

## Review

# Regulation of hematopoietic and leukemic stem cells by the immune system

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Hematopoietic stem cells (HSCs) are rare, multipotent cells that generate via progenitor and precursor cells of all blood lineages. Similar to normal hematopoiesis, leukemia is also hierarchically organized and a subpopulation of leukemic cells, the leukemic stem cells (LSCs), is responsible for disease initiation and maintenance and gives rise to more differentiated malignant cells. Although genetically abnormal, LSCs share many characteristics with normal HSCs, including quiescence, multipotency and self-renewal. Normal HSCs reside in a specialized microenvironment in the bone marrow (BM), the so-called HSC niche that crucially regulates HSC survival and function. Many cell types including osteoblastic, perivascular, endothelial and mesenchymal cells contribute to the HSC niche. In addition, the BM functions as primary and secondary lymphoid organ and hosts various mature immune cell types, including T and B cells, dendritic cells and macrophages that contribute to the HSC niche. Signals derived from the HSC niche are necessary to regulate demand-adapted responses of HSCs and progenitor cells after BM stress or during infection. LSCs occupy similar niches and depend on signals from the BM microenvironment. However, in addition to the cell types that constitute the HSC niche during homeostasis, in leukemia the BM is infiltrated by activated leukemia-specific immune cells. Leukemic cells express different antigens that are able to activate CD4<sup>+</sup> and CD8<sup>+</sup> T cells. It is well documented that activated T cells can contribute to the control of leukemic cells and it was hoped that these cells may be able to target and eliminate the therapy-resistant LSCs. However, the actual interaction of leukemia-specific T cells with LSCs remains ill-defined. Paradoxically, many immune mechanisms that evolved to activate emergency hematopoiesis during infection may actually contribute to the expansion and differentiation of LSCs, promoting leukemia progression. In this review, we summarize mechanisms by which the immune system regulates HSCs and LSCs.

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## Facts

- Hematopoiesis and leukemia are both hierarchically organized processes originating from HSCs and LSCs, respectively.
- LSCs display many features of normal HSCs, including quiescence and self-renewal.
- HSCs and LSCs crucially depend on signals from the BM microenvironment, the so-called niche.
- The BM microenvironment contains innate and adaptive immune cells that regulate hematopoiesis during homeostasis, stress response and infections.
- In leukemia, activated immune cells paradoxically contribute to disease progression.

## Open Questions

- What is the contribution of BM-infiltrating immune cells to the HSC and LSC niche?
- What are the molecular mechanisms of the interaction between immune cells, LSCs and niche cells?
- Do stress-induced alterations in hematopoiesis favor leukemia development and progression?
- How can the knowledge about BM-resident immune cells be exploited to improve immunotherapy for leukemia?

The concept that cancer develops in a hierarchical tree from disease-originating cancer stem cells (CSCs) that self-renew and give rise to more differentiated, non-cancer-initiating cells

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**Abbreviations:** AML, acute myeloid leukemia; BCR/ABL1, break point cluster region/Abelson murine leukemia viral oncogene homolog 1; BM, bone marrow; CLPs, common lymphoid progenitors; CML, chronic myeloid leukemia; CMPs, common myeloid progenitors; CSCs, cancer stem cells; CTLs, cytotoxic effector CD8<sup>+</sup> T cells; CXCL12, C-X-C motif chemokine 12; CXCR4, C-X-C motif chemokine receptor 4; DCs, dendritic cells; ECs, endothelial cells; FLT3-ITD, fms-like tyrosine kinase 3—internal tandem duplication; GM-CSF, granulocyte-macrophage colony stimulating factor; HPCs, hematopoietic progenitor cells; HSCs, hematopoietic stem cells; HSPCs, hematopoietic stem and progenitor cells; IFNs, interferons; IL, interleukin; JC virus, human polyomavirus 2; LCMV, lymphocytic choriomeningitis virus; LEPR, leptin receptor; LMPPs, lymphoid-primed MPPs; LRP, lineage-restricted progenitors; LSCs, leukemic stem cells; mAb, monoclonal antibody; MEP, megakaryocyte-erythrocyte progenitor; MPPs, multipotent progenitors; MSCs, mesenchymal stem/stromal cells; OBs, osteoblasts; OCs, osteoclasts; OLPs, osteolineage progenitor cells; STAT-1, signal transducer and activator of transcription 1; TGF- $\beta$ , transforming growth factor  $\beta$ ; TKIs, tyrosine kinase inhibitors; TLR, Toll-like receptor; TNF $\alpha$ , tumor necrosis factor  $\alpha$ ; TNFR, TNF receptor; TNFRSF1 $\alpha$ , p55 TNF receptor 1  $\alpha$ ; T<sub>regs</sub>, CD4<sup>+</sup> CD25<sup>+</sup> FOXP3<sup>+</sup> regulatory T cells; VCAM-1, vascular cell adhesion molecule-1

