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Hematopoietic Stem Cells, Tumor Cells and Lymphocytes — Party in the Bone Marrow

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1. Introduction

1.1. The hematopoietic stem cell niche

1.1.1. Hematopoietic system development: distinct niches activities

During vertebrate embryogenesis, different anatomical sites are responsible for creating specific conditions to promote hematopoietic stem cells self-renewal, expansion, commitment, and differentiation of the hematopoietic stem cells (HSC) [1,2]. The first hematopoietic cells emerge within the blood islands of the yolk sac (YS), an extra-embryonic site. Most of the cells belong to the primitive erythroid lineage, but a few myeloid cells are also generated [3,4]. In a second wave, hematopoietic progenitors emerge from the mesoderm of the paraaortic-splanchnopleura (Sp), an intra-embryonic site, which later gives rise to the aorta, gonads and mesonephros, and has been named AGM region [1]. Data show that almost all long-term definitive progenitors derive from the AGM region, as those originated in the YS fail to properly reconstitute the adult bone marrow of a lethally irradiated animal [5]. However, when cultured under the right combination of cytokines, cells derived from the mesoderm of the YS, in which blood islands originate, can be instructed to become long-term hematopoietic progenitors [6]. In these conditions, even higher numbers of progenitors could be found in the YS compared to the AGM region. This shows that, *in vivo*, the YS niche does not hold proper conditions to promote full commitment of the hematopoietic progenitors.

After the vascular system is established in the embryo, hematopoietic progenitors migrate and colonize the fetal liver. Fetal liver is the main hematopoietic organ during embryo's development [7,8] and its hematopoiesis requires exogenous colonization. So far, no data indicate that

new progenitors emerge in the fetal liver. All hematopoietic cells are derived from the YS and AGM region [8–10].

At this stage, fetal liver microenvironment is responsible for two very important tasks: full commitment of mesoderm derived progenitors to long-term HSC, and their increase in numbers (higher numbers). Although AGM derived progenitors are able to fully reconstitute a lethally irradiated animal in an experimental model, well-defined HSC can only be observed in the embryo a few days after fetal liver colonization. It has been shown that, to become adult long-term HSC, progenitors derived from the AGM region must go through the fetal liver microenvironment for proper instructions [11]. A few progenitors from the yolk sac become long-term HSC, but those originated from the AGM region are far more predominant. Long-term HSC in the fetal liver are highly proliferative and self-renewable. In mouse embryo, in five days, there could be an increase in 30 times the original number of HSC [7,9]. Different from what would be expected, not a huge number of progenitors colonize the fetal liver. Only a few are necessary. Commitment to HSC and their expansion require two distinct niches in the fetal liver microenvironment, at the same time or at different maturation stages of the liver. This requires further investigation.

The fetal liver remains hematopoietic until birth, or even a short period after. By the time the organ starts to acquire its metabolic properties, HSC are then progressively transferred to their final destination: the bone marrow [9]. In contrast to the fetal liver hematopoietic activity, bone marrow main assignment is blood production – not HSC expansion (only). All types of blood cells are produced in the bone marrow, except for the T lymphocytes, produced in the thymus. Despite its high dynamics, the bone marrow microenvironment is organized, in order to guarantee a finely tuned hierarchical differentiation cascade. Hematopoietic system organization in the marrow cavity follows an also organized distribution of the stromal cells. Different stromal cell types – osteoblasts, reticular cells, perivascular cells, endothelial cells, macrophages – interact with different groups of hematopoietic cells, creating distinct niches in bone marrow microenvironment to harbor. This is the way the differentiation cascade is controlled as hematopoietic cells at different stages of differentiation demand distinct combinations of factors for their proliferation and differentiation [12–15].

Based on cells behavior, at least three niches can be identified in the marrow microenvironment: one responsible for HSC maintenance (self-renewal) throughout life; a second to induce intermediate progenitors expansion; and a third to guarantee hematopoietic cells full commitment and differentiation to the lineages.

In humans, during childhood, almost all bones in our body hold a “hematopoietically” active bone marrow (red bone marrow). After reaching maturity, active bone marrow is restricted to the sternum, ribs, vertebrae, ilium, and femurs’ heads. The rest the bones are filled with “inactive” bone marrow, which is called yellow bone marrow, due to the high number of fat storing cells.

1.1.2. Inside the bone marrow

In spite of its high dynamic, the hematopoietic system, in the bone marrow cavity, is widely hierarchical and hematopoietic cells are not randomly distributed. As mentioned before, specific niches control HSC self-renewal and their engagement to a differentiation cascade.

The concept that different niches would compose the bone marrow microenvironment was envisioned already in the 70's [16]. Based on a stereological study, it was proposed that bone marrow microenvironment could be subdivided into, at least, four niches: endosteal, subendosteal, central, and perisinusoidal [13,16]. Histological and functional assays showed that HSC and primitive progenitors preferentially colonize the endosteal and subendosteal regions – close to the bone surface. Intermediate progenitors and differentiated cells are distributed in the central and perisinusoidal niches, respectively [13,14,16–21]. Due to their close range, endosteal and subendosteal regions are usually identified as one niche, named “endosteal niche”. However, these two regions harbor very distinct stromal cells [15,22,23] and must then be considered as two different niches, as they play distinct roles on HSC behavior.

Based on the expression of different surface markers [24,25] one can isolate the long-term HSC separately from other progenitors. Under physiological conditions, 20%-30% of the HSC are in a quiescent stage. Studies have shown that slow-cycling HSC are found in association with endosteal osteoblasts [19,21,26]. On the other hand, most of the fast-cycling HSC are found in close association with perivascular cells of the blood vessels distributed in the subendosteal zone [27,28]. This has been described *in vivo* in long-term BrdU retaining assays and myeloablation models. In experimental *in vivo* myelosuppressive models, HSC colonizing the vascular niches in the subendosteal region are mostly ablated. Almost all HSC in contact with endosteal osteoblasts are preserved [20,28]. By the time this chapter has been written, the existence of the two separate yet complementary niches is still questioned by a few authors based on technical issues arguments.

