BONE MARROW T CELLS AT THE CENTER STAGE IN IMMUNOLOGICAL MEMORY

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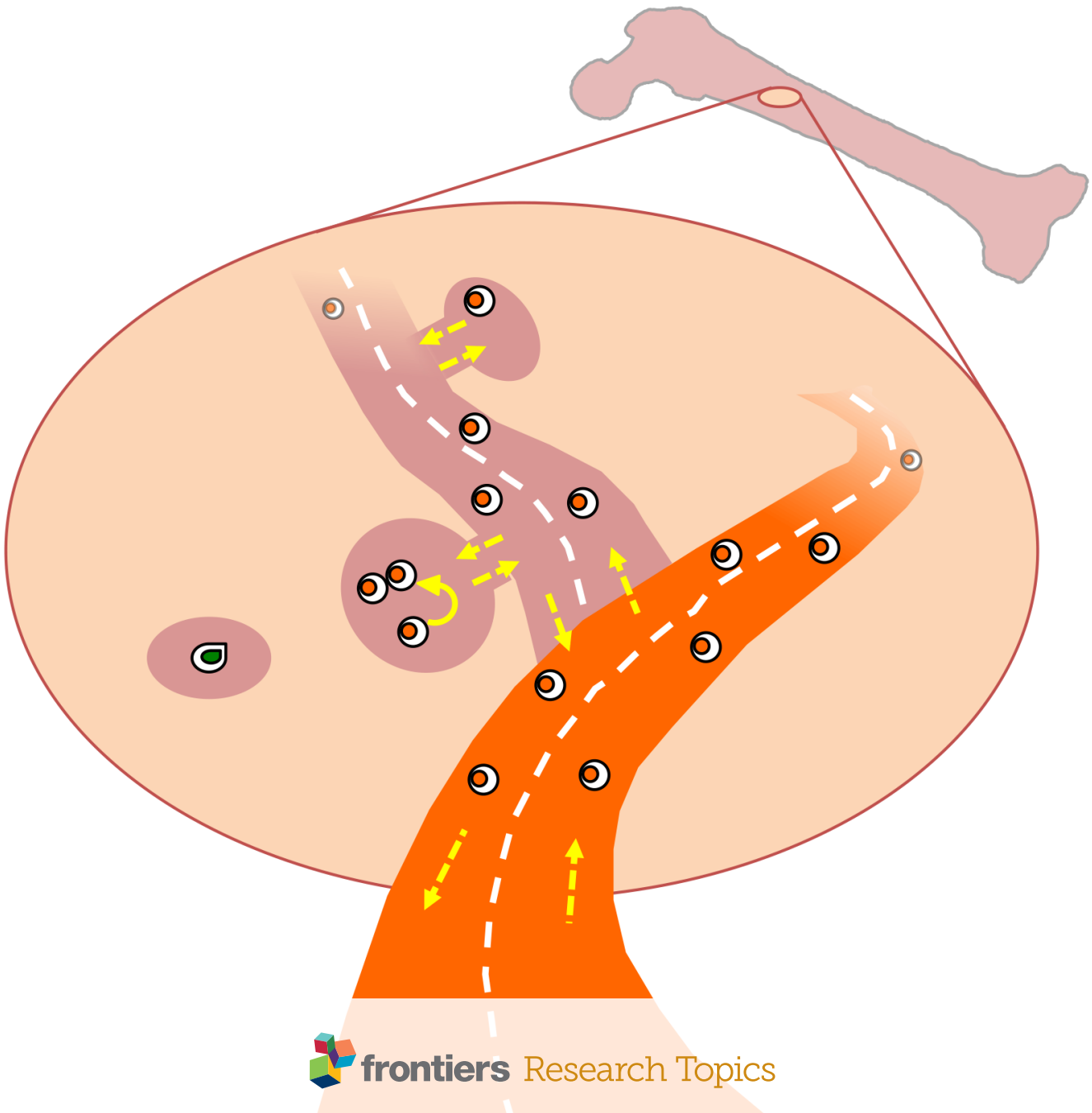

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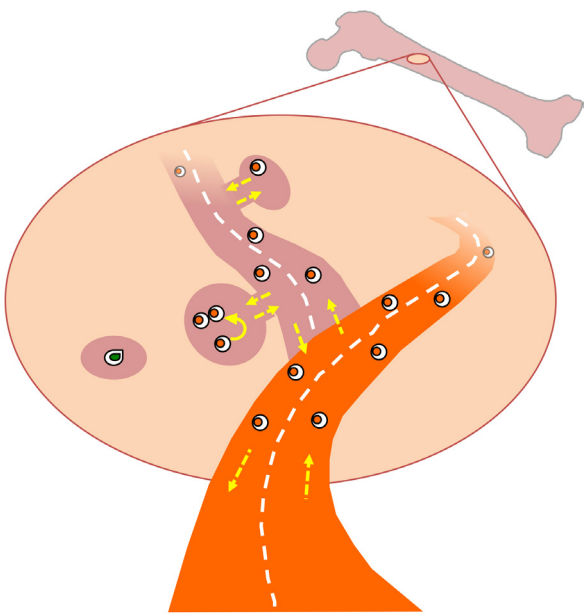
BONE MARROW T CELLS AT THE CENTER STAGE IN IMMUNOLOGICAL MEMORY

Topic Editors:
Francesca Di Rosa, Consiglio Nazionale delle Ricerche, c/o Department of Molecular Medicine, Sapienza University, Italy
Tania H. Watts, University of Toronto, Canada



Stopping-over, passing-through and tissue-resident memory T cells in Bone Marrow.

Under steady state, memory T cells migrate into the Bone Marrow (BM) and then circulate back to the blood, with poorly defined kinetics. It is possible that some recirculating memory T cells quickly transit through the BM parenchyma while others stop over for some time within BM niches. A few memory T cells might stay permanently in BM niches and never return to the blood, representing the equivalent of tissue-resident memory T (TRM) cells identified in other organs.

Figure modified from: Di Rosa F and Gebhardt T (2016) Bone Marrow T Cells and the Integrated Functions of Recirculating and Tissue-Resident Memory T Cells. Front. Immunol. 7:51. doi: 10.3389/fimmu.2016.00051

Increasing evidence supports the notion that bone marrow (BM) represents a relevant player in T cell responses, particularly in its role as a specialized organ for long-term memory. Memory T cells are enriched in the BM over long times after priming, and can be recruited to the periphery upon antigenic challenge. The articles in this research topic include discussions of whether these T cells are passing-through or truly resident, as well as a debate on the extent of proliferation of BM memory T cells. Original research articles in this collection include an analysis of the number of memory T cells found in different bones as well as effects of B cell depletion on T cell memory in the BM. T cells in the BM can influence a number of processes, from bone remodeling, control of cancer, to effects on hemopoiesis or Graft versus Host Disease (GVHD). This research topic contains several contributions to these topics including discussions on how to translate BM T cell knowledge into medicine.

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EDITORIAL
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Editorial: Bone Marrow T Cells at the Center Stage in Immunological Memory

Francesca Di Rosa[1]* *and Tania H. Watts*[2]

[1]Institute of Molecular Biology and Pathology, Consiglio Nazionale delle Ricerche, c/o Department of Molecular Medicine, Sapienza University, Rome, Italy, [2]Department of Immunology, University of Toronto, Toronto, ON, Canada

Keywords: T cells, bone marrow, immunological memory, bone and bones, hematopoiesis, cancer

Editorial on the Research Topic

Bone Marrow T Cells at the Center Stage in Immunological Memory

The notion that bone marrow (BM) T cells give a key contribution to adaptive immunity is increasingly recognized (1–3). Researchers now more often include the BM when analyzing T cell responses in experimental mouse models (4, 5) or when providing an overview of memory T cell compartmentalization (6). Translation of BM T cell knowledge into medicine has begun. Promising results of the first clinical trial using BM T cells in the treatment of multiple myeloma (MM) were reported last year (7). Further applications are expected in the near future, as BM T cells have been involved in a variety of processes, going from normal hematopoiesis to bone resorption in patients affected by hyperparathyroidism (8, 9).

This research topic on BM T cells contains two sections. The first one contains original research contributions on BM memory CD4 and CD8 T cells in mouse models (Hojyo et al.; Geerman et al.) and hosts a debate on the role of BM memory T cells in systemic or localized memory (Di Rosa; Sercan-Alp and Radbruch; Di Rosa and Gebhardt). In the second one, emerging scenarios in translational medicine in different fields (e.g., hematology, oncology, transplantation immunology, osteoimmunology, etc.) are discussed (Wakkach et al.; Borrello and Noonan; Szyska and Na; Pacifici; Bonomo et al.).

MEMORY T CELLS IN THE BM

The BM harbors a high frequency of antigen-specific memory T cells against vaccines, pathogens, and tumors and is considered a major site for the maintenance of memory T cells (reviewed by Di Rosa and Gebhardt). In addition to conventional memory T cells, another class of non-recirculating subsets—the so-called tissue-resident memory T cells (Trm)—has recently been identified in several non-lymphoid organs including skin, gut, and brain (10, 11). These cells, which can provide a first-line defense against reinfection at barrier surfaces, are characterized by expression of CD69 as well as integrins such as CD103 and VLA1, which can contribute to their tissue retention (reviewed in Di Rosa and Gebhardt). BM has a high proportion of $CD69^+$ memory T cells (2, 3, 12), as confirmed in an original report by Geerman et al. in this research topic. However, the expression of CD69 may not be sufficient to define these T cells as "tissue resident." Di Rosa and Gebhardt discuss the evidence that BM T cells are largely circulatory, likely stopping over temporarily in BM niches where they receive survival signals, before re-entering the circulation.

An issue of some debate has been the extent of homeostatic proliferation of the memory T cells in these niches [Di Rosa; Sercan-Alp and Radbruch; (3, 13–16)]. Sercan-Alp and Radbruch have suggested (3) that the level of homeostatic proliferation measured by BrdU is overestimated. However,

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Scott N. Mueller, University of Melbourne, Australia

***Correspondence:**
Francesca Di Rosa
francesca.dirosa@uniroma1.it

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this remains a point of contention. As often found when research groups disagree, the experimental details may offer a solution. One group found, for example, that MyD88 negative mice did not have unexpectedly high rates of BrdU incorporation (Sercan-Alp and Radbruch), suggesting that the BrdU may have been LPS contaminated. Another found that proliferation rates were similar with BrdU and CFSE labels (13). As documented by Di Rosa in her commentary, a variety of experimental approaches have provided evidence that the level of proliferation of memory T cells in the BM, while low, is higher than the level of homeostatic proliferation of T cells in spleen or LN. Thus, it is likely that the niches in the BM that are rich in cytokines such as IL-7 and IL-15, while largely providing survival signals may also induce a low level of proliferation, sufficient to at least partially support homeostasis. A recent hypothesis proposes that memory T cells circulating through the BM may stop to rest for a while in dedicated niches supporting quiescence and/or proliferate in distinct niches for self-renewal, before moving on (16).

In an original research article, Geerman et al. provide evidence that the frequency and phenotype of different subsets of memory T cells as well as their expression of cytokine receptors was similar in different bones in the steady state and after an acute systemic infection with lymphocytic choriomeningitis. This is reassuring for investigators who may wish to use different bones in their studies. Of note, the vertebrae, which contain the most BM cells, also provide the most abundant source of T cells.

In an original research contribution, Hojyo et al. focus on memory CD4 T cells and show that B cell depletion increases the number of $CD49b^{+}Tbet^{+}$ TCR transgenic CD4 memory T cells in the BM. Whether B cell depletion has a direct effect on the CD4 T cells or affects their access to another factor which in turn regulates their expression of $CD49^{+}$ and/or BM localization is not yet clear.

BM T CELLS IN TRANSLATIONAL MEDICINE

The activation state of freshly isolated BM T cells, e.g., resulting from exposure to IL-15 in the organ, together with their prompt response to *in vitro* stimulation makes these cells ideal candidates for adoptive transfers in conditions requiring highly active effectors (17–19). The article by Borrello and Noonan recapitulates concepts and results on the use of marrow-infiltrating lymphocytes (MILs) against MM in humans and discusses the unique opportunity to exploit BM T cells in adoptive T-cell therapy against both hematological and solid cancers. Moreover, MIL transfer might ameliorate bone disease in MM patients, by switching BM T cells from Th17 to Th1 [Borrello and Noonan; (20)].

By contrast, in HSC transplantation (HSCT), donor T cell effector function against host BM stroma is detrimental for donor HSC seeding and hematopoiesis reconstitution. Starting with the recent recognition that BM is a major target organ in GVHD after allogeneic HSCT in leukemic patients (21), Szyska and Na discuss some possible mechanisms underlying this adverse effect, e.g., T-cell-derived cytolytic factors and cytokines can damage osteoblasts, endothelia, and surrounding cells, while replenishment of destroyed niches by hematopoietic cells is impaired.

Two articles link BM T cell-derived TNF-alpha and IL-17 to altered bone metabolism in human diseases. Pacifici discusses the evidence suggesting that catabolic effects of parathyroid hormone on bone in patients affected by hyperparathyroidism relies on Th17 cell-induced RANKL release by osteoblasts and osteocytes, with subsequent osteoclast-mediated bone resorption (9). Wakkach et al. give an overview of the mechanisms supporting bone destruction in inflammatory bowel disease and propose that TNF-alpha-producing Th17 cells in the BM sustain bone loss in patients with Crohn's disease (22).

Bonomo et al. review the evidence that BM T cells are at the cross-roads between immunity, bone metabolism, and hematopoiesis and propose that T cells act as messengers who "bring the news" from the periphery to the BM. According to this view, activated T cells enter the BM and modulate BM-resident cell function, ultimately tuning blood cell production and bone remodeling to the class of peripheral immune response (Bonomo et al.).

AUTHOR CONTRIBUTIONS

TW wrote the paragraph on memory T cells in the BM; FD wrote the paragraph on BM T cells in Translational Medicine; FD and TW together wrote the remaining parts and edited the final text.

REFERENCES

1. Di Rosa F. T-lymphocyte interaction with stromal, bone and hematopoietic cells in the bone marrow. *Immunol Cell Biol* (2009) 87:20–9. doi:10.1038/icb.2008.84
2. Okhrimenko A, Grun JR, Westendorf K, Fang Z, Reinke S, von Roth P, et al. Human memory T cells from the bone marrow are resting and maintain long-lasting systemic memory. *Proc Natl Acad Sci U S A* (2014) 111:9229–34. doi:10.1073/pnas.1318731111
3. Sercan Alp O, Durlanik S, Schulz D, McGrath M, Grun JR, Bardua M, et al. Memory CD8(+) T cells colocalize with IL-7(+) stromal cells in bone marrow and rest in terms of proliferation and transcription. *Eur J Immunol* (2015) 45:975–87. doi:10.1002/eji.201445295
4. Bolinger B, Sims S, Swadling L, O'Hara G, de Lara C, Baban D, et al. Adenoviral vector vaccination induces a conserved program of CD8(+) T cell memory differentiation in mouse and man. *Cell Rep* (2015) 13:1578–88. doi:10.1016/j.celrep.2015.10.034
5. Jung YW, Kim HG, Perry CJ, Kaech SM. CCR7 expression alters memory CD8 T-cell homeostasis by regulating occupancy in IL-7- and IL-15-dependent niches. *Proc Natl Acad Sci U S A* (2016) 113:8278–83. doi:10.1073/pnas.1602899113
6. Farber DL, Yudanin NA, Restifo NP. Human memory T cells: generation, compartmentalization and homeostasis. *Nat Rev Immunol* (2014) 14:24–35. doi:10.1038/nri3567
7. Noonan KA, Huff CA, Davis J, Lemas MV, Fiorino S, Bitzan J, et al. Adoptive transfer of activated marrow-infiltrating lymphocytes induces measurable antitumor immunity in the bone marrow in multiple myeloma. *Sci Transl Med* (2015) 7:288ra78. doi:10.1126/scitranslmed.aaa7014
8. Kim S, Park K, Choi J, Jang E, Paik DJ, Seong RH, et al. Foxp3+ regulatory T cells ensure B lymphopoiesis by inhibiting the granulopoietic activity of

effector T cells in mouse bone marrow. *Eur J Immunol* (2015) 45:167–79. doi:10.1002/eji.201444532
9. Li JY, D'Amelio P, Robinson J, Walker LD, Vaccaro C, Luo T, et al. IL-17A is increased in humans with primary hyperparathyroidism and mediates PTH-induced bone loss in mice. *Cell Metab* (2015) 22:799–810. doi:10.1016/j.cmet.2015.09.012
10. Schenkel JM, Masopust D. Tissue-resident memory T cells. *Immunity* (2014) 41:886–97. doi:10.1016/j.immuni.2014.12.007
11. Mueller SN, Mackay LK. Tissue-resident memory T cells: local specialists in immune defence. *Nat Rev Immunol* (2016) 16:79–89. doi:10.1038/nri.2015.3
12. Shinoda K, Tokoyoda K, Hanazawa A, Hayashizaki K, Zehentmeier S, Hosokawa H, et al. Type II membrane protein CD69 regulates the formation of resting T-helper memory. *Proc Natl Acad Sci U S A* (2012) 109:7409–14. doi:10.1073/pnas.1118539109
13. Parretta E, Cassese G, Santoni A, Guardiola J, Vecchio A, Di Rosa F. Kinetics of in vivo proliferation and death of memory and naive CD8 T cells: parameter estimation based on 5-bromo-2'-deoxyuridine incorporation in spleen, lymph nodes, and bone marrow. *J Immunol* (2008) 180:7230–9. doi:10.4049/jimmunol.180.11.7230
14. Di Rosa F. Maintenance of memory T cells in the bone marrow: survival or homeostatic proliferation? *Nat Rev Immunol* (2016) 16:271. doi:10.1038/nri.2016.31
15. Sercan-Alp O, Radbruch A. The lifestyle of memory CD8(+) T cells. *Nat Rev Immunol* (2016) 16:271. doi:10.1038/nri.2016.32
16. Di Rosa F. Two niches in the bone marrow: a hypothesis on life-long T cell memory. *Trends Immunol* (2016) 37:503–12. doi:10.1016/j.it.2016.05.004
17. Di Rosa F, Santoni A. Bone marrow CD8 T cells are in a different activation state than those in lymphoid periphery. *Eur J Immunol* (2002) 32:1873–80. doi:10.1002/1521-4141(200207)32:7<1873::AID-IMMU1873>3.0.CO;2-P
18. Quinci AC, Vitale S, Parretta E, Soriani A, Iannitto ML, Cippitelli M, et al. IL-15 inhibits IL-7Ralpha expression by memory-phenotype CD8(+) T cells in the bone marrow. *Eur J Immunol* (2012) 42:1129–39. doi:10.1002/eji.201142019
19. Snell LM, Lin GH, Watts TH. IL-15-dependent upregulation of GITR on CD8 memory phenotype T cells in the bone marrow relative to spleen and lymph node suggests the bone marrow as a site of superior bioavailability of IL-15. *J Immunol* (2012) 188:5915–23. doi:10.4049/jimmunol.1103270
20. Noonan K, Marchionni L, Anderson J, Pardoll D, Roodman GD, Borrello I. A novel role of IL-17-producing lymphocytes in mediating lytic bone disease in multiple myeloma. *Blood* (2010) 116:3554–63. doi:10.1182/blood-2010-05-283895
21. Mensen A, Johrens K, Anagnostopoulos I, Demski S, Oey M, Stroux A, et al. Bone marrow T-cell infiltration during acute GVHD is associated with delayed B-cell recovery and function after HSCT. *Blood* (2014) 124:963–72. doi:10.1182/blood-2013-11-539031
22. Ciucci T, Ibanez L, Boucoiran A, Birgy-Barelli E, Pene J, Abou-Ezzi G, et al. Bone marrow Th17 TNFalpha cells induce osteoclast differentiation, and link bone destruction to IBD. *Gut* (2015) 64:1072–81. doi:10.1136/gutjnl-2014-306947

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REVIEW
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Bone Marrow T Cells and the Integrated Functions of Recirculating and Tissue-Resident Memory T Cells

Francesca Di Rosa[1] and Thomas Gebhardt[2]*

[1] Institute of Molecular Biology and Pathology, Consiglio Nazionale delle Ricerche, c/o Department of Molecular Medicine Sapienza University, Rome, Italy, [2] Department of Microbiology and Immunology, Peter Doherty Institute for Infection and Immunity, The University of Melbourne, Melbourne, VIC, Australia

Changes in T cell trafficking accompany the naive to memory T cell antigen-driven differentiation, which remains an incompletely defined developmental step. Upon priming, each naive T cell encounters essential signals – i.e., antigen, co-stimuli and cytokines – in a secondary lymphoid organ; nevertheless, its daughter effector and memory T cells recirculate and receive further signals during their migration through various lymphoid and non-lymphoid organs. These additional signals from tissue microenvironments have an impact on immune response features, including T cell effector function, expansion and contraction, memory differentiation, long-term maintenance, and recruitment upon antigenic rechallenge into local and/or systemic responses. The critical role of T cell trafficking in providing efficient T cell memory has long been a focus of interest. It is now well recognized that naive and memory T cells have different migratory pathways, and that memory T cells are heterogeneous with respect to their trafficking. We and others have observed that, long time after priming, memory T cells are preferentially found in certain niches such as the bone marrow (BM) or at the skin/mucosal site of pathogen entry, even in the absence of residual antigen. The different underlying mechanisms and peculiarities of resulting immunity are currently under study. In this review, we summarize key findings on BM and tissue-resident memory (TRM) T cells and revisit some issues in memory T cell maintenance within such niches. Moreover, we discuss BM seeding by memory T cells in the context of migration patterns and protective functions of either recirculating or TRM T cells.

Keywords: memory T cells, migration, recirculation, bone marrow, tissue-resident T cells, CD8 T cells

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Peter Katsikis, Erasmus University Medical Center, Netherlands

Reviewed by:
David Hildeman, Cincinnati Children's Hospital, USA
Benedita Rocha, Pasteur Institute, France

****Correspondence:***
Francesca Di Rosa
francesca.dirosa@uniroma1.it

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INTRODUCTION

When a pathogen attacks either skin or mucosa, primary immune responses are initiated in the draining lymph nodes (LN) and in some cases in the spleen. In these secondary lymphoid organs, mature antigen-presenting dendritic cells (DC) prime T cells to undergo huge clonal expansion and differentiation into short-lived effector and long-lived memory T cells. Both types of antigen-experienced T cell migrate far from the original priming site, displaying increased capacity to migrate to inflamed tissues as well as to the bone marrow (BM) as compared with naive T cells. Activation-induced changes in T cell membrane expression of chemokine receptors, integrins, and other adhesion molecules underlie this homing behavior. At the end of effective responses, when

there is little or no residual antigen left, effector T cells die, leaving behind a small population of long-lived memory T cells, ready to provide protection in case of challenge with the same antigen. Memory T cells can be found all over the body, with a peculiar enrichment either in the BM or at the port of pathogen entry.

We will examine the evidence showing that memory T cells home to the BM and persist over time within this organ, being in constant exchange with blood T cell pool, whereas memory T cells residing at the port of pathogen entry (skin, etc.) are sessile. Moreover, we will review recent data and speculations on the niches wherein either BM or tissue-resident memory (TRM) T cells are maintained over time. We will discuss how BM memory T cells contribute to systemic memory, while TRM cells participate in local protection.

BONE MARROW MEMORY T CELLS

A "Reservoir" of Memory T Cells in the BM

The BM consists of islets of hematopoietic BM interspersed with fatty areas, all contained within spongy bone and inside central cavities of long bones. It has long been known that in healthy individuals BM contains mature T cells, which can mediate graft-versus-host disease in T-replete BM transplantation settings. T cells represent about 3–8% of total nucleated BM cells, and have a typically reduced CD4/CD8 T cell ratio, as compared with blood (1, 2). BM T cells include also regulatory CD4 T cells (3). No lymphatic drainage is present, thus BM exchanges with the rest of the body occur only through blood circulation.

Upon T cell priming induced via different routes, T cell contraction is less pronounced in the BM than in the spleen and other organs, and is followed by long-lasting persistence of BM antigen-specific memory T cells (4–9). The BM also contains a high proportion of memory-phenotype T cells, i.e., a heterogeneous subset defined by the expression of activation/memory markers, which increases with aging and includes memory T cells specific for previously encountered antigens (10–12). BM memory T cells contain both central memory (TCM) and effector memory (TEM) T cells, two subsets of recirculating memory T cells identified in blood, having respectively high or low expression of the LN homing receptor CCR7 and distinct homing potential (13–16). Thus, the BM is often described as a "reservoir" for long-lived memory T cells (14, 15, 17).

Recirculating Memory T Cells in the BM

Pivotal experiments in sheep showed that T cells labeled *in situ* in the BM migrated out of the organ and reached the spleen and other secondary lymphoid organs (18), suggesting that the BM represents a temporary stopping point for recirculating memory T cells (2). In agreement with this notion, parabiosis experiments showed that about 2 weeks after surgery leading to anastomoses of blood vessels between two CD45-congenic mice, comparable numbers of $CD45.1^+$ and $CD45.2^+$ antigen-specific memory CD8 T cells were found in the BM of each parabiotic mouse (19). Furthermore, intra-vital dynamic imaging studies demonstrated that naive and memory CD8 T cells injected either into the carotid artery or intravenously entered the BM parenchyma of mouse skull and constantly crawled in it (14, 20). Competition among "rival" memory T cells for lodging into the BM was suggested by adoptive transfer experiments showing that memory-phenotype T cells entered BM more easily into young than in thymectomized old mice, where an existing memory T cell pool precluded their free access (11). Such competition with host T cells was lacking when BM T cell recipients were RAG1-deficient mice (21). Thus, it appears that most BM T cells are motile recirculating cells. Some authors argued that the majority if not all of the BM memory T cells are non-migratory cells that permanently inhabit the BM; however, this speculation was based on cell phenotype, activation state, and gene expression analysis (22, 23) and did not take into account the *in vivo* data, including those obtained by *in situ* labeling, parabiosis, intra-vital dynamic imaging, and adoptive transfer (11, 14, 18–20). Nevertheless, the possibility that, similarly to thymus, LN, and spleen (24, 25), the BM also contains a few TRM cells cannot be excluded. For example, parabiosis experiments demonstrated that 3–5% of the antigen-specific memory T cells present in spleen and LN reside permanently in specific locations, i.e., the spleen marginal zone and red pulp and the LN sinuses (25).

In respect to the molecular players of memory T cell homing into the BM, memory CD8 T cells slow down and roll in BM microvessels via L-, P-, and E-selectin-mediated interactions (14). The BM tropism of memory T cells is supported by their high expression of the integrin VLA-4 ($\alpha4\beta1$) and strong response to the BM chemokine CXCL12 (11, 14, 26). Conversely, only a few BM CD8 T cells express cutaneous lymphocyte antigen (CLA) and CCR9, involved in T cell homing to skin and gut, respectively (27). CD4 T cells lodge into the BM via molecular mechanisms at least partially similar to those of CD8 T cells. Expression of β1-integrin by CD4 T cells is required for their retention in the BM (28). In addition, CD4 T cell homing to BM is greatly reduced by anti-α2-integrin antibodies (21), suggesting a pivotal role for α2-integrin-mediated interactions, e.g., between the T cell integrin VLA-2 ($\alpha2\beta1$) and type I collagen, which is highly abundant in bone. Both CD4 and CD8 T cell localization in the BM was compromised when mice lacked the adhesion molecule VCAM-1 (29).

Molecular regulation of T cell egress from the BM involves Sphingosine-1-phosphate (S1P) interaction with its receptor $S1P_1$ (30). S1P levels in the BM are lower than in plasma, so that CD4 and CD8 T cells responding to S1P concentration gradient are normally recruited into the blood, unless $S1P_1$ is pharmacologically inhibited by FTY720 (30). In agreement with the inhibition exerted by CD69 on $S1P_1$ membrane expression and function (31), it was observed that CD69 ko memory CD4 T cells accumulated in lower numbers in the BM as compared with their WT counterparts (32). However, CD69 deficiency did not cause CD4 T cell increase in blood, implying a more complex scenario (32). Since $CD69^+$ cells but not $CD69^-$ cells were associated with $laminin^+$ stromal cells in the BM, it was proposed that CD69 could mediate retention of memory CD4 T cells within the BM (32). Taken together, these results suggest that CD69 regulates local T cell retention in the BM by a variety of mechanisms.

Certain infectious agents and cytokines can modulate BM T cell exchange with blood, for instance in Human Immunodeficiency

Virus (HIV)-infected individuals, CD4 T cells migrate to BM more rapidly than in healthy controls (33). Moreover, after Hematopoietic Stem Cell (HSC)-mobilization procedures, some T cell subsets and especially regulatory T cells increase in the blood, suggesting that they are mobilized from the BM (34, 35). Conversely, high levels of type I IFN induced by acute viral infections block T cell egress from lymphoid organs by a CD69-dependent mechanism (31). Further studies are required to define the rules governing changes in BM T cell recruitment into the blood upon peripheral and systemic immune responses (30, 36).

Antigen and Cytokines for BM Memory T Cells

Memory T cells specific for previously encountered antigens are commonly found in the BM of healthy subjects as well as of individuals affected by infectious, immune-mediated, and neoplastic diseases. For example, human BM from tetanus toxoid-vaccinated individuals contains tetanus-specific CD4 T cells (22, 37), which can be transferred with BM grafts (37). Pathogen-specific CD4 and CD8 T cells were present not only in the BM of immune individuals after resolution of acute infections (22) but also in the BM of subjects infected by persistent viruses, including Cytomegalovirus (CMV), Epstein–Barr Virus (EBV), and Human Hepatitis C Virus (HCV) (16, 27, 38, 39). BM from neoplastic patients contained tumor antigen-specific T cells, even without intentional immunization (40, 41). Moreover, autoreactive T cells were found in the BM from individuals affected by autoimmune diseases (42).

In some of these cases, antigens were expressed in the BM, e.g., in EBV-infected subjects (27) or in idiopathic thrombocytopenia purpura patients (42). Though undetected, tumor antigen-positive micro-metastatic cells could be present in patients with solid tumors (41, 43). However, the presence of antigen in the BM was not a reasonable possibility in other cases, for example long time after vaccination against tetanus (22, 37). In rodents, antigen-primed CD4 and CD8 T cell migration and retention into the BM were observed after peripheral immunization procedures that were unlikely to result in the presence of antigen in the BM (7, 44, 45). Hence, it appears that long-lived antigen-specific memory T cells lodge in the BM during immune responses to a variety of antigens, either localized in the BM or not (2).

Furthermore, mouse BM can be a major site of naive CD4 and CD8 T cell priming under conditions of disrupted lymphocyte trafficking in splenectomized mice (46). Blood-borne antigens can reach the BM and induce CD4 and CD8 T cell priming via BM-resident APC (20, 47). Moreover, circulating DC and even neutrophils can transport antigens to BM (48, 49). Altogether, these results show that, under certain circumstances, BM can function as a secondary lymphoid organ.

T cell contacts with other cells in BM niches and molecular interactions influencing T cell activation, proliferation, or survival in BM niches appear heterogeneous. Microscopy studies of BM sections documented that most mouse memory CD4 and CD8 T cells contacted IL-7$^+$ VCAM-1$^+$ stromal cells (21, 23, 50), whereas some were in proximity to either F4/80$^+$ or CD11c$^+$ cells (21, 51). Clusters of memory T cells and IL-15-producing cells were observed in human BM sections (52) while a population of 4-1BBL-expressing radioresistant stromal cells supporting memory CD8 T cell survival was described in mouse BM (51). In addition to 4-1BBL, other TNF family members have been involved in T cell survival within BM niches, e.g., GITRL (53–57).

Acquisition of a BM-Phenotype by Recirculating Memory T Cells

Elegant cellular barcoding experiments in mice clearly demonstrated that after pathogen infection via a systemic route, pathogen-specific CD8 T cells from the BM and those from blood, spleen, and LN all derive from the same set of precursors (58). BM memory T cells exhibit some phenotypic differences when compared with corresponding cells from lymphoid periphery and blood (7, 8, 22, 56, 59, 60). For example, a high proportion of BM memory CD4 and CD8 T cells express CD69 (22, 23, 32). Moreover, memory CD8 T cells from both mouse and human BM have a lower membrane expression of CD127, i.e., the IL-7Rα chain (8, 22, 59, 61), with the exception of antigen-specific CD8 T cells from lymphocytic choriomeningitis virus (LCMV)-infected mice (23). The TNF-R family member GITR is selectively upregulated by a fraction of mouse BM memory CD8 T cells (56). Downregulation of CD127 and upregulation of GITR were both observed in BM but not in spleen samples from WT mice, while they were lost in IL-15 ko mice, suggesting that they are indirect evidence of IL-15 stimulation in BM (56, 60). In agreement with these observations, adoptively transferred splenic CD8 T cells converted to BM-phenotype after entry into the BM (56, 60). Moreover, phospho-STAT-5 and phospho-p38 MAPK were increased in freshly isolated BM CD8 T cells as compared with corresponding spleen cells, possibly reflecting molecular events in the BM, e.g., signaling by IL-15 and TNF family members (59).

No major differences were found by gene-expression analysis of memory T cell paired samples obtained from either BM or blood/spleen, for example mouse BM and spleen CD127$^+$ memory CD8 T cells (23) and human BM and blood CD69$^-$ memory CD4 T cells (22). This may not be surprising, considering T cell recirculation and the shared ancestry between memory T cells present in BM and in other lymphoid organs (58). After *in vitro* stimulation, memory CD8 T cells freshly isolated from BM strikingly changed their transcriptional profile (23). Of note, BM and spleen memory CD8 T cells had a roughly similar global transcription profile following 2 days of *in vitro* stimulation with anti-CD3+ anti-CD28 beads (23), suggesting that the two types of cell shared a common set of genes poised to be expressed after activation (62, 63).

Taken together, these findings support the view that most BM memory T cells cannot be identified as a distinguished subset. They also suggest that BM memory T cells integrate various signals received in the organ, so that their activation state is different from that of recirculating memory T cells from other sources. However, after either egress from the BM or experimental isolation, memory T cells only transiently retain some traits of the stimulation received in the organ (59).

Contribution of BM T Cells to Memory Maintenance and Secondary Responses

Although the majority of BM memory T cells are quiescent, as demonstrated by staining with the cell cycle marker Ki67 (22, 23), a small percentage of them divides under steady state (8, 9, 60). Indeed, memory T cells have a higher rate of local proliferation in the BM than in spleen and LN, as demonstrated by a variety of experimental approaches (8, 9, 57, 59, 60, 64). For example, by Bromodeoxyuridine, carboxyfluorescein diacetate succinimidyl ester (CFSE), or DNA content assays, BM antigen-specific memory CD8 T cells contained a higher percentage of dividing cells than corresponding cells in spleen, LN, liver, and blood (8, 9). An increased proliferation in the BM was also observed in the case of naive CD8 T cells (59), although as expected their turnover was much lower than that of memory CD8 T cells (65). Estimates of T cell numbers strengthened the view that the BM gives a major contribution to long-term memory T cell maintenance, as well as to the homeostatic regulation of both memory and naive CD8 T cell numbers (2, 8, 9, 66, 67).

CD8 T cell proliferation in the BM is supported by local stimuli, including a dominant role for IL-15, as demonstrated by *in vivo* experiments with IL-15 ko and IL-15Rα ko mice (60). When tested *in vitro*, purified BM CD8 T cells did not exhibit an enhanced proliferative response to IL-7, IL-15, or IL-21 as compared with spleen CD8 T cells (59). Adoptively transferred CD8 T cells originally obtained from either BM or spleen similarly expanded *in vivo* after host injection with Poly I:C, a synthetic dsRNA analog inducing type I IFN and IL-15 (59), as well as upon secondary antigen challenge (68). Taken together, these data support the notion that BM T cells do not exhibit an intrinsic higher proliferative capability, but rather they are constantly stimulated in the BM (59).

Lack of memory T cell lodging into the BM can have a strong impact on memory T cell responses. For example, in CD69 ko mice and in mice treated with anti-α2 integrin antibodies, CD4 T cells were greatly reduced in the BM and help for B cell response was defective (21, 32). Memory CD8 T cells lacking the transcription factor eomesodermin did not populate the BM niche and had impaired long-term persistence and secondary expansion (69). In contrast, β1 integrin-deficient CD4 T cells were not retained in the BM niche but proliferated normally in response to antigenic peptide plus LPS injection (28). Likewise, in the absence of CXCR4, anti-LCMV memory CD8 T cells were defective in migration into the BM, but displayed normal, or even greater, expansion upon secondary challenge with pathogen (70). Further studies are required to clarify these discrepancies, possibly due to pleiotropic effects of key molecule deficiency, and/or differences in the measured aspect of memory response, e.g., T cell self-renewal in the memory phase, T cell expansion upon secondary response, or anti-pathogen protection, after different types of rechallenge.

BM Memory T Cells in Health and Disease

Bone marrow T cells exert potent antigen-specific effector function, as demonstrated by either adoptive transfers in immunodeficient mice (5) or *in vitro* studies (7). T cells from BM of patients with different types of solid and hematological cancers were able to kill autologous tumor cells (40, 71, 72). Adoptive transfer of BM T cells from breast cancer patients in immunodeficient mice induced regression of xenotransplanted autologous tumors, while blood T cells were not as effective (41). Clinical trials with autologous BM T cells have been initiated in various tumors (73), and the first trial in multiple myeloma patients looks very promising (74).

Moreover, it has been proposed that BM-based T cell proliferation is one of the species-specific advantages allowing Simian Immunodeficiency Virus (SIV)-infected Sooty Mangabeys (SM) but not Rhesus Macaques (RM) to maintain normal CD4 T cell counts (75). Notably, SIV infection does not cause immunodeficiency in SM, whereas it progresses to AIDS in RM, similarly to HIV infection in humans (75).

T cell-derived cytokines can modulate hematopoiesis, implying that BM T cells can contribute to shaping hematopoiesis during both acute and chronic infections (76). For example, IFN-γ released by T cells can either induce IL-6 production by BM stromal cells, in turn augmenting myelopoiesis (77), or also act directly on HSC (78–80).

Bone marrow can play a detrimental role in some T-cell mediated diseases by maintaining pathogenic T cells. In mouse models, pathogenic autoreactive T cells were found in the BM in organ-specific autoimmune diseases, e.g., type I diabetes (81) and chronic uveitis (82). Similarly, in mice with inflammatory colitis, pathogenic CD4 T cells were found in the BM (83). Interestingly, maintenance of pathogenic CD4 T cells required IL-7 in the BM, but not in the colon (84). Thus, it was proposed that, in the disease remission phase, colitogenic CD4 T cells persisted in the BM (83, 84).

Moreover, T cell effector function in the BM can stimulate pathological bone resorption, by activating osteoclasts. It is well established that CD4 T cells recruited in joints and periodontal tissue of patients affected by rheumatoid arthritis and periodontitis, respectively, stimulate osteoclastogenesis by producing IL-17 and RANK-L (85–88). Recently, a subset of osteoclastogenic Th17 TNF-α producing cells has been identified in PBMC from patients with Crohn's disease, and it has been proposed that these cells can migrate to the BM and mediate bone loss, in agreement with mouse models (89–91). Notably, in a mouse model of breast cancer, pro-osteoclastogenic BM T cells favored the establishment of skeletal metastases by inducing osteolytic lesions (92).

Finally, T cells regulate physiological processes occurring in the BM, i.e., normal hematopoiesis and bone tissue homeostasis. Surprisingly, the maintenance of normal bone mass and bone mineral density in physiological conditions is promoted by T cells, which stimulate the production of the RANK-L decoy receptor osteoprotegerin by B cells, through CD40L/CD40 interaction (93). A cross-talk between T cells and hematopoietic precursors occurs in the BM in normal healthy conditions (94, 95). For example, it has been shown that BM T cells sustain normal granulopoiesis (94), while regulatory T cells inhibit excessive T cell-production of the granulopoiesis-promoting cytokines GM-CSF, TNF, and IL-6, thus allowing for sufficient B lymphopoiesis (95). Regulatory T cells in the BM are required for HSC engraftment upon transplantation (96, 97), and likewise might protect normal

HSC and their niches from destructive immune responses (97). Taken together, these results suggest that BM T cells are engaged in a complex interplay with other cells in the local environment, contributing to maintain bone and BM integrity and function.

TISSUE-RESIDENT MEMORY T CELLS

A "Reservoir" of Memory T Cells in Non-Lymphoid Tissues

In addition to the BM and secondary lymphoid organs, the body's surfaces such as the linings of the skin, gut, and reproductive tract also harbor large numbers of CD4 and CD8 T cells (6, 98–100). Most of these peripheral T cells are antigen-experienced memory cells and are generally believed to provide specific immunity against renewed infection with previously encountered pathogens. Given their location in close proximity to the external environment, it appears likely that some of these memory T cells also recognize commensal microbiota, and such T cell–microbiota interactions have been proposed to fine-tune peripheral immunity (101, 102).

While it is clear that T cells recirculate between peripheral tissues and the blood via the lymphatic system (103–105), there is recent evidence for a non-recirculating population of memory T cells that remain localized to peripheral tissues and never return to the blood (106, 107). Such TRM cells are best characterized for the CD8 subset and have been described in a large number of peripheral organs, including skin, gut, brain, salivary glands, lungs, female reproductive tract, and others (106, 107). Furthermore, non-recirculating memory T cells also exist in lymphoid organs such as LN and thymus (24, 25).

Sessile TRM Cells Permanently Residing in Tissues

Various experimental strategies, such as organ transplantation, sex-mismatched adoptive T cell transfer, and parabiosis in mice have unequivocally demonstrated that TRM cells can persist in peripheral tissues in disconnection from the pool of recirculating T cells in the blood (108–111). In line with this, CD8 TRM cells are often found in specialized microanatomical compartments such as the epithelial layers of skin and gut where they are sequestered from direct lymphatic drainage by the underlying epithelial basement membrane.

These epithelial CD8 TRM cells commonly express surface receptors such as CD103 (the αE integrin subunit), CD69, and the integrin VLA-1 (α1β1), which are variously involved in T cell retention and persistence (107, 112). For instance, genetic deficiency in CD103 expression results in defective generation of CD8 TRM cells in gut mucosa (113, 114), abolished accumulation of CD8 T cells in islet grafts (115), as well as a gradual loss of TRM cells from skin and lung mucosa (116, 117). Similarly, genetic or functional ablation of VLA-1 causes a dramatic decline in memory CD8 T cell numbers in lung after pulmonary virus infection (118). While these adhesion molecules are likely to mediate the tethering of TRM cells to their microenvironment, integrin binding and ligation may also support T cell survival and functional activity (119, 120). By contrast, the surface molecule CD69 can act to block the functional activity of the tissue exit receptor, $S1P_1$, and thereby inhibit tissue egress of effector T cells with the potential to undergo local differentiation into TRM cells (31, 121). In addition to this posttranslational regulation of $S1P_1$ function, the progressive transcriptional downregulation of $S1P_1$ expression appears to be a critical checkpoint in the TRM maturation pathway in a variety of tissues (117, 122). The high level surface expression of CD69 by fully developed TRM cells further echoes the absence of $S1P_1$ expression, rather than recent T cell receptor activation, as both molecules physically interact with each other, resulting in mutual inhibition of surface expression (123).

In addition to epithelial $CD103^+CD8^+$ TRM cells, phenotypically and anatomically distinct $CD103^-$ $CD8^+$ TRM cells, including those that form clusters at sites of intestinal infection with *Yersinia pseudotuberculosis*, have also been described more recently (100, 124). Likewise, presumably non-recirculating or long-term retained memory T cells have been identified among $CD4^+$ memory T cells in a variety of tissues, including skin, as well as lung, gut, and vaginal mucosa (125–129). Some of these also express markers initially described for $CD8^+$ TRM cells, such as CD69 and CD103, although the role of these molecules in tissue persistence of $CD4^+$ TRM cells remains largely unknown. It is therefore likely that TRM cells exist among both $CD4^+$ and $CD8^+$ memory T cells, and future studies will have to compare the molecular and functional relationship between those different types of TRM cells. Nevertheless, given that the molecular pathways underpinning the maturation and persistence of TRM cells have so far most extensively been studied for $CD103^+$ $CD8^+$ TRM cells, we will focus our attention on this subset for the remainder of this article.

Antigen and Cytokines for $CD103^+$ $CD8^+$ TRM Cells

The highest frequencies of $CD8^+CD103^+$ TRM cells are found in previously infected or inflamed tissue (108, 110, 130), where TRM cells develop from infiltrating effector T cells during lesion resolution (114, 117). Accordingly, peripheral infection can be regarded as the principle inducer of TRM cells in barrier organs such as skin and mucosa. In addition, systemic infections that generate a large pool of memory precursor T cells disseminating throughout the body can give rise to TRM populations in a variety of barrier tissues and internal organs (6, 111, 113, 131). Likewise, repeated immunization has been shown to progressively build up considerable numbers of TRM cells even in non-infected areas of skin (110).

Tissue-resident memory precursor cells are found within the $KLRG1^-$ effector population that also contains the precursors of circulating memory CD8 T cells (114, 117, 132). Of note, TRM precursor effector cells rapidly cease to migrate through peripheral tissues after pathogen clearance (111, 130), meaning that the pool of TRM cells is established early after infection and remains stable with minimal external input thereafter.

The generation of TRM cells in infected skin is regulated by a complex interplay of processes such as peripheral infiltration, tissue exit, local positioning, and survival, which ultimately results in the maturation of a long-lived population of TRM cells in the

epidermal layer. Indeed, epithelial infiltration is critical for TRM formation, at least in skin, since effector T cells that are prevented from epidermal entry by pertussis toxin treatment fail to generate long-lived CD103$^+$ memory cells (117). This compartment-specific generation of T cell memory in skin likely reflects the anatomical restriction of essential differentiation and survival factors such as TGF-β and IL-15 that are expressed at high levels in the epithelial layer. Further supporting this assumption, CD8 T cells fail to acquire a CD103$^+$ TRM phenotype and are lost from skin over time in absence of TGF-β and IL-15 signaling (117).

In addition to these roles of TGF-β and IL-15 in TRM differentiation and survival in skin, the same or other essential cytokines and local factors may be involved in the establishment of TRM populations in an organ- or location-specific manner (113, 114, 116). Local antigen recognition for instance is strictly required for the establishment of long-lived TRM populations in organs such as brain, sensory ganglia, and lungs (120, 130, 133, 134). By contrast, in many other peripheral tissues and internal organs, including skin, gut, kidneys, and salivary glands, local antigen recognition appears to be dispensable for TRM development (113, 130). Nevertheless, it is conceivable that local antigen stimulation enhances TRM accumulation, possibly through the downregulation of tissue exit receptors such as CCR7 or through direct effects on local proliferation and survival of TRM precursor cells.

Tissue-Specific Gene Expression Signature of CD103$^+$CD8$^+$ TRM Cells

Importantly, local memory differentiation by tissue infiltrating effector T cells is accompanied by the progressive acquisition of a specific transcriptional program that distinguishes TRM cells from their TEM and TCM counterparts in the circulation (117, 122). Interestingly, the majority of these TRM-specific transcripts appear to be imprinted by the organ of residency. Skin CD8 TRM cells for instance express CCR8 whereas those in gut mucosa express CCR9 (130), and both chemokine receptors have been implicated in organ-specific homing to the respective anatomical locations. In addition, approximately a quarter of the TRM-specific transcripts are commonly up or downregulated in different CD103$^+$ TRM cell populations from skin, gut, and lungs when compared to recirculating memory cells. This TRM-specific core transcriptional profile across a variety of organs comprises genes encoding for migration and adhesion molecules (e.g., *Itga, Itga1*, and *S1pr1*), transcription factors (e.g., *Eomes*, *Hobit*, and *Litaf*), immunoregulatory molecules (e.g., *Cd244*, *Ctla-4*, and *Icos*), and various enzymes (e.g., *Inapp4b, Cmah*, and *Qpct*) and likely echoes a common molecular program underpinning the formation, persistence, and function of CD103$^+$ CD8 TRM cells in peripheral tissues (130).

Future work will have to define how stably this transcriptional profile is imprinted in TRM cells, and whether TRM cells can adopt alternative transcriptional and functional programs on renewed antigenic challenge or experimental release from the tissue context. Relevant to this, it will be important to test whether the same epigenetic modifications that regulate chromatin accessibility and gene transcriptional programs in recirculating memory T cells (62, 63) also operate in TRM cells. Likewise, the extent to which the CD8 TRM transcriptional signatures and their putative epigenetic regulators overlap with those in CD103$^-$ and/or CD4 TRM cells is currently unknown and needs to be tested. Regardless, there is accumulating evidence that a considerable proportion of T cells in peripheral non-lymphoid tissues adopt a phenotype and transcriptional profile that clearly distinguishes them from recirculating memory T cells.

Contribution of CD103$^+$ CD8$^+$ TRM Cells to Local Immunity

Owing to their strategic location at body surfaces, TRM cells can immediately sense invading pathogens and initiate a rapid local immune response (112). In skin, for instance, TRM cells use dendritic protrusions to probe their environment, a behavior

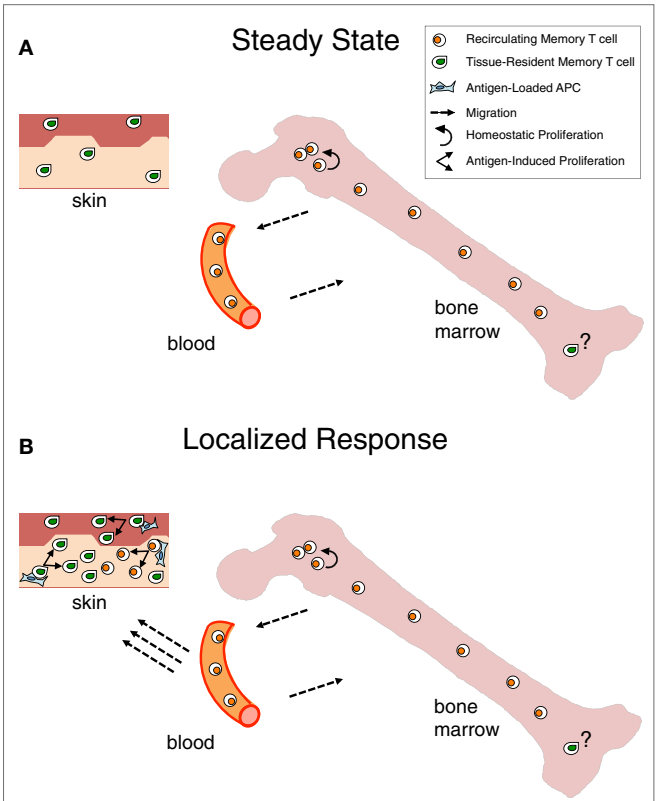

FIGURE 1 | Contribution of bone marrow memory T cells and tissue-resident memory T cells to long-lasting immunity. **(A)** Under steady state, Bone Marrow (BM) memory T cells are in constant exchange with the recirculating memory T cell pool, while tissue resident memory T (TRM) cells are fixed within peripheral organs, e.g., skin, in a quiescent state. Although the majority of BM memory T cells are quiescent, a minor proportion of them self-renew within BM niches, mostly in response to cytokines, e.g., IL-15. This homeostatic proliferation counterbalances losses due to cell death and contributes to maintain constant numbers of recirculating memory T cells over time (8, 9). The putative possibility that a few TRM cells exist in the BM is depicted. **(B)** When infection is localized in peripheral organs, TRM cells represent a first line of defense that is reinforced by the arrival of recirculating memory T cells, acting as a second line of protection. Antigen presented by APC in the peripheral tissue leads to immediate antigen-specific expansion and effector response of the TRM and, with some delay, of the newly recruited memory T cells (163, 164).

that reflects their active contribution to skin immune surveillance (109, 135). Following activation by their pathogen-specific T cell receptors, TRM cells produce a number of proinflammatory mediators, including the cytokines IFN-γ, TNF-α, and IL-2 (112, 136–139). These mediators drive the local activation and recruitment of innate and adaptive immune cells and also act on stromal and parenchymal cells to confer a resistance to pathogen dissemination (112, 136, 138, 139). In addition, TRM cell may directly lyse infected target cells, although the contribution of cytotoxicity in TRM-mediated protection remains to be demonstrated.

Tissue-resident memory cells have been shown to provide an exceptional level of immediate protection from renewed infection with a broad variety of viral and bacterial pathogens (108–110, 114, 134, 135, 138, 140). Furthermore, TRM cells generated by immunization strategies combining T cell priming with local treatment with inflammatory adjuvants provide a level of protection from *de novo* infection in skin and mucosa that is far superior to what can be achieved by circulating memory cells (130). Likewise, local immunizations with human papilloma virus vectors or treatment with T cell-attracting chemokines have been shown to generate protective TRM cells in vaginal mucosa and the female reproductive tract (141, 142).

Since these experiments confirm that TRM cells do not rely on antigen for long-term persistence, unlike other approaches used to generate T-cell immunity in peripheral tissues (143), TRM cells are now regarded as promising mediators of long-lived peripheral immunity for future vaccines (144). Importantly in this respect, a series of landmark papers have demonstrated the existence of Herpes Simplex Virus (HSV)-specific $CD8^+$ memory T cells resident at the dermal-epidermal junction in human genital skin (145–147). Remarkably, these resident T cells share transcriptional commonalities with HSV-specific TRM cells in mice (148) and are involved in preventing genital lesions upon asymptomatic HSV-2 shedding in skin (147). Furthermore, $CD8^+$ memory T cells with a $CD103^+CD69^+VLA\text{-}1^+$ TRM phenotype also exist in other human tissues (129, 149, 150). Such observations strongly argue that TRM cells are critical mediators of peripheral immunity in both mice and humans, and it is has been speculated that

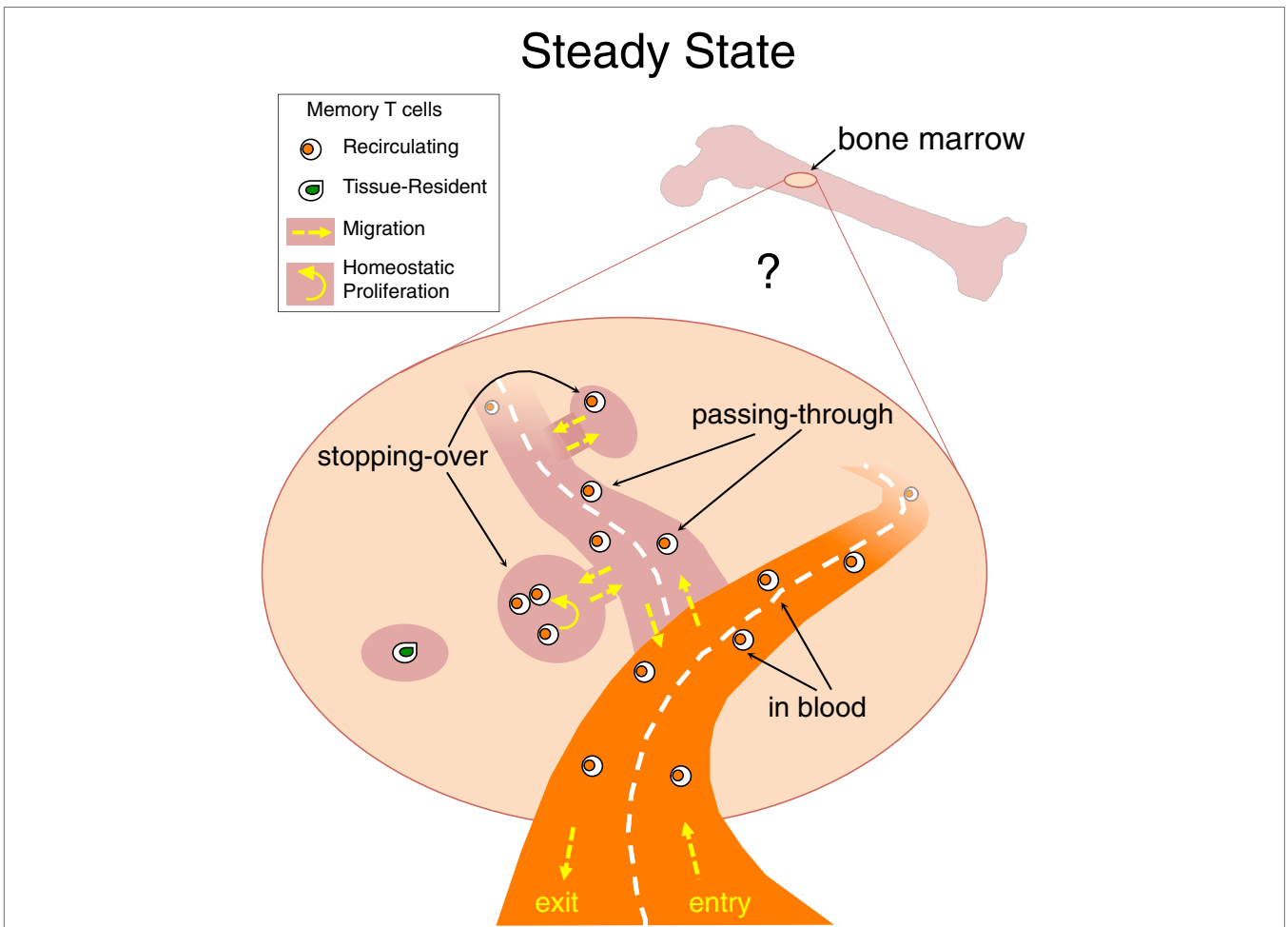

FIGURE 2 | Stopping-over, passing-through and tissue-resident memory T cells in bone marrow. Under steady state, memory T cells migrate into the BM and then circulate back to the blood, with poorly defined kinetics. It is possible that some recirculating memory T cells quickly transit through the BM parenchyma while others stop over for some time within BM niches. A few memory T cells might stay permanently in BM niches and never return to the blood, representing the equivalent of tissue-resident memory (TRM) T cells identified in other organs.

one of their principle functions is in dealing with recurrent or persistent infections in defined anatomical niches such as epithelial or neuronal compartments (151).

While in such cases, TRM cells exert highly beneficial protective functions, pathogenic TRM responses may also cause tissue pathology. Supporting this notion, accumulation and aberrant activation of TRM cells have been described in localized and recurrent human diseases such as skin autoimmunity and transplant rejection (152). Early studies using xenotransplantation of pre-psoriatic human skin onto mice, for instance, have demonstrated that graft-derived human TRM cells, in absence of circulating memory T cells, are sufficient to drive development of psoriasis lesions through localized production of inflammatory cytokines (153, 154). Similarly, IFN-γ producing epidermal TRM cells can initiate skin lesions upon ingestion of drugs that cause recurrent fixed drug eruptions (155), and $CD8^+$ T cells with a TRM phenotype have been described in alopecia and vitiligo lesions (156, 157). Another impressive demonstration of TRM-mediated pathology in humans stems from a recent study that defined donor-derived TRM cells as the major drivers of localized rejection responses after face transplantation (158). Finally, $CD103^+CD8^+$ T cells make up a considerable proportion of tumor-infiltrating lymphocytes in carcinomas and brain tumors, and their peri-tumoural accumulation is associated with improved clinical outcomes (159–162).

