Maintaining memory T-cells: understanding models

Writing Assignment

Student

Reinier K. Groeneveld (4193822)

Utrecht University (UU)
Computational Biology Track
Molecular and Cellular Life Sciences

Examiner

Prof. dr. R.J. (Rob) de Boer

Utrecht University (UU)
Fac. of Beta-Sciences, Dept. of Biology
Theoretical Biology

Second Reviewer

Prof. dr. J.A.M. (José) Borghans

University Medical Centre Utrecht (UMCU)
Center for Translational Immunology

Abstract

Memory T-cells are an important second line of defence against intracellular pathogens, and against microbes capable of chronic or latent infection. Hence, immunological memory is a hallmark of the adaptive immune system. The bone marrow is key to protective immunological memory. In the central dogma, memory is provided by circulating memory T-cells. The memory T cells in BM are part of this. Some challenge this view, and claim memory T cells are also sessile and resting, while others instead claim that there is a proliferative fraction within this quiescent pool. As the role of proliferation in the bone marrow remains ambiguous to this day, mainly because of discrepancy between experiments, methodology, and interpretation, we decide to explore studies that incorporated mathematical models to address the complexity within their data, and more importantly, the uncertainty associated with their results. We first set a basis of the discussion throughout literature to provoke the mindset needed to understand the ambiguity of most recent discussions. We summarize the key results, strengths, limitations, and plausibility of seven modelling studies, in an attempt to forward the discussion on whether proliferation matters, and where possible, how much for long-term maintenance of immunological memory. Despite varying results, their approach, underlying assumptions, and subsequent validations expose the current difficulties of addressing the maintenance of T cell memory.

Keywords: immunological memory, T-cell, CD4+, CD8+, lymphocyte, proliferation.

Layman's summary

Your immune system protects your body from harmful invaders. Two parts are mainly important for the immune system. These are the innate immune system and the adaptive immune system. Together, they protect against harmful substances, germs and foreign cells.

The immune system can be activated by antigens. Examples of antigens are the proteins on the surfaces of bacteria, fungi and viruses, which are recognized to not be a part of the body. After the immune system encounters such a disease-causing invader for the first time, it stores information about the harmful substance. That way, the immune system is able to generate quicker and stronger immune responses to successive encounters with the antigen. This adaptive feature is called immunological memory.

Important for these responses is the presence of memory B and T lymphocytes. Also known as B and T-cells, respectively. These are antigen-specific cells produced during the primary immune response. Memory T-cells are an important second line of defence against intracellular pathogens and infection, where immunological memory can be said to be a hallmark of the immune system.

The bone marrow (BM) contains a major fraction of the memory T cells from the body. These exist of memory CD4+ and memory CD8+ T cells. Because many antigen-specific memory T-cells return to the BM after an infection, the BM is indicated to be important for immunological memory.

In general, it is thought that immunological memory is provided by circulating memory T-cells. This includes the memory T cells in the BM. However, there are groups that do not agree with this and claim that memory T cells are also resting and attached. On the other hand, there are other groups which show that not all memory T-cells are at rest. In their case, a part of the resting cells is even proliferating.

Whether proliferation has a role or not in the BM is still largely debated. Reasons for this is that there are differences between the types of experiments and methods. As a consequence, the results and eventually also the interpretations therefore differ.

In this review, we decided to explore studies that used mathematical models to study the behaviour of T cells. Because the other (experimental) studies do not agree with each other, we try to find out if the results from modelling studies can tell whether or not the memory T cells in the BM proliferate or not. We use seven modelling studies for this and summarize whether proliferation mattered for them or not maintaining immunological memory. Where possible, we also discuss how much.

This research is important because the debate about memory T cells being proliferative or not in the BM needs to be forwarded. Together, the findings show where results overlap in terms of dynamics and shows that further research is still necessary. We find out that the modelling studies also show how difficult it is to addresss the maintenance of memory T-cells in the BM.

Table of contents

Background: immunological memory is adaptive

Immunological memory is the capacity of the immune system to generate quicker and stronger immune responses to successive encounters with an antigen. Important for these responses is the presence of memory B and T lymphocytes (B and T-cells). These are antigen-specific cells produced from activated B and T-cells during the primary immune response, respectively [1]. Upon exposure to their antigen in secondary or subsequent immune responses, maintained memory B or T-cells are (re)activated to differentiate into plasma cells or effector T-cells, respectively [1,2].

The key features of this adaptive immune response are its specificity, and the ability to generate, plus maintain this memory to an antigen (or peptide) [3]. Whilst pre-existing antibodies in the circulation and at the mucosa provide the first line of defence against re-infection by extracellular as well as intracellular pathogens, memory T-cells are an important second line of defence against intracellular pathogens, and in particular against microbes capable of chronic or latent infection [3]. Hence, immunological memory is a hallmark of the adaptive immune system [4].

The bone marrow is actively involved in immunological memory

The bone marrow (BM) is key to protective immunological memory because it accommodates a major fraction of the body's plasma cells, memory CD4+, and memory CD8+ T-cells [45]. Together, they administer immunological protection and effective secondary immune responses to invading pathogens [5]. Many antigen-specific memory T-cells resort to the BM after an infection, further indicating the BM to be an important immunological memory organ [6,29,30]. Also, the BM contains more CD8+ effector memory (~2.8-fold) and more CD8+ central memory T-cells (~1.5 fold) than the spleen [7]. Therefore, the absolute number of all memory T-cells in the BM i.e., effector memory (T_{EM}), central memory (T_{CM}), naïve memory (T_{NV}), tissue-resident memory (T_{RM}); is thought to be substantial as well [45].

What is unique is that the BM accommodates all T-cell subsets irrespective of their activation or memory status [8]. Namely, the BM has been shown to be a preferred residence of (1) memory plasma cells [9,10], of (2) CD4+ memory T-cells [11] which are maintained in the (seeming) absence of antigen [12,13], and (3) naïve CD8+ T-cells. The BM has also been found to be a privileged site for the maintenance of (4) memory CD8+ T-cells [14,15,16]. One of the groups that showed this was the group of Radbruch, whom many agree, have challenged the general dogma of immunology memory [14,17,18,19,20].

In the central dogma, memory is only provided by circulating memory T-cells

The common belief about how immunological memory is being maintained after antigen clearance entails that memory lymphocytes circulate through the body and search cells presenting their cognate antigen [14]. The numbers of these memory lymphocytes are then determined by a balance between (homeostatic) proliferation and cell death. Proliferation primarily takes place in the BM (not everyone agrees, see next paragraph) where it is driven by interleukin-15 (IL-15) with the entire population having an estimated turnover of ~2 months (calculated for mice) [21-24]. Here, memory (CD8+) T-cells from the BM are considered part of the *circulating* memory T-cell population [19],

a pool that is maintained via homeostatic proliferation, driven by cytokines, and potentially persisting antigen [25].