The role of endosteal osteoblasts on the HSC maintenance and self-renewal was first proposed *in vitro* by Taichman and Emerson [14,29,30] and later evidenced *in vivo* by others [31–33]. In transgenic animals, increased numbers of osteoblasts results in an increased number of long-term HSC, without affecting any other hematopoietic subpopulation in the bone marrow [31, 32,34]. Furthermore, when osteoblasts are removed from the marrow cavity, HSC numbers reduces drastically [33]. This is evidence that osteoblasts play a crucial role in HSC maintenance and behavior.

On the same study mentioned before, Lambertsen and Weiss [13] showed that most of the perivascular niches harboring HSC are distributed in the subendosteal zone. In the perivascular niche [19,20,27], HSC reside on the abluminal side of bone marrow sinusoids, and are supported by the endothelial and perivascular reticular cells. HSC residing in the perivascular niche are in close association with reticular cells, which express high levels of CXCL12, a chemokine required for HSC maintenance and lodging [17,27]. Most of the cells creating the proliferative niche express CXCL12. *In situ* observation demonstrated that most of hematopoietic stem cells are concentrated in the trabecular zone of the marrow cavity, which also harbors high numbers of niche osteoblasts, sinusoids, and CXCL12-positive reticular cells.

Nonetheless, HSC maintenance by both endosteal and perivascular niches are, at least in part, mediated by Jagged-Notch and angiopoietin-1-Tie2 interactions [20,27,31,32].

So, all in one thought, in the bone marrow, there are two distinct niches to harbor HSC, referred as to “proliferative niche” and “quiescent niche”, which are composed by perivascular cells and endosteal osteoblasts, respectively [15,22,31,32,35,36]. The real conversation between these niches, and how other elements, such as the immune system, would contribute to the niche formation, organization and dynamics are still to be understood.

The technique to isolate and culture separately endosteal osteoblasts and subendosteal reticular/perivascular cells from the marrow cavity of murine long bones was established [15] and global gene analyses data suggest that both endosteal and subendosteal stroma contribute to the formation of both niches in the marrow.

2. T cells as messengers from the periphery to the hematopoietic bone marrow

2.1. An overview of the immune system

The immune system is composed of hematopoietic cells, which we can be characterized according to the way they recognize and respond to antigens.

The innate immune system, phylogenetically, arises before the adaptive immune system and is so called because its ability to respond to antigens is ready and immediate. Characteristically, the innate immune cells recognize antigen through Pathogen Recognition Receptors (PRR), which are evolutionary conserved and can be common to different cell types. PRRs recognize defined molecular patterns from a pathogen [37] or something that is 'dangerous' to the body [38]. These molecular patterns, which are named Pathogen or Danger Associated Molecular Patterns (PAMPs or DAMPs), are poorly present or even absent in healthy mammals and are rich in or characteristics of bacteria, fungi, virus and so on. The cellular composition of the innate immune system is represented by phagocytes (granulocytes, monocytes/macrophages and dendritic cells) which deals with antigen, ultimately, eliminating it by phagocytosis or secretion of the internal granules content, and some lymphocytes as Natural Killer (NK) cells, $\gamma\delta$ T cells and B1 cells. In common, all these cellular types promptly respond to antigen and will do so in the same time frame and efficiency regardless their previous experience with the same antigen.

The adaptive immune system is so called because its components do not mount an immediate response to antigen. They need to be stimulated in order to mature their effectors functions and these take 3-5 days to happen, and will only be clinically effective after 7 days. Although it takes a while for the adaptive immune response to occur, it does so only once-on the first encounter with the antigen. On the following and subsequent encounters with the same antigen, the response will be fast occurring in less than 24h, revealing the existing memory response. Characteristically, the antigen recognition is done by antigen recognition receptors,

which are diverse at the population level and clonal and unique at individual cell level. These clonal receptors are not conserved and are generated by gene rearrangements during ontogeny of T-and B-lymphocytes, the cellular components of the adaptive immune system.

Innate immune cells and cells from the adaptive immune system mostly differentiate within the adult bone marrow, except for T lymphocytes that differentiate inside the thymus but also arise from hematopoietic progenitors. Although we can didactically separate the immune system into two categories, an effective immune response depends on both innate and adaptive cells. For T cells to be activated, they depend to see antigen complexed to the Major Histocompatibility Molecules (MHC) presented to them by antigen presenting cells (APC), having the dendritic cells (DC) as the most important cell type to initiate the response, or to prime the adaptive immune response. Also, the cytokines secreted by DC at the moment of antigen presentation, will define the fate of the T cell, meaning the cytokines these T cells will present or their specialization on Th1, Th2, Th17, etc for CD4+T cells or Tc1, Tc2, etc for CD8+cells. Also, the antibody class produced by B cells – IgG, IgA, IgE, etc – will be defined by cytokines produced by a given CD4T cell which will 'help' the given B cell at the moment of its activation. So, indirectly, it depends upon the APC which will modulate the CD4 fate. On the other hand, although the cells from the innate immune system can play their role independently, T helper cells can efficiently modulate it and, for example, optimize the microbicidal function of macrophages or even down modulate it. Also, through their role on dictating the immunoglobulin isotype to B cells, T cells will indirectly act on opsonization which will ultimately be effective through the innate system by optimizing phagocytosis and activation of phagocytes and granulocytes, all actors of the so called innate response.

The important point to have in mind here, before getting into the T cells inside the bone marrow, is that the effective immune response depends on T cells and an important part of the immune effector mechanisms rely on the collaboration between adaptive and innate responses, with the innate response being in many cases, the main players at the effector phase.