In summary, an accumulating body of literature implies a critical role of protective as well as pathogenic TRM responses in infectious and inflammatory diseases in humans. While so far this has most intensively been studied in easily accessible organs such as skin, it is tempting to speculate that TRM cells act as central mediators of localized diseases also in lungs, gut, and many other internal organs. Future studies will have to further clarify the role of protective and pathogenic TRM cell responses in humans and will have to explore avenues for their prophylactic or therapeutic manipulation in experimental and clinical settings.

CONCLUDING REMARKS

The postulated division of labor between recirculating memory T cells and TRM cells offers a novel view of both memory maintenance and response to antigenic rechallenge that integrates and broadens the previous perspective based on TCM/TEM paradigm (13).

Long-lived persistence of memory T cells is achieved in the steady state by different mechanisms. Recirculating memory T cells rely on a finely tuned equilibrium between quiescence and homeostatic proliferation, which is mostly achieved within the BM niches wherein these cells temporarily stop. In contrast, TRM cells live permanently as sessile non-migratory cells within skin/mucosal niches, wherein they survive in a quiescent state (**Figure 1A**).

It appears that recirculating memory T cells and TRM cells provide respectively systemic immunity and immediate protection at the port of pathogen entry. Yet, the two types of memory T cells act in concert for tissue protection as recirculating memory T cells are recruited to skin or mucosal sites of secondary challenge, resulting in both more efficient local effector response and boosting of systemic memory (**Figure 1B**). Naturally, recirculating memory T cells arrive with some delay in the tissue, while TRM cells are already there for highly efficient immediate protection.

Taking into consideration that TRM cells have been identified not only at the epithelial barriers of the body but also in lymphoid and/or internal organs, e.g., LN and brain (106, 107), it is likely that each organ harbors some TRM cells and that BM is not an exception to this rule (**Figure 1**). The peculiarity of the BM would be that under steady state most memory T cells in it belong to the recirculating pool. Some heterogeneity might exist in the kinetics of recirculating memory T cell transit into the BM, with some cells just quickly passing through and others stopping over during their journey, thus inhabiting for some time the BM niches wherein they receive signals for survival/homeostatic proliferation (**Figure 2**).

Despite their different migration pathways, positioning and role in immunity, both TRM and BM T cells participate in a local network of cellular and molecular interactions in the organ where they are located, influencing normal tissue homeostasis and organ function. In the case of BM T cells, it has been shown that they normally regulate hematopoiesis, as well as bone metabolism. In respect with TRM cells located in barrier organs, it is conceivable

BOX 1 | Unsolved questions on bone marrow (BM) memory T cells and tissue-resident memory (TRM) T cells.

Tissue-specific signals for differentiation: After naive T cell priming, what are the distinct signals regulating differentiation of either recirculating memory T cells or TRM cells? Do recirculating memory T cells integrate signals for differentiation they receive over time in diverse environments (e.g., within BM, spleen, and LN)? Do TRM cells lodged in diverse tissues receive in each location a specific combination of signals for differentiation?

Tissue-specific signals for survival/homeostatic proliferation: What are the external signals regulating the long-lasting maintenance of recirculating memory T cells within BM niches and/or TRM cells within tissue niches? What is the role of external signals (including antigen, IL-15, TGF-β, etc.) in the quiescence/self-renewal of BM memory T cells and/or in the survival in a quiescent state of TRM cells?

Heterogeneity: If TRM cells receive a specific combination of signals for differentiation and maintenance in each organ or location, and if recirculating memory T cells integrate diverse signals over time, are TRM cells, taken as a whole, expected to be more heterogeneous than recirculating memory T cells? Is it conceivable that several different subsets of location-specific TRM cells do exist?

Intracellular networks orchestrating differentiation: What are the intracellular molecular pathways regulating differentiation and stable genetic imprinting or plasticity of TRM and recirculating memory T cells? How do key intracellular molecules regulate epigenetic marking, gene transcription, protein translation, metabolic state of TRM and of recirculating memory T cells?

Regulation of absolute cell numbers: Is homeostatic maintenance of memory T cell numbers independently regulated for TRM cells and for recirculating memory T cells (including those in BM, spleen, and LN)?

CD4 and CD8 T cells: Do TRM cells resemble recirculating memory T cells in respect to the shared and/or specific protective functions of the CD4 and the CD8 T cell subsets? Are differentiation and maintenance of the CD4 TRM similar to those of the CD8 TRM?

Aging: Are there changes occurring with aging in the distribution of tasks between TRM and recirculating memory T cells?

Normal tissue homeostasis and organ function: Do BM memory T cells and TRM cells play a role in maintaining normal tissue homeostasis and organ function?

that they protect host's health and sometimes contribute to disease in several manners, for example they might shape the gut microbiota composition, with a possible indirect impact on metabolic syndrome, obesity-related disorders, inflammatory bowel disease, and colorectal cancer (101, 165). Some of the unsolved questions on BM memory T cells and TRM cells are listed in **Box 1**.

AUTHOR CONTRIBUTIONS

FR conceptualized the article, prepared the box and the figures, and wrote the section on BM T cells; TG wrote the section on TRM cells; FR and TG worked together on the remaining sections.

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REFERENCES

1. Westermann J, Pabst R. Distribution of lymphocyte subsets and natural killer cells in the human body. *Clin Investig* (1992) **70**:539–44. doi:10.1007/BF00184787
2. Di Rosa F, Pabst R. The bone marrow: a nest for migratory memory T cells. *Trends Immunol* (2005) **26**:360–6. doi:10.1016/j.it.2005.04.011
3. Zou L, Barnett B, Safah H, Larussa VF, Evdemon-Hogan M, Mottram P, et al. Bone marrow is a reservoir for CD4+CD25+ regulatory T cells that traffic through CXCL12/CXCR4 signals. *Cancer Res* (2004) **64**:8451–5. doi:10.1158/0008-5472.CAN-04-1987
4. Benner R, Meima F, van der Meulen GM. Antibody formation in mouse bone marrow. II. Evidence for a memory-dependent phenomenon. *Cell Immunol* (1974) **13**:95–106. doi:10.1016/0008-8749(74)90230-5
5. Slifka MK, Whitmire JK, Ahmed R. Bone marrow contains virus-specific cytotoxic T lymphocytes. *Blood* (1997) **90**:2103–8.
6. Masopust D, Vezys V, Marzo AL, Lefrancois L. Preferential localization of effector memory cells in nonlymphoid tissue. *Science* (2001) **291**:2413–7. doi:10.1126/science.1058867
7. Di Rosa F, Santoni A. Bone marrow CD8 T cells are in a different activation state than those in lymphoid periphery. *Eur J Immunol* (2002) **32**:1873–80. doi:10.1002/1521-4141(200207)32:7<1873::AID-IMMU1873>3.0.CO;2-P
8. Parretta E, Cassese G, Barba P, Santoni A, Guardiola J, Di Rosa F. CD8 cell division maintaining cytotoxic memory occurs predominantly in the bone marrow. *J Immunol* (2005) **174**:7654–64. doi:10.4049/jimmunol.174.12.7654
9. Becker TC, Coley SM, Wherry EJ, Ahmed R. Bone marrow is a preferred site for homeostatic proliferation of memory CD8 T cells. *J Immunol* (2005) **174**:1269–73. doi:10.4049/jimmunol.174.3.1269
10. Price PW, Cerny J. Characterization of CD4+ T cells in mouse bone marrow. I. Increased activated/memory phenotype and altered TCR Vbeta repertoire. *Eur J Immunol* (1999) **29**:1051–6. doi:10.1002/(SICI)1521-4141(199903)29:03<1051::AID-IMMU1051>3.0.CO;2-Y
11. Di Rosa F, Santoni A. Memory T-cell competition for bone marrow seeding. *Immunology* (2003) **108**:296–304. doi:10.1046/j.1365-2567.2003.01593.x
12. Herndler-Brandstetter D, Landgraf K, Tzankov A, Jenewein B, Brunauer R, Laschober GT, et al. The impact of aging on memory T cell phenotype and function in the human bone marrow. *J Leukoc Biol* (2012) **91**:197–205. doi:10.1189/jlb.0611299
13. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* (1999) **401**:708–12. doi:10.1038/44385
14. Mazo IB, Honczarenko M, Leung H, Cavanagh LL, Bonasio R, Weninger W, et al. Bone marrow is a major reservoir and site of recruitment for central memory CD8+ T cells. *Immunity* (2005) **22**:259–70. doi:10.1016/j.immuni.2005.01.008
15. Zhang X, Dong H, Lin W, Voss S, Hinkley L, Westergren M, et al. Human bone marrow: a reservoir for "enhanced effector memory" CD8+ T cells with potent recall function. *J Immunol* (2006) **177**:6730–7. doi:10.4049/jimmunol.177.10.6730
16. Letsch A, Knoedler M, Na IK, Kern F, Asemissen AM, Keilholz U, et al. CMV-specific central memory T cells reside in bone marrow. *Eur J Immunol* (2007) **37**:3063–8. doi:10.1002/eji.200636930
17. Akatsuka Y, Torikai H, Inamoto Y, Tsujimura K, Morishima Y, Kodera Y, et al. Bone marrow may be a reservoir of long-lived memory T cells specific for minor histocompatibility antigen. *Br J Haematol* (2006) **135**:413–4. doi:10.1111/j.1365-2141.2006.06313.x
18. Pabst R, Miyasaka M, Dudler L. Numbers and phenotype of lymphocytes emigrating from sheep bone marrow after in situ labelling with fluorescein isothiocyanate. *Immunology* (1986) **59**:217–22.
19. Klonowski KD, Williams KJ, Marzo AL, Blair DA, Lingenheld EG, Lefrancois L. Dynamics of blood-borne CD8 memory T cell migration in vivo. *Immunity* (2004) **20**:551–62. doi:10.1016/S1074-7613(04)00103-7
20. Milo I, Sapoznikov A, Kalchenko V, Tal O, Krauthgamer R, van Rooijen N, et al. Dynamic imaging reveals promiscuous crosspresentation of blood-borne antigens to naive CD8+ T cells in the bone marrow. *Blood* (2013) **122**:193–208. doi:10.1182/blood-2012-01-401265
21. Tokoyoda K, Zehentmeier S, Hegazy AN, Albrecht I, Grun JR, Lohning M, et al. Professional memory CD4+ T lymphocytes preferentially reside and rest in the bone marrow. *Immunity* (2009) **30**:721–30. doi:10.1016/j.immuni.2009.03.015
22. Okhrimenko A, Grun JR, Westendorf K, Fang Z, Reinke S, von Roth P, et al. Human memory T cells from the bone marrow are resting and maintain long-lasting systemic memory. *Proc Natl Acad Sci U S A* (2014) **111**:9229–34. doi:10.1073/pnas.1318731111
23. Sercan Alp O, Durlanik S, Schulz D, McGrath M, Grun JR, Bardua M, et al. Memory CD8(+) T cells colocalize with IL-7(+) stromal cells in bone marrow and rest in terms of proliferation and transcription. *Eur J Immunol* (2015) **45**:975–87. doi:10.1002/eji.201445295
24. Hofmann M, Oschowitzer A, Kurzhals SR, Kruger CC, Pircher H. Thymus-resident memory CD8+ T cells mediate local immunity. *Eur J Immunol* (2013) **43**:2295–304. doi:10.1002/eji.201343519
25. Schenkel JM, Fraser KA, Masopust D. Cutting edge: resident memory CD8 T cells occupy frontline niches in secondary lymphoid organs. *J Immunol* (2014) **192**:2961–4. doi:10.4049/jimmunol.1400003
26. Cinamon G, Shinder V, Alon R. Shear forces promote lymphocyte migration across vascular endothelium bearing apical chemokines. *Nat Immunol* (2001) **2**:515–22. doi:10.1038/88710
27. Palendira U, Chinn R, Raza W, Piper K, Pratt G, Machado L, et al. Selective accumulation of virus-specific CD8+ T cells with unique homing phenotype within the human bone marrow. *Blood* (2008) **112**:3293–302. doi:10.1182/blood-2008-02-138040
28. DeNucci CC, Shimizu Y. Beta1 integrin is critical for the maintenance of antigen-specific CD4 T cells in the bone marrow but not long-term immunological memory. *J Immunol* (2011) **186**:4019–26. doi:10.4049/jimmunol.1003566

29. Koni PA, Joshi SK, Temann UA, Olson D, Burkly L, Flavell RA. Conditional vascular cell adhesion molecule 1 deletion in mice: impaired lymphocyte migration to bone marrow. *J Exp Med* (2001) **193**:741–54. doi:10.1084/jem.193.6.741
30. Maeda Y, Seki N, Sato N, Sugahara K, Chiba K. Sphingosine 1-phosphate receptor type 1 regulates egress of mature T cells from mouse bone marrow. *Int Immunol* (2010) **22**:515–25. doi:10.1093/intimm/dxq036
31. Shiow LR, Rosen DB, Brdickova N, Xu Y, An J, Lanier LL, et al. CD69 acts downstream of interferon-alpha/beta to inhibit S1P1 and lymphocyte egress from lymphoid organs. *Nature* (2006) **440**:540–4. doi:10.1038/nature04606
32. Shinoda K, Tokoyoda K, Hanazawa A, Hayashizaki K, Zehentmeier S, Hosokawa H, et al. Type II membrane protein CD69 regulates the formation of resting T-helper memory. *Proc Natl Acad Sci U S A* (2012) **109**:7409–14. doi:10.1073/pnas.1118539109
33. Chen JJ, Huang JC, Shirtliff M, Briscoe E, Ali S, Cesani F, et al. CD4 lymphocytes in the blood of HIV(+) individuals migrate rapidly to lymph nodes and bone marrow: support for homing theory of CD4 cell depletion. *J Leukoc Biol* (2002) **72**:271–8.
34. Gurman G, Dilek I, Arslan O, Arat M, Beksac M, Ilhan O, et al. The effect of G-CSF on lymphocyte subsets and CD34+ cells in allogeneic stem cell transplantation. *Transfus Apher Sci* (2001) **24**:23–8. doi:10.1016/S0955-3886(00)00123-5
35. Condomines M, Quittet P, Lu ZY, Nadal L, Latry P, Lopez E, et al. Functional regulatory T cells are collected in stem cell autografts by mobilization with high-dose cyclophosphamide and granulocyte colony-stimulating factor. *J Immunol* (2006) **176**:6631–9. doi:10.4049/jimmunol.176.11.6631
36. Lopez CB, Hermesh T. Systemic responses during local viral infections: type I IFNs sound the alarm. *Curr Opin Immunol* (2011) **23**:495–9. doi:10.1016/j.coi.2011.06.003
37. Vavassori M, Maccario R, Moretta A, Comoli P, Wack A, Locatelli F, et al. Restricted TCR repertoire and long-term persistence of donor-derived antigen-experienced CD4+ T cells in allogeneic bone marrow transplantation recipients. *J Immunol* (1996) **157**:5739–47.
38. Racanelli V, Frassanito MA, Leone P, Brunetti C, Ruggieri S, Dammacco F. Bone marrow of persistently hepatitis C virus-infected individuals accumulates memory CD8+ T cells specific for current and historical viral antigens: a study in patients with benign hematological disorders. *J Immunol* (2007) **179**:5387–98. doi:10.4049/jimmunol.179.8.5387
39. Guerreiro M, Na IK, Letsch A, Haase D, Bauer S, Meisel C, et al. Human peripheral blood and bone marrow Epstein-Barr virus-specific T-cell repertoire in latent infection reveals distinct memory T-cell subsets. *Eur J Immunol* (2010) **40**:1566–76. doi:10.1002/eji.200940000
40. Jahn B, Bergmann L, Weidmann E, Brieger J, Fenchel K, Schwulera U, et al. Bone marrow-derived T-cell clones obtained from untreated acute myelocytic leukemia exhibit blast directed autologous cytotoxicity. *Leuk Res* (1995) **19**:73–82. doi:10.1016/0145-2126(94)00119-U
41. Feuerer M, Beckhove P, Bai L, Solomayer EF, Bastert G, Diel IJ, et al. Therapy of human tumors in NOD/SCID mice with patient-derived reactivated memory T cells from bone marrow. *Nat Med* (2001) **7**:452–8. doi:10.1038/86523
42. Olsson B, Andersson PO, Jernas M, Jacobsson S, Carlsson B, Carlsson LM, et al. T-cell-mediated cytotoxicity toward platelets in chronic idiopathic thrombocytopenic purpura. *Nat Med* (2003) **9**:1123–4. doi:10.1038/nm921
43. Letsch A, Keilholz U, Assfalg G, Mailander V, Thiel E, Scheibenbogen C. Bone marrow contains melanoma-reactive CD8+ effector T cells and, compared with peripheral blood, enriched numbers of melanoma-reactive CD8+ memory T cells. *Cancer Res* (2003) **63**:5582–6.
44. Marshall DR, Turner SJ, Belz GT, Wingo S, Andreansky S, Sangster MY, et al. Measuring the diaspora for virus-specific CD8+ T cells. *Proc Natl Acad Sci U S A* (2001) **98**:6313–8. doi:10.1073/pnas.101132698
45. Isogai S, Miyata S, Taha R, Yoshizawa Y, Martin JG, Hamid Q. CD4+ T cells migrate from airway to bone marrow after antigen inhalation in rats. *J Allergy Clin Immunol* (2004) **113**:455–61. doi:10.1016/j.jaci.2003.11.035
46. Tripp RA, Topham DJ, Watson SR, Doherty PC. Bone marrow can function as a lymphoid organ during a primary immune response under conditions of disrupted lymphocyte trafficking. *J Immunol* (1997) **158**:3716–20.
47. Feuerer M, Beckhove P, Garbi N, Mahnke Y, Limmer A, Hommel M, et al. Bone marrow as a priming site for T-cell responses to blood-borne antigen. *Nat Med* (2003) **9**:1151–7. doi:10.1038/nm914
48. Cavanagh LL, Bonasio R, Mazo IB, Halin C, Cheng G, van der Velden AW, et al. Activation of bone marrow-resident memory T cells by circulating, antigen-bearing dendritic cells. *Nat Immunol* (2005) **6**:1029–37. doi:10.1038/ni1249
49. Duffy D, Perrin H, Abadie V, Benhabiles N, Boissonnas A, Liard C, et al. Neutrophils transport antigen from the dermis to the bone marrow, initiating a source of memory CD8+ T cells. *Immunity* (2012) **37**:917–29. doi:10.1016/j.immuni.2012.07.015
50. Tokoyoda K, Hauser AE, Nakayama T, Radbruch A. Organization of immunological memory by bone marrow stroma. *Nat Rev Immunol* (2010) **10**:193–200. doi:10.1038/nri2727
51. Lin GH, Edele F, Mbanwi AN, Wortzman ME, Snell LM, Vidric M, et al. Contribution of 4-1BBL on radioresistant cells in providing survival signals through 4-1BB expressed on CD8(+) memory T cells in the bone marrow. *Eur J Immunol* (2012) **42**:2861–74. doi:10.1002/eji.201242503
52. Herndler-Brandstetter D, Landgraf K, Jenewein B, Tzankov A, Brunauer R, Brunner S, et al. Human bone marrow hosts polyfunctional memory CD4+ and CD8+ T cells with close contact to IL-15-producing cells. *J Immunol* (2011) **186**:6965–71. doi:10.4049/jimmunol.1100243
53. Pulle G, Vidric M, Watts TH. IL-15-dependent induction of 4-1BB promotes antigen-independent CD8 memory T cell survival. *J Immunol* (2006) **176**:2739–48. doi:10.4049/jimmunol.176.5.2739
54. Sabbagh L, Snell LM, Watts TH. TNF family ligands define niches for T cell memory. *Trends Immunol* (2007) **28**:333–9. doi:10.1016/j.it.2007.06.001
55. Allam A, Conze DB, Giardino Torchia ML, Munitic I, Yagita H, Sowell RT, et al. The CD8+ memory T-cell state of readiness is actively maintained and reversible. *Blood* (2009) **114**:2121–30. doi:10.1182/blood-2009-05-220087
56. Snell LM, Lin GH, Watts TH. IL-15-dependent upregulation of GITR on CD8 memory phenotype T cells in the bone marrow relative to spleen and lymph node suggests the bone marrow as a site of superior bioavailability of IL-15. *J Immunol* (2012) **188**:5915–23. doi:10.4049/jimmunol.1103270
57. Lin GH, Snell LM, Wortzman ME, Clouthier DL, Watts TH. GITR-dependent regulation of 4-1BB expression: implications for T cell memory and anti-4-1BB-induced pathology. *J Immunol* (2013) **190**:4627–39. doi:10.4049/jimmunol.1201854
58. Gerlach C, van Heijst JW, Swart E, Sie D, Armstrong N, Kerkhoven RM, et al. One naive T cell, multiple fates in CD8+ T cell differentiation. *J Exp Med* (2010) **207**:1235–46. doi:10.1084/jem.20091175
59. Cassese G, Parretta E, Pisapia L, Santoni A, Guardiola J, Di Rosa F. Bone marrow CD8 cells down-modulate membrane IL-7R{alpha} expression and exhibit increased STAT-5 and p38 MAPK phosphorylation in the organ environment. *Blood* (2007) **110**:1960–9. doi:10.1182/blood-2006-09-045807
60. Quinci AC, Vitale S, Parretta E, Soriani A, Iannitto ML, Cippitelli M, et al. IL-15 inhibits IL-7Ralpha expression by memory-phenotype CD8(+) T cells in the bone marrow. *Eur J Immunol* (2012) **42**:1129–39. doi:10.1002/eji.201142019
61. Kudernatsch RF, Letsch A, Guerreiro M, Lobel M, Bauer S, Volk HD, et al. Human bone marrow contains a subset of quiescent early memory CD8(+) T cells characterized by high CD127 expression and efflux capacity. *Eur J Immunol* (2014) **44**:3532–42. doi:10.1002/eji.201344180
62. Weng NP, Araki Y, Subedi K. The molecular basis of the memory T cell response: differential gene expression and its epigenetic regulation. *Nat Rev Immunol* (2012) **12**:306–15. doi:10.1038/nri3173
63. Youngblood B, Hale JS, Ahmed R. Memory CD8 T cell transcriptional plasticity. *F1000Prime Rep* (2015) **7**:38. doi:10.12703/P7-38
64. Nemoto Y, Kanai T, Makita S, Okamoto R, Totsuka T, Takeda K, et al. Bone marrow retaining colitogenic CD4+ T cells may be a pathogenic reservoir for chronic colitis. *Gastroenterology* (2007) **132**:176–89. doi:10.1053/j.gastro.2006.10.035
65. Tough DF, Sprent J. Turnover of naive- and memory-phenotype T cells. *J Exp Med* (1994) **179**:1127–35. doi:10.1084/jem.179.4.1127
66. Parretta E, Cassese G, Santoni A, Guardiola J, Vecchio A, Di Rosa F. Kinetics of in vivo proliferation and death of memory and naive CD8 T cells: parameter estimation based on 5-bromo-2'-deoxyuridine incorporation in spleen, lymph nodes, and bone marrow. *J Immunol* (2008) **180**:7230–9. doi:10.4049/jimmunol.180.11.7230
67. Di Rosa F. T-lymphocyte interaction with stromal, bone and hematopoietic cells in the bone marrow. *Immunol Cell Biol* (2009) **87**:20–9. doi:10.1038/icb.2008.84

68. Graef P, Buchholz VR, Stemberger C, Flossdorf M, Henkel L, Schiemann M, et al. Serial transfer of single-cell-derived immunocompetence reveals stemness of CD8(+) central memory T cells. *Immunity* (2014) **41**:116–26. doi:10.1016/j.immuni.2014.05.018
69. Banerjee A, Gordon SM, Intlekofer AM, Paley MA, Mooney EC, Lindsten T, et al. Cutting edge: the transcription factor eomesodermin enables CD8+ T cells to compete for the memory cell niche. *J Immunol* (2010) **185**:4988–92. doi:10.4049/jimmunol.1002042
70. Chaix J, Nish SA, Lin WH, Rothman NJ, Ding L, Wherry EJ, et al. Cutting edge: CXCR4 is critical for CD8+ memory T cell homeostatic self-renewal but not rechallenge self-renewal. *J Immunol* (2014) **193**:1013–6. doi:10.4049/jimmunol.1400488
71. Beckhove P, Feuerer M, Dolenc M, Schuetz F, Choi C, Sommerfeldt N, et al. Specifically activated memory T cell subsets from cancer patients recognize and reject xenotransplanted autologous tumors. *J Clin Invest* (2004) **114**:67–76. doi:10.1172/JCI200420278
72. Noonan K, Matsui W, Serafini P, Carbley R, Tan G, Khalili J, et al. Activated marrow-infiltrating lymphocytes effectively target plasma cells and their clonogenic precursors. *Cancer Res* (2005) **65**:2026–34. doi:10.1158/0008-5472.CAN-04-3337
73. Domschke C, Ge Y, Bernhardt I, Schott S, Keim S, Juenger S, et al. Long-term survival after adoptive bone marrow T cell therapy of advanced metastasized breast cancer: follow-up analysis of a clinical pilot trial. *Cancer Immunol Immunother* (2013) **62**:1053–60. doi:10.1007/s00262-013-1414-x
74. Noonan KA, Huff CA, Davis J, Lemas MV, Fiorino S, Bitzan J, et al. Adoptive transfer of activated marrow-infiltrating lymphocytes induces measurable antitumor immunity in the bone marrow in multiple myeloma. *Sci Transl Med* (2015) **7**:288ra78. doi:10.1126/scitranslmed.aaa7014
75. Paiardini M, Cervasi B, Engram JC, Gordon SN, Klatt NR, Muthukumar A, et al. Bone marrow-based homeostatic proliferation of mature T cells in nonhuman primates: implications for AIDS pathogenesis. *Blood* (2009) **113**:612–21. doi:10.1182/blood-2008-06-159442
76. Takizawa H, Boettcher S, Manz MG. Demand-adapted regulation of early hematopoiesis in infection and inflammation. *Blood* (2012) **119**:2991–3002. doi:10.1182/blood-2011-12-380113
77. Schurch CM, Riether C, Ochsenbein AF. Cytotoxic CD8+ T cells stimulate hematopoietic progenitors by promoting cytokine release from bone marrow mesenchymal stromal cells. *Cell Stem Cell* (2014) **14**:460–72. doi:10.1016/j.stem.2014.01.002
78. Baldridge MT, King KY, Boles NC, Weksberg DC, Goodell MA. Quiescent haematopoietic stem cells are activated by IFN-gamma in response to chronic infection. *Nature* (2010) **465**:793–7. doi:10.1038/nature09135
79. de Bruin AM, Demirel O, Hooibrink B, Brandts CH, Nolte MA. Interferon-gamma impairs proliferation of hematopoietic stem cells in mice. *Blood* (2013) **121**:3578–85. doi:10.1182/blood-2012-05-432906
80. de Bruin AM, Voermans C, Nolte MA. Impact of interferon-gamma on hematopoiesis. *Blood* (2014) **124**(16):2479–86. doi:10.1182/blood-2014-04-568451
81. Li R, Perez N, Karumuthil-Melethil S, Vasu C. Bone marrow is a preferential homing site for autoreactive T-cells in type 1 diabetes. *Diabetes* (2007) **56**:2251–9. doi:10.2337/db07-0502
82. Oh HM, Yu CR, Lee Y, Chan CC, Maminishkis A, Egwuagu CE. Autoreactive memory CD4+ T lymphocytes that mediate chronic uveitis reside in the bone marrow through STAT3-dependent mechanisms. *J Immunol* (2011) **187**:3338–46. doi:10.4049/jimmunol.1004019
83. Nemoto Y, Kanai T, Kameyama K, Shinohara T, Sakamoto N, Totsuka T, et al. Long-lived colitogenic CD4+ memory T cells residing outside the intestine participate in the perpetuation of chronic colitis. *J Immunol* (2009) **183**:5059–68. doi:10.4049/jimmunol.0803684
84. Nemoto Y, Kanai T, Takahara M, Oshima S, Nakamura T, Okamoto R, et al. Bone marrow-mesenchymal stem cells are a major source of interleukin-7 and sustain colitis by forming the niche for colitogenic CD4 memory T cells. *Gut* (2013) **62**:1142–52. doi:10.1136/gutjnl-2012-302029
85. Kong YY, Feige U, Sarosi I, Bolon B, Tafuri A, Morony S, et al. Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. *Nature* (1999) **402**:304–9. doi:10.1038/46303
86. Kotake S, Udagawa N, Takahashi N, Matsuzaki K, Itoh K, Ishiyama S, et al. IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis. *J Clin Invest* (1999) **103**:1345–52. doi:10.1172/JCI5703
87. Teng YT, Nguyen H, Gao X, Kong YY, Gorczynski RM, Singh B, et al. Functional human T-cell immunity and osteoprotegerin ligand control alveolar bone destruction in periodontal infection. *J Clin Invest* (2000) **106**:R59–67. doi:10.1172/JCI10763
88. Sato K, Suematsu A, Okamoto K, Yamaguchi A, Morishita Y, Kadono Y, et al. Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction. *J Exp Med* (2006) **203**:2673–82. doi:10.1084/jem.20061775
89. Ciucci T, Ibanez L, Boucoiran A, Birgy-Barelli E, Pene J, Abou-Ezzi G, et al. Bone marrow Th17 TNFalpha cells induce osteoclast differentiation, and link bone destruction to IBD. *Gut* (2015) **64**:1072–81. doi:10.1136/gutjnl-2014-306947
90. van Hogezand RA, Hamdy NA. Skeletal morbidity in inflammatory bowel disease. *Scand J Gastroenterol* (2006) **243**:59–64. doi:10.1080/00365520600664276
91. Redlich K, Smolen JS. Inflammatory bone loss: pathogenesis and therapeutic intervention. *Nat Rev Drug Discov* (2012) **11**:234–50. doi:10.1038/nrd3669
92. Monteiro AC, Leal AC, Goncalves-Silva T, Mercadante AC, Kestelman F, Chaves SB, et al. T cells induce pre-metastatic osteolytic disease and help bone metastases establishment in a mouse model of metastatic breast cancer. *PLoS One* (2013) **8**:e68171. doi:10.1371/journal.pone.0068171
93. Li Y, Toraldo G, Li A, Yang X, Zhang H, Qian WP, et al. B cells and T cells are critical for the preservation of bone homeostasis and attainment of peak bone mass in vivo. *Blood* (2007) **109**:3839–48. doi:10.1182/blood-2006-07-037994
94. Monteiro JP, Benjamin A, Costa ES, Barcinski MA, Bonomo A. Normal hematopoiesis is maintained by activated bone marrow CD4+ T cells. *Blood* (2005) **105**:1484–91. doi:10.1182/blood-2004-07-2856
95. Kim S, Park K, Choi J, Jang E, Paik DJ, Seong RH, et al. Foxp3+ regulatory T cells ensure B lymphopoiesis by inhibiting the granulopoietic activity of effector T cells in mouse bone marrow. *Eur J Immunol* (2015) **45**:167–79. doi:10.1002/eji.201444532
96. Muller AM, Poyser J, Kupper NJ, Burnett C, Ko RM, Kohrt HE, et al. Donor hematopoiesis in mice following total lymphoid irradiation requires host T-regulatory cells for durable engraftment. *Blood* (2014) **123**:2882–92. doi:10.1182/blood-2013-10-530212
97. Fujisaki J, Wu J, Carlson AL, Silberstein L, Putheti P, Larocca R, et al. In vivo imaging of Treg cells providing immune privilege to the haematopoietic stem-cell niche. *Nature* (2011) **474**:216–9. doi:10.1038/nature10160
98. Clark RA, Chong B, Mirchandani N, Brinster NK, Yamanaka K, Dowgiert RK, et al. The vast majority of CLA+ T cells are resident in normal skin. *J Immunol* (2006) **176**:4431–9. doi:10.4049/jimmunol.176.7.4431
99. Reinhardt RL, Khoruts A, Merica R, Zell T, Jenkins MK. Visualizing the generation of memory CD4 T cells in the whole body. *Nature* (2001) **410**:101–5. doi:10.1038/35065111
100. Steinert EM, Schenkel JM, Fraser KA, Beura LK, Manlove LS, Igyarto BZ, et al. Quantifying memory CD8 T cells reveals regionalization of immunosurveillance. *Cell* (2015) **161**:737–49. doi:10.1016/j.cell.2015.03.031
101. Hand TW, Dos Santos LM, Bouladoux N, Molloy MJ, Pagan AJ, Pepper M, et al. Acute gastrointestinal infection induces long-lived microbiota-specific T cell responses. *Science* (2012) **337**:1553–6. doi:10.1126/science.1220961
102. Naik S, Bouladoux N, Linehan JL, Han SJ, Harrison OJ, Wilhelm C, et al. Commensal-dendritic-cell interaction specifies a unique protective skin immune signature. *Nature* (2015) **520**:104–8. doi:10.1038/nature14052
103. Bromley SK, Yan S, Tomura M, Kanagawa O, Luster AD. Recirculating memory T cells are a unique subset of CD4+ T cells with a distinct phenotype and migratory pattern. *J Immunol* (2013) **190**:970–6. doi:10.4049/jimmunol.1202805
104. Mackay CR, Marston WL, Dudler L. Naive and memory T cells show distinct pathways of lymphocyte recirculation. *J Exp Med* (1990) **171**:801–17. doi:10.1084/jem.171.3.801
105. Yawalkar N, Hunger RE, Pichler WJ, Braathen LR, Brand CU. Human afferent lymph from normal skin contains an increased number of mainly memory/effector CD4(+) T cells expressing activation, adhesion and co-stimulatory molecules. *Eur J Immunol* (2000) **30**:491–7. doi:10.1002/1521-4141(200002)30:2<491::AID-IMMU491>3.0.CO;2-H

106. Gebhardt T, Mueller SN, Heath WR, Carbone FR. Peripheral tissue surveillance and residency by memory T cells. *Trends Immunol* (2013) **34**:27–32. doi:10.1016/j.it.2012.08.008
107. Schenkel JM, Masopust D. Tissue-resident memory T cells. *Immunity* (2014) **41**:886–97. doi:10.1016/j.immuni.2014.12.007
108. Gebhardt T, Wakim LM, Eidsmo L, Reading PC, Heath WR, Carbone FR. Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus. *Nat Immunol* (2009) **10**:524–30. doi:10.1038/ni.1718
109. Gebhardt T, Whitney PG, Zaid A, Mackay LK, Brooks AG, Heath WR, et al. Different patterns of peripheral migration by memory CD4+ and CD8+ T cells. *Nature* (2011) **477**:216–9. doi:10.1038/nature10339
110. Jiang X, Clark RA, Liu L, Wagers AJ, Fuhlbrigge RC, Kupper TS. Skin infection generates non-migratory memory CD8+ T(RM) cells providing global skin immunity. *Nature* (2012) **483**:227–31. doi:10.1038/nature10851
111. Masopust D, Choo D, Vezys V, Wherry EJ, Duraiswamy J, Akondy R, et al. Dynamic T cell migration program provides resident memory within intestinal epithelium. *J Exp Med* (2010) **207**:553–64. doi:10.1084/jem.20090858
112. Gebhardt T, Mackay LK. Local immunity by tissue-resident CD8+ memory T cells. *Front Immunol* (2012) **3**:340. doi:10.3389/fimmu.2012.00340
113. Casey KA, Fraser KA, Schenkel JM, Moran A, Abt MC, Beura LK, et al. Antigen-independent differentiation and maintenance of effector-like resident memory T cells in tissues. *J Immunol* (2012) **188**:4866–75. doi:10.4049/jimmunol.1200402
114. Sheridan BS, Pham QM, Lee YT, Cauley LS, Puddington L, Lefrancois L. Oral infection drives a distinct population of intestinal resident memory CD8(+) T cells with enhanced protective function. *Immunity* (2014) **40**:747–57. doi:10.1016/j.immuni.2014.03.007
115. Feng Y, Wang D, Yuan R, Parker CM, Farber DL, Hadley GA. CD103 expression is required for destruction of pancreatic islet allografts by CD8(+) T cells. *J Exp Med* (2002) **196**:877–86. doi:10.1084/jem.20020178
116. Lee YT, Suarez-Ramirez JE, Wu T, Redman JM, Bouchard K, Hadley GA, et al. Environmental and antigen receptor-derived signals support sustained surveillance of the lungs by pathogen-specific cytotoxic T lymphocytes. *J Virol* (2011) **85**:4085–94. doi:10.1128/JVI.02493-10
117. Mackay LK, Rahimpour A, Ma JZ, Collins N, Stock AT, Hafon ML, et al. The developmental pathway for CD103(+)CD8+ tissue-resident memory T cells of skin. *Nat Immunol* (2013) **14**:1294–301. doi:10.1038/ni.2744
118. Ray SJ, Franki SN, Pierce RH, Dimitrova S, Koteliansky V, Sprague AG, et al. The collagen binding alpha1beta1 integrin VLA-1 regulates CD8 T cell-mediated immune protection against heterologous influenza infection. *Immunity* (2004) **20**:167–79. doi:10.1016/S1074-7613(04)00021-4
119. Le Floc'h A, Jalil A, Vergnon I, Le Maux Chansac B, Lazar V, Bismuth G, et al. Alpha E beta 7 integrin interaction with E-cadherin promotes antitumor CTL activity by triggering lytic granule polarization and exocytosis. *J Exp Med* (2007) **204**:559–70. doi:10.1084/jem.20061524
120. Wakim LM, Woodward-Davis A, Bevan MJ. Memory T cells persisting within the brain after local infection show functional adaptations to their tissue of residence. *Proc Natl Acad Sci U S A* (2010) **107**:17872–9. doi:10.1073/pnas.1010201107
121. Mackay LK, Braun A, Macleod BL, Collins N, Tebartz C, Bedoui S, et al. Cutting edge: CD69 interference with sphingosine-1-phosphate receptor function regulates peripheral T cell retention. *J Immunol* (2015) **195**(5):2059–63. doi:10.4049/jimmunol.1402256
122. Skon CN, Lee JY, Anderson KG, Masopust D, Hogquist KA, Jameson SC. Transcriptional downregulation of S1pr1 is required for the establishment of resident memory CD8+ T cells. *Nat Immunol* (2013) **14**:1285–93. doi:10.1038/ni.2745
123. Bankovich AJ, Shiow LR, Cyster JG. CD69 suppresses sphingosine 1-phosophate receptor-1 (S1P1) function through interaction with membrane helix 4. *J Biol Chem* (2010) **285**:22328–37. doi:10.1074/jbc.M110.123299
124. Bergsbaken T, Bevan MJ. Proinflammatory microenvironments within the intestine regulate the differentiation of tissue-resident CD8(+) T cells responding to infection. *Nat Immunol* (2015) **16**:406–14. doi:10.1038/ni.3108
125. Purwar R, Campbell J, Murphy G, Richards WG, Clark RA, Kupper TS. Resident memory T cells (T(RM)) are abundant in human lung: diversity, function, and antigen specificity. *PLoS One* (2011) **6**:e16245. doi:10.1371/journal.pone.0016245
126. Teijaro JR, Turner D, Pham Q, Wherry EJ, Lefrancois L, Farber DL. Cutting edge: tissue-retentive lung memory CD4 T cells mediate optimal protection to respiratory virus infection. *J Immunol* (2011) **187**:5510–4. doi:10.4049/jimmunol.1102243
127. Iijima N, Iwasaki A. T cell memory. A local macrophage chemokine network sustains protective tissue-resident memory CD4 T cells. *Science* (2014) **346**:93–8. doi:10.1126/science.1257530
128. Watanabe R, Gehad A, Yang C, Scott LL, Teague JE, Schlapbach C, et al. Human skin is protected by four functionally and phenotypically discrete populations of resident and recirculating memory T cells. *Sci Transl Med* (2015) **7**:279ra39. doi:10.1126/scitranslmed.3010302
129. Sathaliyawala T, Kubota M, Yudanin N, Turner D, Camp P, Thome JJ, et al. Distribution and compartmentalization of human circulating and tissue-resident memory T cell subsets. *Immunity* (2013) **38**:187–97. doi:10.1016/j.immuni.2012.09.020
130. Mackay LK, Stock AT, Ma JZ, Jones CM, Kent SJ, Mueller SN, et al. Long-lived epithelial immunity by tissue-resident memory T (TRM) cells in the absence of persisting local antigen presentation. *Proc Natl Acad Sci U S A* (2012) **109**:7037–42. doi:10.1073/pnas.1202288109
131. Masopust D, Vezys V, Wherry EJ, Barber DL, Ahmed R. Cutting edge: gut microenvironment promotes differentiation of a unique memory CD8 T cell population. *J Immunol* (2006) **176**:2079–83. doi:10.4049/jimmunol.176.4.2079
132. Joshi NS, Cui W, Chandele A, Lee HK, Urso DR, Hagman J, et al. Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor. *Immunity* (2007) **27**:281–95. doi:10.1016/j.immuni.2007.07.010
133. Wakim LM, Smith J, Caminschi I, Lahoud MH, Villadangos JA. Antibody-targeted vaccination to lung dendritic cells generates tissue-resident memory CD8 T cells that are highly protective against influenza virus infection. *Mucosal Immunol* (2015) **8**:1060–71. doi:10.1038/mi.2014.133
134. Wu T, Hu Y, Lee YT, Bouchard KR, Benechet A, Khanna K, et al. Lung-resident memory CD8 T cells (TRM) are indispensable for optimal cross-protection against pulmonary virus infection. *J Leukoc Biol* (2014) **95**:215–24. doi:10.1189/jlb.0313180
135. Ariotti S, Beltman JB, Chodaczek G, Hoekstra ME, van Beek AE, Gomez-Eerland R, et al. Tissue-resident memory CD8+ T cells continuously patrol skin epithelia to quickly recognize local antigen. *Proc Natl Acad Sci U S A* (2012) **109**:19739–44. doi:10.1073/pnas.1208927109
136. Ariotti S, Hogenbirk MA, Dijkgraaf FE, Visser LL, Hoekstra ME, Song JY, et al. T cell memory. Skin-resident memory CD8(+) T cells trigger a state of tissue-wide pathogen alert. *Science* (2014) **346**:101–5. doi:10.1126/science.1254803
137. McMaster SR, Wilson JJ, Wang H, Kohlmeier JE. Airway-resident memory CD8 T cells provide antigen-specific protection against respiratory virus challenge through rapid IFN-gamma production. *J Immunol* (2015) **195**:203–9. doi:10.4049/jimmunol.1402975
138. Schenkel JM, Fraser KA, Beura LK, Pauken KE, Vezys V, Masopust D. T cell memory. Resident memory CD8 T cells trigger protective innate and adaptive immune responses. *Science* (2014) **346**:98–101. doi:10.1126/science.1254536
139. Schenkel JM, Fraser KA, Vezys V, Masopust D. Sensing and alarm function of resident memory CD8(+) T cells. *Nat Immunol* (2013) **14**:509–13. doi:10.1038/ni.2568
140. Hofmann M, Pircher H. E-cadherin promotes accumulation of a unique memory CD8 T-cell population in murine salivary glands. *Proc Natl Acad Sci U S A* (2011) **108**:16741–6. doi:10.1073/pnas.1107200108
141. Cuburu N, Graham BS, Buck CB, Kines RC, Pang YY, Day PM, et al. Intravaginal immunization with HPV vectors induces tissue-resident CD8+ T cell responses. *J Clin Invest* (2012) **122**:4606–20. doi:10.1172/JCI63287
142. Shin H, Iwasaki A. A vaccine strategy that protects against genital herpes by establishing local memory T cells. *Nature* (2012) **491**:463–7. doi:10.1038/nature11522
143. Hansen SG, Vieville C, Whizin N, Coyne-Johnson L, Siess DC, Drummond DD, et al. Effector memory T cell responses are associated with protection of rhesus monkeys from mucosal simian immunodeficiency virus challenge. *Nat Med* (2009) **15**:293–9. doi:10.1038/nm.1935
144. Kupper TS. Old and new: recent innovations in vaccine biology and skin T cells. *J Invest Dermatol* (2012) **132**:829–34. doi:10.1038/jid.2011.400

145. Zhu J, Hladik F, Woodward A, Klock A, Peng T, Johnston C, et al. Persistence of HIV-1 receptor-positive cells after HSV-2 reactivation is a potential mechanism for increased HIV-1 acquisition. *Nat Med* (2009) **15**:886–92. doi:10.1038/nm.2006
146. Zhu J, Koelle DM, Cao J, Vazquez J, Huang ML, Hladik F, et al. Virus-specific CD8+ T cells accumulate near sensory nerve endings in genital skin during subclinical HSV-2 reactivation. *J Exp Med* (2007) **204**:595–603. doi:10.1084/jem.20061792
147. Zhu J, Peng T, Johnston C, Phasouk K, Kask AS, Klock A, et al. Immune surveillance by CD8alphaalpha+ skin-resident T cells in human herpes virus infection. *Nature* (2013) **497**:494–7. doi:10.1038/nature12110
148. Peng T, Zhu J, Phasouk K, Koelle DM, Wald A, Corey L. An effector phenotype of CD8+ T cells at the junction epithelium during clinical quiescence of herpes simplex virus 2 infection. *J Virol* (2012) **86**:10587–96. doi:10.1128/JVI.01237-12
149. Piet B, de Bree GJ, Smids-Dierdorp BS, van der Loos CM, Remmerswaal EB, von der Thusen JH, et al. CD8(+) T cells with an intraepithelial phenotype upregulate cytotoxic function upon influenza infection in human lung. *J Clin Invest* (2011) **121**:2254–63. doi:10.1172/JCI44675
150. Woodberry T, Suscovich TJ, Henry LM, August M, Waring MT, Kaur A, et al. Alpha E beta 7 (CD103) expression identifies a highly active, tonsil-resident effector-memory CTL population. *J Immunol* (2005) **175**:4355–62. doi:10.4049/jimmunol.175.7.4355
151. Bevan MJ. Memory T cells as an occupying force. *Eur J Immunol* (2011) **41**:1192–5. doi:10.1002/eji.201041377
152. Clark RA. Resident memory T cells in human health and disease. *Sci Transl Med* (2015) **7**:269rv1. doi:10.1126/scitranslmed.3010641
153. Boyman O, Hefti HP, Conrad C, Nickoloff BJ, Suter M, Nestle FO. Spontaneous development of psoriasis in a new animal model shows an essential role for resident T cells and tumor necrosis factor-alpha. *J Exp Med* (2004) **199**:731–6. doi:10.1084/jem.20031482
154. Conrad C, Boyman O, Tonel G, Tun-Kyi A, Laggner U, de Fougerolles A, et al. Alpha1beta1 integrin is crucial for accumulation of epidermal T cells and the development of psoriasis. *Nat Med* (2007) **13**:836–42. doi:10.1038/nm1605
155. Mizukawa Y, Yamazaki Y, Teraki Y, Hayakawa J, Hayakawa K, Nuriya H, et al. Direct evidence for interferon-gamma production by effector-memory-type intraepidermal T cells residing at an effector site of immunopathology in fixed drug eruption. *Am J Pathol* (2002) **161**:1337–47. doi:10.1016/S0002-9440(10)64410-0
156. Harris JE, Harris TH, Weninger W, Wherry EJ, Hunter CA, Turka LA. A mouse model of vitiligo with focused epidermal depigmentation requires IFN-gamma for autoreactive CD8(+) T-cell accumulation in the skin. *J Invest Dermatol* (2012) **132**:1869–76. doi:10.1038/jid.2011.463
157. Xing L, Dai Z, Jabbari A, Cerise JE, Higgins CA, Gong W, et al. Alopecia areata is driven by cytotoxic T lymphocytes and is reversed by JAK inhibition. *Nat Med* (2014) **20**:1043–9. doi:10.1038/nm.3645
158. Lian CG, Bueno EM, Granter SR, Laga AC, Saavedra AP, Lin WM, et al. Biomarker evaluation of face transplant rejection: association of donor T cells with target cell injury. *Mod Pathol* (2014) **27**:788–99. doi:10.1038/modpathol.2013.249
159. Cresswell J, Robertson H, Neal DE, Griffiths TR, Kirby JA. Distribution of lymphocytes of the alpha(E)beta(7) phenotype and E-cadherin in normal human urothelium and bladder carcinomas. *Clin Exp Immunol* (2001) **126**:397–402. doi:10.1046/j.1365-2249.2001.01652.x
160. Djenidi F, Adam J, Goubar A, Durgeau A, Meurice G, de Montpreville V, et al. CD8+CD103+ tumor-infiltrating lymphocytes are tumor-specific tissue-resident memory T cells and a prognostic factor for survival in lung cancer patients. *J Immunol* (2015) **194**:3475–86. doi:10.4049/jimmunol.1402711
161. Webb JR, Milne K, Nelson BH. Location, location, location: CD103 demarcates intraepithelial, prognostically favorable CD8 tumor-infiltrating lymphocytes in ovarian cancer. *Oncoimmunology* (2014) **3**:e27668. doi:10.4161/onci.27668
162. Webb JR, Milne K, Watson P, Deleeuw RJ, Nelson BH. Tumor-infiltrating lymphocytes expressing the tissue resident memory marker CD103 are associated with increased survival in high-grade serous ovarian cancer. *Clin Cancer Res* (2014) **20**:434–44. doi:10.1158/1078-0432.CCR-13-1877
163. Wakim LM, Gebhardt T, Heath WR, Carbone FR. Cutting edge: local recall responses by memory T cells newly recruited to peripheral nonlymphoid tissues. *J Immunol* (2008) **181**:5837–41. doi:10.4049/jimmunol.181.9.5837
164. Wakim LM, Waithman J, van Rooijen N, Heath WR, Carbone FR. Dendritic cell-induced memory T cell activation in nonlymphoid tissues. *Science* (2008) **319**:198–202. doi:10.1126/science.1151869
165. Marchesi JR, Adams DH, Fava F, Hermes GD, Hirschfield GM, Hold G, et al. The gut microbiota and host health: a new clinical frontier. *Gut* (2015) **65**(2):330–9. doi:10.1136/gutjnl-2015-309990

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Quantitative and Qualitative Analysis of Bone Marrow CD8$^+$ T Cells from Different Bones Uncovers a Major Contribution of the Bone Marrow in the Vertebrae

*Sulima Geerman[1,2], Sarah Hickson[1,2], Giso Brasser[1,2], Maria Fernanda Pascutti[1,2] and Martijn A. Nolte[1,2]**

[1] Department of Hematopoiesis, Sanquin Research, Amsterdam, Netherlands, [2] Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, Amsterdam, Netherlands

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***Correspondence:**
Martijn A. Nolte
m.nolte@sanquin.nl

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Bone marrow (BM) plays an important role in the long-term maintenance of memory T cells. Yet, BM is found in numerous bones throughout the body, which are not equal in structure, as they differ in their ratio of cortical and trabecular bone. This implies that BM cells within different bones are subjected to different microenvironments, possibly leading to differences in their frequencies and function. To address this, we examined BM from murine tibia, femur, pelvis, sternum, radius, humerus, calvarium, and the vertebrae and analyzed the presence of effector memory (T_{EM}), central memory (T_{CM}), and naïve (T_{NV}) CD8$^+$ T cells. During steady-state conditions, the frequency of the total CD8$^+$ T cell population was comparable between all bones. Interestingly, most CD8$^+$ T cells were located in the vertebrae, as it contained the highest amount of BM cells. Furthermore, the frequencies of T_{EM}, T_{CM}, and T_{NV} cells were similar between all bones, with a majority of T_{NV} cells. Additionally, CD8$^+$ T cells collected from different bones similarly expressed the key survival receptors IL-7Rα and IL-15Rβ. We also examined BM for memory CD8$^+$ T cells with a tissue-resident memory phenotype and observed that approximately half of all T_{EM} cells expressed the retention marker CD69. Remarkably, in the memory phase of acute infection with the lymphocytic choriomeningitis virus (LCMV), we found a massive compositional change in the BM CD8$^+$ T cell population, as the T_{EM} cells became the dominant subset at the cost of T_{NV} cells. Analysis of Ki-67 expression established that these T_{EM} cells were in a quiescent state. Finally, we detected higher frequencies of LCMV-specific CD8$^+$ T cells in BM compared to spleen and found that BM in its entirety contained fivefold more LCMV-specific CD8$^+$ T cells. In conclusion, although infection with LCMV caused a dramatic change in the BM CD8$^+$ T cell population, this did not result in noticeable differences between BM collected from different bones. Our findings suggest that in respect to CD8$^+$ T cells, BM harvested from a single bone is a fair reflection of the rest of the BM present in the murine body.

Keywords: vertebrae, bone marrow, CD8$^+$ T cells, memory, LCMV, CD69, tissue-resident, Ki-67

INTRODUCTION

The bone marrow (BM) acts as the primary site for the formation of all mature blood cells through the process of hematopoiesis. The complex hematopoietic process that gives rise to these cells takes place in the red (hematopoietic) part of the BM. At birth, BM primarily consists of red marrow, but with age, the red marrow decreases and is replaced by yellow (adipocytic) marrow (1). In adults (>25 years of age), red marrow is predominantly located in the tips (epiphysis), whereas yellow marrow is mostly found in the shafts (diaphysis) of the long bones. The epiphysis primarily consists of trabecular (spongy) bone, whereas the diaphysis consists of cortical (compact) bone (2). These differences in the composition of bone have been shown to influence the function of the BM. Human hematopoietic stem cells (HSCs) isolated from trabecular marrow of long bones have superior regenerative and self-renewal capacity compared to HSCs from the cortical marrow harvested from the shaft area and have also been shown to be molecularly distinct (3). Farrell et al. (4) found similar numbers of human HSCs and myeloid progenitor cells (GM-CFCs) in trochanter marrow (region between the epiphysis and diaphysis of the femur) compared to marrow in the femoral epiphysis. However, they observed a decline in numbers of femoral marrow-derived GM-CFCs in aged individuals, while the numbers for GM-CFCs derived from trochanter marrow did not change. In mice, substantial heterogeneity has been found in bone remodeling activity, blood volume fraction, and hypoxia between epiphysis, diaphysis, and calvarium, which were also shown to affect HSC function (5). These data indicate that distinct compartments within the BM are different, leading to functional differences for the cells that reside in these specific niches.

Next to its important function as a primary lymphoid organ, the BM has also gained recognition for its role as a secondary lymphoid organ. Dendritic cells in the BM can take up and present blood-borne antigens and thereby activate local naïve T cells (6). Neutrophils can capture and transport virus from the dermis into the BM, leading to priming of virus-specific CD8+ T cells by local antigen presenting cells (7). Additionally, the BM is also actively involved in immunological memory. Effector T cells that survive antigen clearance develop into memory T cells and home to the BM. Here, they provide life-long protection against reinfection (8–10). Studies in mice lacking IL-7 and IL-15 or their receptors IL-7Rα (CD127) and IL-15Rβ (CD122) have shown that these two cytokines are vital for the maintenance of memory CD8+ T cells, as they affect both their generation and survival (11–13). These effects could be direct, but they could also be mediated indirectly through the induction of costimulatory molecules that control memory T cell survival (14, 15). It has recently been shown that BM memory CD8+ T cells acquire IL-7 by docking to IL-7-producing reticular stromal cells (16). Additionally, human memory CD8+ T cells have been shown to be in close contact with a variety of IL-15-producing BM cells. These BM resident cells displayed morphological characteristics of stromal cells, dendritic cells, and monocytes (17).

Thus, it is now clear that BM is important for long-term memory maintenance and is therefore more frequently included in studies of (adaptive) immune responses [reviewed in Ref. (18, 19)]. However, little is known about quantitative and qualitative differences between various bones regarding T cell maintenance. Most information on BM T cells has been obtained from single cell suspensions prepared from crushed or flushed tibia and/or femurs. Hence, the BM has been conceptually and also practically regarded as a single organ. However, this view may not be justified, as bones throughout the body are diverse in their composition of cortical and trabecular bone depending on their mechanical or organ protection function (2), already leading to functional differences at the level of HSCs. Here, we examined if anatomical differences exist in BM, by assessing the CD8+ T cell population in BM harvested from murine tibia, femur, pelvis, sternum, radius, humerus, calvarium, and vertebrae and compared this to the spleen. We found that both in steady state and after infection with acute lymphocytic choriomeningitis virus (LCMV), BM located in distinct bones have comparable frequencies of CD8+ T cell subsets. Furthermore, by calculating the total number of BM CD8+ T cells found in the entire body, we demonstrate that BM is superior to spleen in harboring memory CD8+ T cells, and that this is attributed to the major contribution of the memory CD8+ T cells present in the vertebrae.

MATERIALS AND METHODS

Mice

Wild-type (WT) C57BL/6J mice were kept under specific pathogen-free conditions in the animal facility of the Academic Medical Center (Amsterdam, The Netherlands) or Netherlands Cancer institute (Amsterdam, The Netherlands). Female or male mice between the age of 13 and 17 weeks were used for steady-state experiments. For LCMV experiments, mice that were 9–16 weeks old were injected intraperitoneally with 2.0×10^5 PFU of the Armstrong strain, kindly provided by Dr. Ramon Arens (LUMC, Leiden, The Netherlands) in 200 µl PBS. Mice were sacrificed during the memory phase (>42 days post injection). Mice received chow and acidified drinking water *ad libitum*. Animal experiments were performed in accordance with the institutional and national guidelines and approved by the Experimental Animal Committees of both animal facilities.

Sample Collection and Preparation

Tibia, femur, pelvis, radius together with humerus were collected from every mouse. Additionally, we harvested sternum, calvarium, and the vertebrae (cervical vertebrae C1–sacral vertebrae S5). Bones were cleaned and crushed in MACS buffer (PBS + 1% FCS + 2 mM EDTA) using a mortar and pestle. BM cell suspensions were filtered through a 40-µm cell strainer to remove bone debris. Single splenocyte suspensions were prepared by crushing the spleen through a 40-µm cell strainer with the plunger of a syringe. For several LCMV experiments, whole spleen and BM cells were enriched for CD8+ T cells with CD8α microbeads (Miltenyi Biotec) and MACS LS columns (Miltenyi Biotec). Erythrocytes were lysed with red blood cell lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 127 mM EDTA). White blood cells were counted with CASY Cell Counter and Analyzer (Roche).

Flow Cytometry Analysis

The following antibodies were used in this study: CD3ε-eFlour 450 (145-2C11, eBioscience), CD3ε-APC-eFluor 780 (17A2, eBioscience), CD8α-APC-eFluor 780 (53-6.7, eBioscience), CD8α-BV605 (53-6.7, Biolegend), CD8α-PerCP-Cy5.5 (53-6.7, eBioscience), CD44-PE-Cy7 (IM7, Biolegend), CD62L-APC (MEL-14, eBioscience), CD62L-BV510 (MEL-14, Biolegend), CD69eFluor 450 (H1.2F3, eBioscience) CD69-biotin (H1.2F3, eBioscience), CD122-FITC (TM-B1, BD Biosciences), CD127-BV605 (A7R34, Biolegend), and Streptavidin PerCP-Cy5.5 (BD Biosciences). The MHC class I tetramers H2-D^{b}GP$_{33-41}$ APC and H2-D^{b}NP$_{396-404}$ PE were kind gifts from Dr. Ramon Arens (LUMC, Leiden, The Netherlands). Cells were fixed with Foxp3/Transcription Factor Staining buffer set (eBioscience) and stained with Ki-67 PE or Ki-67 FITC (B56, BD Biosciences). Samples were acquired with the LSR Fortessa (BD) and analyzed with FlowJo software (Tree Star, Inc.).