Some show that the BM also maintains memory as resting memory T-cells

Radbruch's group showed that *mouse* memory CD4+ T-cells generated in immune responses are resting in the BM: the cells were maintained exclusively in the BM, disappeared from secondary lymphoid organs, and were resting in terms of transcriptional activity, proliferation, and mobility (absent from the periphery) [26]. Later, also *human* memory CD4+ T-cells were shown to reside in the BM [30]. More specific, the comparison of memory CD4+ T-cell repertoires from the blood and BM of individual donors revealed that memory T-cells specific for systemic childhood pathogens in many donors, were only and readily detected in the BM but not in the blood, which implies that both compartments are separate (instead of the same) and that these BM memory CD4+ T-cells are resting residents of the BM (more detailed analysis still needed).

Therefore, they and others [30], offer an alternative scenario for the maintenance of systemic and local immunological memory wherein aside from the circulating memory T-cells, memory is also provided by populations of memory T-cells residing in the BM. They argue that these memory T-cells are resting in terms of proliferation and mobility after antigen clearance, since a subset of 30% and of 60% of human CD4+ and CD8+ memory T-cells from BM, respectively, expressed CD69 [14,27] and resemble T_{RM} cells [14,16,19,30]. Briefly, a high proportion of CD4+ and CD8+ T_{EM} expressed CD69, a small percentage of CD4+ and CD8+ T_{CM} cells expressed CD69, whereas expression was absent from T_N cells [27].

The role for CD8 memory T-cells in the BM is still ambiguous

In contrast to CD4+ T-cells, the situation for BM residency of memory CD8+ T-cells is much less clear. First, for the BM memory CD8+ T-cells (1) cell cycle analysis by propidium iodide (PI) staining showed that more than 99% of the memory CD8+ T-cells in human BM are either in the G0 or G1 phase of the cell cycle, and (2) Ki-67 staining showed that 98–99.5% of the cells are indeed in G0 (resting in terms of proliferation) [30]. Similarly, for mouse memory CD8+ T-cells, more than 99% of the cells were in G0 or G1, according to PI staining, with 95% of them being in G0 according to Ki67 staining [14,6].

Second, these methods are arguably non-invasive and do not involve the manipulation of cells *in vivo*, in contrast to other methods like bromodeoxyuridine (BrdU) or carboxyfluorescein succinimidyl ester (CFSE) labelling [21-24]. Note that deuterium labelling is also non-invasive and finds short-lived average life spans [40]. Moreover, the demonstration that BrdU can induce proliferation of memory T-cells [14], together with the cell cycle analysis and Ki67 staining from above, has led to the claim that homeostatic proliferation has been overestimated previously [21-24], with possibly no role at all in the maintenance memory CD4+ and CD8+ T-cells [14,19].

Third, because up to 60% of the human BM memory CD8+ T-cells express CD69, a molecule that in CD4+ T-cells is essential for retention in the BM [14,16,30], and because their phenotype resembles T_{RM} cells with respect to the expression of CD69,

sphingosine-1-phosphate receptor 1 (*S1PR1*) and Kruppel-like factor 2 (*KLF2*), it was suggested that (1) bone marrow memory CD8+ T-cells are sessile and (2) similarity exist between BM memory CD8+ T-cells and non-migratory T_{RM} cells. Furthermore, these (60%) memory CD8+ T-cells express CD69 but not *S1PR1*: thus, they are not attracted to enter the blood circulation [30]. Finally, it has been recently shown that all memory CD69+CD8+ T-cells in the lung, spleen and lymph nodes are tissue-resident, non-circulating cells [31,32]. Therefore, that the BM instead provides survival signals for resident memory CD8+ T-cells, as it does for plasma cells [33,34,35].

Others find that the BM also maintains actively proliferating memory T-cells

The conclusion that CD8+ memory T-cells in the BM are quiescent and do not proliferate, opposes studies from other groups regarding the maintenance of CD8+ memory T-cells in the BM. For instance, the group of Francesca di Rosa has shown that BM CD8+ memory T-cells contain a higher percentage of proliferating cells than their counterparts in either the spleen or lymph nodes, and proposed that the BM acts as a niche for antigen-independent proliferation of CD8+ memory T-cells [21,23]. Two other studies, of which one was in non-human primates, concluded the same, that homeostatic proliferation of memory CD8+ T-cells are most profound in the BM [22,36].

In opposition, Di Rosa first argues it might be misleading to chiefly consider BM memory T-cells as non-circulating and non-dividinotng cells [18], and despite the fact that the frequency of dividing memory CD8+ T-cells in the BM is low, the absolute numbers of proliferating memory CD8+ T-cells is much higher in the BM than in the spleen and lymph nodes [22,21]. Second, BrdU-related artefacts may occur at high BrdU doses [14] but seem uncommon at the standard BrdU dose that was used in bone marrow T-cell studies [21-23].

Third, they argue that global transcription data, and CD69 expression profiles (and colocalization with stromal cells shown in tissue sections) are not appropriate for addressing *in vivo* T-cell migration. They point out that *in situ*-labelling studies and parabiosis experiments have shown that memory T-cells do recirculate to and from the bone marrow [37,38] (which does not exclude the existence of a separate resident population in those organs according to Radbruch [26,5]). Hence, the available evidence supports the view that the bone marrow is a 'stopping point' where recirculating memory CD8+ T-cells are stimulated to proliferate before continuing to move around the body [14,21,23,39].

Finally, an eventual revisitation (by Di Rosa) shows that data on proliferation agree [17]. The summary of published data on memory CD8+ T-cell proliferation in the BM reveals that proliferation results on *total* CD8+ and memory CD8+ T-cells were all higher in BM in comparison with corresponding cells from lymphoid periphery/blood: in all reports, spleen, LN, and blood were all lower than the BM over different experimental methods (DNA content, BrDU, CFSE) [17]. Therefore, discrepancies remain in the interpretations only [18,19].

