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In vivo deuterium labelling in mice supports a dynamic model for memory T-cell maintenance in the bone marrow

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ABSTRACT

The maintenance and dynamics of memory T-cells in the bone marrow are a matter of ongoing debate. It has been suggested that memory T-cells in the bone marrow are maintained as long-lived, quiescent cells. We have recently shown that memory T-cells isolated from goat bone marrow undergo self-renewal and recirculate *via* the blood and lymph. Using the well-established memory T-cell markers CD44 and CD62L we here show very similar results in mice. This provides further support for the concept that memory T-cells are continuously self-renewing and recirculating between blood, bone marrow, spleen and lymph nodes.

1. Introduction

There is increasing evidence that the bone marrow (BM) plays a central role in the maintenance of memory T-cells. T-cells with a memory phenotype are abundantly present in the BM, and antigen-specific T-cells home there after priming to be recruited to the periphery upon re-challenge [1–5]. The maintenance and dynamics of memory T-cells in the BM are heavily debated. While several studies have suggested that the BM is the preferential site for memory T-cell self-renewal [1,6], other studies have proposed that memory T-cells located in the BM are sessile and rest in terms of proliferation [3,7]. This last hypothesis originated from the observation that memory T-cells isolated from murine or human BM consistently showed lower levels of cell division than T-cells isolated from murine spleen and human blood, as measured by Ki-67 expression.

Recently, we simultaneously quantified the *in vivo* dynamics of memory-like T-cells in BM, blood, and lymph nodes (LN) in goats [8]. We analysed the proliferation of memory $CD4^+$ and $CD8^+$ T-cells from blood, BM and LN by Ki-67 staining, and quantified their production rates using *in vivo* deuterium labelling. In line with previous studies [3,4,7], we found that the percentage of Ki-67^+ cells was significantly lower in BM compared to blood. Nevertheless, *in vivo* deuterium labelling revealed that memory T-cells isolated from BM have similar expected lifespans as those isolated from blood and LN [8]. Our results suggested that the majority of memory T-cells are maintained by constant self-renewal and are recirculating between the blood, BM, and LN.

Although, the goat model gave us the opportunity to obtain paired samples of blood, BM and LN to perform deuterium enrichment analysis, it also presented a major disadvantage, since the phenotype of memory T-cells in goats was not previously defined. Here, we sought experimental confirmation for our recent results, by performing a small-scale deuterium labelling study in mice. Using a similar experimental approach, we isolated T-cells from blood, BM, spleen and LN, characterized the naive and memory T-cell compartment in the different tissues based on CD44 and CD62 L expression, analysed T-cell proliferation by Ki-67 staining, and quantified the turnover rate of naive and memory T-cells using *in vivo* deuterium labelling.

2. Material and methods

2.1. Mice

129S2/SvPas Crl (129 Sv) mice (N = 10) were purchased from Charles River (Sulzfeld, Germany). Mice were housed and handled in accordance with good animal practice as defined by the Society of Laboratory Animals. All animal experiments were approved by the Animal Experiments Committee of Utrecht University, IVD Utrecht, The Netherlands; DEC AVD115002016714.

2.2. Stable isotope labelling

Ten 25-week old mice received 8% deuterated water (99.8% D_2O, Cambridge Isotope Laboratories) in their drinking water for 28 days. At day 4, mice were given an intra-peritoneal boost injection of 15 ml/kg