Statistical Analysis

Statistical analyses were performed with Prism (GraphPad Software, Inc.) using an unpaired *t* test followed by Welch's correction or a one-way ANOVA followed by Tukey's correction. Significance is indicated by $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, and $^{\#}p < 0.05$ between spleen and all the different bones.

RESULTS

BM Contains More Memory CD8+ T Cells than the Spleen

To examine whether the composition of the CD8+ T cell population in a single bone is representative of all other bones, we examined the CD8+ T cells in BM from tibia, femur, pelvis, sternum, radius, humerus, calvarium, and vertebrae and compared this to the spleen. We observed that the frequencies of CD3+ cells, and also of CD8+ T cells, were significantly lower in all bones compared to the spleen (**Figures 1A,B**). Regarding the presence of the classical CD8+ T cell subsets, i.e., effector memory (T_{EM}; CD44+CD62L−), central memory (T_{CM}; CD44+CD62L+), and naïve (T_{NV}; CD44−CD62L+) T cells (20, 21), we found that all bones consisted primarily (~65%) of T_{NV} cells (**Figures 1C,D**). Strikingly, the frequencies of T_{EM}, T_{CM}, and T_{NV} cells between the different bones were highly comparable. We observed that in comparison to spleen, all bones had higher frequencies of CD8+ T_{EM} cells (**Figure 1E**). A similar pattern was observed for CD8+ T_{CM} cells (**Figure 1H**). In absolute numbers, the majority of BM memory CD8+ T cells was located in the vertebrae (**Figures 1F,I**), as these contained the highest amount of BM cells, almost equivalent to the spleen (**Table 1**). BM, in its totality, contained ~2.8-fold more T_{EM} and ~1.5 more T_{CM} cells compared to spleen (**Figures 1G,J**). Taken together, we conclude that in the steady state, BM is quite distinct from spleen regarding frequencies of CD3+ and CD8+ T cells, but comparable between the different bones. Additionally, we show that BM accumulates, also in absolute numbers, more memory CD8+ T cells compared to spleen.

The Expression of CD122 and CD127 on CD8+ T Cells Is Similar between Different Bones

The cytokines IL-7 and IL-15 are important for the development and maintenance of BM CD8+ T cells (11–13). In the murine BM, the expression of these cytokines is primarily confined to stromal cells (22, 23). As bones differ in structure, and possibly also in composition of IL-7 and IL-15 producing stromal cells, we tested if BM CD8+ T cells regulate the expression of the IL-7 and IL-15 receptors, according to their current location. This is particularly interesting, as IL-15 can regulate the expression of the receptor for IL-7 (24). Therefore, we compared the presence of CD122 and CD127 on CD8+ T cells collected from different bones. The highest expression of CD122 was found on the T_{CM} cells, whereas T_{NV} cells had the lowest expression of CD122 (**Figure 2A**). In contrast, T_{NV} cells expressed the highest levels of CD127, while T_{EM} cells expressed the lowest levels of CD127 (**Figure 2E**). Despite these marked differences in expression levels between the CD8+ T cell subsets, we observed similar expression of CD122 (**Figures 2B–D**) and CD127 (**Figures 2F–H**) by CD8+ T cells harvested from different bones. It remains to be investigated whether the lack of anatomical differences in respect to receptor levels also reflects similar concentrations of IL-7 and IL-15 in BM in different bones.

BM Effector Memory CD8+ T Cells Strongly Increase after Infection with LCMV

We did not observe differences in CD8+ T cell frequencies in BM collected from different bones in the steady state. Thus, we questioned whether this would change after an infection that elicits a large influx of memory T cells. Therefore, we infected mice with the Armstrong strain of LCMV. This acute systemic infection is cleared from the BM within 8 days due to a strong CD8+ T cell response (25, 26). We analyzed the BM in the memory phase (>42 days) and found that the frequencies of CD3+ cells and CD8+ T cells were lower when compared to the spleen, but still similar between bones (data not shown). Interestingly, we found that the frequency of the CD8+ T_{EM} subset strongly increased, ranging from ~10% in the steady state to ~60% after LCMV (**Figures 3A,B**). The increase in T_{EM} cells corresponded with a decrease in T_{NV} cells, whereas the T_{CM} subset was largely unaffected (**Figures 3C–E**). This was comparable between all bones. The T_{EM} subset also increased in the spleen, although here the majority (~55%) of the CD8+ T cells still exhibited a naïve phenotype. Both BM and spleen T_{EM} cells were primarily Ki-67−, indicating that they are in the G0 phase of cell cycle, and are thus resting memory CD8+ T cells (**Figure 3F**). This was similar for all the different bones (data not shown). In summary, we show that even after resolved infection with LCMV, no anatomical differences occur in the BM regarding CD8+ T cell frequencies.

BM Contains CD8+ T Cells with a Tissue-Resident Memory Phenotype

Apart from being a primary and secondary lymphoid organ, BM, like any other tissue, is susceptible to viral infections (27,

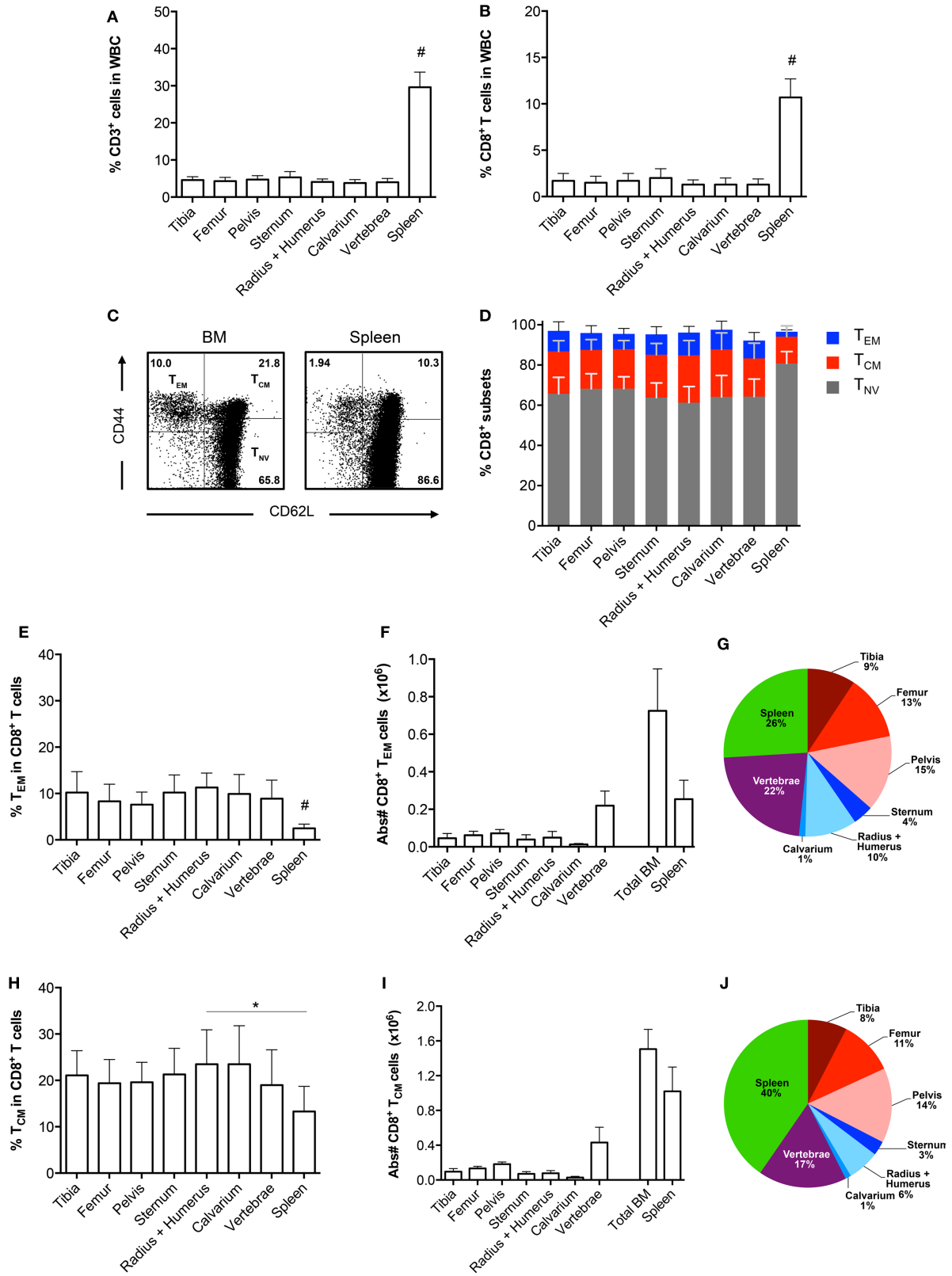

FIGURE 1 | Comparable CD8+ T cell frequencies in BM obtained from different bones.

(Continued)

FIGURE 1 | Continued

(A) Frequency of CD3+ and **(B)** CD8+ T cells in total white blood cells (WBC). **(C)** Representative FACS plots showing expression of CD44 and CD62L in CD8+ T cells for BM (radius + humerus) and spleen. **(D)** Frequency of T_{EM}, T_{CM}, and T_{NV} cells within the total CD8+ T cell population. **(E)** Frequency and **(F)** absolute numbers of T_{EM} cells within the total CD8+ T cell population. **(G)** Distribution of T_{EM} cells (based on absolute numbers). **(H)** Frequency and **(I)** absolute numbers of T_{CM} cells within the total CD8+ T cell population. **(J)** Distribution of T_{CM} cells (based on absolute numbers). The double amount of cells was taken into account for estimating the contribution of tibia, femur, pelvis, and radius + humerus for the calculation of "Total BM" in **(F,I)** and for their contribution in **(G,J)**. Graphs show mean ± SD of each bone ($n = 4$–8), pooled from the three independent experiments. Statistical analysis was performed with a one-way ANOVA followed by Tukey's correction. Significance is indicated by $^{*}p < 0.05$ or $^{\#}p < 0.05$ between spleen and all bones.

TABLE 1 | BM yield from different bones.

Absolute number of white blood cells ($\times 10^6$)	
	Steady state ($n = 5$)
Tibia	13.7 ± 8.0
Femur	25.3 ± 7.5
Pelvis	25.6 ± 10.1
Sternum	7.9 ± 2.6
Radius + humerus	14.4 ± 2.2
Calvarium	6.0 ± 2.0
Vertebrae	97.5 ± 16.9
Spleen	99.3 ± 9.9

White blood cell count per bone and spleen. Mean ± SD are shown (n = 5).

28). Interestingly, over the past few years, it has been reported that following an infection, a subset of memory CD8+ T cells has the ability to take up residence in a particular tissue and provide tissue-specific immunity. These tissue-resident memory T cells (T_{RM}) have been identified in skin, female genital tract, intestinal mucosa, kidney, pancreas, stomach, heart, salivary glands, and the brain (29–34). These cells are characterized by the expression of the C-type lectin CD69, which inhibits the expression of the egress receptor sphingosine-1-phosphate receptor 1 (S1PR1) (35, 36). Moreover, T_{RM} cells are identified by the absence of CD62L, making them a subgroup of the T_{EM} subset (37). Here, we examined the expression of CD69 by BM T_{EM} cells and compared these frequencies with the CD69 expression in other BM CD8+ T cell subsets. We found that during the steady state, approximately half (~47%) of BM T_{EM} cells and ~20% of BM T_{CM} expressed CD69 (**Figures 4A,B**). The T_{NV} subset barely expressed CD69 (data not shown). The frequencies of CD69 in each CD8+ T cell subset were similar for all bones (data not shown). Furthermore, the frequencies in the BM memory CD8+ T cells subsets were much higher than in the equivalent memory CD8+ T cell subsets located in the spleen (**Figures 4A–D**). Of specific note, after the infection with LCMV, the frequency of BM and spleen CD69+ cells decreased in the T_{EM} subset, while the frequency of BM CD69+ cells in the T_{CM} subset remained the same. However, we did not observe differences in absolute numbers of CD69+ T_{EM} cells before and after infection (data not shown). This indicates that the decreased frequency of CD69+ T_{EM} cells after infection results from a massive influx of CD69− T_{EM} cells. In conclusion, BM contains a significant number of CD8+ T cells with a T_{RM} phenotype, though an LCMV infection elicits an influx of mostly conventional T_{EM} cells.

BM Harbors a Significant Portion of LCMV-Specific CD8+ T Cells

In order to analyze CD8+ T cells generated specifically against LCMV, we pooled BM from different bones to obtain sufficient cell numbers (26). BM from the vertebrae was analyzed separately because of its high cellularity. We enriched for CD8+ T cells and stained with MHC-I tetramers loaded with the LCMV epitopes GP_{33-41} and $NP_{396-404}$. We observed that the spleen had lower frequencies of LCMV-specific CD8+ T cells compared to either BM compartment (**Figures 5A,D**). We did not observe differences in frequencies of LCMV-specific CD8+ T cells between the BM from vertebrae and the other bones (**Figures 5B,E**). Based on the absolute numbers, the vertebrae itself contained ~35% of all GP_{33}-tetramer+ and 37% of all NP_{396}-tetramer+ CD8+ T cells present in the total BM. Furthermore, as we harvested the majority of the BM from the body, we could calculate that BM harbored 1.5×10^5 GP_{33}-tetramer+ and 1.4×10^5 NP_{396}-tetramer+ CD8+ T cells, which was fivefold to sixfold more than the spleen (**Figures 5C,F**), thereby emphasizing the role of the BM as memory T cell organ. LCMV-specific CD8+ T cells in BM primarily (~75%) had a T_{EM} phenotype, while the remainder exhibited a T_{CM} phenotype (**Figure 5G**). Additionally, LCMV-specific CD8+ T cells barely expressed CD69 or Ki-67, indicating that they were not actively cycling, but also not T_{RM} cells (**Figure 5H**). Our results suggest that the distribution of LCMV-specific CD8+ T cells is comparable between BM in the vertebrae and the rest of the bones, and that these cells are phenotypically similar to the rest of the memory CD8+ T cells. Our data also suggest that infection with LCMV does not result in substantial generation of LCMV-specific CD8+ T cells with a T_{RM} phenotype.

DISCUSSION

In the present study, we examined BM from murine tibia, femur, pelvis, sternum, radius, humerus, calvarium, and vertebrae and addressed if anatomical differences exist at the level of BM CD8+ T cells. Here, we show that during the steady state, BM derived from different bones had similar CD8+ T cell frequencies. Furthermore, the frequencies of the T_{EM}, T_{CM}, and T_{NV} subsets were also comparable between all the bones. We also examined BM during the memory phase of a LCMV infection. This virus is cleared primarily by CD8+ T cells and results in the generation of virus-specific memory CD8+ T cells, which remain detectable long after the initial infection (25, 26). Similarly to the steady state, we did not observe anatomical differences in BM after

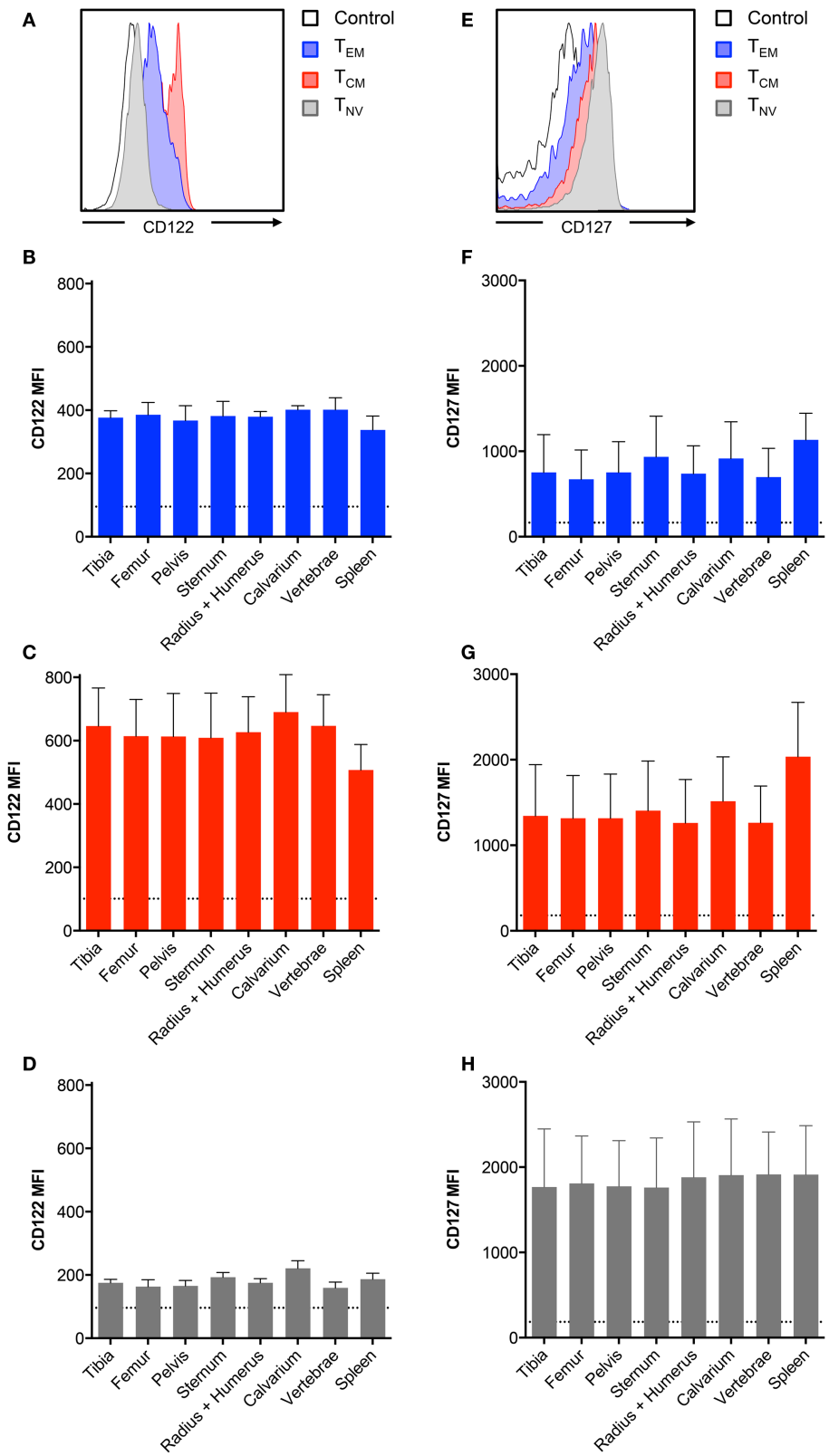

FIGURE 2 | BM memory CD8+ T cell subsets differ in their expression of survival receptors. (A,E) Representative histograms showing expression of CD122 and CD127 in CD8+ T_{EM}, T_{CM}, and T_{NV} subsets. Black line indicates intensity of unstained cells. **(B–D)** MFI of CD122 or **(F–H)** MFI of CD127 on T_{EM}, T_{CM}, and T_{NV} subsets. Dotted line indicates MFI of unstained cells. Graphs show mean ± SD for each bone (n = 5–8).

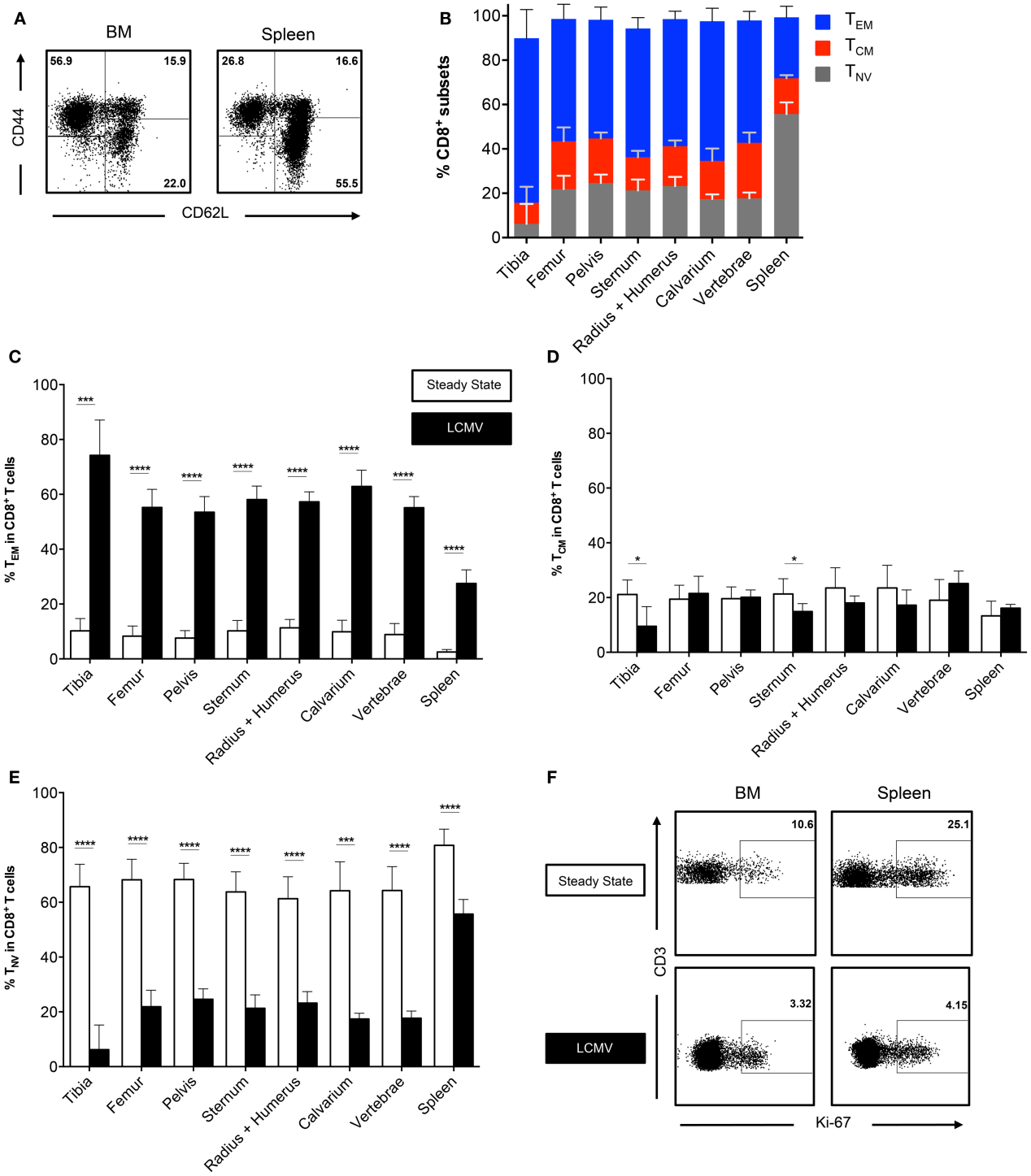

FIGURE 3 | The CD8⁺ T cell population consists primarily of memory cells after infection with LCMV. (A) Representative FACS plots showing expression of CD44 and CD62L in CD8⁺ T cells for BM (tibia) and spleen. **(B)** Frequency of T_{EM}, T_{CM}, and T_{NV} subsets in CD8⁺ T cells. **(C–E)** Frequency of T_{EM}, T_{CM}, and T_{NV} subsets in the CD8⁺ T cell population during steady state and after infection with LCMV. White bars = steady state and black bars = LCMV. Data for **(C–E)** are identical to that for **Figure 1D** and **(B)**. **(F)** Representative FACS plots showing expression of Ki-67 on T_{EM} cells in spleen and BM during steady state (femur) and after infection with LCMV (vertebrae). Graphs show mean ± SD of each bone (n = 4–8), pooled from the three independent steady states and the two independent LCMV experiments. Statistical analysis was performed with unpaired t test followed by Welch's correction. Significance is indicated by $^{*}p < 0.05$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

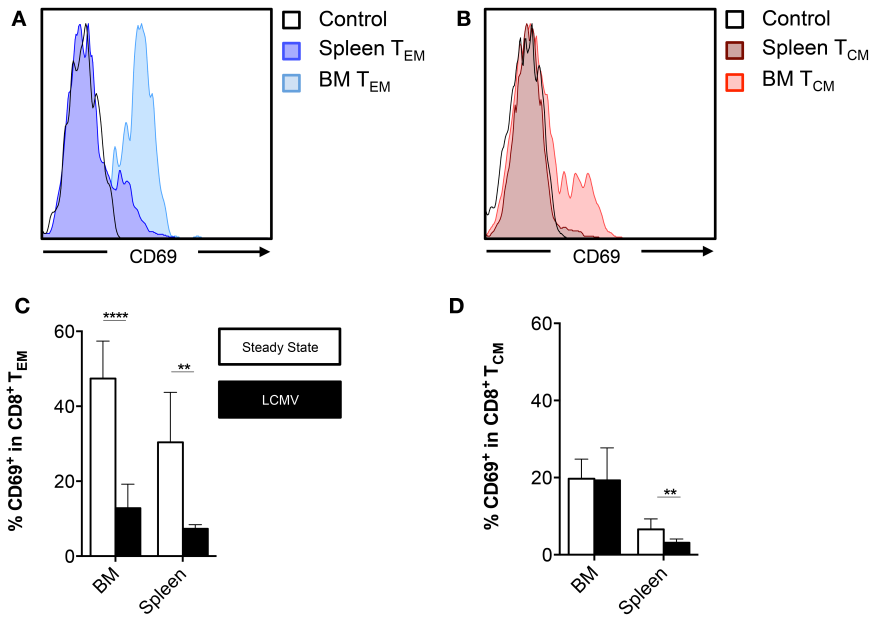

FIGURE 4 | BM memory CD8⁺ T cell subsets contain cells that express CD69. (A,B) Representative histograms showing expression of CD69 on T_{EM} or T_{CM} cells in BM (femur) and spleen. **(C,D)** Frequency of CD69⁺ cells in the CD8⁺ T_{EM} or T_{CM} subsets in BM (vertebrae) and spleen. Graphs show mean ± SD of the vertebrae (n = 4–8), pooled from the three independent steady states and the two independent LCMV experiments. White bars = steady state and black bars = LCMV. Statistical analysis was performed with unpaired t test followed by Welch's correction. Significance is indicated by $^{**}p < 0.01$ and $^{****}p < 0.0001$.

infection with LCMV. To date, only a limited number of studies have addressed the possible anatomical differences in the BM. These studies primarily focused on the functional differences within different regions inside a bone, but not necessarily between different bones. The majority of the studies found functional, but limited differences in frequencies of HSCs (3–5). It remains to be determined if BM T cells derived from different bones are also functionally distinct. Results obtained from a study performed with human BM suggest that this might not be the case. Pritz et al. (38) compared the phenotype and function of T cells derived from iliac crest and the femoral shaft and found no differences between the distribution of T cell populations and their cytokine production. Interestingly, although we found no differences between bones, we did observe that both during the steady state and after infection with LCMV, the majority of CD8⁺ T cells were located in the vertebrae, a collection of bones that has not been well studied and is not frequently included during sample preparation. From both a practical and ethical point of view, inclusion of the vertebrae can limit the amount of mice required for any given experiment, as it holds more than a third of all BM present in the murine body.

Here, we also demonstrated that BM substantially changes after infection with LCMV. The decline in frequency of T_{NV} cells coincided with the increased frequency of T_{EM} cells. As we did not observe differences in absolute numbers of total CD8⁺ T cells between steady state and LCMV-infected mice, our results suggest that the space in the BM is limited, resulting in one subset being replaced by another. Sercan Alp et al. (16) demonstrated that memory CD8⁺ T cells colocalize with IL-7-producing reticular stromal cells, in a 1:1 ratio in the BM. This, combined with our results and the fact that CD8⁺ T_{NV} cells primarily depend on IL-7 for survival [reviewed in Ref. (39)], suggests that CD8⁺ T_{NV} cells were outcompeted or blocked from entering these IL-7-rich niches, as these became occupied by memory CD8⁺ T cells. Yet, we show that BM memory CD8⁺ T cells express the receptors to respond to both IL-7 and IL-15, which could indicate that naïve and memory CD8⁺ T cells reside in different niches. If this is indeed the case, our results suggest that after infection with LCMV, the BM microenvironment changed and became less favorable for naïve CD8⁺ T cells and/or a more advantageous for memory CD8⁺ T cells. As LCMV has been shown to infect BM stromal fibroblast and endothelial cells (27), it could well be that the cellular sources of IL-7 and IL-15 in the BM are severely affected by the infection. Alternatively, there could also be a role for hematopoietic cells, as dendritic cells can increase their IL-15 production in response to inflammatory signals (40). Further studies are required to show how the BM niches that maintain CD8⁺ T cells adapt in order to accommodate the substantial amount of memory CD8⁺ T cells generated after infection with LCMV and how this relates to the spleen. Moreover, it remains unclear why the BM harbors so many T_{EM} cells long after the infection has been resolved and whether their presence affects the hematopoietic function of the BM, as activated immune cells have been shown to directly influence HSC function and hematopoiesis [reviewed in Ref. (41)].

Furthermore, we demonstrated that BM contained CD8⁺ T cells with a T_{RM} ($CD44^+CD62L^-CD69^+$) phenotype. In the past, the surface molecule CD69 was associated with the "recently

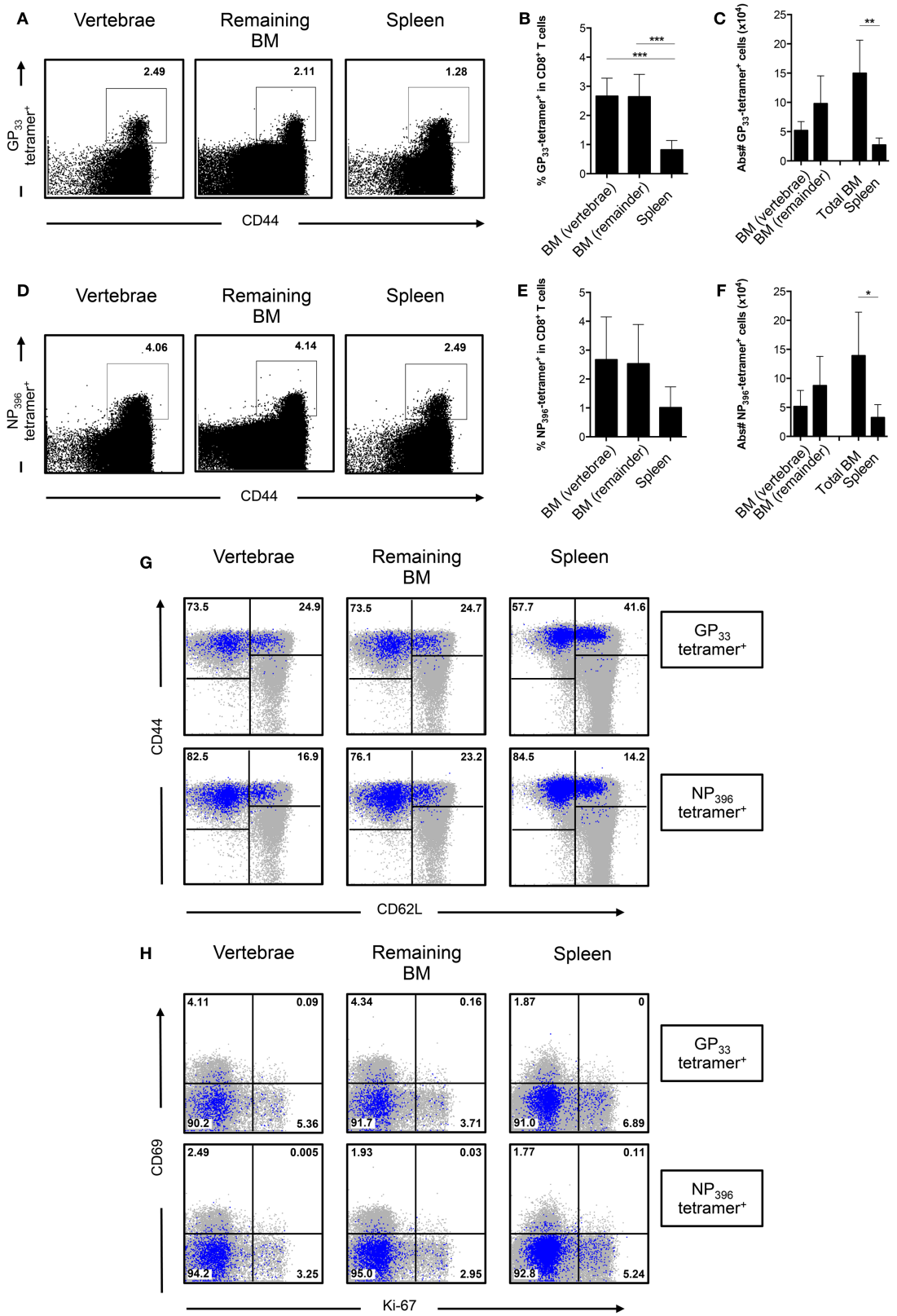

FIGURE 5 | The frequency of LCMV-specific CD8+ T cells is similar between BM in vertebrae and the remaining bones.

(Continued)

FIGURE 5 | Continued
(A) Representative FACS plots showing staining of GP_{33}-tetramer in CD8+ T cells. **(B)** Frequency and **(C)** absolute numbers of GP_{33}-tetramer+ cells in CD8+ T cells. **(D)** Representative FACS plots showing staining of NP_{396}-tetramer in CD8+ T cells. **(E)** Frequency and **(F)** absolute numbers of NP_{396}-tetramer+ cells in CD8+ T cells. **(G)** Representative FACS plots showing expression of CD44 and CD62L of GP_{33}-tetramer+ or NP_{396}-tetramer+ CD8+ T cells. **(H)** Representative FACS plots showing expression of CD69 and Ki-67 of GP_{33}-tetramer+ or NP_{396}-tetramer+ CD8+ T cells. For **(G,H)**, tetramer+ cells (blue) are superimposed on all CD8+ T cells (gray). Percentages shown in **(G,H)** reflect the frequencies of tetramer+ CD8+ T cells. Graphs show mean ± SD of vertebrae or remaining BM (n = 3–6), pooled from the two independent experiments. Statistical analysis was performed with one-way ANOVA followed by Tukey's correction or with unpaired t test followed by Welch's correction. Significance is indicated by $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

activated" status of T cells. More recently, this surface molecule has become important for its role in tissue retention, as it down-regulates S1PR1 and thereby blocks the egress of lymphocytes from tissues (35). Currently, it is unclear if CD69 alone is sufficient for identification of T_{RM} cells in BM, as it has not been unequivocally demonstrated that CD69+ BM memory CD8+ T cells are non-circulating cells. Additionally, it has been postulated that T_{RM} cells may reside in the CD69− fraction of memory T cells (42). Nonetheless, BM memory CD8+ T cells, which express CD69, resemble T_{RM} cells in other tissues, as they have low expression of S1PR1 (16). Furthermore, in accordance with our findings, BM memory CD8+ T cells are not activated, but rather quiescent in terms of proliferation and gene expression (16). It is thus highly likely that the CD69+ memory CD8+ T cells that we identified in BM are resident, rather than recently activated T cells. Currently, it is understood that T_{RM} cells reside in tissues where the initial infection took place and are positioned as the first line of defense, in order to accelerate pathogen elimination during secondary encounters (37). Whether T_{RM} cells in the BM are also strategically positioned to fulfill a similar function and protect the BM from invading pathogens remains to be investigated.

In summary, our findings suggest that in respect to the frequency of CD8+ T cells, BM harvested from one bone is representative of the BM found in all bones located throughout the body. Our results reinforce the notion that BM is a major immunological organ, as it is quantitatively superior to the spleen in accumulation and accommodation of memory CD8+ T cells, and should therefore be included when studying (adaptive) immune responses and memory T cell maintenance.

AUTHOR CONTRIBUTIONS

SG, SH, GB, and MP conducted the experiments; SG analyzed data; MP and MN supervised the project; and SG and MN wrote the manuscript.

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REFERENCES

1. Bianco P. Bone and the hematopoietic niche: a tale of two stem cells. *Blood* (2011) **117**:5281–9. doi:10.1182/blood-2011-01-315069
2. Gurkan UA, Akkus O. The mechanical environment of bone marrow: a review. *Ann Biomed Eng* (2008) **36**:1978–91. doi:10.1007/s10439-008-9577-x
3. Guezguez B, Campbell CJV, Boyd AL, Karanu F, Casado FL, Di Cresce C, et al. Regional localization within the bone marrow influences the functional capacity of human HSCs. *Cell Stem Cell* (2013) **13**:175–89. doi:10.1016/j.stem.2013.06.015
4. Farrell TL, McGuire TR, Bilek LD, Brusnahan SK, Jackson JD, Lane JT, et al. Changes in the frequencies of human hematopoietic stem and progenitor cells with age and site. *Exp Hematol* (2014) **42**:146–54. doi:10.1016/j.exphem.2013.11.003
5. Lassailly F, Foster K, Lopez-onieva L, Currie E, Bonnet D. Multimodal imaging reveals structural and functional heterogeneity in different bone marrow compartments: functional implications on hematopoietic stem cells. *Blood* (2013) **122**:1730–40. doi:10.1182/blood-2012-11-467498
6. Feuerer M, Beckhove P, Garbi N, Mahnke Y, Limmer A, Hommel M, et al. Bone marrow as a priming site for T-cell responses to blood-borne antigen. *Nat Med* (2003) **9**:1151–7. doi:10.1038/nm914
7. Duffy D, Perrin H, Abadie V, Benhabiles N, Boissonnas A, Liard C, et al. Neutrophils transport antigen from the dermis to the bone marrow, initiating a source of memory CD8+ T cells. *Immunity* (2012) **37**:917–29. doi:10.1016/j.immuni.2012.07.015
8. Di Rosa F, Santoni A. Memory T-cell competition for bone marrow seeding. *Immunology* (2003) **108**:296–304. doi:10.1046/j.1365-2567.2003.01593.x
9. Mazo IB, Honczarenko M, Leung H, Cavanagh LL, Bonasio R, Weninger W, et al. Bone marrow is a major reservoir and site of recruitment for central memory CD8+ T cells. *Immunity* (2005) **22**:259–70. doi:10.1016/j.immuni.2005.01.008
10. Tokoyoda K, Zehentmeier S, Hegazy AN, Albrecht I, Grün JR, Löhning M, et al. Professional memory CD4+ T lymphocytes preferentially reside and rest in the bone marrow. *Immunity* (2009) **30**:721–30. doi:10.1016/j.immuni.2009.03.015
11. Lodolce JP, Boone DL, Chai S, Swain RE, Dassopoulos T, Trettin S, et al. IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation. *Immunity* (1998) **9**:669–76. doi:10.1016/S1074-7613(00)80664-0
12. Kennedy MK, Glaccum M, Brown SN, Butz EA, Viney JL, Embers M, et al. Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. *J Exp Med* (2000) **191**:771–80. doi:10.1084/jem.191.5.771
13. Schluns KS, Kieper WC, Jameson SC, Lefrançois L. Interleukin-7 mediates the homeostasis of naïve and memory CD8 T cells in vivo. *Nat Immunol* (2000) **1**:426–32. doi:10.1038/80868

14. Pulle G, Vidric M, Watts TH. IL-15-dependent induction of 4-1BB promotes antigen-independent CD8 memory T cell survival. *J Immunol* (2006) **176**:2739–48. doi:10.4049/jimmunol.176.5.2739
15. Snell LM, Lin GHY, Watts TH. IL-15-dependent upregulation of GITR on CD8 memory phenotype T cells in the bone marrow relative to spleen and lymph node suggests the bone marrow as a site of superior bioavailability of IL-15. *J Immunol* (2012) **188**:5915–23. doi:10.4049/jimmunol.1103270
16. Sercan Alp Ö, Durlanik S, Schulz D, McGrath M, Grün JR, Bardua M, et al. Memory CD8+ T cells colocalize with IL-7+ stromal cells in bone marrow and rest in terms of proliferation and transcription. *Eur J Immunol* (2015) **45**(4):975–87. doi:10.1002/eji.201445295
17. Herndler-Brandstetter D, Landgraf K, Jenewein B, Tzankov A, Brunauer R, Brunner S, et al. Human bone marrow hosts polyfunctional memory CD4+ and CD8+ T cells with close contact to IL-15-producing cells. *J Immunol* (2011) **186**:6965–71. doi:10.4049/jimmunol.1100243
18. Di Rosa F, Pabst R. The bone marrow: a nest for migratory memory T cells. *Trends Immunol* (2005) **26**:360–6. doi:10.1016/j.it.2005.04.011
19. Tokoyoda K, Radbruch A. Signals controlling rest and reactivation of T helper memory lymphocytes in bone marrow. *Cell Mol Life Sci* (2012) **69**:1609–13. doi:10.1007/s00018-012-0969-6
20. Sallusto F, Lenig D, Förster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* (1999) **401**:708–12. doi:10.1038/44385
21. Weninger W, Crowley MA, Manjunath N, von Andrian UH. Migratory properties of naive, effector, and memory CD8(+) T cells. *J Exp Med* (2001) **194**:953–66. doi:10.1084/jem.194.7.953
22. Cui G, Hara T, Simmons S, Wagatsuma K, Abe A, Miyachi H, et al. Characterization of the IL-15 niche in primary and secondary lymphoid organs in vivo. *Proc Natl Acad Sci U S A* (2014) **111**:1915–20. doi:10.1073/pnas.1318281111
23. Hara T, Shitara S, Imai K, Miyachi H, Kitano S, Yao H, et al. Identification of IL-7-producing cells in primary and secondary lymphoid organs using IL-7-GFP knock-in mice. *J Immunol* (2012) **189**:1577–84. doi:10.4049/jimmunol.1200586
24. Quinci AC, Vitale S, Parretta E, Soriani A, Iannitto ML, Cippitelli M, et al. IL-15 inhibits IL-7R**α** expression by memory-phenotype CD8+ T cells in the bone marrow. *Eur J Immunol* (2012) **42**:1129–39. doi:10.1002/eji.201142019
25. Slifka MK, Matloubian M, Ahmed R. Bone marrow is a major site of long-term antibody production after acute viral infection. *J Virol* (1995) **69**:1895–902.
26. Slifka MK, Whitmire JK, Ahmed R. Bone marrow contains virus-specific cytotoxic T lymphocytes. *Blood* (1997) **90**:2103–8.
27. Binder BD, Fehr J, Hengartner H, Zinkernagel RM. Virus-induced transient bone marrow aplasia: major role of interferon-alpha/beta during acute infection with the noncytopathic lymphocytic choriomeningitis virus. *J Exp Med* (1997) **1997**(185):517–30. doi:10.1084/jem.185.3.517
28. Subramanian A, Hegde S, Porayette P, Yon M, Hankey P, Paulson RF. Friend virus utilizes the BMP4-dependent stress erythropoiesis pathway to induce erythroleukemia. *J Virol* (2008) **82**:382–93. doi:10.1128/JVI.02487-06
29. Gebhardt T, Wakim LM, Eidsmo L, Reading PC, Heath WR, Carbone FR. Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus. *Nat Immunol* (2009) **10**:524–30. doi:10.1038/ni.1718
30. Tang VA, Rosenthal KL. Intravaginal infection with herpes simplex virus type-2 (HSV-2) generates a functional effector memory T cell population that persists in the murine genital tract. *J Reprod Immunol* (2010) **87**:39–44. doi:10.1016/j.jri.2010.06.155
31. Masopust D, Choo D, Vezys V, Wherry EJ, Duraiswamy J, Akondy R, et al. Dynamic T cell migration program provides resident memory within intestinal epithelium. *J Exp Med* (2010) **207**:553–64. doi:10.1084/jem.20090858
32. Casey KA, Fraser KA, Schenkel JM, Moran A, Abt MC, Beura LK, et al. Antigen-independent differentiation and maintenance of effector-like resident memory T cells in tissues. *J Immunol* (2012) **188**:4866–75. doi:10.4049/jimmunol.1200402
33. Hofmann M, Pircher H. E-cadherin promotes accumulation of a unique memory CD8 T-cell population in murine salivary glands. *Proc Natl Acad Sci U S A* (2011) **108**:16741–6. doi:10.1073/pnas.1107200108
34. Wakim LM, Woodward-Davis A, Bevan MJ. Memory T cells persisting within the brain after local infection show functional adaptations to their tissue of residence. *Proc Natl Acad Sci U S A* (2010) **107**:17872–9. doi:10.1073/pnas.1010201107
35. Shiow LR, Rosen DB, Brdicková N, Xu Y, An J, Lanier LL, et al. CD69 acts downstream of interferon-alpha/beta to inhibit S1P1 and lymphocyte egress from lymphoid organs. *Nature* (2006) **440**:540–4. doi:10.1038/nature04606
36. Mackay LK, Braun A, Macleod BL, Collins N, Tebartz C, Bedoui S, et al. Cutting edge: CD69 interference with sphingosine-1-phosphate receptor function regulates peripheral T cell retention. *J Immunol* (2015) **194**(5):2059–63. doi:10.4049/jimmunol.1402256
37. Schenkel JM, Masopust D. Tissue-resident memory T cells. *Immunity* (2014) **41**:886–97. doi:10.1016/j.immuni.2014.12.007
38. Pritz T, Landgraf-Rauf K, Herndler-Brandstetter D, Rauf R, Lair J, Gassner R, et al. Bone marrow T cells from the femur are similar to iliac crest derived cells in old age and represent a useful tool for studying the aged immune system. *Immun Ageing* (2013) **10**:17. doi:10.1186/1742-4933-10-17
39. Cox MA, Kahan SM, Zajac AJ. Anti-viral CD8 T cells and the cytokines that they love. *Virology* (2013) **435**:157–69. doi:10.1016/j.virol.2012.09.012
40. Mattei F, Schiavoni G, Belardelli F, Tough DF. IL-15 is expressed by dendritic cells in response to type I IFN, double-stranded RNA, or lipopolysaccharide and promotes dendritic cell activation. *J Immunol* (2001) **167**:1179–87. doi:10.4049/jimmunol.167.3.1179
41. Libregts SFWM, Nolte MA. Parallels between immune driven-hematopoiesis and T cell activation: 3 signals that relay inflammatory stress to the bone marrow. *Exp Cell Res* (2014) **329**(2):239–47. doi:10.1016/j.yexcr.2014.09.016
42. Steinert EM, Schenkel JM, Fraser KA, Beura LK, Manlove LS, Igyártó BZ, et al. Quantifying memory CD8 T cells reveals regionalization of immunosurveillance. *Cell* (2015) **161**:737–49. doi:10.1016/j.cell.2015.03.031

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Commentary: Memory CD8$^+$ T cells colocalize with IL-7$^+$ stromal cells in bone marrow and rest in terms of proliferation and transcription

*Francesca Di Rosa**

Institute of Molecular Biology and Pathology, Consiglio Nazionale delle Ricerche, c/o Department of Molecular Medicine, Sapienza University, Rome, Italy

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A commentary on

Memory CD8$^+$ T cells colocalize with IL-7$^+$ stromal cells in bone marrow and rest in terms of proliferation and transcription

*by Sercan Alp O, Durlanik S, Schulz D, McGrath M, Grun JR, Bardua M, et al. Eur J Immunol (2015) **45**:975–87. doi: 10.1002/eji.201445295*

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Stephen Philip Schoenberger, La Jolla Institute for Allergy and Immunology, USA

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Charles Surh, The Scripps Research Institute, USA
Kimberly Sue Schluns, University of Texas MD Anderson Cancer Center, USA

***Correspondence:**
Francesca Di Rosa
francesca.dirosa@uniroma1.it

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Several studies have shown that the bone marrow (BM) is implicated in the long-lasting persistence of memory CD8 T cells [see Ref. (1) and references therein]. Generally, it has been thought that the BM accomplishes this by sustaining a higher level of homeostatic proliferation of recirculating memory CD8 T cells than do spleen and lymph nodes (LN) in the steady state. This slow intermittent cell division would counteract cell death, thus contributing to the stable maintenance of memory T cell numbers over time. In a recent article entitled "Memory CD8$^+$ T cells colocalize with IL-7$^+$ stromal cells in bone marrow and rest in terms of proliferation and transcription," Sercan Alp and coworkers challenge this view (2). They emphasize that results on memory CD8 T cell proliferation are discrepant and propose that the BM instead provides survival signals for resident memory CD8 T cells, as it does for plasma cells (3–5). They show that BM memory CD8 T cells colocalize with stromal cells, expressing the prosurvival cytokine IL-7. Moreover, they demonstrate that CD69, i.e., a typical marker of tissue-resident memory T cells, is expressed by a higher proportion of memory CD8 T cells in the BM than in the spleen. Finally, they show that freshly isolated BM memory CD8 T cells have a predominant resting transcriptional profile, in comparison with *in vitro*-activated CD8 T cells (2).

Starting from the article by Sercan Alp et al., this commentary revisits the data published so far on memory CD8 T cell proliferation in the BM and suggests that apparent discrepancies can be reconciled by a detailed analysis (see **Table 1** and references therein). In respect to the interplay between memory CD8 T cells and other cells within BM niches and the possibility that BM memory T cells represent a pool of tissue-resident memory T cells, the reader is referred to another article in this issue (6).

Sercan Alp and coworkers examined memory CD8 T cell proliferation or quiescence in mice by three methods, i.e., DNA content analysis, bromodeoxyuridine (BrdU) incorporation, and Ki67 staining (2).

DNA content analysis measures the percentage of cells in S/G_2/M phase of cell cycle at a given time, thus providing a static index of proliferation in untreated individuals (16). By this method, Sercan Alp et al. found that the frequency of dividing cells within memory-phenotype CD44^{high}

TABLE 1 | Summary of published results on total CD8 and memory CD8 T cell proliferation in bone marrow, grouped according to experimental methods.

	CD8 T cells	BM	Lymphoid periphery/blood	Reference
(A) DNA content				
Species				
Mouse	$CD8^+$	↑	Spleen and PLN	Parretta et al. (7)
Mouse	$CD8^+$ Ag-specific P14 (LCMV)	↑	Spleen, total LN, and blood	Becker et al. (8)
Mouse	$CD8^+$ $CD44^{hi}$	↑	Spleen	Sercan Alp et al. (2)
Human	$CD8^+$ $CD45RA^-$ $CD45R0^+$	↑	Blood	Okhrimenko et al. (9)
(B) BrdU				
BrdU administration				
1 i.v. injection, 1 h before analysis	$CD8^+$	↑	Spleen, PLN, MLN, CLN, and blood	Westermann et al. (10)
In drinking water for 3 d	$CD8^+$	↑	Spleen, PLN, and MLN	Parretta et al. (7)
	$CD8^+$ $CD44^{hi}$	↑	Spleen, PLN, and MLN	
	$CD8^+$ Ag-specific (OVA)	↑	Spleen and PLN	
	$CD8^+$ Ag-specific OT-I (OVA)	↑	Spleen and PLN	
1 i.v. injection, 1 d before analysis	$CD8^+$ $CD44^{hi}$	↑	Spleen	Becker et al. (8)
	$CD8^+$ Ag-specific P14 (LCMV)	↑	Spleen, total LN, and blood	
In drinking water for 3 d	$CD8^+$ $CD122^{hi}$	↑	Spleen, PLN, and MLN	Cassese et al. (11)
In drinking water for 3 d (tx mice)	$CD8^+$	↑	Spleen and PLN	Parretta et al. (12)
In drinking water for either 14 or 42 d (tx mice)	$CD8^+$ $CD44^{hi}$	↑	Spleen and PLN	Parretta et al. (12)
In drinking water for either 5 or 8 d	$CD8^+$	↑	Spleen	Snell et al. (13)
In drinking water with sugar for 3 d	$CD8^+$ $CD44^{hi}$	↑[a]	Spleen	Sercan Alp et al. (2)
(C) CFSE				
CFSE-labeled cell transfer				
Splenocyte transfer, either 15 or 25 d before analysis	$CD8^+$ $CD44^{hi}$	↑	Spleen, total LN, and blood	Becker et al. (8)
	$CD8^+$ Ag-specific P14 (LCMV)	↑	Spleen, total LN, and blood	
Splenic $CD8^+$ $CD44^{hi}$ cell transfer, 7 d before analysis	$CD8^+$ $CD44^{hi}$	↑	Spleen and PLN	Quinci et al. (14)
In vitro primed OT-I splenocyte transfer, 21 d before analysis	$CD8^+$ Ag-specific OT-I (OVA)	↑	Spleen and PLN	Lin et al. (15)

The table summarizes published proliferation results on total CD8 and memory CD8 T cells in BM, in comparison with corresponding cells from lymphoid periphery/blood. The arrow ↑ indicates that results in BM were higher than those in lymphoid periphery/blood. Lymphoid periphery comprised spleen and LN, as indicated. Please, note that in all reports, spleen, LN, and blood were all concordantly lower than the BM. All results were obtained by flow cytometry, except for Westermann et al. (10), in which cell suspension analysis was performed by microscopy. (A) DNA content results are expressed as percentages of cells having $2n < DNA \leq 4n$; (B) BrdU results are expressed as percentages of $BrdU^+$ cells after a BrdU pulse or BrdU continuous labeling in drinking water, as indicated; and (C) CFSE results are expressed as percentages of donor $CFSE^+$ cells with decreased CFSE staining intensity, after CFSE-labeled cell transfer, as indicated. All BrdU and CFSE experiments were performed in mice, except for experiments by Westermann et al. (10), which were performed in rats.

Ag, antigen; BM, bone marrow; BrdU, bromodeoxyuridine; CFSE, carboxyfluorescein diacetate succinimidyl ester; CLN, cervical lymph nodes; d, day; h, hour; i.v., intravenous; LCMV, lymphochoriomeningitis virus; LN, lymph nodes; MLN, mesenteric lymph nodes; OT-I, TCR transgenic cells expressing a TCR against OVA; OVA, ovalbumin; PLN, peripheral lymph nodes; P14, TCR transgenic cells expressing a TCR against LCMV; Ref., reference; tx, thymectomized.

[a]*Indicates that an abnormal proliferation was observed upon BrdU treatment (see text for details).*

CD8 T cells in the BM was only about 0.4%. However, this low percentage was still three to eight times higher than that found in corresponding spleen samples [(2), BM 0.32–0.47% and spleen 0.05–0.10%; see Figures 4E and S3], in line with what has been seen in other studies by comparing CD8 T cells from BM with those from blood, spleen, or LN (7–9).

Sercan Alp et al. showed that assessment of CD8 T cell proliferation by BrdU incorporation may be misleading (2). BrdU is a thymidine analog that labels cells during S phase, thus marking the cells undergoing division in the course of BrdU treatment. Depending on dose and length of treatment, BrdU may have toxic effects, potentially leading to artifacts (17). In mice, BrdU is either injected or administered in drinking water, sometimes with sugar addition, a stratagem used to overcome the unpleasant taste of the analog (18, 19). Sugar can increase water consumption, e.g. in 4 hours mice drink 0.5–1.5 ml tap water and 2–4 times more water containing 10% sugar (20). However, total water intake is not usually measured in BrdU experiments, leaving actual BrdU dose undetermined. In the study by Sercan Alp et al., the mice were treated with 1 mg/ml BrdU plus 10% sugar in drinking water for 3 days, and there was an artificial rise – especially in the BM – of dividing memory CD8 T cell frequency, as measured by a BrdU-independent method [(2), Figure 4E]. Based on these results, the authors suggest that previous studies had greatly overestimated the extent of memory CD8 T cell proliferation (2).

However, other authors have used lower doses of BrdU without sugar (7, 12, 21–23), and Parretta et al. found little difference in proliferation (when tested by a BrdU-independent assay) between mice treated with BrdU or not (12). In more details, to compare the two groups of mice, Parretta et al. measured CD8

T cell proliferation by carboxyfluorescein diacetate succinimidyl ester (CFSE), a cytoplasmic dye that is equally distributed between daughter cells upon division. They reported that the proportion of dividing (i.e., $CFSE^{low}$) CD8 T cells in spleen, LN, and BM in response to PolyI:C injection was similar when mice were either untreated or treated with 0.8 mg/ml BrdU in drinking water for 3 days (12), a standard protocol (24). PolyI:C treatment might have masked the toxic effects of BrdU (12); nevertheless, the dose of BrdU plus sugar is a major difference between the Sercan Alp et al.'s study and those of other groups. Indeed, the percentage of $BrdU^+$ cells within spleen $CD44^{high}$ CD8 T cells reported by Sercan Alp et al., i.e., about 30%, was definitely higher in comparison with previous data reported by several authors, all obtained with 0.8 mg/ml BrdU in drinking water for 3 days and no sugar (7, 21, 23). For example, Parretta et al. found that the fraction of $BrdU^+$ cells within $CD44^{high}$ CD8 T cells was 6% in spleen, 5% in LN, and 13% in BM, on average (7). Taking everything into account, it could be argued that the confounding effect of BrdU on BM CD8 T cell proliferation was dose dependent and limited to the study by Sercan Alp and coworkers (2).

Finally, Sercan Alp et al. analyzed CD8 T cells by intracellular staining for Ki67, a cell-cycle marker. They showed that on average, 93–95% of the memory CD8 T cells are negative for Ki67 (i.e., in G_0 phase) in the BM and 88–94% in the spleen [(2), Figures 4B,D]. This indicates that the vast majority of the cells are quiescent and non-dividing at a given time, with a slight difference between BM and spleen. However, it should not be overlooked that the Ki67 assay does not give any information on frequency of dividing cells (i.e., in $S/G_2/M$), since all cells in $G_1/S/G_2/M$ score positively for Ki67. It appears that rather than being in contrast with previous findings on proliferation obtained by other methods (see **Table 1** and references therein), the Ki67 results in the Sercan Alp et al.'s study simply report on a different aspect of the cell cycle.

Table 1 is a summary of published findings on total CD8 and memory CD8 T cell proliferation in BM, grouped according to the experimental methods. In addition to DNA content and BrdU, some authors used CFSE. For example, Quinci et al. found that in 1 week the fraction of $CFSE^{low}$ cells within $CD44^{high}$ CD8 T cells was 17% in spleen, 17% in LN, and 27% in BM, on average (14). All data in **Table 1** show a higher percentage of proliferating cells within memory CD8 T cells in the BM than in lymphoid periphery, i.e., spleen, LN, and blood.

