blood

2013 122: 2205-2212
Prepublished online August 14, 2013;
doi:10.1182/blood-2013-03-488411

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Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.

Regular Article

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Closing the gap between T-cell life span estimates from stable isotope-labeling studies in mice and humans

Liset Westera,[1] Julia Drylewicz,[1,2] Ineke den Braber,[1] Tendai Mugwagwa,[2] Iris van der Maas,[1] Lydia Kwast,[1] Thomas Volman,[1] Elise H. R. van de Weg-Schrijver,[1] István Bartha,[2-5] Gerrit Spierenburg,[1] Koos Gaiser,[1] Mariëtte T. Ackermans,[6] Becca Asquith,[7] Rob J. de Boer,[2] Kiki Tesselaar,[1] and José A. M. Borghans[1]

[1]Laboratory for Translational Immunology, University Medical Center Utrecht, Utrecht, The Netherlands; [2]Theoretical Biology & Bioinformatics, Department of Biology, Utrecht University, Utrecht, The Netherlands; [3]Institute of Biology, Eötvös Loránd University, Budapest, Hungary; [4]School of Life Sciences, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland; [5]Institute of Microbiology, University Hospital and University of Lausanne, Lausanne, Switzerland; [6]Department of Clinical Chemistry, Laboratory of Endocrinology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands; and [7]Department of Immunology, Imperial College, London, United Kingdom

Key Points

- Life span estimates can be sensitive to the duration of stable isotope label administration, explaining discrepancies in the literature.
- Multiexponential models are needed to obtain reliable leukocyte life span estimates.

Quantitative knowledge of the turnover of different leukocyte populations is a key to our understanding of immune function in health and disease. Much progress has been made thanks to the introduction of stable isotope labeling, the state-of-the-art technique for in vivo quantification of cellular life spans. Yet, even leukocyte life span estimates on the basis of stable isotope labeling can vary up to 10-fold among laboratories. We investigated whether these differences could be the result of variances in the length of the labeling period among studies. To this end, we performed deuterated water-labeling experiments in mice, in which only the length of label administration was varied. The resulting life span estimates were indeed dependent on the length of the labeling period when the data were analyzed using a commonly used single-exponential model. We show that multiexponential models provide the necessary tool to obtain life span estimates that are independent of the length of the labeling period. Use of a multiexponential model enabled us to reduce the gap between human T-cell life span estimates from 2 previously published labeling studies. This provides an important step toward unambiguous understanding of leukocyte turnover in health and disease. (*Blood*. 2013;122(13):2205-2212)

Introduction

Quantitative insights into leukocyte turnover is vital to a better understanding of immune function in health and disease.[1,2] These insights help understand the pathogenesis and treatment of clinical conditions of leukocyte depletion, such as HIV infection,[3] bone marrow transplantation, or chemotherapy; and leukocyte excess, including leukemia.[4] In vivo stable isotope labeling with deuterated (heavy) water (^2H_2O) or deuterated glucose is one of the most reliable ways to measure leukocyte turnover, because deuterium labeling can be safely applied in humans and does not interfere with cell turnover at the doses used.[2,5] Nevertheless, T-cell life span estimates can differ up to 10-fold among stable isotope-labeling studies.[2] The cause of this discrepancy has yet to be elucidated.

A meta-analysis of different stable isotope-labeling studies revealed a positive correlation between the estimated T-cell life span and the duration of label administration.[2] Studies based on deuterated glucose, which is typically administered for shorter periods than ^2H_2O, have consistently yielded shorter average life spans than studies based on heavy water.[2] Although it cannot be excluded that a difference between the ^2H-labeled compounds may have an influence,[1] the correlation between the expected life span and the length of the labeling period remained when comparing life span estimates derived from glucose labeling or water labeling separately.[2] This finding suggests that at least some of the discrepancy between estimated T-cell life spans from stable isotope-labeling studies is attributable to the duration of label administration.

Mathematical modeling is essential for the interpretation of stable isotope-labeling data. Typically, these models are differential equations, which are based on the assumption that cellular events such as division and death are distributed exponentially. Depending on the complexity of the population structure of the model, its solution involves 1 to several exponentials. Therefore, we refer to these models as exponential models. A major step forward was made by Asquith et al,[6] who argued that if a cell population is kinetically heterogeneous (ie, a population comprising multiple subpopulations with different turnover rates, or a population in which recently divided cells and quiescent cells have different life expectancies), the rate of label uptake during the labeling period may not be equal to the rate at which label is lost after label cessation, because the kinetics of labeled cells may not be representative of the cell population as a whole. To account for this heterogeneity, a model was

Submitted March 5, 2013; accepted August 2, 2013. Prepublished online as *Blood* First Edition paper, August 14, 2013; DOI 10.1182/blood-2013-03-488411.

L.W., J.D., and I.d.B. contributed equally to this study.

R.J.d.B., K.T., and J.A.M.B. contributed equally to this study.

The online version of this article contains a data supplement.

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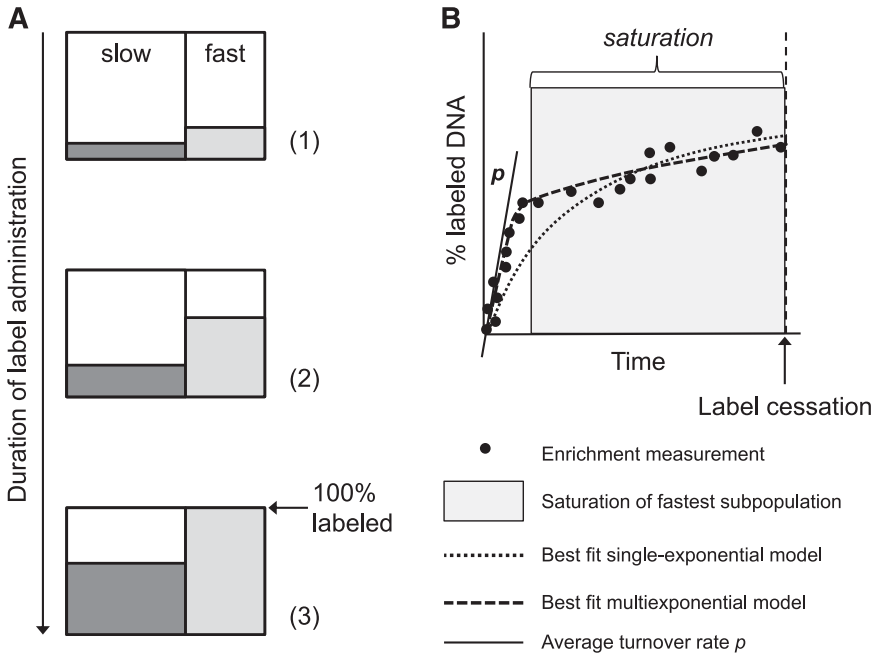

Figure 1. The influence of the length of the labeling period on the estimated turnover rate *p*. Consider an artificial long-term labeling experiment of a kinetically heterogeneous cell population, in which the labeled fractions of a slow (dark gray) and a fast subpopulation (light gray) gradually increase with the duration of label administration (A). During labeling, samples are obtained, and the percentage of labeled DNA is determined at several time points (B, black circles). During the labeling phase, the initial accrual of label (the slope nearby the origin, as indicated by the black tangent line) reflects *p* of the kinetically heterogeneous population (A, situations 1 and 2; B, white area). If labeling is continued, the enrichment level of the fastest subpopulation may start to saturate (A, situation 3). Although cells of the fastest subpopulation continue to divide after this point, this is no longer reflected by a corresponding increase in their enrichment level. If sampling is continued (B, gray area), any further increase in labeled DNA is largely the result of cell production in the slow subpopulation, reflected by a second, flatter slope of the labeling curve (B). If the label enrichment data are fitted using a single-exponential model (dotted black curve), the model seeks a compromise between the initial, steep increase and the later, slower increase of label enrichment. As a result, the model fit is forced to bend downward from the initial slope, and the average turnover rate will become increasingly underestimated with increasing duration of label administration. In contrast, the multiexponential model corrects for this effect by allowing for multiple slopes during the labeling phase (B, dashed black curve), and thereby yields a reliable estimate of the average turnover rate, independent of the length of the labeling period.

proposed that distinguishes 2 parameters: the average turnover rate (p), and the average disappearance rate of labeled cells (d^*). This model is now commonly used[4,7-11] and has stressed the importance of deducing average life spans from p. The rate of d^* has previously been shown to be dependent on the length of the labeling period: the shorter the labeling period, the stronger the bias toward rapidly proliferating cells in the labeled fraction, and hence the faster the loss of label d^*.[6] To date, why also p is higher (and hence the average life span shorter) in short-term compared with longer-term labeling studies[2] has not been experimentally addressed.

Unlike delabeling curves, which may vary according to the length of the labeling period, the shape of the uplabeling curve is independent of the length of the labeling period. It is determined by the weighted average of turnover rates of all subpopulations, and its initial slope should reflect p (Figure 1A). Here, we test the hypothesis that during long-term labeling, the label uptake of cells with fast turnover may start to saturate[7] (Figure 1A). If label administration is continued beyond this point, subsequent label accrual occurs mainly because of cells with relatively slow kinetics. Because single-exponential models cannot capture the saturation behavior and, instead, are forced to make a compromise, p could become increasingly underestimated as the length of the labeling period increases (Figure 1B). This mechanism might explain the positive correlation between the estimated T-cell life span and the length of the labeling period observed in the literature.

