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HIGHLIGHTS

Basic

COMMENTARY

Maintenance of memory CD8 T cells: Divided over division

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Once generated during an infection, memory CD8$^+$ T cells can provide long-lasting protection against reinfection with an intracellular pathogen, but the longevity of this defense depends on the ability of these pathogen-specific memory cells to be maintained. It is generally believed that the bone marrow plays an important role in this respect, where memory CD8 T cells receive reinvigorating signals from cytokines that induce homeostatic proliferation. However, in the current issue of the *European Journal of Immunology*, Siracusa et al. (Eur. J. Immunol. 2017. 47: 1900–1905) argue against this dogma, as they provide evidence that CD8 memory T cells in murine bone marrow are not proliferating, but largely quiescent, which protects them from elimination by the cytostatic drug Cyclophosphamide. Interestingly, this is in sharp contrast to the proliferating cell counterparts in the spleen, which are eliminated by this treatment. Here, we will discuss the impact of these results, how they relate to opposing findings by others in the field, and what the relevance of these findings is for humans and clinical applications.

Keywords: Bone marrow · Cellular proliferation · Chemotherapy · Cyclophosphamide · Memory cells

See accompanying article by Siracusa et al.

The body's ability to keep a fraction of pathogen-specific T cells alive after an infection is a key feature of the adaptive immune system, as these memory cells provide rapid protection upon reinfection with the same pathogen. Long-term maintenance of these memory T cells occurs in the absence of antigen [1, 2] and is considered to depend on homeostatic proliferation driven by cytokines such as IL-7 and IL-15 [3–5]. Although memory T cells are present throughout the body, both in lymphoid and non-lymphoid organs, the bone marrow (BM) is a major player in this respect [6–8]. The BM contains many stromal cells that express IL-7 and IL-15 [9–11] and it was concluded, based on in vivo Bromodeoxyuridine (BrdU) labeling experiments and adoptive transfer of CFSE-labeled memory T cells, that homeostatic proliferation of memory CD8 T cells is most profound in the BM [12]. However, this dogma has been challenged by the group of Andreas Radbruch. This group first described that memory CD8 T cells in the BM express only very low levels of the Ki-67 protein, which indicates that these cells are quiescent, rather than actively cycling [10]. Furthermore, they showed that BrdU-incorporation not only reflects, but could also induce cell cycle progression, which provides a proper explanation for their opposing results with previous reports. In a new manuscript in the current issue of the *European Journal of Immunology* [13], the same group corroborates their findings using the cytostatic drug Cyclophosphamide (CyP), which induces apoptosis in proliferating cells due to DNA crosslinking [14]. They show that treatment of mice with CyP induces DNA crosslinking in memory CD8 T cells in the BM, but this does not kill the cells unless proliferation is induced by TCR triggering [13]. This is in strong contrast with the non-quiescent memory CD8 T cells in the spleen,

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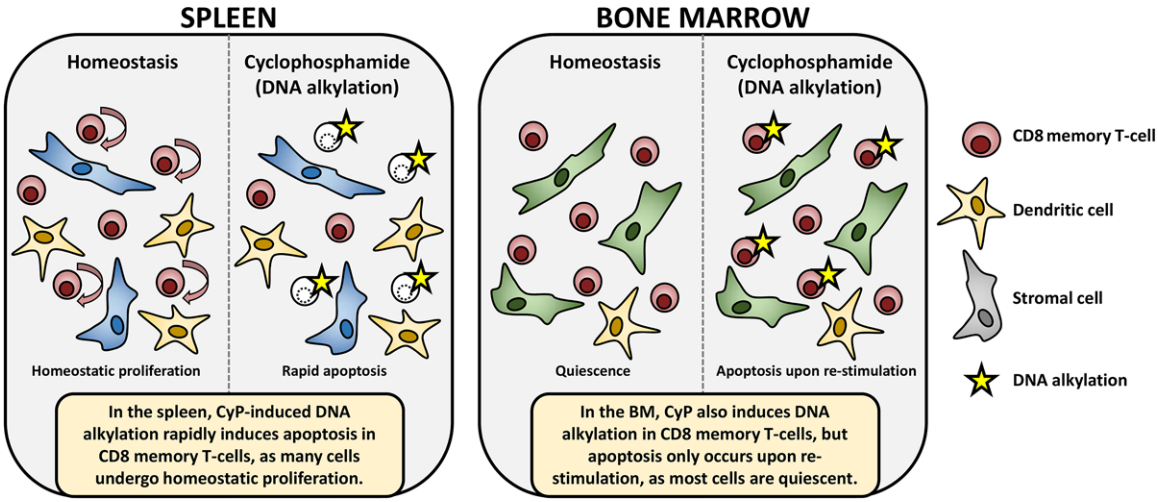