Thus, the data on proliferation are in agreement, while discrepancies remain in interpretations (25, 26). The main point of contention is how much the proliferation occurring in the BM contributes to the long-term maintenance of memory CD8 T cells. Sercan Alp et al. focused their attention on the paucity of proliferating cells in their BM samples (2), ignoring that this is, nevertheless, a higher proportion than that found in spleen and LN. It could be argued that such difference in proliferating cell frequencies should not be neglected, in light of the fact that BM memory CD8 T cells are a large population. Indeed, the BM is a huge organ and, moreover, after the peak of an acute response, antigen-specific CD8 T cell contraction is often less pronounced in the BM than in other organs, resulting in a high number of memory CD8 T cells in the BM in the memory phase (7, 8, 27, 28). For example, in the contraction phase of the response against the M-45 epitope of murine cytomegalovirus (MCMV), the frequency of antigen-specific CD8 T cells dropped 14–20 times in the blood, liver, and lung, and only about five times in the BM (28). Moreover, at late times (6–10 weeks) after immunization against the model antigen ovalbumin, the number of antigen-specific memory CD8 T cells in the BM was 2–3 times higher than that in the spleen and 3–11 times higher than that in total LN (7). However, enrichment of antigen-specific CD8 T cells in the BM in the memory phase was not observed in other types of responses. For example, at late times after infection, antigen-specific memory CD8 T cell frequency in the BM was not higher than that in blood, liver, or lung in the inflationary response against the M-38 epitope of MCMV (28) or in the response against vaccinia virus induced by skin scarification, which mostly elicited antigen-specific tissue-resident memory CD8 T cells in the skin (29).

Taking everything into account, the BM plays a preferential role in sustaining the homeostatic proliferation of antigen-specific memory CD8 T cells following classical acute responses resulting in the long-term systemic memory (1, 7, 8, 30).

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The author confirms being the sole contributor of this work and approved it for publication.

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REFERENCES

1. Di Rosa F. T-lymphocyte interaction with stromal, bone and hematopoietic cells in the bone marrow. *Immunol Cell Biol* (2009) **87**:20–9. doi:10.1038/icb.2008.84
2. Sercan Alp O, Durlanik S, Schulz D, McGrath M, Grun JR, Bardua M, et al. Memory CD8(+) T cells colocalize with IL-7(+) stromal cells in bone marrow and rest in terms of proliferation and transcription. *Eur J Immunol* (2015) **45**:975–87. doi:10.1002/eji.201445295
3. Manz RA, Thiel A, Radbruch A. Lifetime of plasma cells in the bone marrow. *Nature* (1997) **388**(6638):133–4. doi:10.1038/40540
4. Slifka MK, Antia R, Whitmire JK, Ahmed R. Humoral immunity due to long-lived plasma cells. *Immunity* (1998) **8**:363–72. doi:10.1016/S1074-7613(00)80541-5
5. Cassese G, Arce S, Hauser AE, Lehnert K, Moewes B, Mostarac M, et al. Plasma cell survival is mediated by synergistic effects of cytokines and adhesion-dependent signals. *J Immunol* (2003) **171**:1684–90. doi:10.4049/jimmunol.171.4.1684

6. Di Rosa F, Gebhardt T. Bone marrow T cells and the integrated functions of recirculating and tissue-resident memory T cells. *Front Immunol* (2016) **7**:51. doi:10.3389/fimmu.2016.00051
7. Parretta E, Cassese G, Barba P, Santoni A, Guardiola J, Di Rosa F. CD8 cell division maintaining cytotoxic memory occurs predominantly in the bone marrow. *J Immunol* (2005) **174**:7654–64. doi:10.4049/jimmunol.174.12.7654
8. Becker TC, Coley SM, Wherry EJ, Ahmed R. Bone marrow is a preferred site for homeostatic proliferation of memory CD8 T cells. *J Immunol* (2005) **174**:1269–73. doi:10.4049/jimmunol.174.3.1269
9. Okhrimenko A, Grun JR, Westendorf K, Fang Z, Reinke S, von Roth P, et al. Human memory T cells from the bone marrow are resting and maintain long-lasting systemic memory. *Proc Natl Acad Sci U S A* (2014) **111**:9229–34. doi:10.1073/pnas.1318731111
10. Westermann J, Ronneberg S, Fritz FJ, Pabst R. Proliferation of lymphocyte subsets in the adult rat: a comparison of different lymphoid organs. *Eur J Immunol* (1989) **19**:1087–93. doi:10.1002/eji.1830190619
11. Cassese G, Parretta E, Pisapia L, Santoni A, Guardiola J, Di Rosa F. Bone marrow CD8 cells down-modulate membrane IL-7R{alpha} expression and exhibit increased STAT-5 and p38 MAPK phosphorylation in the organ environment. *Blood* (2007) **110**:1960–9. doi:10.1182/blood-2006-09-045807
12. Parretta E, Cassese G, Santoni A, Guardiola J, Vecchio A, Di Rosa F. Kinetics of in vivo proliferation and death of memory and naive CD8 T cells: parameter estimation based on 5-bromo-2′-deoxyuridine incorporation in spleen, lymph nodes, and bone marrow. *J Immunol* (2008) **180**:7230–9. doi:10.4049/jimmunol.180.11.7230
13. Snell LM, Lin GH, Watts TH. IL-15-dependent upregulation of GITR on CD8 memory phenotype T cells in the bone marrow relative to spleen and lymph node suggests the bone marrow as a site of superior bioavailability of IL-15. *J Immunol* (2012) **188**:5915–23. doi:10.4049/jimmunol.1103270
14. Quinci AC, Vitale S, Parretta E, Soriani A, Iannitto ML, Cippitelli M, et al. IL-15 inhibits IL-7Ralpha expression by memory-phenotype CD8(+) T cells in the bone marrow. *Eur J Immunol* (2012) **42**:1129–39. doi:10.1002/eji.201142019
15. Lin GH, Snell LM, Wortzman ME, Clouthier DL, Watts TH. GITR-dependent regulation of 4-1BB expression: implications for T cell memory and anti-4-1BB-induced pathology. *J Immunol* (2013) **190**:4627–39. doi:10.4049/jimmunol.1201854
16. Hellerstein MK. Measurement of T-cell kinetics: recent methodologic advances.*ImmunolToday*(1999)**20**:438–41.doi:10.1016/S0167-5699(99)01529-7
17. Reome JB, Johnston DS, Helmich BK, Morgan TM, Dutton-Swain N, Dutton RW. The effects of prolonged administration of 5-bromodeoxyuridine on cells of the immune system. *J Immunol* (2000) **165**:4226–30. doi:10.4049/jimmunol.165.8.4226
18. Astrakhan A, Sather BD, Ryu BY, Khim S, Singh S, Humblet-Baron S, et al. Ubiquitous high-level gene expression in hematopoietic lineages provides effective lentiviral gene therapy of murine Wiskott-Aldrich syndrome. *Blood* (2012) **119**:4395–407. doi:10.1182/blood-2011-03-340711
19. Kolhatkar NS, Brahmandam A, Thouvenel CD, Becker-Herman S, Jacobs HM, Schwartz MA, et al. Altered BCR and TLR signals promote enhanced positive selection of autoreactive transitional B cells in Wiskott-Aldrich syndrome. *J Exp Med* (2015) **212**:1663–77. doi:10.1084/jem.20150585
20. Bulwa ZB, Sharlin JA, Clark PJ, Bhattacharya TK, Kilby CN, Wang Y, et al. Increased consumption of ethanol and sugar water in mice lacking the dopamine D2 long receptor. *Alcohol* (2011) **45**:631–9. doi:10.1016/j.alcohol.2011.06.004
21. Tough DF, Sprent J. Turnover of naive- and memory-phenotype T cells. *J Exp Med* (1994) **179**:1127–35. doi:10.1084/jem.179.4.1127
22. Zhang X, Sun S, Hwang I, Tough DF, Sprent J. Potent and selective stimulation of memory-phenotype CD8+ T cells in vivo by IL-15. *Immunity* (1998) **8**:591–9. doi:10.1016/S1074-7613(00)80564-6
23. Krieg C, Boyman O, Fu YX, Kaye J. B and T lymphocyte attenuator regulates CD8+ T cell-intrinsic homeostasis and memory cell generation. *Nat Immunol* (2007) **8**:162–71. doi:10.1038/ni1418
24. Tough DF, Sprent J. Measurement of T and B cell turnover with bromodeoxyuridine. *Curr Protoc Immunol* (1996) **Chapter 4**:Unit4.7.
25. Di Rosa F. Maintenance of memory T cells in the bone marrow: survival or homeostatic proliferation? *Nat Rev Immunol* (2016). doi:10.1038/nri.2016.31
26. Alp OS, Radbruch A. The lifestyle of memory CD8$^+$ T cells. *Nat Rev Immunol* (2016). doi:10.1038/nri.2016.32
27. Masopust D, Vezys V, Marzo AL, Lefrancois L. Preferential localization of effector memory cells in nonlymphoid tissue. *Science* (2001) **291**:2413–7. doi:10.1126/science.1058867
28. Bolinger B, Sims S, Swadling L, O'Hara G, de Lara C, Baban D, et al. Adenoviral vector vaccination induces a conserved program of CD8(+) T cell memory differentiation in mouse and man. *Cell Rep* (2015) **13**:1578–88. doi:10.1016/j.celrep.2015.10.034
29. Jiang X, Clark RA, Liu L, Wagers AJ, Fuhlbrigge RC, Kupper TS. Skin infection generates non-migratory memory CD8+ T(RM) cells providing global skin immunity. *Nature* (2012) **483**:227–31. doi:10.1038/nature10851
30. Di Rosa F, Pabst R. The bone marrow: a nest for migratory memory T cells. *Trends Immunol* (2005) **26**:360–6. doi:10.1016/j.it.2005.04.011

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Response: Commentary: Memory CD8$^+$ T Cells Colocalize with IL-7$^+$ Stromal Cells in Bone Marrow and Rest in Terms of Proliferation and Transcription

*Özen Sercan-Alp and Andreas Radbruch**

Department of Cell Biology, German Rheumatism Research Center, Leibniz Institute, Berlin, Germany

Keywords: CD8 T cells, bone marrow, memory, Ki-67, homeostatic proliferation, BrdU, DNA content

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Tania H. Watts,
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Charles Surh,
Scripps Research Institute, USA

***Correspondence:**
Andreas Radbruch
radbruch@drfz.de

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In her commentary to our paper (1), Francesca Di Rosa very nicely summarizes past and recent findings in the field of bone marrow (BM)-resident memory CD8 T cells (2) and discusses the lifestyle of memory CD8 T cells in the BM, as circulating cells temporarily staying and proliferating in the BM. Here, in our response, we argue that the available evidence strongly suggests that the vast majority (>90%) of the memory CD8 T cells in BM are resting in terms of proliferation.

We have recently analyzed proliferation of memory CD8 T cells in murine and human BM, murine spleen, and human blood, according to expression of Ki-67, incorporation of Bromodeoxyuridine (BrdU), and DNA content analysis by propidium iodide (PI) staining. Ki-67 is a protein reported to be expressed by cells that are in any stage of the cell cycle, from G1 to M phases, but not in the G0 phase (3). In the memory phase of an immune response, on average, more than 94% of the Ag-specific memory CD8 T cells in murine BM did not express Ki-67 (1) indicating that only a few percent (less than 6%) of the cells are proliferating, if at all. Indeed, staining of the cells for their DNA content with PI revealed that less than 1% of the cells were actively proliferating, i.e., in the S/G2/M phases of cell cycle at any given time point. Similar results for Ki-67 expression of memory CD8 cells in murine BM have been published in the meantime by Geerman and colleagues (4). For memory CD8 cells from human BM and blood, we had described before that, on average, less than 2% of BM or 5% of blood memory CD8 T cells expressed Ki-67 (5). We would like to emphasize that staining for Ki-67 and for DNA content are non-invasive methods, in all likelihood, reflecting the situation *in vivo*.

In previous analyses, the groups of Rafi Ahmed and Francesca Di Rosa had used BrdU and carboxyfluorescein succinimidyl ester (CFSE) to analyze proliferation of BM memory CD8 T cells *in vivo* (6–8). BrdU was fed to mice in drinking water or injected intravenously. After 1 day of BrdU injection, about 7% of the BM memory CD8 T cells proliferated incorporating BrdU into their DNA (6). When the mice were fed with BrdU in drinking water for a longer time period such as 14 days, about 50% of the memory phenotype CD8 T cells became BrdU$^+$ (8). Extrapolation suggested a complete turnover of the memory CD8 T cell pool of BM within 1–2 months (8, 9). When we noticed the apparent discrepancy between our Ki-67 and PI stainings and the previously published BrdU-incorporation data, we combined all three approaches. We fed mice with BrdU in drinking water and then stained their memory CD8 T cells for BrdU and Ki-67 or analyzed their DNA content. After 3 days of BrdU feeding, about 60% of the memory phenotype CD8 T cells

of BM had incorporated BrdU. Ki-67 staining revealed that almost all of the BrdU$^+$ cells, hence 60% of all memory CD8 T cells, also became Ki-67$^+$ as opposed to less than 6% in mice not fed with BrdU. Furthermore, the frequency of cells in S/G2/M increased to 5.4%, as compared to 0.4% in mice, which did not receive BrdU in drinking water (1). This result shows that BrdU can induce proliferation of resting memory CD8 T cell of the BM. By comparing BrdU incorporation and CFSE dilution of adoptively transferred CFSE-labeled cells, Parretta and her colleagues showed that CD8 T cell proliferation that is assessed by the loss of CFSE staining was comparable between BrdU-treated and untreated mice (8). However, it should be noted that mice receiving CFSE-labeled CD8 memory T cells were also injected with Polyinosinic:polycytidylic acid (poly:IC), activating the MyD88 pathway. The induction of proliferation of memory CD8 T cells by BrdU is MyD88 dependent, since MyD88 deficient mice do not show Ki-67 upregulation upon BrdU feeding (unpublished data). Thus, in this particular, highly invasive experiment, both poly I:C and BrdU could have induced proliferation. We agree with Francesca Di Rosa that it is difficult, if not impossible, to standardize the uptake of BrdU of individual mice, if the BrdU is provided in the drinking water. Nonetheless, BrdU is clearly no reliable marker for proliferation and results warrant confirmation by alternative (non-invasive) measures.

In summary, recent data from our group and the group of Nolte, using non-invasive measures of proliferation, strongly suggest that proliferation of CD8 memory T cells in the memory phase of an immune response is very low, if it occurs at all (1, 4, 5). What is the nature of the very few memory CD8 T cells, which are proliferating in steady state, as detected by non-invasive analyses? Could they be cross-reactive to self-antigens? Analyzing the entire pool of memory CD8 T cells is more complicated due to the presence of recently activated cells. An indication for this is that, in human BM and blood, some of the Ki-67$^+$ memory T cells have downregulated the expression of CD127, the receptor for interleukin 7, indicating their recent activation (5). Nevertheless, by far, the vast majority of memory T cells are resting in terms of proliferation. A fundamental question comes up, namely, whether (homeostatic) proliferation does play any role in the maintenance of CD8 memory T cells, CD4 memory T cells (10), and memory B cells.

AUTHOR CONTRIBUTIONS

ÖS-A and AR wrote the text and approved the final submission.

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REFERENCES

1. Sercan Alp O, Durlanik S, Schulz D, McGrath M, Grun JR, Bardua M, et al. Memory CD8(+) T cells colocalize with IL-7(+) stromal cells in bone marrow and rest in terms of proliferation and transcription. *Eur J Immunol* (2015) 45(4):975–87. doi:10.1002/eji.201445295
2. Di Rosa F. Commentary: memory CD8(+) T cells colocalize with IL-7(+) stromal cells in bone marrow and rest in terms of proliferation and transcription. *Front Immunol* (2016) 7:102. doi:10.3389/fimmu.2016.00102
3. Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U, Stein H. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *J Immunol* (1984) 133(4): 1710–5.
4. Geerman S, Hickson S, Brasser G, Pascutti MF, Nolte MA. Quantitative and qualitative analysis of bone marrow CD8(+) T cells from different bones uncovers a major contribution of the bone marrow in the vertebrae. *Front Immunol* (2015) 6:660. doi:10.3389/fimmu.2015.00660
5. Okhrimenko A, Grun JR, Westendorf K, Fang Z, Reinke S, von Roth P, et al. Human memory T cells from the bone marrow are resting and maintain long-lasting systemic memory. *Proc Natl Acad Sci U S A* (2014) 111(25):9229–34. doi:10.1073/pnas.1318731111
6. Becker TC, Coley SM, Wherry EJ, Ahmed R. Bone marrow is a preferred site for homeostatic proliferation of memory CD8 T cells. *J Immunol* (2005) 174(3):1269–73. doi:10.4049/jimmunol.174.3.1269
7. Parretta E, Cassese G, Barba P, Santoni A, Guardiola J, Di Rosa F. CD8 cell division maintaining cytotoxic memory occurs predominantly in the bone marrow. *J Immunol* (2005) 174(12):7654–64. doi:10.4049/jimmunol.174.12.7654
8. Parretta E, Cassese G, Santoni A, Guardiola J, Vecchio A, Di Rosa F. Kinetics of in vivo proliferation and death of memory and naive CD8 T cells: parameter estimation based on 5-bromo-2'-deoxyuridine incorporation in spleen, lymph nodes, and bone marrow. *J Immunol* (2008) 180(11):7230–9. doi:10.4049/jimmunol.180.11.7230
9. Choo DK, Murali-Krishna K, Anita R, Ahmed R. Homeostatic turnover of virus-specific memory CD8 T cells occurs stochastically and is independent of CD4 T cell help. *J Immunol* (2010) 185(6):3436–44. doi:10.4049/jimmunol.1001421
10. Tokoyoda K, Zehentmeier S, Hegazy AN, Albrecht I, Grun JR, Lohning M, et al. Professional memory CD4+ T lymphocytes preferentially reside and rest in the bone marrow. *Immunity* (2009) 30(5):721–30. doi:10.1016/j.immuni.2009.03.015

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B Cells Negatively Regulate the Establishment of CD49b$^+$T-bet$^+$ Resting Memory T Helper Cells in the Bone Marrow

*Shintaro Hojyo[1], Jana Sarkander[1], Christian Männe[1], Mathias Mursell[1], Asami Hanazawa[1], David Zimmel[1,2], Jinfang Zhu[3], William E. Paul[3], Simon Fillatreau[1,4,5,6], Max Löhning[1,2], Andreas Radbruch[1] and Koji Tokoyoda[1]**

[1] Deutsches Rheuma-Forschungszentrum Berlin, Leibniz Institute, Berlin, Germany, [2] Experimental Immunology and Osteoarthritis Research, Department of Rheumatology and Clinical Immunology, Charité-Universitätsmedizin Berlin, Berlin, Germany, [3] Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA, [4] INSERM U1151-CNRS UMR 8253, Institut Necker-Enfants Malades, Paris, France, [5] Faculté de Médecine, Université Paris Descartes, Sorbonne Paris Cité, Paris, France, [6] Assistance Publique-Hôpitaux de Paris, Hôpital Necker Enfants Malades, Paris, France

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Edited by:
Francesca Di Rosa,
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Reviewed by:
David Gray,
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Joshy Jacob,
Emory University, USA

***Correspondence:**
Koji Tokoyoda
tokoyoda@drfz.de

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During an immune reaction, some antigen-experienced CD4 T cells relocate from secondary lymphoid organs (SLOs) to the bone marrow (BM) in a CD49b-dependent manner and reside and rest there as professional memory CD4 T cells. However, it remains unclear how the precursors of BM memory CD4 T cells are generated in the SLOs. While several studies have so far shown that B cell depletion reduces the persistence of memory CD4 T cells in the spleen, we here show that B cell depletion enhances the establishment of memory CD4 T cells in the BM and that B cell transfer conversely suppresses it. Interestingly, the number of antigen-experienced CD4 T cells in the BM synchronizes the number of CD49b$^+$T-bet$^+$ antigen-experienced CD4 T cells in the spleen. CD49b$^+$T-bet$^+$ antigen-experienced CD4 T cells preferentially localize in the red pulp area of the spleen and the BM in a T-bet-independent manner. We suggest that B cells negatively control the generation of CD49b$^+$T-bet$^+$ precursors of resting memory CD4 T cells in the spleen and may play a role in bifurcation of activated effector and resting memory CD4 T cell lineages.

Keywords: resting memory, CD4 T helper cells, B cells, bone marrow, T-bet

INTRODUCTION

One of the greatest characteristics of the immune system is to memorize previously encountered antigens and mount rapid recall responses. It is well established that CD4 T cells play an essential role in regulating the generation of high-affinity memory B cells and long-lived plasma cells (1–3) as well as the maintenance and expansion of memory CD8 T cells during secondary immune responses (4–7). However, the potential role and whereabouts of memory CD4 T cells were debated in the

Abbreviations: BM, bone marrow; DC, dendritic cell; LCMV, lymphocytic choriomeningitis virus; PALS, periarterial lymphatic sheaths; SLO, secondary lymphoid organ; Tfh, T follicular helper.

field for a long time. Memory CD4 T cells are generated from antigen-experienced CD4 T cells during the down-sizing of an immune reaction and are maintained in the absence of antigen by survival signals and homeostatic proliferation (8–11). While the bone marrow (BM) has been known to host long-lived plasma cells (12, 13), memory CD4 T cells were thought to constantly circulate throughout the body. Recently, we have uncovered that murine BM is a home of resting memory CD4 T cells (14). Some activated CD4 T cells migrate into the BM in a CD69- and CD49b (integrin α2)-dependent manner and reside and rest as memory CD4 T cells in the survival niches composed of IL-7$^+$collagen-XI$^+$ stromal cells (14–17). Interestingly, the repertoire of memory CD4 T cells in human BM is also significantly enriched for systemic pathogens compared to blood memory T cells (18, 19). While the vast majority of activated CD4 T cells undergo apoptosis after clearance of the antigen, it is still poorly understood which subpopulation in antigen-specific activated CD4 T cells has the potential to develop memory Th cells in the BM.

B cells are considered as potent antigen-presenting cells nearly as effective as dendritic cells (DCs) (20). While DCs play an essential role for priming naive CD4 T cells in the periarterial lymphatic sheaths (PALS) of spleen, B cells contribute to the generation of follicular helper T (Tfh) cells (21). Several reports provided direct evidence of the requirement of B cells for the establishment of memory CD4 T cells in the spleen, comparing the expansion and maintenance of antigen-specific CD4 T cells from B cell-depleted or -deficient and control mice (22–26). In case of a lymphocytic choriomeningitis virus (LCMV) infection model, B cell-deficient mice rapidly lost memory CD4 T cells in the spleen during the contraction phase, despite normal expansion after acute infection (26). Thus, B cells are important for the formation of splenic memory CD4 T cells in vaccination and infection models. However, all these reports focused on memory CD4 T cells in the spleen, but not in the BM.

In this study, we investigated whether B cells are involved in the establishment of resting CD4 T cell memory in the BM. We examined the accumulation of antigen-specific activated CD4 T cells in the BM of B cell-depleted or B cell-deficient mice in the early phase of an immune reaction. We here show that B cells make a negative impact on the accumulation of CD49b$^+$T-bet$^+$ resting memory CD4 T cells in the BM, suggesting that B cells contribute to a quantitative balance of the commitment to effector Tfh cell and BM resting memory CD4 T cell lineage.

MATERIALS AND METHODS

Mice

Lymphocytic choriomeningitis virus–TCR tg [SMARTA (27)], *Tbx21*-KO (28), JHT (29), T-bet-ZsGreen reporter (30), or *Rag1*-KO mice were used. In all experiments, the mice were used at 6–16 weeks of age and were maintained under specific pathogen-free conditions. All mouse experiments were performed in accordance with the German law for animal protection and with permission from the local veterinary offices, and in compliance with the guidelines of the Institutional Animal Care and Use Committee. For immunizations, mice were injected intraperitoneally (i.p.) with LCMV $GP_{61–80}$ peptide (synthesized by Genecust) plus lipopolysaccharide (LPS, O111:B4).

Flow Cytometry and Cell Sorting

Single-cell suspensions were prepared from the spleen, BM, and blood of individual mice. The viability of cells was assessed by trypan blue exclusion. For cell staining, cells were stained for 15 min at 4°C with monoclonal antibodies against CD4 (RM4–5), CD19 (6D5), CD25 (PC61.5), CD44 (IM7), B220 (RA3-6B2), CD49b (HMa2), CD62L (MEL-14), NK1.1 (PK136), Thy1.1 (OX-7), CXCR4 (L276F12), CXCR5 (L138D7), CCR7 (4B12), and T-bet (4B10) and isotype controls. To exclude dead cells, we stained the cells with 1 μg/ml propidium iodide (Sigma). Intracellular staining for transcription factors was performed using Foxp3/transcription factor staining buffer kit (eBioscience) according to the manufacturer's protocol.

Real-Time PCR Analysis

The total RNA was extracted from splenocytes, and reverse-transcribed with High Capacity RNA-to-cDNA Kit (Life Technologies). Real-time PCR analysis was performed using the SYBR Green Master Mix (Life Technologies). Samples were normalized to the *Hprt* expression. *Cxcl12* primer sequences: forward 5′-AAA CCA GTC AGC CTG AGC TAC C-3′, reverse 5′-GGC TCT GGC GAT GTG GC-3′; *Hprt* primer sequences: forward 5′-TCC TCC TCA GAC CGC TTT T-3′, reverse 5′-CAT AAC CTG GTT CAT CAT CGC-3′.

Cell Sorting and Adoptive Transfer

For positive sorting of splenic CD4 T cells, the Fab fragments of anti-CD4 antibody and Streptavidin MicroBeads (Miltenyi Biotec) were used. For negative sorting of splenic B cells, splenocytes were stained with FITC-conjugated anti-Mac1 (M1/70), anti-CD4 (GK1.5), and anti-CD8a (53–6.7) antibodies and then with anti-FITC and anti-Thy1.2 MicroBeads (Miltenyi Biotec) and were sorted by a magnetic cell separation system (MACS, Miltenyi Biotec). Approximately $0.5–1 \times 10^6$ purified LCMV–TCR tg CD4 T cells were transferred intravenously (i.v.) into C57BL/6, JHT, or *Rag1*-KO mice, if any, with 1×10^7 purified splenic B cells.

B Cell Depletion

B cells were depleted by antibody-mediated antigen-receptor cross-linking, as previously described (31). Mice were injected i.p. with either 200 μg of anti-IgD (11.26c) or isotype control followed by injection of 200 μg of mouse anti-rat IgG antibody (MAR18.5). The efficiency of B cell depletion was examined by flow cytometric analysis using anti-B220 (RA3-6B2) and anti-CD19 (6D5) antibodies.

Immunofluorescent Staining and Confocal Microscopy

For immunofluorescence staining, samples were fixed in 4% paraformaldehyde and equilibrated in 30% sucrose. Cryostat sections of adult spleen were stained with monoclonal antibodies against Thy1.1 (HIS51), B220 (RA3-6B2), and CD4 (RM4–5). All histological analyses were carried out with a confocal laser microscope (LSM710, Carl Zeiss).

Statistical Analyses

All statistical analyses were performed using two-tailed Student's *t*-test.

RESULTS

B Cell Depletion Enhances the Accumulation of Antigen-Experienced CD4 T Cells in the BM

B cell depletion inhibits the generation of memory CD4 T cells in the spleen, while it does not affect the expansion of CD4 T cells (22–26). To examine the effect of B cell depletion on the generation of resting memory CD4 T cells in the BM, we transferred LCMV–TCR tg CD4 T cells into mice pretreated with anti-IgD or isotype control followed by treatment of anti-rat IgG. The depletion protocol reduced more than 80% of B220^{hi}CD19^{+} mature B cells in the spleen and mesenteric lymph nodes (mLN), and around 70% in BM, but did not affect BM B cell precursors, including pro-B cells, which may compete for IL-7-expressing stromal niches with memory CD4 T cells (Figure S1 in Supplementary Material) (32, 33). Next, we immunized mice with LCMV peptide. Although LCMV-specific CD4 T cells in the spleen of B cell-depleted and control mice expanded similarly, there were more CD4 T cells in the BM of B cell-depleted mice compared to the BM of B cell-sufficient mice (**Figure 1A**). A similar result was obtained in the BM from *Rag1*$^{-/-}$ host mice that had received antigen-specific CD4 T cells (**Figure 1B**). By contrast, when wild-type splenic polyclonal B cells were cotransferred with LCMV-specific CD4 T cells into *Rag1*$^{-/-}$ mice followed by immunization with LCMV peptide (Figure S2A in Supplementary Material), the accumulation of antigen-specific CD4 T cells in the BM was dramatically decreased, despite normal expansion in the spleen (**Figure 1C**). Furthermore, in B cell-deficient JHT mice transferred with LCMV–TCR tg CD4 T cells, despite smaller expansion in the spleen compared to C57BL/6 host mice, the equivalent number of antigen-specific CD4 T cells was detected in the BM, involving in an increased ratio of antigen-specific CD4 T cells in the BM compared to the spleen (Figure S2B in Supplementary Material). Collectively, these results indicate that B cells suppress the accumulation of antigen-experienced CD4 T cells in the BM.

B Cell Depletion Enhances the Induction of CD49b^{+}T-bet^{+} Antigen-Specific CD4 T Cells

How does B cell depletion affect antigen-specific CD4 T cells in the spleen? CD49b^{+} antigen-specific activated CD4 T cells in

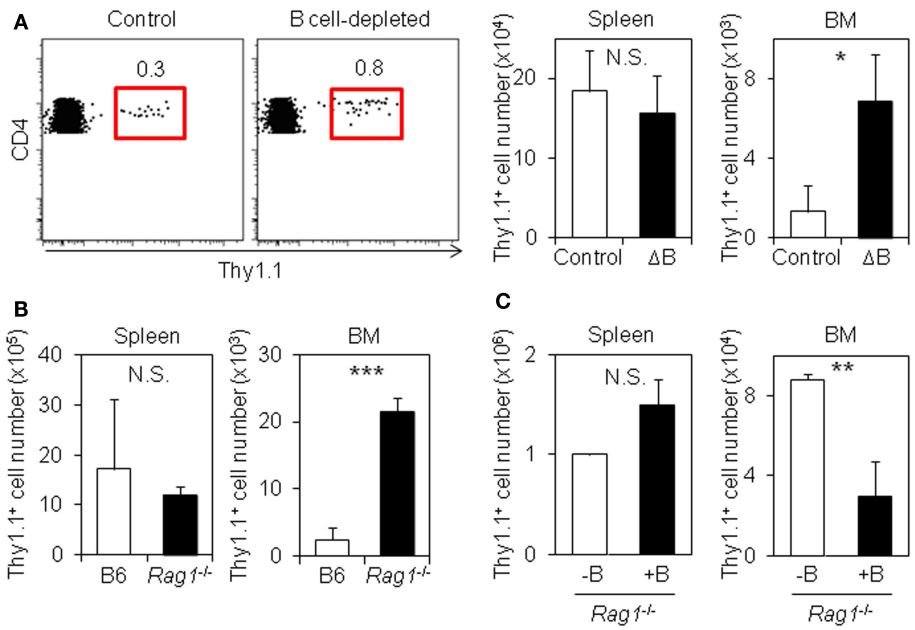

FIGURE 1 | B cells suppress the accumulation of antigen-specific CD4 T cells in the BM. (A) B cell depletion promotes the accumulation of antigen-specific CD4 T cells in the BM. C57BL/6 mice were treated with isotype-matched control antibody or anti-IgD antibody followed by injection of anti-rat IgG antibody. Two days later, purified Thy1.1^{+} LCMV–TCR CD4 T cells were transferred into the antibody-treated mice, and then immunized with LCMV $GP_{61–80}$ plus LPS. On day 6, the Thy1.1^{+} CD4 T cells in the spleen and BM were analyzed by flow cytometry and enumerated. Gating plots show the Thy1.1^{+} cell population in CD4^{+}B220^{-}NK1.1^{-}PI^{-} cells of the BM. Bar charts represent the cell numbers. Data represent the mean ± SD; $^{*}p < 0.05$; $N = 4$. **(B)** Accumulation of antigen-specific CD4 T cells in the BM is also promoted in *Rag1*-KO host mice. Purified Thy1.1^{+} LCMV–TCR CD4 T cells were transferred into C57BL/6 or *Rag1*-KO mice followed by immunization with LCMV $GP_{61–80}$ plus LPS. On day 4, the Thy1.1^{+} CD4 T cells in the spleen and BM were analyzed by flow cytometry and enumerated. Bar charts represent the cell numbers. Data represent the mean ± SD; $^{***}p < 0.001$; $N = 4$. **(C)** B cell-cotransfer suppresses the accumulation of antigen-specific CD4 T cells into the BM. Purified Thy1.1^{+} LCMV–TCR CD4 T cells were transferred into *Rag1*-KO mice with or without B cells followed by immunization with LCMV $GP_{61–80}$ plus LPS. On day 6, the Thy1.1^{+} CD4 T cells in the spleen and BM were analyzed by flow cytometry and enumerated. Bar charts represent the cell numbers. Data represent the mean ± SD; $^{**}p < 0.01$; $N = 4$.

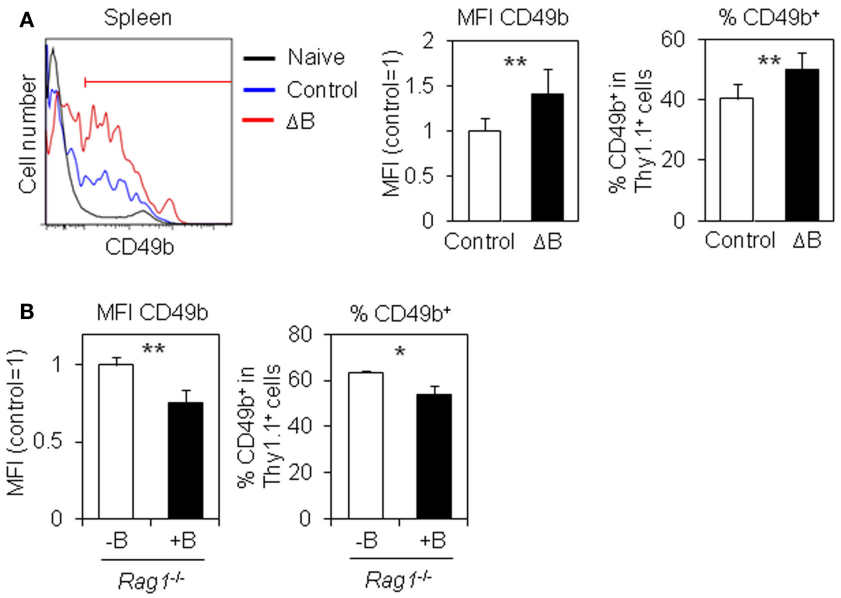

FIGURE 2 | CD49$^+$ antigen-specific CD4 T cells are increased by B cell-depletion. (A) CD49b$^+$ antigen-specific CD4 T cells are increased in the spleen of B cell-depleted mice. Purified Thy1.1$^+$ LCMV–TCR CD4 T cells were transferred into B cell-depleted or control C57BL/6 mice followed by immunization with LCMV $GP_{61–80}$ plus LPS. On day 6, the CD49b$^+$ population in the spleen was analyzed by flow cytometry. Histograms show CD49b expression in CD4$^+$Thy1.1$^+$B220$^-$NK1.1$^-$PI$^-$ cells and CD4$^+$Thy1.1$^-$B220$^-$NK1.1$^-$PI$^-$ (naive) cells in the spleen. Gated line in the histogram represents a CD49b$^+$ population. Bar charts represent the relative ratio of mean fluorescent intensity (MFI) of CD49b (left) and CD49b$^+$ population (right) in CD4$^+$Thy1.1$^+$B220$^-$NK1.1$^-$PI$^-$ cells in the spleen. Data represent the mean ± SD; $^{**}p < 0.01$; $N = 7$. **(B)** CD49b$^+$ antigen-specific CD4 T cells are decreased in the spleen by B cell cotransfer. A CD49b$^+$ population in the spleen of mice described in **Figure 1C** was analyzed by flow cytometry. Bar charts represent the relative ratio of MFI of CD49b (left) and CD49b$^+$ population (right) in CD4$^+$Thy1.1$^+$B220$^-$NK1.1$^-$PI$^-$ cells in the spleen. Data represent the mean ± SD; $^*p < 0.05$, $^{**}p < 0.01$; $N = 3$.

the spleen preferentially migrate into the BM (15). We speculated that B cell depletion affects the expression of CD49b in antigen-specific activated CD4 T cells in the spleen. Notably, splenic antigen-specific CD4 T cells in B cell-depleted mice expressed significantly more CD49b compared to control mice (**Figure 2A**) and conversely the CD4 T cells in B cell-transferred *Rag1*$^{-/-}$ mice decreased its expression (**Figure 2B**), suggesting that B cells negatively regulate the expression of CD49b and consequently reduce the accumulation of antigen-specific CD4 T cells in the BM.

Misumi and Whitmire have reported that B cell depletion affects the expression of Ly-6C and T-bet in antigen-specific CD4 T cells (23). Therefore, in order to examine the expression of Ly-6C and T-bet in antigen-specific CD4 T cells, we used anti-Ly-6C antibodies and T-bet ZsGreen BAC tg mice that express ZsGreen fluorescence protein under control of *Tbx21* regulatory elements (30). Ly-6C$^+$ cells were slightly but significantly increased in splenic antigen-specific CD4 T cells from B cell-depleted mice (**Figure 3A**). T-bet$^+$ antigen-specific CD4 T cells were significantly increased in the spleen and blood after B cell depletion, while the population in the BM was likely saturated (**Figure 3B**). We also detected three times more T-bet$^+$ cells among antigen-specific CD4 T cells in the spleen of B cell-depleted mice using a T-bet-specific antibody (Figure S3 in Supplementary Material). Intriguingly, CD49b$^+$T-bet$^+$ antigen-specific CD4 T cells in immunized host mice were detected at the lowest percentage in the spleen (14%), at the midst in the blood (34%), and at the highest in the BM (53%) (**Figure 3C**), suggesting that CD49b$^+$T-bet$^+$ antigen-specific CD4 T cells selectively migrate from the spleen into the BM *via* blood. These results argue that CD49b$^+$T-bet$^+$ antigen-specific CD4 T cells are the potential precursors of BM resting memory CD4 T cells.

T-bet$^+$ Antigen-Specific CD4 T Cells Preferentially Localize in Red Pulp of Spleen

The precursors of long-lived plasma cells egress from splenic B cell follicles toward the BM *via* splenic red pulp and blood (34). To examine the localization of T-bet$^+$ antigen-specific CD4 T cells in the spleen, we performed a histological analysis and assessed the localization of T-bet$^+$ and T-bet$^-$ antigen-specific CD4 T cells (**Figure 4**). On day 6 after immunization, while most of T-bet$^-$ antigen-specific CD4 T cells remained in the white pulp, including B cell follicles and PALS, T-bet$^+$ cells significantly stayed in the red pulp. These data suggest that T-bet$^+$ resting memory CD4 T cell precursors preferentially localize in splenic red pulp and blood. It is well known that the precursors of long-lived plasma cells migrate into the BM in a CXCL12-dependent manner (33, 35). To examine the involvement of some chemokines in the localization of the resting memory CD4 T cell precursors, the expression of *Cxcl12* mRNA in the spleen tissue and CXCR4, CXCR5, and CCR7 proteins on antigen-specific CD4 T cells in the spleen and BM from B-cell

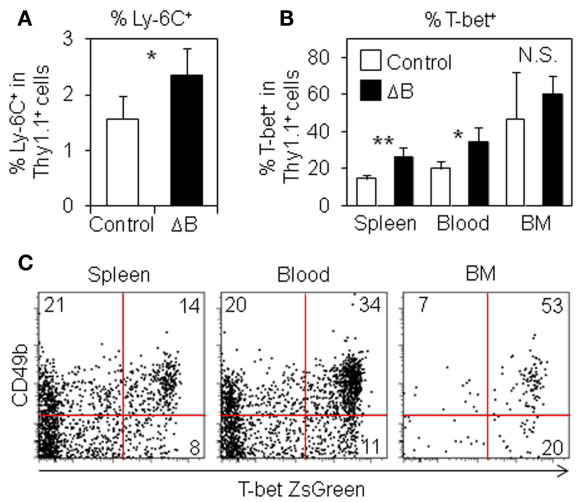

FIGURE 3 | Ly-6C$^+$ and T-bet$^+$ antigen-specific CD4 T cells are increased by B cell-depletion. (A) Ly-6C$^+$ antigen-specific CD4 T cells are increased in the spleen from B cell-depleted mice. Purified Thy1.1$^+$ T-bet-ZsGreen reporter LCMV–TCR CD4 T cells were transferred into B cell-depleted or control C57BL/6 mice followed by immunization with LCMV $GP_{61–80}$ plus LPS. On day 6, Ly-6C$^+$ population in the spleen was analyzed by flow cytometry. Bar chart represents the Ly-6C$^+$ population in CD4$^+$Thy1.1$^+$B220$^-$NK1.1$^-$PI$^-$ cells in the spleen. Data represent the mean ± SD; $^*p < 0.05$; $N = 4$. **(B)** T-bet$^+$ antigen-specific CD4 T cells are increased in the spleen and blood from B cell-depleted mice. In **(A)**, T-bet$^+$ (ZsGreen$^+$) population in the spleen, blood, and BM was analyzed by flow cytometry. Bar chart represents T-bet$^+$ (ZsGreen$^+$) population in CD4$^+$Thy1.1$^+$B220$^-$NK1.1$^-$PI$^-$ cells in the spleen, blood, and BM. Data represent the mean ± SD; $^*p < 0.05$, $^{**}p < 0.01$; $N = 4$. **(C)** CD49b$^+$T-bet$^+$ antigen-specific CD4 T cells are detected at the highest percentage in the BM. Purified Thy1.1$^+$ T-bet-ZsGreen reporter LCMV–TCR CD4 T cells were transferred into C57BL/6 mice followed by immunization with LCMV $GP_{61–80}$ plus LPS. On day 6, CD49b$^+$T-bet$^+$ (ZsGreen$^+$) population in CD4$^+$Thy1.1$^+$B220$^-$NK1.1$^-$PI$^-$ cells in the spleen, blood, and BM was analyzed by flow cytometry.

depleted and control mice were analyzed. However, their expression profiles were not influenced by B cell depletion (Figure S4 in Supplementary Material).

T-bet Is Not Required for the Accumulation of Antigen-Specific CD4 T Cells in the BM

Finally, to examine the physiological impact of T-bet on the accumulation of antigen-specific CD4 T cells in the BM, we compared the accumulation of T-bet$^{+/+}$ or T-bet$^{-/-}$ LCMV–TCR tg CD4 T cells in the BM. On day 6 after immunization, T-bet-deficient CD4 T cells accumulated in the BM equally as well as T-bet-sufficient cells (**Figure 5A**). Furthermore, T-bet deficiency did not affect surface CD49b expression (**Figure 5B**). Consistent with these observations, when we compared the number of CD44^{hi} memory-phenotype CD4 T cells in T-bet-sufficient and -deficient backgrounds, both groups showed similar numbers of CD44^{hi} CD4 T cells in the spleen and BM (Figure S5 in Supplementary Material). Taken together, T-bet is dispensable for the expression of CD49b to facilitate the accumulation of antigen-specific CD4 T cells in the BM.

DISCUSSION

B cells have been described as a positive regulator to generate CD4 T cell memory in the spleen (22–26). However, we here showed that the depletion or deficiency of B cells actually promotes the accumulation of antigen-specific activated CD4 T cells in the BM, and in contrast the pretransfer of B cells suppresses their accumulation, suggesting that B cells are a negative regulator to generate CD4 T cell memory in the BM. We also found that B cell depletion facilitates the upregulation of the expression of CD49b, a homing receptor of CD4 T cells to the BM, and T-bet, the lineage-specifying transcription factor of Th1 cell differentiation, in the activated CD4 T cells in the spleen. Moreover, the T-bet$^+$ cell population preferentially localizes in the red pulp of the spleen and in the BM during an immune reaction. We suggest that CD49b$^+$T-bet$^+$ antigen-specific CD4 T cells in the spleen are the precursors of BM resting memory CD4 T cells.

We demonstrated that B cells negatively control the expression of T-bet and CD49b in antigen-experienced CD4 T cells in the spleen. It remains unclear at the molecular level how B cells downregulate the expression of these molecules and whether a T-bet-inducing signal is directly involved in CD49b expression in activated CD4 T cells. Intriguingly, a loss of T-bet did not alter the expression level of CD49b in activated CD4 T cells and hardly affected their accumulation in the BM. The result suggests that T-bet itself plays a dispensable role in the migration of activated CD4 T cells. Rather, the upstream signaling pathway (T-bet-inducing signal) might be critical in this process in parallel with the control of CD49b expression. Naive T cells do not express T-bet and CD49b. Once they are stimulated *via* the TCR, while T-bet expression is induced *via* IFNγR and IL-12R signaling pathways (28, 30, 36, 37), CD49b expression is induced as well as VLA-2 on Th1 cells *in vitro* in an IFNγ- and IL-12-independent manner (38). Hence, opposite to the upregulation of T-bet and CD49b by DCs, B cell-mediated signals may commonly downregulate the expression of these molecules. This notion is supported by recent findings (39, 40). They have described that ICOS costimulation *via* B cells is crucial to maintain the Tfh phenotype through the downregulation of transcription factor KLF2 and that the blockade of ICOS ligand on B cells increases *Tbx21* transcripts in antigen-specific CD4 T cells. In a KLF2-transduced HUVEC cell line, CD49b is upregulated compared to a control cell line at the transcriptional level (41). It remains to be further clarified whether B cells suppress T-bet and CD49b in activated CD4 T cells in a KLF2-dependent manner.

Our data suggest that DCs may activate CD4 T cells and license them to differentiate into resting memory cells in the BM during primary immune response, while some activated CD4 T cells contact bystander B cells as follow-up antigen-presenting cells through cognate interaction and further differentiate into effector Tfh cells (42–45). Thus, additional activation by B cells following DCs may induce the differentiation of effector Tfh cell lineage (21), suppressing the differentiation program for a BM resting memory cell lineage. Resting memory CD4 T cells in the BM are more functional *in vitro* and *in vivo* compared to spleen-resident memory cells (14). Adoptively transferred memory CD4 T cells from the BM can efficiently help B cells

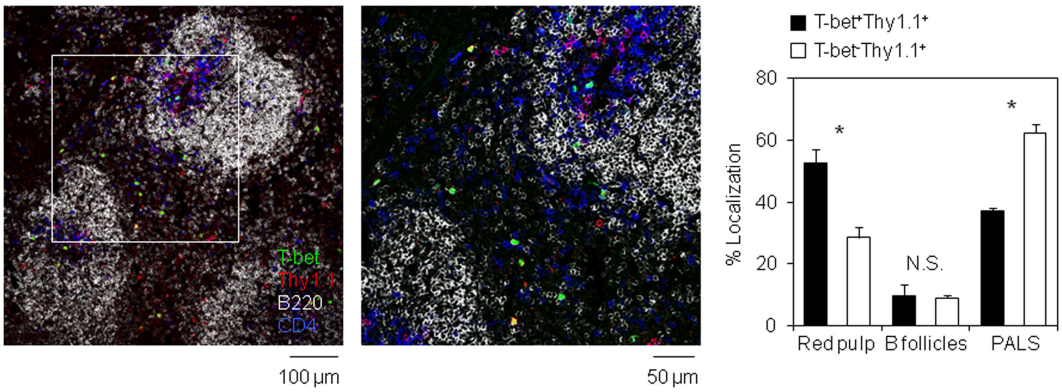

FIGURE 4 | T-bet-expressing antigen-specific CD4 T cells preferentially egress from white pulp into red pulp. Localization of T-bet$^+$ (ZsGreen$^+$) Thy1.1$^+$ CD4 T cells in the spleen section. Frozen section prepared from the spleen of host mouse in **Figure 3C** was stained with anti-Thy1.1 (red), anti-B220 (gray), and anti-CD4 (blue) antibodies. Bar chart represents the localization of T-bet$^+$Thy1.1$^+$ and T-bet$^-$Thy1.1$^+$ cells in each area of the spleen. Data represent the mean ± SD. $^*p < 0.05$.

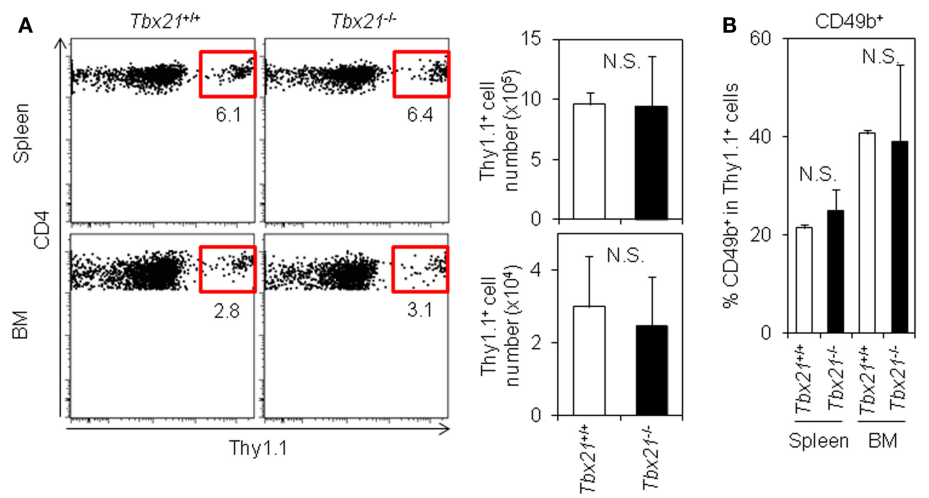

FIGURE 5 | T-bet is not required for the accumulation of antigen-specific CD4 T cells in the BM. (A) T-bet-deficient CD4 T cells normally accumulate in the BM. Purified *Tbx21*$^{+/+}$ or *Tbx21*$^{-/-}$ Thy1.1$^+$ LCMV–TCR CD4 T cells were transferred into C57BL/6 mice followed by immunization with LCMV GP_{61-80} plus LPS. On day 6, the Thy1.1$^+$ CD4 T cells in the spleen and BM were analyzed by flow cytometry and enumerated. Gating plots show a Thy1.1$^+$ population in CD4$^+$B220$^-$PI$^-$ cells in the spleen and BM. Bar charts represent the cell numbers. Data represent the mean ± SD; $N = 3$. **(B)** T-bet-deficiency does not alter surface CD49b expression. In **(A)**, CD49b$^+$ population in antigen-specific CD4 T cells was analyzed by flow cytometry. Bar chart represents CD49b$^+$ population in CD4$^+$Thy1.1$^+$B220$^-$PI$^-$ cells in the spleen and BM. Data represent the mean ± SD; $N = 3$.

to produce high-affinity antibodies, suggesting that BM memory CD4 T cells can differentiate into Tfh cells during recall response. We have previously shown that a persistent antigen with adjuvants of oil and aluminum hydroxide enhances the expansion of antigen-specific CD4 T cells in the secondary lymphoid organs (SLOs), but not the BM (46). BM resting memory CD4 T cells are unaffected by persistence of antigen (46), while splenic Tfh cells, probably also memory Tfh cells, are sustained by persistence of antigen (47). Thus, the ratio of splenic Tfh cells and BM resting memory cells is markedly affected by antigen persistence. We suggest that the length of antigen persistence may define the quantitative balance of effector and resting memory, i.e., whether the CD4 T cells should contribute to the long-lasting reaction to exclude the persistent antigen or store their ability for recall response.

AUTHOR CONTRIBUTIONS

SH, JS, and KT designed the research; SH, JS, CM, MM, and DZ performed the research; AH, JZ, WP, SF, and ML contributed new reagents/analytic tools; and SH, ML, AR, and KT wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2016.00026

REFERENCES

1. Crotty S, Kersh EN, Cannons J, Schwartzberg PL, Ahmed R. SAP is required for generating long-term humoral immunity. *Nature* (2003) **421**:282–7. doi:10.1038/nature01318
2. Francus T, Francus Y, Siskind GW. Memory T cells enhance the expression of high-avidity naive B cells. *Cell Immunol* (1991) **134**:520–7. doi:10.1016/0008-8749(91)90323-4
3. Gershon RK, Paul WE. Effect of thymus-derived lymphocytes on amount and affinity of anti-hapten antibody. *J Immunol* (1971) **106**:872–4.
4. Janssen EM, Lemmens EE, Wolfe T, Christen U, von Herrath MG, Schoenberger SP. CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. *Nature* (2003) **421**:852–6. doi:10.1038/nature01441
5. Shedlock DJ, Shen H. Requirement for CD4 T cell help in generating functional CD8 T cell memory. *Science* (2003) **300**:337–9. doi:10.1126/science.1082305
6. Sun JC, Bevan MJ. Defective CD8 T cell memory following acute infection without CD4 T cell help. *Science* (2003) **300**:339–42. doi:10.1126/science.1083317
7. Sun JC, Williams MA, Bevan MJ. CD4+ T cells are required for the maintenance, not programming, of memory CD8+ T cells after acute infection. *Nat Immunol* (2004) **5**:927–33. doi:10.1038/ni1105
8. Boyman O, Letourneau S, Krieg C, Sprent J. Homeostatic proliferation and survival of naive and memory T cells. *Eur J Immunol* (2009) **39**:2088–94. doi:10.1002/eji.200939444
9. Kassiotis G, Garcia S, Simpson E, Stockinger B. Impairment of immunological memory in the absence of MHC despite survival of memory T cells. *Nat Immunol* (2002) **3**:244–50. doi:10.1038/ni766
10. Seddon B, Tomlinson P, Zamoyska R. Interleukin 7 and T cell receptor signals regulate homeostasis of CD4 memory cells. *Nat Immunol* (2003) **4**:680–6. doi:10.1038/ni946
11. Swain SL, Hu H, Huston G. Class II-independent generation of CD4 memory T cells from effectors. *Science* (1999) **286**:1381–3. doi:10.1126/science.286.5443.1381
12. Manz RA, Thiel A, Radbruch A. Lifetime of plasma cells in the bone marrow. *Nature* (1997) **388**:133–4. doi:10.1038/40540
13. Slifka MK, Antia R, Whitmire JK, Ahmed R. Humoral immunity due to long-lived plasma cells. *Immunity* (1998) **8**:363–72. doi:10.1016/S1074-7613(00)80541-5
14. Tokoyoda K, Zehentmeier S, Hegazy AN, Albrecht I, Grun JR, Lohning M, et al. Professional memory CD4+ T lymphocytes preferentially reside and rest in the bone marrow. *Immunity* (2009) **30**:721–30. doi:10.1016/j.immuni.2009.03.015
15. Hanazawa A, Hayashizaki K, Shinoda K, Yagita H, Okumura K, Lohning M, et al. CD49b-dependent establishment of T helper cell memory. *Immunol Cell Biol* (2013) **91**:524–31. doi:10.1038/icb.2013.36
16. Kassiotis G, Gray D, Kiafard Z, Zwirner J, Stockinger B. Functional specialization of memory Th cells revealed by expression of integrin CD49b. *J Immunol* (2006) **177**:968–75. doi:10.4049/jimmunol.177.2.968
17. Shinoda K, Tokoyoda K, Hanazawa A, Hayashizaki K, Zehentmeier S, Hosokawa H, et al. Type II membrane protein CD69 regulates the formation of resting T-helper memory. *Proc Natl Acad Sci U S A* (2012) **109**:7409–14. doi:10.1073/pnas.1118539109
18. Herndler-Brandstetter D, Landgraf K, Jenewein B, Tzankov A, Brunauer R, Brunner S, et al. Human bone marrow hosts polyfunctional memory CD4+ and CD8+ T cells with close contact to IL-15-producing cells. *J Immunol* (2011) **186**:6965–71. doi:10.4049/jimmunol.1100243
19. Okhrimenko A, Grun JR, Westendorf K, Fang Z, Reinke S, von Roth P, et al. Human memory T cells from the bone marrow are resting and maintain long-lasting systemic memory. *Proc Natl Acad Sci U S A* (2014) **111**:9229–34. doi:10.1073/pnas.1318731111
20. Yuseff MI, Pierobon P, Reversat A, Lennon-Dumenil AM. How B cells capture, process and present antigens: a crucial role for cell polarity. *Nat Rev Immunol* (2013) **13**:475–86. doi:10.1038/nri3469
21. Crotty S. T follicular helper cell differentiation, function, and roles in disease. *Immunity* (2014) **41**:529–42. doi:10.1016/j.immuni.2014.10.004
22. Linton PJ, Harbertson J, Bradley LM. A critical role for B cells in the development of memory CD4 cells. *J Immunol* (2000) **165**:5558–65. doi:10.4049/jimmunol.165.10.5558
23. Misumi I, Whitmire JK. B cell depletion curtails CD4+ T cell memory and reduces protection against disseminating virus infection. *J Immunol* (2014) **192**:1597–608. doi:10.4049/jimmunol.1302661
24. Mollo SB, Zajac AJ, Harrington LE. Temporal requirements for B cells in the establishment of CD4 T cell memory. *J Immunol* (2013) **191**:6052–9. doi:10.4049/jimmunol.1302033
25. van Essen D, Dullforce P, Brocker T, Gray D. Cellular interactions involved in Th cell memory. *J Immunol* (2000) **165**:3640–6. doi:10.4049/jimmunol.165.7.3640
26. Whitmire JK, Asano MS, Kaech SM, Sarkar S, Hannum LG, Shlomchik MJ, et al. Requirement of B cells for generating CD4+ T cell memory. *J Immunol* (2009) **182**:1868–76. doi:10.4049/jimmunol.0802501
27. Oxenius A, Bachmann MF, Zinkernagel RM, Hengartner H. Virus-specific MHC-class II-restricted TCR-transgenic mice: effects on humoral and cellular immune responses after viral infection. *Eur J Immunol* (1998) **28**:390–400. doi:10.1002/(SICI)1521-4141(199801)28:01<390::AID-IMMU390>3.3.CO;2-F
28. Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* (2000) **100**:655–69. doi:10.1016/S0092-8674(00)80702-3
29. Gu H, Zou YR, Rajewsky K. Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-loxP-mediated gene targeting. *Cell* (1993) **73**:1155–64. doi:10.1016/0092-8674(93)90644-6
30. Zhu J, Jankovic D, Oler AJ, Wei G, Sharma S, Hu G, et al. The transcription factor T-bet is induced by multiple pathways and prevents an endogenous Th2 cell program during Th1 cell responses. *Immunity* (2012) **37**:660–73. doi:10.1016/j.immuni.2012.09.007
31. Tsubata T, Murakami M, Honjo T. Antigen-receptor cross-linking induces peritoneal B-cell apoptosis in normal but not autoimmunity-prone mice. *Curr Biol* (1994) **4**:8–17. doi:10.1016/S0960-9822(00)00003-8
32. Hardy RR, Kincade PW, Dorshkind K. The protean nature of cells in the B lymphocyte lineage. *Immunity* (2007) **26**:703–14. doi:10.1016/j.immuni.2007.05.013
33. Tokoyoda K, Egawa T, Sugiyama T, Choi BI, Nagasawa T. Cellular niches controlling B lymphocyte behavior within bone marrow during development. *Immunity* (2004) **20**:707–18. doi:10.1016/j.immuni.2004.05.001
34. Manz RA, Arce S, Cassese G, Hauser AE, Hiepe F, Radbruch A. Humoral immunity and long-lived plasma cells. *Curr Opin Immunol* (2002) **14**:517–21. doi:10.1016/S0952-7915(02)00356-4

35. Hargreaves DC, Hyman PL, Lu TT, Ngo VN, Bidgol A, Suzuki G, et al. A coordinated change in chemokine responsiveness guides plasma cell movements. *J Exp Med* (2001) **194**:45–56. doi:10.1084/jem.194.1.45
36. Afkarian M, Sedy JR, Yang J, Jacobson NG, Cereb N, Yang SY, et al. T-bet is a STAT1-induced regulator of IL-12R expression in naive $CD4^+$ T cells. *Nat Immunol* (2002) **3**:549–57. doi:10.1038/ni794
37. Schulz EG, Mariani L, Radbruch A, Hofer T. Sequential polarization and imprinting of type 1 T helper lymphocytes by interferon-gamma and interleukin-12. *Immunity* (2009) **30**:673–83. doi:10.1016/j.immuni.2009.03.013
38. Sasaki K, Tsuji T, Jinushi T, Matsuzaki J, Sato T, Chamoto K, et al. Differential regulation of VLA-2 expression on Th1 and Th2 cells: a novel marker for the classification of Th subsets. *Int Immunol* (2003) **15**:701–10. doi:10.1093/intimm/dxg066
39. Lee JY, Skon CN, Lee YJ, Oh S, Taylor JJ, Malhotra D, et al. The transcription factor KLF2 restrains CD4(+) T follicular helper cell differentiation. *Immunity* (2015) **42**:252–64. doi:10.1016/j.immuni.2015.01.013
40. Weber JP, Fuhrmann F, Feist RK, Lahmann A, Al Baz MS, Gentz LJ, et al. ICOS maintains the T follicular helper cell phenotype by down-regulating Kruppel-like factor 2. *J Exp Med* (2015) **212**:217–33. doi:10.1084/jem.20141432
41. Parmar KM, Larman HB, Dai G, Zhang Y, Wang ET, Moorthy SN, et al. Integration of flow-dependent endothelial phenotypes by Kruppel-like factor 2. *J Clin Invest* (2006) **116**:49–58. doi:10.1172/JCI24787
42. Crotty S, Johnston RJ, Schoenberger SP. Effectors and memories: Bcl-6 and Blimp-1 in T and B lymphocyte differentiation. *Nat Immunol* (2010) **11**:114–20. doi:10.1038/ni.1837
43. Hale JS, Youngblood B, Latner DR, Mohammed AU, Ye L, Akondy RS, et al. Distinct memory CD4+ T cells with commitment to T follicular helper- and T helper 1-cell lineages are generated after acute viral infection. *Immunity* (2013) **38**:805–17. doi:10.1016/j.immuni.2013.02.020
44. Marshall HD, Chandele A, Jung YW, Meng H, Poholek AC, Parish IA, et al. Differential expression of Ly6C and T-bet distinguish effector and memory Th1 CD4(+) cell properties during viral infection. *Immunity* (2011) **35**:633–46. doi:10.1016/j.immuni.2011.08.016
45. Pepper M, Pagan AJ, Igyarto BZ, Taylor JJ, Jenkins MK. Opposing signals from the Bcl6 transcription factor and the interleukin-2 receptor generate T helper 1 central and effector memory cells. *Immunity* (2011) **35**:583–95. doi:10.1016/j.immuni.2011.09.009
46. Hanazawa A, Lohning M, Radbruch A, Tokoyoda K. CD49b/CD69-dependent generation of resting t helper cell memory. *Front Immunol* (2013) **4**:183. doi:10.3389/fimmu.2013.00183
47. Baumjohann D, Preite S, Reboldi A, Ronchi F, Ansel KM, Lanzavecchia A, et al. Persistent antigen and germinal center B cells sustain T follicular helper cell responses and phenotype. *Immunity* (2013) **38**:596–605. doi:10.1016/j.immuni.2012.11.020

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Marrow-Infiltrating Lymphocytes – Role in Biology and Cancer Therapy

Ivan Borrello and Kimberly A. Noonan*

Department of Oncology, Johns Hopkins University, Baltimore, MD, USA

The past several years have witnessed the acceptance of immunotherapy into the mainstream of therapies for patients with cancer. This has been driven by the clinical successes of antibodies to the checkpoint inhibitors, CTLA-4 and PD-1, capable of imparting long-term remissions in several solid tumors as well as Hodgkin's lymphoma (1) and the therapeutic successes of adoptive T-cell transfer with chimeric antigen receptors (2) or modified T-cell receptors (3) that have mostly utilized peripheral T-cells. One emerging area of therapeutic T cell intervention has been the utilization of marrow-infiltrating lymphocytes (MILs) – a novel form of adoptive T-cell therapy. This approach was initially developed to increase the likelihood of a precursor T-cell population with an enhanced tumor specificity in bone marrow (BM)-derived malignancies. However, the unique attributes of BM T-cells and their interaction with their microenvironment provide significant rationale to utilize these cells therapeutically in diseases that extend beyond hematologic malignancies.