Here, we have investigated this hypothesis by performing ^2H_2O labeling experiments in mice in which only the duration of label administration was varied. We confirm that life spans estimated by fitting single-exponential models to stable isotope labeling data are sensitive to the length of the labeling period. When using a multi-exponential model (describing label accrual with more than 1 exponential), which explicitly captures kinetic heterogeneity within a cell population[12] (Figure 1B), we found that life span estimates became independent of the duration of label administration. By labeling mice in utero to have all leukocytes of newborn mice equally labeled, we confirmed that the life span estimates that were obtained with the multiexponential model were reliable. Application of the model to published human deuterium-labeling data reduced the difference between life span estimates on the basis of glucose and ^2H_2O labeling studies.[10,13] Both our findings and approach present a major step toward consensus on how long different types of leukocytes live in health and disease.

Methods

Mice

C57Bl/6 mice were maintained by in-house breeding at the Central Animal Facility at Utrecht University, Utrecht, The Netherlands, in accordance with institutional and national guidelines.

^2H_2O labeling

For finite-term labeling experiments, ~12-week-old male mice were given a boost intraperitoneal injection of 15 mL/kg of ^2H_2O (99.8%; Cambridge Isotopes) in phosphate-buffered saline and received 4% ^2H_2O for 1, 4, or 8 weeks. For prenatal labeling experiments, to obtain mice in which all cells were labeled to the same extent, female mice were given a boost injection

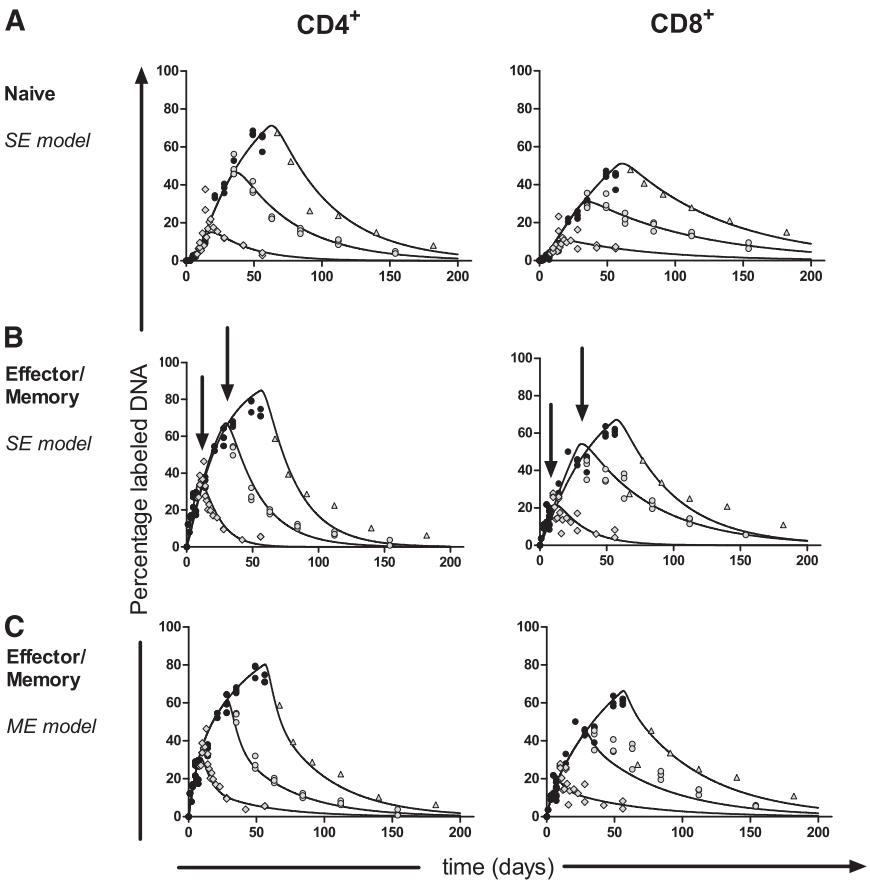

Figure 2. Best fits of the single-exponential model and the multiexponential model to labeling experiments of different duration. At different time points during and after labeling, the percentage of labeled DNA of splenic (A) naive and (B-C) effector/memory (E/M) $CD4^+$ and $CD8^+$ T cells was determined. Dots represent measurements (ie, individual mice) at different time points during labeling for 1, 4, and 8 weeks (●, black circles), and during delabeling after 1 week of labeling (◇, gray diamonds), 4 weeks of labeling (○, gray circles), or 8 weeks of labeling (△, gray triangles). (A-B) Data were fitted separately for each labeling period using the single-exponential (SE) model to estimate p of the cells and d^* of the labeled cells for the corresponding labeling period. For naive T cells, a delay was added in the model as described in supplemental Materials equations 9 and 10 and was estimated to be 4 days (95% CI, 2-6 days). For effector/memory T cells (B), the best-fitting curves during the labeling period were not identical for the different labeling periods (indicated by arrows). (C) When the data were fitted separately for each labeling period using the multiexponential (ME) model (describing 2 kinetically different subpopulations; the addition of more subpopulations did not change the average turnover rate), the best-fitting curves during the labeling period were almost identical. Label enrichment was corrected for ^2H_2O enrichment in plasma (supplemental Figure 1A) and was scaled between 0% and 100% by normalizing for the maximal percentage of labeled DNA as measured in thymocytes (supplemental Figure 1B).

of 15 mL/kg of 99.8% ^2H_2O in phosphate-buffered saline. They were placed together with male mice and drank 4% ^2H_2O until they gave birth. After weaning, male pups received 4% ^2H_2O until age 16 weeks.

The 9-week labeling data in humans have been published previously,[10] and the enrichment in total $CD4^+$ T cells was derived from naive and memory T cells as described in supplemental Materials (available on the *Blood* Web site).

Cell preparation and sorting

Spleens were isolated at different time points for further sorting. Thymocytes were isolated as a cell population with rapid turnover to determine the maximal level of label enrichment. Blood was collected in EDTA vials and was spun down to isolate plasma. Single-cell suspensions were obtained as described previously.[10] Splenocytes were stained with CD62L-FITC, CD44-eFluor450 (eBioScience, San Diego, CA), CD4-APC-H7, and CD8-PerCP (BD PharMingen, San Jose, CA) in the presence of a 2.4G2-blocking antibody. Within $CD4^+CD8^-$ and $CD4^-CD8^+$ splenocytes, naive T cells were defined as $CD62L^+CD44^-$ and effector/memory T cells as $CD44^+$. Cells were sorted using a FACSAria cell sorter and FACSdiva software (BD PharMingen). Genomic DNA was isolated according to the manufacturer's instructions (Nucleospin Blood QuickPure; Macherey-Nagel, Duren, Germany).

Measurement of deuterium enrichment in DNA and body water

Deuterium enrichment in DNA was measured according to the method described by Neese et al[14] with minor modifications, as described previously.[10] Both natural enrichment and concentration dependence (abundance sensitivity) were controlled for, using a naturally enriched background sample or standards of known isotopic enrichments. To determine deuterium enrichment in body water, plasma samples were measured using gas chromatography-mass spectrometry.[15,16] Mathematical modeling is described in supplemental Materials.

Statistical analysis

Analysis-of-variance tests were performed to compare the different estimates using Prism 5 (GraphPad). Nested mathematical models were compared using an F test. Differences with a P value of $< .05$ were considered significant.

Results

Life span estimates can be influenced by the duration of label administration

The observed correlation in the literature between average life span estimates and the duration of label administration[2] prompted us to investigate whether we could reproduce this correlation within a single experiment, in which only the duration of label administration was varied. Twelve-week-old C57Bl/6 mice were given a bolus of ^2H_2O and subsequently 4% ^2H_2O in the drinking water for 1, 4, or 8 weeks. Splenic effector/memory ($CD44^+$) T cells and naive ($CD62L^+CD44^-$) T cells were isolated at different time points during ^2H_2O administration (labeling phase), and after ^2H_2O administration (delabeling phase), and deuterium enrichment in the DNA was measured. Labeling curves for naive (Figure 2A) and effector/memory (Figure 2B) $CD4^+$ and $CD8^+$ T-cell subsets of the 1-week, 4-week, and 8-week labeling experiments were fitted separately with the single-exponential model proposed for interpreting deuterated glucose experiments,[6] which we have previously adapted for use with ^2H_2O labeling[10] (supplemental Materials).