2.2. Bone marrow : A hospitable environment for T cells

After maturation inside the thymus, T cells gain the peripheral blood circulation and enter the secondary lymphoid organs (SLO) - lymph nodes and spleen - where they can be activated. Classically, these two SLO are considered the sites of naive T cell activation given their architecture, which allows concentration of antigen, DCs and naive T cells in the same neighborhood. This architecture is extremely important given the low frequency of antigen specific T cells making it difficult to meet with antigen, by chance, anywhere in the body.

Primed T cells will generate effectors cells, which will deal with the incoming antigen in the short-term response and will be vanished after antigen clearance. Primed T cells will also generate memory cells, which will be kept, even in the absence of antigen. Memory cells can be found in the SLO and in tissues as different memory cell subpopulations. Those in the tissues, are the effectors memory cells, which respond rather quickly after antigen exposure and those in the SLO are considered the central memory cells, responsible for keeping the memory pool and they take a little longer than effector memory cells to respond to antigen [39].

However, the above mentioned circulation pattern and activation sites of mature T cells had been challenged and revitalized by studies on BM T cells in the last decade.

T cells account for only 3-8% of total BM cells, what seems a small number, but in fact it is estimated to be close to or even higher than the number of T cells in the spleen when all hematopoietic bones are considered [40]. Moreover, the CD4 to CD8 ratio is 1:2 instead of the 2:1 ratio found in peripheral blood, indicating a local microenvironmental regulation of these cell subsets. Interestingly, these cells do not seem to be BM resident cells nor depend on antigen presence for its location in the bone marrow. Naive as well as memory T cells carry CXCR4, a receptor for CXCL12 (SDF1) a critical chemokine produced by stromal cells in the bone marrow, which play a significant role on HSC migration into the BM and its specific niche. Although both, memory and naive cells can respond to CXCL12 in migration assays, memory or activated cells respond more efficiently [41]. Besides, parabiont studies had shown that activated/memory CD4 and CD8 T cells recirculate and distribute equally through SLO and BM between the two animals [42,43] indicating that T cells recirculate through the BM.

Antigen recognition in the bone marrow could be one important requirement to keep them there not only as spectators but as active cells influencing the microenvironment. Of note is the fact BM CD8 T cells are extremely active, with a proliferation rate in vivo higher than the ones in spleen and lymph nodes [44,45]. Similarly, BM CD4 T cells produce high amounts of cytokine in the absence of intentional stimulation [46,47]. However, in the case of CD8 cells, when taken out from the BM, their behavior in vitro is similar to the one from splenic cells, indicating that this is not an intrinsic characteristic of BM cells, but is a modulation imposed by the BM microenvironment [40]. The presence of antigen is actually possible, as bone marrow DC were shown to present blood born antigens to naive CD4 and CD8 T cells [48]. Moreover, not only DCs, but other myeloid cells can also present antigen to naive BM T cells, what is not observed in spleen where T cell priming depends mostly on DCs [49]. Another curious fact about the bone marrow environment and T cells is that antigen specific cells are found in several diseases but do not always relate to the presence of antigen, neither in the bone marrow nor in the periphery [40]. Memory CD8 T cells are maintained by IL-7 and IL-15 which are produced in copious amounts by stromal cells in the BM. On the other hand, memory CD4 cells do not need recognition of MHC with the cognate peptide, but depend on the presence of MHC and IL-7 to be maintained.

So, it seems that the BM environment have all the requirements to attract and eventually keep T cells active: BM DC and other myeloid cells can present antigen and prime T cells, the stroma produces IL-7 and IL-15 necessary for memory CD8 maintenance, and hematopoietic cells in the marrow express MHC molecules fulfilling the requirements to maintain memory CD4 cells.

But what are these cells doing in the bone marrow, since the majority of infections are not BM specific, and in pathological conditions such as cancer, antigen specific cells are found there in the absence of the pathogenic antigen? (although the antigen peptide might be presented by BM DCs as mentioned above)

2.3. T cell help for hematopoiesis

BM is specialized in generating hematopoietic cells and T cells localize in the hematopoietic niche, on the perivascular regions where pericytes are present being one of the stem cell niches [50]. These data show that T cells are in close physical contact with the hematopoietic environment. In infectious situations, it appears that T cell amplification of hematopoiesis is required to clear pathogens [51–53]. These can be achieved by local secretion of cytokines by T cells (at the expense of antigen recognition *in situ*) including GM-CSF, IL-3, IL-4, IL-5, IL-6, IL-13, IL17 and oncostatin M, which all contribute to amplify granulocyte generation inside the bone marrow. However, the role of T cells in "normal" hematopoiesis has not been extensively considered as normal hematopoiesis is considered an innate immune phenomena independent of antigen recognition.

The relationship between hematopoiesis and T cells was first suggested almost 40 years ago when it was shown that 1-day thymectomized mice were anemic, showed arrested erythroid maturation and reduction in the number of spleen colony-forming units in the bone marrow and spleen [54,55]. In addition, intravenous injection of live thymocytes accelerated hematopoietic reconstitution in sublethally irradiated mice [56]. In the 90's it was suggested that singeneic T cells could stimulate the growth of hematopoietic progenitors [57]. Much more recently, it was clearly shown that T cell deficient mice (nude and SCID mice) have a severe reduction in the number of granulocytes in peripheral blood, despite the high frequency of granulo-monocytic progenitors in the bone marrow. By injecting CD4 T cells into these animals, the peripheral cytopenia was corrected and the number of progenitors accumulated in the bone marrow diminished to levels similar to the ones found in normal euthymic animals [46]. Moreover, studies with TCR transgenic mice in the RAGKO background, i.e., in the absence of endogenous gene rearrangements to guarantee that the only T cell specificity in the animals was the one from the transgenic receptor, evidenced the same altered hematopoiesis present in the T cell deficient mice: accumulation of immature myeloid-monocytic progenitors in the BM and granulopenia in the peripheral blood. Also, their BM T cells did not show the characteristic activated phenotype found in regular animals. Strikingly after injection of the cognate antigen, BM T cells became activated and the abnormal hematopoietic phenotype was corrected.