Keywords: marrow-infiltrating lymphocytes, bone marrow, cancer immunotherapy

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Tania H. Watts,
University of Toronto, Canada

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Naoto Hirano,
University of Toronto, Canada
Jean-Sébastien Delisle,
Centre de recherche de l'Hôpital Maisonneuve-Rosemont, Canada

****Correspondence:***
Ivan Borrello
iborrell@jhmi.edu

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INTRODUCTION

Most adoptive T-cell therapy has utilized the circulating pool of peripheral blood lymphocytes (PBLs). This is likely due to the relative ease of obtaining these cells through large volume pheresis. An alternative approach pioneered by Dr. Rosenberg and colleagues at the National Cancer Institute has focused on the use of tumor infiltrating lymphocytes (TILs) in patients with metastatic melanoma (4). The rationale for TILs is that tumor-specific T-cells are more likely to be found in proximity of the tumor. This has proven to be true, and several clinical studies have demonstrated appreciable tumor specificity with measurable antitumor efficacy. However, limitations exist with the TIL approach. First, in contrast to PBLs, TILs are not present in all individuals, they require a surgical procedure for harvesting, and the expansion protocols are often both lengthy and costly.

In hematologic malignancies, MILs likely offer the same benefits of TILs. Being obtained from the tumor microenvironment in myeloma as well as several other hematologic malignancies, these cells demonstrate heightened tumor specificity upon activation and expansion (5). However, the unique immunologic properties of bone marrow (BM) impart essential properties that could possibly make them an even better source of T-cells for adoptive therapy approaches than their PBL counterparts.

RATIONALE FOR USE OF MILs IN ADOPTIVE T CELL THERAPY

Effective adoptive T-cell therapy requires T-cells to possess important attributes. At a minimum, they must (1) possess endogenous tumor specificity, (2) be capable of trafficking to the tumor site upon infusion, (3) kill the tumor upon encounter, and (4) persist over time. MILs possess these properties in large part because of the unique immune environment present within the BM.

T-cells compose only 3–8% of the population within the BM (6). However, the BM plays a critical role in priming naive T-cells (7), serving as a reservoir of antigen experienced CD8 memory T cells (8), and the site of homeostatic proliferation of both CD4 and CD8 T-cells (9, 10). While the BM lacks the organized structure of the lymph node or spleen, it does provide an environment that supports appropriate T-cell development in the absence of the thymus (11) and is capable of forming lymphoid follicles which increase with inflammatory, autoimmune, or infectious states (12). Despite this immune function, the BM lacks lymphatic vessels and is vascularized only by blood vessels. The absence of lymphatic vessels likely enables the massive lymphocyte recirculation that occurs daily. However, entrance of central memory T_{CM} cells into the BM is a more tightly regulated process mediated by an interaction between L-, P-, and E-selectins, whereas arrest within the BM requires the VCAM-1/α4β1 pathway (13).

Stromal-derived factor-1/CXCL12 is highly expressed in the BM, and its cognate receptor, CXCR4, is upregulated on memory T cells by IL-15 and increases adhesion of T_{CM} to the BM microvessels (13, 14). Furthermore, memory T-cells have higher expression of CXCR4 compared to naive T-cells as its expression appears to be affected by the presence of antigen and various cytokines (14). In myeloma, we have confirmed the expression of CXCR4 on MILs, whereas no detectable expression was appreciated on PBLs from the same patients (**Figure 1**) (5). Of note, expression was even greater following activation with anti-CD3/CD28 beads. In hematologic malignancies, this expression may provide important advantages to MILs, such as maximizing trafficking of the T-cells to the tumor microenvironment and thus further increase their therapeutic benefit in adoptive T-cell therapy. In addition to the possibility that CXCR4 expression on MILs can increase their trafficking to the BM, its increased expression on memory T-cells also provides another justification for the use of MILs in adoptive T-cell therapy. Specifically, CXCR4 is also involved in promotion of CD8 T_{CM} homeostatic proliferation and maintenance (15). CD8 T_{CM} imparts long-term memory and are essential to maximizing the overall efficacy of adoptive T-cell therapy (16). The combined increased expression of CXCR4 on memory T-cells, the increased trafficking of these T-cells in response to CXCL12 to the BM, and the subsequent enrichment of memory T cells within the BM all underscore the unique attributes of BM resident T-cells that make them an ideal source for adoptive T-cell therapeutic approaches. Trafficking to the tumor site, BM, and persistence over time are two important properties for T-cells in the treatment of hematologic malignancies.

ROLE OF BM T CELLS IN DISEASE REGULATION AND SYSTEMIC IMMUNITY

It is logical to assume that T-cells within a certain microenvironment most likely possess an enhanced antigenic specificity for the cells residing within that organ. This is certainly the rationale behind the therapeutic use of TILs for the treatment of metastatic melanoma and other diseases, in which they have been employed and was the primary assumption used in the clinical development of MILs in multiple myeloma by our group. However, as outlined above, the BM represents a unique organ with several important immune attributes, which can be employed for therapeutic purposes but can also contribute to the pathology of disease.

Hematologic Malignancies

The BM has been described as a reservoir for Tregs (17). Interestingly, this observation was made in healthy hosts, and our findings in myeloma demonstrated the opposite – more Tregs present in the peripheral blood than BM (18). This paradox was ultimately resolved by our demonstration that the large amounts of IL-6 produced by the myelomatous plasma cells in combination with TGF-β (as well as IL-1β and IL-21) were responsible for the skewing of CD4 away from a Treg phenotype toward a Th17 phenotype that was responsible for the activation of osteoclasts and the development of lytic bone lesions (18) as well as

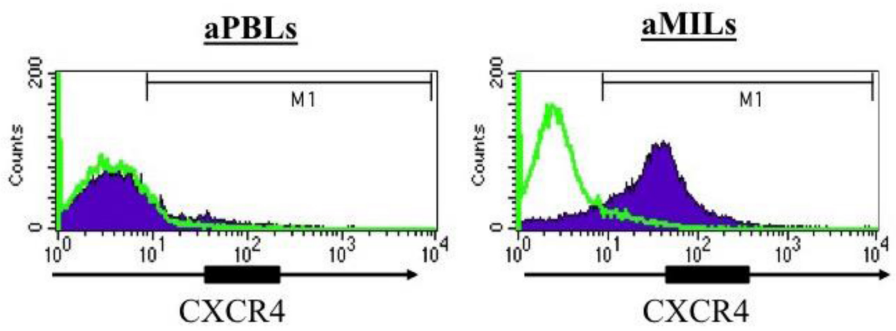

FIGURE 1 | CXCR4 expression in activated PBLs compared to activated MILs. The T cells were activated with anti-CD3/CD28 beads, and CXCR4 expression was then assessed by flow cytometry.

the promotion of myeloma cell growth (19). Underscoring the importance of the immune composition of the BM, it was the extent of the Th17 phenotype of the BM (but not blood) T-cells and not the myeloma cell concentration that correlated to the extent of bone disease (18). Interestingly, activation of MILs with anti-CD3/CD28 resulted in a significant skewing toward a Th1 phenotype and the addition of activated MILs to an osteoclast differentiation assay significantly reduced the outgrowth of osteoclasts (18). Taken together, these data not only provide an explanation for the immune-mediated contribution to lytic bone disease in myeloma but also suggest that an adoptive T cell approach, such as the utilization of Th1-skewed-activated MILs, could possibly also exert a benefit in terms of reducing bone disease.

Viral Responses

The role of the BM in serving as a reservoir of antigen-experienced T-cells is likely also mediated by the ability of the BM to effectively present antigen. In fact, it has been demonstrated that differentiated dendritic cells (DCs) traffic and home to the BM where they then form stable antigen-dependent interactions with T_{CM} (20). This interaction is likely one of the key mediators responsible for the maintenance of central memory in the BM. It is this attribute that also enables the BM to enrich for viral-specific memory T-cells. In a murine LCMV model, it was shown that no significant differences existed between the precursor frequency of LCMV-specific T-cells in the spleen and BM during the acute infection. Furthermore, long-term immunity was found in the BM and the spleen, and the adoptive transfer of BM from LCMV-infected mice was able to effectively protect against viral infection (21). Chronic viral infections, such as EBV, also demonstrate an increase in BM-specific T-cells to lytic antigens compared to the blood, which was independent of viral load (22). More importantly, that study showed a unique expression of homing receptors present on T cells obtained from the BM and not observed on T cells from other compartments.

Solid Tumors

In contrast to the two abovementioned conditions, in solid tumors (especially in the non-metastatic setting), it is unlikely that the BM will contain clinically detectable amounts of tumor. In breast cancer, tumor-specific T-cells have been identified in the BM to a greater extent than in the blood even in conditions in which tumor could not be detected by nested PCR (23). Similar results were also observed in melanoma and pancreatic cancer; although in the former, BM T-cell recognition was more commonly seen in patients with metastatic disease (24, 25). Based on these results, studies are currently ongoing examining the ability to utilize MILs as a therapeutic source of T cells in various solid tumors.

THERAPEUTIC ROLE OF MILs

The abovementioned attributes underscore the uniqueness of the MILs as more than simply the TILs of hematologic malignancies. In fact, the demonstration by various groups that antigen-specific cells can be found even in the absence of known tumor or viral involvement of the BM provides the rationale for their therapeutic use in those disease settings as well.

Multiple Myeloma

Marrow-infiltrating lymphocytes do appear to possess many of the essential features that make them the ideal for adoptive T cell therapy and more specifically, for hematologic malignancies. They have an endogenous antigenic specificity that is broad and targets antigens present on both the mature myelomatous plasma cells as well as their clonogenic precursors. In NOD/SCID models, MILs have shown the ability to traffic to the BM, to persist with an activated phenotype, and to exert measurable antitumor activity (**Figure 2**). Our clinical data demonstrate a direct correlation between the ability to achieve a complete remission (CR), following the adoptive transfer of activated MILs and the presence of significant tumor-specific immunity to myeloma cell lysate (26). Studies to specifically determine the specific antigenic recognition are ongoing. However, the antigen-specific immunity appears to be both CD4- and CD8 mediated.

These initial studies have led to the development of clinical trials for myeloma in the autologous transplant setting. This involved the bedside harvest of 200 ml of BM, which was well tolerated with conscious sedation and from which MILs were then expanded in all patients. Of note, MILs were not isolated prior to activation but rather were expanded within the BM microenvironment because the presence of antigen during the T-cell expansion was shown to be critical to maintaining the tumor specificity of the MILs while subjected to the potent polyclonal expansion of CD3/CD28 stimulation. However, underscoring the unique attribute of MILs, we have demonstrated that it is more than the presence of antigen during the expansion that is responsible for the antigenic specificity as PBLs expanded within the BM environment showed only a slight increase in the tumor specificity but significantly less could be achieved with MILs (5). In our clinical study, we observed an increase in the tumor specificity of the MILs in all patients. However, the magnitude of this specificity varied significantly post-expansion. Interestingly, a direct correlation existed between the extent of the tumor specificity obtained *ex vivo* and the clinical outcomes of the patients – higher tumor specificity and increased production of both IL-2 and IFNγ of the *ex vivo* product was associated with CRs (26). Several properties of MILs at baseline also seemed to correlate to clinical outcomes. Specifically, patients whose pre-expansion MILs had significant percentage of CD8 T_{CM} and low spontaneous IFNγ production were more likely to achieve a CR to therapy. The demonstration of a direct correlation between the immune parameters of the T-cell product and clinical outcomes speaks to the endogenous tumor specificity of MILs.

Marrow-infiltrating lymphocytes were successfully administered without the development of a cytokine release syndrome or significant lymphocytosis. This differs significantly from the infusion of activated PBL which, even when unmodified, demonstrated a significant lymphocytosis *in vivo*. The absence

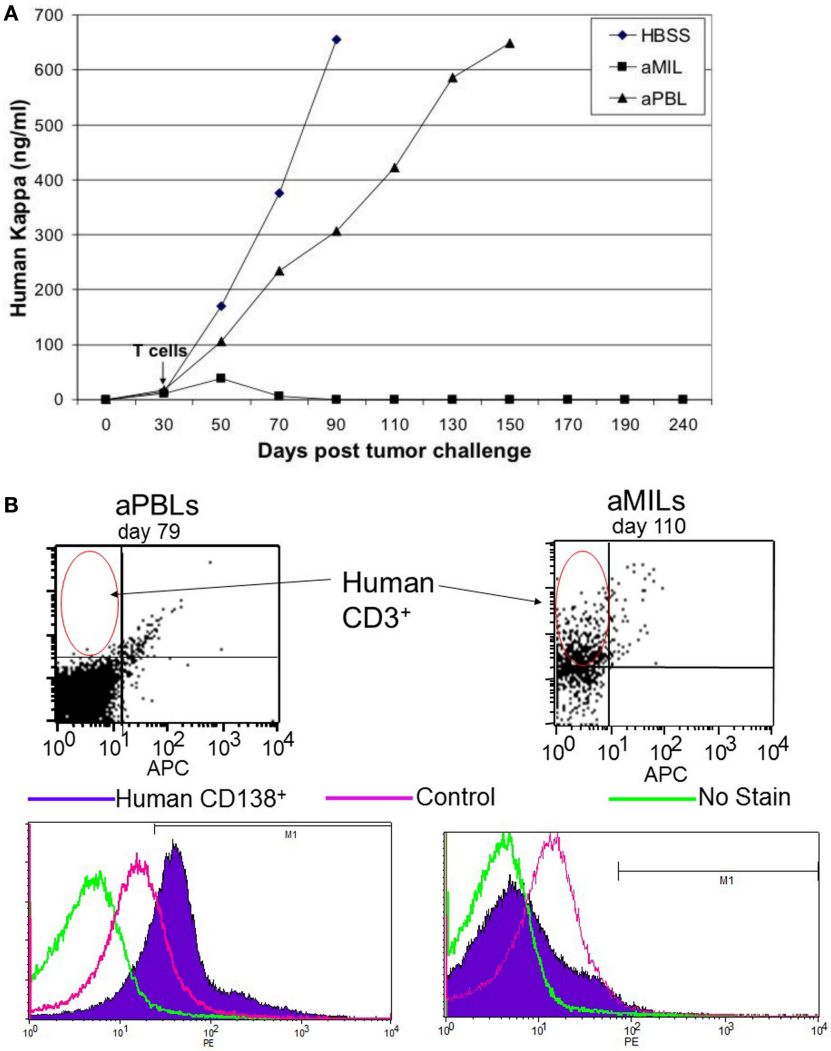

FIGURE 2 | **Activated MILs exert a potent antitumor MILs**. **(A)** NOD/SCID mice (10 mice per group) were challenged with the H929 myeloma cell line and followed until a detectable kappa light chain was present in the blood consistent with engraftment. The mice were challenged with either activated PBLs or activated MILs and followed for overall survival. **(B)** To determine the ability of these T cells to traffic and exert antimyeloma activity, the mice were sacrificed at the indicated time points. When mice that received aPBL died or conversely a mouse that received aMILs was sacked on D110 to analyze the BM was stained for either human CD3 cells or CD138+ myeloma. As shown, the mice that received MILs showed significant T cell trafficking to the BM and no detectable myeloma.

of these effects, following the infusion of MILs, could be related to the infusion of a cell dose that was below the threshold for expansion or could reflect a different trafficking pattern of MILs. However, we did observe the development of a rash consistent with an acute autologous graft-vs.-host disease in roughly 20% of patients. This was mostly limited to the skin and resolved spontaneously. No long-term sequelae were evident in patients who received MILs.

A major objective of these studies was to determine the extent of immune responsiveness within the tumor microenvironment. For this reason, the immune monitoring was only performed on BM samples obtained at time points pre- and posttransplant. In addition to the direct correlation observed between the *ex vivo* expanded product and clinical outcomes, the MILs obtained from the patients up to 1 year posttransplant also demonstrated a direct correlation between the tumor specificity and the clinical response to therapy. MILs with the greatest antimyeloma specificity were observed in patients who achieved a CR. The CR patients also demonstrated an increase in CD8 cytotoxic activity as measured by both granzyme B and perforin production, an increase in IFNγ production, and a decrease in IL-6 production. One obstacle for many non-gene-modified T-cell studies has been the absence of persistence of *ex vivo* activated T-cells. We attempted to determine this in our study by vaccinating our patients the polyvalent pneumococcal vaccine (PCV) prior to harvesting the MILs and then reinfusing the MILs into a host

with a T-cell-depleted graft where the MILs constituted more than 95% of the adoptively transferred T cells. As such, it is fair to assume that the vast majority of PCV-specific MILs were those adoptive transferred. Our ability to detect PCV-specific MILs up to 1 year posttransplant, especially in patients who achieved a CR strongly, suggests their persistence.

Ongoing clinical studies are addressing approaches aimed at increasing the efficacy of MILs adoptive cell therapy. These have included the addition of a cellular allogeneic myeloma vaccine (myeloma-GVAX) as well as the use of the lenalidomide following immune reconstitution. The former attempts to increase the precursor frequency of myeloma-specific MILs, and the latter attempts to maintain the activation state of the MILs.

Post-Allogeneic Transplant MILs

A major unmet need in allogeneic stem cell transplantation remains the difficulty of dealing with relapse disease following the transplant. The current standard approach is the use of donor lymphocyte infusions (DLI), which has a limited efficacy but is associated with a significant incidence of graft-vs.-host disease (GVHD) that can be in excess of 50% (27). The recent development at our center are allogeneic transplants with the use of posttransplant cyclophosphamide (PTCy), which has significantly reduced the incidence of GVHD and has enabled transplants to be performed across HLA barriers without significant increases in the morbidity or mortality compared to HLA-identical transplants (28). Because of the PTCy elimination of alloreactive T-cells, we hypothesized that MILs obtained from these patients posttransplant could be utilized therapeutically as a modified version of DLI with a better toxicity profile and heightened tumor specificity. The rationale is that the PTCy would have eliminated the alloreactive T-cells and the BM would possess the donor-derived tumor-specific T-cells. As such, we should be able to obtain MILs from these patients in the post-allogeneic transplant setting, activate, and expand these cells. The expectation is that the MILs would have heightened tumor specificity, effectively traffic to the BM, eradicate existing disease, and cause minimal, if any, GVHD. In preclinical experiments, the tumor specificity of MILs, following anti-CD3/CD28 activation, was observed in all patients examined, and one patient in whom a tetramer existed for the HLA-A2$^+$ PR-1 peptide, the precursor frequency of MILs went from 1 to 17.8% following expansion (unpublished data). Considering the polyclonal expansion achieved with anti-CD3/CD28 stimulation, the theoretical ability to increase the antigenic frequency to numerous antigens by this percentage with minimal associated toxicity offers significant promise as an approach that should significantly improve the therapeutic outcomes for patients relapsing following an allogeneic BMT.

SOLID TUMORS

The positive prognostic value in terms of overall survival of TILs in many solid tumors points to a clear role of the immune system in regulating cancer growth (29–31). This has been further evidenced by the clinical benefits observed in many metastatic melanoma patients treated with TILs (32). However, a major limitation to such an approach is that not all patients have TILs that can be harvested, and the harvesting is a surgical procedure with all its limitations.

The presence of tumor-specific T-cells in the BM of patients with solid tumors opens up the prospect of utilizing MILs for therapeutic adoptive T-cell therapy in this setting, despite the presence of tumor within the BM. This approach has been examined in patients with metastatic breast cancer (33). In that study, 16 HLA-A2$^+$ patients had MILs cocultured with autologous DCs and an allogeneic breast cancer cell line, MCF7. An average of 2×10^7 T-cells were infused without a lymphoablative preparative regimen. There was a direct correlation between the number of tumor-specific infused T-cells, the presence of a memory phenotype, and the absence of a Th2 phenotype with the overall response. Interestingly, patients with evidence of tumor-specific immunity in PBL showed a significantly longer median survival compared to non-responders (58.6 vs. 13.6 months; $p = 0.009$) (34).

CHIMERIC ANTIGEN RECEPTOR-MODIFIED T CELLS

Chimeric antigen receptor (CAR) T-cells have demonstrated the ability to eradicate significant tumor burdens in patients with chronic lymphocytic leukemia (35) and acute lymphoblastic leukemia (36, 37). While the response rates are impressively around 70–90% in ALL with a 6-month event-free survival of 67%, the overall mechanisms of relapse are not completely understood. The development of antigen-escape variants are clearly observed in a fraction of the patients (38). One approach being developed to overcome this problem is to utilize an additional CAR, which in the case of ALL has been developed to target CD22 (39). An alternative approach could be to utilize a cell with an intrinsically broader antigenic specificity than that found on a PBL. MILs would appear to possess such properties and as such could potentially serve as a better source of T-cells for CAR-based adoptive T-cell therapy.

CONCLUSION

The BM represents a unique immunologic environment that serves both as a reservoir of memory T-cells and can effectively prime naive T-cells. This is, in large part, due to the efficient processing and presentation of antigens by resident APCs. The major benefit of this biology is that MILs possess many essential properties that make them ideal for adoptive T-cell therapy: they are enriched for T_{CM}, they possess a broad antigenic specificity, effectively traffic to the BM upon reinfusion, and are capable of significant cytotoxicity. Furthermore, the presence of antigen-specific T-cells in the BM in patients with certain viral infections, as well as solid tumors, potentially provides the rationale to utilize MILs for adoptive T-cell therapy.

AUTHOR CONTRIBUTIONS

IB and KN contributed to the design of experiments, analysis, and writing of the manuscript.

REFERENCES

1. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer* (2012) **12**(4):252–64. doi:10.1038/nrc3239
2. Sadelain M. CAR therapy: the CD19 paradigm. *J Clin Invest* (2015) **125**(9):3392–400. doi:10.1172/JCI80010
3. Bonini C, Mondino A. Adoptive T-cell therapy for cancer: the era of engineered T cells. *Eur J Immunol* (2015) **45**(9):2457–69. doi:10.1002/eji.201545552
4. Dudley ME, Wunderlich JR, Robbins PF, Yang JC, Hwu P, Schwartzentruber DJ, et al. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science* (2002) **298**(5594):850–4. doi:10.1126/science.1076514
5. Noonan K, Matsui W, Serafini P, Carbley R, Tan G, Khalili J, et al. Activated marrow-infiltrating lymphocytes effectively target plasma cells and their clonogenic precursors. *Cancer Res* (2005) **65**(5):2026–34. doi:10.1158/0008-5472.CAN-04-3337
6. Schirrmacher V, Feuerer M, Fournier P, Ahlert T, Umansky V, Beckhove P. T-cell priming in bone marrow: the potential for long-lasting protective anti-tumor immunity. *Trends Mol Med* (2003) **9**(12):526–34. doi:10.1016/j.molmed.2003.10.001
7. Feuerer M, Beckhove P, Garbi N, Mahnke Y, Limmer A, Hommel M, et al. Bone marrow as a priming site for T-cell responses to blood-borne antigen. *Nat Med* (2003) **9**(9):1151–7. doi:10.1038/nm914
8. Feuerer M, Beckhove P, Bai L, Solomayer EF, Bastert G, Diel IJ, et al. Therapy of human tumors in NOD/SCID mice with patient-derived reactivated memory T cells from bone marrow. *Nat Med* (2001) **7**(4):452–8. doi:10.1038/86523
9. Di Rosa F, Santoni A. Bone marrow CD8 T cells are in a different activation state than those in lymphoid periphery. *Eur J Immunol* (2002) **32**(7):1873–80. doi:10.1002/1521-4141(200207)32:7<1873::AID-IMMU1873>3.0.CO;2-P
10. Parretta E, Cassese G, Santoni A, Guardiola J, Vecchio A, Di Rosa F. Kinetics of in vivo proliferation and death of memory and naive CD8 T cells: parameter estimation based on 5-bromo-2'-deoxyuridine incorporation in spleen, lymph nodes, and bone marrow. *J Immunol* (2008) **180**(11):7230–9. doi:10.4049/jimmunol.180.11.7230
11. Dejbakhsh-Jones S, Jerabek L, Weissman IL, Strober S. Extrathymic maturation of alpha beta T cells from hemopoietic stem cells. *J Immunol* (1995) **155**(7):3338–44.
12. Zhao E, Xu H, Wang L, Kryczek I, Wu K, Hu Y, et al. Bone marrow and the control of immunity. *Cell Mol Immunol* (2012) **9**(1):11–9. doi:10.1038/cmi.2011.47
13. Mazo IB, Honczarenko M, Leung H, Cavanagh LL, Bonasio R, Weninger W, et al. Bone marrow is a major reservoir and site of recruitment for central memory CD8+ T cells. *Immunity* (2005) **22**(2):259–70. doi:10.1016/j.immuni.2005.01.008
14. Jourdan P, Vendrell JP, Huguet MF, Segondy M, Bousquet J, Pene J, et al. Cytokines and cell surface molecules independently induce CXCR4 expression on CD4+ CCR7+ human memory T cells. *J Immunol* (2000) **165**(2):716–24. doi:10.4049/jimmunol.165.2.716
15. Chaix J, Nish SA, Lin WH, Rothman NJ, Ding L, Wherry EJ, et al. Cutting edge: CXCR4 is critical for CD8+ memory T cell homeostatic self-renewal but not rechallenge self-renewal. *J Immunol* (2014) **193**(3):1013–6. doi:10.4049/jimmunol.1400488
16. Berger C, Jensen MC, Lansdorp PM, Gough M, Elliott C, Riddell SR. Adoptive transfer of effector CD8+ T cells derived from central memory cells establishes persistent T cell memory in primates. *J Clin Invest* (2008) **118**(1):294–305. doi:10.1172/JCI32103
17. Zou L, Barnett B, Safah H, Larussa VF, Evdemon-Hogan M, Mottram P, et al. Bone marrow is a reservoir for CD4+CD25+ regulatory T cells that traffic through CXCL12/CXCR4 signals. *Cancer Res* (2004) **64**(22):8451–5. doi:10.1158/0008-5472.CAN-04-1987
18. Noonan K, Marchionni L, Anderson J, Pardoll D, Roodman GD, Borrello I. A novel role of IL-17-producing lymphocytes in mediating lytic bone disease in multiple myeloma. *Blood* (2010) **116**(18):3554–63. doi:10.1182/blood-2010-05-283895
19. Prabhala RH, Pelluru D, Fulciniti M, Prabhala HK, Nanjappa P, Song W, et al. Elevated IL-17 produced by TH17 cells promotes myeloma cell growth and inhibits immune function in multiple myeloma. *Blood* (2010) **115**(26):5385–92. doi:10.1182/blood-2009-10-246660
20. Cavanagh LL, Bonasio R, Mazo IB, Halin C, Cheng G, van der Velden AW, et al. Activation of bone marrow-resident memory T cells by circulating, antigen-bearing dendritic cells. *Nat Immunol* (2005) **6**(10):1029–37. doi:10.1038/ni1249
21. Slifka MK, Whitmire JK, Ahmed R. Bone marrow contains virus-specific cytotoxic T lymphocytes. *Blood* (1997) **90**(5):2103–8.
22. Palendira U, Chinn R, Raza W, Piper K, Pratt G, Machado L, et al. Selective accumulation of virus-specific CD8+ T cells with unique homing phenotype within the human bone marrow. *Blood* (2008) **112**(8):3293–302. doi:10.1182/blood-2008-02-138040
23. Feuerer M, Rocha M, Bai L, Umansky V, Solomayer EF, Bastert G, et al. Enrichment of memory T cells and other profound immunological changes in the bone marrow from untreated breast cancer patients. *Int J Cancer* (2001) **92**(1):96–105. doi:10.1002/1097-0215(200102)9999:9999<::AID-IJC1152>3.0.CO;2-Q
24. Muller-Berghaus J, Ehlert K, Ugurel S, Umansky V, Bucur M, Schirrmacher V, et al. Melanoma-reactive T cells in the bone marrow of melanoma patients: association with disease stage and disease duration. *Cancer Res* (2006) **66**(12):5997–6001. doi:10.1158/0008-5472.CAN-04-0484
25. Schmitz-Winnenthal FH, Volk C, Z'Graggen K, Galindo L, Nummer D, Ziouta Y, et al. High frequencies of functional tumor-reactive T cells in bone marrow and blood of pancreatic cancer patients. *Cancer Res* (2005) **65**(21):10079–87. doi:10.1158/0008-5472.CAN-05-1098
26. Noonan KA, Huff CA, Davis J, Lemas MV, Fiorino S, Bitzan J, et al. Adoptive transfer of activated marrow-infiltrating lymphocytes induces measurable antitumor immunity in the bone marrow in multiple myeloma. *Sci Transl Med* (2015) **7**(288):288ra78. doi:10.1126/scitranslmed.aaa7014
27. Luznik L, Fuchs EJ. Donor lymphocyte infusions to treat hematologic malignancies in relapse after allogeneic blood or marrow transplantation. *Cancer Control* (2002) **9**(2):123–37.
28. Luznik L, Jones RJ, Fuchs EJ. High-dose cyclophosphamide for graft-versus-host disease prevention. *Curr Opin Hematol* (2010) **17**(6):493–9. doi:10.1097/MOH.0b013e32833eaf1b
29. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* (2006) **313**(5795):1960–4. doi:10.1126/science.1129139
30. Zhang L, Yang N, Conejo-Garcia JR, Katsaros D, Mohamed-Hadley A, Fracchioli S, et al. Expression of endocrine gland-derived vascular endothelial growth factor in ovarian carcinoma. *Clin Cancer Res* (2003) **9**(1):264–72.
31. Salgado R, Denkert C, Campbell C, Savas P, Nucifero P, Aura C, et al. Tumor-infiltrating lymphocytes and associations with pathological complete response and event-free survival in HER2-positive early-stage breast cancer treated with lapatinib and trastuzumab: a secondary analysis of the NeoALTTO Trial. *JAMA Oncol* (2015) **1**(4):448–54. doi:10.1001/jamaoncol.2015.0830
32. Hinrichs CS, Rosenberg SA. Exploiting the curative potential of adoptive T-cell therapy for cancer. *Immunol Rev* (2014) **257**(1):56–71. doi:10.1111/imr.12132
33. Schuetz F, Ehlert K, Ge Y, Schneeweiss A, Rom J, Inzkirweli N, et al. Treatment of advanced metastasized breast cancer with bone marrow-derived tumour-reactive memory T cells: a pilot clinical study. *Cancer Immunol Immunother* (2009) **58**(6):887–900. doi:10.1007/s00262-008-0605-3
34. Domschke C, Ge Y, Bernhardt I, Schott S, Keim S, Juenger S, et al. Long-term survival after adoptive bone marrow T cell therapy of advanced metastasized breast cancer: follow-up analysis of a clinical pilot trial. *Cancer Immunol Immunother* (2013) **62**(6):1053–60. doi:10.1007/s00262-013-1414-x
35. Porter DL, Levine BL, Kalos M, Bagg A, June CH. Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. *N Engl J Med* (2011) **365**(8):725–33. doi:10.1056/NEJMoa1103849
36. Maude SL, Frey N, Shaw PA, Aplenc R, Barrett DM, Bunin NJ, et al. Chimeric antigen receptor T cells for sustained remissions in leukemia. *N Engl J Med* (2014) **371**(16):1507–17. doi:10.1056/NEJMoa1407222

37. Davila ML, Riviere I, Wang X, Bartido S, Park J, Curran K, et al. Efficacy and toxicity management of 19-28z CAR T cell therapy in B cell acute lymphoblastic leukemia. *Sci Transl Med* (2014) **6**(224):224ra25. doi:10.1126/scitranslmed.3008226
38. Maude SL, Teachey DT, Porter DL, Grupp SA. CD19-targeted chimeric antigen receptor T-cell therapy for acute lymphoblastic leukemia. *Blood* (2015) **125**(26):4017–23. doi:10.1182/blood-2014-12-580068
39. Haso W, Lee DW, Shah NN, Stetler-Stevenson M, Yuan CM, Pastan IH, et al. Anti-CD22-chimeric antigen receptors targeting B-cell precursor acute lymphoblastic leukemia. *Blood* (2013) **121**(7):1165–74. doi:10.1182/blood-2012-06-438002

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Bone Marrow GvHD after Allogeneic Hematopoietic Stem Cell Transplantation

*Martin Szyska[1] and Il-Kang Na[1,2]**

[1] Experimental and Clinical Research Center (ECRC), Berlin, Germany, [2] Department of Hematology, Oncology and Tumor Immunology, Charité – Universitätsmedizin Berlin, Berlin, Germany

The bone marrow is the origin of all hematopoietic lineages and an important homing site for memory cells of the adaptive immune system. It has recently emerged as a graft-versus-host disease (GvHD) target organ after allogeneic stem cell transplantation (alloHSCT), marked by depletion of both hematopoietic progenitors and niche-forming cells. Serious effects on the restoration of hematopoietic function and immunological memory are common, especially in patients after myeloablative conditioning therapy. Cytopenia and durable immunodeficiency caused by the depletion of hematopoietic progenitors and destruction of bone marrow niches negatively influence the outcome of alloHSCT. The complex balance between immunosuppressive and cell-depleting treatments, GvHD and immune reconstitution, as well as the desirable graft-versus-tumor (GvT) effect remains a great challenge for clinicians.

Keywords: GvHD, bone marrow, alloHSCT, memory niche, minor antigen, GvL, GvT, bone marrow stroma

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Tania H. Watts,
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Chiara Bonini,
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***Correspondence:**
Il-Kang Na
il-kang.na@charite.de

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For many decades, allogeneic stem cell transplantation (alloHSCT) has been used for the treatment of hematological malignancies. Alloreactive T cells contained within the donor bone marrow preparations have eventually been recognized as both causative for the life-threatening graft-versus-host disease (GvHD) and the beneficial graft-versus-tumor (GvT) effect (1, 2). Great scientific effort has since been put into further delineating the impact of T cell subpopulations and associated effector functions on GvHD development in order to segregate GvHD from GvT but no clinically feasible solution to this apparent dilemma has yet emerged.

Skin, liver, and intestine are regarded as the principal target organs of GvHD that can be affected to varying degrees or not at all. Individual outcomes of alloHSCT are hardly predictable because the complex interplay of multiple factors is just starting to be understood. GvHD is commonly correlated with long-term cytopenic conditions, resulting in mortality due to infections and bleeding complications (3). Besides toxicity resulting from conditioning treatment, alloreactivity in the bone marrow has been deemed responsible for the observed defects in hematopoiesis. Recent studies conclusively demonstrated niche-forming cells in the bone marrow as targets of GvHD (4, 5).

Here, we address the molecular and cellular causes of GvHD in general and focus next on the sequence of events leading to hematopoietic failure and immunodeficiency as a consequence of alloreactivity in the bone.

SOURCE OF ALLOREACTIVITY

In both GvHD and GvT, donor T cells react against host cells expressing alloantigens. In major histocompatibility complex (MHC)-mismatched alloHSCT, a large fraction of donor T cells targets monomorphic host proteins presented as peptides in the context of recipient MHC molecules.

In donors, developing T cells are negatively selected exclusively against proteins presented as peptides bound to self-MHC molecules. Therefore, a large fraction of these T cells express T cell receptors with high affinity for host MHC molecules or the presented peptides in their context (6). Due to the comparably large number of reactive T cell clones (7), the ensuing GvHD response in MHC-mismatched settings is usually very severe and can be difficult to control despite the application of intensive immunosuppressive treatments.

In MHC-matched transplant settings, donor T cells target minor histocompatibility antigens (MiHAs), polymorphic genes presented *via* MHC molecules as processed peptides. Negative selection against these antigens is absent in the donor thymus due to lack of expression. Therefore, T cell receptors with high affinity to recipient MiHAs exist in small frequencies within the donor T cell repertoire. Respective T cell clones can become activated in an inflammatory environment as caused by pretransplant regimens and may trigger GvHD or react against MiHA-expressing tumor tissue. Whereas in MHC-mismatched alloHSCT, alloantigens are exclusively presented by host antigen-presenting cells (APCs), in an MHC-matched setting they can additionally be of donor origin due to cross presentation after uptake of host cell fragments (8, 9). Although only a fraction of polymorphic genes can be presented as peptide in a given MHC combination and single MiHA differences are not regarded as sufficient for the induction of GvHD in clinical settings, novel tools such as global genome association studies and *in silico* prediction have been widely used to identify an ever-growing set of clinically relevant MiHAs among thousands of polymorphic genes (10, 11), explaining the high incidence of GvHD even in MHC-matched transplantations.

Alloreactive donor T cells exert their effector function *via* both soluble and cell-contact-dependent cytotoxic factors. Upon activation by APCs, mainly CD4 T cells produce Th1-type cytokines, including interferon-γ (IFN-γ), tumor-necrosis factor-α (TNF-α), and interleukin-1 (IL-1). These soluble factors are systemically transported through the blood to GvHD target organs and locally act by rendering various cell types more susceptible to the ensuing alloreactive T cell response.

Antigen-specific target cell killing is principally mediated by the perforin–granzyme pathway and Fas–Fas ligand (FasL) interaction, both of which are employed by both CD4 and CD8 cytotoxic T lymphocytes (CTLs). Upon binding to their cognate antigen, CTLs can secrete perforin and granzyme, which in combination leads to lysis and rapid apoptosis of target cells. In an inflammatory context, Fas can be upregulated on target cells making them susceptible for cytotoxic killing by FasL-expressing T cells (12).

Due to broad functional overlap, complex differential expression of Fas in various organs under different pretreatment conditioning and incompatible GvHD models used in respective studies, the individual impact of FasL and perforin–granzyme pathways from CD4 and CD8 effectors is still under discussion (13). However, the FasL–Fas pathway appears to be more associated with the establishment of donor chimerism and GvHD severity and more important for CD4 effector function, whereas GvT is suggested to be more attributable to perforin–granzyme cytotoxicity without any salient T cell subset preference (14–16).

Ultimately, even a complete lack of both cytolytic pathways does not abolish GvHD, clearly demonstrating the existence of additional cytotoxic effectors in the T cell arsenal (17). TNF-related apoptosis-inducing ligand (TRAIL) and TNF-like weak inducer of apoptosis (TWEAK) are both expressed by T cells and have been assumed to partially compensate under these conditions by signaling through their cognate receptors death receptor 4 (DR4) or DR5 and TWEAK receptor (CD266), respectively (18).

GvHD AND IMMUNE RECONSTITUTION

In order to facilitate bone marrow engraftment and to diminish tumor burden, patients are treated with conditioning prior to alloHSCT, leading to tissue damage and immunosuppression. Whereas acute GvHD (aGvHD) occurs in the early phase after alloHSCT or immediately after termination of immunosuppressive regimen, chronic GvHD (cGvHD) emerges later, is clinically less defined and shares many characteristics with autoimmune diseases, including *de novo* generation of autoreactive T cell clones and the development of autoantibody titers (19, 20).

In affected patients, harsh treatment with immunosuppressive drugs can control the effects of GvHD, though at the cost of delayed immune reconstitution and mitigation of desired GvT effects. Even without immunosuppression, outcomes of sublethal GvHD include generalized cytopenia and a dramatically delayed immune reconstitution of all lymphoid lineages (21). Consequently, lethal GvHD and opportunistic infections are responsible for high mortality in relapse-free patients within the first year after alloHSCT (22, 23).

The discovery of strong alloreactivity against thymic tissues has suggested a link between aGvHD and impaired T cell reconstitution after alloHSCT (24). Allogeneic T cells targeting host T cells and also the thymic architecture can easily be understood as causative for T cell deficiencies with additional implications for B cell immunity, since both B cell effector function and memory formation largely depend on interaction with CD4 T cells (25). Thymic GvHD also adds a new layer of understanding on the frequently observed autoimmune traits of ensuing cGvHD. The thymus is the principal organ of T lymphocyte development, in charge of generating an extremely diverse set of T cell clones, while eliminating autoreactive clones (26). Arguably, the stringent and finely tuned T cell selection process in the thymus can be unhinged by destruction of self-APCs, which could allow potentially autoreactive clones to escape into the periphery and to cause autoimmune symptoms.

Using more refined mouse models of both MHC-matched and mismatched HSCT, the bone marrow has been established as an additional target of GvHD. In these studies, GvHD generally affected hematopoiesis and lymphoid development (27). However, serial bone marrow transfer experiments strongly suggest that GvHD progression depends on the targeting of non-hematopoietic cells of the bone marrow (5). Infiltrating T cells of donor origin were clearly associated with both impaired hematopoiesis and destruction of specialized niche-forming

cells, including osteoblasts and sinusoidal vascular endothelial cells (28). In several studies, GvHD effects seemed to selectively impair B cell development (14, 29). For the first time, Mensen et al. could translate these findings to the human setting by correlating impaired immune reconstitution after HSCT with both GvHD and T cell infiltration into the bone marrow and by demonstrating a striking reduction in osteoblasts in these patients (4). This closely resembles the findings in mouse models of GvHD allowing a generalized view on immunodeficiency after alloHSCT.

IMPLICATIONS OF BONE MARROW NICHE DESTRUCTION

In adult individuals, the bone marrow is both the origin of hematopoiesis and the ultimate harbor of immune cells comprising the immunological memory, namely, long-lived plasma cells and memory CD4 and CD8 T cells (30–34). Furthermore, hematological malignancies either originate or later become manifest in the bone marrow. All immunological functions strictly depend on a complex organization of niche-forming stromal cells of mesenchymal and endothelial origin providing important developmental cues to hematopoietic progenitors or crucial survival signals to memory cells.

Figure 1 proposes a model of bone marrow GvHD by bringing together data from clinical studies and various scientific investigations using mouse models or *in vitro* culture systems. **Figure 1** (left) depicts bone marrow homeostasis with a focus on niche constituents and points out cells susceptible to standard preconditioning treatment. **Figure 1** (right) shows cell types and effector mechanisms involved in acute bone marrow GvHD and clarifies how multiple niches are impacted by alloreactivity as explained in the following section.

A mutual starting point for all hematopoietic lineages is the endosteum, where self-renewing hematopoietic stem cells (HSCs)

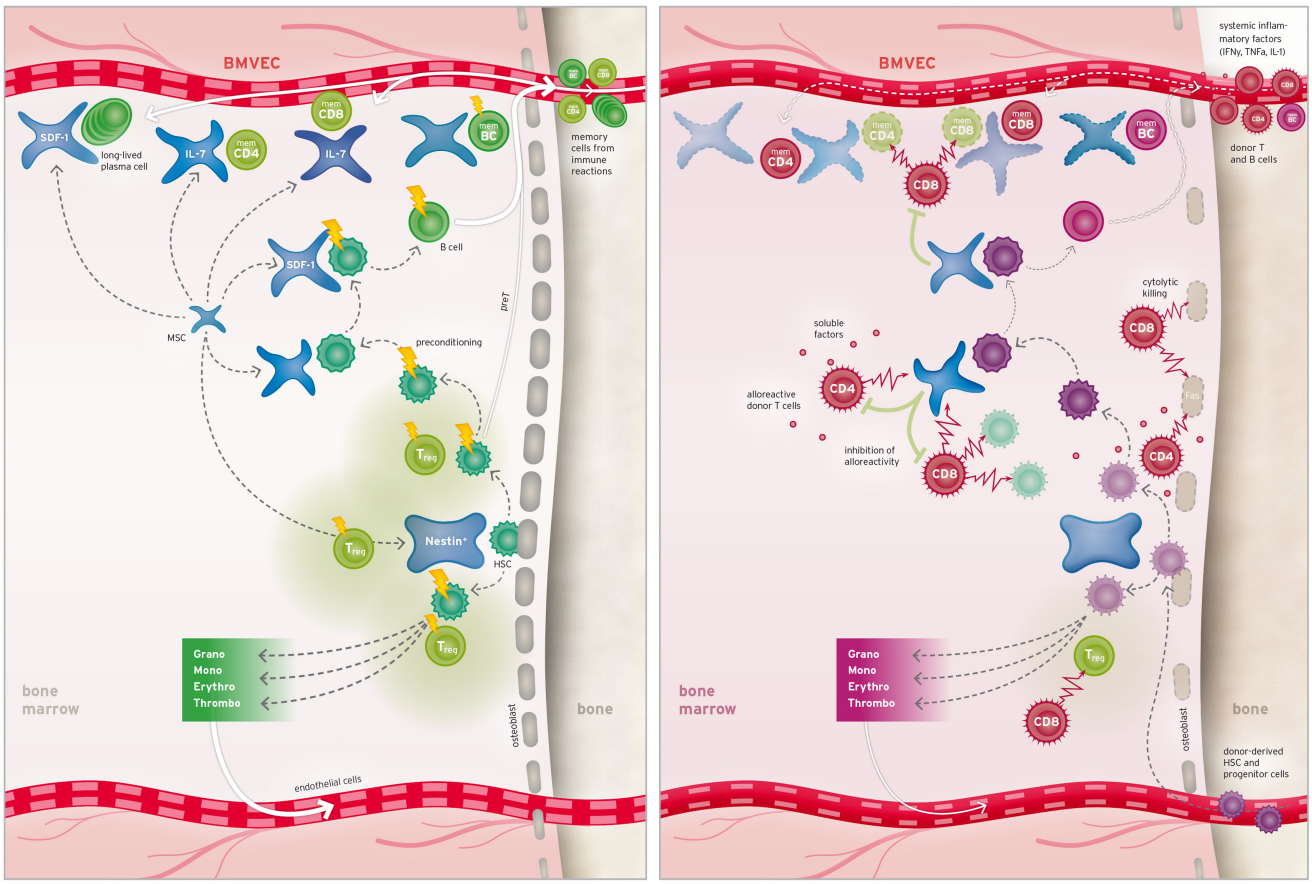

FIGURE 1 | **Bone marrow niches and their response to preconditioning and alloHSCT**. Left: niches for hematopoietic stem cells (HSCs) are located at the endosteal border and comprise osteoblasts and specialized mesenchymal cells partly immunoprotected by adjacent regulatory T cells (Treg). Alternatively, HSCs can seed into perivascular niches (not shown). B cell progenitors and recirculating memory T, B (memCD4, memCD8, and memBC), and plasma cells occupy additional specialized perivascular niches. Upon preconditioning, bone marrow cells are differently affected by the treatment as indicated by the flash size. Right: after alloHSCT, alloreactive T cells are massively activated (not shown) leads to systemic influx of soluble inflammatory factors into the bone marrow where they cause upregulation of Fas on various cell types and harm donor and recipient HSCs alike. Infiltrating alloreactive donor T cells deplete residual host hematopoietic cells and support disintegration of endostial and perivascular niches by means of cytolytic and soluble factors. Consequently, efflux of hematopoietic lineages and seeding capacity for donor-derived hematopoietic stem and memory cells is diminished.

reside in niches made up of specialized osteoblasts and nestin-expressing MSCs (35, 36) presumed to be osteogenic progenitors. Alternatively, HSCs can also be maintained in perivascular niches made up of CXCL12-expressing MSCs adjacent to vascular endothelial cells of bone marrow sinusoids (37, 38). Although both the endosteal and the perivascular niche seem to equally support HSC maintenance, the interplay between both niches has not been established yet (39). During proliferation of niche-derived cells, individual lineage decisions are made in a step-wise fashion during migration from the endosteum toward the marrow sinusoids, where cells ultimately exit the bone marrow and enter the blood circulation. For B cells, the maturation from HSC to transitional B cell exiting the bone marrow requires multiple dedicated stromal cells providing stage-specific signals (40).

Stromal and hematopoietic cells are differently affected by the conditioning treatment prior to alloHSCT. HSCs, hematopoietic progenitor cells, B cells, myeloid cells, and, to a lesser extent, T cells are depleted by irradiation and/or anti-mitotic drugs (27).

In contrast, mesenchymal and endothelial cells as well as memory T and B cells are resting cells and were shown to be comparably resistant to depletion (41, 42). Danger signals mediated by tissue damage trigger APC maturation and increased presentation of alloantigens *via* MHCI and II.

Upon alloHSCT, donor T cells, contained within the bone marrow preparations, circulate into secondary lymphoid organs and become activated mainly *via* interaction with dendritic cells (DC), APCs that express alloantigens along with high levels of the costimulatory molecules CD80 and CD86 upon preconditioning (9). At least for CD8 T cells, the initiation of aGvHD is strictly dependent on host APC activity (43). Activated T cells start proliferating and secrete large amounts of inflammatory cytokines and thereby initiate the acute phase of GvHD.

In the bone marrow, systemic inflammation leads to drastic changes in bone marrow-resident cells. TNF-α and IL-1 signaling stimulates upregulation of MHCII, CD40, and adhesion signals in endothelial cells, and blood vessel walls become more permeable, facilitating activation and entrance of innate immune cells and alloreactive lymphocytes into the bone marrow (44). Furthermore, prolonged systemic levels of IFN-γ in combination with TNF-α initiate endothelial cell death, which can be expected to impair maintenance of perivascular niches for memory and stem cells (45).

Osteoblasts constitutively express MHCI and potentially respond to inflammatory signals by upregulation of MHCII, Fas, and CD40, making them easy targets for alloreactive T cell response (46, 47). Mesenchymal cells also respond to IFN-γ by upregulation of MHCI and MHCII but demonstrate a striking inability to activate alloreactive T cells (48, 49). Even more, mesenchymal cells are capable of actively inhibiting T cell effector function by direct and indirect mechanisms, including interference with DC maturation and secretion of IL-10 (50). However, it is unclear whether MSCs themselves have the potential to resist strong alloreactivity. In a likely scenario, perivascular MSCs constituting important survival niches for memory and HSCs would at least display reduced niche capacities when adjacent endothelial cells became apoptotic.

Concomitantly, donor HSCs and progenitor cells enter the bone marrow *via* sinusoids and migrate to cell-specific niches that have been made available by depletion of host hematopoietic cells. However, HSC survival and proliferative capacity is also affected by soluble inflammatory factors, resulting in reduced HSC seeding under aGvHD conditions (51, 52).

Subsequently, activated alloreactive donor T cells infiltrate the bone marrow and exert their cytolytic effector functions by attacking cells presenting host alloantigens. The outcome of the ensuing immune response is impacted by multiple factors, including the immunogenic strength, level of presentation and number of individual alloantigens on host cells, the degree of inflammation caused by conditioning treatment, the size of the residual host T cell population capable of mounting a host-versus-graft response, and the naive repertoire of donor T cells.

Ultimately, the remaining hematopoietic host cells, including HSCs and memory cells, are depleted mainly *via* CD8-derived Fas–FasL and complete donor chimerism is established (14, 27). At that stage, the patient's immunological memory should be deleted with grave implications for immunity against recurring pathogens. As recipient APCs of hematopoietic origin are depleted, alloantigen is largely presented by donor-derived APCs. However, due to the poor engraftment potential of donor-derived mesenchymal cells, the majority of niche-forming cells in the bone marrow remain host mesenchymal and endothelial cells continuously expressing alloantigens (53). Osteoblasts and endothelial cells present MHCI and II at least under inflammatory conditions and therefore, most niche-forming cells of the bone marrow, albeit poor antigen presenters, constitute targets of alloreactivity under harsh conditions such as preconditioning and aGvHD (47). Additionally, continuous presentation of phagocytosed alloantigens by donor-derived APCs *via* MHCII, and to a lesser extent *via* cross presentation to MHCI, can support indirect niche destruction mainly *via* alloreactive CD4 T cells secreting soluble factors.

In sum, bone marrow GvHD leads to gradual reduction of aforementioned niches, which diminishes hematopoiesis and seeding of donor-derived memory cells into their respective bone marrow niches (**Figure 1**, right). Although the endosteal niche has recently been reported to be immune-privileged by means of regulatory T cells (54), steep reduction in osteoblast numbers argues against any protective environment at least under GvHD conditions. The bone marrow niche size for distinct cell populations is strictly limited by the number of respective niche-forming cells; and hence, any reduction in their numbers directly decreases the specific niche-capacity of the bone marrow. IL-7-expressing perivascular stroma cells in the bone marrow comprise the niche for memory T cells and loss of these cells due to alloreactivity negatively impacts donor memory T cell seeding and prolongs the preexisting immunosuppressive state of patients receiving alloHSCT (31, 55). Furthermore, it is not surprising that the B cell developmental program, requiring several distinct populations of niche-forming cells for individual maturation steps, proves to be the most affected cell population (29, 40). Hypothetically, the tightly regulated process of central tolerance that removes potentially autoreactive B cell clones could readily be unhinged by GvHD-mediated niche damage, possibly leading to secondary autoimmune symptoms synonymous with the chronic form of GvHD. Interestingly, cGvHD has been shown to

be associated with autoantibody titers (56). All factors described above work in combination at manifesting a stage of prolonged immunosuppression.

FUTURE DIRECTIONS

The rationale of using alloHSCT for the treatment of hematological disorders is the rapid replacement of the patient's defective hematopoietic system, whereas harnessing the alloreactivity of donor T cells for rejection of persistent tumor. However, these very T cells are also responsible for GvHD and accelerated immunodeficiency, a price we are willing to pay for a chance of relapse-free survival. However, the hidden cost is obviously much higher, considering that donor T cells are also responsible for bone marrow GvHD, which potentially leads to extensive destruction of niche-forming cells by not yet fully understood mechanisms. This greatly influences the kinetics of comprehensive immune reconstitution, because the replenishment of destroyed niches is apparently very slow. Besides broad effects on hematopoiesis, the seeding capacity of recirculating memory T cells into the bone marrow might be impacted by bone marrow GvHD, as we could show for B cell development. In consequence, a large fraction of treated patients remains vulnerable to otherwise harmless infections for months or years after treatment. Further research is needed to better protect dedicated bone marrow niches from GvHD. Alternatively, novel transplantation protocols should demonstrate improved seeding capacity of donor mesenchymal stem cells to rapidly replenish destroyed host niche-forming cells and to harness their unique immunosuppressive properties (57).

AUTHOR CONTRIBUTIONS

MS wrote the mini review. IN supervised, edited and approved the final version for publication.