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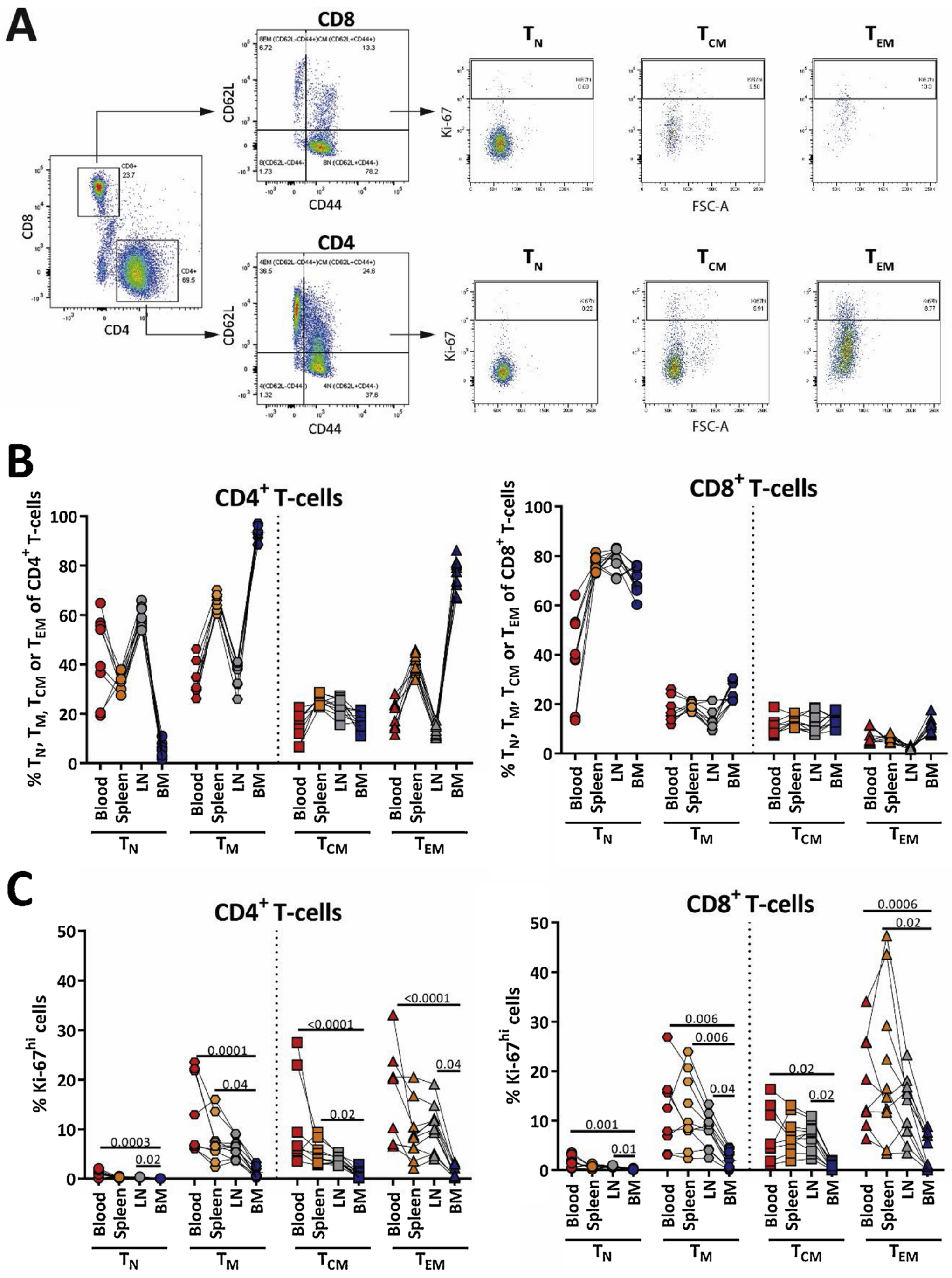

Fig. 1. Tissue distribution of naive and memory T-cell subsets and their respective expression of Ki-67. (A) Gating strategy used to identify T_N ($CD62L^+ CD44^-$), T_M ($CD44^+$), T_{CM} ($CD62L^+ CD44^+$) and T_{EM} ($CD62L^- CD44^+$) cell subsets within $CD3^+CD4^+$ and $CD3^+ CD8^+$ T-cells, and Ki-67 positive cells within the different T-cell subsets. A representative FACS staining from the spleen is shown. For the analysis in panel C the fraction of Ki-67^{hi} cells was used. (B) Frequency of T_N, T_M, T_{CM} and T_{EM} cells within $CD4^+$ (left panel) and $CD8^+$ (right panel) T-cells in blood (red), spleen (orange), LN (grey) and BM (blue). (C) Fraction of Ki-67^{hi} cells within T_N, T_M, T_{CM} and T_{EM} $CD4^+$ (left panel) and $CD8^+$ (right panel) T-cells in blood (red), spleen (orange), LN (grey) and BM (blue). Representative FACS staining of the different tissues can be found in Sup. Fig. 1, representative FACS staining and the gating strategy for Ki-67^{hi} cells can be found in Sup. Fig. 2 (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

D_2O in phosphate-buffered saline (PBS). To determine deuterium enrichment in the body water, EDTA-plasma was collected during the up- and down-labelling phase, and was frozen and stored at −80 °C until analysis.