All these results show that T cells, as antigen recognition entities present in the periphery, act as messengers to the hematopoietic bone marrow. They traffic to the bone mainly after activation, recognize antigen within the bone cavity and help hematopoiesis so that it acquire the so-called 'normal' configuration. Normal hematopoiesis is not a phenomenon independent of the adaptive immune response, but is a response to an immunological insult, instructed by T cells (Figure 1).

At the end, this makes a lot of sense since the optimal effector immune mechanisms relies on innate immune cells acting on its best with components of the adaptive responses. And if memory response need to be fast and precise, T cells need to be rapidly activated and find their way to the bone marrow to instruct hematopoiesis to produce more of effectors. In the absence of enough phagocytes, and these in the absence of T cell help and immunoglobulins, the response will not be as efficient as necessary to counteract an invasive pathogen.

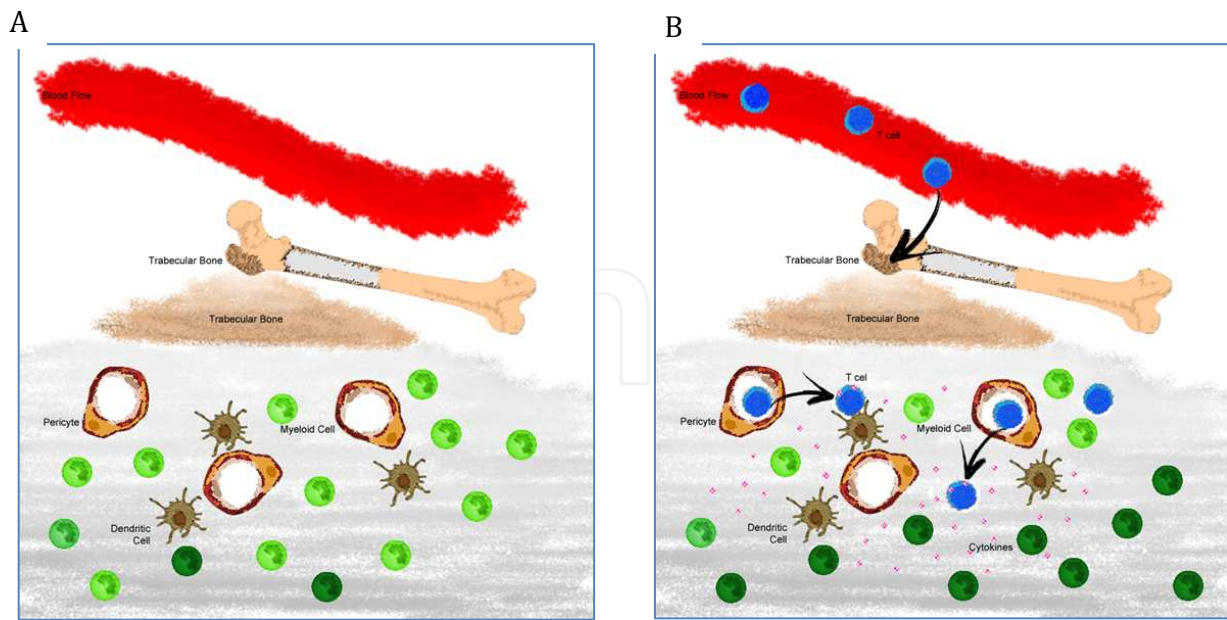


Figure 1. T cells help myeloid cell differentiation-In A, in the absence of activated T cells, myeloid progenitor cells (light green) accumulate in the bone marrow. When T cells are activated and migrate to the bone cavity, as shown in B, they most probably interact with APC such as DCs, secrete cytokines which will help the terminal differentiation of myeloid cells (dark green cells) giving rise to the normal cells counts in the peripheral blood.

3. Roommates in the bone cavity: Tumor cells, HSC and T cells

3.1. The crosstalk between T cells and bone: An overview of osteoimmunology

First of all, bone marrow is in close contact with bone tissue formed by the organized deposits of type I collagen and hydroxyapatite, a calcium phosphate salt, in which bone cells are distributed. Rather than being an inert matrix, bone undergoes a continuous turnover: osteoblast activity resulting in bone deposition is counteracted by osteoclast mediated bone resorption. Osteoblasts are cells of mesenchymal origin, whereas osteoclasts are of hemato-poietic origin — multinucleated giant cells, derived from monocytes/macrophages progenitors expressing CD11b–c, CD14 and receptor activator of nuclear factor, (RANK). Curiously, several factors regulating bone homeostasis are also molecular players of the immune response. For example, the TNF family member RANK ligand (RANKL) (also called TRANCE, OPGL, ODF), a potent regulator of osteoclast activation and differentiation, is expressed not only by osteoblasts, but also by monocytes, neutrophils, dendritic cells, B cells and activated CD4 and CD8 T cells [58–61]. RANKL mediates its biological effects by binding to RANK, expressed by osteoclast progenitors, mature osteoclasts, DCs and neutrophils [60,62]. RANKL can also bind to the soluble protein osteoprotegerin (OPG), which acts as an inhibitory decoy receptor and can be produced by osteoblasts, DCs and B cells [58–61]. By binding to RANK, RANKL strongly stimulates bone resorption, contributes to lymph node organogenesis, prolongs DC survival and augments DC adjuvant properties [63].

Once both immune and skeletal systems share many regulatory elements, including some which are key in bone remodelling, it seems reasonable to think that these two systems interact with each other. Indeed, the new and complex interdisciplinary field of osteoimmunology implies the concept that bone, and its cavity, crosstalk with the immune system. Osteoimmunology investigates the interactions between these two systems, since bone marrow stromal cells express surface molecules essential for hematopoiesis— from which all cells of the mammalian immune system derive — and stimulate immune cells, which produce various regulatory cytokines that influence the bone fate [58,64].

