Human neutrophil kinetics: modeling of stable isotope labeling data supports short blood neutrophil half-lives

Running title: Modeling in vivo human neutrophil kinetics

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Scientific Category: Granulocytes and Myelopoiesis

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Word count main text – 3,983

Word count abstract - 238

Number of Figures: 3

Number of Tables: 3

Key points

- Mechanistic modeling of stable isotope labeling verifies human neutrophil half-lives of 13-19 hours in contrast to recent estimates of >3days
- Human neutrophil kinetics can be measured using a single dose deuterium-labeled glucose protocol

Abstract

Human neutrophils have traditionally been thought to have a short half-life in blood; estimates vary from 4-18 hours. This dogma was recently challenged by stable isotope labeling studies with heavy water which yielded estimates in excess of 3 days. To investigate this disparity we generated new stable isotope labeling data in healthy adult subjects using both heavy water (n=4) and deuterium-labeled glucose (n=9), a compound with more rapid labeling kinetics. To interpret results we developed a novel mechanistic model. We applied this model to both previously-published (n=5) and newly-generated data. We initially constrained the ratio of the blood neutrophil pool to the marrow precursor pool (R=0.26, from published values). Analysis of heavy water datasets yielded turnover rates consistent with a short blood half-life, but parameters, particularly marrow transit-time, were poorly-defined. Analysis of glucose-labeling data yielded more precise estimates of half-life, 0.79 ± 0.25 days (19 hours), and marrow transit-time, 5.80 ± 0.42 days. Substitution of this marrow transit-time in the heavy water analysis gave a better-defined blood half-life, 0.77 ± 0.14 days (18.5 hours), close to glucose-derived values. Allowing R to vary yielded a best-fit value, R=0.19. Reanalysis of the previously-published model and data also revealed the origin of their long estimates for neutrophil half-life, an implicit assumption that R is very large, which is physiologically untenable. We conclude that stable isotope labeling in healthy humans is consistent with a blood neutrophil half-life of less than one day.

Introduction

Maintenance of adequate numbers of circulating neutrophils is critical for survival, but our understanding of how this is achieved is incomplete. Some aspects of neutrophil development, such as the origins, development and maturation of bone marrow precursors,[1-3] are well-characterized; whilst others, such as the dynamics of cell proliferation and blood turnover, are more controversial.[4] It is known that there is a clear separation between the precursor expansion phase, which terminates when cells become metamyelocytes,[1] and the maturation phase, which normally lasts about 5-6 days before release of cells into the circulation,[5] but the fate of cells once released into the circulation is more controversial.

Traditional dogma maintains that cells circulate for less than a day before leaving the circulation; this may be even faster during infection or trauma. Estimated half-lives in healthy individuals vary between 4.3 and 17.5 hours.[4-6] However, this dogma dates back to early experiments with cell transfer models and/or toxic or radioactive tracers.[5,7-9] Interpretation of such experiments may have been confounded by cell death (particularly since neutrophils are very susceptible to apoptosis with any *ex vivo* manipulation) or direct effects from high-energy radioactive tracers.[4]

A recent study by Pillay *et al* using *in vivo* stable isotope labeling with heavy (deuterium-labeled) water (^2H_2O) - which is non-toxic and does not require cell manipulation - concluded that the previous estimates were incorrect; their estimate for the *in vivo* half-life of circulating neutrophils was about 3.7 days (corresponding to a lifespan of 5.4 days), an order of magnitude higher than previous estimates.[10] If true, this would represent a major paradigm shift in our understanding of neutrophil kinetics. One potential explanation for this discrepancy between historic and recent studies might be excess cell toxicity in historic data leading to an overestimate of cell disappearance in these earlier studies. A second potential explanation is the mathematical model used to interpret the recent stable-isotope labeling curves.[11] The one-compartment model of Pillay *et al* may have

failed to adequately describe the behavior of a complex population which proliferates in a remote (unmeasured) compartment (i.e. bone marrow), and which is lost from the sampled compartment (i.e. blood). Furthermore, rapid cell fluxes may be difficult to capture with heavy water, which labels and delabels slowly (turnover rate ~5 %/day).

One way to resolve this uncertainty would be to sample bone marrow following deuterium-labeling;[12] although undoubtedly revealing, this would require repeated marrow aspirations. We propose here an alternative, less-invasive approach, combining a novel mathematical model of granulopoiesis with the acquisition of new complementary data using the short-lived label, deuterium-labeled glucose. To investigate why Pillay *et al.* obtained such large estimates we also acquired new data using their label of choice (heavy water) and, in addition, performed a reanalysis of their published data.

This aims of this study were: (a) to test whether a short-pulse labeling protocol with deuterium-labeled glucose could be developed for analysis of *in vivo* human neutrophil kinetics, (b) to develop a physiological yet parsimonious model of neutrophil proliferation and loss, and (c) to re-evaluate quantitative parameters of neutrophil kinetics (proliferation rate, marrow transit-time and blood half-life) using data from both published water-labeling data and new glucose- and heavy water-labeling studies.