For naive $CD4^+$ and $CD8^+$ T cells, d^* (Figure 3A) was somewhat higher than the estimated p (Figure 3B), especially in the 1-week

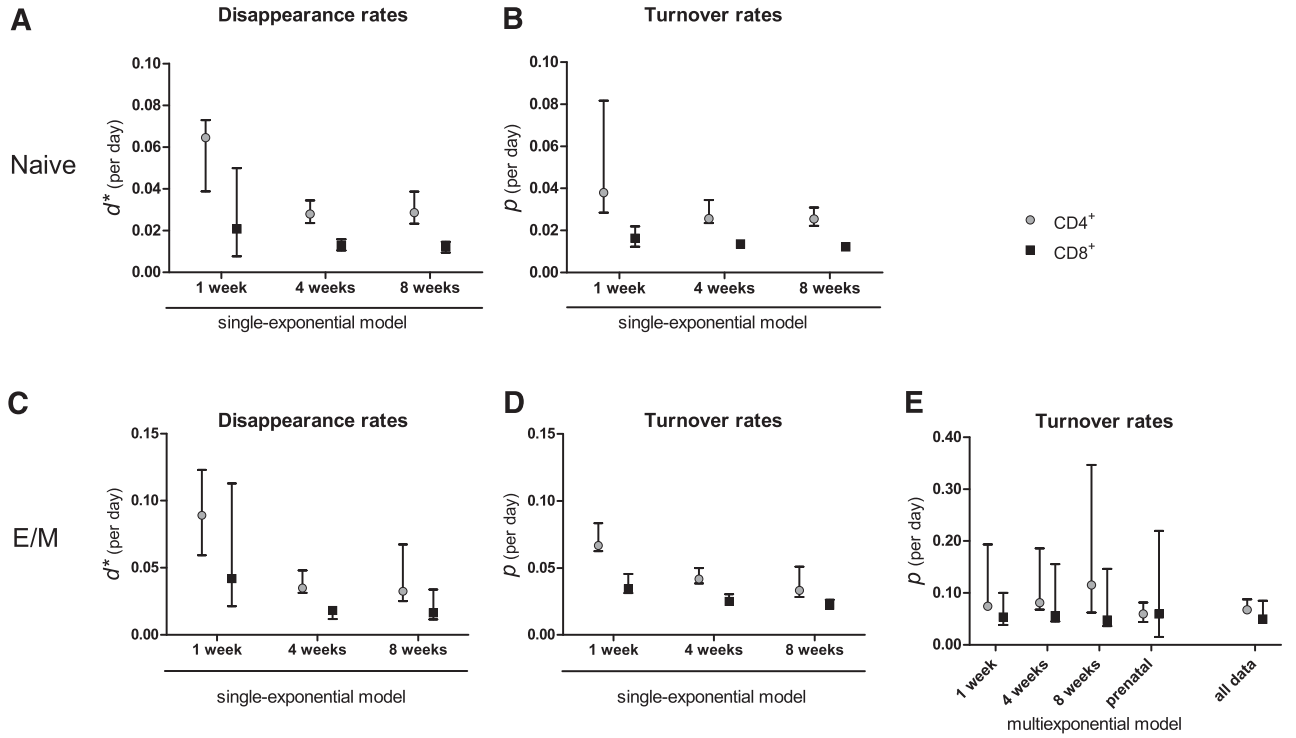

Figure 3. Summary of parameter estimates. Estimates of d^* (A,C) and the average turnover rate p (B,D) of naive (A-B) and effector/memory (C-D) $CD4^+$ (○, gray circles) and $CD8^+$ (■, black squares) T cells, obtained by fitting the single-exponential model to the data collected during 1, 4, or 8 weeks of labeling. (E) Estimates of the average turnover rate of $CD4^+$ (○, gray circles) and $CD8^+$ (■, black squares) effector/memory T cells obtained by fitting a multiexponential model (describing 2 subpopulations) to the individual data sets (labeling for 1, 4, or 8 weeks; or prenatal labeling) and simultaneously to all data combined. Bars with whiskers represent the 95% CIs of the estimates obtained by bootstrapping the residuals.

labeling experiment, and tended to decrease with the length of the labeling period, although not significantly ($P = .07$ for $CD4^+$ and $P = .19$ for $CD8^+$; Figure 3A). The estimated p (and hence the average life span) of naive T cells was similar for the 3 durations of label administration ($P = .30$ for $CD4^+$ and $P = .41$ for $CD8^+$; Figure 3B). For $CD4^+$ and $CD8^+$ effector/memory T cells, d^* decreased as the length of the labeling period increased ($P < 10^{-4}$; Figure 3C), and unlike what was observed for naive T cells, even the estimated average turnover rate p decreased significantly as the duration of label administration increased ($P < 10^{-4}$; Figure 3D). Hence, within a single experiment, we reproduced the previously observed correlation in the literature between the duration of label administration and the estimated average life span.[2]

Inspection of the best fits to the data shows that the discrepancy in the estimated average turnover rate of effector/memory T cells is caused by the model that was fitted to the data. Although the curves of the 3 labeling experiments should be identical during labeling (as we observed for naive T cells, Figure 2A), the separate fits to the 1-, 4-, and 8-week labeling data of effector/memory T cells differed during labeling (Figure 2B, indicated by arrows). Apparently, the model could not capture saturation of the fastest cells in the effector/memory pool and thereby underestimated the average turnover rate during long-term labeling (Figure 1).

Multiexponential models correct for the influence of the length of the labeling period

Because the correlation between p and the duration of label administration was most evident for the $CD4^+$ and $CD8^+$ effector/memory T-cell pools, we used the labeling data from those cell populations to investigate how life spans can be determined reliably when cell populations are kinetically heterogeneous. We propose using a multiexponential model that explicitly accounts for kinetic heterogeneity,[12] by describing multiple subpopulations each with their own production and disappearance rate. Each subpopulation is assumed to be in equilibrium, that is, its production equals loss (supplemental Materials). In contrast to the single-exponential models that are typically used, a multiexponential model describes both the labeling and the delabeling phase by a multiexponential function.[12] Because a very similar model is obtained in populations with temporal heterogeneity (eg, consisting of quiescent and activated cells),[17] we here use the multiexponential model generally to account for heterogeneity in the population. Although the number of kinetically different subpopulations within a cell population may not be known, one can increase the number of subpopulations in the model until the estimated average turnover rate no longer markedly changes, provided sufficient data are available (supplemental Materials).

To test whether a multiexponential model would correct for the influence of the length of label administration on the estimated average turnover rate, we fitted the individual labeling curves of T cells from the 1-, 4-, and 8-week labeling experiments separately using a multiexponential model. Because the multiexponential model did not improve the fits of the naive T-cell data for any duration of label administration (not shown) but did influence the average life span estimates of effector/memory T cells, we decided to focus on the latter. The labeling data from the $CD4^+$ and $CD8^+$effector/memory T-cell pools were well described by a model describing 2 kinetically different subpopulations (the addition of more subpopulations did not change the average turnover rate). In contrast to the single-exponential model (Figure 2B), the multiexponential model described the 1-, 4-, and 8-week labeling data with largely overlapping curves during labeling (Figure 2C) and thus yielded 3

similar turnover rates ($P = .08$ for $CD4^+$ and $P = .60$ for $CD8^+$) that were independent of the length of the labeling period (Figure 3E). As expected, the estimates obtained by the single- and multiexponential model differed most for the longer labeling periods, and the multiexponential model gave significantly better fits to the data than the single-exponential model (8 weeks of labeling: $P < .0001$ for $CD4^+$ and $P = .0028$ for $CD8^+$; 4 weeks of labeling: $P < .0001$ for $CD4^+$). For the 1-week data, both models behaved similarly.

It should be noted that the 4-week labeling data of $CD8^+$ effector/memory T cells were not fitted well during the delabeling phase, and that the multiexponential model did not describe the 4-week labeling data significantly better than the single-exponential model ($P = .08$). Importantly, our estimates of the average turnover rate of $CD8^+$ effector/memory T cells do not depend on the 4-week labeling data, as using only 1-week and 8-week labeling data (separately or combined) yielded consistent estimates.

Hence, the correlation of published turnover rates with the length of the labeling period may be the result of kinetic heterogeneity that was not fully accounted for by the models that were used to fit the data. Mathematical models that explicitly capture such kinetic heterogeneity yield average turnover rates that do not depend on the duration of label administration.

Prenatal labeling experiments yield similar life span estimates

Next, we sought to obtain independent confirmation of the turnover rates that we estimated when fitting the multiexponential model to the 1-, 4-, and 8-week labeling data. Because the main difficulty in the interpretation of "finite-term" labeling experiments is caused by the difference between cells that are and are not labeled during the experiment, we designed a labeling experiment in which, at cessation of label administration, all cells were labeled. Female mice received a bolus of ^2H_2O and were subsequently fed with 4% ^2H_2O in the drinking water before conception and throughout pregnancy. Female mice thus gave birth to pups that had been labeled in utero (referred to as "prenatal labeling") and in which all cells were equally labeled. Pups received ^2H_2O until age 16 weeks, after which ^2H_2O was withdrawn from the drinking water. They were euthanized at different time points after labeling to measure the loss of deuterium enrichment in the DNA of their T cells. The resulting delabeling curves were used to deduce d, which, in this case, reflects the cell population as a whole and can directly be interpreted as the average turnover rate.