It is clear that immune cells producing pro-inflammatory cytokines contribute to bone damage by potentiating the effects of RANK/RANKL/OPG pathway. The cytokines TNF- α , interleukin (IL)-1, IL-3, IL-6, IL-7, IL-11, IL-15 IL-17A/F, and prostaglandin-E2 (as well hormones and related peptides as parathyroid hormone, parathyroid hormone-related protein, and glucocorticoids, besides 1,25(OH) $_2$ vitamin D3) potentiate bone loss either by increasing osteoclast generation and activation or by inducing RANKL expression by the osteoblasts [60]. On the other hand, IL-4, IL-5, IL-10, IL-12, IL-13, IL-18 and interferon (IFN)- α , IFN- β and IFN- γ are inhibitors of osteoclastogenesis by blocking RANKL signalling, either directly or indirectly [58,59]. Interestingly, IL-1 is a stimulator of TRAF6 expression on the osteoclast, thereby potentiating RANK/RANKL signaling cascade, whereas IFN- γ is known to downregulate TRAF6 by proteosomal degradation aborting osteoclast formation [58,65]. In contrast with this effect, IFN- γ has also been implicated in osteoclast formation and bone resorption, underlining the controversial role of IFN- γ in osteoclastogenesis [59,61,63,66].

The crosstalk mechanisms between T cells and osteoclasts have been extensively documented. The key role of Th 17 CD4 T cells, — an osteoclastogenic subclass of T cells expressing membrane and soluble RANKL, IL-17A/F and TNF α — on exacerbated and uncontrolled function of osteoclasts, has been investigated in models of inflammatory diseases, such as autoimmune rheumatoid arthritis [58,59], periodontitis [67], multiple myeloma [61] and breast tumor skeletal metastasis [68]. Nevertheless, the literature of this field has also been showing that these exacerbated osteoclast pattern, might also be controlled by other T cells subsets as regulatory CD4 T (Treg) cells. T reg cells produce anti-osteoclastogenic cytokines such as IL-4, IL-10 and TGF- β and express CTLA-4 inhibiting bone destruction [69,70]. Besides that, recently, it was showed that osteoclasts can present antigenic peptides to CD8 T cells, apart from CD4 T cells, resulting in FoxP3 expression. In this way, CD8 FoxP3+cells function as CD8 Treg cells, able to cause an inappropriate activation of the immune response through reciprocal interactions between CD137/CD137L and RANK/RANKL pathways. CD137, expressed on T cells, is a co-stimulatory molecule induced by TCR activation and its ligand, CD137L, is expressed on DCs and osteoclasts precursors. Once T cell CD137 binds to CD137L on osteoclasts precursors, multinucleation of osteoclasts is suppressed.. However, CD137/CD137L will signal simultaneously with RANKL/RANK on the Tcell/Osteoclast pair and this might lead to increased apoptosis by T cells [71]. Therefore, in pathological conditions, the effects of T cells on osteoclastogenesis will depend on the balance between positive and negative factors that they express.

In addition to inflammatory pathological conditions, increasing evidence supports the notion that T cells are also involved in post-menopausal osteoporosis [66]. Experiments in mice showed that, in the absence of estrogens, higher numbers of TNF- α producing T cells were found in the bone marrow, stimulating directly osteoclasts activity and augmenting their response to RANKL. By comparing peripheral blood mononuclear cells from pre and post-menopausal women, it was observed that estrogen deficiency was associated with an increased production of TNF- α . The action of TNF- α is not limited to the induction of local inflammation, but is both directly and indirectly involved in the activation of osteoclasts. Although further work is necessary to clarify the complex changes leading to post-menopausal osteoporosis in women, a pro-osteoclastogenetic contribution of T cells has to be taken into account.

Finally, it was documented that T cells have a protective role on bone turnover under physiological conditions [66,72]. Hints that this modulation may occur came from in vitro studies showing that osteoclastogenesis was inhibited by CD8 T cells. Moreover, after CD3 and CD28 activation, mouse lymph node CD8 T cells showed a delayed kinetics of RANKL expression, as compared with corresponding CD4 T cells. Culture of bone marrow cells from CD4 and CD8 T cell depleted mice showed enhanced osteoclastogenesis in response to 1,25(OH)₂ vitamin D₃ stimulation, suggesting that T cells had a suppressive effect in this system [6]. Moreover, the protective role of T cells on bone metabolism was also documented by in vivo studies, showing that both B cell- and T cell-deficient mice have decreased bone mineral density. A detailed analysis demonstrated that osteoporosis was prevented by osteoprotegerin produced by bone marrow resident B cells stimulated by T cells through CD40L/CD40 interactions. In contrast, IL-17A does not play any relevant role in physiological bone homeostasis, as IL-17A-deficient mice show normal bone mineral density and skeletal development [6]. Taken together, these findings support the notion that bone marrow derived CD4 and CD8 T cells play a protective role in physiological bone homeostasis, using pathways different from those associated with inflammatory bone diseases.

3.2. Inflammatory bone diseases: Rheumatoid arthritis as a model of T cell involvement in bone diseases

Rheumatoid arthritis (RA) is the prototype of chronic inflammatory joint diseases and is characterized by persistent inflammation and progressive bone erosions, leading to functional disability and high morbidity. In this disease it is clear that the pro-inflammatory cytokines IL-17A, TNF- α , IL-1 and IL-6 are involved in the perpetuation of the inflammatory condition. The RANK/RANKL/OPG pathway is also strongly involved in RA pathology and it was observed that the RANKL/OPG ratio is increased, leading to bone erosions [58,59]. This effect is mainly dependent on osteoclastic activity and is expressed by two main mechanisms: i) destruction of the organic matrix (type I collagen) by osteoclast cathepsin K, and ii) dissolution of the mineralized component (hydroxyapatite crystals) by the acidic microenvironment generated by the osteoclastic proton pump.