Methods

1. *In vivo* glucose-labeling

Nine healthy adult subjects (age 20-52; 6M:3F; Table 1) received [6,6-$^{2}H_2$]-glucose (Cambridge Isotopes, Cambridge MA) as an oral solution. In initial studies, 60g was given over 10 hours as half-hourly aliquots, preceded by a priming dose to rapidly achieve steady-state labeling. Subsequent studies were modified to develop the simplest workable protocol; dosing was progressively reduced (Table 1) to a single oral dose of 20g. Blood glucose enrichment was monitored during and after oral labeling, as previously described.[13] At selected time-points after labeling, neutrophils were isolated from blood by gradient centrifugation with *Polymorphprep* (Alere Technologies AS, Oslo, Norway); purity and yield were confirmed by flow cytometry. In two subjects, C59 and C60, aliquots were further purified by CD16 antibody-coated magnetic bead adhesion (Miltenyi Biotec, UK) and analyzed in parallel with cells purified by gradient-separation alone. DNA was extracted and deuterium enrichment measured by gas chromatography-mass spectrometry (GC-MS) as previously described.[13,14]

2. *In vivo* heavy water labeling

Four further healthy subjects (age 29-83; 3M:1F) received heavy water ($^{2}H_2O$) for 7 weeks: 50ml 70% $^{2}H_2O$ (Cambridge Isotopes, Cambridge MA) three-times daily for one week, then twice-daily for 6 weeks, as previously described.[14] During labeling and de-labeling periods, saliva samples were collected to measure the deuterium content in body water. Neutrophils were prepared from the buffy coat layer below a Ficoll density gradient and DNA processed and analyzed as above.

All subjects gave written informed consent following Ethical Review Board approval (Ref 10/H0803/102 and 13/LO/0022); all interventions were performed according to the principles of the Declaration of Helsinki.

3. Analysis of previously published *in vivo* heavy water labeling data

Previously published[15] *in vivo* heavy water labeling data from five subjects were re-analyzed using the new model (below). Experimental details have been published elsewhere.[15] Briefly, healthy young humans (age 20-25) received heavy water for 9 weeks comprising a prime of 10ml ^2H_2O /kg body weight over 24h, followed by 1.25ml ^2H_2O /kg body weight daily. Cell samples were taken at 14 time points between week 0 and 16; urine samples were measured for body water enrichment. DNA enrichments in neutrophils were measured by GC-MS.

4. Modeling

To interpret the experimental data, we constructed a new model based on the known physiology of the granulopoietic system (Figure 1). Implicit are two premises: (i) that mature circulating neutrophils acquire label as a consequence of cell division during the mitotic stage in the bone marrow, i.e. as myelocytes, promyelocytes, myeloblasts etc.), and (ii) that after the last mitosis, metamyelocytes go through a maturation/transit process before release into the circulation, resulting in a delayed appearance of labeled cells in blood. Model equations are as follows:

$$\frac{dN_p(t)}{dt} = p \cdot N_p(t) - q \cdot N_p(t) \qquad (1a)$$

$$\frac{dN_b(t)}{dt} = s \cdot N_p(t-\Delta) \; - z \cdot N_b(t) \qquad (1b)$$

where N_p are proliferating bone marrow neutrophils and N_b are blood neutrophils. N_p proliferate at a rate p and leave the proliferating pool at rate q into the post-mitotic maturation/transit pool where they reside for time Δ. After the transit time Δ, neutrophils enter into the circulation at rate s and exit the circulation at rate z.

In the presence of label, deuterium is incorporated into neutrophil DNA during division of the N_p pool in the bone marrow. Assuming that the amount of DNA per cell is conserved (amount of DNA proportional to the number of neutrophils) the model equations can be written as follows:

$$\frac{dNL_p(t)}{dt} = p \cdot N_p(t) \cdot U_t \cdot b_{w/g} - q \cdot NL_p(t) \qquad (2a)$$

$$\frac{dNL_b(t)}{dt} = s \cdot NL_p(t - \Delta) \; - z \cdot NL_b(t) \qquad (2b)$$

where *NL* are labeled neutrophils, $b_{w/g}$ is the normalizing factor of either water or glucose [13,14,16,17] and U_t, representing label enrichment (instantaneous exposure to label) at time *t* approximated by the experimental plasma label enrichment. U_t was described by an empirical curve for water labeling studies [15]; by a square-pulse approach with exponential tail for the 3h, 4h and 10h labeling protocols and by interpolation between data points for the single oral bolus approach (C59,C60) (Supplementary Text 1).

Assuming a constant number of cells in each pool, the steady-state constraint requires that the net flow of the system equals 0 ($p \cdot N_p(t) = q \cdot N_p(t) = s \cdot N_p(t-\Delta) = z \cdot N_b(t)$), from equations 1a and 1b. Using these constraints to eliminate *p, q* and *s* and dividing equations 2a and 2b by the number of cells in each pool gives equations 3a and 3b, where *L* is the fraction of labeled neutrophils in the proliferating pool (L_p) and in blood (L_b), and *R* is the ratio of blood neutrophils to mitotic neutrophil precursors in marrow $R = \frac{N_b}{N_p}$.

$$\frac{dL_p(t)}{dt} = z \cdot R \cdot U_t \cdot b_{w/g} - z \cdot R \cdot L_p(t) \qquad (3a)$$

$$\frac{dL_b(t)}{dt} = z \cdot L_p(t - \Delta) \; - z \cdot L_b(t) \qquad (3b)$$

This model thus has up to three free parameters (z, R and Δ, see following subsection).

Estimation of the ratio of blood neutrophils to bone marrow mitotic precursors (R)

We defined R as the ratio of the number of neutrophils in blood to the number of mitotic neutrophils in marrow. Initially we fixed R using estimates derived from the literature, R=0.26 (Box 1). This value was used when fitting each individual separately. In later analysis we allowed R to be a free parameter which we estimated from fitting the model to all individuals simultaneously (R, z and Δ were population parameters).