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by asymmetric division was first documented in leukemia two decades ago.<sup>1</sup> The CSC hypothesis is now widely accepted and was extended and adapted to several solid tumors.<sup>2</sup> Since the first description of leukemic stem cells (LSCs), our knowledge about their biology grew substantially and nowadays, LSCs are phenotypically well characterized in chronic myeloid leukemia (CML) and in some forms of acute myeloid leukemia (AML).<sup>3</sup> From a clinical point of view, LSCs are of fundamental interest as they are resistant against most of our current cancer treatments such as irradiation and chemotherapy and probably also against more targeted therapies such as tyrosine kinase inhibitors and immunotherapy.<sup>4</sup> Therefore, LSCs are the main reason for treatment failure and disease relapse. Different mechanisms may contribute to the resistance of LSCs to current therapies. LSCs express drug efflux proteins that lead to multidrug resistance.<sup>5</sup> In addition, most cytotoxic drugs and irradiation depend on cell division in order to induce cell death but LSCs are largely quiescent. Many stem cell characteristics including quiescence are determined by interactions with the niche. Growing evidence suggests that LSCs depend on similar niche signals as their normal counterpart, the hematopoietic stem cells (HSCs).<sup>6</sup> Although HSCs are mobile and recirculate in the blood, most of them are found in the trabecular bone area of the bone marrow (BM),<sup>7,8</sup> where they reside in close proximity to sinusoids and other blood vessels.<sup>9</sup> Endothelial and perivascular cells produce C-X-C motif chemokine 12 (CXCL12) and stem cell factor that are necessary for HSC and LSC maintenance.<sup>10–12</sup> The role of other cell populations present in the BM in the regulation of HSC function is less clear. However, the sympathetic nervous system, adipocytes, macrophages and cells of the adaptive immune system have been shown to regulate hematopoietic stem and progenitor cells (HSPCs).<sup>13,14</sup>

In a healthy individual, CD4<sup>+</sup> and CD8<sup>+</sup> T cells represent approximately 1.5% and 2.5% of the total BM cellularity, respectively. Up to 30% of all BM-resident CD4<sup>+</sup> T cells are CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> regulatory T cells (T<sub>regs</sub>).<sup>15</sup> Interestingly, BM T cells including T<sub>regs</sub> are also localized in the trabecular bone area in proximity to sinusoids. BM CD4<sup>+</sup> and CD8<sup>+</sup> T cells have a memory phenotype and secrete cytokines that are necessary for HSC maintenance, such as interleukin 3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF).<sup>16</sup> Therefore, BM-resident T cells may contribute to the formation of the perivascular HSC niche. In response to an infection or BM stress, the cellular composition of the microenvironment as well as the cytokine milieu change fundamentally in order to meet the organism's requirement for demand-adapted hematopoiesis.<sup>17</sup>

Similarly, leukemia induces an adaptive and innate immune response and causes an inflammatory environment in the BM. Various leukemia antigens have been characterized and activated leukemia-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells have been documented.<sup>3,18</sup> Therefore, the BM microenvironment and the LSC niche changes dramatically with leukemia-specific effector T cells infiltrating the BM. These infiltrating effector T cells may potentially recognize and eliminate LSCs. However, mechanisms that evolved to protect normal HSCs from elimination and to regulate demand-adapted responses during inflammation most likely protect LSCs from

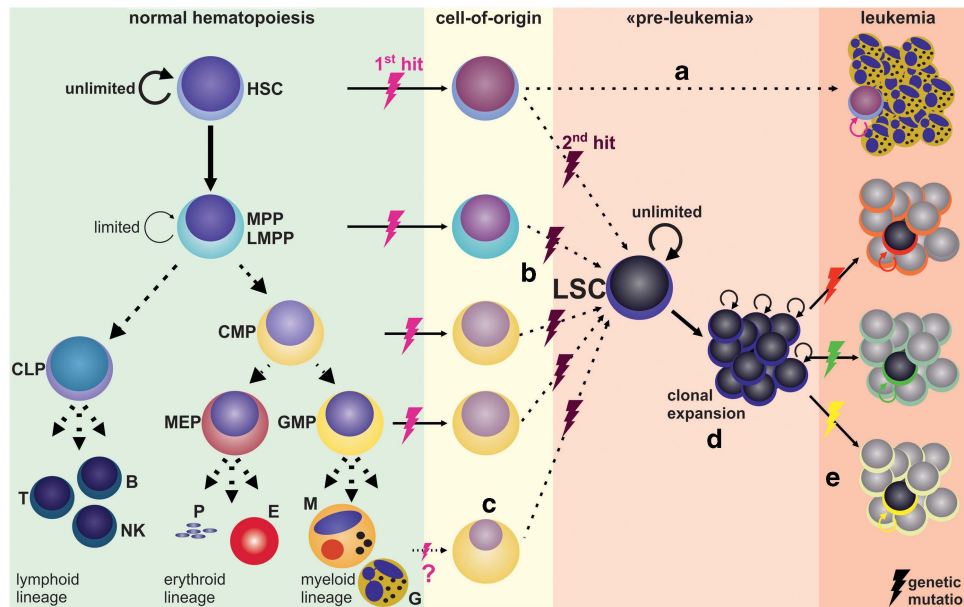
immune-mediated elimination and may even contribute to the expansion of LSCs and leukemia progression. The understanding of these mechanisms may help to develop novel immunotherapies that allow targeting LSCs specifically.