In RA, T cell derived RANKL was initially proposed to be the main contributor to exacerbated osteoclastogenesis, but Th17 RANKL⁺ subset T cells from RA joints also produce IFN- γ , an anti-osteoclastogenic cytokine, which counterbalance the action of RANKL [58–61]. Thus, in

such model, it is not clear whether Th17 RANKL⁺ subset T cells exert a direct effect on osteoclastogenesis. It is more likely that Th17 RANKL⁺ subset T cells contribute indirectly through IL-17A activity over synovial fibroblasts which produce RANKL and directly stimulates osteoclastogenesis. Another T cell shown to be potentially involved is the CD4 Treg subset. In fact, an increase of Treg CD4 T cell number improves clinical signs of arthritis and suppressed local and systemic bone destruction. Synovial tissues of patients with RA also produce many factors regulating bone resorption, such as TNF- α , IL-1 and IL-6, which amplify osteoclast differentiation, activation and consequent bone destruction. Inhibitors that target TNF- α , IL-1 and IL-17A pro-inflammatory and osteoclastogenic cytokines have been approved for the treatment of RA.

More recently, investigators also demonstrated that RANKL plus macrophage colony-stimulating factor can induce transdifferentiation of immature dendritic cells into the osteoclastogenic lineage and that this process is significantly enhanced by RA synovial fluid [73]. Dendritic cells are antigen presenting cells, but they could function as osteoclasts precursors in inflammatory conditions. We can conclude that since dendritic cells modulate T cell activity through the RANK/RANKL pathway and other cytokines associated with osteoclastogenesis, as mentioned earlier, it can function as an osteoimmune interface, contributing to bone loss in inflammatory diseases.

Although T cells clearly contribute to RA pathology, they do so in the outer face of the bone, outside the bone marrow cavity. By the same token, periodontal disease, which had been shown to be dependent of Th17 RANKL⁺ T cells activated by bacteria present in the oral cavity also lead to extra medullary lesions [67]. Similar mechanisms might act in pathological situations arising within the bone marrow cavity such as post-menopausal osteoporosis and cancers, as myeloma and solid tumor metastasis. In either case it is clear the potential for the adaptive immune system to interact with the bone remodelling system.

3.3. Cancer: Multiple myeloma as an example of bone marrow derived tumors

Multiple myeloma (MM) is a clonal B-cell malignancy characterized by an accumulation of mature plasma cells in the bone marrow, leading to bone destruction and failure of normal hematopoiesis. However the cancer induced osteolytic disease in this case, may count on T cell activity inside the bone marrow, in addition to the presence of the tumor itself [61]. In multiple myeloma patients with lytic bone disease, it was observed an increase in bone marrow Th17 T cells expressing high levels of RANKL that can directly stimulate osteoclasts [49,74]; moreover, increased production of T cell derived IL-3, occurring in this disease, can inhibit osteoblast generation and facilitate hematopoiesis.

This is one the few, if not the only, malignant bone marrow disease associated with bone loss where T cell activity has been studied, and actually shown to be concordant with osteolytic activity. So, instead of having the osteolytic disease induced by cancer cells only, it is proposed the participation of Th17 cells in the pathogenesis of lytic lesions in bone marrow malignancies. In fact, as reported for other human malignancies [75] and in accordance to the phenotype of BM T cells, the number of memory/activated T cells in MM patients is increased, their activity is enhanced and they proliferate much more efficiently than blood derived T cells. These data

suggest that BM T cells in pathological, non-infectious conditions such as cancer, can also migrate to the bone marrow and in addition to its effects over hematopoiesis, as discussed above, they can also influence bone remodelling.

3.4. Breast tumor skeletal metastasis: the case of osteolytic bone disease in the absence of tumor cells

Bone metastases, present in 70% of patients with metastatic breast cancer, lead to skeletal disease, fractures and intense pain, which are all believed to be mediated by tumor cells. Engraftment of tumor cells is supposed to be preceded by changes in the target tissue to create a permissive microenvironment, the pre-metastatic niche, for the establishment of the metastatic foci. In bone metastatic niche, metastatic cells stimulate bone consumption resulting in the release of growth factors that feed the tumor, establishing a vicious cycle between the bone remodelling system and the tumor itself [76]. Yet, how the pre-metastatic niches arise in the bone tissue remains unclear.

As already mentioned before, CD4 and CD8 T cells have been shown to unbalance the bone remodeling process in inflammatory osteolytic diseases, however, little is known about their role in cancer induced bone disease, a process that differs from inflammatory diseases as in the former it happens in the bone cavity and not on the periosteal surface.

It had been shown, in an experimental model, that tumor specific T cells have a pro-osteoclastogenic phenotype, i.e., Th17 producers of IL-17F and RANKL among others, when tumors are highly metastatic. On the other hand, the T cell phenotype was not pro-osteoclastogenic, and even rich in anti-osteoclastogenic cytokines as IFN- γ and IL-10, if the tumor was localized to the breast and incapable of sending metastasis to any distant organ, including the BM. This suggest that T cells activity is modulated by the tumor since sibling cell lines, with different metastatic characteristics and sharing the same cognate T cell antigen, trigger different T cell phenotypes.

The pro-osteoclastogenic T cell phenotype observed with metastatic tumor was evident inside the BM, and preceded bone metastatic colonization. Also, osteolytic lesions were already present very early on disease evolution, and again, before metastatic colonization. By transferring BM T cells from animals bearing the highly aggressive tumors, before metastasis started, to athymic mouse which never saw tumors, led to an intense osteolytic disease, similar in kinetics and intensity to the one observed in the tumor bearing donor animals. These indicate that T cells can induce osteolytic disease which precedes metastatic colonization. In vivo inhibition of RANKL production by Th17 CD4 T cells, but not of IL-17F, completely protects mice from osteolytic disease and, surprisingly, completely abolishes the development of bone metastases, suggesting that CD4 T cells prepare the metastatic niche for further establishment of tumor cells. In conclusion, these results unveil an unexpected role for RANKL derived from T cells in setting the pre-metastatic niche and promoting tumor spread, an extra role for T cells only recently explored (Figure 2).