Fitting procedure and expression of results

Equation 3a and 3b were fit to the experimental data by minimizing the sum of squared residuals between prediction and observation using the pseudorandom algorithm in the FME package in R.[18-20] Standard errors on estimates were calculated using the asymptotic covariance matrix method.[21]

When comparing our conclusions with other estimates in the literature, the reader should be aware that we have quoted half-life values ($t_{½}=ln(2)/z$), which will be a factor of 1.44 less than "lifespan" values, (lifespan=$1/z$).

Box 1: Estimation of R from published literature

We define R as the ratio of the number of neutrophils in blood to the number of mitotic neutrophil precursors in bone marrow. We assume R is constant for all individuals. We estimate R by estimating each pool size from values in the published literature.

Total blood neutrophil pool size

The mean count of circulating blood neutrophils for the Caucasian population has been estimated to be 4.2×10^9cells per liter.[22] The blood volume of the 73kg reference man is 5.3L.[23] This gives an estimate for the circulating pool of 3.05×10^8 cells/kg body weight (BW). In addition to the circulating pool the total blood pool also includes the marginal pool; since this is in fast equilibrium with the circulating pool, we consider them to be a single kinetically homogeneous population.[8] The size of the marginal pool has been estimated by different methods. Labeling studies suggest a value of between 43% and 58% of the total blood granulocyte pool,[5,7,8] but these studies were performed under conditions that might affect neutrophil kinetics. Studies using epinephrine (which mobilizes the marginal blood pool without affecting the total blood pool size[7,24]), suggest that the marginal pool size accounts for 50-55% of the total neutrophil blood pool.[25,26] Taking the more conservative value (50%) gives an estimate of the total blood granulocyte pool (marginal and circulating) of 6.1×10^8 cells/kg BW, the value used in this study. Notably, it agrees closely with the estimate of 7×10^8cells/kg BW published by Cartwright *et al*.[8]

Bone marrow mitotic neutrophil pool size

Mitotic neutrophils include myeloblasts, promyelocytes and myelocytes -with a minor contribution from the stem cell pool.[27] Flow cytometry and microscopy studies suggest that mitotic neutrophils in marrow account for 17-19% of total marrow cellularity.[2,28,29] The cellularity of bone marrow has been extensively studied in mice[30-32] and humans[33-37]; the median of these different estimates is 13.05×10^9

cells/kg (Supplementary Table 1). Taking a value of 17% for the marrow neutrophil precursor mitotic pool gives an estimate of 2.35×10^9 cells/kg (the value used in this study), in line with the estimate provided by Dancey *et al* of 2.11×10^9 cells/kg.[5]

Estimation of the ratio of blood neutrophils to bone marrow mitotic precursors (R)

Combining these values, a total blood neutrophil pool of 6.1×10^8 cells/kg and a marrow neutrophil precursor mitotic pool of 2.35×10^9 cells/kg, yields an estimate of R of 0.26 blood neutrophils per mitotic neutrophil precursor.

Results

1. Experimental exploration of neutrophil kinetics using deuterium-labeled glucose.

Neutrophil labeling after dosing with deuterium-labeled glucose showed a consistent pattern (Figure 2) characterized by a lag period of 5-7 days with no appreciable labeling, a rapid increase in the proportion of labeled cells, then a slower disappearance. Since deuterium enrichments in the DNA of circulating neutrophils in initial experiments with a 60g 10-hour dosing schedule were high in the measurement range, we sought to develop the simplest, least-intrusive, and most cost-effective protocol by progressively reducing the dose and duration of labeling. Our final protocol, 20g given as a single dose (Table 1), yielded enrichments in the readily-measurable range, typically 0.5 atoms percent enrichment (APE); further dosage reductions may be feasible whilst still obtaining reliable results. Subject acceptability was good, although repeated post-labeling measurements (we estimate at least six time-points) were required to capture the lag, peak and disappearance phases adequately.

2. Modeling of heavy water and deuterium-labeled glucose derived datasets

We developed a two compartment model in which neutrophils acquire label as a consequence of cell division during the mitotic stage in the bone marrow, i.e. as myelocytes, promyelocytes, myeloblasts etc.) and then go through a maturation/transit phase (metamyelocytes) before release into the circulation (Figure 1). The ratio of the size of the total blood neutrophil pool to the bone marrow neutrophil precursor mitotic pool (R) was initially fixed at a value derived from published literature (R= 0.26; Box 1); later, we explored the implications of allowing R to be a free parameter. We fitted this model to the experimental data to estimate the rate of neutrophil disappearance from the

blood (z) and the bone marrow transit time (Δ). The model fitted the new water and glucose data and the published heavy water data well with the exception of one individual (C42) whose data was not consistent with other datasets (Figures 2 and 3). Parameters estimates are given in Table 2. Glucose-labeling experiments yielded values for the median marrow transit time of 5.72 days (mean ± SD 5.80 ± 0.42 days) with a mean neutrophil half-life in blood of 0.79 ± 0.25 days (19 ± 6 hours, equivalent to a "lifespan" of 1.05 days). Heavy water studies gave not dissimilar values for the half-life in blood of 1.11 ± 0.36 days but parameter estimates were poorly resolved, especially the post-mitotic transit-time which had a wide inter-individual range and large standard errors (range 0.41-5.91 days; mean ± SD 3.4 ± 2.09 days; Supplementary Figure 1, ids A-E and DW04-DW11); similarly a wide range of values of z fitted the data equally well (Supplementary Figure 2, identifiers A-E and DW04-DW11). In contrast, estimates from glucose labeling were better resolved (Table 2; Supplementary Figures 1 and 2, C36R-C60).