In line with the finite-term labeling experiments, fitting the multiexponential model (describing 2 kinetically different subpopulations; the addition of more subpopulations did not change the average turnover rate, Figure 4A) to the effector/memory delabeling data gave a significantly better description of the data ($P < .01$) than the single-exponential model (not shown). Fitting the prenatal data yielded average turnover rates for effector/memory $CD4^+$ and $CD8^+$ T cells that were similar to the estimates obtained in the 1-, 4-, and 8-week labeling experiments ($P = .68$ for $CD4^+$ and $P = .54$ for $CD8^+$; Figure 3E). Indeed, we found that delabeling of a fully labeled population behaved similarly to labeling of an unlabeled population. For all cell subsets analyzed, both the average turnover rate and the number of exponentials required to describe the data were similar for the finite-term labeling data and the corresponding prenatal labeling data. This independent experiment confirmed the turnover estimates obtained by fitting the multiexponential model to the finite-term labeling data above.

Simultaneously fitting the model to the complete data set we had collected (ie, the 1-, 4-, and 8-week labeling experiments and the prenatal labeling experiments together, Figure 4B-C) revealed that mouse $CD4^+$ and $CD8^+$ effector/memory T cells have average turnover rates of 0.068 (95% confidence interval [CI], 0.065-0.088) and 0.050 (95% CI, 0.045-0.085) per day (Figure 3E), corresponding to average life spans of 15 days (95% CI, 11-15 days) for $CD4^+$, and 20 days (95% CI, 12-22 days) for $CD8^+$ effector/memory T cells (Table 1).

The multiexponential model reduces the difference between human T-cell life span estimates

Next, we applied the multiexponential model to previously published human ^2H_2O labeling data[10] to obtain human T-cell life span estimates that are independent of the length of the labeling period. For each individual labeled with ^2H_2O for 9 weeks, the multiexponential model (describing 2 kinetically different subpopulations; the addition of more subpopulations did not change the average turnover rate) fitted the memory $CD4^+$ and $CD8^+$ T-cell data significantly better ($P < .001$) and, in general, yielded higher average turnover rates p (and therefore shorter average life spans) than the single-exponential model (Figure 5A; supplemental Figure 4). Hence, single-exponential models have overestimated the average life span of memory T cells in long-term labeling studies. According to the multiexponential model, human memory $CD4^+$ and $CD8^+$ T cells have a median p of 0.0061 (range, 0.0020-0.0141) and 0.0064 (range, 0.0043-0.089) per day, corresponding to median life spans of 164 days (range,71-500 days) for $CD4^+$ and 157 days (range, 113-231 days) for $CD8^+$ memory T cells.

Finally, we investigated whether the multiexponential model could resolve the discrepancy between previously published human stable isotope-labeling studies. We confined our analysis to 2 data sets that clearly differed in the length of the labeling period, and had a sufficient number of data points: a 1-week deuterated glucose-labeling experiment[13] and a 9-week ^2H_2O-labeling experiment.[10] Because the glucose-labeling experiment reported deuterium enrichment in total $CD4^+$ T cells, and the ^2H_2O-labeling experiment distinguished between naive and memory T cells, we first recalculated the corresponding levels of deuterium enrichment in total $CD4^+$ T cells for the 9-week ^2H_2O-labeling experiment (supplemental Materials). When we fitted a single-exponential model to the data, the life spans estimated from the 9-week labeling experiment were longer than the life spans based on the 1-week labeling experiment (Figure 5B; individual fits in supplemental Figure 5), in line with the positive correlation between estimated life spans and the duration of label administration in the literature.[2] The multiexponential model reduced the estimated life spans from the 9-week labeling experiment and hence reduced the differences between the studies (Figure 5B). Although the discrepancies between the 2 studies were not entirely resolved, largely because of a single outlier in the ^2H_2O study whose memory turnover was much lower than in the other individuals[10] (supplemental Figure 4D), correction of the length-of-labeling effect revealed that the current best estimate of the average life span of $CD4^+$ T cells in healthy human adults (based on the median values of both studies) lies between 270 and 469 days.

Discussion

The quantification of leukocyte life spans from stable isotope-labeling data relies on the use of mathematical models. The finite-term

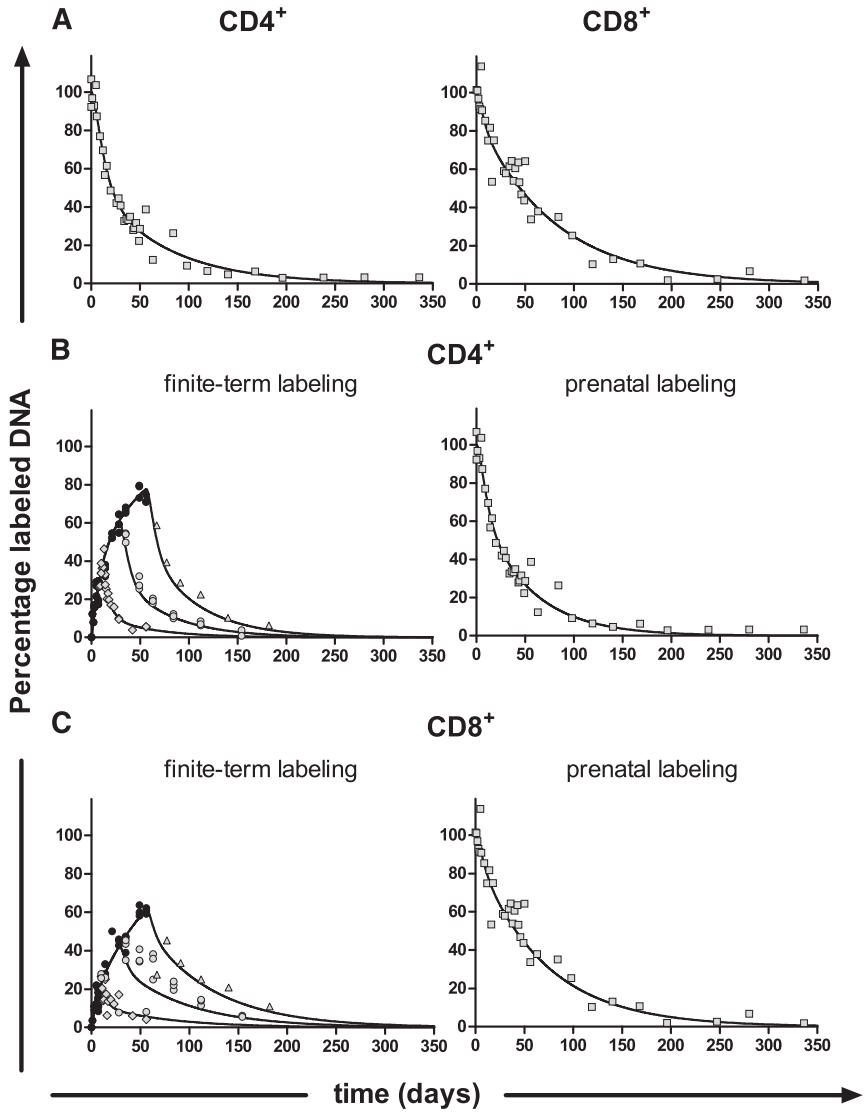

Figure 4. Best fits of the multiexponential model to effector/memory T-cell data from prenatal and finite-term labeling experiments. (A) Mice were labeled prenatally and drank ^2H_2O until age 16 weeks. At different time points after label cessation, the percentage of labeled DNA of splenic effector/memory CD4$^+$ (left) and CD8$^+$ T cells (right) was determined. Gray squares (□) represent measurements (ie, individual mice) at different time points after labeling. Data were fitted with the multiexponential model (describing 2 subpopulations) to estimate the average turnover rate of the total cell population. (B-C) Effector/memory CD4$^+$ (B) and CD8$^+$ (C) T-cell labeling data from the finite-term (left) and prenatal labeling (right) experiments were simultaneously fitted with the multiexponential model to estimate the average turnover rate. Black circles (●) represent measurements (ie, individual mice) at different time points during labeling; during delabeling after 1 week of labeling (◇, gray diamonds), 4 weeks of labeling (○, gray circles), or 8 weeks of labeling (△, gray triangles); and during delabeling of prenatally labeled mice (□, gray squares).

labeling experiments that we performed in mice show that single-exponential models fail to correctly describe the dynamics of kinetically heterogeneous cell populations and thereby yield average life span estimates that depend on the duration of label administration. Longer labeling periods gave rise to longer estimated average life spans, confirming the correlation observed in the literature.[2] These analyses suggest that a considerable part of the discrepancy in published T-cell life spans arises from differences in the duration of label administration. Here, we show that the use of a multiexponential model resolves the dependence on the length of the labeling period and thereby yields reliable turnover parameters.

Table 1. Life span estimates of CD4$^+$ and CD8$^+$ effector/memory T cells from different labeling experiments

	Life span, days (95% CIs)	
	CD4$^+$effector/memory T cells	CD8$^+$ effector/memory T cells
No. of weeks		
1	13.46 (5.17-15.02)	18.68 (10.00-26.30)
4	12.30 (5.38-14.75)	17.82 (6.43-22.06)
8	8.67 (2.88-16.05)	20.94 (6.82-27.56)
Prenatal	16.85 (12.29-22.64)	16.82 (4.55-66.53)
All data	14.78 (11.39-15.39)	20.14 (11.73-22.04)

It has been proposed before that a multiexponential model describing all subpopulations would be the ideal way to model kinetically heterogeneous cell populations, but that this would lead to models with too many parameters.[6] The mathematical model proposed by Asquith et al[6] was a pragmatic solution that captured kinetic heterogeneity by allowing p to be different from d*. However, the fact that single-exponential models fail to describe labeling curves that are identical during labeling but different after label cessation stresses the need for a multiexponential model to obtain reliable estimates of turnover rates, particularly when the labeling period is long.