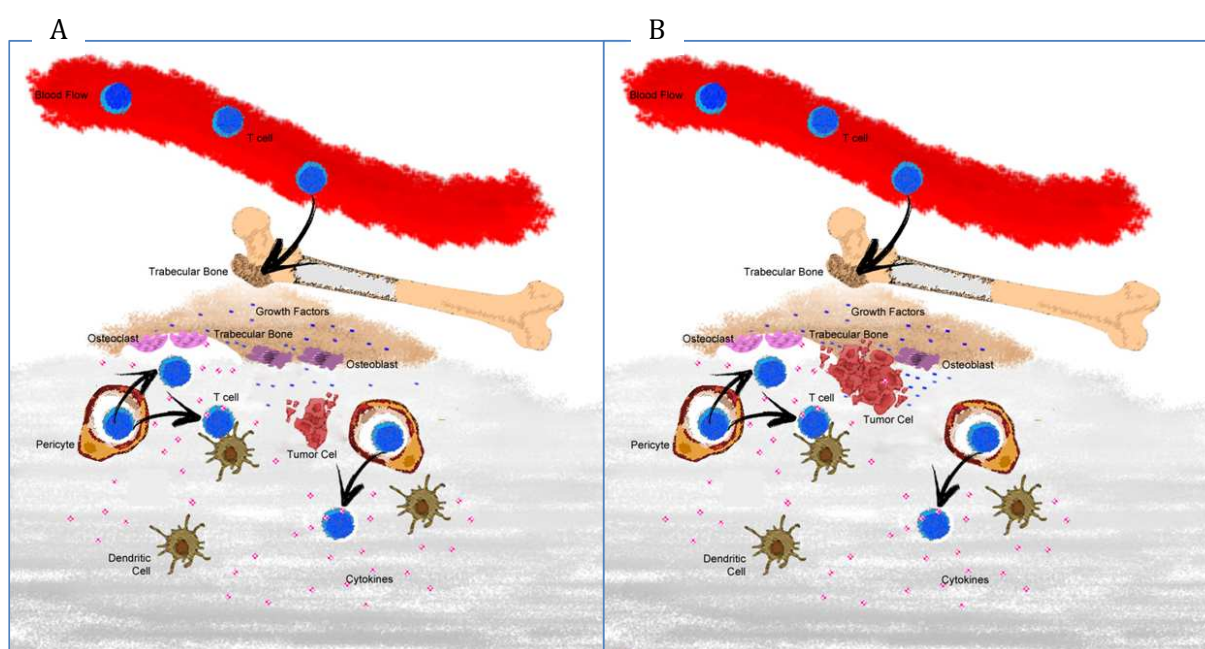


Figure 2. T cells induce osteolytic disease which is critical for bone metastasis establishment. In A ; Tumor primed T cells, inside the BM, produce pro-osteoclastogenic cytokines (mainly IL-17F and RANKL) which promotes osteoclastogenesis and activate osteoclasts. Osteoclast activity generates a pro-metastatic environment, with the release of growth factors from the bone matrix. In B, with the hospitable environment prepared, tumor cells can establish themselves in the marrow, and after that, they will progress by themselves, directly regulating bone remodeling.

In summary, for tumor cells to first establish in the bone marrow niche, growth factors need to be available favoring its hostage, in other words, a pre-metastatic niche needs to be prepared. On that sense, again, T lymphocytes work as messengers from the periphery as they migrate to the BM after they get primed by tumor antigens. In the BM, they signal and alter bone homeostasis as to prepare the pre-metastatic niche instructed by the tumor modulation of T cell phenotype in the periphery [68].

4. Conclusions and perspectives

From all of the above it can be depicted that once the hematopoietic niche depends upon the bone lining osteoblasts, it is reasonable to expect an interplay between bone and hematopoietic regulation. On the other hand, it is known that T lymphocytes also communicate with the hematopoietic and bone tissues adding more complexity to the whole balance of these systems.

T cells in the bone marrow are compatible with memory cells and found in their activated state. In the absence of T cells, hematopoiesis is altered and a maturation arrest is observed in the bone marrow, where high numbers of immature myelo-monocytic progenitors are found accompanied by peripheral cytopenia. When T cells are replenished, the bone marrow arrested progenitors progress, differentiate and migrate to the periphery, correcting the myelogram and the peripheral cytopenia. This is conceptually important since what we use to understand

as "normal" hematopoiesis, which should be an antigen independent activity, is already the result of the adaptive immune response, which, in fact, need the innate immune cells to operate!

The localization of active T cells within the marrow cavity coincides with the "proliferative niche" of adult HSC, or the perivascular niche. In fact, the evidences favor a T cell function on the proliferative/differentiative phase of myelopoiesis and not on stem cell maintenance, as stated above.

Hematopoietic stem cells are supported and regulated by stromal cells covering the inner surface of bones, or the endosteum. The endosteum also supports bone remodeling and osteoblasts are present there. The subendosteal region harbors pre-osteoblast, reticular cells and mesenchymal stem cells, with higher hematopoietic supporting role, characterizing different niches involved in different activities that might be cross-regulated somehow.

Activated T cells are able to interact with hematopoietic system, apparently on the proliferative niche, and this is in close contact with the bone remodeling system. When activated, BM T cells can increase osteoclastogenesis and this will favor, in case of a bone metastatic disease, the establishment of bone colonization by the malignant cell. Curiously, metastatic cells in the BM use the same niche as hematopoietic progenitor cells [77] and might well be regulated by antigen specific activated T cells, which are able to prepare the pre-metastatic niche and interfere with the hematopoietic niche. At one side, increase in osteoclasts numbers might be the result of increased proliferation of myeloid progenitors, but it also result from direct T cell signaling to stimulate osteoclast differentiation (figure 3).