REFERENCES

1. Korngold R, Sprent J. Lethal graft-versus-host disease after bone marrow transplantation across minor histocompatibility barriers in mice. Prevention by removing mature T cells from marrow. *J Exp Med* (1978) **148**:1687–98. doi:10.1084/jem.148.6.1687
2. Porter DL, Roth MS, McGarigle C, Ferrara J, Antin JH. Induction of graft-versus-host disease as immunotherapy for relapsed chronic myeloid leukemia. *N Engl J Med* (1994) **330**:100–6. doi:10.1056/NEJM199401133300204
3. Bruno B, Gooley T, Sullivan KM, Davis C, Bensinger WI, Storb R, et al. Secondary failure of platelet recovery after hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant* (2001) **7**:154–62. doi:10.1053/bbmt.2001.v7.pm11302549
4. Mensen A, Jöhrens K, Anagnostopoulos I, Demski S, Oey M, Stroux A, et al. T-cell infiltration of the human bone marrow during acute GvHD is associated with impaired B-cell reconstitution and function after allogeneic-HSCT. *Blood* (2014) **124**:963–72. doi:10.1182/blood-2013-11-539031
5. Shono Y, Ueha S, Wang Y, Abe J, Kurachi M, Matsuno Y, et al. Bone marrow graft-versus-host disease: early destruction of hematopoietic niche after MHC-mismatched hematopoietic stem cell transplantation. *Blood* (2010) **115**:5401–11. doi:10.1182/blood-2009-11-253559
6. Felix NJ, Allen PM. Specificity of T-cell alloreactivity. *Nat Rev Immunol* (2007) **7**:942–53. doi:10.1038/nri2200
7. Suchin EJ, Langmuir PB, Palmer E, Sayegh MH, Wells AD, Turka LA. Quantifying the frequency of alloreactive T cells in vivo: new answers to an old question. *J Immunol* (2001) **166**:973–81. doi:10.4049/jimmunol.166.2.973
8. Joffre OP, Segura E, Savina A, Amigorena S. Cross-presentation by dendritic cells. *Nat Rev Immunol* (2012) **12**:557–69. doi:10.1038/nri3254
9. Anderson BE, McNiff JM, Jain D, Blazar BR, Shlomchik WD, Shlomchik MJ. Distinct roles for donor- and host-derived antigen-presenting cells and costimulatory molecules in murine chronic graft-versus-host disease: requirements depend on target organ. *Blood* (2005) **105**:2227–34. doi:10.1182/blood-2004-08-3032
10. Van Bergen CA, Rutten CE, Van Der Meijden ED, Van Luxemburg-Heijs SA, Lurvink EG, Houwing-Duistermaat JJ, et al. High-throughput characterization of 10 new minor histocompatibility antigens by whole genome association scanning. *Cancer Res* (2010) **70**:9073–83. doi:10.1158/0008-5472.CAN-10-1832
11. DeLuca DS, Eiz-Vesper B, Ladas N, Khattab BA-M, Blasczyk R. High-throughput minor histocompatibility antigen prediction. *Bioinformatics* (2009) **25**:2411–7. doi:10.1093/bioinformatics/btp404
12. Shustov A, Nguyen P, Finkelman F, Elkon KB, Via CS. Differential expression of Fas and Fas ligand in acute and chronic graft-versus-host disease: up-regulation of Fas and Fas ligand requires CD8+ T cell activation and IFN-γ production. *J Immunol* (1998) **161**:2848–55.
13. Van den Brink MRM, Burakoff SJ. Cytolytic pathways in haematopoietic stem-cell transplantation. *Nat Rev Immunol* (2002) **2**:273–81. doi:10.1038/nri775
14. Baker MB, Riley RL, Podack ER, Levy RB. Graft-versus-host-disease-associated lymphoid hypoplasia and B cell dysfunction is dependent upon donor T cell-mediated Fas-ligand function, but not perforin function. *Proc Natl Acad Sci U S A* (1997) **94**:1366–71. doi:10.1073/pnas.94.4.1366
15. Schmaltz C, Alpdogan O, Horndasch KJ, Muriglan SJ, Kappel BJ, Teshima T, et al. Differential use of Fas ligand and perforin cytotoxic pathways by donor T cells in graft-versus-host disease and graft-versus-leukemia effect. *Blood* (2001) **97**:2886–95. doi:10.1182/blood.V97.9.2886
16. Lin L, Couturier J, Yu X, Medina MA, Kozinetz CA, Lewis DE. Granzyme B secretion by human memory CD4 T cells is less strictly regulated compared to memory CD8 T cells. *BMC Immunol* (2014) **15**:36. doi:10.1186/s12865-014-0036-1
17. Marks L, Altman NH, Podack ER, Levy RB. Donor T cells lacking Fas ligand and perforin retain the capacity to induce severe GvHD in minor histocompatibility antigen mismatched bone-marrow transplantation recipients. *Transplantation* (2004) **77**:804–12. doi:10.1097/01.TP.0000110416.96307.D5
18. Kaplan MJ, Ray D, Mo R-R, Yung RL, Richardson BC. TRAIL (Apo2 ligand) and TWEAK (Apo3 ligand) mediate CD4+ T cell killing of antigen-presenting macrophages. *J Immunol* (2000) **164**:2897–904. doi:10.4049/jimmunol.164.6.2897
19. Parkman R. Clonal analysis of murine graft-vs-host disease. I. Phenotypic and functional analysis of T lymphocyte clones. *J Immunol* (1986) **136**:3543–8.
20. Tyndall A, Dazzi F. Chronic GVHD as an autoimmune disease. *Best Pract Res Clin Haematol* (2008) **21**:281–9. doi:10.1016/j.beha.2008.03.003
21. Pulanic D, Lozier JN, Pavletic SZ. Thrombocytopenia and hemostatic disorders in chronic graft versus host disease. *Bone Marrow Transplant* (2009) **44**:393–403. doi:10.1038/bmt.2009.196
22. Goker H, Haznedaroglu IC, Chao NJ. Acute graft-vs-host disease: pathobiology and management. *Exp Hematol* (2001) **29**:259–77. doi:10.1016/S0301-472X(00)00677-9
23. Gratwohl A, Brand R, Frassoni F, Rocha V, Niederwieser D, Reusser P, et al. Cause of death after allogeneic haematopoietic stem cell transplantation (HSCT) in early leukaemias: an EBMT analysis of lethal infectious complications and changes over calendar time. *Bone Marrow Transplant* (2005) **36**:757–69. doi:10.1038/sj.bmt.1705140
24. Holländer GA, Widmer B, Burakoff SJ. Loss of normal thymic repertoire selection and persistence of autoreactive T cells in graft vs host disease. *J Immunol* (1994) **152**:1609–17.

25. Swain SL, McKinstry KK, Strutt TM. Expanding roles for CD4$^+$ T cells in immunity to viruses. *Nat Rev Immunol* (2012) **12**:136–48. doi:10.1038/nri3152
26. Klein L, Hinterberger M, Wirnsberger G, Kyewski B. Antigen presentation in the thymus for positive selection and central tolerance induction. *Nat Rev Immunol* (2009) **9**:833–44. doi:10.1038/nri2669
27. Müller AMS, Linderman JA, Florek M, Miklos D, Shizuru JA. Allogeneic T cells impair engraftment and hematopoiesis after stem cell transplantation. *Proc Natl Acad Sci U S A* (2010) **107**:14721–6. doi:10.1073/pnas.1009220107
28. Yao Y, Song X, Cheng H, Tang G, Hu X, Zhou H, et al. Dysfunction of bone marrow vascular niche in acute graft-versus-host disease after MHC-haploidentical bone marrow transplantation. *PLoS One* (2014) **9**:e104607. doi:10.1371/journal.pone.0104607
29. Storek J, Wells D, Dawson MA, Storer B, Maloney DG. Factors influencing B lymphopoiesis after allogeneic hematopoietic cell transplantation. *Blood* (2001) **98**:489–91. doi:10.1182/blood.V98.2.489
30. Manz RA, Thiel A, Radbruch A. Lifetime of plasma cells in the bone marrow. *Nature* (1997) **388**:133–4. doi:10.1038/40540
31. Tokoyoda K, Zehentmeier S, Hegazy AN, Albrecht I, Grün JR, Löhning M, et al. Professional memory CD4+ T lymphocytes preferentially reside and rest in the bone marrow. *Immunity* (2009) **30**:721–30. doi:10.1016/j.immuni.2009.03.015
32. Di Rosa F, Pabst R. The bone marrow: a nest for migratory memory T cells. *Trends Immunol* (2005) **26**:360–6. doi:10.1016/j.it.2005.04.011
33. Becker TC, Coley SM, Wherry EJ, Ahmed R. Bone marrow is a preferred site for homeostatic proliferation of memory CD8 T cells. *J Immunol* (2005) **174**:1269–73. doi:10.4049/jimmunol.174.3.1269
34. Mazo IB, Honczarenko M, Leung H, Cavanagh LL, Bonasio R, Weninger W, et al. Bone marrow is a major reservoir and site of recruitment for central memory CD8+ T cells. *Immunity* (2005) **22**:259–70. doi:10.1016/j.immuni.2005.01.008
35. Zhang J, Niu C, Ye L, Huang H, He X, Tong WG, et al. Identification of the haematopoietic stem cell niche and control of the niche size. *Nature* (2003) **425**:836–41. doi:10.1038/nature02041
36. Méndez-Ferrer S, Michurina TV, Ferraro F, Mazloom AR, Macarthur BD, Lira SA, et al. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* (2010) **466**:829–34. doi:10.1038/nature09262
37. Kiel MJ, Yilmaz OH, Iwashita T, Yilmaz OH, Terhorst C, Morrison SJ. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell* (2005) **121**:1109–21. doi:10.1016/j.cell.2005.05.026
38. Sugiyama T, Kohara H, Noda M, Nagasawa T. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. *Immunity* (2006) **25**:977–88. doi:10.1016/j.immuni.2006.10.016
39. Kiel MJ, Morrison SJ. Uncertainty in the niches that maintain haematopoietic stem cells. *Nat Rev Immunol* (2008) **8**:290–301. doi:10.1038/nri2279
40. Nagasawa T. Microenvironmental niches in the bone marrow required for B-cell development. *Nat Rev Immunol* (2006) **6**:107–16. doi:10.1038/nri1780
41. Li HH, Wang YW, Chen R, Zhou B, Ashwell JD, Fornace AJ Jr. Ionizing radiation impairs T cell activation by affecting metabolic reprogramming. *Int J Biol Sci* (2015) **11**:726–36. doi:10.7150/ijbs.12009
42. Sugrue T, Lowndes NF, Ceredig R. Mesenchymal stromal cells: radio-resistant members of the bone marrow. *Immunol Cell Biol* (2013) **91**:5–11. doi:10.1038/icb.2012.61
43. Shlomchik WD, Couzens MS, Tang CB, McNiff J, Robert ME, Liu J, et al. Prevention of graft versus host disease by inactivation of host antigen-presenting cells. *Science* (1999) **285**:412–5. doi:10.1126/science.285.5426.412
44. Pober JS, Sessa WC. Evolving functions of endothelial cells in inflammation. *Nat Rev Immunol* (2007) **7**:803–15. doi:10.1038/nri2171
45. Li JH, Pober JS. The cathepsin B death pathway contributes to TNF plus IFN-γ-mediated human endothelial injury. *J Immunol* (2005) **175**:1858–66. doi:10.4049/jimmunol.175.3.1858
46. Tsuboi M, Kawakami A, Nakashima T, Matsuoka N, Urayama S, Kawabe Y, et al. Tumor necrosis factor-α and interleukin-1β increase the Fas-mediated apoptosis of human osteoblasts. *J Lab Clin Med* (1999) **134**:222–31. doi:10.1016/S0022-2143(99)90201-9
47. Schrum LW, Bost KL, Hudson MC, Marriott I. Bacterial infection induces expression of functional MHC class II molecules in murine and human osteoblasts. *Bone* (2003) **33**:812–21. doi:10.1016/S8756-3282(03)00265-5
48. Tse WT, Pendleton JD, Beyer WM, Egalka MC, Guinan EC. Suppression of allogeneic T-cell proliferation by human marrow stromal cells: implications in transplantation. *Transplantation* (2003) **75**:389–97. doi:10.1097/01.TP.0000045055.63901.A9
49. Fasslrinner F, Wobus M, Duryagina R, Müller K, Stopp S, Wehner R, et al. Differential effects of mixed lymphocyte reaction supernatant on human mesenchymal stromal cells. *Exp Hematol* (2012) **40**:934–44. doi:10.1016/j.exphem.2012.07.011
50. Ryan JM, Barry FP, Murphy JM, Mahon BP. Mesenchymal stem cells avoid allogeneic rejection. *J Inflamm Lond Engl* (2005) **2**:8. doi:10.1186/1476-9255-2-8
51. Okamoto T, Kanamaru A, Kakishita E, Nagai K. Stromal fibroblastic and hematopoietic progenitors in patients with graft-versus-host disease (GVHD). *Int J Hematol* (1991) **54**:299–306.
52. de Bruin AM, Demirel Ö, Hooibrink B, Brandts CH, Nolte MA. Interferon-γ impairs proliferation of hematopoietic stem cells in mice. *Blood* (2013) **121**:3578–85. doi:10.1182/blood-2012-05-432906
53. Awaya N, Rupert K, Bryant E, Torok-Storb B. Failure of adult marrow-derived stem cells to generate marrow stroma after successful hematopoietic stem cell transplantation. *Exp Hematol* (2002) **30**:937–42. doi:10.1016/S0301-472X(02)00821-4
54. Fujisaki J, Wu J, Carlson AL, Silberstein L, Putheti P, Larocca R, et al. In vivo imaging of Treg cells providing immune privilege to the haematopoietic stem-cell niche. *Nature* (2011) **474**:216–9. doi:10.1038/nature10160
55. Sercan Alp Ö, Durlanik S, Schulz D, McGrath M, Grün JR, Bardua M, et al. Memory CD8+ T cells colocalize with IL-7+ stromal cells in bone marrow and rest in terms of proliferation and transcription. *Eur J Immunol* (2015) **45**:975–87. doi:10.1002/eji.201445295
56. Cutler C, Miklos D, Kim HT, Treister N, Woo SB, Bienfang D, et al. Rituximab for steroid-refractory chronic graft-versus-host disease. *Blood* (2006) **108**:756–62. doi:10.1182/blood-2006-01-0233
57. Ringdén O, Uzunel M, Rasmusson I, Remberger M, Sundberg B, Lönnies H, et al. Mesenchymal stem cells for treatment of therapy-resistant graft-versus-host disease. *Transplantation* (2006) **81**:1390–7. doi:10.1097/01.tp.0000214462.63943.14

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The Role of IL-17 and TH17 Cells in the Bone Catabolic Activity of PTH

Roberto Pacifici[1,2]*

[1]Division of Endocrinology, Metabolism and Lipids, Department of Medicine, Emory University, Atlanta, GA, USA, [2]Immunology and Molecular Pathogenesis Program, Emory University, Atlanta, GA, USA

Osteoimmunology is field of research dedicated to the study of the interactions between the immune system and bone. Among the cells of the immune system that regulate the skeleton in health and disease are T lymphocytes, T cells secrete inflammatory/osteoclastogenic cytokines such as RANKL, TNF, and IL-17, as well as factors that stimulate bone formation, including Wnt ligands. In addition, T cells regulate the differentiation and life span of stromal cells via CD40L and other costimulatory molecules expressed on their surface. Consensus exists that parathyroid hormone (PTH) induces bone loss by increasing the production of RANKL by osteocytes and osteoblast. However, new evidence suggests that PTH expands Th17 cells and increases IL-17 levels in mice and humans. Studies in the mouse of further shown that Th17 cell produced IL-17 acts as an "upstream cytokine" that increases the sensitivity of osteoblasts and osteocytes to PTH. As a result, PTH stimulates osteocytic and osteoblastic release of RANKL. Therefore, PTH cause bone loss only in the presence of IL-17 signaling. This article reviews the evidence that the effects of PTH are mediated not only by osteoblasts and osteocytes, but also T cells and IL-17.

Keywords: T cells, PTH, IL-17, osteoblasts, osteocytes, bone

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***Correspondence:**
Roberto Pacifici
roberto.pacifici@emory.edu

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INTRODUCTION

Parathyroid hormone (PTH) is an important regulator of calcium and phosphorus concentrations in extracellular fluid. Physiologic levels of circulating PTH are essential for maintaining serum and urinary calcium levels within their normal range. Chronic excessive PTH production is a cause of skeletal and extra skeletal disease. Secondary hyperparathyroidism has been implicated in the pathogenesis of senile osteoporosis (1), while primary hyperparathyroidism (PHPT) is associated with accelerated bone loss (2) and osteoporosis (3–5).

Primary and secondary hyperparathyroidism are mimicked by continuous PTH (cPTH) infusion. cPTH and PHPT increase bone turnover in trabecular and cortical bone, as evidenced by elevations in histomorphometric and biochemical markers of resorption and formation (6–8), whereas PHPT and cPTH treatment cause cortical bone loss by enhancing endosteal resorption through stimulation of osteoclast formation, activity, and life span (3, 8, 9). Severe chronic elevations of PTH levels may also lead to trabecular bone loss (3, 8), although PHPT and cPTH treatment often induce a modest increase in cancellous bone (4–6, 10).

The effects of cPTH on bone result from its binding to the PTH/PTH-related protein (PTHrP) receptor (PPR or PTHR1), which is expressed not only on BM stromal cells (SCs), osteoblasts, and osteocytes (11, 12) but also on T cells (13) and macrophages (14). SCs and osteoblasts were the first targets of PTH to be identified, and earlier consensus developed that the catabolic effect of

cPTH is mostly mediated by enhanced production of RANKL and decreased production of OPG by SCs and osteoblasts (15–17). More recent studies in mice with deletion and/or overexpression of PPR and RANKL in osteocytes (12, 18–20) lead to the recognition that osteocytes represent essential targets of PTH in bone, and that increased production of RANKL by osteocytes plays an important role in cPTH-induced bone loss (12, 19). However, some reports have ascribed a key role to OB produced RANKL (21). Moreover, studies have also shown that PPR signaling in T cells stimulates the release of TNF (22), and that deletion of T cells, T cell production of TNF, or PPR signaling in T cells prevents cPTH-induced bone loss (22, 23), as effectively as deletion of PPR signaling in osteocytes. Because of these reports, T cells are now recognized as a second critical target of PTH in bone. Controversy remains on the relative relevance of T cells, osteocytes, and osteoblasts for the activity of PTH. However, new evidence suggests that PTH expands Th17 cells and increases IL-17 levels in mice and humans (24). Studies in the mouse of further shown that Th17 cell-produced IL-17 acts as an "upstream cytokine" that increases the sensitivity of osteoblasts and osteocytes to PTH. As a result, PTH stimulates osteocytic and osteoblastic release of RANKL, and thus cause bone loss, only in the presence of intact IL-17 signaling. This article focuses on the role of Th17 cell-produced IL-17 in the mechanism of action of PTH in bone.

TH17 CELLS AND PTH-INDUCED BONE LOSS

The discovery that T lymphocytes express functional PPR (13) and respond to PTH (25) prompted investigations on the role of T cells as mediators of the effects of cPTH in bone. Early studies revealed that levels of PTH typically found in PHPT require the presence of T cells to induce bone loss (26, 27), whereas conditions that cause extreme elevations in PTH levels induce bone loss via T cell-independent mechanisms (28–31).

T cells exert complex activities that are relevant for the effects of PTH in bone, including stimulating the production of TNF by both $CD4^+$ and $CD8^+$ T cells (22). Since $CD8^+$ cells are more abundant in the BM than $CD4^+$ cells, most of the TNF produced in the BM in response to cPTH originates from $CD8^+$ cells (22). TNF stimulates osteoclast formation and activity via multiple mechanisms, which include increased production of RANKL by all osteoblastic cells including osteocytes. Attesting to the relevance of T cell produced TNF, cPTH fails to induce bone loss and stimulate bone resorption in mice specifically lacking T cell TNF production (22). PTH induces T cell production of TNF via direct activation of PPR signaling in T cells (22). Conditional silencing of the PTH receptor PPR in T cells blunts the stimulation of bone resorption induced by cPTH without affecting bone formation, thus blocking cortical bone loss and converting the effects of cPTH in trabecular bone from catabolic to anabolic (22). These findings demonstrate the critical relevance of direct PPR signaling in T cells.

cPTH stimulates bone cells and immune cells to release growth factors and cytokines. Among them are TGFβ, IL-6, and TNF (22, 32–34). TGFβ and IL-6 direct the differentiation of naive $CD4^+$ cells into Th17 cells (35–37).

Th17 cells are the most osteoclastogenic subsets of T cells (38). Th17 cells are defined by their capacity to produce the cytokine IL-17. Th17 cells are constitutively present at mucosal surfaces, especially in the intestinal lamina propria (39). Th17 cells play a pivotal role in the bone loss of inflammatory conditions such as psoriasis, rheumatoid arthritis, periodontal disease, and IBD (40, 41). Th17 cells potently induce osteoclastogenesis by secreting IL-17, RANKL, TNF, IL-1, and IL-6, along with low levels of IFNγ (42–44). IL-17 stimulates the release of RANKL by osteoblasts and osteocytes (24, 38) and potentiates the osteoclastogenic activity of RANKL by upregulating RANK (45). IL-17 provides an important connection between T cells and osteocytes as this T cell cytokine regulates osteocytic RANKL production (24), which is one key effect of PTH on osteocytes (12, 19).

Studies with agents neutralizing TNF have implicated TNF in the generation of Th17 cells in rodents and humans (46–48). Moreover, PTH binding to PPR activates the G protein-coupled receptor subunit GαS, leading to the generation of cAMP (49). Accumulation of cAMP in $CD4^+$ cells and the resulting Ca^{2+} influx further promote Th17 cell differentiation and activity (50). Therefore, cPTH could expand Th17 cells via several mechanisms. This reasoning prompted investigations on the relationship between cPTH treatment and Th-17. Murine studies have revealed that cPTH treatment increases the relative and absolute frequency of $Th17^+$ cells and the levels of IL-17 in peripheral blood, spleen, and BM (24). Detailed analysis of samples of peripheral blood revealed that cPTH increased IL-17 levels in purified peripheral blood $CD4^+$ cells and unfractionated peripheral blood nucleated cells, but not in $CD4^+$ cell-depleted peripheral blood nucleated cells, indicating that $CD4^+$ cells represent the major source of IL-17 mRNA in peripheral blood cells. Moreover, cPTH also increases the mRNA levels of the Th17-inducing transcription factors RORα and RORγt in peripheral blood, spleen, and BM $CD4^+$ T cells. This effect of cPTH is specific for Th17 cells because cPTH does not expand murine Th1 cells, Th2 cells, and regulatory T cells.

Mechanistic studies have disclosed that cPTH increases Th17 cell differentiation in the BM and the spleen. By contrast, cPTH does not stimulate Th17 cell proliferation (24). Surprisingly, these studies have shown that cPTH expands Th17 cells and increases the production of IL-17 via TNF, and more specifically, the pool of TNF produced by conventional $CD4^+$ and $CD8^+$ T cells. This conclusion is based on the fact that cPTH failed to expand BM and splenic Th17 calls in TNF-null mice and in T cell-null mice previously subjected to adoptive transfer of $TNF^{-/-}$ T cells. The latter experimental model is particularly significant, because the host mice possess all physiologic sources of TNF except for T cells. Yet, specific ablation of T cell produced TNF is sufficient to block the capacity of cPTH to expand Th17 cells, increase BM IL-17 levels and prevent bone loss (24). Additional T cell reconstitution studies revealed that TNF directly targets Th17 precursors. To reach this conclusions, investigators reconstituted T cell-null mice with $CD4^+$ cells from $TNFR1^{-/-}$ and $TNFR2^{-/-}$ mice and then treated the host mice with cPTH. Under these conditions, cPTH expanded BM Th17 cells and increase BM IL-17 levels in

mice with TNFR2$^{-/-}$ T cells but not in those with TNFR1$^{-/-}$ T cells, thus demonstrating that TNFR1 signaling is required for cPTH to induce the differentiation of CD4$^+$ cells into Th17 cells. In addition to IL-6 TGFβ and TNF, several cytokines are known to promote Th17 cell expansion. Among them are the T cell produced factor IL-21 and the macrophage/dendritic cell produced cytokine IL-23. cPTH treatment increases the BM levels of IL-21, and IL-23R in WT mice. By contrast, cPTH did not increase the levels of these cytokines in TNF$^{-/-}$ mice. Thus, IL-21 and IL-23 are likely to contribute to expansion of Th17 cells induced by cPTH. However, cPTH upregulates these factors via TNF.

As stated above, PTH receptor signaling activates GαS, leading to the generation of cAMP (49), which further promotes TH17 cell differentiation via Ca^{2+} influx (50). One mechanism by which activation of GαS in CD4$^+$ cells could promote Th17 cell differentiation is increased sensitivity to TNF. To investigate this hypothesis, investigators used GαS$^{\Delta CD4,8}$ mice, a strain characterized by silenced GαS signaling in CD4$^+$ and CD8$^+$ T cells. Pivotal experiments revealed that cultures of CD4$^+$ cells from cPTH-treated control mice yielded a higher number of Th17 cells as compared to those from vehicle-treated mice (24). By contrast, cultures of CD4$^+$ cells from vehicle and cPTH-treated GαS$^{\Delta CD4,8}$ mice yielded similar numbers of Th17 cells, demonstrating that cPTH increases the sensitivity of nascent Th17 cells to TNF via GαS signaling in CD4$^+$ cells. Attesting to the relevance of GαS signaling in CD4$^+$ cells for Th17 cell generation, cPTH was found not to expand BM and splenic Th17 cells and not to exerts its bone catabolic activity in GαS$^{\Delta CD4,8}$ mice, demonstrate that silencing of Gαs in T cells prevents the expansion of Th17 cells and the bone loss induced by cPTH (24).

Signaling events downstream of GαS include cAMP generation (49) and activation of L-type calcium channels (51), which promote Th1 and Th17 cell differentiation (50). This evidence suggests the possibility that treatment with the L-type calcium channel blocker diltiazem may blunt the differentiation of CD4$^+$ cells into Th17 cells (50) and prevent the bone loss induced by cPTH.

This hypothesis was tested in murine studies that revealed that diltiazem blocks the expansion of Th17 cells, the increase in bone resorption, and the loss of cortical and trabecular bone induced by cPTH. The finding may suggest a potential therapeutic role for L-type calcium channel blockers in the treatment of hyperparathyroidism.

NEUTRALIZATION OF IL-17A OR SILENCING OF IL-17RA BLOCK CPTH-INDUCED BONE LOSS

The finding that cPTH increases the levels of IL-17 does not demonstrate that IL-17 plays a role in the bone catabolic activity of cPTH. To demonstrate the relevance of IL-17 in the mechanism by which cPTH alters skeletal homeostasis mice were treated with cPTH and a neutralizing antibody directed against murine IL-17 (IL-17 Ab). These studies revealed that IL-17 Ab completely prevents the loss of cortical and trabecular bone induced by cPTH. Analysis of biochemical and histomorphometric indices of bone turnover revealed that neutralization of IL-17 blunts the bone catabolic activity of cPTH by decreasing bone resorption (24).

To confirm these findings, additional experiments were conducted utilizing IL-17RA-null mice, a strain lacking the heterodimeric receptor IL-17RA/IL-17RC known as IL-17RA (52, 53). IL-17 signaling is silenced in IL-17RA$^{-/-}$ mice (54). These studies disclosed that cPTH stimulates bone resorption and indices bone loss in control mice but not in IL-17RA mice (24), thus demonstrating that silencing of IL-17 signaling prevents the bone catabolic activity of cPTH.

IL-17 IS AN UPSTREAM CYTOKINE REQUIRED FOR CPTH TO INCREASE RANKL RELEASE BY OSTEOBLASTS AND OSTEOCYTES

Osteocytes and the pool of RANKL produced by osteocytes are crucial for the activity of cPTH (12, 19, 20). On the other hand, studies form our laboratory have shown T cells are an additional important target of PTH (55). The fact that silencing of PPR signaling in T cells and osteocytes induces similar bone sparing effects is in keeping with a "serial circuit" regulatory model, where signals from one population affect the response to cPTH of the other. Since T cells and osteocytes have limited physical contacts, the cross talk between these populations is likely mediated by a soluble factor. IL-17A is a probable candidate because it is a potent inducer of RANKL in organ cultures containing osteoblasts and osteocytes (56). In support of this hypothesis, investigations have shown that neutralization of IL-17 via treatment with IL-17 Ab and deletion of IL-17RA block the capacity of cPTH to increase the production of RANKL by osteocytes and osteoblasts (24). These data indicate that IL-17A increases the sensitivity of osteoblasts and osteocytes to cPTH, thus enabling these lineages to release RANKL when stimulated by cPTH. Therefore, IL-17 mediates the bone catabolic activity of cPTH by upregulating the production of RANKL by osteocytes and osteoblasts (**Figure 1**).

It is important to underscore that the available published data suggest that T cells, osteoblasts, and osteocytes are all required for cPTH and by extension, PHPT, to induce bone loss. By contrast, osteocytes, but not T cells and IL-17, are required for physiologic levels of endogenous PTH to regulate bone remodeling. In fact, mice lacking PPR signaling in osteocytes have high baseline bone volume (12), while IL-17RA-null mice and those lacking PPR signaling in T cells (22, 57) have a normal bone volume.

INCREASED PRODUCTION OF IL-17A IN HUMANS AFFECTED BY PRIMARY HYPERPARATHYROIDISM

While numerous studies have investigated the role of immune cells and cytokines in the mechanism of action of PTH in animal models, little information is available in humans.

To investigate the effects of PHPT on the production of cytokines, unfractionated peripheral blood nucleated cells were obtained from 57 healthy controls and 20 similar subjects affected by PHPT. In PHPT patient's blood samples were obtained before surgery and 1 month after successful resolution of PHPT by parathyroidectomy. This study revealed (**Figure 2**) that the mRNA

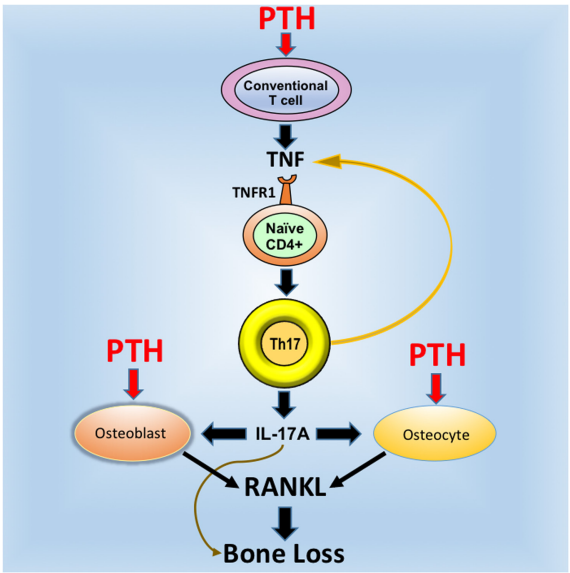

FIGURE 1 | Schematic representation of the mechanism of action of cPTH in bone. PTH binds to the PTH receptor PPR expressed in conventional CD4⁺ and Cd8⁺ T cells and induces the secretion of TNF. This cytokines induces the differentiation of naive CD4⁺ cells into Th17 cells via TNFR1 signaling. Th17 cells release additional TNF, which further stimulates Th17 differentiation. More importantly, Th17 cells secrete IL-17, which targets osteocytes and osteoblasts, thus increasing their sensitivity to TNF. In the presence of IL-17, PPR activation in osteocytes and osteoblasts stimulates these cells to release RANKL, which stimulates bone resorption and induces bone loss. Silencing of IL-17 or IL-17RA signaling blocks the capacity of cPTH to stimulate the production of RANKL by osteocytes and osteoblasts. Reproduced with permission from Ref. (24).

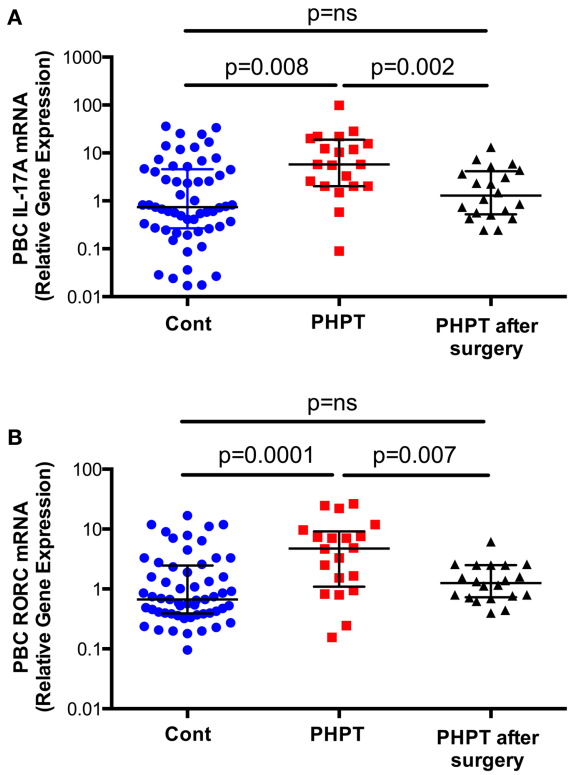

FIGURE 2 | Levels (Median ± interquartile range) of IL-17A (Panel A) and RORC mRNAs (Panel B) in healthy controls ($n = 57$) and subjects with PHPT before ($n = 20$) and after parathyroidectomy ($n = 20$). Data were analyzed by Mann–Whitney (healthy controls vs. PHPT before surgery and healthy controls vs. PHPT after surgery) and Wilcoxon matched pairs signed rank tests (PHPT vs. PHPT after surgery) as the data were not normally distributed according to the Shapiro–Wilk normality test. Reproduced with permission from Ref. (24).

levels of IL-17A in unfractionated peripheral blood nucleated cells were approximately threefold higher in PHPT patients than in healthy controls (24). Moreover, surgical restoration of normal parathyroid function was associated with the normalization of IL-17A levels. Furthermore, the mRNA levels of the IL-17-inducing transcription factor RORC were approximately threefold higher in PHPT patients before surgery than in healthy controls and parathyroidectomy was followed by a decrease in RORC mRNA levels. PTH levels were directly correlated with IL-17A level and the differences in IL-17A and RORC levels between healthy controls and PHPT patients remained significant even after adjustment for age and gender by a multiple regression model. These findings suggest that increased IL-17A gene expression in PHPT patients is due to increased levels of circulating PTH.

CONCLUSION

Remarkable progress has been made in understanding how T cells participate in the regulation of bone remodeling in health and disease. An impressive amount of work published in the last 10 years has led to the recognition that T cells play an unexpected role in the regulation of bone resorption and bone formation through a variety of mechanisms and the involvement of specialized cell lineages such as Th17 cells and Tregs. Work remains to be done to fully understand the cross-talk between bone cells and immune cells.

Some confirmation of the relevance of T cells in human bone diseases has now been reported but much remains to be done. Most of the human evidence has been accrued in studies on the pathogenesis of postmenopausal osteoporosis. For example, evidence begins to emerge in favor of a role of T cell produced TNF in postmenopausal bone loss in women (58, 59) and that in humans estrogen deficiency expands RANKL-expressing T cells and B cells (60, 61). Moreover, a role for IL-1 and TNF in humans is supported by reports that menopause increases the levels of these factors (62–66), while treatment with inhibitors of IL-1 and TNF prevents the increase in bone resorption induced by estrogen deficiency (67). A recent report from our laboratory show that PHPT increases IL-17 production in humans, an abnormality which is resolved by successful parathyroidectomy. Our studies show that in mice, the bone loss induced by cPTH is prevented by the calcium channel blocker diltiazem, and IL-17 Ab. Direct clinical applications of these finding arise because L-type calcium channel blocker are available, while anti-human IL-17 Abs and IL-17 receptor Abs are under investigation as therapeutic agents in psoriasis and spondyloarthropathy (68–71).

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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REFERENCES

1. Riggs BL, Melton LJ. Medical progress: involutional osteoporosis. *N Eng J Med* (1986) **314**:1676–84. doi:10.1056/NEJM198606263142605
2. Grey AB, Stapleton JP, Evans MC, Reid IR. Accelerated bone loss in postmenopausal women with mild primary hyperparathyroidism. *Clin Endocrinol (Oxf)* (1996) **44**:697–702. doi:10.1046/j.1365-2265.1996.744565.x
3. Potts J. Primary hyperparathyroidism. In: Avioli LV, Krane S, editors. *Metabolic Bone Diseases*. (Vol. **1**), San Diego: Academic Press (1998). p. 411–42.
4. Parisien M, et al. Histomorphometric analysis of bone in primary hyperparathyroidism. In: Belezikian JP, editor. *The Parathyroids. Basic and Clinical Concepts*. San Diego: Academic Press. (2001). p. 423–36.
5. Silverberg SJ, Shane E, de la Cruz L, Dempster DW, Feldman F, Seldin D, et al. Skeletal disease in primary hyperparathyroidism. *J Bone Miner Res* (1989) **4**:283–91. doi:10.1002/jbmr.5650040302
6. Zhou H, Shen V, Dempster DW, Lindsay R. Continuous parathyroid hormone and estrogen administration increases vertebral cancellous bone volume and cortical width in the estrogen-deficient rat. *J Bone Miner Res* (2001) **16**:1300–7. doi:10.1359/jbmr.2001.16.7.1300
7. Dempster DW, Parisien M, Silverberg SJ, Liang XG, Schnitzer M, Shen V, et al. On the mechanism of cancellous bone preservation in postmenopausal women with mild primary hyperparathyroidism. *J Clin Endocrinol Metab* (1999) **84**:1562–6. doi:10.1210/jcem.84.5.5652
8. Iida-Klein A, Lu SS, Kapadia R, Burkhart M, Moreno A, Dempster DW, et al. Short-term continuous infusion of human parathyroid hormone 1-34 fragment is catabolic with decreased trabecular connectivity density accompanied by hypercalcemia in C57BL/J6 mice. *J Endocrinol* (2005) **186**:549–57. doi:10.1677/joe.1.06270
9. Lotinun S, Evans GL, Bronk JT, Bolander ME, Wronski TJ, Ritman EL, et al. Continuous parathyroid hormone induces cortical porosity in the rat: effects on bone turnover and mechanical properties. *J Bone Miner Res* (2004) **19**:1165–71. doi:10.1359/JBMR.040404
10. Hock JM, Gera I. Effects of continuous and intermittent administration and inhibition of resorption on the anabolic response of bone to parathyroid hormone. *J Bone Miner Res* (1992) **7**:65–72. doi:10.1002/jbmr.5650070110
11. Calvi LM, Sims NA, Hunzelman JL, Knight MC, Giovannetti A, Saxton JM, et al. Activated parathyroid hormone/parathyroid hormone-related protein receptor in osteoblastic cells differentially affects cortical and trabecular bone. *J Clin Invest* (2001) **107**:277–86. doi:10.1172/JCI11296
12. Saini V, Marengi DA, Barry KJ, Fulzele KS, Heiden E, Liu X, et al. Parathyroid hormone (PTH)/PTH-related peptide type 1 receptor (PPR) signaling in osteocytes regulates anabolic and catabolic skeletal responses to PTH. *J Biol Chem* (2013) **288**:20122–34. doi:10.1074/jbc.M112.441360
13. Terauchi M, Li JY, Bedi B, Baek KH, Tawfeek H, Galley S, et al. T lymphocytes amplify the anabolic activity of parathyroid hormone through Wnt10b signaling. *Cell Metab* (2009) **10**:229–40. doi:10.1016/j.cmet.2009.07.010
14. Cho SW, Soki FN, Koh AJ, Eber MR, Entezami P, Park SI, et al. Osteal macrophages support physiologic skeletal remodeling and anabolic actions of parathyroid hormone in bone. *Proc Natl Acad Sci U S A* (2014) **111**(4):1545–50. doi:10.1073/pnas.1315153111
15. Weir EC, Lowik CW, Paliwal I, Insogna KL. Colony stimulating factor-1 plays a role in osteoclast formation and function in bone resorption induced by parathyroid hormone and parathyroid hormone-related protein. *J Bone Miner Res* (1996) **11**:1474–81. doi:10.1002/jbmr.5650111014
16. Ma YL, et al. Catabolic effects of continuous human PTH (1 – 38) in vivo is associated with sustained stimulation of RANKL and inhibition of osteoprotegerin and gene-associated bone formation. *Endocrinology* (2001) **142**:4047–54. doi:10.1210/en.142.9.4047
17. Qin L, Raggatt LJ, Partridge NC. Parathyroid hormone: a double-edged sword for bone metabolism. *Trends Endocrinol Metab* (2004) **15**:60–5. doi:10.1016/j.tem.2004.01.006
18. Powell WF Jr, Barry KJ, Tulum I, Kobayashi T, Harris SE, Bringhurst FR, et al. Targeted ablation of the PTH/PTHrP receptor in osteocytes impairs bone structure and homeostatic calcemic responses. *J Endocrinol* (2011) **209**:21–32. doi:10.1530/JOE-10-0308
19. Xiong J, Piemontese M, Thostenson JD, Weinstein RS, Manolagas SC, O'Brien CA. Osteocyte-derived RANKL is a critical mediator of the increased bone resorption caused by dietary calcium deficiency. *Bone* (2014) **66C**:146–54. doi:10.1016/j.bone.2014.06.006
20. Ben-awadh AN, Delgado-Calle J, Tu X, Kuhlenschmidt K, Allen MR, Plotkin LI, et al. Parathyroid hormone receptor signaling induces bone resorption in the adult skeleton by directly regulating the RANKL gene in osteocytes. *Endocrinology* (2014) **155**:2797–809. doi:10.1210/en.2014-1046
21. Fumoto T, Takeshita S, Ito M, Ikeda K. Physiological functions of osteoblast lineage and T cell-derived RANKL in bone homeostasis. *J Bone Miner Res* (2014) **29**:830–42. doi:10.1002/jbmr.2096
22. Tawfeek H, Bedi B, Li JY, Adams J, Kobayashi T, Weitzmann MN, et al. Disruption of PTH Receptor 1 in T cells protects against PTH-induced bone loss. *PLoS One* (2010) **5**:e12290. doi:10.1371/journal.pone.0012290
23. Gao Y, Wu X, Terauchi M, Li JY, Grassi F, Galley S, et al. T cells potentiate PTH-induced cortical bone loss through CD40L signaling. *Cell Metab* (2008) **8**:132–45. doi:10.1016/j.cmet.2008.07.001
24. Li JY, D'Amelio P, Robinson J, Walker LD, Vaccaro C, Luo T, et al. IL-17A is increased in humans with primary hyperparathyroidism and mediates PTH-induced bone loss in mice. *Cell Metab* (2015) **22**:799–810. doi:10.1016/j.cmet.2015.09.012
25. Stojceva-Taneva O, Fadda GZ, Smogorzewski M, Massry SG. Parathyroid hormone increases cytosolic calcium of thymocytes. *Nephron* (1993) **64**:592–9. doi:10.1159/000187406
26. Hory BG, Roussanne MC, Rostand S, Bourdeau A, Drüeke TB, Gogusev J. Absence of response to human parathyroid hormone in athymic mice grafted with human parathyroid adenoma, hyperplasia or parathyroid cells maintained in culture. *J Endocrinol Invest* (2000) **23**:273–9. doi:10.1007/BF03343723
27. Strieth S, von Johnston V, Eichhorn ME, Enders G, Krasnici S, Thein E, et al. A new animal model to assess angiogenesis and endocrine function of parathyroid heterografts in vivo. *Transplantation* (2005) **79**:392–400. doi:10.1097/01.TP.0000151633.92173.75
28. Schachter P, Christy MD, Leight GS Jr, Lobaugh B. Function in athymic nude mice of parathyroid heterografts from patients with primary hyperparathyroidism and secondary hyperparathyroidism. *Surgery* (1990) **108**:1040–6.
29. Guise TA, Chirgwin JM, Favarato G, Boyce BF, Mundy GR. Chinese hamster ovarian cells transfected with human parathyroid hormone-related protein cDNA cause hypercalcemia in nude mice. *Lab Invest* (1992) **67**:477–85.
30. Mehdizadeh S, Alaghband-Zadeh J, Gusterson B, Arlot M, Bradbeer JN, Loveridge N. Bone resorption and circulating PTH-like bioactivity in an animal model of hypercalcaemia of malignancy. *Biochem Biophys Res Commun* (1989) **161**:1166–71. doi:10.1016/0006-291X(89)91364-8
31. Uy HL, Guise TA, De La Mata J, Taylor SD, Story BM, Dallas MR, et al. Effects of parathyroid hormone (PTH)-related protein and PTH on osteoclasts and osteoclast precursors in vivo. *Endocrinology* (1995) **136**:3207–12. doi:10.1210/en.136.8.3207
32. Lowik CWGM, et al. Parathyroid hormone (PTH) and PTH-like protein (PLP) stimulate IL-6 production by osteogenic cells: a possible role of interleukin-6 in osteoclastogensis. *Biochem Biophys Res Commun* (1989) **162**:1546–52. doi:10.1016/0006-291X(89)90851-6
33. Koh AJ, Novince CM, Li X, Wang T, Taichman RS, McCauley LK. An irradiation-altered bone marrow microenvironment impacts anabolic actions of PTH. *Endocrinology* (2011) **152**:4525–36. doi:10.1210/en.2011-1515
34. Cho SW, Pirih FQ, Koh AJ, Michalski M, Eber MR, Ritchie K, et al. The soluble interleukin-6 receptor is a mediator of hematopoietic and skeletal actions of parathyroid hormone. *J Biol Chem* (2013) **288**(10):6814–25. doi:10.1074/jbc.M112.393363

35. Bettelli E, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* (2006) **441**:235–8. doi:10.1038/nature04753
36. Mangan PR, Harrington LE, O'Quinn DB, Helms WS, Bullard DC, Elson CO, et al. Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature* (2006) **441**:231–4. doi:10.1038/nature04754
37. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* (2006) **24**:179–89. doi:10.1016/j.immuni.2006.01.001
38. Sato K, Suematsu A, Okamoto K, Yamaguchi A, Morishita Y, Kadono Y, et al. Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction. *J Exp Med* (2006) **203**:2673–82. doi:10.1084/jem.20061775
39. Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, et al. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell* (2006) **126**:1121–33. doi:10.1016/j.cell.2006.07.035
40. Miossec P, Korn T, Kuchroo VK. Interleukin-17 and type 17 helper T cells. *N Engl J Med* (2009) **361**:888–98. doi:10.1056/NEJMra0707449
41. Martinez GJ, Nurieva RI, Yang XO, Dong C. Regulation and function of proinflammatory TH17 cells. *Ann N Y Acad Sci* (2008) **1143**:188–211. doi:10.1196/annals.1443.021
42. Komatsu N, Takayanagi H. Autoimmune arthritis: the interface between the immune system and joints. *Adv Immunol* (2012) **115**:45–71. doi:10.1016/B978-0-12-394299-9.00002-3
43. Waisman A. T helper cell populations: as flexible as the skin? *Eur J Immunol* (2011) **41**:2539–43. doi:10.1002/eji.201141938
44. Jovanovic DV, Di Battista JA, Martel-Pelletier J, Jolicoeur FC, He Y, Zhang M, et al. IL-17 stimulates the production and expression of proinflammatory cytokines, IL-beta and TNF-alpha, by human macrophages. *J Immunol* (1998) **160**:3513–21.
45. Adamopoulos IE, Chao CC, Geissler R, Laface D, Blumenschein W, Iwakura Y, et al. Interleukin-17A upregulates receptor activator of NF-kappaB on osteoclast precursors. *Arthritis Res Ther* (2010) **12**:R29. doi:10.1186/ar2936
46. Nakae S, Suto H, Berry GJ, Galli SJ. Mast cell-derived TNF can promote Th17 cell-dependent neutrophil recruitment in ovalbumin-challenged OTII mice. *Blood* (2007) **109**:3640–8. doi:10.1182/blood-2006-09-046128
47. Chen DY, et al. Increasing levels of circulating Th17 cells and interleukin-17 in rheumatoid arthritis patients with an inadequate response to anti-TNF-alpha therapy. *Arthritis Res Ther* (2011) **13**:R126. doi:10.1186/ar3431
48. Sugita S, et al. Inhibition of Th17 differentiation by anti-TNF-alpha therapy in uveitis patients with Behcet's disease. *Arthritis Res Ther* (2012) **14**:R99. doi:10.1186/ar3824
49. Datta NS, Abou-Samra AB. PTH and PTHrP signaling in osteoblasts. *Cell Signal* (2009) **21**:1245–54. doi:10.1016/j.cellsig.2009.02.012
50. Li X, et al. Divergent requirement for Galphas and cAMP in the differentiation and inflammatory profile of distinct mouse Th subsets. *J Clin Invest* (2012) **122**:963–73. doi:10.1172/JCI59097
51. Hell JW. Beta-adrenergic regulation of the L-type Ca2+ channel Ca(V)1.2 by PKA rekindles excitement. *Sci Signal* (2010) **3**:e33. doi:10.1126/scisignal.3141pe33
52. Iwakura Y, Ishigame H, Saijo S, Nakae S. Functional specialization of interleukin-17 family members. *Immunity* (2011) **34**:149–62. doi:10.1016/j.immuni.2011.02.012
53. Zepp J, Wu L, Li X. IL-17 receptor signaling and T helper 17-mediated autoimmune demyelinating disease. *Trends Immunol* (2011) **32**:232–9. doi:10.1016/j.it.2011.02.007
54. Ye P, Rodriguez FH, Kanaly S, Stocking KL, Schurr J, Schwarzenberger P, et al. Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. *J Exp Med* (2001) **194**:519–27. doi:10.1084/jem.194.4.519
55. Pacifici R. Role of T cells in the modulation of PTH action: physiological and clinical significance. *Endocrine* (2013) **44**:576–82. doi:10.1007/s12020-013-9960-8
56. Nakashima T, Kobayashi Y, Yamasaki S, Kawakami A, Eguchi K, Sasaki H, et al. Protein expression and functional difference of membrane-bound and soluble receptor activator of NF-kappaB ligand: modulation of the expression by osteotropic factors and cytokines. *Biochem Biophys Res Commun* (2000) **275**:768–75. doi:10.1006/bbrc.2000.3379
57. Bedi B, Li JY, Tawfeek H, Baek KH, Adams J, Vangara SS, et al. Silencing of parathyroid hormone (PTH) receptor 1 in T cells blunts the bone anabolic activity of PTH. *Proc Natl Acad Sci U S A* (2012) **109**:E725–33. doi:10.1073/pnas.1120735109
58. D'Amelio P, Grimaldi A, Di Bella S, Brianza SZ, Cristofaro MA, Tamone C, et al. Estrogen deficiency increases osteoclastogenesis up-regulating T cells activity: a key mechanism in osteoporosis. *Bone* (2008) **43**:92–100. doi:10.1016/j.bone.2008.02.017
59. Adeel S, Singh K, Vydareny KH, Kumari M, Shah E, Weitzmann MN, et al. Bone loss in surgically ovariectomized premenopausal women is associated with T lymphocyte activation and thymic hypertrophy. *J Investig Med* (2013) **61**:1178–83. doi:10.231/JIM.0000000000000016
60. Eghbali-Fatourechi G, Khosla S, Sanyal A, Boyle WJ, Lacey DL, Riggs BL. Role of RANK ligand in mediating increased bone resorption in early postmenopausal women. *J Clin Invest* (2003) **111**:1221–30. doi:10.1172/JCI200317215
61. Taxel P, Kaneko H, Lee SK, Aguila HL, Raisz LG, Lorenzo JA. Estradiol rapidly inhibits osteoclastogenesis and RANKL expression in bone marrow cultures in postmenopausal women: a pilot study. *Osteoporos Int* (2008) **19**:193–9. doi:10.1007/s00198-007-0452-7
62. Pacifici R, Rifas L, McCracken R, Vered I, McMurtry C, Avioli LV, et al. Ovarian steroid treatment blocks a postmenopausal increase in blood monocyte interleukin 1 release. *Proc Natl Acad Sci U S A* (1989) **86**:2398–402. doi:10.1073/pnas.86.7.2398
63. Pacifici R, Brown C, Puscheck E, Friedrich E, Slatopolsky E, Maggio D, et al. Effect of surgical menopause and estrogen replacement on cytokine release from human blood mononuclear cells. *Proc Natl Acad Sci U S A* (1991) **88**:5134–8. doi:10.1073/pnas.88.12.5134
64. Cohen-Solal ME, Graulet AM, Denne MA, Gueris J, Baylink D, de Vernejoul MC. Peripheral monocyte culture supernatants of menopausal women can induce bone resorption: involvement of cytokines. *J Clin Endocrinol Metab* (1993) **77**:1648–53. doi:10.1210/jcem.77.6.8263153
65. Cohen-Solal ME, Graulet AM, Gueris J, Denne MA, Bergot C, Morieux C, et al. Bone resorption at the femoral neck is dependent on local factors in nonosteoporotic late postmenopausal women: an in vitro-in vivo study. *J Bone Miner Res* (1995) **10**:307–14. doi:10.1002/jbmr.5650100219
66. Bernard-Poenaru O, Roux C, Blanqué R, Gardner C, de Vemejoul MC, Cohen-Solal ME. Bone-resorbing cytokines from peripheral blood mononuclear cells after hormone replacement therapy: a longitudinal study. *Osteoporos Int* (2001) **12**:769–76. doi:10.1007/s001980170054
67. Charatcharoenwitthaya N, et al. Effect of blockade of tumor necrosis factor-alpha and interleukin-1 action on bone resorption in early postmenopausal women. *J Bone Miner Res* (2007) **22**(5):724–9. doi:10.1359/jbmr.070207
68. Leonardi C, Matheson R, Zachariae C, Cameron G, Li L, Edson-Heredia E, et al. Anti-interleukin-17 monoclonal antibody ixekizumab in chronic plaque psoriasis. *N Engl J Med* (2012) **366**:1190–9. doi:10.1056/NEJMoa1109997
69. Mease PJ, Genovese MC, Greenwald MW, Ritchlin CT, Beaulieu AD, Deodhar A, et al. Brodalumab, an anti-IL17RA monoclonal antibody, in psoriatic arthritis. *N Engl J Med* (2014) **370**:2295–306. doi:10.1056/NEJMoa1315231
70. Yeremenko N, Paramarta JE, Baeten D. The interleukin-23/interleukin-17 immune axis as a promising new target in the treatment of spondyloarthritis. *Curr Opin Rheumatol* (2014) **26**:361–70. doi:10.1097/BOR.0000000000000069
71. Martin DA, Churchill M, Flores-Suarez L, Cardiel MH, Wallace D, Martin R, et al. A phase Ib multiple ascending dose study evaluating safety, pharmacokinetics, and early clinical response of brodalumab, a human anti-IL-17R antibody, in methotrexate-resistant rheumatoid arthritis. *Arthritis Res Ther* (2013) **15**:R164. doi:10.1186/ar4347

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Osteoimmune Interactions in Inflammatory Bowel Disease: Central Role of Bone Marrow Th17 TNFα Cells in Osteoclastogenesis

Abdelilah Wakkach[1,2], Matthieu Rouleau[1,2] and Claudine Blin-Wakkach[1,2]*

[1] CNRS, UMR 7370, Laboratoire de PhysioMédecine Moléculaire (LP2M), Faculté de Médecine, Nice, France, [2] University Nice Sophia Antipolis, Nice, France

Osteoimmunology is an interdisciplinary research field dedicated to the study of the crosstalk between the immune and bone systems. CD4+ T cells are central players in this crosstalk. There is an emerging understanding that CD4+ T cells play an important role in the bone marrow (BM) under physiological and pathological conditions and modulate the differentiation of bone-resorbing osteoclasts. However, identification of the mechanisms that maintain CD4+ T cells in the BM is still a matter of investigation. This article describes the CD4+ T cell populations of the BM and reviews their role as osteoclastogenic population in inflammatory bowel disease.

Keywords: Th17 Cells, osteoclasts, bone marrow, inflammatory bowel diseases, osteoporosis, osteoimmunology

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****Correspondence:***
Abdelilah Wakkach
wakkach@unice.fr

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Bone marrow (BM) has long been known to play an important role in the immune system as a central hematopoietic organ. However, its function in T cell-mediated tolerance or immunity remains elusive. In the past, most of immunological studies were focused on T and B cells in the thymus, the lymph nodes, and the spleen considered as the central reservoirs of adaptive immunity. Over time, it became apparent that the immune system has a far more decentralized governing function. Recently, the BM was shown to play several unexpected roles, serving as an important reservoir for memory T cells including pathogenic cells and long-lived plasma cells involved in the maintenance of long-term immunity and pathogenicity.

BONE MARROW RESIDENT CD4+ T CELLS

Memory CD4+ T cells provide rapid and highly effective protective immunity against previously encountered pathogens and can recognize a wide variety of antigens. The concept of systemic memory consists of two major subsets: central memory T (T_{CM}) cells and effector memory T (T_{EM}) cells. Importantly, this identification was done in the blood (1). T_{CM} cells express the chemokine receptor CCR7 and the vascular addressing L selectin (CD62L), which enables them to migrate from the blood to the lymph nodes. T_{EM} cells express low levels of CCR7 and CD62L but have receptors that allow them to access peripheral tissues as, for example, the E-selectin ligand cutaneous lymphocyte antigen (CLA), which grants them access to the skin, and α4β7, an integrin that allows them access to the gut. These memory T cells are called tissue-resident memory T cells (T_{RM}) (2).

In the BM, T cells represent about 3–8% of total nucleated cells. BM T cells reach the BM from the blood and, after homing to the BM, can move back to the blood to migrate to other lymphoid organs (3). The rules governing cell migration to the BM have been investigated mainly

in the case of hematopoietic stem cells (HSCs) and revealed the dominant role of the CXCR4–CXCL12 axis in this migration. Analysis of $CD4^+$ T cells from the BM of normal mice or mice affected with inflammatory bowel diseases (IBDs) showed the presence of both T_{CM} and T_{EM} cells that are characterized by a high expression of the chemokine receptors CXCR4 and CCR6 (4, 5). However, the mechanism of recruitment of these T cells in the BM remains elusive. Moreover, the degree to which memory cells are resident (T_{RM}) within the BM versus transiting through it is less clear.

In order for T_{RM} cells to be maintained in the BM, they must adapt to the local environment and ignore signals that may induce their egress outside of the BM. The mechanisms and cells involved in the maintenance of BM T cells represent a matter of active research. BM stroma includes mesenchymal stromal cells (MSCs), endothelial cells, osteoblasts (OBLs), and adipocytes. This stromal cell heterogeneity complicates the understanding of the implication of these cells in the maintenance of immunological memory. It is well recognized that BM stromal cells support hematopoiesis by establishing specialized niches. These niches regulate the fate of HSCs in terms of quiescence, migration, and differentiation (6). The major components of the HSC niches include several MSC populations [CXC12-abundant reticular (CAR) cells and $Nestin^+$ cells] (7), OBLs (8, 9), and endothelial cells (10, 11). In addition, regulatory T cells (12), macrophages (13), and osteoclasts (OCLs) (14, 15) were shown to contribute to the regulation of the HSC niches. MSCs have also been involved in the retention of T cells in the BM. Tokoyoda et al. showed that memory $CD4^+$ T cells are located close to BM stromal cells expressing IL-7 and VCAM1 (16). This was confirmed by Nemoto et al. who reported that in IBD, pathogenic $CD4^+$ T cells are retained in the BM through interactions with IL-7-producing MSCs (17). However, the exact nature of the $VCAM1^+$ and $IL\text{-}7^+$ stromal cells and their role in the maintaining of memory $CD4^+$ T cells remain to be elucidated. Adoptive transfer of B and T lymphocytes in mice led to seeding of dendritic cell (DC) clusters with grafted cells in perivascular domains, which were referred to as BM immune niches (18). Overall, these observations suggested the existence of $CD4^+$ T cell niches that remain to be characterized in term of cell composition and regulation. The identification of BM immune niches raises many new questions. Which molecules regulate T cell migration? How are memory $CD4^+$ T cells maintained and for how long? How do they interact with BM stroma under physiological and inflammatory conditions? The identification of BM-specific factors that control T cell functions is likely to have a major impact on translational medicine.

