in mice [90]. Initial studies showed limited ability of memory T cells in tissues such as brain  
 396 to recirculate between two parabiotic mice [90]. A more thorough follow up revealed an inability  
 397 of LCMV-specific memory CD8 T cells residing in most nonlymphoid tissues to recirculate between

398 parabiotic mice [91]. Interestingly, some of such tissue-resident memory T cells were also found  
399 in secondary lymphoid tissues such as lymph nodes and spleen [92]. Because many of such non-  
400 recirculating T cells reside in peripheral, non-lymphoid tissues, perhaps, it is not totally surprising  
401 that they are not able to recirculate. However, non-recirculating cells could even be found in the  
402 blood, e.g., crawling along sinusoids in the liver [93, 94]. It is important to realize, however, that  
403 many of studies documenting “non-recirculatory” nature of lymphocytes have been performed for a  
404 relatively short-time period and deeper, mathematical modeling-assisted analyses of the data from  
405 such parabiosis experiments are needed in order to accurately quantify the residency time of such  
406 tissue-resident lymphocytes.

## 407 Summary

408 There have been multiple studies documenting lymphocyte migration kinetics via secondary lym-  
409 phoid organs of mice, rats, pigs, and sheep. Many studies involved simple or complex mathematical  
410 models to estimate residence times of lymphocytes in different tissues and in different conditions. A  
411 quick comparison reveals that these estimates while being approximately similar still vary dramat-  
412 ically in absolute values (Table 1). It is unclear at present if such variability in estimates is simply  
413 due to differences in experimental methodologies involved, in mathematical modeling approaches,  
414 or both. Future studies should attempt to determine whether estimates of the residence times are  
415 robust to the choice of mathematical model. It is often expected (and was found in this analysis) that  
416 conclusions arising from models being fitted to data, for example, estimates of model parameters,  
417 can be model-dependent [75]. It is also possible that there may not be such a universal parameter  
418 such as lymphocyte (e.g., naive CD8 T cell) residency time in a LN or spleen. Residency time may  
419 depend on the environment lymphocyte is in (resting vs. inflamed LN), previous history of the  
420 lymphocyte, or other factors. Future studies will have to become more mechanistic and instead of  
421 simply measuring/estimating lymphocyte migration kinetics, should attempt to determine why some  
422 lymphocytes spend 10 hours in tissues while other lymphocytes only 5 hours. Fundamental under-  
423 standing of lymphocyte recirculation kinetics (or absence of thereof for tissue-resident lymphocytes)  
424 should allow to improve therapies that involve lymphocytes such as cancer immunotherapies.

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