Figure 1. Siracusa et al. [13] show that in the spleen, CD8 memory T cells are rapidly depleted upon CyP treatment, as they undergo homeostatic proliferation. In contrast, CD8 memory T cells in the BM are mostly quiescent, and thus protected from immediate depletion by CyP. These differences could be due to the supporting cell types that are present in spleen and bone marrow (e.g, more dendritic cells in the spleen), and/or differential expression of homeostatic cytokines.

which are rapidly killed by CyP treatment (Fig. 1). Importantly, by treating mice with the drug FTY720, which inhibits egress of lymphocytes from lymphoid organs, the authors demonstrate that maintenance of memory CD8 T cells in the BM upon CyP treatment is not caused by a compensatory influx of peripheral CD8 T cells. A caveat of the study is that CyP also has some non-specific side-effects: CyP can deplete proliferating Tregs that in turn affect T cell homeostasis [15], and CyP has been shown to induce type 1 interferon and thereby increase proliferation of $CD8^+$ memory T cells [16]. Nevertheless, the findings of Siracusa et al. [13] challenge our view on how memory CD8 T cells are maintained and suggest that the underlying mechanism may differ per organ.

The conclusion that CD8 memory T cells in the BM are quiescent and do not proliferate, opposes previous studies from other groups regarding the maintenance of CD8 memory T cells in the BM. The group of Francesca di Rosa has published two papers showing that BM CD8 memory T cells contain a higher percentage of proliferating cells than their counterparts in either the spleen or lymph nodes, and this group proposed that the BM acts as a niche for antigen-independent proliferation of CD8 memory T cells [17, 18]. Two other studies, of which one was in non-human primates, came to the same conclusion that homeostatic proliferation of memory CD8 T cells is most profound in the BM [12, 19]. The discrepancies between the results of the Radbruch group and other groups regarding the proliferation of CD8 memory T cells in the BM remain unresolved, and could be due to many different factors. One key issue is the suspected induction of proliferation by BrdU treatment [10, 18]. Most studies that have claimed higher proliferation of CD8 memory T cells in the BM as compared to the spleen, have done so based on BrdU incorporation by CD8 memory T cells [12, 17, 18]. In their 2015 study, Radbruch and colleagues showed that a 3 day treatment of 1 mg/mL BrdU in drinking water supplemented with sugar (which increases water consumption [20]) is sufficient to induce CD8 memory T-cell proliferation, with up to 75% of all CD8 memory T cells in the BM and spleen proliferating [10]. Of note, these percentages were much higher than those reported by the aforementioned studies [12, 17, 18]. In contrast, Di Rosa and colleagues showed that their standard treatment regimen of 3 days with 0.8 mg/mL BrdU in the drinking water does not increase the total number of divided CD8 memory T cells [18]. However, their readout for proliferation was the fraction of T cells that had diluted their CFSE content, rather than the Ki-67 expression measured by Radbruch and colleagues [10], which could also make a difference. Thus, BrdU might affect proliferation of CD8 memory T cells, but the threshold for and/or the extent of the effect may depend on the dosage and, most likely, the duration of BrdU treatment. The effect of BrdU on cell proliferation is not limited to CD8 memory T cells as it also induces the cell cycle progression of quiescent HSCs [21] and possibly affects a myriad of other cell types as well. Therefore, it is of great importance to control for the direct effect of BrdU on proliferation and to also calculate the BrdU intake in individual mice in future proliferation studies. Alternatively, using DNA-labeling agents with lower toxicity, or incorporating deuterated glucose in the DNA of dividing cells, may prove more reliable to measure CD8 memory T-cell proliferation [22–24].