Fitting multiexponential models not only reveals the average life span of a cell population but also reveals quantitative insights into the sizes α_i and turnover rates p_i of its subpopulations. The uncertainty on the latter parameters is, however, generally much larger than on p, because of the strong correlation between the size of a subpopulation and its turnover rate (supplemental Figure 6). Therefore, the biological interpretation of the parameters describing the kinetically different subpopulations used in the model is not straightforward. It is important to realize that the number of kinetically different subpopulations that is sufficient to describe the data may, in fact, be lower than the actual number of subpopulations, and that the subpopulations need not even reflect phenotypically different subsets. Moreover, if the use of a multiexponential model

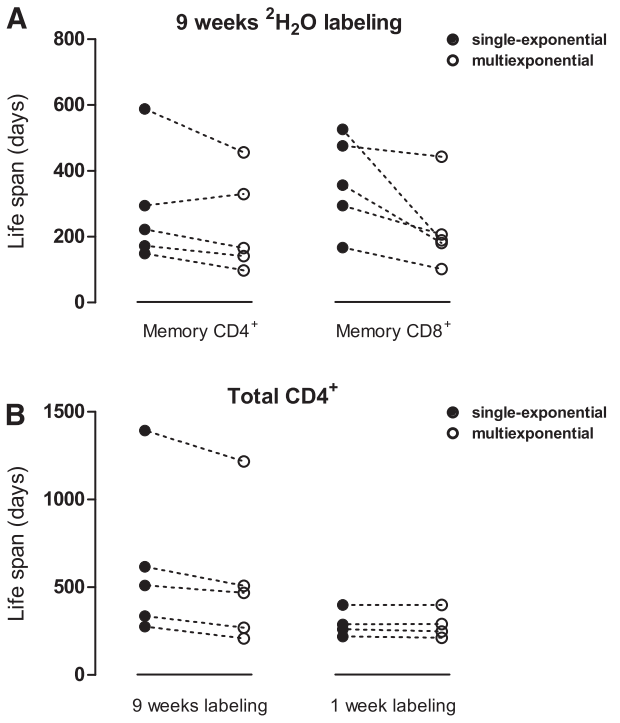

Figure 5. Average T-cell life spans re-estimated from published labeling experiments in humans. Data from 2 published experiments were used: 9-week ^2H_2O labeling in 5 healthy individuals,[10] and 1-week deuterated glucose labeling in 4 healthy individuals.[13] For each individual, the life span of the indicated cell population was estimated using a single-exponential model (●, closed circles) or a multiexponential model (○, open circles, describing 2 kinetically different subpopulations; the addition of more subpopulations did not change the average turnover rate). Estimates obtained for a single individual using the 2 models are connected by a dashed line (---). (A-B) For each individual in the 9-week ^2H_2O-labeling experiment, the average life span of $CD4^+$ and $CD8^+$ memory T cells was estimated using both models. Individual fits of the multiexponential model to the memory T-cell data are shown in supplemental Figure 4. (B) The average life span of total $CD4^+$ T cells was re-estimated by fitting the single-exponential model (●, closed circles) or the multiexponential model (○, open circles) to the deuterium-labeling data of individuals labeled with deuterated glucose for 1 week,[13] or ^2H_2O for 9 weeks.[10] For the 9-week labeling experiment, individual fits of the multiexponential model are shown in supplemental Figure 5. For the 1-week glucose-labeling data, both models included a 1-day delay.[17,22]

significantly improves the fit to the data, an alternative interpretation is that cells transiently have different turnover rates (ie, that there is so-called temporal heterogeneity). For example, resting cells and cells that have recently been produced or activated may have different life expectancies, an illustrative example being activation-induced cell death.[7,17]

Our analyses demonstrate that the use of single-exponential models may lead to overestimation of the average life span of kinetically heterogeneous cell populations, especially in long-term labeling studies. This problem can be overcome by implementing a multiexponential model. Hence, both short-term and long-term labeling can be considered, and the decision should be based on the population of interest (slow or fast turnover) and on practical and/or ethical considerations. Although short-term labeling studies are less prone to underestimate cellular turnover rates, long-term labeling can also have advantages. First, longer labeling periods allow more frequent sampling during the labeling phase, which is the essential phase to estimate average turnover rates. It also allows better spreading of blood withdrawals with time, keeping the burden of blood sampling for patients relatively low. Second, prolonged exposure to labeling allows even cells with relatively slow turnover rates, such as naive T cells, to become sufficiently labeled to reliably estimate their turnover. Third, longer labeling gives recently produced, and hence labeled, cells ample time to appear in the blood, where most measurements are generally taken. Naturally, the shorter the labeling period, the less saturation of subpopulations is expected to occur, and the smaller the requirement for a multiexponential model. However, even during short-term labeling, saturation may already be present. Therefore, it may be good to always use the multiexponential model, both for short-term and long-term labeling periods. As long as label accrual reflects the average population turnover (before any signs of saturation), the multiexponential model will behave like a single-exponential model, and the fitting procedure will set the contribution of the extra exponential(s) to zero. It will hence yield the same average turnover rate.

Remarkably, 2 earlier studies have reported longer life span estimates for murine memory $CD8^+$ T cells than the 20-day average that we found. Labeling experiments with 5-bromo-2'-deoxyuridine in thymectomized mice revealed a $CD8^+$ effector/memory T-cell half-life of 63 days (corresponding to a life span of 91 days),[18] and later Choo et al[19] showed that adoptively transferred lymphocytic choriomeningitis virus–specific memory $CD8^+$ T cells and bulk $CD44^{hi}$ T cells had an intermitotic time of ~50 days. A major advantage of stable-isotope labeling is that one can study cell turnover under physiological circumstances, without affecting immune homeostasis. This could be different for the (more manipulative) approaches used in the earlier studies. More in agreement with our results is a study by Younes et al,[20] who proposed that the $CD4^+$ memory pool is heterogeneous, comprising both slowly dividing "authentic" antigen-experienced memory cells as well as rapidly dividing "memory-phenotype" cells that arise from an antigen-independent mechanism. The level of 5-bromo-2′-deoxyuridine incorporation that they measured in $CD4^+$ ($CD44^{hi}$) memory-phenotype cells corresponds to a $CD4^+$ memory T-cell life span of 14 to 22 days.[21] This estimate is very similar to our $CD4^+$ memory T-cell life span estimate of 15 days.

In humans, the current best estimates are that memory $CD4^+$ and $CD8^+$ T cells live 164 and 157 days, respectively. Again, these estimates are considerably shorter than our previous estimates of 222 and 357 days for memory $CD4^+$ and $CD8^+$ T cells, respectively.[10] Thus, discrepancies in the literature on human T-cell life span estimates[10,13] may have, to a large extent, been caused by the use of single-exponential models, which led to overestimation of T-cell life spans in long-term labeling studies. We cannot exclude that other, yet unidentified factors may cause differences in life span estimates, such as an intrinsic difference between ^2H_2O and deuterated glucose, which may underlie the remaining differences between the estimated life spans of the 1-week deuterated glucose experiment and the 9-week ^2H_2O experiment (Figure 5B). The part of the variation that is due to the length of the labeling period has at least been resolved thanks to the use of a multiexponential model.

Acknowledgments

The authors thank David Ho and Hiroshi Mohri for sharing their published data, and Linda McPhee for comments on the manuscript.

This research was supported by the Landsteiner Foundation for Blood Transfusion Research (grant 0210); the Netherlands Organisation for Scientific Research grants 927.50.029, 917.96.350, and 016.096.350; and visitor's grant 040.11.128); the Virgo Consortium (Netherlands Genomics Initiative, BB.000342.1); and the Research Council for Earth and Life Sciences, with financial aid

from the Netherlands Organisation for Scientific Research (grant 836.07.002).

Authorship

Contribution: L.W., J.D., B.A., R.J.d.B., K.T., and J.A.M.B. wrote the manuscript; L.W., I.d.B., and K.T. designed the experiments; L.W., I.d.B., L.K., T.V., E.H.R.v.d.W.-S., G.S., K.G., and M.T.A. performed the experiments; and J.D., T.M., I.v.d.M., I.B., R.J.d.B., and J.A.M.B. performed mathematical modeling.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: J.A.M. Borghans, Laboratory for Translational Immunology, University Medical Center Utrecht, WKZ Room KC 02.085.2, PO Box 85090, 3508 AB Utrecht, The Netherlands; e-mail: J.Borghans@umcutrecht.nl.