We hypothesized that the slightly longer half-life estimates for the water dataset could be an artefact of the poor estimates of the transit-times. Hence, we repeated the fits to the water datasets with a fixed transit-time of 5.7 days, a value consistent both with historical data[5,16,38] and our estimates from glucose labeling. The resulting estimates for the half-life of neutrophils, mean 0.77 ± 0.14 days, were not only better resolved but also remarkably consistent with the glucose-based estimates (Table 3). The quality of the fit (deviation between observation and prediction) with free and fixed transit times were very similar. Therefore, if R is fixed, based upon estimates from the literature, and if a glucose/literature-derived value for transit-time is used for heavy water modeling, both approaches give almost identical estimates of the key kinetics of neutrophil homeostasis, and provide good fits to the experimental data.

3. Validation of the ratio of blood neutrophils to bone marrow mitotic precursors (R)

Our initial approach relied upon estimating R from published literature. To investigate the accuracy of this estimate, we simultaneously fitted the data from the previously-published 9-week heavy water labeling, and our 3-hour and 10-hour oral glucose labeling datasets; we let z, transit time and R be free population parameters; C42 was excluded at this point since data was not consistent with other individuals and we were concerned this may have a distorting effect on the estimate of R. In order to minimize the number of free parameters, b_g and b_w were fixed; b_g to 0.73[13] and b_w to 1 by normalizing the water data to 100%.[15] The latter normalization could not be carried out for the 7-week labeling dataset due to the uncertainty in having reached 100% of the attainable label in neutrophil DNA[15]; for this reason the 7-week dataset was excluded from estimates of R. We found two scenarios that fitted the data equally well (Supplementary Figure 3), as previously hypothesized;[11] one with an R of 0.19 ± 0.00 (transit time 5.72 days; half-life 13h), and another one with R=5.49 ± 1.26 (transit time 5.73 days; half-life 3 days). In the former scenario, marrow maturation/transit time is the rate-limiting step, whereas in the latter, the cell disappearance from the blood is also slow, as previously described.[11] Focusing on the first scenario, which produces estimates of R similar to those in the literature, we see that our estimates of neutrophil half-life are reasonably robust to the assumed value of R, being only slightly lower with a free value of R than with a fixed value. This analysis also showed that a second scenario was numerically possible but physiologically unlikely as R of the order of 5 is incompatible with what is known about the relative sizes of the marrow and blood compartments.

4. Comparison of our estimates of neutrophil half-life with those obtained by Pillay *et al.*

Our estimates of neutrophil half-life are consistent with a short half-life of less than one day, whether we fit to our newly-obtained data or the data presented by Pillay *et al*[10] and whichever label we use. Thus, the difference in estimated half-life must be attributable to model choice not data. Comparison of our model with that of Pillay *et al* (Supplementary Text 2) shows that Pillay *et al* implicitly assume that R is large, an assumption which is at odds with current knowledge of the system. Furthermore, it can be seen (Supplementary Figure 3) that as R is increased the estimate of the circulating half-life increases, explaining why Pillay *et al* obtained such large estimates for the half life. We suggest that as R is thought to be small (of the order of 0.2) then our, shorter, estimates of neutrophil half-life are more likely to be accurate.

Discussion

Neutrophil proliferation and maturation/transit in marrow, egression to blood, and loss rates from blood have recently received unprecedented attention. Revised estimates for neutrophil lifespan in blood, generated from heavy water experiments (mean lifespan 5.4 days, corresponding to a half-life of 3.7 days) conflict with the widely-accepted dogma that neutrophils spend only a very short period in blood (generally considered to be about 11h, corresponding to a half-life of 7.6h[5]). Li *et al*[11] observed that this disparity could result from data interpretation using a single-compartment model. Applying a multi-compartment model to the tritium-labeled thymidine data of Dancey *et al*,[5] they showed that two distinct scenarios are mathematically plausible: one where the time taken for proliferation and transit in marrow is rate-limiting, and another in which the circulatory half-life is the rate-limiting step.[11] We confirmed the mathematical plausibility of these two scenarios using a two-compartment model with deuterium-labeling experimental data, albeit with slightly longer half-lives in both situations. Crucially, we observed that a pivotal difference between the two scenarios correlates with different relative sizes of the marrow-precursor and blood neutrophil pools. We hypothesized that focusing on this aspect might allow resolution of the dilemma without recourse to invasive bone-marrow sampling experiments. We also hypothesized that parameter estimates should be independent of the kinetics of the tracer used and so should yield similar conclusions whether derived from heavy water or deuterium-labeled glucose when interpreted using a two compartment model. Our studies have enabled us to draw several conclusions.

Firstly, in terms of experimental approaches, we found that deuterium-labeled glucose labeling allowed estimation of the key parameters of neutrophil kinetics with a simple, short, readily-executed protocol. Administration of a single oral dose achieves adequate enrichment for quantitation, although repeated follow-up blood sampling is required. As neutrophils are highly-labeled, minor cellular impurities have a relatively small effect and cell sorting by dual-gradient density centrifugation appears adequate; parallel studies with and without further magnetic bead

purification in two subjects yielded almost identical enrichment results (Supplementary Figure 4). The single-dose $^{2}H_{2}$-glucose approach with two-compartment modeling appears to represent a potentially widely-applicable method for the study of human neutrophil kinetics *in vivo*.