Besides in vivo administered DNA-labeling agents, proliferation can also be measured *ex vivo* by staining for Ki-67, which labels all cells in all phases of the cell cycle except those in G_0 [25], and/or a staining for DNA content that detects cells actively proliferating in $S/G_2/M$ phase. Of note, both the group of Radbruch [10, 12] and an earlier study by Becker et al. [10, 12] show that the frequency of actively dividing cells is in fact higher in the BM than the spleen in mice not treated with BrdU. Interestingly, proliferation of CD8 memory T cells is also markedly higher in the BM than in the spleen and lymph nodes when homeostasis is disturbed by high doses of BrdU or with the double-stranded RNA-mimic polyI:C [10, 12]. Thus, although most CD8 memory T cells in the

BM rest in terms of proliferation under homeostatic conditions, the BM seems particularly capable of supporting their proliferation when homeostasis is disturbed. Herein may also lie an explanation for the high proliferation rates of CD8 memory T cells in the BM that other groups have reported [12, 17, 18] as compared with the findings from the group of Radbruch [10], as mice from different housing facilities have a differential microbial make-up, and the microbiome has substantial influence on the murine T-cell compartment [26].

Beyond the proliferation issue, Siracusa et al. [13] show that when T-cell recirculation is blocked with FTY720, CD8 memory T-cell numbers in the BM remain unaltered by CyP treatment. From these findings they conclude that both $CD69^+$ and $CD69^-$ CD8 memory T cells, as well as $CCR7^+$ and $CCR7^-$ $CD8^+$ memory T cells, must be resident [13]. However, this conclusion can be questioned, as FTY720 blocks egress of T cells from various tissues, including the BM [27]. Therefore, an alternative explanation for these findings is that there is an equilibrium between BM entry and exit under homeostatic conditions, and that blocking T-cell circulation merely provides a snapshot of CD8 memory T cells present in the BM at a specific moment. In fact, there is ample evidence for continuous migration of memory T cells to and from the BM, and this has been demonstrated to be important for homeostatic maintenance [28–30]. The recirculation of memory T cells from the BM to the lymph nodes or spleen is probably also important for efficient recall responses, as their residency in the BM will most likely not be beneficial for their protective role upon re-infection.

The observed difference in CyP-sensitivity between CD8 memory T cells in the BM versus spleen [13] raises the question why these cells should undergo homeostatic proliferation in the spleen rather than the BM. It is conceivable that quantitative and/or qualitative differences exist between these organs in supporting cell types and expression of the homeostatic cytokines on which memory T cells depend. IL-15 is a potent proliferative agent for CD8 memory T cells [31], whereas IL-7 rather ensures T-cell survival [32]. It could thus be that CD8 memory T cells in the BM are mostly exposed to IL-7, whereas a splenic environment may be richer in IL-15 and induce proliferation. Indeed, $CD8^+$ memory T cells in the BM are located close to VCAM-1^+ IL-7^+ stromal cells [10]; however, these cells also express IL-15 [11]. Jung et al. showed that the opposite is in fact true and that IL-7 is important for CD8 memory T cells in the spleen, whereas IL-15 plays a major role in the BM [33]. Furthermore, in human BM most CD8 memory T cells localize close to IL-15 producing cells [34]. This means that the difference in homeostatic proliferation between memory CD8 T cells in the BM and spleen cannot simply be explained by differential expression of IL-7 and IL-15. However, the context in which these cytokines are presented could also be important, as it has been suggested that memory CD8 T cells may depend for their homeostasis on IL-15 that is trans-presented by dendritic cells [35], which are more abundant in the spleen than BM [36] (Fig. 1). Taking all these considerations into account, it would be interesting to examine the impact of CyP on CD8 memory T cells in the spleen versus BM in IL-$7^{-/-}$ or IL-$15^{-/-}$ mice. Other cytokines could also be of interest, such as IL-10, which has an incompletely understood positive effect on CTL responses in humans and mice [37–39]; in particular IL-10 strongly enhances IL-15-driven, TCR-independent "homeostatic" proliferation of human CD8+ memory T cells [40], although it is not known whether there are differences in IL-10 signaling in $CD8^+$ memory T cells in the BM vs spleen. Another thought-provoking concept is that memory T cells not only migrate to the BM to receive maintenance signals, but that they actually have a purpose there and that they are able to modulate the local blood-forming process. T-cell activation can directly affect the differentiation and proliferation of hematopoietic stem and progenitor cells (HSPCs), whereas memory CD8 T cells can support HSPC engraftment upon transplantation [41]. Moreover, unpublished observations from the group of Nolte indicate that memory CD8 T cells have a beneficial effect on the survival and maintenance of HSPCs. Addressing the importance of the interplay between adaptive immunity and hematopoiesis in the BM will be an exciting new avenue of future research.