The role of proliferation in the bone marrow is still undecisive

An attempt to interpret all these seemingly clashing results from the previous paragraphs has become the "Two Niches in the Bone Marrow" hypothesis (also by Di Rosa) which utilizes mechanisms from hematopoietic stem cells (HSCs) [42]. In brief,

the model proposes that recirculating memory T-cells are maintained within two separate types of BM niches. One is (a) quiescent and another is (b) proliferative (self-renewing). This division of labour between niches would result in (a) maintenance of quiescent T-cells and (b) support of actively cycling cells: the (a) 'quiescence niche' would protect from replicative senescence, as well as accumulation of DNA damage with aging, while the (b) 'self-renewal niche' would support division [43,44]. This possibly explains how a stable memory T-cell pool in the BM is maintained over time whilst allowing rapid secondary responses when needed [45]. Though experimentally unproven, some support this idea [45] of kinetical similarity between CD8+ T-cells and HSCs [46,47] because of the impact of T-cells on the hematopoietic process.

Further quantifications are needed to forward the discussion

It is evident at this point that further quantification is required to better understand how immunological memory lasts and is subsequently maintained. The main question is: How much does proliferation contribute to the long-term maintenance of memory T-cells? Particularly for the memory CD8+ T-cells in the BM [17]. The discrepancies between the results regarding the proliferation of CD8 memory T-cells in the BM remain unresolved [48]. However, their necessary disagreements and even most recent debates [41,49] have been insightful for showing that the differences in homeostatic proliferation are difficult to explain and need additional quantitation.

There is a limited number of modelling studies that analyse the dynamics of memory T-cell populations, their kinetics, and stochasticity, to determine whether and how proliferation maintains a stable memory T-cell population. Although they do not all focus on the BM or incorporate human specimen, the underlying assumptions of such studies, and the additional fitting of parameter values to experimental data, can provide a better understanding of factors contributing to long-term maintenance of immunological memory. Especially because most other experiments only deliver snapshots of a very dynamic environment [45].

We therefore next assess a selection of seven modelling studies chosen in the interest of their approach. They acquire experimental data and explore more than one data type or mathematical option to narrow down their interpretation about T-cell proliferation i.e., test stochastic versus deterministic nature [50]; fit four different proliferative models [51]; fit model to three types of experimental data and test heterogeneity against homogeneity [52]; test six states for cells transiting through S phase (or not) during differentiation [56]; fit to leukocyte subsets from three anatomical parts and test heterogeneity against homogeneity [58]; fit three lifespan models and test homogeneity against heterogeneity [59]; fit five proliferative models to independent short and long term data [61].

We summarize their mathematical model to understand what their findings imply for long-term maintenance of memory T-cells and whether their assumptions and parameters provide additional insight regarding the question addressed above i.e., proliferative, quiescent, homogenous, heterogeneous populations? We mainly focus on the simulated data that resulted from the fitting of mathematical models to experimental data and discuss, where relevant, key results, strong or weak points, and interpretations. For convenience, the sections of each study contain an "M", "H", or "G", indicating whether it involves a murine, human, or goat study, respectively.

Absence of memory CD4 T-cells does not affect memory CD8 T-cell proliferation (M)

To address whether or not CD4+ T-cells assist in the homeostatic turnover and long-term maintenance of the overall quality of memory CD8+ T-cells, Choo et al. (2010) transferred lymphocytic choriomeningitis virus (LCMV)-specific memory CD8+ T-cells into naive WT mice and longitudinally analysed in vivo division kinetics of cell turnover in the individual mice [50]. By fitting their mathematical model to CFSE profiles of memory CD8+ T-cells they first determined that the percentage of undivided cells decreased exponentially (>60 days post-infection). This suggested that there was a single homogeneous population of memory T-cells, and that the recruitment into division was stochastic (they hypothesize that a separate population of nondividing memory CD8 T-cells would not result in this exponential decrease; we note however that this population average perhaps does not reflect its sub-population kinetics).

Second, the number of divisions of CD8+ T-cells at a given time followed a Poisson distribution, where the *mean number* of divisions and the *variance* in the number of divisions both increased linearly with time, indicating that the division rate does not change over time, and in turn confirming a stochastic nature of memory CD8+ T-cell turnover; cells do not spend exactly the same time between successive divisions and the probability that a cell divides does not depend on its previous history.

Third, this stochastic turnover of memory CD8+ T-cells resulted in an average rate of division of 0.02 divisions per day, equivalent to an intermitotic interval time (mean time between two cell cycles) of approx. 50 days. This leads to the conclusion that adoptively transferred memory CD8+ T-cells into antigen-independent recipients are steadily maintained and actively undergo slow homeostatic turnover which is important for the long-term maintenance of memory CD8+ T-cells.

Next, to rule out a specific role for CD4+ T-cells, naive $CD4^{-/-}$ mice which lack CD4+ T-cells after deletion of the CD4 gene, were used as recipients for the adoptive transfer experiments instead of WT mice. Analysis of the associated CFSE profiles showed that the homeostatic turnover of memory cells was unaffected by the absence of CD4 T-cell help. Moreover, similarly to WT recipients, transferred memory CD8+ T-cells in $CD4^{-/-}$ animals were maintained steadily in their total number while retaining phenotypic expression and functions for the entire duration of the experiments. This suggests that CD4+ T-cell help is not required for the long-term maintenance of memory CD8+ T-cells in terms of quantity (see paragraph above) and quality.

Finally, to also rule out contamination of $CD4^{-/-}$ mice with MHC class II–restricted CD4- T-cells that may substitute the behaviour of the traditional helper CD4+ T-cells, they adoptively transferred memory CD8+ T-cells into uninfected MHC $II^{-/-}$ mice which also lack CD4+ T-cells. These donor memory CD8+ T-cells exhibited homeostatic turnover comparable to the WT and $CD4^{-/-}$ recipients. Both the phenotype of the donor memory CD8+ T-cells and their recall response (ability to respond to secondary challenges) remained unchanged, supporting the $CD4^{-/-}$ animal findings from above, that the homeostatic turnover and the long-term maintenance of the quality of memory CD8+ T-cells is independent of CD4+ T-cell help.

Interesting here is that the homeostatic proliferation of fully formed memory CD8+ T-cells does not depend on CD4+ T-cells for undergoing and for maintaining their function in the setting of an acute viral infection, and that CD8+ memory T-cells exhibit a stochastic nature while doing so. The authors note however that their interval time of approx. 50 days can slightly differ from other estimates throughout literature from colleagues [23] because of different cell labelling techniques or the type of memory cells

studied. Another caution they point out for comparing the study with another analysis, is the initial precursor frequency of antigen specific CD8+ T_N cells, since this may influence CD8+ T-cell response to an infection.