CROSSTALK BETWEEN $CD4^+$ T CELLS AND BONE CELLS

The crosstalk between the immune and bone systems led to the emergence of an interdisciplinary field called osteoimmunology (19). This field emerged from clinical observations reporting that patients presenting an overactivation of the immune system, such as chronic infections and chronic inflammatory diseases, also display bone loss (20–22). The importance of this crosstalk was further confirmed with the identification of the role of activated $CD4^+$ T cells in pathological osteoclastogenesis (23). Its scope has been extended to encompass a wide range of molecular and cellular interactions, including those between immune cells and bone cells, and between bone cells and hematopoietic cells. These interactions are essential for the development of the immune and bone systems (15, 24).

Bone remodeling is a highly regulated process involving complex interactions between the activity of the bone-forming OBLs and the activity of the bone-resorbing OCLs. OCLs are multinucleated cells from the myeloid lineage (monocytes and DCs) (25) that degrade the bone matrix and release growth factors that contribute to the coupling between OCLs and OBLs (26). The mesenchymal-derived OBLs migrate to the eroded sites and initiate new bone formation by the secretion of an extracellular matrix and its calcification. In a physiological state, bone remodeling provides an adequate environment for the development of the immune system and the protection of HSCs (6).

The BM represents a reservoir for memory T cells among which 25% are $Foxp3^+$ regulatory T cells (27). It is also a preferential site for the migration or the selective retention and function of regulatory T cells. This finding provides evidence for an unidentified role of the BM in T-cell homeostasis. Moreover, Tokoyoda et al. have shown that in adult mice, more than 80% of antigen specific memory $CD4^+$ T cells rest in the BM for more than 90 days after immunization and do not proliferate (16). From these observations, we can hypothesize that the immunosuppressive activity of BM regulatory T cells participates in the quiescence of memory T cells as recently shown for $CD8^+$ memory T cells (28). In this study, regulatory T cells suppress proliferation and effector programs during the memory differentiation of $CD8^+$ T cells in the lymph nodes (28). In addition to regulatory T cells, MSCs are also generally considered as immunosuppressive cells. MSCs may suppress T-lymphocyte proliferation and functions both *in vitro* and *in vivo* (29, 30). They produce soluble factors, including TGF-β, able to mediate suppression of T-cell proliferation (31). MSCs can also inhibit T cell proliferation by increasing IL-10 secretion (31). These properties suggest that the immunosuppressive effect of MSCs may participate to the preservation of $CD4^+$ memory T cells in the BM. However, MSCs are a heterogeneous population of cells and their immune suppressive activity has mainly been explored in pathological conditions. It is therefore important to better understand this function to clarify how MSCs may control the quiescence and niche of $CD4^+$ T cells.

To date, the role of $CD4^+$ regulatory T cells and memory T cells on bone cells in physiological conditions remains controversial. It has been reported that $Rag1^{-/-}$ mice lacking T cells have a normal bone phenotype (32), whereas T cell-deficient nude mice display, with age, an increased bone resorption and a decreased bone mineral density (33). T cells are capable of mediating anti-osteoclastogenic signals, as depletion of CD4 and CD8 T lymphocytes in mice *in vivo* enhances vitamin D3-stimulated OCL formation by blocking the production of osteoprotegerin (OPG) by B cells (34). These observations revealed the difficulty to conclude on the role of $CD4^+$ T cells in steady state osteoclastogenesis because they can have direct and indirect effects on

OCL precursors and also because of their heterogeneity. Indeed, Th1, Th2, and Th17 cells have been reported to have opposite effects on OCL differentiation *in vitro*. Th1 and Th2 cells inhibit OCL formation through their production of INF-γ and IL-4, respectively, whereas Th17 cells have an osteoclastogenic helper effect *in vitro* mediated by MSCs (35).

OSTEOIMMUNOLOGY INTERACTIONS IN INFLAMMATORY BOWEL DISEASE

Inflammatory bowel diseases are inflammatory diseases that affect the gastrointestinal tract. There are two main clinical forms of IBD: Crohn's disease which affects any part of gastrointestinal tract and ulcerative colitis in which the pathology mostly affects the colonic mucosa (36). Several factors including genetic factors, gut microbiota, and the host immune system have been described to contribute to IBD (36). Moreover, these diseases are often associated with extra-intestinal manifestations, in particular, bone loss. Indeed, more than 40% of patients with IBD also present bone loss that remains a major extra-intestinal cause of morbidity leading to an impaired quality of life and productivity (37). The prevalence of osteopenia and osteoporosis in patients presenting with IBD ranges from 22–77% to 17–41%, respectively, depending on the population location or the study design. According to the WHO, osteoporosis is defined as a systemic skeletal disease that occurs when bone resorption exceeds bone formation. In low-turnover osteoporosis, bone resorption is normal whereas the synthesis of bone tissue is reduced. In contrast, in high-turnover osteoporosis, the activity of OCL is increased. Osteoporosis associated with chronic inflammation usually follows the high-turnover pattern, whereas corticosteroid-induced osteoporosis is usually of the low-turnover pattern (38).

The association between chronic inflammation and bone destruction has been recognized a long time ago, leading to the hypothesis of the participation of immune cells in the control of bone remodeling (19). A seminal work published in 1999 by Kong et al. established the role of $CD4^+$ T cells in inflammatory osteoclastogenesis. In this study, the authors demonstrated that activated $CD4^+$ T cells produce RANKL and induce the differentiation of OCLs *in vitro* (23). Besides that, $ctla4^{-/-}$ mice, in which T cells are spontaneously activated, displayed a severe osteoporosis. Interestingly, transfer of $CD4^+$ T cells from $ctla4^{-/-}$ mice into lymphocyte-deficient $rag1^{-/-}$ mice caused bone destruction that can be reversed through inhibition of RANKL by OPG (23). One additional study confirmed these observations and identified a common causal link between intestinal inflammation and bone loss showing that activated T cells producing RANKL accumulate in the BM during intestinal inflammation (39). Taken together, these studies suggest that pathogenic $CD4^+$ T cells present in the BM during IBD are potentially osteoclastogenic.

Using a mouse model of colitis, induced in SCID mice by injection of $CD4^+CD45RB^{high}$ naive T cells, Nemoto et al. found that a large number of $CD4^+CD44^+CD62L^-$ memory T cells resides in the BM. Transfer of BM memory $CD4^+$ T cells into new recipient SCID mice reproduced colitis. These BM $CD4^+$ cells of colitic mice have therefore been described as "colitogenic memory T cells" (5). Importantly, these resident BM $CD4^+$ memory T cells are closely associated with IL-7-producing stromal cells, and they cannot induce colitis when transferred into $IL\text{-}7^{-/-} \times Rag1^{-/-}$ mice, suggesting that IL-7 plays an essential role in their maintenance or survival in the BM (5). Recently, the same group has demonstrated that BM MSCs are the major source of IL-7 and play a pathological role in IBD by forming the niche for these colitogenic $CD4^+$ memory T cells (17). However, their osteoclastogenic effect has not been explored.

Although IBD has traditionally been assumed to be a Th1-dependent disease, there is controversy over the role of Th1 on bone homeostasis. IFN-γ has been shown to directly inhibit osteoclastogenesis by interfering with the RANKL–RANK signaling pathway (40). Moreover, *in vitro* differentiated $CD4^+$ Th1 cells have been shown to inhibit OCL formation through their canonical production of IFN-γ (35). In contrast, observations in humans suggested that IFN-γ may promote osteoclastogenesis because it improves bone resorption in osteopetrotic patients treated with IFN-γ (41). IFN-γ is also a physiologic inducer of MHC class II expression by APCs resulting in the activation of T cells that induce bone resorption by their secretion of RANKL and TNF-α (42). Together, these data suggest that IFN-γ inhibits OCL formation through direct targeting of maturing OCLs, while it promotes osteoclastogenesis indirectly by stimulating T cell activation.

Nowadays, it is well known that the inflamed gastrointestinal mucosa of patients with IBD is massively infiltrated with Th17 cells and that Th17-related cytokines are produced in excess (43). Based on this, it is obvious that Th17 cells play an important role in the pathogenesis of IBD, which was previously solely attributed to Th1 cells. However, although a pathogenic role in intestine inflammation has been ascribed to Th17 cells, administration of neutralizing anti-IL-17A antibody to patients with Crohn's disease did not show any therapeutic benefit (44). Moreover, in some patients, it exacerbated the disease suggesting a protective role of IL-17A (44). Thus, despite the role of Th17 cytokines is important in many aspects of intestinal homeostasis and protection from mucosal pathogens, their role in the pathogenesis of IBD remains ambiguous. However, IL-17 represents a potent osteoclastogenic cytokine, and its receptors are expressed by many cell types, including OBLs and OCLs (45). Kotake et al. reported that IL-17 is abundant in rheumatoid synovial fluid and that IL-17 stimulates OCL differentiation by inducing RANKL expression by OBLs (46). It should be noted that the effect of IL-17 is not limited to this direct action on the OBLs. IL-17 facilitates local inflammation by recruiting and activating immune cells, which leads to an abundance of inflammatory cytokines such as TNF-α (46). IL-17-deficient mice are resistant to bone destruction induced by LPS (35). Otherwise, Oostlander et al. suggested a particular role for IL-17 in osteoclastogenesis in Crohn's disease patients (47). More recently, we have shown that BM Th17 T cells expressing high levels of TNFα were able to induce OCL differentiation in IBD mice. This T-cell-induced OCL differentiation could be inhibited by IL-17 blockade (4) suggesting the importance of IL-17A in osteoporosis.

A picture emerged from the literature (48, 49) to define what we believe to be osteoclastogenic T cells: first, osteoclastogenic T cells should not produce a large amount of IFN-γ. Second, they should trigger local inflammation and the production of inflammatory cytokines, including TNF-α, that induce RANK-L expression on MSCs. Third, they should express RANK-L and might directly participate in the increase of osteoclastogenesis.

Recent data indicate that in IBD, Th17 cells-producing TNF-α represent the long-sought-after osteoclastogenic T cell subset that fulfills all the criteria mentioned above (4, 50). In murine models of IBD associated with bone loss, we have shown that Th17-producing TNF-α cells accumulate in the BM, the colon, and spleen and have a potent capacity to induce OCL differentiation without addition of any exogenic osteoclastogenic factors (4). Through their production of RANK-L and TNFα, Th17 cells directly induce the differentiation of precursors into OCLs (4), but they also have a major effect on MSCs through their production of IL-17. Indeed, IL-17 increases RANK-L expression in MSCs leading to increased OCL formation (4, 35, 46). Moreover, in the context of IBD, Th17 cells increase the expression of monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein 1α (MIP-1α) by MSCs, which may promote the recruitment of inflammatory monocytes (OCL precursors) in the BM and their differentiation into OCLs (4). To translate these finding into human disease, IL-17-producing T cells from the blood of IBD patients are osteoclastogenic cells *in vitro* and increase MIP-1α and MCP-1 expression by human MSCs (4, 47), suggesting their participation to osteoporosis in these patients. Therefore, Th17 cells represent a key target for innovative therapeutic approaches for IBD-associated bone destruction.

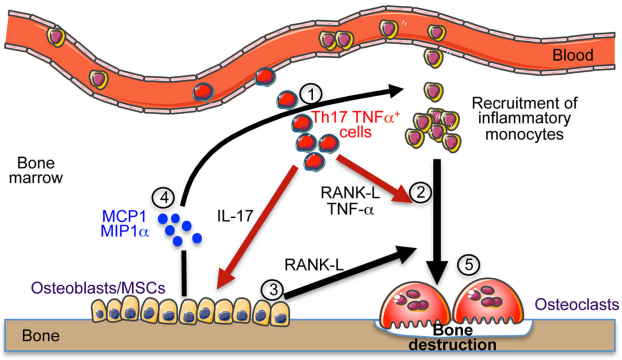

FIGURE 1 | Th17 cells induce bone destruction in IBD. In IBD, Th17 TNFα cells migrate and accumulate in the bone marrow (1). They express RANK-L and TNF-α that participate to osteoclast differentiation (2). They also express IL-17 that stimulates the local inflammation and activates osteoblasts to produce RANK-L inducing osteoclast formation (3) and MCP-1 and MIP-1α chemokines (4) participating to the recruitment of osteoclast precursor cells (monocytes) in the BM that contribute to the increased osteoclastogenesis (5) and to bone destruction.

CONCLUDING REMARKS

Recent advances have contributed to our understanding of the biology of CD4+ T cells in the BM. These T cells fulfill both homeostatic and pathological functions with respect to the bone system. IBD is an immune-mediated disease characterized by systemic Th1 and Th17 responses and bone destruction. Recent studies have revealed that Th17 cells are not only required for the initiation of systemic immune response as they are critical regulators in the chronic inflammation associated with bone destruction, particularly in rheumatoid arthritis. Our recent findings extend these observation to IBD in which the site of inflammation is far from the bone and provide the first characterization of osteoclastogenic Th17 TNF-α+ cells in the BM linking IBD and bone destruction.

Collectively, we propose that these osteoclastogenic cells, once in the BM, enable the secretion of chemokines and RANKL by stromal cells. This enhances the recruitment of inflammatory monocytes and DCs that differentiate into OCLs and increase the bone resorption leading to osteoporosis as illustrated in **Figure 1**.

AUTHOR CONTRIBUTIONS

AW wrote and reviewed the manuscript. CB-W and MR reviewed the manuscript.

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REFERENCES

1. Sallusto F, Lenig D, Förster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* (1999) **401**:708–12. doi:10.1038/44385
2. Mueller SN, Gebhardt T, Carbone FR, Heath WR. Memory T cell subsets, migration patterns, and tissue residence. *Annu Rev Immunol* (2013) **31**:137–61. doi:10.1146/annurev-immunol-032712-095954
3. Di Rosa F, Pabst R. The bone marrow: a nest for migratory memory T cells. *Trends Immunol* (2005) **26**:360–6. doi:10.1016/j.it.2005.04.011
4. Ciucci T, Ibáñez L, Boucoiran A, Birgy-Barelli E, Pène J, Abou-Ezzi G, et al. Bone marrow Th17 TNFα cells induce osteoclast differentiation, and link bone destruction to IBD. *Gut* (2015) **64**:1072–81. doi:10.1136/gutjnl-2014-306947
5. Nemoto Y, Kanai T, Makita S, Okamoto R, Totsuka T, Takeda K, et al. Bone marrow retaining colitogenic CD4+ T cells may be a pathogenic reservoir for chronic colitis. *Gastroenterology* (2007) **132**:176–89. doi:10.1053/j.gastro.2006.10.035
6. Scadden DT. Nice neighborhood: emerging concepts of the stem cell niche. *Cell* (2014) **157**:41–50. doi:10.1016/j.cell.2014.02.013
7. Schajnovitz A, Itkin T, D'Uva G, Kalinkovich A, Golan K, Ludin A, et al. CXCL12 secretion by bone marrow stromal cells is dependent on cell contact and mediated by connexin-43 and connexin-45 gap junctions. *Nat Immunol* (2011) **12**:391–8. doi:10.1038/ni.2017

8. Calvi LM, Adams GB, Weibrecht KW, Weber JM, Olson DP, Knight MC, et al. Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature* (2003) **425**:841–6. doi:10.1038/nature02040
9. Zhang J, Niu C, Ye L, Huang H, He X, Tong W-G, et al. Identification of the haematopoietic stem cell niche and control of the niche size. *Nature* (2003) **425**:836–41. doi:10.1038/nature02041
10. Butler JM, Nolan DJ, Vertes EL, Varnum-Finney B, Kobayashi H, Hooper AT, et al. Endothelial cells are essential for the self-renewal and repopulation of Notch-dependent hematopoietic stem cells. *Cell Stem Cell* (2010) **6**:251–64. doi:10.1016/j.stem.2010.02.001
11. Ding L, Saunders TL, Enikolopov G, Morrison SJ. Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature* (2012) **481**:457–62. doi:10.1038/nature10783
12. Fujisaki J, Wu J, Carlson AL, Silberstein L, Putheti P, Larocca R, et al. In vivo imaging of Treg cells providing immune privilege to the haematopoietic stem-cell niche. *Nature* (2011) **474**:216–9. doi:10.1038/nature10160
13. Chow A, Lucas D, Hidalgo A, Méndez-Ferrer S, Hashimoto D, Scheiermann C, et al. Bone marrow CD169+ macrophages promote the retention of hematopoietic stem and progenitor cells in the mesenchymal stem cell niche. *J Exp Med* (2011) **208**:261–71. doi:10.1084/jem.20101688
14. Kollet O, Dar A, Shivtiel S, Kalinkovich A, Lapid K, Sztainberg Y, et al. Osteoclasts degrade endosteal components and promote mobilization of hematopoietic progenitor cells. *Nat Med* (2006) **12**:657–64. doi:10.1038/nm1417
15. Mansour A, Abou-Ezzi G, Sitnicka E, Jacobsen SEW, Wakkach A, Blin-Wakkach C. Osteoclasts promote the formation of hematopoietic stem cell niches in the bone marrow. *J Exp Med* (2012) **209**:537–49. doi:10.1084/jem.20110994
16. Tokoyoda K, Zehentmeier S, Hegazy AN, Albrecht I, Grün JR, Löhning M, et al. Professional memory CD4+ T lymphocytes preferentially reside and rest in the bone marrow. *Immunity* (2009) **30**:721–30. doi:10.1016/j.immuni.2009.03.015
17. Nemoto Y, Kanai T, Takahara M, Oshima S, Nakamura T, Okamoto R, et al. Bone marrow-mesenchymal stem cells are a major source of interleukin-7 and sustain colitis by forming the niche for colitogenic CD4 memory T cells. *Gut* (2013) **62**:1142–52. doi:10.1136/gutjnl-2012-302029
18. Sapoznikov A, Pewzner-Jung Y, Kalchenko V, Krauthgamer R, Shachar I, Jung S. Perivascular clusters of dendritic cells provide critical survival signals to B cells in bone marrow niches. *Nat Immunol* (2008) **9**:388–95. doi:10.1038/ni1571
19. Arron JR, Choi Y. Osteoimmunology: bone versus immune system. *Nature* (2000) **408**:535–6. doi:10.1038/35046196
20. Stellon AJ, Davies A, Compston J, Williams R. Bone loss in autoimmune chronic active hepatitis on maintenance corticosteroid therapy. *Gastroenterology* (1985) **89**:1078–83.
21. Feldmann M, Brennan FM, Maini RN. Role of cytokines in rheumatoid arthritis. *Annu Rev Immunol* (1996) **14**:397–440. doi:10.1146/annurev.immunol.14.1.397
22. Piepkorn B, Kann P, Forst T, Andreas J, Pfützner A, Beyer J. Bone mineral density and bone metabolism in diabetes mellitus. *Horm Metab Res* (1997) **29**:584–91. doi:10.1055/s-2007-979106
23. Kong Y-Y, Feige U, Sarosi I, Bolon B, Tafuri A, Morony S, et al. Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. *Nature* (1999) **402**:304–9. doi:10.1038/46303
24. Blin-Wakkach C, Wakkach A, Quincey D, Carle GF. Interleukin-7 partially rescues B-lymphopoiesis in osteopetrotic oc/oc mice through the engagement of B220+ CD11b+ progenitors. *Exp Hematol* (2006) **34**:851–9. doi:10.1016/j.exphem.2006.04.003
25. Wakkach A, Mansour A, Dacquin R, Coste E, Jurdic P, Carle GF, et al. Bone marrow microenvironment controls the in vivo differentiation of murine dendritic cells into osteoclasts. *Blood* (2008) **112**:5074–83. doi:10.1182/blood-2008-01-132787
26. Tang Y, Wu X, Lei W, Pang L, Wan C, Shi Z, et al. TGF-beta1-induced migration of bone mesenchymal stem cells couples bone resorption with formation. *Nat Med* (2009) **15**:757–65. doi:10.1038/nm.1979
27. Zou L, Barnett B, Safah H, Larussa VF, Evdemon-Hogan M, Mottram P, et al. Bone marrow is a reservoir for CD4+CD25+ regulatory T cells that traffic through CXCL12/CXCR4 signals. *Cancer Res* (2004) **64**:8451–5. doi:10.1158/0008-5472.CAN-04-1987
28. Kalia V, Penny LA, Yuzefpolskiy Y, Baumann FM, Sarkar S. Quiescence of memory CD8(+) T cells is mediated by regulatory T cells through inhibitory receptor CTLA-4. *Immunity* (2015) **42**:1116–29. doi:10.1016/j.immuni.2015.05.023
29. Nauta AJ, Fibbe WE. Immunomodulatory properties of mesenchymal stromal cells. *Blood* (2007) **110**:3499–506. doi:10.1182/blood-2007-02-069716
30. Anderson P, Souza-Moreira L, Morell M, Caro M, O'Valle F, Gonzalez-Rey E, et al. Adipose-derived mesenchymal stromal cells induce immunomodulatory macrophages which protect from experimental colitis and sepsis. *Gut* (2013) **62**:1131–41. doi:10.1136/gutjnl-2012-302152
31. Sioud M. New insights into mesenchymal stromal cell-mediated T-cell suppression through galectins. *Scand J Immunol* (2011) **73**:79–84. doi:10.1111/j.1365-3083.2010.02491.x
32. Anginot A, Dacquin R, Mazzorana M, Jurdic P. Lymphocytes and the Dap12 adaptor are key regulators of osteoclast activation associated with gonadal failure. *PLoS One* (2007) **2**:e585. doi:10.1371/journal.pone.0000585
33. Li Y, Toraldo G, Li A, Yang X, Zhang H, Qian W-P, et al. B cells and T cells are critical for the preservation of bone homeostasis and attainment of peak bone mass in vivo. *Blood* (2007) **109**:3839–48. doi:10.1182/blood-2006-07-037994
34. Grcević D, Lee SK, Marusić A, Lorenzo JA. Depletion of CD4 and CD8 T lymphocytes in mice in vivo enhances 1,25-dihydroxyvitamin D3-stimulated osteoclast-like cell formation in vitro by a mechanism that is dependent on prostaglandin synthesis. *J Immunol* (2000) **165**:4231–8.
35. Sato K, Suematsu A, Okamoto K, Yamaguchi A, Morishita Y, Kadono Y, et al. Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction. *J Exp Med* (2006) **203**:2673–82. doi:10.1084/jem.20061775
36. Maloy KJ, Powrie F. Intestinal homeostasis and its breakdown in inflammatory bowel disease. *Nature* (2011) **474**:298–306. doi:10.1038/nature10208
37. Tilg H, Moschen AR, Kaser A, Pines A, Dotan I. Gut, inflammation and osteoporosis: basic and clinical concepts. *Gut* (2008) **57**:684–94. doi:10.1136/gut.2006.117382
38. Raisz LG. Pathogenesis of osteoporosis: concepts, conflicts, and prospects. *J Clin Invest* (2005) **115**:3318–25. doi:10.1172/JCI27071
39. Ashcroft AJ, Cruickshank SM, Croucher PI, Perry MJ, Rollinson S, Lippitt JM, et al. Colonic dendritic cells, intestinal inflammation, and T cell-mediated bone destruction are modulated by recombinant osteoprotegerin. *Immunity* (2003) **19**:849–61. doi:10.1016/S1074-7613(03)00326-1
40. Takayanagi H, Ogasawara K, Hida S, Chiba T, Murata S, Sato K, et al. T-cell-mediated regulation of osteoclastogenesis by signalling cross-talk between RANKL and IFN-gamma. *Nature* (2000) **408**:600–5. doi:10.1038/35046102
41. Key LL, Rodriguiz RM, Willi SM, Wright NM, Hatcher HC, Eyre DR, et al. Long-term treatment of osteopetrosis with recombinant human interferon gamma. *N Engl J Med* (1995) **332**:1594–9. doi:10.1056/NEJM199506153322402
42. Gao Y, Grassi F, Ryan M, Pacifici R. IFN-gamma stimulates osteoclast formation and bone loss in vivo via antigen-driven T cell activation. *J Clin Invest* (2007) **117**(1):122–32. doi:10.1172/JCI30074
43. Ray S, De Salvo C, Pizarro TT. Central role of IL-17/Th17 immune responses and the gut microbiota in the pathogenesis of intestinal fibrosis. *Curr Opin Gastroenterol* (2014) **30**:531–8. doi:10.1097/MOG.0000000000000119
44. Hueber W, Sands BE, Lewitzky S, Vandemeulebroecke M, Reinisch W, Higgins PDR, et al. Secukinumab, a human anti-IL-17A monoclonal antibody, for moderate to severe Crohn's disease: unexpected results of a randomised, double-blind placebo-controlled trial. *Gut* (2012) **61**:1693–700. doi:10.1136/gutjnl-2011-301668
45. Van bezooijen RL, Farih-Sips HC, Papapoulos SE, Löwik CW. Interleukin-17: a new bone acting cytokine in vitro. *J Bone Miner Res* (1999) **14**:1513–21. doi:10.1359/jbmr.1999.14.9.1513
46. Kotake S, Udagawa N, Takahashi N, Matsuzaki K, Itoh K, Ishiyama S, et al. IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis. *J Clin Invest* (1999) **103**:1345–52. doi:10.1172/JCI5703
47. Oostlander AE, Everts V, Schoenmaker T, Bravenboer N, van Vliet SJ, van Bodegraven AA, et al. T cell-mediated increased osteoclast formation from

peripheral blood as a mechanism for Crohn's disease-associated bone loss. *J Cell Biochem* (2012) **113**:260–8. doi:10.1002/jcb.23352
48. Takayanagi H. Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems. *Nat Rev Immunol* (2007) **7**:292–304. doi:10.1038/nri2062
49. Jones D, Glimcher LH, Aliprantis AO. Osteoimmunology at the nexus of arthritis, osteoporosis, cancer, and infection. *J Clin Invest* (2011) **121**:2534–42. doi:10.1172/JCI46262
50. Syrbe U, Siegmund B. Bone marrow Th17 TNFα cells induce osteoclast differentiation and link bone destruction to IBD. *Gut* (2015) **64**:1011–2. doi:10.1136/gutjnl-2014-308436

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HYPOTHESIS AND THEORY
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A T Cell View of the Bone Marrow

Adriana Bonomo[1,2], Ana Carolina Monteiro[2], Triciana Gonçalves-Silva[2,3], Eric Cordeiro-Spinetti[4], Rômulo Gonçalves Galvani[1,2,5] and Alex Balduino[4,6]*

[1] Cancer Program (Fio-Cancer), Oswaldo Cruz Foundation, Rio de Janeiro, Brazil, [2] Laboratory on Thymus Research, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil, [3] Immunology and Inflammation Graduate Program, Paulo de Góes Microbiology Institute, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil, [4] Cell Biology and Technology Laboratory, Veiga de Almeida University, Rio de Janeiro, Brazil, [5] Microbiology Graduate Program, Paulo de Góes Microbiology Institute, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil, [6] Excellion Laboratory, Amil/UnitedHealth Group, Petrópolis, Brazil

The majority of T cells present in the bone marrow (BM) represent an activated/memory phenotype and most of these, if not all, are circulating T cells. Their lodging in the BM keeps them activated, turning the BM microenvironment into a "memory reservoir." This article will focus on how T cell activation in the BM results in both direct and indirect effects on the hematopoiesis. The hematopoietic stem cell niche will be presented, with its main components and organization, along with the role played by T lymphocytes in basal and pathologic conditions and their effect on the bone remodeling process. Also discussed herein will be how "normal" bone mass peak is achieved only in the presence of an intact adaptive immune system, with T and B cells playing critical roles in this process. Our main hypothesis is that the partnership between T cells and cells of the BM microenvironment orchestrates numerous processes regulating immunity, hematopoiesis, and bone remodeling.

Keywords: T cells, bone remodeling, hematopoiesis, B cells, osteoclast, osteoblast

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Viktor Umansky, German Cancer Research Center (DKFZ), Germany

****Correspondence:***
Adriana Bonomo
acbonomo@gmail.com, adriana.bonomo@fiocruz.br

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The presence of T cells inside the bone marrow (BM) cavity has been reported mostly for central memory T cells (Tcm). It is suggested that hematopoietic stem cells (HSCs) niches serve as "hubs" for optimal T cell maintenance, as T cells can survive in the absence of antigen, in an environment rich in interleukin-7 (IL-7) and IL-15 (1), and other mediators believed to be important to T cell memory maintenance (2). Naive T cells can also be primed, get activated, and develop into effector cells inside the BM. Altogether, the BM can be seen as a primary lymphoid organ, which supports primary and secondary T cell responses (3). However, in the BM, the hematopoietic and the bone remodeling systems share the same microenvironment, together with T cells, which come from the periphery. These three components are maintained in equilibrium under homeostatic conditions. Nonetheless, when T cells are activated, this equilibrium is disrupted, and the result depends on the class of T cell response and its impact on hematopoiesis and bone remodeling.

The next sections will describe and discuss the interactions of BM T cells with the hematopoietic and bone remodeling systems. The first section starts describing the development and organization of the BM hematopoietic microenvironment, discussing the perivascular and endosteal niches and their players. In the second section, the role of T cell on shaping hematopoiesis and the correlation between the class of the adaptive response and type of innate immunity stimulated by specific T cells is presented as a cooperative ensemble. In the third section, T cell influence on bone remodeling is described, as well as the T cell-mediated interactions occurring either in physiological conditions or in two bone pathologies (arthritis and cancer metastasis). The main molecular axis that controls bone remodeling, RANK–RANK-L–OPG, is shared with T and B cells, and is discussed as the molecular basis for functional interaction between the skeletal and the adaptive immune systems. Finally, in

the fourth section, T cell activities on both hematopoietic and bone remodeling systems are presented and the ability of T cells to orchestrate both systems is discussed according to the class of the response.

HEMATOPOIETIC STEM CELL NICHE IN THE BM: CLOSE TO THE BONE SURFACE, NOT FAR FROM THE BLOOD VESSEL

Establishment of the HSC Niche in the BM

During embryo development, first HSCs emerge in the blood islands in the extra-embryonic mesoderm of the yolk sac (4–6). Classified as a primitive population of HSCs; HSCs differentiate into nucleated erythrocytes and monocyte-like myeloid cells. In a second wave of hematopoiesis, termed definitive hematopoietic, HSCs emerge from the intra-embryonic mesoderm of the para-aortic-splanchnopleura/aorta-gonad-mesonephros (AGM) region. These cells, but not those derived from the yolk sac, are capable of fully reconstituting the BM of a lethally irradiated adult animal for 6 months or longer, indicating their definitive properties (4–8).

After blood circulation is established in the embryo, HSCs from both the yolk sac and the AGM region migrate and colonize the fetal liver. The fetal liver microenvironment is responsible for expanding the numbers of HSCs and promoting full commitment of the mesoderm-derived hematopoietic progenitors (9–13). Fetal liver HSC numbers progressively increase, prior to the migration of the hematopoietic system to the recently formed BM cavities. Once established in the BM cavity, most primitive hematopoietic progenitors and stem cells reside in the endosteal and subendosteal regions. Committed progenitors and differentiated cells derived from stem cells will then occupy the central and perisinusoidal regions of the BM cavity, respectively (**Figure 1**) (14–18).

The BM vasculature is composed of a dense network of blood vessels widely spread within the cavity. Along the bone diaphysis axis, the central artery carries in arterial blood to supply BM cells demand, while, in the same region, the central sinus carries out venous blood and differentiated hematopoietic cells into the blood circulation. Finer arteries and arterioles branch out from the central artery prior to reaching the endosteal surfaces, where they create a web-like structure to provide nutrients to the HSC niche (19–21). Conversely, sinusoidal blood venules cross the BM cavity and coalesce into wider veins as they drain into a central sinus. Approximately, one-fifth of all long-term HSCs in the BM, identified by the expression of CD150, c-Kit, and Sca-1, and absence of lineage markers, are found in close association with endosteal osteoblasts (18, 22–32) The remaining 80% dwell in the abluminal surface of the subendosteal sinusoids, interacting with pericytes and endothelial cells, both contributing to HSC self-renewal (28, 33–35). These data derived from different groups indicate that the niche formed by endosteal osteoblasts and the subendosteal sinusoidal perivascular cells coexist and are referred "quiescent niche" and "proliferative niche," respectively. *In vitro* and *in vivo*

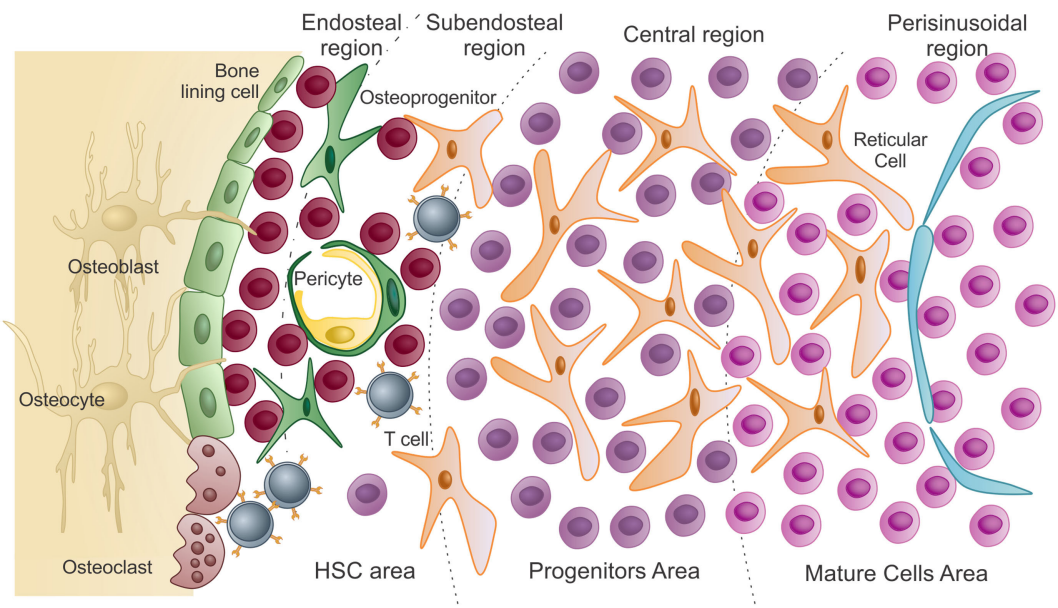

FIGURE 1 | Schematic presentation of the bone marrow microenvironment under "homeostatic" conditions. As previously described by Lambertsen and Weiss (15), HSC area, which harbors hematopoietic stem cells and uncommitted progenitors, comprises both endosteal and subendosteal niches. Committed progenitors and differentiated cells are distributed in the central and perisinusoidal niches, respectively. Quiescent hematopoietic stem cells are in close association with endosteal osteoblasts and bone-lining cells. As HSCs exit quiescence to proliferative states, they migrate and colonize the subendosteal perivascular niche, interacting with both endothelial cells and pericytes. Subendosteal sinusoid-derived pericytes serve as source for new osteoprogenitors, which will differentiate into osteoblasts during bone remodeling (adapted from Journal of Cellular Biochemistry, 2014, Cordeiro-Spinetti et al. with permission from John Wiley & Sons).

studies suggest that endosteal osteoblasts in the niche stimulate self-renewal and quiescence of the HSC (24, 29, 31, 32, 36, 37). Such that in transgenic animals, increased or decreased numbers of osteoblasts result in increased or decreased numbers of long-term HSC, respectively, without affecting any of the other hematopoietic lineages in the BM (24, 31, 32, 38). *In situ* observations show that most of the HSC population is concentrated in the trabecular bone zone, which also concentrates high numbers of osteoblasts, sinusoids, and CXCL12-positive pericyte-like cells (35, 39). In either case, HSCs are supported by highly expressing CXCL12-positive cells (40). The existence of the two niches remains controversial.

In the BM cavity, the microenvironment organization must match the hematopoietic system differentiation cascade, and each niche composition depends upon quality and number of specific types of stromal cell populations. Stromal cells comprise almost all non-hematopoietic cells found in the BM cavity – osteoblasts, endothelial cells, perivascular cells, mesenchymal cells, and nerve cells – which assemble in an organized network to physically and biologically properly support hematopoiesis (16, 18, 23, 24, 32, 41). Among all stromal cells, mesenchymal population represents around 90%, including pericytes, mesenchymal stem cells (MSCs) and progenitors, perivascular cells, non-perivascular reticular cells, adventitial cells, and all types of myofibroblasts.

Adaptive Immune Cells in the BM

Translating the spatial organization of niches in conjunction with hematopoietic differentiation, it is conceivable that blood cell production follows a radial pattern within the BM cavity, with most primitive cells residing close to the bone surface and differentiated cells exiting the BM cavity through a central venous sinus (15, 16) (**Figure 1**). A few differentiated hematopoietic cells leave the BM cavity, circulate in the peripheral blood, differentiate inside other organs as T cells in the thymus, and then return to the BM cavity, migrating to specific niches and playing significant roles as "stromal cells." As such, T cells participate in a paracrine and juxtacrine signaling to contribute to hematopoiesis. B and T lymphocytes are among those cells with the ability to return to the BM after a quick trip to peripheral blood and lymphoid tissues. After BM cavity entering, memory B-lymphocytes, plasma cells, and T cells concentrate in the perivascular niche of subendosteal sinusoids (**Figure 1**). They will dwell in close association with CXCL12 highly producing cells, from where they produce and secrete cytokines to help maintain hematopoiesis homeostasis as we know it (23, 42–46).

Recirculating memory B-lymphocytes and active plasma cells secrete considerable levels of IL-6 and TGF-β, contributing to a myelopoietic balance (45, 46). $CD4^+$ T cells express CXCR4, which promotes their migration and colonization close to CXCL12 highly producing subendosteal sinusoid perivascular cells. CXCR4 expressing T cells provide substantial assistance to differentiating B-lymphocytes and produce membrane bound and secreted cytokines that regulate myelopoiesis (42, 45, 46). Of note, recirculating B and T cells will colonize those niches identified as HSCs proliferative niche.

Adipocytes in the BM

Among those cells composing the stromal population, BM adipocytes apparently present the potential to contribute to both local and systemic metabolism (47, 48). BM adipose tissue (MAT) accumulation occurs during aging and other clinical conditions, including osteoporosis, diabetes, and gonadal dysfunction (48, 49), and interventions, such as chemotherapy and radiation (47, 49).

Osteoblasts, hematopoietic-supporting reticular cells, and BM adipocytes derive from a common precursor, named BM MSC, also known as skeletal stem cell or stromal stem cell (47–52). Clinical and histomorphometric studies have shown a correlation between MAT accumulation and decreased bone formation and increased bone loss (48), as hormones, corticoids, and local and peripheral factors might induce mesenchymal progenitors to follow the adipogenic differentiation path at the expense of the osteogenic one. Recent data, however, indicate that the hypothesis of a direct impact of MAT accumulation on skeletal tissue balance disruption is controversial, as adipocytes in the BM may present distinctive phenotypes, resembling either brown or white adipocytes, with potential different interactions with the skeletal tissue (53, 54).

Marrow adipose tissue accumulation and bone loss during aging and other conditions is usually accompanied by a decrease in HSCs number and blood cells production (55–59). In humans, when MAT areas mature in the yellow BM, adipocytes become densely packed and leave no room for active hematopoiesis (48, 60). In humans, yellow BM adipocytes appear at or right before birth, increasing up to the eighth week of age. Larger animals have more yellow BM as compared to smaller animals, which also extends farther into the bone cavities (60–63).

Adipocytes in the red BM do not become packed, but several findings implicate them as negative regulators of hematopoiesis. Adipocyte-derived soluble factors inhibit both B lymphopoiesis and granulopoiesis *in vitro* by blocking committed progenitors differentiation (64–67). When co-cultured with BM adipocytes, HSCs show reduced self-renewal capacity as compared to those co-cultured with other stromal cells (23, 68–71). *In vivo*, BM cavity areas rich in adipocytes most likely hold lower numbers of HSCs and short-term repopulating cells (72). Moreover, wild-type HSCs present enhanced repopulation ability when transplanted into animals with reduced numbers of BM adipocytes (73, 74). It was also observed that the balance between osteoblast and adipocyte differentiation influences hematopoietic recovery after irradiation. Hematopoietic precursor numbers increase when Runx2 expression peaks and PPAR gamma expression decreases, suggesting that osteogenesis but not adipogenesis supports hematopoiesis (73). All data published so far support a negative effect of BM adipocytes in hematopoiesis, even though further investigation is definitely necessary.

In pathological conditions, primary or metastatic tumor cells can grow in the BM. In respect of the niches of tumor cells in the BM, the role played by BM adipocytes is not clear yet. Apparently, BM adipocytes might secrete factors, which will modulate hematopoietic, immune, and tumor cells behavior in the niche. However, it is also very likely that the disruption in the osteoblast/adipocyte differentiation balance results in a new combination of

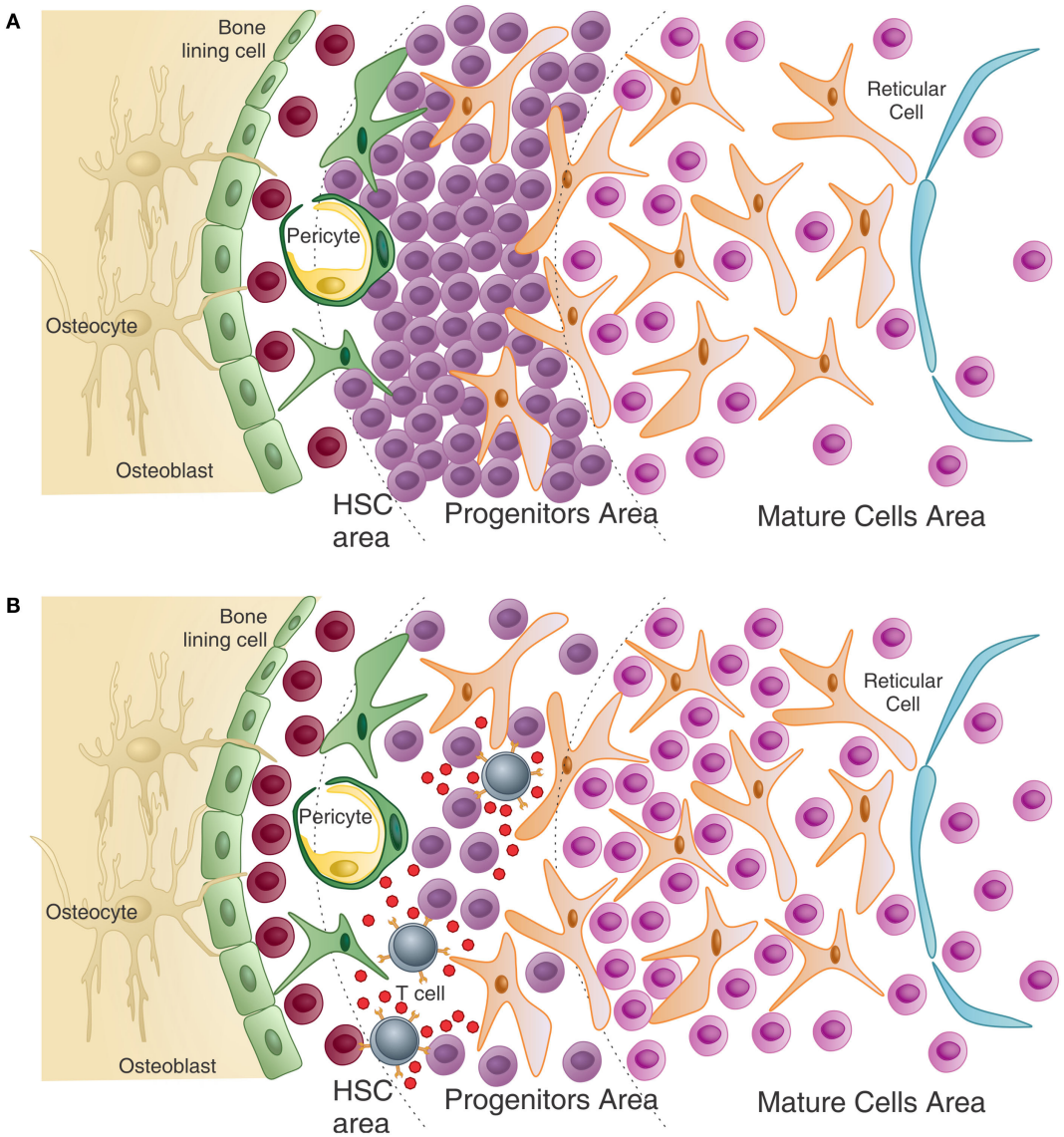

FIGURE 2 | T cells help normal hematopoiesis – (A) in the absence of T cells, or in the presence of non-activated T cells (not represented), immature progenitors cells, particularly of the myeloid lineage, accumulate in the bone marrow resulting in peripheral granulopenia. (B) When activated T cells are present, they can see antigen in several hematopoietic cells and release growth factors, in return. These cytokines can directly and indirectly affect maturation of myeloid progenitors. Also, T cell cytokines modulate the stroma, which in turn can modulate HSC maintenance and myeloid differentiation. The number of HSC is also higher than in the T-less environment (adapted from Journal of Cellular Biochemistry, 2014, CordeiroSpinetti et al. with permission from John Wiley & Sons).

stromal cells and formation of different categories of niches in the BM cavity.

T CELL IN HEMATOPOIESIS: ORCHESTRATING THE PERIPHERAL DEMAND

T Cell Help Keeps "Normal" Hematopoiesis in the BM

One of the first links between T lymphocytes and hematopoiesis emerges during development, and the relationship persists throughout life (75, 76). *In vitro*, it was shown that T cells could instruct splenic hematopoiesis in response to syngeneic stimulation (77). Also, patients receiving T cell depleted allogeneic BM transplantation have higher rates of graft failure (78, 79). Nonetheless, when $CD3^+$ cells were co-administered with allogeneic BM stem cells, improvement and optimization of hematopoietic cells engraftment is observed (80).

Using athymic mice, our data suggest that T cell interactions with hematopoietic precursor populations maintain hematopoiesis. These athymic mice exhibit granulocytopenia in the peripheral blood, as immature granulocytes accumulate in the BM (**Figure 2A**). Importantly, immature hematopoietic

cells in the BM have no intrinsic defect that prevents their differentiation, since *in vitro* stimulation with growth factors induces their differentiation and maturation. Thymus graft or $CD4^+$ T cells' adoptive transfer, but not $CD8^+$ T cells, rescues normal hematopoiesis and restores peripheral granulocytes counts. These $CD4^+$ T lymphocytes present in the BM are predominantly activated and produce IL-4 and IFN-γ, even in the absence of any intentional external antigen stimulation (42, 81). However, if T cells are virgin, meaning they have yet to interact with their cognate antigen (as in TCR transgenic mice specific for an external antigen), peripheral granulopenia with accumulation of myeloid blasts in the BM is observed (42). "Normal" hematopoiesis is restored, however, if T cells are activated with their cognate antigen (**Figure 2B**). This work demonstrates that, among its functions, T cell cross talk with hematopoiesis plays a critical role in "normal" hematopoiesis.

One critical demonstration of this relationship comes from studies with hyper IgM immunodeficiency patients. These patients have a mutation in the CD40L gene, and their activated T cells do not assist B cells and macrophages in their activities, even in the presence of cognate antigen (82–84). However, these patients are also granulopenic, suggesting that CD40L is also important for hematopoiesis. In fact, CD40L engagement induces the production of Flt3L by different cell types and thrombopoietin by BM stromal cells (85). Moreover, human $CD34^+$ hematopoietic progenitor cell are CD40 positive and when CD40L binds to it, proliferation and myeloid differentiation take place, mainly on the dendritic cell (DC) pathway (86).

BM *In Situ* Signaling and Delivery of Cytokines: T Cells Bring the News

Studies on hematopoietic cells from the BM and the spleen of mice with diverse T helper cell polarization profiles provided clues on how T cells influence normal hematopoiesis. In mice with targeted deletion of STAT-4, which are biased to the Th2 phenotype, the number and cycling status of hematopoietic cells are significantly decreased compared to wild-type mice. By contrast, STAT-6 knockout mice, which are biased to Th1 response, demonstrate increased numbers of progenitors and cycling cells. This phenomenon is attributed to oncostatin M (OSM) secreted by Th1 T cells, since treatment of STAT-4-deficient mice with OSM recovers the number of cycling progenitors in the BM (87). From these observations, it becomes clear that activated BM T cells play an important role in hematopoiesis.

Further examples of T cells regulating hematopoiesis abound. For example Rådinger and colleagues (88, 89) reported that $CD8^+$ T lymphocytes have a regulatory role in the control of eosinopoiesis in the BM. Transgenic mice overexpressing IL-5 in $CD3^+$ cells and deficient in $CD8^+$ cells show a significant decrease in BM eosinophils in steady state, what is not observed in the partner knockout mice to $CD4^+$. In addition, adoptive transfer of naive CD8 T lymphocytes into immunodeficient mice (SCID-bg) restores normal levels of eosinophils in the BM. By contrast, in airway allergen challenge both T CD4 and CD8 subsets seem to be important in BM and bronchoalveolar lavage (BAL) eosinophilia (88, 89). Another example of hematopoiesis regulation by T cells comes from adoptive transfer of activated antigen-specific CD8 T lymphocytes, leading to increased numbers of multipotent and committed hematopoietic progenitor cells, especially myeloid lineage. These CD8 T cells secrete IFN-γ, which in turn acts on BM mesenchymal stromal cells, inducing these cells to release hematopoietic cytokines, especially IL-6. IL-6 modulates the expression of transcription factors essential to myeloid commitment and promotes accumulation of myeloid cells in the periphery (90).

Another molecule that implicates T lymphocytes in hematopoiesis is granulocyte macrophage colony-stimulating factor (GM-CSF). GM-CSF is a hematopoietic growth factor that stimulates proliferation of myeloid progenitor cells. It induces survival and activation of mature myeloid cells, and has a pro-inflammatory role in different diseases (91, 92). Interestingly, the hematopoietic system of GM-CSF knockout mice is normal (93). However, these mice when infected with *Mycobacterium avium* (94) or *Listeria monocytogenes* (95) have severe depletion of BM hematopoietic cell numbers, a deficient inflammatory response and are unable to increase their colony-forming cells in BM. This suggests that GM-CSF is essential for a hematopoietic response emergency, but not required for normal hematopoiesis. Interestingly, Th1 and Th17 lymphocytes are able to produce and secrete GM-CSF when activated by different stimuli (96–99). Recently, GM-CSF producing T cells (Th-GM) were reported. Generation of $GM\text{-}CSF^+/IL3^+$ CD4 lymphocytes is mediated by IL-7/STAT-5 signaling, and Th-GMs do not express the transcription factors T-bet and ROR-γt, suggesting that these cells belong to a distinct subset of Th cells (100, 101).

The effects of IL-17 in hematopoiesis appear to be largely indirect through the induction of cytokine secretion, including GM-CSF, G-CSF, SCF, NO, IL-6, prostaglandins, and other chemokines (102–104). BM stromal cells are the main target of IL-17 as stromal and MSCs, express a high level of IL-17RA (105, 106).

Treg Inhibits Differentiation and Preserves the HSC Pool

Recently, Treg lymphocytes involvement in hematopoiesis was investigated. The frequency of FoxP3 Treg cells in CD4 T cells is higher in the BM (around 20%) than that in the spleen and lymph nodes (10–15%) (107, 108). Treg cells suppress the function of CD4 and CD8 T cells. In a murine model of allogeneic hematopoietic stem cell transplantation (HSCT) into non-irradiated recipients, Tregs co-localized with HSCTs and accumulated close to endosteum surface in the calvaria and trabecular BM. When Treg cells are depleted, a 70% reduction in the number of surviving donor cells, with concomitant increase in levels of TNF-α and IL-4 producing $CD4^+FoxP3^-$ and $CD8^+$ T BM cells is observed. These data suggest that Tregs provide an immune privilege mechanism that enables transplanted allo-HSPCs to escape allogeneic rejection in the BM (107). However, using a direct approach, Urbieta and colleagues (109) demonstrated that active Tregs are able to prevent the differentiation of IL-3/SCF colony-forming units (CFU) progenitor cells *in vitro* and this inhibition depends upon T cell antigen recognition and TGF-β, but not on FasL and perforin. Moreover, in syngeneic HSCT, Treg depletion either in host mice or in donor cell population increases

splenic CFU-IL3 after transplantation indicating that donor and host Tregs can modulate hematopoiesis. In line with these results, addition of Tregs to T-cell-depleted transplants diminishes splenic and BM-derived CFU and peripheral neutrophil counts (109). It seems that Tregs indeed protect immature progenitors from differentiating and, most probably, help to keep the stem cell pool, although this deserves a more detailed investigation.

Peripheral Demand and Central Hematopoiesis Holding Hands

During infections and inflammatory conditions, immune effector cells, especially neutrophils, are highly demanded and rapidly consumed in peripheral tissues. Although adaptive immune cells (T and B lymphocytes) respond to efficient antigen presentation with activation and proliferation, innate immune cells need to be replenished to maintain the progenitor cells pool. The hematopoietic response to this need is named demand-adapted hematopoiesis or emergency hematopoiesis.

T lymphocytes fit well with the definition of "messengers from the periphery to the BM" (**Figure 2**). The beauty of the system is that the pleiotropic effect of T cell interaction with multiple systems coordinates the immune response "class" in the adaptive as well as in the innate compartment. Th2 responses are the example prototype, with IL-4 being the master cytokine orchestrating the immune response. In the periphery, Th2 cells help B cells with a consequent switch in immunoglobulin class to IgE. In the BM, IL-4 induces mast cell differentiation. IgE has no function alone and works in pair with mast cells, made possible by the stimulation of IgE production by B cells and mast cells differentiation from BM progenitors, in a coordinated manner. Similar to this, Th1 responses induce IgG2a class switch in B cells and activate macrophages through IFN-γ, which inhibits osteoclast (OC) differentiation favoring the monocyte differentiation pathway (110). IgG2a is a good opsonin to which macrophages have high-affinity receptors. Th1 activity in the BM increases differentiation of monocytes, which have high-affinity receptors for IgG2a, and the immunoglobulin isotype is also stimulated by Th1 cells. In parallel to what was described above for Th2 cells, Th1 cells coordinate hematopoietic activity with adaptive immunity, with the immunoglobulin class switch improving innate immunity. Less obvious is the Th17 response, which is closely related to indirect actions, but signals on epithelial and stromal cells licensing those for production of cytokines, which will stimulate granulopoiesis and neutrophil migration to the damaged tissue. These cells are short lived and highly demanded, especially in the first phase of the response, needing to be replaced and produced in accelerated rates. This can be achieved after the Th17-specific cell engages its TCR on the surface of an antigen-presenting cell (APC) in the BM (42, 111, 112). Th17 cells secrete IL-17 and GM-CSF and stimulate BM stromal cells to produce G-CSF, modifying hematopoiesis based on antigen-specific peripheral demand.

There are still many points to be considered and investigated in the relationship between T lymphocytes and the hematopoietic system. However, the data gathered here provide strong evidence that this is not a simple and static relationship; it is modulated according to BM microenvironment, as well as by changes in the external environment sensed with the help of activated T cells.

T CELL AND THE BONE: REGULATING THE BONE REMODELING SYSTEM

Bone Remodeling: The Players

The skeletal system renews itself completely every 18 years, indicating its dynamic metabolism. Subendosteal perivascular cells serve as source for new osteoprogenitors in the BM cavity (68, 113, 114). As bone remodels, several factors released from the matrix after OC-mediated bone resorption will recruit bone-lining cells to become activate osteoblasts (113, 115). Osteoblasts will then deposit new bone matrix and further mineralize it. During bone deposition, a few osteoblasts get trapped in the matrix and become fully differentiated osteogenic cells, turning into osteocytes (113, 115).

Osteoclasts, on the other hand, derive from the hematopoietic lineage. OCs are bone resident cells involved in physiological and pathological remodeling of bone tissue. Mature OCs are tartrate-resistant acid phosphatase (TRAP), calcitonin receptor, cathepsin K, αβ3-integrin, and adenosine triphosphate-dependent proton pump subunits positive, multinucleated giant cells that are generated from the fusion of precursors derived from the monocyte/macrophage lineage (116). Mature OCs polarize and adhere to the bone matrix, acidify the bone surface, release osteolytic enzymes, and resorb bone. Two factors appear as crucial for OC differentiation: macrophage colony-stimulating factor (M-CSF), which induces monocyte differentiation, and RANKL, which is essential for stimulating pre-OC differentiation and its merge into multinucleated cells. Both factors are believed to derive mainly from osteoprogenitor cells and pre-osteoblast. Osteoblasts, on the other hand, produce and secrete osteoprotegerin (OPG) that is a RANK decoy receptor and inhibits OC formation and bone resorption (116).

Under physiological conditions, the balance between bone formation and resorption is finely tuned and can be disrupted under non-homeostatic conditions (**Figure 3A**).

RANK–RANKL–OPG Axis: Linking the Immune and Skeletal System

RANKL (also known as ODF, TRANCE, OPGL, and TNFSF11) and its receptor RANK (also known as TRANCE-R and TNFRSF11A) central players in bone metabolism (117) were first described as molecules expressed in T cells and DCs. OPG, the soluble receptor/antagonist for RANKL, initially characterized as an inhibitor factor of OC development and bone resorption, expressed by osteoblasts (116, 118), and B cells (119, 120), has also been described as a crucial regulator of B cell maturation and development of antibody responses (121). In fact, the RANK/RANKL system also controls lymph node organogenesis and development of thymic medullary epithelial cells and lymphocytes (122–124). The RANK–RANKL–OPG axis also has important functions in other systems, such as the lactating breast, and in the thermo-regulatory system (125). Regulation of tumor cell migration to the bone as well as primary mammary tumor growth had also been reported and relate to physiological activities of these molecules (126).