Secondly, in terms of modeling, we confirmed that estimation of parameters is critically related to the relationship between the size of the marrow-precursor and the blood neutrophil pool. Fixing this ratio of pool sizes at 0.26 (from published values for physiological variables, Box 1) allowed us to obtain unique best fits for key parameters in neutrophil kinetics. Poor parameter estimates for heavy water could be attributed to the difficulty in estimating the marrow transit-time using a tracer with a slow "on" and "off" dynamic. When externally-derived values for transit-time were fixed for the heavy water data, much more precise estimates for the key parameters were obtained, which were remarkably close to those obtained from glucose-labeling experiments.

Additionally, we were able to demonstrate the source of the very high estimates of circulatory half-life previously calculated by Pillay *et al* (see Supplementary Text 2). It seems that the major problem of the previously-described one-compartment model is the implicit assumption that the number of proliferating neutrophil precursors in marrow is small compared to the number of neutrophils in blood leading to a very high estimate of the neutrophil half-life. This assumption clashes with current knowledge of neutrophil cell population sizes in bone marrow and blood.

Thirdly, in terms of the physiological implications of these findings, we found that, for fixed R, stable isotope labeling data was consistent with the traditional dogma of a rapid turnover of blood neutrophils. Mean values for the circulatory half-lives were 19h and 18h, for glucose and water, respectively. We further confirmed that in healthy individuals, the post-mitotic transit-time is about 5.7 days. If R is allowed to be a free parameter we estimate a slightly shorter neutrophil half-life of 13h. These estimates are in good agreement with previous half-life estimates obtained both by labeling [5,7-9] and an independent (label-free) method based on fitting a model of myelosuppression to antitumor-treatment datasets[39]. If the model presented here, with an R equal to 0.26, is fitted to

$[^3H]_3$-thymidine data available in the literature,[1,5] a blood half-life of 13-16h is obtained, which is slightly smaller than the estimates obtained from heavy water and glucose using the same value of R. It is well known that *ex vivo* manipulation of neutrophils or the use of toxic compounds such as DFP32, BrdU, and $[^3H]_3$thymidine may affect the viability of cells, affect their kinetics, plasticity and/or increase their sequestration in organs.[40-44] As discussed elsewhere,[4] this may explain these remaining discrepancies.

Although more complex models can readily be developed they did not change our basic conclusion that neutrophil half-life is short, though precise estimates did vary (3h-16h depending on model). None of the models provided a better fit to the labeling data than our current model choice (results not shown). Other scenarios considered included concatenated series of mitotic pools in marrow, a discrimination between marginal/circulating blood pools, egression from marrow defined by different probability density functions and age-dependent loss in blood. The current model assumes a steady state – application to non-steady state situations such as trauma and sepsis may be attained by the inclusion of a feed-back loop affecting proliferation, as has been done before in studies of myelosupression upon anti-tumor treatment.[39,45,46] Similarly, circadian oscillations are not included within the steady-state model. Blood neutrophil counts follow a circadian rhythm,[47,48] which may be due to changes in proliferation, transit/release or death/disappearance. Heavy water labels throughout the 24-hour cycle whilst glucose labeling was always performed in day-light hours (photophase); samples were consistently taken in the early-morning (0800-1000). The fact that photophase labelling with glucose and continual (photophase & scotophase) labelling with water gave similar estimates for neutrophil proliferation and dwell time suggests that there are probably not very large variations in these parameters with phase of the circadian clock. A more detailed analysis of the potential impact of circadian oscillations is, however, beyond the scope of this paper.

In summary, application of a mechanistic, biologically-meaningful model demonstrates that *in vivo* deuterium labeling data, whether obtained using heavy water or glucose is consistent with a half-life

for neutrophils in blood of less than a day. We have shown how longer half-lives may be mathematically consistent with the data, but only if the relative sizes of the blood and marrow pools are allowed to assume unphysiological values. This model has wider potential applications, including investigation of cells such as some T-cell populations, leukemic cells and monocytes, where proliferation and sampling occur in different compartments.

Tables

Identifier	Age (years)	Gender	Label	Total dose	Labeling time
C36R	52	M	$^{2}H_2$-Glucose	60 g	10h
C41	28	F	$^{2}H_2$-Glucose	60 g	10h
C42	44	F	$^{2}H_2$-Glucose	30 g	4h
C46	28	F	$^{2}H_2$-Glucose	20 g	3h
C48	36	M	$^{2}H_2$-Glucose	20 g	3h
C49	30	M	$^{2}H_2$-Glucose	20 g	3h
C50	27	M	$^{2}H_2$-Glucose	20 g	3h
C59	20	M	$^{2}H_2$-Glucose	20 g	bolus
C60	21	M	$^{2}H_2$-Glucose	20 g	bolus
DW04	83	M	Heavy water	5250 ml	7 weeks
DW09	47	M	Heavy water	5250 ml	7 weeks
DW10	34	M	Heavy water	5250 ml	7 weeks
DW11	29	F	Heavy water	5250 ml	7 weeks

Table 1. Participants and dosing for human deuterium-labeled glucose and heavy water studies
Total volume of heavy water is shown as ml of 70% $^{2}H_2O$; dosing was 50ml three-times daily for one week, then twice-daily for 6 weeks.