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Supplemental Materials

Closing the gap between T-cell life span estimates from stable isotope-labeling studies in mice and men

Liset Westera*, Julia Drylewicz*, Ineke den Braber[*], Tendai Mugwagwa, Iris van der Maas, Lydia Kwast, Thomas Volman, Elise HR van de Weg-Schrijver, István Bartha, Gerrit Spierenburg, Koos Gaiser, Mariëtte T Ackermans, Becca Asquith, Rob J de Boer[#], Kiki Tesselaar[#], José AM Borghans[#]

Calculation of enrichment in total $CD4^+$ T cells

To be able to compare data from a previously published 9-week heavy water ^2H_2O labeling experiment, which distinguished naive ($CD27^+CD45RO^-$) from memory ($CD45RO^+$) T cells[1], and a 1-week deuterated glucose labeling experiment on total $CD4^+$ T cells[2] (data kindly provided by David Ho and Hiroshi Mohri), we calculated the corresponding enrichment level of total $CD4^+$ T cells from the heavy water ^2H_2O labeling experiment using the relative sizes and the enrichment levels of the naive and memory $CD4^+$ T-cell subsets[1]. This could be done for $CD4^+$ T cells because the fraction of $CD27^-CD45RO^-$ cells is negligible in the $CD4^+$ T-cell pool, but not for $CD8^+$ T cells. The enrichment levels of memory and total $CD4^+$ T cells were fitted with a single-exponential[3] and a multiexponential model describing two kinetically different subpopulations (the addition of more subpopulations did not change the average turnover rate).

Mathematical modeling of deuterium labeling data. Following Vrisekoop et al.[1], the availability of heavy water at any moment in time was calculated by fitting the following equations to the deuterium enrichment in the plasma:

(i) For finite-term labeling experiments:

$$S(t) = f(1 - e^{-\delta t}) + S_0 e^{-\delta t} \quad \text{during label intake } (t \le \tau), \qquad [1]$$

$$S(t) = \left[f(1 - e^{-\delta \tau}) + S_0 e^{-\delta \tau} \right] e^{-\delta (t - \tau)} \quad \text{after label intake } (t > \tau). \qquad [2]$$

(ii) For prenatal labeling experiments: $S(t) = \beta e^{-\delta t}$. [3]

In these equations, *S(t)* represents the fraction of ^2H_2O in plasma at time *t* (in days), *f* is the fraction of deuterium in the drinking water, labeling was stopped at $t = \tau$ days in finite-term labeling experiments, and at t=0 days in the prenatal labeling experiments, δ represents the turnover rate of body water per day, S_0 is the plasma enrichment level attained after the i.p. ^2H_2O boost at day 0 of finite-term labeling experiments, and β the plasma enrichment level at the start of the de-labeling period in the prenatal labeling experiments. The best fits to the plasma data are shown in Fig. S1A and Fig. S2.

To model the label enrichment of adenosine in the DNA of cells, we used both single-exponential and multiexponential models. In the single-exponential model[3], cells have an average turnover rate *p* (meaning that a fraction *p* of cells is renewed per day; life spans are obtained by inverting *p*), and labeled cells are lost at a rate *d** per day. We previously extended this model to include the dependence on the actual enrichment of the body water (as described by *S(t)*)[1]. We also introduced a parameter *c* which accounts for the fact that the adenosine deoxyribose (dR) moiety contains multiple hydrogen atoms that can be replaced by deuterium. Basically one writes that each adenosine residue replicates at rate *p* per day and will incorporate a deuterium atom with probability *cS(t)*. The fraction of labeled DNA (*L*) at any given time is hence given by:

$$\frac{dL}{dt} = pcS(t) - d^* L. \qquad [4]$$

To determine the maximum level of label incorporation that could possibly be attained in finite-term labeling experiments, we fitted this model to labeling data from mouse thymocytes (Fig. S1B), which are known to have a high rate of turnover[4, 5]. Turnover parameters for the different T-cell subsets were determined as described before [1]. Because we observed a lag in the appearance of labeled naive T cells in the spleen after start of (finite-term) labeling, suggesting that cells had divided in the thymus and then migrated to the spleen, we allowed for a time delay Δ with which labeled cells appear in the spleen. When fitting naive T-cell data, Δ was treated as a free parameter, while it was fixed to Δ=0 when fitting effector/memory T-cell data. The corresponding equations are:

$$\text{if } t \le \Delta: \; L(t) \; = \; 0, \qquad [5]$$

if $\Delta < t \le \tau + \Delta$:

$$L(t) \; = \; \frac{cpf}{\delta - d^*}\left[\frac{\delta}{d^*}(1 - \mathrm{e}^{(-d^*(t-\Delta))}) - (1 - \mathrm{e}^{-\delta(t-\Delta)}) + \frac{S_0}{f}(\mathrm{e}^{-d^*(t-\Delta)} - \mathrm{e}^{-\delta(t-\Delta)})\right], \qquad [6]$$

if $t > \tau + \Delta$:

$$L(t) \; = \; \frac{cpf}{\delta - d^*}\left[\frac{\delta}{d^*}(\mathrm{e}^{-d^*(t-\Delta-\tau)} - \mathrm{e}^{-d^*(t-\Delta)}) - (\mathrm{e}^{-\delta(t-\Delta-\tau)} - \mathrm{e}^{-\delta(t-\Delta)}) + \frac{S_0}{f}(\mathrm{e}^{-d^*(t-\Delta)} - \mathrm{e}^{-\delta(t-\Delta)})\right]. \qquad [7]$$

Alternatively, labeling data from the different T-cell subsets were fitted using a multiexponential model in which each subpopulation *i* contains a fraction α_i of cells with turnover rate p_i per day. Assuming a steady state for each kinetic subpopulation, the fraction of labeled deoxyribose residues of adenosine in the DNA of each subpopulation *i* was modeled by the following differential equation:

$$\frac{dL_i}{dt} = p_i c S(t) - p_i L_i . \qquad [8]$$

For naive T cells, T-cell production may occur both in the thymus and in the periphery. The fraction of labeled DNA in the total T-cell population under investigation was subsequently derived from $L(t) = \sum_i \alpha_i L_i(t)$ and the average turnover rate p was calculated as $p = \sum_i \alpha_i p_i$.

For finite-term labeling experiments, the analytical solutions are:

if $t \le \Delta$: $L_i(t) = 0$,

$$\text{if } \Delta < t \le \tau + \Delta: \; L_i(t) = \frac{c}{\delta - p_i}\left[p_i(S_0 \mathrm{e}^{-p_i(t-\Delta)} - S(t-\Delta)) + f(1 - \mathrm{e}^{-p_i(t-\Delta)})\right], \qquad [9]$$

$$\text{if } t > \tau + \Delta: \; L_i(t) = \frac{p_i c}{\delta - p_i}\left[S(\tau)\mathrm{e}^{-p_i(t-\Delta-\tau)} - S(t-\Delta)\right] + L_i(\tau)\mathrm{e}^{-p_i(t-\Delta-\tau)}. \qquad [10]$$

Again, Δ was fixed to 0 when fitting effector/memory T-cell data, while Δ was a free parameter when fitting naive T-cell data.

For prenatal labeling experiments, the analytical solution is: $L_i(t) = \frac{\beta c}{\delta - p_i}\left[\delta \mathrm{e}^{-p_i t} - p_i \mathrm{e}^{-\delta t}\right]$.

Best fits were determined by minimizing the sum of squared residuals using the R function nlminb[6]. The 95% confidence intervals were determined using a bootstrap method where the residuals to the optimal fit were resampled 500 times.

The effect of the number of kinetically different subpopulations on the estimated average turnover rate. To estimate the average turnover rate using the multiexponential model, one has to consider a given number of kinetically different subpopulations. We investigated the effect of model specification by creating artificial data and fitting this data set

using different models with increasing numbers of kinetically different subpopulations. We consider the following models during label intake:

$$\frac{dL_i}{dt} = p_i - p_i L_i \qquad (M_i) \text{ for i=1…6.}$$

And after label intake:

$$\frac{dL_i}{dt} = -p_i L_i \qquad (M_i) \text{ for i=1…6.}$$

Note that in the artificial data we consider perfect labeling, i.e., $cS(t)$=1 during label uptake and 0 after. Each subpopulation i contains a fraction α_i of cells with turnover rate p_i per day. The fraction of labeled DNA in the total T-cell population is subsequently derived from $L(t) = \sum_i \alpha_i L_i(t)$ and the average turnover rate p is calculated as $p = \sum_i \alpha_i p_i$. For model M_1 we consider two variants (M_{1a} and M_{1b} in Fig. S3). In model M_{1a} we set $\alpha_1 < 1$ to make it equivalent to the model proposed by Asquith et al [2], and model M_{1b} is a single-exponential model because we set $\alpha_1 = 1$. The models M_2 to M_6 are multiexponential models including 2 to 6 kinetically different subpopulations (i.e., with 2 to 6 turnover rates).

Artificial data were generated using the model (M_2) (assuming two kinetically different subpopulations) and Gaussian white noise was added. The duration of label administration was assumed to be 90 days and the label enrichment was measured every 7 days until day 150. The data were fitted with the eight proposed models using Mathematica. The 95% confidence intervals were determined using a bootstrap method.