Stem cell-like memory T-cells require ongoing proliferation for maintenance (H)

To investigate how stem cell-like memory T (T_{SCM}) cells, an intermediate during differentiation from T_N to memory T-cell, are maintained in healthy humans in blood, Ahmed et al. (2016) measured the in vivo turnover of T_{SCM} cells using stable isotope labelling with heavy water (long-term, 7-weeks) [51]. Their approach involved the fitting of four mathematical models to the labelling data of sorted CD4+ T_N, CD4+ T_{SCM}, CD8+ T_N, and CD8+ T_{SCM} cells. They measure the source of label enrichment in each pool to describe the flux from a precursor compartment, to T_N, and eventually to T_{SCM} cells.

More specifically, in each of these four models a proliferative precursor compartment replenishes T_N cells. This T_N pool does not proliferate and only differentiates into T_{SCM}. Two scenarios are constituted for T_{SCM} cells: dividing (proliferation) or non-dividing, and two scenarios for T_N cells: differentiation is accompanied by division or not accompanied by division (one T_N cell gives two T_{SCM} cells or one T_{SCM} cell, respectively). These four models were also tested for a proliferative T_N pool, instead of only a differentiating pool, and with an eliminated precursor compartment that normally would replenish the T_N cells.

The results of the original four models yielded better predictions than the latter two subsequent variants. From the original four, a model in which both T_N and T_{SCM} cells do not proliferate, plus wherein T_N differentiation into T_{SCM} is not accompanied by division, was also excluded (visually bad fit). It was however not possible to separate the three other remaining models. These all indicated considerable replacement rates for the T_{SCM} population across lineages and subjects: median 0.02 p. day (inter-quartile range 0.016–0.037 p. day). Suggesting that the T_{SCM} pool is sustained by ongoing proliferation and cellular turnover rather than relatively dormant T_{SCM} cells.

To reinforce their findings, the authors show that T_{SCM} cells in vivo produce higher turnover rates compared to T_N cells by measuring Ki-67 expression (indicates active phases of the cell cycle) and telomere lengths (indicative of replicative history). Besides (limited) telomere length erosion, the T_{SCM} cells displayed higher levels of telomerase activity than either T_N or other memory T-cells, and high expression levels of Ki-67. Again, indicative of considerable turnover within the T_{SCM} compartment during self-maintenance.

Unfortunately, they do not show numbers for distinct sub-populations that might co-exist within one cell-type compartment. As they themselves also point out, there is possibility that their rapid turnover of T_{SCM} cells at the whole-population level reflects a composite of kinetically distinct subsets. Hence, it is impossible to state whether the T-cell differentiation pathway is linear or bifurcated e.g., a single T_N cell producing short-lived effector and long-lived memory T-cell, rather than producing only one product.

A sub-population of T_{SCM} cell show long lived maintenance of T-cell memory (H)

The study in the previous section [51] could not address if there was kinetical differentiation within the T_{SCM} population itself. For the sake of clarification, the same group produced a follow-up study by Costa Del Amo (2018) to investigate if the dynamics

within the T_{SCM} pool in healthy humans are compatible with maintenance of immunological memory. Their mathematical model incorporates three types of CD8+ T-cell data. In addition to stable isotope (heavy water) labelling, and telomere length, they decided to add a third dataset of cross-sectional T_{SCM} cell data from YFV vaccine recipients [55], to constrain the space of possible models, improve parameter identifiability, and to rule out ambiguous scenarios that produce equal levels of label in the T_{SCM} population of their experiment.

At first, only the isotope labelling and telomere length data were fitted simultaneously, to either memory CD4+ or memory CD8+ T-cell data, to find evidence for kinetic heterogeneity of sub-populations within the T_{SCM} pool. This fitting was done for a homogeneous model of constant population where the input rate (proliferation T_{SCM} + differentiation from T_N) of the whole T_{SCM} population equalled the disappearance rate of labelled T_{SCM} cells. Afterwards, in a heterogeneous version of the model, this restriction was taken out, permitting kinetic heterogeneity in the T_{SCM} pool.

Constraining the T_{SCM} population to be homogeneous resulted in poor fit to the data providing evidence to reject the assumption of homogeneity. Conversely, in the T_N cell pool, no evidence was found to reject the null hypothesis of homogeneity. They subsequently used the heterogeneous model to impose kinetic homogeneity on the T_{SCM} pool, which indicated that the average half-life of a T_{SCM} clone was short (< 1 year: 430 days). Despite that this explained the average turnover observed for the bulk T_{SCM} population (see previous section), they concluded that this was too short for a memory-maintaining-stem-cell-population. The average clonal half-life and total precursor lifespans were much lower compared to studies where the recall response to a previously encountered antigen has half-life in the order of decades (8–15-year) [53,54].

The model used up to this point allows for heterogeneity (or perhaps rather asymmetry) but only reports population averages i.e., the proliferation rate and clonal half-life are averaged across the whole T_{SCM} population. The authors hypothesize that averaging may disguise a small, long-lived population within the bulk short-lived population and therefore made a more complex system. This so-called explicit description of heterogeneity however provided no further information (data not shown).

Therefore, the authors fitted this explicit heterogeneity version of the T_N and T_{SCM} model to the additional dataset of CD8+ T_{SCM} cell response in humans (see first paragraph). Thus, three types of CD8+ T-cell data. Subsequently, evidence was found for at least 2 kinetically distinct sub-populations of CD8+ T_{SCM} cells that turn over at different rates (results for CD4+ not shown).

From the T_{SCM} cells that were generated upon clonal expansion of T_N cells, the majority differentiated into a sub-population characterised by rapid replacement with a half-life of 5 months, which explained the rapid average turnover of the bulk T_{SCM}. However, there was also a remaining fraction that entered a long-lived sub-population with a median half-life of 9 years, consistent with the recall response (see third paragraph). An additional Gillespie analysis further showed that despite small, that this latter population was not subject to excessive stochasticity. Hence, dynamics of a sub-population of T_{SCM} cells compatible with a putative role in the maintenance of T-cell memory.

A disadvantage however is that many of the model parameters were poorly identifiable. Nonetheless, the authors defend that the firm conclusions about clonal longevity and self-renewal can be still drawn. Another disadvantage is the usage of vaccination data generated using the YFV-17D vaccine, known for its ability to generate an exceptional

CD8+ T-cell response, and therefore perhaps not representative of average immunity. Note that the authors repeat the analysis without reference to YFV vaccination data to address this and confirm the conclusion that the T_{SCM} population consists of sub-populations with different. Finally, they notify that their study was confined to circulating T_{SCM} cells, and therefore, that interpretation needs caution because it has been shown that memory T-cell dynamics can vary across different anatomical compartments in mice e.g., spleen versus BM [49]. In short, although the role of the short-lived T_{SCM} sub-population is unclear, it shows that a subset of T_{SCM} cells, in healthy humans in vivo, have the dynamic properties of self-renewal, and that clonal longevity necessary to maintain long-lived immune memory.