Identifier	Normalization Factor, $b_{g/w}$	Transit-time, Δ (days)	Loss rate from blood, z (day $^{-1}$)	Proliferation rate, p (day $^{-1}$)	Blood half-life (days)
Deuterium-labeled glucose ($^2H_2$-glucose)					
C36R	0.73*	5.91 ± 0.07	1.03 ± 0.04	0.268	0.67
C41	0.73*	5.56 ± 0.00	1.06 ± 0.19	0.276	0.65
C42	0.73*	5.38 ± 0.00	0.52 ± 0.08	0.135	1.34
C46	0.73*	5.72 ± 0.00	1.09 ± 0.05	0.283	0.63
C48	0.73*	5.29 ± 0.07	0.72 ± 0.12	0.187	0.97
C49	0.73*	5.88 ± 0.00	0.92 ± 0.06	0.239	0.76
C50	0.73*	6.60 ± 0.72	1.42 ± 0.29	0.369	0.49
C59	0.73*	5.61 ± 0.00	0.82 ± 0.00	0.213	0.85
C60	0.73*	6.24 ± 0.01	0.98 ± 0.00	0.255	0.71
Mean ± SD		5.80 ± 0.42	0.95 ± 0.29	0.247 ±0.066	0.79 ± 0.25
Median		5.72	0.98	0.255	0.71
Heavy water (^2H_2O)					
A	4.32 ± 0.03	4.49 ± 0.00	0.74 ± 0.02	0.192	0.94
B	4.74 ± 0.05	4.69 ± 0.62	0.77 ± 0.09	0.200	0.90
C	4.89 ± 0.07	5.25 ± 0.35	0.78 ± 0.00	0.203	0.89
D	3.69 ± 0.11	1.77 ± 0.07	0.47 ± 0.04	0.122	1.49
E	3.98 ± 0.06	0.41 ± 0.00	0.53 ± 0.03	0.138	1.31
DW04	4.40 ± 0.31	5.91 ± 0.00	1.18 ± 0.09	0.307	0.59
DW09	3.83 ± 0.19	0.84 ± 2.82	0.40 ± 0.11	0.104	1.74
DW10	4.38 ± 0.00	5.07 ± 1.97	0.73 ± 0.00	0.190	0.96
DW11	3.18 ± 0.14	2.20 ± 2.29	0.57 ± 0.17	0.148	1.21
Mean ± SD	4.16 ± 0.54	3.40 ± 2.09	0.68 ± 0.23	0.178 ±0.060	1.11 ± 0.36
Median	4.32	4.49	0.73	0.190	0.96

Table 2. Best fit parameters derived from deuterium labeling experiments

Results from deuterium-labeled glucose labeling studies (ids C36R, C41, C42, C46, C48, C50, C59 and C60) and heavy water labeling studies (A, B, C, D, E, DW04, DW09, DW10 and DW11) are shown. Normalization factors are b_g and b_w for glucose and water respectively: * denotes a fixed value. Transit-time, Δ is the time from the last mitosis in marrow until cell egression to blood; z is the loss rate of blood neutrophils. Parameter estimates are shown with their standard error estimated from the asymptotic covariance matrix; mean values are shown with their standard deviation. The

proliferation rate of mitotic neutrophils, p, is not a free parameter; it is calculated from the other model parameters p=z.R. Half-life is calculated from the loss rate z as ln2/z.

Heavy water (^2H_2O)

Identifier	Normalization Factor b_w	Transit-time, Δ (days)	Loss rate from blood, z (day $^{-1}$)	Proliferation rate, p (day $^{-1}$)	Blood half-life (days)
A	4.30 ± 0.03	5.7*	0.93 ± 0.00	0.242	0.75
B	4.72 ± 0.02	5.7*	0.96 ± 0.01	0.250	0.72
C	4.88 ± 0.07	5.7*	0.84 ± 0.00	0.218	0.82
D	3.59 ± 0.12	5.7*	0.84 ± 0.00	0.218	0.82
E	3.89 ± 0.04	5.7*	1.34 ± 0.16	0.348	0.52
DW04	4.40 ± 0.15	5.7*	1.13 ± 0.24	0.294	0.62
DW09	3.65 ± 0.20	5.7*	0.68 ± 0.00	0.177	1.01
DW10	4.37 ± 0.25	5.7*	0.80 ± 0.19	0.208	0.87
DW11	3.14 ± 0.14	5.7*	0.92 ± 0.19	0.239	0.75
Mean ± SD	4.10 ± 0.57		0.94 ± 0.19	0.244 ± 0.051	0.77 ± 0.14
Median	4.30		0.92	0.239	0.75

Table 3. Revised parameter estimates for water datasets after fixing the transit time

Parameters as in Table 2. * denotes the fixed value of transit-time, Δ, in this model.