Fig. S3 presents the average turnover rates estimated by the different models from one generated data set. The models M_{1a} and M_{1b} clearly underestimate the average turnover rate, whereas adding more kinetically different subpopulations than really presented leads to

accurate (and similar) estimates. In practice, if there are too many subpopulations, the algorithm chooses either to put in a very small fraction for a subpopulation, or to assign the same turnover rate to two or more subpopulations. Similar results were found for data generated from different sets of parameters. Note that this result is conditional to the sampling design as well as the noise level in the data, which were all set to mimic true experiments.

To determine the number of kinetically different subpopulations to include in the model we recommend a stepwise selection procedure, adding a new kinetically different subpopulation into the model and stopping when the average turnover rate no longer markedly changes provided sufficient data are available.

Finally, one may wonder whether a multiexponential model with 2 kinetically different subpopulations (model M_2) can describe labeling experiments from biological populations composed of several kinetically different subpopulations. We tested one example creating artificial data with model M_4, setting α_i=0.25 and p_i=1, 0.5, 0.25, 0.125 for i=1,2,3,4. In a similar setup as in supplemental Figure 3, model M_2 performed reasonably in estimating the average turnover rate: $p_{estimated}$= 0.40 (95%CI= 0.20;0.78) for the "true" turnover rate p_{true}= 0.46 (not shown).

Table S1. Estimates for deuterium enrichment in plasma and thymocytes.

Parameter		Estimate (95% confidence interval)	
		Finite-term labeling	Prenatal labeling
Plasma	S_0	0.015 (0.012-0.018)	0.0248 (0.0238-0.0260)
	δ (per day)	0.261 (0.230-0.272)	0.2242 (0.2034-0.2439)
	f (per day)	0.024 (0.023-0.025)	n/a
Thymocytes	p (per day)	0.416 (0.377-0.468)	n/a
	c	3.093 (2.974-3.161)	3.755 (3.596-3.984)*

*c is estimated directly from the level of enrichment of thymocytes at day 0 in prenatal labeling experiments. n/a: not applicable

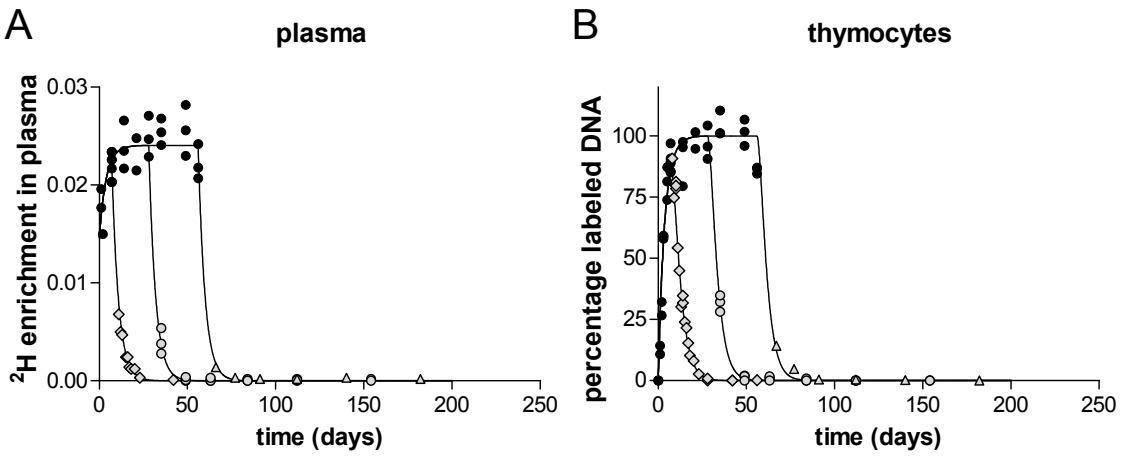

Fig. S1. Deuterium enrichment in plasma (A) and thymocytes (B) of mice labeled for 1, 4, or 8 weeks. Best fits are given by black lines. Thymocytes were used to determine the maximum percentage of labeled DNA that could possibly be attained (see Methods). Dots represent measurements (i.e., individual mice) at different time points during up-labeling (● black) and different points post labeling, after 1 week (◇ gray diamonds), after 4 weeks (○ gray circles), and after 8 weeks (△ gray triangles) of labeling, respectively. Parameter estimates are given in Table S1.

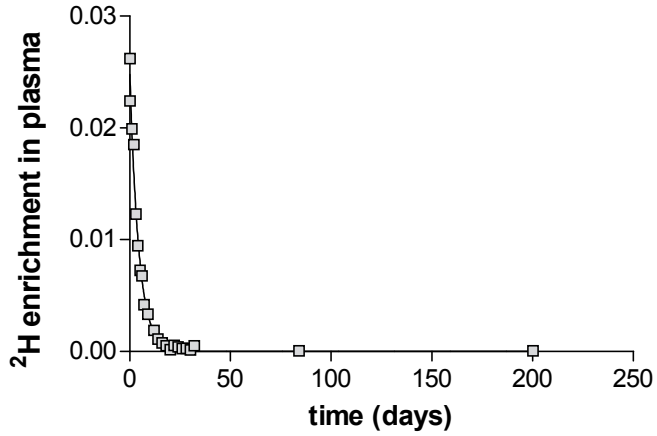

Fig. S2. Deuterium enrichment in plasma of prenatally labeled mice from the moment ^2H_2O was withdrawn from the drinking water (i.e., at 16 weeks of age). Gray squares (□) represent plasma deuterium enrichment measurements (i.e., individual mice); the black curve represents the best fit of the model to the experimental data. The turnover rate of plasma (body water) in prenatal labeling experiments is somewhat slower than the turnover rate in finite-term labeling experiments (see Table S1), which is natural because deuterium is fully enriched in all available compartments in prenatally labeled mice.

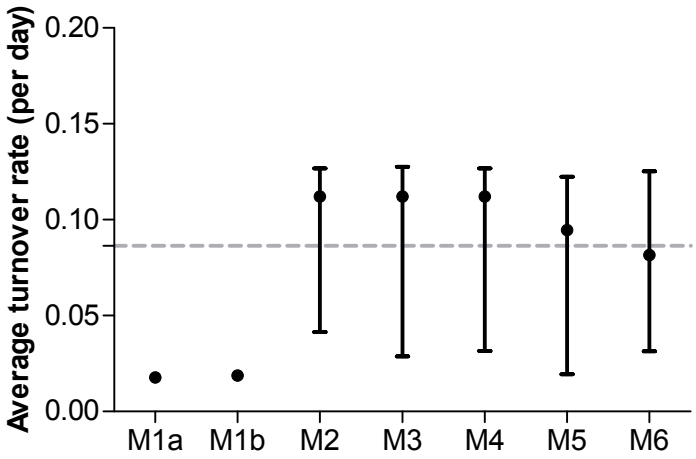

Fig. S3. Average turnover rates and 95% confidence intervals estimated with the 7 different models. Model M_{1a} is the one proposed by Asquith et al. [2] where only a fraction α of cells is turning over, while the rest of the cell population is kinetically inert. Model M_{1b} assumes that all cells have the same turnover rate (i.e., form a homogeneous population). Models M_2 to M_6 are kinetic-heterogeneity models including 2 to 6 kinetically different subpopulations (i.e., with 2 to 6 turnover rates). Artificial labeling data were generated with model M_2 for $p_1 = 0.720$, $p_2 = 0.016$ and $\alpha_1 = 0.10$, corresponding to an average turnover rate $p = 0.0864$ (---- dashed gray horizontal line), and after adding a Gaussian white noise, these data were fitted with the 7 different models.

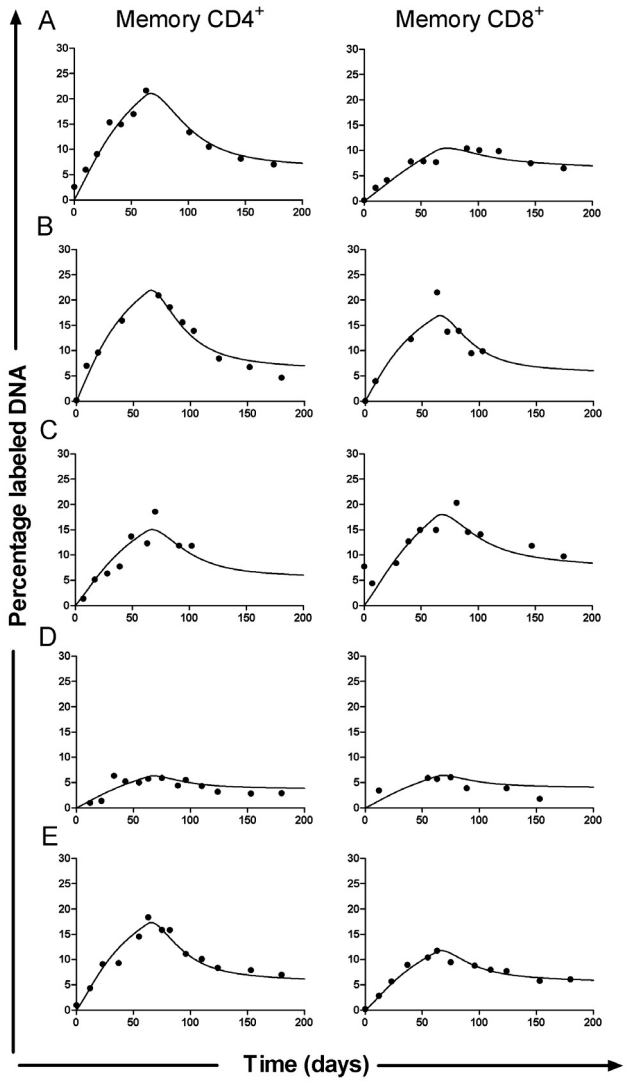

Fig. S4. Best fits of the multiexponential model to deuterium enrichment data in memory $CD4^+$ and $CD8^+$ T cells from five healthy individuals (A to E). During 9 weeks of ^2H_2O labeling, $CD4^+$ (left) and $CD8^+$ (right) memory T cells were isolated at different time points during and post-labeling, and deuterium enrichment of the DNA was determined and previously published [1]. The resulting labeling curves were fitted with a multiexponential model describing two kinetically different subpopulations (the addition of more subpopulations did not change the average turnover rate).