Whether or not alterations on bone remodeling are directly linked to alterations in hematopoiesis and vice-versa, and the dependency on the T cell adaptive immune response is still a theme of debate.

Studies in germ free mice might elucidate the subject, since the absence of intentional stimulation is not synonymous of no stimulation making it difficult to address the immune regulation of blood and bone.

It is now believed that the commensal microbiota plays an important role on basically every system related to immune response. Very recently, it was shown that Germ Free mice are cytopenic not only in the peripheral blood, but also in the bone marrow. The cytopenia is reversed after intestinal colonization with commensal bacteria [78] and this provide an optimal tune to the immune system to fight infection. On the other hand, Germ Free mice were recently shown to be osteopetrotic and this is reversed by colonization with commensal microbiota [79]. Moreover, in this report, the number and activation state of immune cells was analyzed and in the BM, the number of T cells was diminished in GF mice and the frequency of osteoclast precursors was also deficient. These are in accordance with the view that in the steady state, recognition of antigen by T cells tune the bone remodelling system and this might be related to hematopoietic activity.

Altogether, we provide evidence that, inside the BM there are at least two co-existing systems- bone and hematopoietic-which can be regulated by T cells as they bring messages from the periphery to the BM, resulting in hematopoietic/cancer niche and bone remodeling regulation

(figure 3). Whether or not, regulation of one system interferes with the other one, and most important, to which extend, is still a matter of debate.

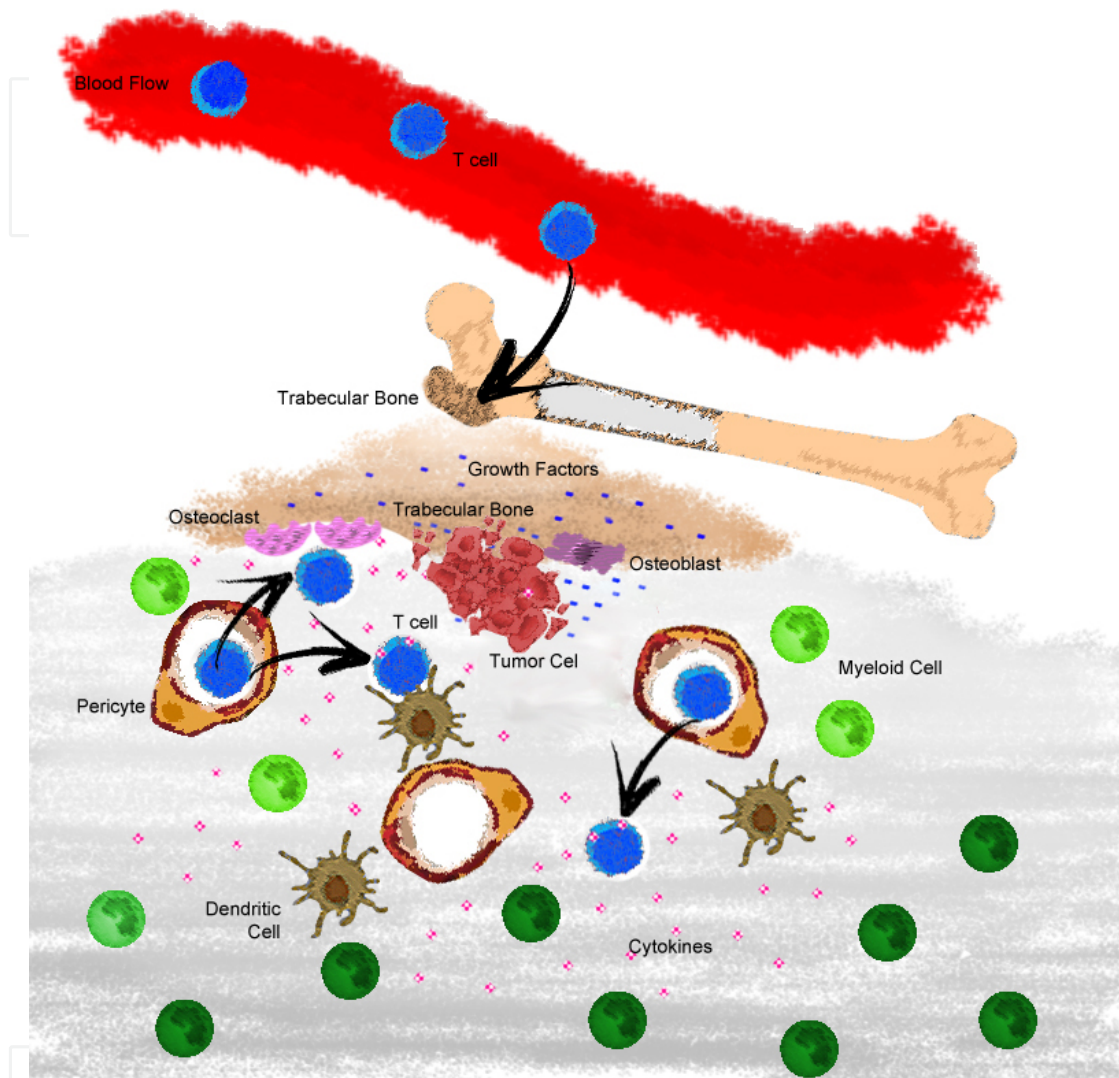


Figure 3. T cells modulate the bone and hematopoietic system after activation: Antigen primed T cells, inside the BM, produce cytokines which can act in both, hematopoiesis and bone remodeling. Depending on the stimuli, each of the two systems can receive positive or negative signals. In the case exemplified here, positive signals for osteolytic disease and myeloid cell expansion and maturation is shown. On this case, T cell help metastasis establishment and boost hematopoiesis. (dark green : mature myeloid cells, light green: immature myeloid cells, Blue: T cells, brown: dendritic cells, pink: osteoclasts, red: tumor cells, purple: osteoblasts).

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