Figure Legends

Figure 1. Physiology of granulocyte turnover translated to a model

We consider mitotic neutrophil precursors as a single pool *Np* ("proliferating neutrophils"). The *Np* pool consists mainly of myelocytes, promyelocytes and myeloblast, but also contains earlier precursors. Cells in the *Np* population proliferate at a mean rate *p*. After the last mitosis, cells enter the post-mitotic maturation/transit pool at a rate *q*. Transit neutrophils remain in the post-mitotic pool for a period of 4-6 days,[5,16,49] referred to as the transit-time (Δ), before egressing from bone marrow into the blood pool. Most egressing cells are segmented neutrophils, but some cells leave as band neutrophils. In the blood, neutrophils exist in a freely circulating pool and a marginal pool (cells retained in proximity to the endothelium). Since the circulating and marginal pool are considered to be in rapid dynamic equilibrium,[7,8] we consider them as a single kinetically-homogeneous pool N_B. Blood neutrophils are lost from the circulation at a rate *z* primarily to the bone marrow, liver and spleen.[50-52] This loss is generally considered to be a random, irreversible process.[5,8,49,53,54]

Figure 2. Best model fits to neutrophil DNA enrichment data in subjects labeled with deuterium-labeled glucose

Subjects were labeled with deuterium-labeled glucose (6,6-$^2H_2$-glucose) for 10 hours (C36R, C41), 4 hours (C42), 3 hours (C46, C48, C49 and C50), or as a single oral dose (C59 and C60). Dots represent measured fractional enrichment of deoxyadenosine in blood neutrophil DNA; lines represent best fits of the model (equations 3a and 3b) to the data.

Figure 3. Best model fits to neutrophil DNA enrichment data in subjects labeled with heavy water

Subjects were labeled with heavy water (^2H_2O) for 9 weeks (individuals A, B, C, D, E from [15]) or 7 weeks (DW04, DW09, DW10 and DW11 – new data). Dots represent measured fractional enrichment

of deoxyadenosine in blood neutrophil DNA; lines represent best fits of the model (equations 3a and 3b) to the data.

Acknowledgements, Authorship Contributions and Disclosure of Conflicts of Interest

Acknowledgements:

The research leading to these results has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement 317040 (QuanTI). B.A. is a Wellcome Trust Investigator (103865) and is funded by the Medical Research Council UK (J007439 and G1001052), the European Union Seventh Framework Programme (FP7/2007–2013) under grant agreement 317040 (QuanTI) and Leukemia and Lymphoma Research (15012). J.L.B. is funded by the European Union Seventh Framework Programme (FP7/2007–2013) under grant agreement 317040 (QuanTI). DCM received funding from the Medical Research Council UK (G1001052).

The Imperial College High Performance Computing Service: (http://www.imperial.ac.uk/ict/services/teachingandresearchservices/highperformancecomputing) was used for this work.

Authorship Contributions:

Designed research: DM, BA, ME, JLB, CN

Performed research: JLB, ME, RA, AS, YZ

Collected data: RA, AS, YZ

Analyzed and interpreted data: DM, BA, JLB, ME

Wrote the manuscript: BA, DM, JLB, ME, CN, MB

Conflicts of Interest:

None

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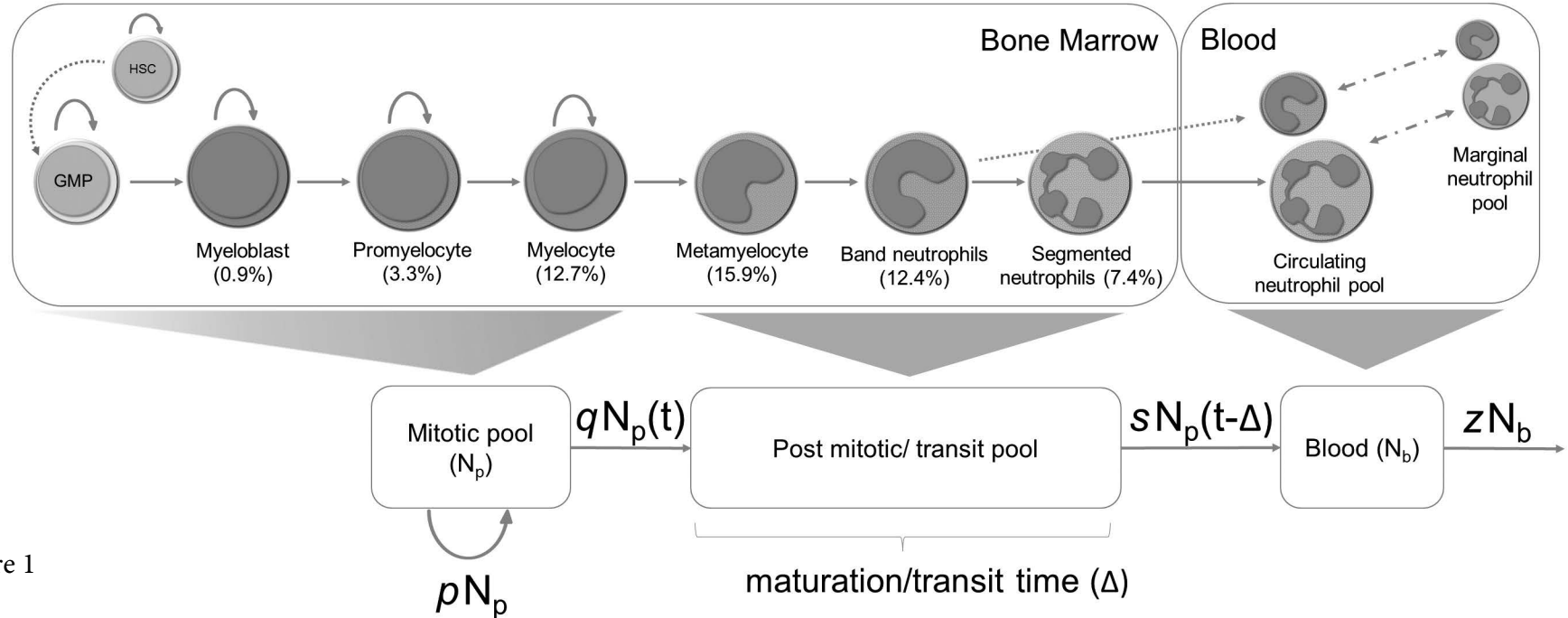
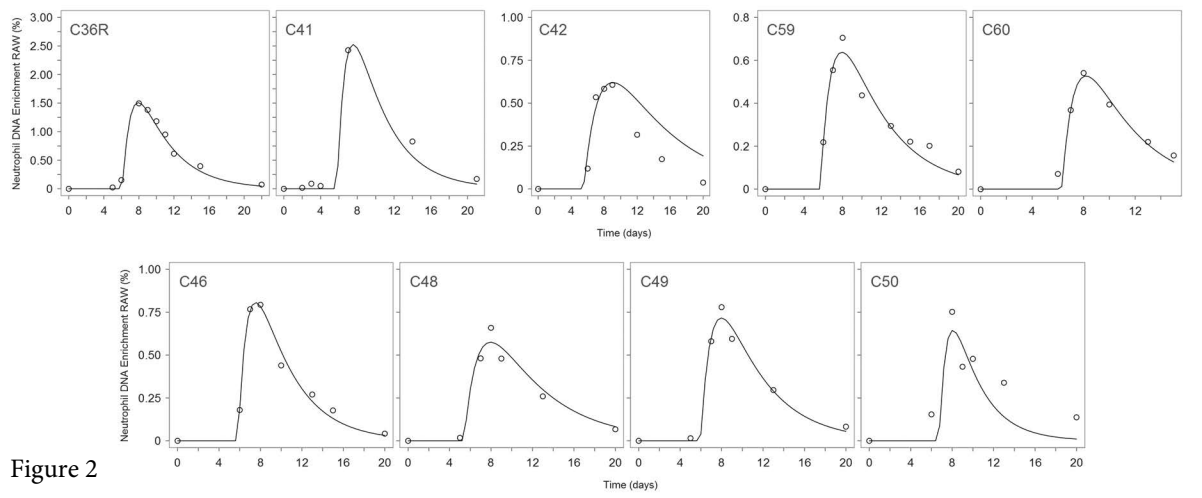