What are the implications of the concept proposed by Radbruch and colleagues for the maintenance of human memory T cells and for the clinic? There is clear evidence from vaccinia virus-experienced individuals that human T-cell memory can be maintained in the absence of antigen for several decades, i. e. for a lifetime. This is not a trivial finding, because human beings are continuously exposed to pathogens that trigger T-cell responses, and memory T cells with new specificities need therefore to be continuously added to the memory pool. How this is achieved is rather difficult to address in the human system, but the available evidence suggests that the mechanisms controlling T-cell homeostasis in humans and mice are similar. Thus, early studies have shown that human blood $CD8^+$ memory T cells from healthy individuals proliferate with IL-7 and IL-15 in a TCR-independent manner in vitro, and some spontaneously incorporated BrdU *ex vivo*, suggesting that human $CD8^+$ memory T cells also proliferate in the steady state in vivo [42]. Consistently, in an elegant study with deuterated glucose to label DNA of dividing cells in vivo, it was shown that human blood $CD8^+$ memory T cells proliferated in healthy individuals, and proliferation dramatically increased in patients with acute EBV infection [24]. It is, however, unclear if this turn-over in the steady state is driven exclusively by homeostatic cytokines or due to antigens from persistent pathogens. Human $CD8^+T_{CM}$ and T_{EM} subsets express different levels of IL-7Rα and IL-2/15Rβ [42] suggesting that they respond preferentially to IL-7 and IL-15, respectively. This notion is supported by the different proliferation rates of CCR7-deficient and -sufficient CTL in mice that lack either IL-7 or IL-15, respectively [33]. Notably, T_{EM} and T_{CM} in human peripheral blood had a higher turnover as compared with the terminally differentiated T_{EMRA} subset [42], and similar findings were reported for $CD8^+$ T-cell subsets analyzed by Ki-67 staining in human BM [43]. Radbruch and colleagues reported that the fraction of Ki-67^+ proliferating cells was significantly higher in $CD4^+$ and $CD8^+$ human memory T cells in peripheral blood, compared to the BM of the same individuals [44], and more prominent in cells that lacked IL-7Rα or CD69 expression. These data suggest that also in humans the majority

of memory cells in the BM are resident cells that survive in the absence of proliferation with IL-7, but it could also be explained by the two-niche hypothesis proposed by di Rosa [45]. Intriguingly, in the same study the authors reported that $CD4^+$ memory T cells responding to systemic pathogens were enriched in the BM [44]. However, the increased responsiveness of human T cells in the BM to antigenic stimulation [46] is a possible caveat here, and MHC multimer staining for antigen-specific T cells should be performed to corroborate this important concept.

Finally, it should be stressed that the question of whether the maintenance of T-cell memory requires proliferation is not a purely academic debate, but is also a relevant issue for the clinic. For example, it has obvious implications for protective memory against pathogens in cancer patients undergoing chemotherapy [47]. Moreover, depleting proliferating T-lymphocytes with CyP could prevent graft-versus-host disease following transplantations [48]. In addition, cladribine, a drug that depletes proliferating lymphocytes and leukemia cells, has been recently approved as a therapy for relapsing-remitting multiple sclerosis [49]. As the BM contains also auto-reactive T cells, the presence of resting, but potentially pathogenic, T cells [50] in the BM could lead to long-term resistance to this new promising therapy. In conclusion, the results of Siracusa et al. [13] are important for stimulating discussion and research in the field to resolve these key issues.

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