Brief report

In vivo labeling with ^2H_2O reveals a human neutrophil lifespan of 5.4 days

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Neutrophils are essential effector cells of the innate immune response and are indispensable for host defense. Apart from their antimicrobial functions, neutrophils inform and shape subsequent immunity. This immune modulatory functionality might however be considered limited because of their generally accepted short lifespan (< 1 day). In contrast to the previously reported short lifespans acquired by ex vivo labeling or manipulation, we show that in vivo labeling in humans with the use of ^2H_2O under homeostatic conditions showed an average circulatory neutrophil lifespan of 5.4 days. This lifespan is at least 10 times longer than previously reported and might lead to reappraisal of novel neutrophil functions in health and disease. (*Blood*. 2010;116(4):625-627)

Introduction

Neutrophils are indispensable for host defense.[1] In addition, these cells play a detrimental role in the pathogenesis of many acute and chronic inflammatory diseases. They can cause tissue damage through aspecific activation of their repertoire of antimicrobial mechanisms. Neutrophils also inform and shape subsequent immunity[2] and can prolong inflammation by release of cytokines[3] and chemokines.[4] There is an emerging concept that neutrophils directly influence adaptive immune responses through pathogen shuttling to draining lymph nodes,[5,6] antigen presentation,[7] and modulation of T helper 1/T helper 2 responses.[8] Along this line, neutrophils have been reported to be an important component of myeloid-derived suppressor cells mediating lymphocyte suppression in various experimental models of acute[9] and chronic inflammation.[10]

Targeting neutrophils in disease has mainly been focused on limiting their damaging capacity or directing their cytotoxic machinery to tumors.[11] Their immune modulatory functions have received little attention as potential targets in inflammatory diseases. This may at least in part be due to the current paradigm that these functions are of limited importance because of the generally accepted short circulatory half-life of neutrophils. Neutrophil lifespans have mainly been assessed by determination of ex vivo lifespans in culture (< 24 hours) and by transfer studies of ex vivo–manipulated neutrophils. The latter studies showed an estimated circulating half-life of approximately 8 hours in humans.[12] Ex vivo manipulation has been shown to have dramatic effects on neutrophil redistribution in vivo.[13] In mice, half-lives of 8 to 10 hours were reported when neutrophils were labeled in vivo.[14] In contrast, ex vivo labeling in mice showed that after transfer 90% of labeled neutrophils were cleared from the circulation within 4 hours, resulting in a half-life of less than 1.5 hours.[15] These differences between in vivo and ex vivo labeling strengthen our hypothesis that neutrophil transfer experiments may lead to an underestimation of neutrophil lifespan. The activation during ex vivo manipulation has probably led to retention in the lungs,[16] liver, spleen, and bone marrow (BM),[15] which may drastically reduce their circulatory half-life. To circumvent the complications introduced by ex vivo manipulation, we labeled the neutrophil pool in vivo in healthy mice and humans by administration of ^2H_2O in drinking water. Acquisition of label and appearance of labeled neutrophils in the circulation is characterized by (1) the rate of division in the mitotic pool (MP) in the BM, (2) the transit time of newly formed neutrophils through the postmitotic pool (PMP) in the BM, and (3) the delay in mobilization of neutrophils from the PMP to the blood. With the use of a combination of gas chromatography and mass spectrometry the fraction of ^2H-labeled adenosine in the DNA of the proliferating neutrophil pool was measured, and the kinetics of the neutrophil pool was determined.

Study design

Human volunteers

Five healthy male volunteers (characteristics described previously[17]) were included in the study after giving written informed consent in accordance with the Declaration of Helsinki. The labeling protocol and sample collection are described (supplemental Methods, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). This study was approved by the medical ethical committee of the Academic Medical Center Amsterdam.