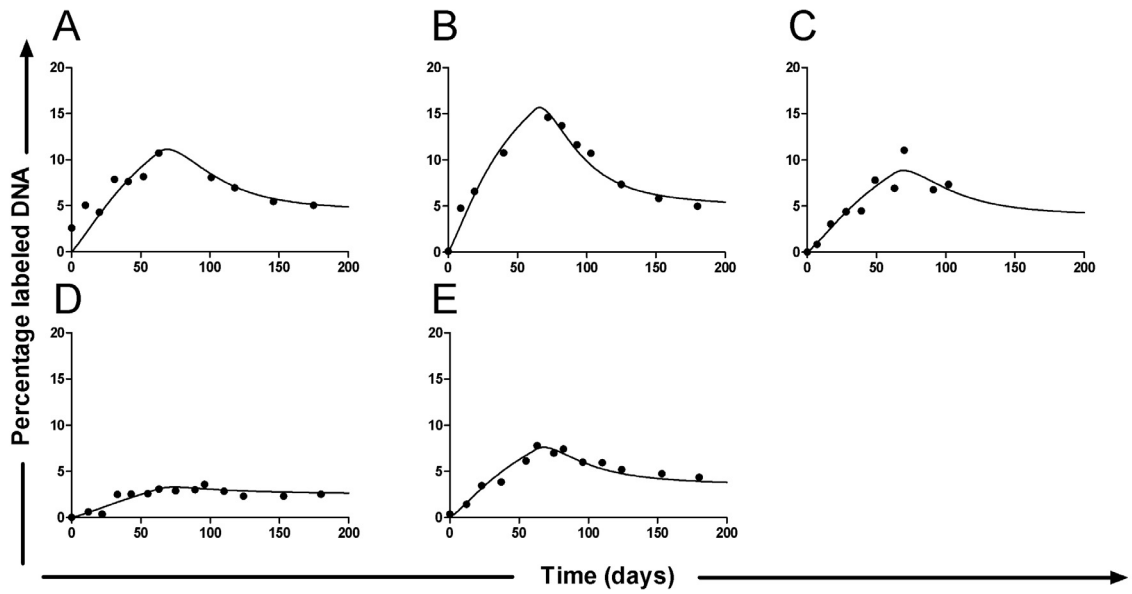

Fig. S5. Best fits of the multiexponential model to deuterium enrichment data in total $CD4^+$ T cells from five healthy individuals (A to E). During 9 weeks of ^2H_2O labeling, $CD4^+$ naive and memory T cells were isolated at different time points during and post-labeling, and deuterium enrichment was determined, and previously published [1]. From this the deuterium enrichment in total $CD4^+$ T cells at every time point was recalculated, and the resulting labeling curves were fitted with a multiexponential model describing two kinetically different subpopulations (the addition of more subpopulations did not change the average turnover rate).

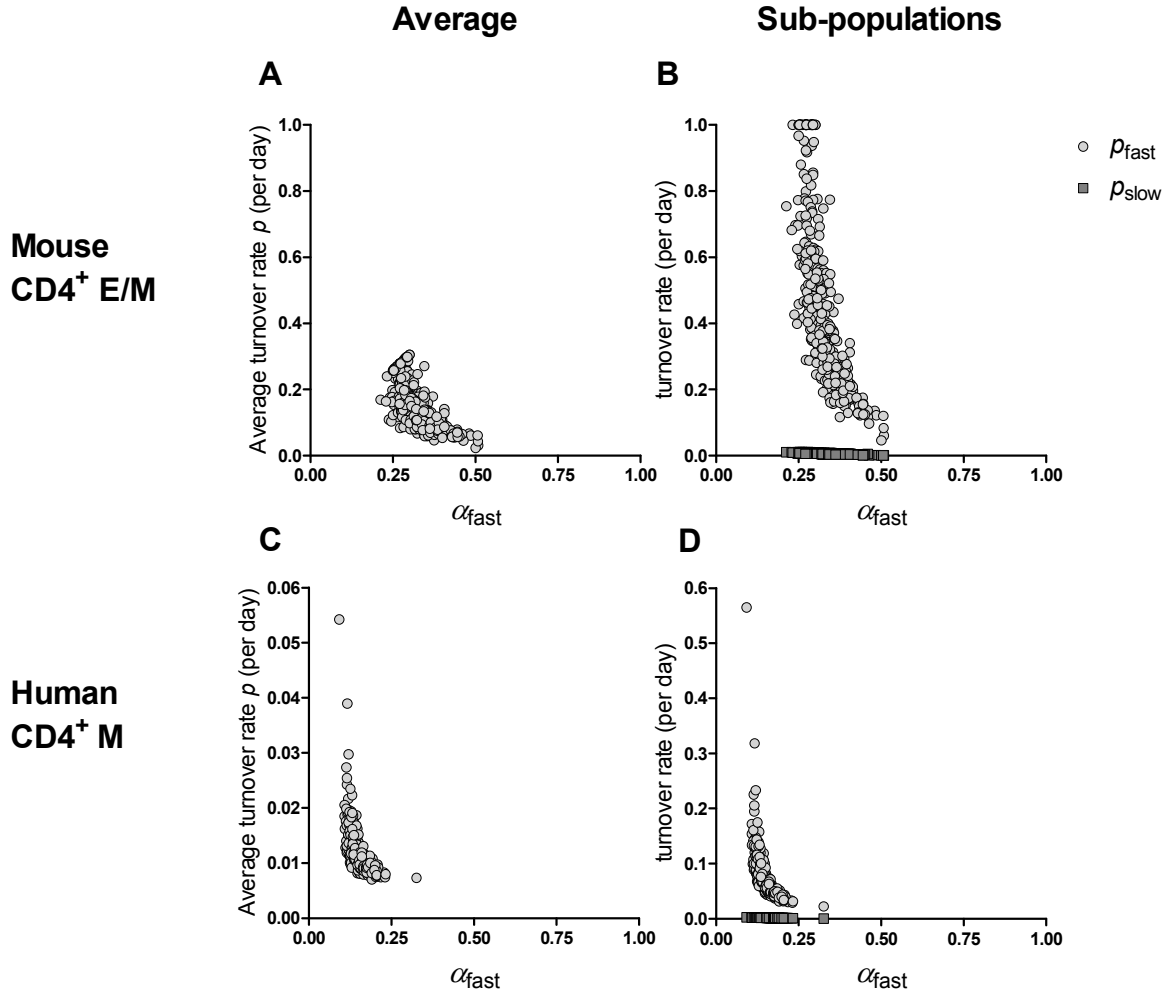

Fig. S6. Relationship between parameter estimates describing the individual subpopulations. As an illustration of the ranges of, and the relationship between, the sizes of the different subpopulations, the average turnover rate (panels A and C), and the individual turnover rates of the subpopulations (panels B and D), we selected two data sets: 8-week labeling of $CD4^+$ effector/memory (E/M) T cells in mice (panels A and B), and labeling of human $CD4^+$ memory T cells (individual A of Vrisekoop et al. [1], panels C and D, note the different scales!). For both data sets we plotted the 500 parameter sets obtained by the bootstrap analysis of Figure 2. We ordered the results of the bootstrap analysis such that the turnover rate of the fastest (p_{fast}) and the slowest (p_{slow}) subpopulations can be plotted as a function of the size of the fastest subpopulation (α_{fast}). The examples illustrate that there is quite a strong correlation

between the size of a subpopulation and its rate of turnover (panels B and D). As a result, the confidence limits on the turnover rate of the fastest subpopulation (panels B and D) are much less restricted than on the average turnover rate *p* (panels A and C). Nevertheless, the data do provide some bounds on the sizes and turnover rates of the subpopulations. The mouse data show that the fastest subpopulation makes up between 24% and 51% of the $CD4^+$ E/M pool, while in humans it represents only 11-20% of the $CD4^+$ memory pool. In the mouse data set, the turnover rate of the fast subpopulation cannot be estimated as it varies from almost 0 to 1 (panel B), while the human labeling data reveal that the turnover rate of the fast subpopulation lies between 0.03 and 0.17 per day, and that of the slow subpopulation between 0.001 and 0.002 per day (panel D).

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