The role of the adaptive immune system in what is known as the equilibrium between bone consumption and deposition in

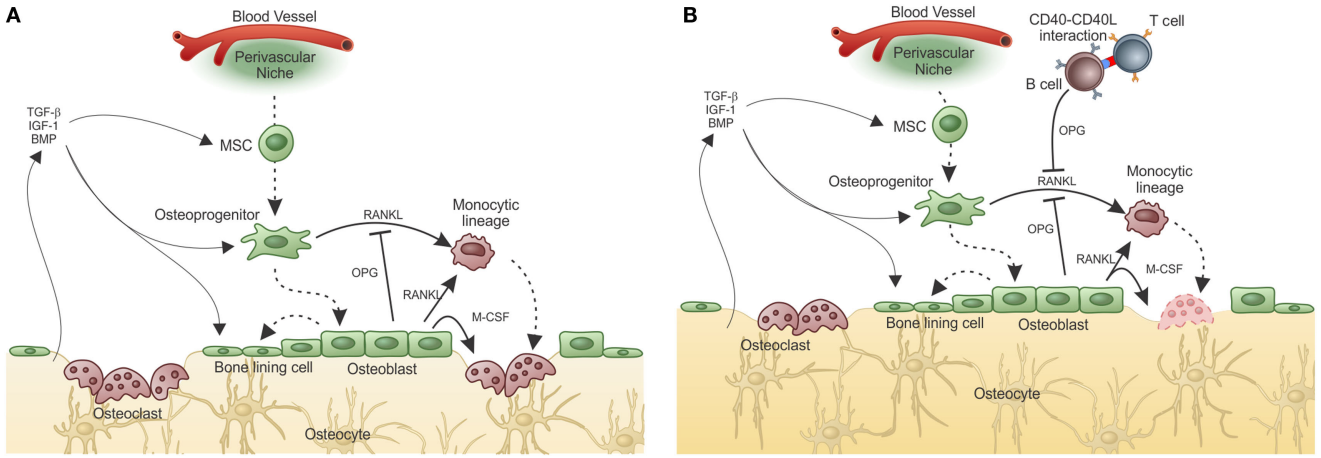

FIGURE 3 | Adaptive immunity participates in physiological bone remodeling – (A) bone remodeling relies on osteoclasts (OC), which in physiological situations differentiate from OC precursors of the monocytic lineage after signaling through RANK in its surface and RANKL produced by osteoprogenitor cells. As the bone is resorbed, growth factors and other molecules stored in the extracellular matrix are released, including TGF-β that stimulates osteoblastogenesis from MSC, which reside in the perivascular niche (as in the representation) or in the endosteal niche (not represented). As a consequence, new osteoblasts are formed resulting in bone formation. OPG, a decoy receptor for RANKL, is produced by osteoblasts and participate modulating the bone remodeling system. **(B)** When activated T cells are present, they activate B cells in a CD40–CD40L-dependent manner with OPG production in large quantities by B cells. This leads to bone accumulation as the RANK–RANKL axis is disrupted by the high amounts of OPG produced by B cells. This is what defines the "normal" bone mass in physiological situation, in SPF condition. Dashed lines represent cellular differentiation; filled line represents cytokine action.

healthy animals was addressed by Li et al. (119). They showed that acquisition of normal bone mass depends on the presence of OPG produced by mature activated B cells. However, in the absence of T cells, B cells do not produce OPG and bone remodeling is unbalanced toward bone consumption due to lack of the inhibitory OPG–RANKL loop. Moreover, OPG production by B cells was dependent upon CD40L from activated T cells, showing that T–B interaction is required for "normal" bone density maintenance (**Figure 3B**).

Evidence related to hormonal (estrogen) regulation of TNF-α production by T cells have also been published, and data link adaptive immunity to bone activity (127). However, the results vary depending on the model used (128). In the presence of B cells, but in the absence of T cells, bone mass decreases after ovariectomy, in accordance to Li et al. (119). In this case, B cells are present, but are not activated by T cells. However, if both, B and T cells are absent, as in RAG-deficient mice, bone mass increases after ovariectomy making any conclusion about the role of estrogen in regulation of T cell activity over the bone difficult at the present moment.

Apart from hormones, another important evidence of T cell participation in physiological bone remodeling comes from studies in germ-free (GF) mice (127, 129). These animals have lower numbers of T cells in the BM and these are not activated. Also, GF mice have low frequency of OCs and are osteopetrotic. Coincidentally, colonization with commensal microbiota reverses the osteopetrotic phenotype and restores normal numbers of activated T cells, as well as of OC precursors (130) These data are in accordance with the view that in steady-state healthy conditions, recognition of antigen by T cells tunes the bone remodeling system.

T-cell-derived RANKL can contribute to bone loss in some pathological conditions, as it was first proposed by Kong and colleagues in a model of inflammatory bone disease (131). Later studies showing reversal of RANKL-dependent osteopetrosis by hyper-expression of RANKL in T and B cells (132) reinforced the interplay between adaptive immune cell-derived RANKL and bone metabolism. On the other hand, Sato et al. better described the involvement of T-cell-derived IL-17A and interaction with RANKL in synoviocyte and macrophages in the inflamed joint (133, 134).

Generally, the cross talk between both systems has been studied in models of arthritis, in which the action of T cells in an inflamed joint stimulates osteoclastogenesis and bone resorption through the indirect action of T-cell-derived IL-17A and RANKL (134–137). However, in the arthritis models, T cells reach the bone through the articular cavity after an inflammatory injury, which destroys the cartilage and exposes the periosteal bone surface.

In contrast to these mechanisms observed in arthritis, in other conditions, the T cells normally present inside the BM and in contact with the bone might contribute to pathological bone remodeling. In fact, we have recently shown that RANKL production by tumor-primed BM $CD4^+$ T cells induces pre-metastatic osteolytic bone disease that licenses metastatic colonization (138). We reached this conclusion by showing that the 4T1 breast tumor induces production of pro-osteoclastogenic cytokines, IL-17F, and RANKL by BM $CD4^+$ T cells, leading to OC formation and activation *in vitro* and *in vivo*. Surprisingly, osteolytic disease was observed even before tumor cells colonized the bone and was induced by T cell transfer, showing that T cells prepare the metastatic niche for further establishment of tumor cells

(**Figure 4A**). Thus, the T cell mediated pre-metastatic osteolytic disease adds an extra step to the vicious cycle hypothesis, which established that bone colonization by tumor cells culminate in the release of growth factors from the bone matrix as a consequence of OC activity (**Figure 4B**) (139, 140).

Osteoclasts and Dendritic Cells: Modulators of the Bone Remodeling Gear

Interactions between monocytes/macrophages and OCs has long been studied and was appreciated in the pioneering work of Horton et al. in the early 1970s (141). The set of soluble factors secreted from antigen-stimulated peripheral blood mononuclear cells was initially described as an "OC-activating factor," which subsequently was identified as predominantly interleukin-1 (IL-1) and TNF (142). In addition, numerous macrophage-derived cytokines influence OC differentiation and/or function, such as M-CSF, which are essential for the development and survival of the monocyte/macrophage lineage as well as OC differentiation (136).

One important cell type also derived from monocytic progenitors and precursors in the BM is the DC, the most potent APC, responsible for activating naive T cells, and orchestrating the immune responses (143). Since DCs are derived from the same monocyte/macrophage OC precursor lineage, it is not surprising that splenic immature DCs can be developed into OCs (OC–DC), when cultured with osteoclastogenic factors M-CSF and RANKL (144–146).

Accumulated evidence indicates that mature OCs can function as APCs to both $CD4^+$ and $CD8^+$ T cells *in vitro*. They express MHC I and II, CD80, CD86, CD40, IL-10, TGF-β, IL-6, and TNF-α, all molecules that act on T cell activation and class switch (147). It has been suggested that the monocyte population used to differentiate OCs carry contaminant DCs or monocyte themselves, which could explain the antigen-presenting capabilities of OCs. OCs can in fact be derived from immature DCs *in vivo* and *in vitro* (148–150), although the antigen-presenting capability of these OC–DCs had never been addressed.

Circulating DCs are normally recruited to inflamed tissues (149, 151) and IL-23-producing DCs play an indirect role in osteoclastogenesis by stimulating T cell expression of RANKL and IL-1, IL-6, IL-17, and TNF-α that augment the release of TRAP and cathepsin K by resident OCs (149). A still-open question is whether the same growth factors that control normal osteoclastogenesis are also involved in generating OCs in response to inflammation with potent DC activity. Participation of OC–DC in bone remodeling was suggested in Langerhans cell histiocytosis (LCH). LCH is a multi-symptom disease with associated bone resorption and granuloma formation, and soft tissue inflammatory lesions (152). Bone pathology appears to rely on DCs and monocytes, which produce high levels of IL-17A and induce DC–OC differentiation (150, 153). Also it was shown that DCs could induce osteolysis when transferred to regular or osteopetrotic mice (154). Osteolytic activity *in vivo* was inhibited with OPG and bisphosphonates, implying a role for RANKL in the pathogenic DC–OC pathway (154).

Since it was shown that DC can prime T cells inside the BM (155), it can be reasoned that a DC can acquire OC characteristics while keeping its antigen-presenting capabilities. If this is true, then DCs can work as messengers from the periphery to the BM where they maintain T cell activation/memory and interfere with bone metabolism, in addition to participating in hematopoiesis (**Figure 5**).

FINAL REMARKS: TUNING THE BM MULTISYSTEM

The skeletal system provides vital functions, as mechanical structure, movement, protection, calcium storage, endocrine

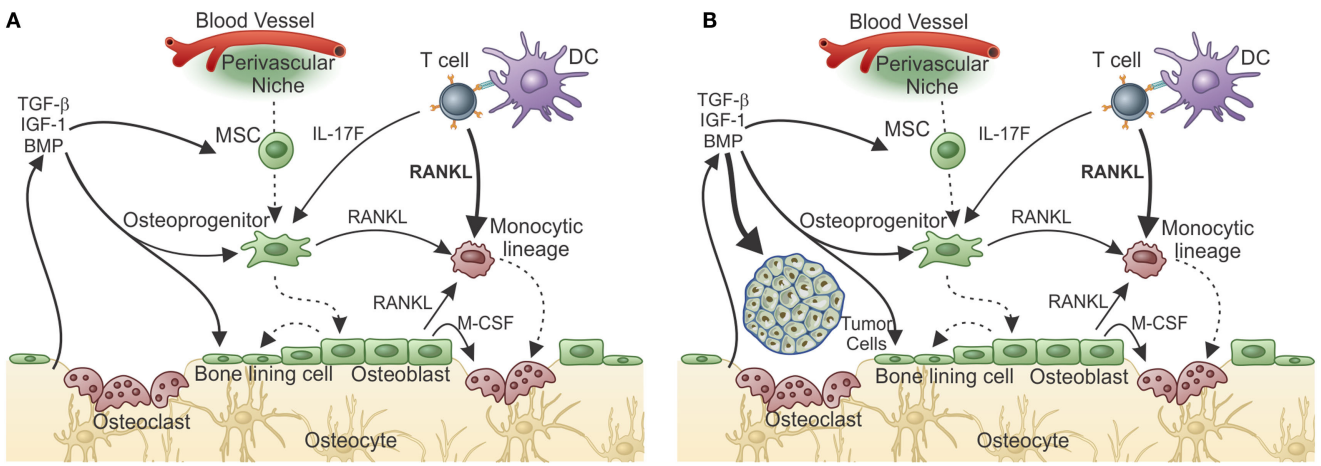

FIGURE 4 | T-cell-derived RANKL prepares the bone pre-metastatic niche. (A) After responding T cells meet tumor antigen and get activated, they migrate to the bone marrow where, after encountering antigen, they release cytokines. In the case of a metastatic tumor, responding T cells produce IL-17 and RANKL. Although IL-17F from T cells can indirectly influences osteoclastogenesis, T-cell-derived RANKL is the central cytokine stimulating osteoclastogenesis and bone consumption resulting in pre-metastatic niche organization with growth factor release from the resorbed matrix. **(B)** The growth factor-rich environment will nourishes the arriving tumor cells allowing metastatic colonization. In the absence of T-cell-derived RANKL, metastatization into the bone does not take place.

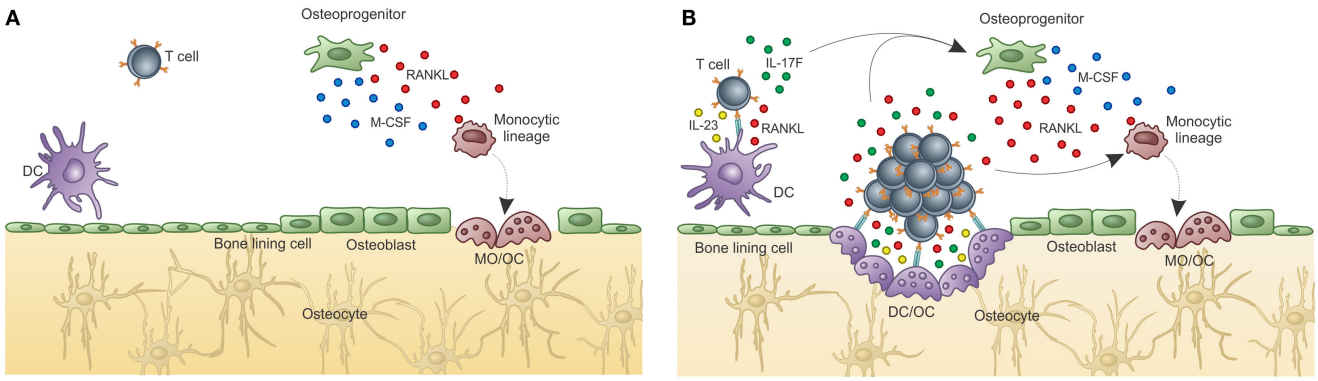

FIGURE 5 | Osteoclasts are optimal antigen-presenting cells when derived from DCs, but not from BM progenitors. Osteoclasts are usually derived from bone marrow progenitors or monocytic precursors (OC), but can also derive from dendritic cells under certain conditions (OC–DC). **(A)** Classical "constitutive" OC. OC are not good antigen presenters, cannot activate T cells, and do not maintain T cell activation in the BM. Thus, OCs do not contribute to T cell activation in the BM. **(B)** Activation induced OC–DC. Under the influence of RANKL among other cytokines, OC–DCs are induced. If OC–DCs are as good as fresh DCs in presenting antigen to T cells and if they provide IL-23, a positive loop will be built, which can amplify the osteoclastogenic potential of T cell stimulation. In this context, the bone-resorbing activity of both DC–OC and classical OC will result in decreased bone mass (represented by the light color of the matrix). Whether both OCs dwells in the same resorbing pit, or are segregated into separate subniches, as represented in B, is unknown.

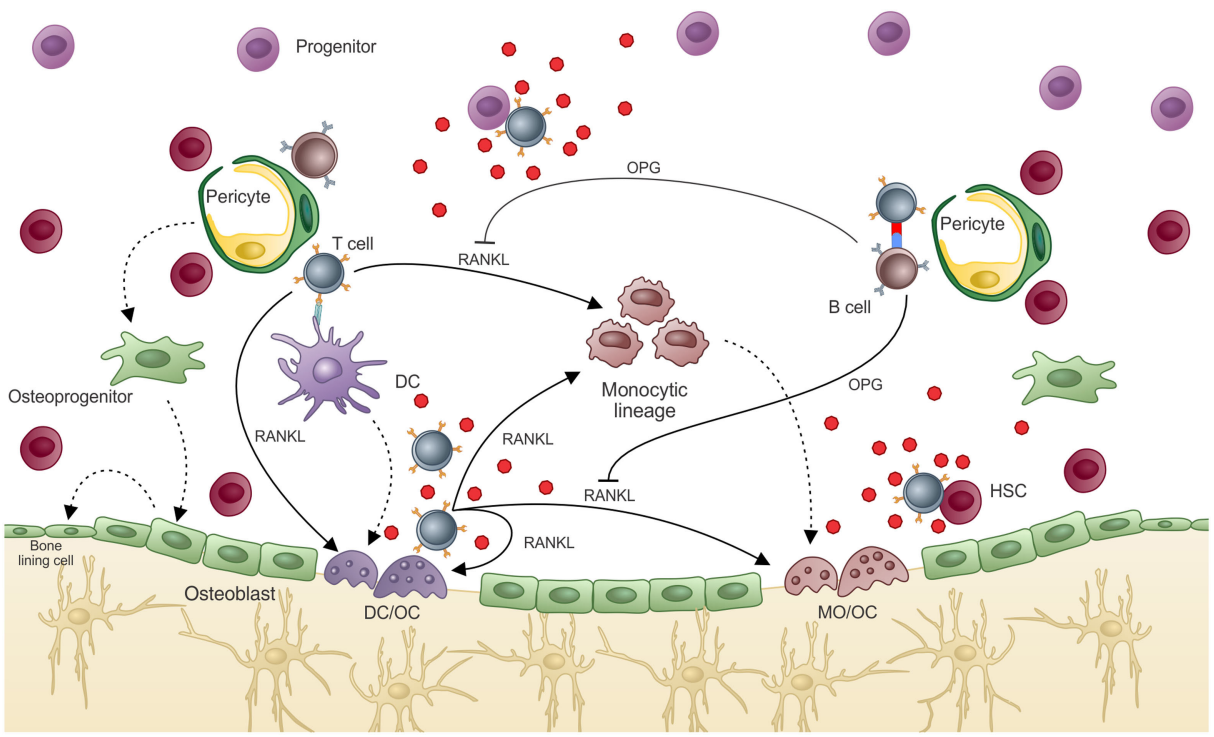

FIGURE 6 | T cell regulation of bone marrow environment. T cell participation in the BM environment promotes what we know as "normal hematopoiesis" and "normal bone mass." In hematopoiesis, T cell release of hematopoietic cytokines after activation allows terminal differentiation of hematopoietic progenitors/precursors, which will compose the "normal" peripheral pool. "Normal" bone density is also dependent on T cell activation, which in a normal state activates B cells to produce OPG, balancing bone resorption by OCs and mineral deposition by OBs. If T cells are highly activated or are turned into an osteoclastogenic phenotype, excessive bone resorption can lead to osteolytic disease. Moreover, some of the cytokines produced by T cells that can interfere with the bone remodeling system can also affect hematopoiesis, such as IL-17F.

regulation, and support for the whole hematopoietic system. Blood cell production is a very finely controlled process, which depends upon stromal cells and their factors, and cell intrinsic conditions. In the BM cavity, HSCs and progenitors reside close to the bone surfaces, which suggests that the two systems are integrated.

Bone remodeling's influence over hematopoiesis is not clear, although there are reports in the literature, indicating that osteoblast activity increases the number of primitive progenitors (24, 31, 32, 38) and also that increased numbers of OCs might induce HSC mobilization (28, 32, 115, 156–158). It is possible that OC mobilization and activation together disrupt HSC niches as the stem cells reside close to the bone surface and in close contact to osteoblasts. As both OCs and OBs are involved in bone remodeling, stem cell niches are disrupted and the immature progenitors leave the quiescent niche and are enabled to enter the proliferation and differentiation pathway. T cells participate on maintaining or disrupting the balance of both systems depending on their activity. In the case of pro-osteolytic activity, when T cells get inside the BM, they can see antigen on IL-23$^+$ DCs, keeping their Th17 profile, producing RANKL and inducing osteolysis. This will disrupt hematopoiesis through osteoclastogenesis stimulation, which disturbs the HSC niche. At the same time, T cells can produce IL-17A, F, and G-CSF (98, 99), which indirectly stimulates neutrophil maturation, providing "help" for the immature myeloid progenitors recently released from the stem cell niche.

In summary, we believe that T cells pave the road that unravels the partnership of the three systems (i.e., immunity, hematopoiesis, bone homeostasis) by acting as messengers from the periphery to the bone cavity. It is clear that inside the BM, T cells bring information from the periphery. They produce cytokines and express membrane molecules, which will act in an antigen-specific manner. Although it remains unclear as to the need for a cognate antigen inside the BM, it is clear that only after *in vivo* priming can BM T cells acquire an activated phenotype. Whether or not T cell regulation of bone homeostasis requires recognition of self-antigens by DCs or OCs inside the BM, or if antigen-loaded APCs do the job or both, is still not clear. However, it is clear that if BM T cells recognize antigen (self or not) on APCs, they will exert their effector functions "helping" the bone remodeling and hematopoietic system. The final effect on hematopoiesis and bone remodeling depends on the class of the T cell response (159), identified by the cytokine package produced and released by the T cell subset (**Figure 6**).

AUTHOR CONTRIBUTIONS

ABo, AM, TG-S discussed the literature anrd wrote about T cells inside the bone marrow; EC-S and ABa discussed and wrote about the bone marrow organization and its niches. RG prepared the illustrations, discussed and reviewd the paper. ABo and ABa revised and put together the niches, in the bone marrow and the adaptive immune system.

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REFERENCES

1. Mazo IB, Honczarenko M, Leung H, Cavanagh LL, Bonasio R, Weninger W, et al. Bone marrow is a major reservoir and site of recruitment for central memory CD8+ T cells. *Immunity* (2005) **22**:259–70. doi:10.1016/j.immuni.2005.01.008
2. Sabbagh L, Snell LM, Watts TH. TNF family ligands define niches for T cell memory. *Trends Immunol* (2007) **28**:333–9. doi:10.1016/j.it.2007.06.001
3. Di Rosa F, Pabst R. The bone marrow: a nest for migratory memory T cells. *Trends Immunol* (2005) **26**:360–6. doi:10.1016/j.it.2005.04.011
4. Godin I, Cumano A. The hare and the tortoise: an embryonic haematopoietic race. *Nat Rev Immunol* (2002) **2**:593–604. doi:10.1038/nri857
5. Ikuta K, Weissman IL. Evidence that hematopoietic stem cells express mouse c-kit but do not depend on steel factor for their generation. *Proc Natl Acad Sci U S A* (1992) **89**:1502–6. doi:10.1073/pnas.89.4.1502
6. Moore MA, Metcalf D. Ontogeny of the haemopoietic system: yolk sac origin of in vivo and in vitro colony forming cells in the developing mouse embryo. *Br J Haematol* (1970) **18**:279–96. doi:10.1111/j.1365-2141.1970.tb01443.x
7. Cumano A, Ferraz JC, Klaine M, Di Santo JP, Godin I. Intraembryonic, but not yolk sac hematopoietic precursors, isolated before circulation, provide long-term multilineage reconstitution. *Immunity* (2001) **15**:477–85. doi:10.1016/S1074-7613(01)00190-X
8. Medvinsky A, Dzierzak E. Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell* (1996) **86**:897–906. doi:10.1016/S0092-8674(00)80165-8
9. Christensen JL, Wright DE, Wagers AJ, Weissman IL. Circulation and chemotaxis of fetal hematopoietic stem cells. *PLoS Biol* (2004) **2**:E75. doi:10.1371/journal.pbio.0020075
10. Ema H, Nakauchi H. Expansion of hematopoietic stem cells in the developing liver of a mouse embryo. *Blood* (2000) **95**:2284–8.
11. Inlay MA, Serwold T, Mosley A, Fathman JW, Dimov IK, Seita J, et al. Identification of multipotent progenitors that emerge prior to hematopoietic stem cells in embryonic development. *Stem Cell Rep* (2014) **2**:457–72. doi:10.1016/j.stemcr.2014.02.001
12. Kieusseian A, Brunet de la Grange P, Burlen-Defranoux O, Godin I, Cumano A. Immature hematopoietic stem cells undergo maturation in the fetal liver. *Development* (2012) **139**:3521–30. doi:10.1242/dev.079210
13. Morrison SJ, Hemmati HD, Wandycz AM, Weissman IL. The purification and characterization of fetal liver hematopoietic stem cells. *Proc Natl Acad Sci U S A* (1995) **92**:10302–6. doi:10.1073/pnas.92.22.10302
14. Cordeiro-Spinetti E, Taichman RS, Balduino A. The bone marrow endosteal niche: how far from the surface? *J Cell Biochem* (2015) **116**:6–11. doi:10.1002/jcb.24952
15. Lambertsen RH, Weiss L. A model of intramedullary hematopoietic microenvironments based on stereologic study of the distribution of endocloned marrow colonies. *Blood* (1984) **63**:287–97.
16. Lord BI, Testa NG, Hendry JH. The relative spatial distributions of CFUs and CFUc in the normal mouse femur. *Blood* (1975) **46**:65–72.
17. Nilsson SK, Simmons PJ. Transplantable stem cells: home to specific niches. *Curr Opin Hematol* (2004) **11**:102–6. doi:10.1097/01.moh.0000133651.06863.9c
18. Taichman RS. Blood and bone: two tissues whose fates are intertwined to create the hematopoietic stem-cell niche. *Blood* (2005) **105**:2631–9. doi:10.1182/blood-2004-06-2480
19. Fei J, Jia F, Peyrin F, Françoise P, Malaval L, Vico L, et al. Imaging and quantitative assessment of long bone vascularization in the adult rat using microcomputed tomography. *Anat Rec (Hoboken)* (2010) **293**:215–24. doi:10.1002/ar.21054
20. Nombela-Arrieta C, Pivarnik G, Winkel B, Canty KJ, Harley B, Mahoney JE, et al. Quantitative imaging of haematopoietic stem and progenitor cell

localization and hypoxic status in the bone marrow microenvironment. *Nat Cell Biol* (2013) **15**:533–43. doi:10.1038/ncb2730
21. Skawina A, Litwin JA, Gorczyca J, Miodoński AJ. The vascular system of human fetal long bones: a scanning electron microscope study of corrosion casts. *J Anat* (1994) **185**(Pt 2):369–76.
22. Arai F, Hirao A, Ohmura M, Sato H, Matsuoka S, Takubo K, et al. Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell* (2004) **118**:149–61. doi:10.1016/j.cell.2004.07.004
23. Balduino A, Mello-Coelho V, Wang Z, Taichman RS, Krebsbach PH, Weeraratna AT, et al. Molecular signature and in vivo behavior of bone marrow endosteal and subendosteal stromal cell populations and their relevance to hematopoiesis. *Exp Cell Res* (2012) **318**:2427–37. doi:10.1016/j.yexcr.2012.07.009
24. Calvi LM, Adams GB, Weibrecht KW, Weber JM, Olson DP, Knight MC, et al. Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature* (2003) **425**:841–6. doi:10.1038/nature02040
25. Kiel MJ, Iwashita T, Yilmaz OH, Morrison SJ. Spatial differences in hematopoiesis but not in stem cells indicate a lack of regional patterning in definitive hematopoietic stem cells. *Dev Biol* (2005) **283**:29–39. doi:10.1016/j.ydbio.2005.03.037
26. Kiel MJ, Radice GL, Morrison SJ. Lack of evidence that hematopoietic stem cells depend on N-cadherin-mediated adhesion to osteoblasts for their maintenance. *Cell Stem Cell* (2007) **1**:204–17. doi:10.1016/j.stem.2007.06.001
27. Nilsson SK, Johnston HM, Coverdale JA. Spatial localization of transplanted hemopoietic stem cells: inferences for the localization of stem cell niches. *Blood* (2001) **97**:2293–9. doi:10.1182/blood.V97.8.2293
28. Song J, Kiel MJ, Wang Z, Wang J, Taichman RS, Morrison SJ, et al. An in vivo model to study and manipulate the hematopoietic stem cell niche. *Blood* (2010) **115**:2592–600. doi:10.1182/blood-2009-01-200071
29. Taichman RS, Emerson SG. The role of osteoblasts in the hematopoietic microenvironment. *Stem Cells* (1998) **16**:7–15. doi:10.1002/stem.160007
30. Taichman R, Reilly M, Verma R, Ehrenman K, Emerson S. Hepatocyte growth factor is secreted by osteoblasts and cooperatively permits the survival of haematopoietic progenitors. *Br J Haematol* (2001) **112**:438–48. doi:10.1046/j.1365-2141.2001.02568.x
31. Visnjic D, Kalajzic Z, Rowe DW, Katavic V, Lorenzo J, Aguila HL. Hematopoiesis is severely altered in mice with an induced osteoblast deficiency. *Blood* (2004) **103**:3258–64. doi:10.1182/blood-2003-11-4011
32. Zhang J, Niu C, Ye L, Huang H, He X, Tong W-G, et al. Identification of the haematopoietic stem cell niche and control of the niche size. *Nature* (2003) **425**:836–41. doi:10.1038/nature02041
33. Boulais PE, Frenette PS. Making sense of hematopoietic stem cell niches. *Blood* (2015) **125**:2621–9. doi:10.1182/blood-2014-09-570192
34. Kunisaki Y, Bruns I, Scheiermann C, Ahmed J, Pinho S, Zhang D, et al. Arteriolar niches maintain haematopoietic stem cell quiescence. *Nature* (2013) **502**:637–43. doi:10.1038/nature12612
35. Morrison SJ, Scadden DT. The bone marrow niche for haematopoietic stem cells. *Nature* (2014) **505**:327–34. doi:10.1038/nature12984
36. Taichman RS, Emerson SG. Human osteoblasts support hematopoiesis through the production of granulocyte colony-stimulating factor. *J Exp Med* (1994) **179**:1677–82. doi:10.1084/jem.179.5.1677
37. Taichman RS, Reilly MJ, Emerson SG. Human osteoblasts support human hematopoietic progenitor cells in vitro bone marrow cultures. *Blood* (1996) **87**:518–24.
38. Kuznetsov SA, Riminucci M, Ziran N, Tsutsui TW, Corsi A, Calvi L, et al. The interplay of osteogenesis and hematopoiesis: expression of a constitutively active PTH/PTHrP receptor in osteogenic cells perturbs the establishment of hematopoiesis in bone and of skeletal stem cells in the bone marrow. *J Cell Biol* (2004) **167**:1113–22. doi:10.1083/jcb.200408079
39. Bourke VA, Watchman CJ, Reith JD, Jorgensen ML, Dieudonnè A, Bolch WE. Spatial gradients of blood vessels and hematopoietic stem and progenitor cells within the marrow cavities of the human skeleton. *Blood* (2009) **114**:4077–80. doi:10.1182/blood-2008-12-192922
40. Greenbaum A, Hsu Y-MS, Day RB, Schuettpelz LG, Christopher MJ, Borgerding JN, et al. CXCL12 in early mesenchymal progenitors is required for haematopoietic stem-cell maintenance. *Nature* (2013) **495**:227–30. doi:10.1038/nature11926
41. Ding L, Saunders TL, Enikolopov G, Morrison SJ. Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature* (2012) **481**:457–62. doi:10.1038/nature10783
42. Monteiro JP, Benjamin A, Costa ES, Barcinski MA, Bonomo A. Normal hematopoiesis is maintained by activated bone marrow CD4+ T cells. *Blood* (2005) **105**:1484–91. doi:10.1182/blood-2004-07-2856
43. Nemoto Y, Kanai T, Makita S, Okamoto R, Totsuka T, Takeda K, et al. Bone marrow retaining colitogenic CD4+ T cells may be a pathogenic reservoir for chronic colitis. *Gastroenterology* (2007) **132**:176–89. doi:10.1053/j.gastro.2006.10.035
44. Slifka MK, Matloubian M, Ahmed R. Bone marrow is a major site of long-term antibody production after acute viral infection. *J Virol* (1995) **69**:1895–902.
45. Tokoyoda K, Egawa T, Sugiyama T, Choi B-I, Nagasawa T. Cellular niches controlling B lymphocyte behavior within bone marrow during development. *Immunity* (2004) **20**:707–18. doi:10.1016/j.immuni.2004.05.001
46. Tokoyoda K, Zehentmeier S, Hegazy AN, Albrecht I, Grün JR, Löhning M, et al. Professional memory CD4+ T lymphocytes preferentially reside and rest in the bone marrow. *Immunity* (2009) **30**:721–30. doi:10.1016/j.immuni.2009.03.015
47. Cawthorn WP, Scheller EL, Learman BS, Parlee SD, Simon BR, Mori H, et al. Bone marrow adipose tissue is an endocrine organ that contributes to increased circulating adiponectin during caloric restriction. *Cell Metab* (2014) **20**:368–75. doi:10.1016/j.cmet.2014.06.003
48. Scheller EL, Rosen CJ. What's the matter with MAT? Marrow adipose tissue, metabolism, and skeletal health. *Ann N Y Acad Sci* (2014) **1311**:14–30. doi:10.1111/nyas.12327
49. Fazeli PK, Horowitz MC, MacDougald OA, Scheller EL, Rodeheffer MS, Rosen CJ, et al. Marrow fat and bone – new perspectives. *J Clin Endocrinol Metab* (2013) **98**:935–45. doi:10.1210/jc.2012-3634
50. Bianco P. Minireview: the stem cell next door: skeletal and hematopoietic stem cell "niches" in bone. *Endocrinology* (2011) **152**:2957–62. doi:10.1210/en.2011-0217
51. Caplan AI. Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. *J Cell Physiol* (2007) **213**:341–7. doi:10.1002/jcp.21200
52. Motyl KJ, McCabe LR. Leptin treatment prevents type I diabetic marrow adiposity but not bone loss in mice. *J Cell Physiol* (2009) **218**:376–84. doi:10.1002/jcp.21608
53. Bethel M, Chitteti BR, Srour EF, Kacena MA. The changing balance between osteoblastogenesis and adipogenesis in aging and its impact on hematopoiesis. *Curr Osteoporos Rep* (2013) **11**:99–106. doi:10.1007/s11914-013-0135-6
54. Scheller EL, Doucette CR, Learman BS, Cawthorn WP, Khandaker S, Schell B, et al. Region-specific variation in the properties of skeletal adipocytes reveals regulated and constitutive marrow adipose tissues. *Nat Commun* (2015) **6**:7808. doi:10.1038/ncomms8808
55. Beghé C, Wilson A, Ershler WB. Prevalence and outcomes of anemia in geriatrics: a systematic review of the literature. *Am J Med* (2004) **116**(Suppl):3S–10S. doi:10.1016/j.amjmed.2003.12.009
56. Gruver AL, Hudson LL, Sempowski GD. Immunosenescence of ageing. *J Pathol* (2007) **211**:144–56. doi:10.1002/path.2104
57. Guralnik JM, Eisenstaedt RS, Ferrucci L, Klein HG, Woodman RC. Prevalence of anemia in persons 65 years and older in the United States: evidence for a high rate of unexplained anemia. *Blood* (2004) **104**:2263–8. doi:10.1182/blood-2004-05-1812
58. Hakim FT, Gress RE. Immunosenescence: deficits in adaptive immunity in the elderly. *Tissue Antigens* (2007) **70**:179–89. doi:10.1111/j.1399-0039.2007.00891.x
59. Linton PJ, Dorshkind K. Age-related changes in lymphocyte development and function. *Nat Immunol* (2004) **5**:133–9. doi:10.1038/ni1033
60. Emery JL, Follett GF. Regression of bone marrow haemopoiesis from the terminal digits in the foetus and infant. *Br J Haematol* (1964) **10**:485–9. doi:10.1111/j.1365-2141.1964.tb00725.x
61. Bigelow CL, Tavassoli M. Fatty involution of bone marrow in rabbits. *Acta Anat (Basel)* (1984) **118**:60–4. doi:10.1159/000145823
62. Kricun ME. Red-yellow marrow conversion: its effect on the location of some solitary bone lesions. *Skeletal Radiol* (1985) **14**:10–9. doi:10.1007/BF00361188

63. Tavassoli M, Watson LR, Khademi R. Retention of hemopoiesis in tail vertebrae of newborn rats. *Cell Tissue Res* (1979) **200**:215–22. doi:10.1007/BF00236414
64. Belaid-Choucair Z, Lepelletier Y, Poncin G, Thiry A, Humblet C, Maachi M, et al. Human bone marrow adipocytes block granulopoiesis through neuropilin-1-induced granulocyte colony-stimulating factor inhibition. *Stem Cells* (2008) **26**:1556–64. doi:10.1634/stemcells.2008-0068
65. Belaid Z, Hubint F, Humblet C, Boniver J, Nusgens B, Defresne M-P. Differential expression of vascular endothelial growth factor and its receptors in hematopoietic and fatty bone marrow: evidence that neuropilin-1 is produced by fat cells. *Haematologica* (2005) **90**:400–1.
66. Bilwani FA, Knight KL. Adipocyte-derived soluble factor(s) inhibits early stages of B lymphopoiesis. *J Immunol* (2012) **189**:4379–86. doi:10.4049/jimmunol.1201176
67. Kennedy DE, Knight KL. Inhibition of B lymphopoiesis by adipocytes and IL-1-producing myeloid-derived suppressor cells. *J Immunol* (2015) **195**:2666–74. doi:10.4049/jimmunol.1500957
68. Balduino A, Hurtado SP, Frazão P, Takiya CM, Alves LM, Nasciutti L-E, et al. Bone marrow subendosteal microenvironment harbours functionally distinct haemosupportive stromal cell populations. *Cell Tissue Res* (2005) **319**:255–66. doi:10.1007/s00441-004-1006-3
69. Cheng Y-H, Chitteti BR, Streicher DA, Morgan JA, Rodriguez-Rodriguez S, Carlesso N, et al. Impact of maturational status on the ability of osteoblasts to enhance the hematopoietic function of stem and progenitor cells. *J Bone Miner Res* (2011) **26**:1111–21. doi:10.1002/jbmr.302
70. Chitteti BR, Cheng Y-H, Poteat B, Rodriguez-Rodriguez S, Goebel WS, Carlesso N, et al. Impact of interactions of cellular components of the bone marrow microenvironment on hematopoietic stem and progenitor cell function. *Blood* (2010) **115**:3239–48. doi:10.1182/blood-2009-09-246173
71. Chitteti BR, Bethel M, Voytik-Harbin SL, Kacena MA, Srour EF. In vitro construction of 2D and 3D simulations of the murine hematopoietic niche. *Methods Mol Biol* (2013) **1035**:43–56. doi:10.1007/978-1-62703-508-8_5
72. Naveiras O, Nardi V, Wenzel PL, Hauschka PV, Fahey F, Daley GQ. Bone-marrow adipocytes as negative regulators of the haematopoietic microenvironment. *Nature* (2009) **460**:259–63. doi:10.1038/nature08099
73. Poncin G, Beaulieu A, Humblet C, Thiry A, Oda K, Boniver J, et al. Characterization of spontaneous bone marrow recovery after sublethal total body irradiation: importance of the osteoblastic/adipocytic balance. *PLoS One* (2012) **7**:e30818. doi:10.1371/journal.pone.0030818
74. Zhu R-J, Wu M-Q, Li Z-J, Zhang Y, Liu K-Y. Hematopoietic recovery following chemotherapy is improved by BADGE-induced inhibition of adipogenesis. *Int J Hematol* (2013) **97**:58–72. doi:10.1007/s12185-012-1233-4
75. Resnitzky P, Zipori D, Trainin N. Effect of neonatal thymectomy on hemopoietic tissue in mice. *Blood* (1971) **37**:634–46.
76. Trainin N, Resnitzky P. Influence of neonatal thymectomy on cloning capacity of bone marrow cells in mice. *Nature* (1969) **221**:1154–5. doi:10.1038/2211154a0
77. Bonomo AC, el-Cheikh MC, Borojevic R, Cavalcante LA, DosReis GA. Comparative analysis of splenic cell proliferation induced by interleukin 3 and by syngeneic accessory cells (syngeneic mixed leukocyte reaction): evidence that autoreactive T-cell functioning instructs hematopoietic phenomena. *Cell Immunol* (1990) **125**:210–24. doi:10.1016/0008-8749(90)90075-3
78. Ho VT, Soiffer RJ. The history and future of T-cell depletion as graft-versus-host disease prophylaxis for allogeneic hematopoietic stem cell transplantation. *Blood* (2001) **98**:3192–204. doi:10.1182/blood.V98.12.3192
79. Martin PJ, Hansen JA, Torok-Storb B, Moretti L, Press O, Storb R, et al. Effects of treating marrow with a CD3-specific immunotoxin for prevention of acute graft-versus-host disease. *Bone Marrow Transplant* (1988) **3**:437–44.
80. Kaufman CL, Colson YL, Wren SM, Watkins S, Simmons RL, Ildstad ST. Phenotypic characterization of a novel bone marrow-derived cell that facilitates engraftment of allogeneic bone marrow stem cells. *Blood* (1994) **84**:2436–46.
81. Monteiro JP, Bonomo A. Linking immunity and hematopoiesis by bone marrow T cell activity. *Braz J Med Biol Res* (2005) **38**:1475–86. doi:10.1590/S0100-879X2005001000004
82. Banchereau J, Bazan F, Blanchard D, Brière F, Galizzi JP, van Kooten C, et al. The CD40 antigen and its ligand. *Annu Rev Immunol* (1994) **12**:881–922. doi:10.1146/annurev.iy.12.040194.004313
83. Foy TM, Shepherd DM, Durie FH, Aruffo A, Ledbetter JA, Noelle RJ. In vivo CD40-gp39 interactions are essential for thymus-dependent humoral immunity. II. Prolonged suppression of the humoral immune response by an antibody to the ligand for CD40, gp39. *J Exp Med* (1993) **178**:1567–75. doi:10.1084/jem.178.5.1567
84. Nonoyama S, Hollenbaugh D, Aruffo A, Ledbetter JA, Ochs HD. B cell activation via CD40 is required for specific antibody production by antigen-stimulated human B cells. *J Exp Med* (1993) **178**:1097–102. doi:10.1084/jem.178.3.1097
85. Solanilla A, Déchanet J, El Andaloussi A, Dupouy M, Godard F, Chabrol J, et al. CD40-ligand stimulates myelopoiesis by regulating flt3-ligand and thrombopoietin production in bone marrow stromal cells. *Blood* (2000) **95**:3758–64.
86. Flores-Romo L, Björck P, Duvert V, van Kooten C, Saeland S, Banchereau J. CD40 ligation on human cord blood CD34+ hematopoietic progenitors induces their proliferation and differentiation into functional dendritic cells. *J Exp Med* (1997) **185**:341–9. doi:10.1084/jem.185.2.341
87. Broxmeyer HE, Bruns HA, Zhang S, Cooper S, Hangoc G, McKenzie ANJ, et al. Th1 cells regulate hematopoietic progenitor cell homeostasis by production of Oncostatin M. *Immunity* (2002) **16**:815–25. doi:10.1016/S1074-7613(02)00319-9
88. Rådinger M, Sergejeva S, Johansson A-K, Malmhäll C, Bossios A, Sjöstrand M. Regulatory role of CD8+ T lymphocytes in bone marrow eosinophilopoiesis. *Respir Res* (2006) **7**:83. doi:10.1186/1465-9921-7-83
89. Rådinger M, Bossios A, Alm A-S, Jeurink P, Lu Y, Malmhäll C, et al. Regulation of allergen-induced bone marrow eosinophilopoiesis: role of CD4+ and CD8+ T cells. *Allergy* (2007) **62**:1410–8. doi:10.1111/j.1398-9995.2007.01509.x
90. Schürch CM, Riether C, Ochsenbein AF. Cytotoxic CD8(+) T cells stimulate hematopoietic progenitors by promoting cytokine release from bone marrow mesenchymal stromal cells. *Cell Stem Cell* (2014) **14**:460–72. doi:10.1016/j.stem.2014.01.002
91. Fleetwood AJ, Cook AD, Hamilton JA. Functions of granulocyte-macrophage colony-stimulating factor. *Crit Rev Immunol* (2005) **25**:405–28. doi:10.1615/CritRevImmunol.v25.i5.50
92. Hamilton JA, Anderson GP. GM-CSF biology. *Growth Factors* (2004) **22**:225–31. doi:10.1080/08977190412331279881
93. Stanley E, Lieschke GJ, Grail D, Metcalf D, Hodgson G, Gall JA, et al. Granulocyte/macrophage colony-stimulating factor-deficient mice show no major perturbation of hematopoiesis but develop a characteristic pulmonary pathology. *Proc Natl Acad Sci U S A* (1994) **91**:5592–6. doi:10.1073/pnas.91.12.5592
94. Zhan Y, Cheers C. Haemopoiesis in mice genetically lacking granulocyte-macrophage colony stimulating factor during chronic infection with Mycobacterium avium. *Immunol Cell Biol* (2000) **78**:118–23. doi:10.1046/j.1440-1711.2000.00891.x
95. Zhan Y, Lieschke GJ, Grail D, Dunn AR, Cheers C. Essential roles for granulocyte-macrophage colony-stimulating factor (GM-CSF) and G-CSF in the sustained hematopoietic response of Listeria monocytogenes-infected mice. *Blood* (1998) **91**:863–9.
96. El-Behi M, Ciric B, Dai H, Yan Y, Cullimore M, Safavi F, et al. The encephalitogenicity of T(H)17 cells is dependent on IL-1- and IL-23-induced production of the cytokine GM-CSF. *Nat Immunol* (2011) **12**:568–75. doi:10.1038/ni.2031
97. Grifka-Walk HM, Giles DA, Segal BM. IL-12-polarized Th1 cells produce GM-CSF and induce EAE independent of IL-23. *Eur J Immunol* (2015) **45**:2780–6. doi:10.1002/eji.201545800
98. McGeachy MJ. GM-CSF: the secret weapon in the T(H)17 arsenal. *Nat Immunol* (2011) **12**:521–2. doi:10.1038/ni.2044
99. McWilliams IL, Rajbhandari R, Nozell S, Benveniste E, Harrington LE. STAT4 controls GM-CSF production by both Th1 and Th17 cells during EAE. *J Neuroinflammation* (2015) **12**:128. doi:10.1186/s12974-015-0351-3
100. Herndler-Brandstetter D, Flavell RA. Producing GM-CSF: a unique T helper subset? *Cell Res* (2014) **24**:1379–80. doi:10.1038/cr.2014.155
101. Sheng W, Yang F, Zhou Y, Yang H, Low PY, Kemeny DM, et al. STAT5 programs a distinct subset of GM-CSF-producing T helper cells that is essential for autoimmune neuroinflammation. *Cell Res* (2014) **24**:1387–402. doi:10.1038/cr.2014.154

102. Cordeiro-Spinetti E, Taichman RS, Balduino AJ. The bone marrow endosteal niche: how far from the surface? *J. Cell Biochem* (2015) **116**:6–11. doi:10.1002/jcb.24952
103. Krstić A, Santibanez JF, Okić I, Mojsilović S, Kocić J, Jovčić G, et al. Combined effect of IL-17 and blockade of nitric oxide biosynthesis on haematopoiesis in mice. *Acta Physiol* (2010) **199**:31–41. doi:10.1111/j.1748-1716.2010.02082.x
104. Jovčić G, Bugarski D, Petakov M, Krstić A, Vlaški M, Stojanović N, et al. In vivo effects of interleukin-17 on haematopoietic cells and cytokine release in normal mice. *Cell Prolif* (2004) **37**:401–12. doi:10.1111/j.1365-2184.2004.00322.x
105. Silva WA, Covas DT, Panepucci RA, Proto-Siqueira R, Siufi JLC, Zanette DL, et al. The profile of gene expression of human marrow mesenchymal stem cells. *Stem Cells* (2003) **21**:661–9. doi:10.1634/stemcells.21-6-661
106. Mojsilović S, Jauković A, Santibañez JF, Bugarski D. Interleukin-17 and its implication in the regulation of differentiation and function of hematopoietic and mesenchymal stem cells. *Mediators Inflamm* (2015) **2015**:470458. doi:10.1155/2015/470458
107. Fujisaki J, Wu J, Carlson AL, Silberstein L, Putheti P, Larocca R, et al. In vivo imaging of Treg cells providing immune privilege to the haematopoietic stem-cell niche. *Nature* (2011) **474**:216–9. doi:10.1038/nature10160
108. Zhao E, Xu H, Wang L, Kryczek I, Wu K, Hu Y, et al. Bone marrow and the control of immunity. *Cell Mol Immunol* (2012) **9**:11–9. doi:10.1038/cmi.2011.47
109. Urbieta M, Barao I, Jones M, Jurecic R, Panoskaltsis-mortari A, Blazar BR, et al. Hematopoietic progenitor cell regulation by CD4+CD25+T cells. *Blood* (2010) **115**:4934–43. doi:10.1182/blood-2009-04-218826
110. Takayanagi H, Ogasawara K, Hida S, Chiba T, Murata S, Sato K, et al. T-cell-mediated regulation of osteoclastogenesis by signalling cross-talk between RANKL and IFN-gamma. *Nature* (2000) **408**:600–5. doi:10.1038/35046102
111. Mirantes C, Passegué E, Pietras EM. Pro-inflammatory cytokines: emerging players regulating HSC function in normal and diseased hematopoiesis. *Exp Cell Res* (2014) **329**:248–54. doi:10.1016/j.yexcr.2014.08.017
112. Di Rosa F. T-lymphocyte interaction with stromal, bone and hematopoietic cells in the bone marrow. *Immunol Cell Biol* (2009) **87**:20–9. doi:10.1038/icb.2008.84
113. Kristensen HB, Andersen TL, Marcussen N, Rolighed L, Delaisse JM. Increased presence of capillaries next to remodeling sites in adult human cancellous bone. *J Bone Miner Res* (2013) **28**:574–85. doi:10.1002/jbmr.1760
114. Taichman RS, Wang Z, Shiozawa Y, Jung Y, Song J, Balduino A, et al. Prospective identification and skeletal localization of cells capable of multilineage differentiation in vivo. *Stem Cells Dev* (2010) **19**:1557–70. doi:10.1089/scd.2009.0445
115. Jang M-G, Lee JY, Yang J-Y, Park H, Kim JH, Kim J-E, et al. Intermittent PTH treatment can delay the transformation of mature osteoblasts into lining cells on the periosteal surfaces. *J Bone Miner Metab* (2015). doi:10.1007/s00774-015-0707-x
116. Raggatt LJ, Partridge NC. Cellular and molecular mechanisms of bone remodeling. *J Biol Chem* (2010) **285**:25103–8. doi:10.1074/jbc.R109.041087
117. Anderson DM, Maraskovsky E, Billingsley WL, Dougall WC, Tometsko ME, Roux ER, et al. A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. *Nature* (1997) **390**:175–9. doi:10.1038/36593
118. Simonet W, Lacey D, Dunstan C, Kelley M, Chang M-S, Lüthy R, et al. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* (1997) **89**:309–19. doi:10.1016/S0092-8674(00)80209-3
119. Li Y, Toraldo G, Li A, Yang X, Zhang H, Qian WP, et al. B cells and T cells are critical for the preservation of bone homeostasis and attainment of peak bone mass in vivo. *Blood* (2007) **109**:3839–48. doi:10.1182/blood-2006-07-037994
120. Yun TJ, Chaudhary PM, Shu GL, Frazer JK, Ewings MK, Schwartz SM, et al. OPG/FDCR-1, a TNF receptor family member, is expressed in lymphoid cells and is up-regulated by ligating CD40. *J Immunol* (1998) **161**:6113–21.
121. Yun TJ, Tallquist MD, Aicher A, Rafferty KL, Marshall AJ, Moon JJ, et al. Osteoprotegerin, a crucial regulator of bone metabolism, also regulates B cell development and function. *J Immunol* (2001) **166**:1482–91. doi:10.4049/jimmunol.166.3.1482
122. Dougall WC, Glaccum M, Charrier K, Rohrbach K, Brasel K, De Smedt T, et al. RANK is essential for osteoclast and lymph node development. *Genes Dev* (1999) **13**:2412–24. doi:10.1101/gad.13.18.2412
123. Kong YY, Yoshida H, Sarosi I, Tan HL, Timms E, Capparelli C, et al. OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature* (1999) **397**:315–23. doi:10.1038/16852
124. Rossi SW, Kim M-Y, Leibbrandt A, Parnell SM, Jenkinson WE, Glanville SH, et al. RANK signals from CD4(+)3(-) inducer cells regulate development of Aire-expressing epithelial cells in the thymic medulla. *J Exp Med* (2007) **204**:1267–72. doi:10.1084/jem.20062497
125. Fata JE, Kong YY, Li J, Sasaki T, Irie-Sasaki J, Moorehead RA, et al. The osteoclast differentiation factor osteoprotegerin-ligand is essential for mammary gland development. *Cell* (2000) **103**:41–50. doi:10.1016/S0092-8674(00)00103-3
126. Jones DH, Nakashima T, Sanchez OH, Kozieradzki I, Komarova SV, Sarosi I, et al. Regulation of cancer cell migration and bone metastasis by RANKL. *Nature* (2006) **440**:692–6. doi:10.1038/nature04524
127. Roggia C, Gao Y, Cenci S, Weitzmann MN, Toraldo G, Isaia G, et al. Up-regulation of TNF-producing T cells in the bone marrow: a key mechanism by which estrogen deficiency induces bone loss in vivo. *Proc Natl Acad Sci U S A* (2001) **98**:13960–5. doi:10.1073/pnas.251534698
128. Lee S-K, Kadono Y, Okada F, Jacquin C, Koczon-Jaremko B, Gronowicz G, et al. T lymphocyte-deficient mice lose trabecular bone mass with ovariectomy. *J Bone Miner Res* (2006) **21**:1704–12. doi:10.1359/jbmr.060726
129. Charles JF, Ermann J, Aliprantis AO. The intestinal microbiome and skeletal fitness: connecting bugs and bones. *Clin Immunol* (2015) **159**:163–9. doi:10.1016/j.clim.2015.03.019
130. Sjögren K, Engdahl C, Henning P, Lerner UH, Tremaroli V, Lagerquist MK, et al. The gut microbiota regulates bone mass in mice. *J Bone Miner Res* (2012) **27**:1357–67. doi:10.1002/jbmr.1588
131. Kong YY, Feige U, Sarosi I, Bolon B, Tafuri A, Morony S, et al. Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. *Nature* (1999) **402**:304–9. doi:10.1038/46303
132. Kim N, Odgren PR, Kim DK, Marks SC, Choi Y. Diverse roles of the tumor necrosis factor family member TRANCE in skeletal physiology revealed by TRANCE deficiency and partial rescue by a lymphocyte-expressed TRANCE transgene. *Proc Natl Acad Sci U S A* (2000) **97**:10905–10. doi:10.1073/pnas.200294797
133. Sato K, Suematsu A, Okamoto K, Yamaguchi A, Morishita Y, Kadono Y, et al. Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction. *J Exp Med* (2006) **203**:2673–82. doi:10.1084/jem.20061775
134. Takayanagi H. Osteoimmunology and the effects of the immune system on bone. *Nat Rev Rheumatol* (2009) **5**:667–76. doi:10.1038/nrrheum.2009.217
135. Arron JR, Choi Y. Bone versus immune system. *Nature* (2000) **408**:535–6. doi:10.1038/35046196
136. Takayanagi H. Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems. *Nat Rev Immunol* (2007) **7**:292–304. doi:10.1038/nri2062
137. Takayanagi H. New developments in osteoimmunology. *Nat Rev Rheumatol* (2012) **8**:684–9. doi:10.1038/nrrheum.2012.167
138. Monteiro AC, Leal AC, Gonçalves-Silva T, Mercadante ACT, Kestelman F, Chaves SB, et al. T cells induce pre-metastatic osteolytic disease and help bone metastases establishment in a mouse model of metastatic breast cancer. *PLoS One* (2013) **8**:e68171. doi:10.1371/journal.pone.0068171
139. Mundy GR. Metastasis to bone: causes, consequences and therapeutic opportunities. *Nat Rev Cancer* (2002) **2**:584–93. doi:10.1038/nrc867
140. Roodman GD. Mechanisms of bone metastasis. *N Engl J Med* (2004) **350**:1655–64. doi:10.1056/NEJMra030831
141. Horton JE, Raisz LG, Simmons HA, Oppenheim JJ, Mergenhagen SE. Bone resorbing activity in supernatant fluid from cultured human peripheral blood leukocytes. *Science* (1972) **177**:793–5. doi:10.1126/science.177.4051.793
142. Dewhirst FE, Stashenko PP, Mole JE, Tsurumachi T. Purification and partial sequence of human osteoclast-activating factor: identity with interleukin 1 beta. *J Immunol* (1985) **135**:2562–8.
143. Merad M. The dendritic cellelineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. *Ann Rev Imunnol* (2013) **31**:563–604. doi:10.1146/annurev-immunol-020711-074950
144. Alnaeeli M, Penninger JM, Teng YA. Immune interactions with CD4+ cells promote the development of functional osteoclasts from murine

CD11c+ dendritic cells. *J Immunol* (2006) **177**:3314–26. doi:10.4049/jimmunol.177.5.3314
145. Rivollier A, Mazzorana M, Tebib J, Piperno M, Aitsiselmi T, Rabourdin-Combe C, et al. Immature dendritic cell transdifferentiation into osteoclasts: a novel pathway sustained by the rheumatoid arthritis microenvironment. *Blood* (2004) **104**:4029–37. doi:10.1182/blood-2004-01-0041
146. Speziani C, Rivollier A, Gallois A, Coury F, Mazzorana M, Azocar O, et al. Murine dendritic cell transdifferentiation into osteoclasts is differentially regulated by innate and adaptive cytokines. *Eur J Immunol* (2007) **37**:747–57. doi:10.1002/eji.200636534
147. Li H, Hong S, Qian J, Zheng Y, Yang J, Yi Q. Crosstalk between the bone and immune systems: osteoclasts function as antigen-presenting cells and activate CD4+ and CD8+ T cells. *Blood* (2010) **116**:210–7. doi:10.1182/blood-2009-11-255026
148. Alnaeeli M, Teng Y-TA. Dendritic cells differentiate into osteoclasts in bone marrow microenvironment in vivo. *Blood* (2009) **113**:264–5. doi:10.1182/blood-2008-09-180836
149. Alnaeeli M, Park J, Mahamed D, Penninger JM, Teng Y-T. Dendritic cells at the osteo-immune interface: implications for inflammation-induced bone loss. *J Bone Miner Res* (2007) **22**:775–80. doi:10.1359/jbmr.070314
150. Coury F, Annels N, Rivollier A, Olsson S, Santoro A, Speziani C, et al. Langerhans cell histiocytosis reveals a new IL-17A-dependent pathway of dendritic cell fusion. *Nat Med* (2008) **14**:81–7. doi:10.1038/nm1694
151. Tran Janco JM, Lamichhane P, Karyampudi L, Knutson KL. Tumor-infiltrating dendritic cells in cancer pathogenesis. *J Immunol* (2015) **194**:2985–91. doi:10.4049/jimmunol.1403134
152. Badalian-Very G, Vergilio J-A, Degar BA, Rodriguez-Galindo C, Rollins BJ. Recent advances in the understanding of Langerhans cell histiocytosis. *Br J Haematol* (2012) **156**:163–72. doi:10.1111/j.1365-2141.2011.08915.x
153. Lourda M, Olsson-Åkefeldt S, Gavhed D, Björnfot S, Clausen N, Hjalmars U, et al. Detection of IL-17A-producing peripheral blood monocytes in Langerhans cell histiocytosis patients. *Clin Immunol* (2014) **153**:112–22. doi:10.1016/j.clim.2014.04.004
154. Grosjean F, Nasi S, Schneider P, Chobaz V, Liu A, Mordasini V, et al. Dendritic cells cause bone lesions in a new mouse model of histiocytosis. *PLoS One* (2015) **10**:e0133917. doi:10.1371/journal.pone.0133917
155. Feuerer M, Beckhove P, Garbi N, Mahnke Y, Limmer A, Hommel M, et al. Bone marrow as a priming site for T-cell responses to blood-borne antigen. *Nat Med* (2003) **9**:1151–7. doi:10.1038/nm914
156. Kollet O, Dar A, Shivtiel S, Kalinkovich A, Lapid K, Sztainberg Y, et al. Osteoclasts degrade endosteal components and promote mobilization of hematopoietic progenitor cells. *Nat Med* (2006) **12**:657–64. doi:10.1038/nm1417
157. Mansour A, Anginot A, Mancini SJC, Schiff C, Carle GF, Wakkach A, et al. Osteoclast activity modulates B-cell development in the bone marrow. *Cell Res* (2011) **21**:1102–15. doi:10.1038/cr.2011.21
158. Moutsatsos IK, Turgeman G, Zhou S, Kurkalli BG, Pelled G, Tzur L, et al. Exogenously regulated stem cell-mediated gene therapy for bone regeneration. *Mol Ther* (2001) **3**:449–61. doi:10.1006/mthe.2001.0291
159. Matzinger P, Kamala T. Tissue-based class control: the other side of tolerance. *Nat Rev Immunol* (2011) **11**:221–30. doi:10.1038/nri2940

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