Mice

C57Bl/6 mice were maintained under specific pathogen-free conditions in accordance with institutional and national guidelines. Twelve-week-old
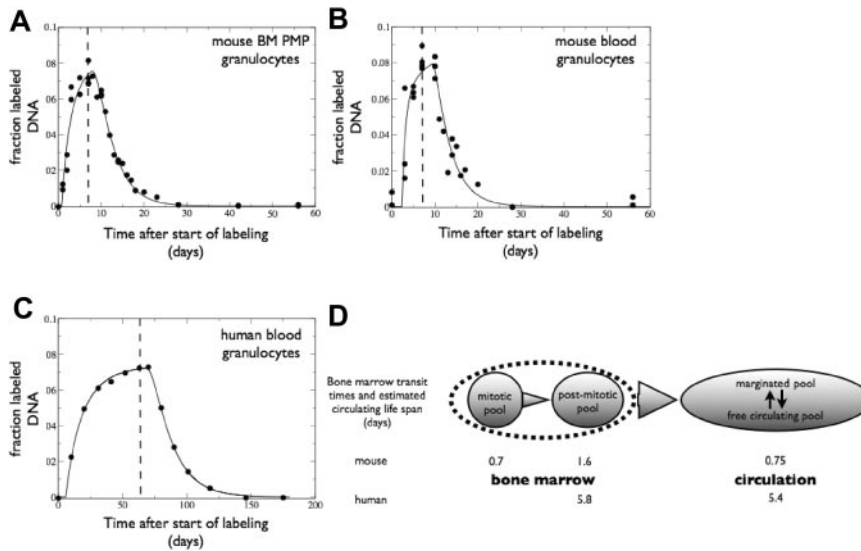
The online version of the article contains a data supplement.

Figure 1. Analysis of neutrophil turnover by in vivo ^2H_2O labeling. Cross-sectional up- and down-labeling of murine (A) BM PMP neutrophils and (B) blood neutrophils. (C) Representative example of longitudinal up- and down-labeling of human blood neutrophils; parameter values of 5 volunteers are given in Table 1. Dashed vertical lines indicate the time of label cessation on day 7 (A-B) or day 63 (C). Curves were fitted as described in "Methods," taking into account the actual level of label enrichment of plasma in mice or urine in humans (supplemental Figure 1). (D) Estimated median neutrophil lifespans and transit times (in days) of mice and humans. Murine estimates for transit times in the BM could be calculated directly; from the BM labeling data we calculated that labeled cells entered the PMP with a delay of $\Delta = 0.7$ days and that they had an expected lifespan in the PMP of $1/d = 1.6$ days (see "Mathematical modeling"). The total transit time in the BM of $0.7 + 1.6$ days matches the estimated $\Delta = 2.3$ days that resulted from analysis of the murine blood-labeling data. BM transit times in humans were estimated from the delay Δ with which labeled neutrophils were observed in the blood, whereas the circulating lifespan was calculated from $1/d$.

mice obtained a boost injection (intraperitoneally) of 16.5 mL/kg 90% ^2H_2O in phosphate-buffered saline (PBS; Cambridge Isotopes), followed by feeding with 4% ^2H_2O in drinking water for 1 week.

Neutrophil isolation

Neutrophils were isolated as previously described (supplemental Methods).

Mathematical modeling

Neutrophil lifespans were estimated by mathematical modeling, taking into account the availability of ^2H_2O in urine or plasma for men and mice, respectively (supplemental Methods).

On the basis of the assumption that in homeostasis neutrophils are a kinetically homogeneous population and that, as a consequence, the rate at which neutrophils are produced and enter the blood equals the rate at which labeled neutrophils are lost in a random manner from the circulation, we fitted the level of deuterium-enrichment of the DNA of neutrophils to the solution of the following formula: $dL(t)/dt = dcU(t-\Delta) - dL(t)$ in which $L(t)$ is the fraction of labeled DNA in neutrophils at time t (in days), c is an amplification factor that is required because of the multiple hydrogen atoms in a single adenosine deoxyribose moiety that can be replaced by deuterium, d is the average turnover rate of neutrophils and $U(t-\Delta)$ is the ^2H_2O enrichment of body water as measured from the urine or serum of men and mice, respectively.[17] We allowed for a time delay of Δ days between production of neutrophils in the MP of the BM and measurement of labeled DNA in the PMP or the blood.

Results and discussion

We first compared the dynamics of neutrophils in BM and blood of mice. We administered ^2H_2O to mice for 7 days and measured the

accrual and loss of label in plasma, BM-derived, and blood-derived murine neutrophils. Isolation of the BM PMP showed a transit time of 1.6 days in the PMP, in addition to 0.7 days of residence time in the MP (Figure 1A). The resulting combined delay of 2.3 days in the BM was confirmed by mathematical modeling of the blood data, which showed an estimated delay of 2.3 days (Figure 1B). The average half-life of circulating neutrophils in mice was estimated to be 12.5 hours, corresponding to an expected lifespan of 0.75 days (Figure 1B).