T-cell proliferation and EdU labelled cells correlate by a linear 'signature' (M)

To assess the impact of genetics and aging on the immune system, Vibert et al. (2017) investigated the proliferation dynamics of T-cells from early differentiation in thymus to final maturation in spleen in two different strains of mice [56]. They used multicolour flow cytometry analysis during in vivo pulse/chase periods with EdU injection (a thymidine analogue) to reveal active DNA labelling of cells during S phase of the cell cycle. Manual gating allowed identification of unlabelled cells into either G0/G1 (G), S, or G2/M (M) that have *not* transited through S phase during pulse, and labelled cells (G'+S'+M') that did transit through S phase.

Through time, the percentage of EdU+ labelled cells were assessed according to differentiation stages, from most immature (CD3-CD4-CD8-) to the single positive mature stage (CD4+CD3+ or CD8+CD3+). Their mathematical model was fitted to this by means of a cytometry bi-dimensional dot plot that reveals the DNA content and intensity of EdU label. However, since their experiment stops with a pulse phase, the dot plot only permits delimitation of three groups: EdU labelled, G0/G1 unlabelled, and G2/M unlabelled cells. Thus, no unlabelled cells in S phase unfortunately. Further, the duration of G0/G1 phase, S, and G2/M gives the *inter-mitotic time* (an indication of the mean time between two cell cycles) and *proliferation rate* is defined as the number of dividing cells in G2/M phase (both unlabelled and labelled cells).

Their results for proliferation and intermitotic time were quite divergent. Both strains of mice showed a two-fold decrease of proliferation rates in thymus with aging whereas the opposite was true for the spleen, where the proliferation rates increase with age (percentage doubled in one strain and quadrupled in the other). Moreover, the duration of G0/G1 (and G2/M) phase also showed contrasting behaviour. In the thymus, the time spent in G0/G1 (and G2/M) increased with age, whilst decreased for the spleen, for both strains.

Likewise, the duration values were also quite heterogeneous e.g., G2/M duration varied from 2 hours in young thymocytes to 63 hours in young splenocytes. Moreover, with aging (2 vs 18 months old), the above-mentioned decrease in inter-mitotic time for thymocytes is found to result in a decrease of the total number of thymocytes in both strains. Note that the total number of dividing cells for thymocytes was not shown for splenocytes. We could therefore not assess if splenocytes also decrease with aging. Nevertheless, based on these findings the authors conclude that genetics and age must influence T-cell dynamics (likely memory as well).

Later, their model was also validated against another pulse-chase experiment from literature [57] to confirm plausibility of biological parameters obtained from fitting. As

a result, a 'signature' that links lymphocyte proliferation according to strains and age was found: a linear correlation emerged between the percentages of EdU+ cells and the proliferation rates per day. It turned out that this relation was very similar in the two strains of mice and was independent of age.

It is interesting, but perhaps not unexpected, that genetic origin (different strains) and age (old and young) revealed a specific signature, driven by T-cell proliferation and cell cycle phase duration, across multiple T-cell lineages and maturation stages. Nevertheless, few sidenotes must be taken. First, the authors point out that a correct understanding of kinetic heterogeneity within the whole population requires further identification into sub-populations, and that the estimated parameter values must be considered as averages over the total CD4 and CD8 population studied (like Costa Del Amo [52]).

Second, DNA content does not allow for individual estimates of G2 and M phases, and perhaps more problematically, for G0 and G1 phases: G0/G1 phase duration is an average between G0 and G1 durations, weighted by relative importance in numbers of cells (Ki-67 labelling studies to separate G0 from G1 cells are apparently in preparation (P. Loap et al.).

Third, the earlier mentioned linear 'signature', is most likely the result of mice that are in steady state; they argue that the possibility of transit to another compartment by labelled cells is limited, because their sixteen-hour long pulse/chase/pulse experiment is too short for this (samples from the thymus and spleen samples were taken half an hour after the final EdU injection). As a result, the number of labelled cells is proportional to the rate of entry into S phase and the proliferation rate.

Finally, the authors show a scheme of their model where exit of cells is represented either by death, differentiation, or migration. Yet, they later confusingly discuss that intermitotic time values are only indicative because cell death is not integrated in the mathematical model; they can only quantify the numbers of dead thymocyte cells at instant time (and not estimate rates/day). Dead cells were identified as apoptotic cells in sub G0/G1 and as cells with a DNA content >4, corresponding to macrophages engulfing dying cells. Nonetheless, despite that inter-mitotic time and cell phase duration are only indicative, they defend that the relative differences shown between genetic background and age remain valid.

Memory T-cells in BM are short-lived, self-renew, and continuously recirculate (G)

To investigate whether the BM plays an important role in the long-term maintenance of memory T-cells, Baliu-Piqué et al. (2018) used in vivo stable isotope (4 weeks deuterium) labelling in goats to simultaneously quantify the average turnover rates of memory CD4+ and CD8+ T-cells in BM, blood, and lymphoid organs (LN) [58]. They use the relatively large size of the goat to their advantage to obtain enough T-cells from paired samples of blood, BM, and LNs.

Although the T-cell subsets of the goat as an animal model are less well defined, their analysis differentiate CD4+ and CD8+ T-cell characteristics of memory T-cells (i.e., CCR7− T-cells) from T_N like features (i.e., CCR7+ T-cells). Their approach involves a mathematical model that was fitted to the labelling data (label enrichment of adenosine in the DNA of each sub-population) and allowed for kinetic heterogeneity between cells of the same population. Because population sizes hardly changed during the labelling and de-labelling phase, they assumed a steady state for each kinetic sub-population.

The fitting of the model to the deuterium enrichment in the DNA of memory CD4+ and CD8+ T-cells revealed no significant differences in the average turnover rates of memory T-cells isolated from BM (average lifespan of 50 days for CD4+ and 54 days for CD8+ T-cells), blood (44 days for CD4+ and 32 days for CD8+) and LN (54 days for CD4+ and 136 days for CD8+). On average, memory T-cells, even the ones located in BM and LN, were short lived, with an average lifespan of about 50 days. Suggesting similar turnover rates of CD4+ and CD8+ memory T-cells obtained from blood, BM, and LN.