Bone Marrow
Blood
HSC
GMP
Myeloblast
(0.9%)
Promyelocyte
(3.3%)
Myelocyte
(12.7%)
Metamyelocyte
(15.9%)
Band neutrophils
(12.4%)
Segmented
neutrophils (7.4%)
Circulating
neutrophil pool
Marginal
neutrophil
pool
Mitotic pool
(N_p)
$qN_p(t)$
Post mitotic/ transit pool
$sN_p(t-\Delta)$
Blood (N_b)
zN_b
pN_p
maturation/transit time (Δ)

Figure 1


C36R
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C42
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C46
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C49
C50
Neutrophil DNA Enrichment RAW (%)
Time (days)

Figure 2

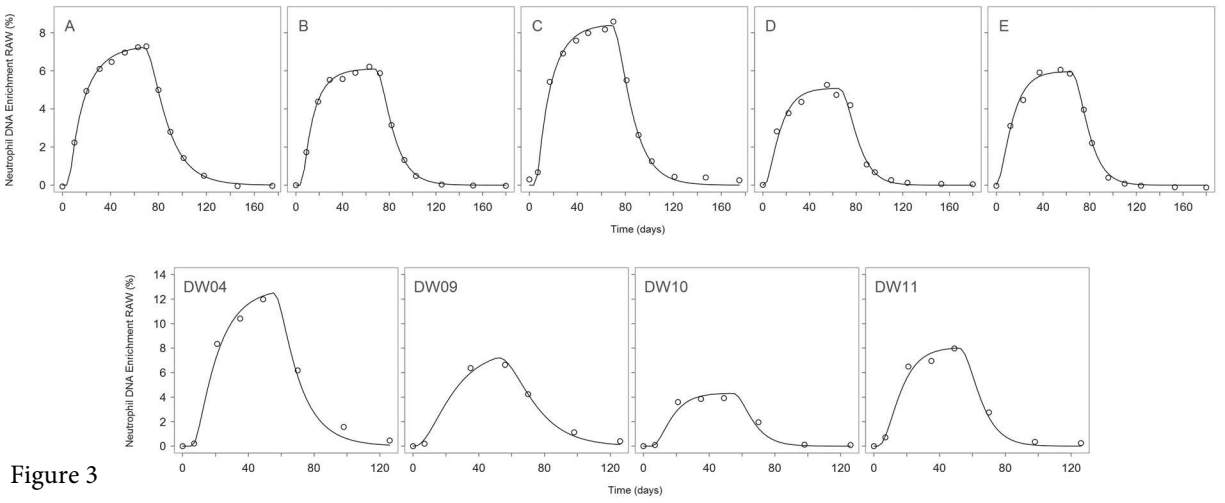
A
B
C
D
E
Neutrophil DNA Enrichment RAW (%)
0
2
4
6
8
0
40
80
120
160
Time (days)
DW04
DW09
DW10
DW11
Neutrophil DNA Enrichment RAW (%)
0
2
4
6
8
10
12
14
0
40
80
120
Time (days)

Figure 3