These estimates are in perfect agreement with results from previous in vivo studies on neutrophil lifespans in the blood and BM of mice.[14]

We next evaluated the dynamics of neutrophils in the peripheral blood of healthy human volunteers. We applied our mathematical model for ^2H_2O labeling to data of 5 healthy volunteers and estimated a median half-life of 3.8 days for circulating neutrophils, corresponding to an expected lifespan of 5.4 days in peripheral blood and a delayed exit from the BM of 5.8 days (Table 1; Figure 1C). The narrow confidence intervals of our fits support the reliability of these estimates. The estimated delay is in line with the transit time through the PMP of 6 to 7 days that was previously reported according to in vivo ^3H-thymidine or ^2H-glucose labeling of BM in humans.[12,18] In summary, our estimated half-lives of murine circulating neutrophils and human and murine BM neutrophils are in good agreement with previously reported half-lives based on in vivo labeling.[12,14,18] Our estimated human circulating neutrophil half-life of approximately 90 hours, however, is at least 10-fold longer than previous estimates that were based on ex vivo–labeled neutrophils.[12]

Table 1. Best-fitting parameters for the neutrophil labeling data of 5 healthy volunteers

Volunteer	d (days^{-1})	Half-life (days)	Lifespan (days)	Δ (days)
A	0.186 (0.156-0.241)	3.73	5.38	5.80 (4.92-6.91)
B	0.197 (0.160-0.250)	3.52	5.08	6.01 (5.13-6.84)
C	0.185 (0.145-0.218)	3.75	5.41	6.14 (5.22-6.54)
D	0.119 (0.077-0.192)	5.83	8.40	3.94 (0.19-6.61)
E	0.135 (0.106-0.211)	5.13	7.41	2.33 (0.23-4.70)
Median	0.185	3.75	5.41	5.80

d represents the average turnover rate of neutrophils (which represents their production and loss rate in steady state conditions). Δ represents the delay with which labeled cells from the BM reach the blood. Average lifespans were calculated as $1/d$ or were converted to half-lives as $\ln2/d$. Values in parentheses are 95% confidence intervals as determined by bootstrapping.

We investigated whether the discrepancies between the current and previous estimates for human neutrophil lifespans could be due to our assumption that neutrophils are kinetically homogeneous. To exclude the possibility that the longer estimated half-life could be due to a small contaminating population of relatively long-lived eosinophils, we fitted our data to a model including a second kinetic population (see supplemental Methods) with a circulating lifespan of 12 days, which represented 5% of the isolated cell population. This hardly affected the estimated half-lives of the remaining 95% of neutrophils (not shown). Conversely, when forcing one subpopulation of neutrophils to have an estimated lifespan as short as 12 hours, we found that to be compatible with our labeling data, either (1) this fast population had to be very small while the other neutrophils still had an expected lifespan of approximately 5 days, or (2) there had to be considerable heterogeneity between neutrophils, such that a second, slower, population of neutrophils would form a significant part of the cell population with estimated lifespans of up to approximately 10 to 20 days (see supplemental Table 1). Fits in which all neutrophils had an expected lifespan of 12 hours and BM neutrophils entered the blood with a delay of 7 days were incompatible with the labeling data. Our data therefore suggest that the expected lifespan of nonactivated neutrophils under homeostatic conditions is much longer than previously thought and that previous studies that used ex vivo manipulation have underestimated the circulating half-life of neutrophils because of activation and homing of these cells. In addition, clinical data, such as neutrophil kinetics after myeloablation and granulocyte colony-stimulating factor administration, probably have underestimated normal neutrophil circulatory half-lives, because of neutrophil activation and differential homing under these clinical conditions.[15]

These results have important implications for neutrophils during immune homeostasis. First, the general paradigm that murine and human neutrophil half-lives are similar is apparently incorrect. Second, combined with the novel view that human neutrophils can perform various immune modulatory functions, their relatively long estimated circulatory half-life may provide the incentive to target neutrophils to modulate immunity in cancer, autoimmune disorders, and vaccine development.

Authorship

Contribution: J.P. performed murine experiments, collected data, and wrote the manuscript, with contributions from the other authors as appropriate; I.d.B., N.V., and L.M.K performed murine and human labeling experiments; R.J.d.B. and J.A.M.B. developed and applied the mathematical modeling; and K.T. and L.K designed and coordinated the study. All authors discussed the results and commented on the manuscript.

Conflict-of-interest disclosure: J.P. receives a research grant from GlaxoSmithKline. L.K. has received a grant for a public-private partnership between the University of Utrecht, Dutch Government, and GlaxoSmithKline, Nycomed, AstraZeneca, and Danone ($1 000 000 for the period 2008-2012) and has a patent pending regarding active FcγRII as marker for acute and chronic inflammation. The remaining authors declare no competing financial interests.

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