Furthermore, the model suggested that the memory CD4+ T-cell pool in blood, LN, and BM, and the memory CD8+ T-cell pool in blood, was composed of at least two 2 kinetically different sub-populations, whereas labelling curves of memory CD8+ T-cells from the LN and BM were well described by a kinetically *homogeneous* model. The authors point out that even though they cannot exclude the scenario where a sub-population of memory cells of very long-lived cells was not picked up during deuterium labelling in the 4-week labelling period, that the data still indicate such a population does not preferentially reside in the BM (long-term in vivo labelling experiment still needed).

What is interesting however is that the authors beforehand showed that the percentage of Ki-67+ cells (indicates active and recent division) was higher in memory T-cells from blood compared to BM and LN ($p<1e-4$): average fraction of Ki-67+ memory T-cells from blood cells were 3.2% (CD4+) and 3.9% (CD8+), from BM were 1.1% (CD4+) and 1.3% (CD8+), and from LN were 1.1% (CD4+) and 1.3% (CD8+). Which thus contrast with their findings of the deuterium enrichment levels from above (the turnover rate), wherein in CD4+ and CD8+ memory (CCR7–) T-cells from blood, BM, and LN were instead very similar.

The authors also warn about Ki+67 interpretation, arguing that Ki-67 is a snapshot that inherently provides no information on the longevity of the cells, which in turn indicates that the source of T-cells taken as a reference impacts data interpretation. The authors point out that they have circumvented this problem by simultaneously comparing the kinetics of memory T-cells from BM, blood and LN earlier on (during fitting of mathematical model in the second paragraph). Where memory T-cells from BM (1) are shown to be maintained by continuous low-level proliferation by an average lifespan of 50 days, and (2) show no evidence for a long lifespan of either CD4+ or CD8+ memory T-cells (in BM) i.e., no significant differences were found in deuterium labelling, and hence in cellular lifespans, between memory T-cells isolated from the blood, BM, and LN (significance level not shown).

Finally, in CD8+ memory T-cells from BM and blood there was no enrichment of T_{RM} core transcriptional-signature-genes, indicating that most BM memory T-cells were not sessile (CD69 was not included). Moreover, because only 2% of these signature-genes were differentially expressed between CD8+ memory T-cells from BM and blood (thus quite similar), they argue that BM memory T-cells in goats continuously recirculate.

In brief, their findings indicate no evidence for a long lifespan of either CD4+ or CD8+ memory T-cells in BM, where the latter takes hold as a homogeneous and short-lived population, maintained by self-renewal, and continuously recirculates. The authors acknowledge that a similar in vivo deuterium labelling experiment would have to be done in humans, emphasizing that the use of different techniques and the comparison to different organs in general seem to lead to conflicting results.

Heterogeneous-age-independency depicts memory T-cell proliferation in vivo (H)

To investigate if T-cell proliferation in healthy humans depend on cell age, Costa del Amo et al. (2020) combined stable isotope labelling in vivo (both with heavy water and deuterated glucose) with stochastic, agent-based mathematical modelling [59]. Three paradigms were modelled to find out if this choice of model makes a significant difference for estimating parameters of T-cell kinetics (with age defined as time since division): (1) the cyton model, in which the probability of a cell dying or dividing increases with its age, (2) the risk model, in which the probability of death or division decrease as the cell ages;(3) the age-independent model, in which the probability of death or division is independent of cell age. All three were fitted to experimental data from a published stable isotope-labelling study [60] and compared for a homogeneous cell population or a heterogeneous population of two kinetically distinct sub-populations (at least two exponentials required to capture their dynamics).

The best description of stable isotope labelling data for CD4+ and CD8+ T_N cells was delivered by the *homogeneous* version of the age-independent model, whereas memory CD4+ and CD8+ T-cells by the *heterogeneous* version of the age-independent model. A note of care is that the homogeneous age-independent model was preferred when data was limited, which as the author remark, is owing to excess complexity of the heterogeneous models (i.e., the data are not sufficiently rich to support a more complex models).

As a point of concern, they therefore also criticize the generally used Corrected Akaike Information Criterion (AICc) that they use to compare the quality of model fits. Despite the fact that the AICc does identify the minimal model needed for describing the data, it can be debated whether this is the definition of the "true" model; the winning model is that with least number of parameters. Nonetheless, in support of their simulations, they argue that since the age-independent heterogeneous model (3 parameters) might be skipped in the model identification process, because is relatively more complicated than the cyton model (2 parameters) and the homogeneous age-independent model (1 parameter). When data becomes sparse, and there is insufficient data to support more complex models, the winning model thus becomes the homogeneous age-independent model.

Next, their approach was validated (1) by generating *in silico* data with noise and reverse-identifying each model, and (2) by using an alternative approach allowing parameters the freedom to take up the three paradigms. However, when assessed for a model's ability to predict new data [independent data that the authors generate themselves by using Annexin V binding to later quantify the fraction of labelled DNA in Annexin V+ and Annexin V– CD4+(CD45RO+) T-cells], only three of the six models provided successful signature prediction: the heterogeneous age-independent, and both the homogeneous and heterogeneous risk models correctly predicted that the fraction of Annexin V+ cells (early indicator for apoptosis) was significantly higher amongst labelled cells than non-labelled cells.

What is striking is that eventually no evidence is found to support the cyton model as a description of T-cell dynamics in humans in vivo. Not a single version ever provided the best description of in vivo heavy water labelling. This was true for all four T-cell populations considered: naïve CD4+ T-cells, naïve CD8+ T-cells, memory CD4+ T-cells and memory CD8+ T-cells. Moreover, an alternative approach which was independent of model complexity (fitting an unconstrained gamma or lognormal distribution) also found no evidence for the cyton model, and the cyton models all failed to predict patterns

in the above-mentioned independent and newly generated dataset. The authors remark that based on the evidence that the cyton model in vitro is so strong, it is surprising that it fails to explain lymphocyte dynamics in vivo.

They put forward three explanations. Firstly, the difference may relate to the dissimilarity between conditions in vitro and in vivo. Secondly, the difference may relate to contrasts between mice and humans. Thirdly, the difference might be related to stimulation, because in modelled scenarios there was no infection, whilst in earlier work that support the cyton model, lymphocytes were always stimulated exogenously. Finally, all participants were aged under 35 years (21-33 years old), perhaps indicating the need of older individuals (no reference from literature provided to support this).