2.3. Sampling and cell preparation

Spleen, BM, LN, thymus and blood were isolated at five different time points (N = 2 per time point) during and after label administration. Blood was collected in EDTA tubes. Single cell suspensions from spleen, BM, LN, thymus and blood were obtained as described previously [1].

2.4. Flow cytometry and cell sorting

Samples were stained for 30 min at 4 °C with anti-CD3-V500 (clone 500A2; BD), anti-CD4-APC-H7 (GK1.5; BD), anti-CD8a-BV786 (clone 53-6.7; BD), anti-CD44-Alexa Fluor450 (IM7; eBioscience), anti-CD62L-FITC (MEL-14; eBioscience) monoclonal antibodies. For intracellular staining, cells were subsequently fixed for 20 min at room temperature with 100 μl fixation/permeabilisation buffer of the FoxP3 Transcription factor staining set (eBioscience), permeabilised for 15 min at room temperature in 100 μl permeabilisation buffer, and stained with Ki-67-PE (clone 16A8, Biolegend) in 100 μl permeabilisation buffer for 30 min at room temperature. Cells were analysed on an LSR-Fortessa using FACS Diva software (BD Biosciences) and FlowJo software (version 9.8.3). Naive (T_N; $CD62L^+CD44^-$) and memory (T_M; $CD44^+$) $CD4^+$ and $CD8^+$ T-cells were sorted from spleen, BM and LN on a FACS Aria III (BD Biosciences) (Sup. Fig. 1). Representative FACS staining and the gating strategy for Ki-67^{hi} cells can be found in Sup. Fig. 2 and Sup. Fig. 3. The percentage Ki-67^{hi} cells was considered for the analysis of Ki-67 expression from Fig. 1C.

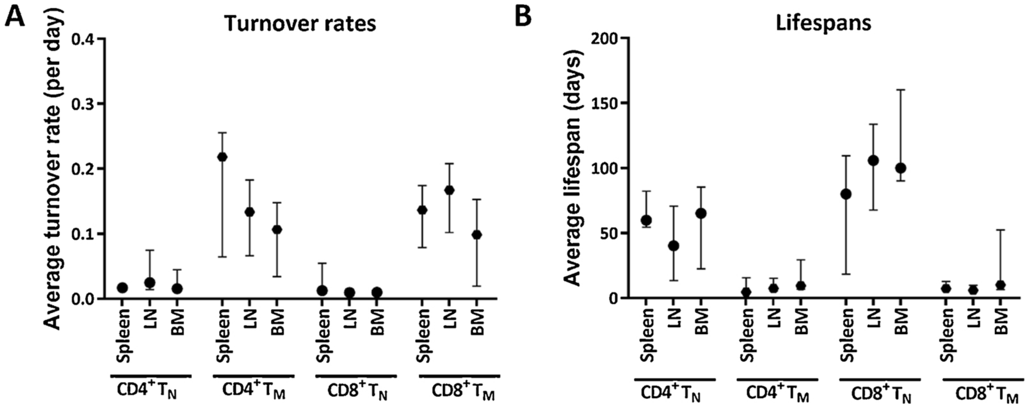

Fig. 2. Average turnover rates and expected lifespans of CD4$^+$ and CD8$^+$ T-cells. Estimated turnover rates (p; left panel, Sup. Table 2) and lifespans (1/p; right panel) of T_N (CD62L$^+$CD44$^-$) and T_M (CD44$^+$) CD4$^+$ and CD8$^+$ T-cells isolated from spleen, LN and BM.

2.5. DNA extraction

DNA was extracted from total thymocytes and from sorted T-cell subsets by suspending the frozen cell pellets in 200ul PBS, and subsequently boiling the samples for 10 min in a boiling water bath [10]. Samples were stored at −20 °C until further analysis.

2.6. Measurement of ^2H_2O enrichment in body water and DNA

Deuterium enrichment in plasma and DNA was analysed by gas-chromatography/mass-spectrometry (GC/MS) using an Agilent 5973/6890 GC/MS system (Agilent Technologies) as described previously [8,11].

2.7. Mathematical modelling of plasma and DNA enrichment data

We fitted a previously described model [11], which allows for heterogeneity between cells within an isolated population, to the labelling data of the different T-cell subsets. The labelling curves of T_M CD4$^+$ and CD8$^+$ T-cells from spleen and LN were significantly better described by a model including two kinetically different subpopulations while the other populations required only one subpopulation (for the fits of the model to the data see Sup. Fig. 4 and Sup. Fig. 5).