Nonetheless, they argue that age likely determines cell fate in vitro but not in vivo, and that in the latter, cells receive external stimuli from other cells, antigen and cytokines, and thus extrinsic factors rather than age are more likely to determine cell fate in homeostasis. Additionally, simplifications in assumptions might affect results yet are necessary to avoid overparameterization. Overall, they conclude that the choice of model paradigm has a large impact on parameter estimates, and that they find no evidence to support the cyton model in humans in vivo to describe T-cell fate. We note however, that the data in this study was on slow renewal, whilst cyton has been mostly fitted to fast clonal expansion [64].

A power law predicts stabilizing memory T-cells and a decrease in death rate (H)

To analyse how immunological memory lasts a lifetime, Zarnitsyna et al. (2021) recently quantified (1) the division rate, and (2) the rate of cell death of Yellow Fever Vaccine (YFV)-specific CD8+ T-cells after immunization [61]. Their analyses incorporated data from one short term study (42 days - 1 year after immunization) [62] and another long-term study (0.3 month – 30 years after immunization) [63] where vaccination with the yellow fever virus vaccine (YFV-17D) resulted in long-term memory.

A set of five mathematical models were developed to explore the dynamics and turnover of the YFV-specific CD8+ T-cells: (1) one simple exponential model, with one population, and constant rates for division and death, (2) one bi-exponential model, with two populations, and two different sets of constant division and death rates, allowing an early expanding stem cell like population, (3) three different variations of a progressive quiescence model, with three different combinations of division and/or death rates that can change from an initial value at day 42 to an asymptotic value in the long-term.

These models were first fit to short-term data from Akondy [62] and used to estimate how the turnover rate of the YFV-specific CD8+ T-cell population declines during the early phase after vaccination. After individually being assessed for consistency, the best models were investigated and tested on how well they predict the long-term data from Marraco [63] to assess out of sample predictions up to three decades after vaccination.

By using the first year of data only, two quiescent progressive models and a simple power law accurately predicted T-cell frequencies up to 30 years post-vaccination, wherein division rates of YFV-specific CD8+ T-cells drop and stabilize at a low level of 0,1% p. day (0.001 p. day) after one year following vaccination. The death rates however continued to gradually decline from 0.18% per day during the first three years (half-life approx. 1 year) till 0.009% per day after 10 years. These YFV-specific CD8+ T-cells thus gradually live longer. Furthermore, the long-term decline in the number of YFV-specific CD8+ T-cells showed poor support for models where the division rate asymptotes to

zero in the long-term i.e., in model where cells stop dividing in the long-term, confirming that long-term memory in humans is maintained by a population undergoing turnover.

Finally, the authors claim the stabilizing decrease of the division rate can possibly be the result of a T_{SCM} population, but also that such interpretation needs caution. This assumes that changes in the populations in the blood are representative of changes elsewhere; data was obtained from cells circulating in the blood and thus their analysis might not apply to resident memory populations. Another caveat is that turnover of the YFV-specific CD8+ T cells is considered as a whole, thus does not provide insight to differentiation pathways between different sub-populations also likely to have different division and death rates. Also, memory is associated with changes in both the number of antigen specific CD8+ T cells and a change in their phenotype, and the study focuses only on the former. Still, the study exposes that a power law fit to the first year of data accurately predicted the initial decline and long-term maintenance of memory CD8+ T-cells over three decades.

Recap of the most relevant results

Altogether, the seven modelling studies handle diverse topics, and consequently a variation of results. Fortunately, all groups focussed on experiments performed under in vivo conditions, allowing for an interpretation of in vivo kinetics. To recap, we next summarize if results mattered for short- or long-term homeostatic proliferation, and if so, where and how much. Afterwards we relate key findings for the discussion of proliferation in the BM that we have provoked earlier on.

Choo et al. (2010) [50] confirmed that proliferation of memory CD8+ T-cells is observed in the peripheral blood of mice and that this characteristic, in terms of quantity and quality, is maintained in antigen-independent recipients, and even in the absence of memory CD4+ T-cells. They report a turnover of stochastic nature for memory CD8+ T-cells of on average 0.02 divisions per day, equivalent to an intermitotic interval time of ~50 days, which is a slow homeostatic turnover according to them.

Ahmed et al. (2016) [51] ratified that a flux of from a precursor compartment, to TN, and eventually to T_{SCM} cells, is best described by a T_{SCM} pool, that is sustained by ongoing proliferation with T-cell turnover rather than by relatively dormant T-cells, for both the memory CD4+ and CD8+ T-cells. The replacement has a median rate of 0.02 per day for the T_{SCM} population in peripheral blood of healthy humans, which they state to be a considerable rate.

Costa Del Amo et al. (2018) [52] found evidence for at least 2 kinetically distinct sub-populations of CD8+ T_{SCM} cells within an enveloping T_{SCM} pool, from peripheral blood in healthy humans. The was a large group characterised by rapid replacement of half-life 5 months, and another smaller long-lived sub-population with a median half-life of 9 years which was not subject to excessive stochasticity. Constriction to a homogeneous model on beforehand rejected the null-hypothesis of homogeneity in the T_{SCM} pool, whereas no evidence was found to reject the null hypothesis of homogeneity in the T_N cell pool.

Vibert et al. (2017) [56] produced a linear correlation between the number of cells labelled by EdU and proliferation rate, that emerges as a type of ‘signature’, regardless of mouse strains and age, for CD4+ and CD8+ T-cells. With aging, proliferation is shown to decrease for thymocytes whilst increase for splenocytes, whereas the duration of

G0/G1 (and G2/M) phase increased for thymocytes and decreased for splenocytes with age. Duration values varied from 2 to 63 hours in young splenocytes.

Baliu-Piqué et al. (2018) [58] obtained lifespans of on average ~50 days for memory CD4+ and CD8+ T-cells in BM of goats. They indicate this is short-lived and measure similar turnover rates of memory CD4+ and CD8+ T-cells between blood, BM, and LN. In the BM, memory CD4+ T-cell were composed of at least two 2 kinetically different sub-populations, but memory CD8+ T-cells from BM were better described by a kinetically *homogeneous* model. Memory CD8+ T-cells from BM and blood did not resemble T_{RM} core transcriptional-genes, hence arguably both continuously recirculate.

Costa del Amo et al. (2020) [59] depicted that the choice of model paradigm, in terms of cell fate, impacts parameter estimates heavily in vivo in blood of healthy humans. A *homogeneous*-age-independent version paradigm was suited for CD4+ and CD8+ T_N cells whilst a *heterogeneous*-age-independent paradigm was better for *memory* CD4+ and CD8+ T-cells. Correct predictions for early apoptosis were only delivered by the heterogeneous age-independent, and the homogeneous- and heterogeneous-risk models. No evidence was found for an increased probability of cell death or division with age.

Zarnitsyna et al. (2021) [61] found that a power law and two other quiescent progressive models can accurately fit short-term human immunization data of memory CD8+ T-cells from blood, and successfully predict long-term stabilization of memory CD8+ T-cells over three decades. Division rates stabilize eventually at a low level of 0,1% per day (0.001 per day) one year following vaccination, but the rates of cell death continue to decline gradually till 0.009% per day after 10 years, such that cells stop dividing in the long-term was not supported i.e., long-term memory in humans is steadily maintained by a population undergoing turnover.

Implications

Since the most direct results for the BM were given by Baliu-Piqué et al. (2018) [58] we take their study as a starting point to relate all other results. It is the only study to directly measure memory CD4+ and CD8+ T-cells from the BM, and revealed a short-lived memory CD4+ and CD8+ T-cell population in the BM. Despite the fact that the findings were for goats; their kinetical findings find overlap with some of the other results.

First, of interest is that they find evidence of at least two 2 kinetically different sub-populations of the memory CD4+ T-cell pool in blood, LN, and BM. According to the results that we have discussed of Choo et al. (2010) [50] the presence of these CD4+ T-cells should not be decisive in in terms of support and hence proliferation of memory CD8+ T-cells, at least for those in the blood, quantity and quality-wise. We thus next continue only for CD8+ T-cells.

Second, Baliu-Piqué et al. showed that the memory CD8+ T-cell pool in blood contained evidence for at least two kinetically different sub-populations. Of particular interest is that this was not shown to be the case in the counterparts from LN and BM. Namely, the memory CD8+ T-cells from the LN and BM were well described by a kinetically *homogeneous* model instead. This kinetic difference is in disagreement with the results from other studies that assess blood specimen, because it confirms that changes in the populations in the blood are indeed unrepresentative of changes elsewhere. Hence, we carry on for blood specimen and later discuss BM.

Third, Costa del Amo et al. (2020) [59] depicted that the choice of age model paradigm impacts parameter estimates heavily. They assessed cell fate in blood from healthy humans and indicated that a *heterogeneous* (age-independent) model paradigm was best suited for describing memory CD8+ T-cells. Likewise, Costa Del Amo et al. (2018) [52] found evidence for at least 2 kinetically distinct sub-populations in the blood. Hence, this provides additional support: despite the fact that the latter was shown for CD8+ T_{SCM} cells rather than memory CD8+ T-cells, both studies [59,52] agree with Baliu-Piqué et al., and show 'kinetical heterogeneity' in the CD8+ memory T-cells population in blood. We note however that Baliu-Piqué et al. provided information about the whole population only, not about the sub-populations associated with kinetic heterogeneity. We therefore next relate for 'averaged' population dynamics.

Fourth, lifespans of memory CD8+ T-cells from blood were argued to be relatively short-lived by Baliu-Piqué et al (32 days). This was comparable with the considerable replacement rates for the T_{SCM} population by Ahmed et al. (0.02 p. day; ~50 days) [51]. Similarly, the stochastic turnover of memory CD8+ T-cells by Choo et al. (2010) [50] were in a comparable range (0.02 p. day; ~50 days). Despite the fact that these rates were reported for goats, humans, and mice, respectively, all three quantifications indicate ongoing proliferation of memory CD8+ T-cells.

Additional confirmation for this ongoing turnover is published by Zarnitsyna et al. (2021) [61]. They revealed that here was poor support for models where the division rate of YFV-specific CD8+ T-cells drops to zero in the long-term. Altogether, these results agree that long-term immunological memory in the blood is maintained by continuous proliferation of memory CD8+ T-cells, similar to Baliu-Piqué et al. We next extend the discussion to quiescence.

Fifth, although kinetic heterogeneity is supported (see third comparison paragraph), we remark that small renewal rates, and the rates from previous paragraph as well, might still be compatible with a relatively quiescent and resident sub-population. This possibility of a (relatively) quiescent fraction is supported by the results from Costa Del Amo et al. (2018) [52]. They showed evidence for 2 kinetically distinct sub-populations of CD8+ T_{SCM} cells, within an enveloping T_{SCM} pool, from peripheral blood in healthy humans. One of these was a larger fast-circulating pool (half-life 5 months), whereas the other was a smaller slow-proliferating sub-population (half-life 9 years).

The same could be implied for the long-term decline in YFV-specific CD8+ T-cells by Zarnitsyna et al. This showed a gradual and continued decrease in the rate of cell death and therefore suggested that cells seem to eventually live longer. However, this numbers were given for the population as a whole. A model allowing a subsequent division of this population might permit a (relatively) quiescent second sub-population, even for small rates at late stage. We next move to recirculation.

Sixth, according to Baliu-Piqué et al., memory CD8+ T-cells from BM were better described by a kinetically *homogeneous* model. Striking is that a major fraction of memory CD8+ T-cells from BM and blood did not resemble T_{RM} core transcriptional-genes. Also, compared to each other, memory CD8+ T-cells from BM and blood transcriptionally differed by only 2% in terms of these T_{RM} signature-genes. Almost all memory CD8+ T-cells from blood and BM thus arguably continuously recirculate, were non-sessile, and show proliferation.

We finish by mentioning that it is unfortunate that Vibert et al. (2017) [56] did not prove insightful for our analyses on BM or memory CD8+ T-cells and on long-term data (although their introduction and discussion seemed to indicate so). Nonetheless, they

illustrate clearly, like Baliu-Piqué et al, that the source of T-cells heavily impacts data interpretation. We next finalize our discussion.

To conclude, whilst there is (1) overlap with regards to heterogenous dynamics for memory CD8+ T-cells from the blood, in which (2) memory CD8+ T-cells showed support for ongoing proliferation, and (3) YFV-specific CD8+ even showed poor support for cells that stop dividing in the long-term, we cannot rule out possible scenarios involving quiescence or dormancy. Hence, since (4) Baliu-Piqué et al. simultaneously compared the kinetics of memory T-cells from BM, blood and LN, to prevent bias into interpretation, and the (5) memory CD8+ T-cells from BM and blood transcriptionally did not resemble T_{RM} core-genes, yet (6) at the same time did resemble each other in terms of these T_{RM} core transcriptional-genes; we therefore choose to at least follow the postulations from Baliu-Piqué et al., that most memory CD8+ T cells from BM continuously recirculate, show signs of self-renewal, are accompanied by a heterogeneous memory CD8+ T cell population in the blood, where there might still be a quiescent sub-population.

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