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism. Pairwise comparisons between two and more groups were performed using Kruskal-Wallis and Dunn's multiple comparisons test. P-values < 0.05 were considered significant. Deuterium-enrichment data were fitted with the function nlm in R. The 95% confidence intervals were determined using a bootstrap method where the residuals to the optimal fit were resampled 500 times.

3. Results and discussion

3.1. The fraction of Ki-67 positive T-cells in BM is lower than in their blood counterparts

We characterized the T-cell subset composition in each tissue by flow cytometric analysis of well-established T-cell markers in 129 sv mice. CD3$^+$CD4$^+$ and CD8$^+$ T-cells were further defined by the expression of CD62 L and CD44 as naive T-cells (T_N, CD62L$^+$CD44$^-$) and memory T-cells (T_M, CD44$^+$); within the memory T-cell population we further distinguished central memory T-cells (T_{CM}, CD62L$^+$CD44$^+$) from effector memory T-cells (T_{EM}, CD62L-CD44$^+$) (Fig. 1A). The subset composition of different tissues was different for CD4$^+$ and CD8$^+$ T-cells. For CD4$^+$ T-cells, T_{EM} was the predominant subset in BM (median of 78%), while the median fraction of T_{EM} in blood, spleen and LN was only 20, 40 and 14%, respectively (Fig. 1B, left panel). For CD8$^+$ T-cells, the T_N subset predominated in spleen, BM and LN (70–80%), with lower frequencies (15–60%) in blood (Fig. 1B, right panel).

Next, we determined the fraction of Ki-67^{hi} cells within the different CD4$^+$ and CD8$^+$ T-cell populations in paired samples from blood, spleen, BM and LN. T_M cells showed a higher proportion of Ki-67^{hi} cells compared to T_N cells at all sites. Both for CD4$^+$ and CD8$^+$ T_M cells, the frequency of Ki-67^{hi} cells was highest in blood, followed by spleen and LN, and lowest in BM (0.1%–3%) (Fig. 1C). These results are in line with our previous findings on Ki67 expression of memory T-cells in goats [8], as well as with previous findings in mice and humans [3,4,7,12]. We also analysed Ki-67 expression in T_{CM} and T_{EM} cells and again found the lowest frequencies of Ki-67^{hi} cells in the bone marrow (Fig. 1C).

3.2. Memory T-cells from BM, spleen and LN have similar turnover rates

Next, we analysed the turnover of T-cells at different sites in the body using *in vivo* deuterium labelling. Because the amount of blood is limiting in mice studies, we focused our analyses on T-cells isolated from spleen, LN and BM. The percentage of ^{2}H incorporation in the DNA of sorted T_N and T_M CD4$^+$ and CD8$^+$ T-cells was very similar for T-cells isolated from spleen, BM and LN (Sup. Fig. 4). Fitting the multi-exponential model to the labelling data revealed that for CD4$^+$ T-cells, we estimated that T_M cells live on average 5 days for the spleen, 9 days for the BM, and 8 days for the LN; for CD8$^+$ T-cells the estimated lifespan of T_M cells was on average 7 days for the spleen, 10 days for the BM, and 6 days for the LN (Fig. 2). The average turnover rates, and hence expected lifespans, of memory T-cells were not significantly different for cells derived from spleen, BM and LN (Fig. 2).

The fact that the T_M cells isolated from BM, spleen and LN turn over at very similar rates, while the fraction of Ki-67^{hi} T_M cells is remarkably low in BM as compared to spleen and LN, suggests that T_M cells continuously recirculate between these compartments. Our findings in mice thereby confirm our previous results in goats [8], and suggest that the constant turnover and recirculation of BM T_M cells is not specific for goats. We thereby provide further support for the view that, during homeostasis, memory T-cells are continuously self-renewing and recirculating between blood, BM, spleen and LN. Importantly, our conclusion is independent of the ongoing discussion whether T-cell proliferation occurs preferentially in the BM or not. Resolving the latter issue is complicated by the fact that in a system where cells are continuously recirculating, snapshot markers such as Ki-67 do not necessarily represent ongoing proliferation at a particular site.

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Author contributions

M.B.P., J.A.M.B. and K.T. wrote the manuscript; and M.B.P. and S.A.O. performed the experiments.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imlet.2019.04.004.

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