## HSCs and LSCs

HSCs are multipotent and self-renewing tissue-specific stem cells that initiate and maintain life-long hematopoiesis, the production of mature blood cells of all lineages.<sup>19</sup> HSCs are rare cells that only comprise approximately 0.001–0.01% of total BM cells in mice<sup>20</sup> and approximately 0.01–0.2% of total BM mononuclear cells in humans.<sup>21</sup> Being at the top of the hematopoietic hierarchy, HSCs divide infrequently, giving rise to transient-amplifying multipotent (MPPs) and lineage-restricted progenitors that proliferate extensively and differentiate toward mature blood cells (Figure 1). Quiescence, also known as dormancy, warrants the genomic integrity of HSCs, as frequent chromosomal replications may introduce oncogenic DNA mutations. Dormancy also protects HSCs from uncontrolled proliferation, which would result in exhaustion.<sup>22</sup> In addition, HSCs can undergo asymmetrical division, assuring that always one daughter cell remains an HSC. This mechanism, referred to as self-renewal, keeps the HSC pool constant. However, HSCs possess a non-exhaustive replication and proliferation capacity that can be initiated in stress situations, such as after cytotoxic chemotherapy, irradiation or during infections.<sup>22</sup> Moreover, HSCs express receptors for cytokines, chemokines and danger-associated molecular patterns, allowing them to respond to signals from mature immune cells and to sense pathogens directly during inflammation or infection to adapt their cycling and differentiation behavior.<sup>17</sup>

Leukemia is a paradigmatic disease of CSCs. According to the CSC hypothesis, tumors are composed of a bulk of cancer cells displaying marked morphological, genetic and functional heterogeneity. Within this bulk resides a small population of cells with stem cell characteristics that propagates the disease.<sup>2,23</sup> In leukemia, LSCs are thought to reside at the top of the leukemic hierarchy, like HSCs in hematopoiesis (Figure 1). LSCs produce more differentiated, heterogenic leukemic blasts that feature a high proliferative potential, a block in terminal differentiation and defective apoptosis or senescence mechanisms, leading to blast accumulation and clinical disease. Stem cell features, such as quiescence, the expression of high levels of ATP-binding cassette pumps and the localization in distinct niches, render CSCs resistant to all kinds of therapy.<sup>4,6</sup> Thus, cure of cancer implies the elimination of CSCs and persisting CSCs are a main cause of disease relapse.

In contrast to differentiated hematopoietic cells that are removed after fulfilling their functions, self-renewing HSCs persist for long periods of time, allowing accumulation of genetic damage and malignant transformation. Therefore, it has been suggested that HSCs serve as the cancer-initiating cells (cell-of-origin) for LSCs (Figure 1).<sup>24,25</sup> Experimental evidence supporting this theory came from seminal studies by John Dick and colleagues who first demonstrated in xenotransplants that all clonogenic capacity resided in lin<sup>−</sup>CD34<sup>+</sup>CD38<sup>−</sup> AML cells, whereas lin<sup>−</sup>CD34<sup>+</sup>CD38<sup>+</sup> or lin<sup>+</sup> AML



**Figure 1** The leukemic stem cell model. In normal hematopoiesis, rarely dividing hematopoietic stem cells (HSCs) with unlimited self-renewal capacity (indicated by circle arrow) give rise to transient-amplifying multipotent progenitors (MPPs) that have only limited self-renewal capacity.<sup>20</sup> MPPs further differentiate toward oligopotential lineage-restricted progenitors (LRPs), such as common lymphoid and myeloid progenitors (CLPs, CMPs) and granulocyte-macrophage progenitors (GMPs) that have lost self-renewal capacity. LRP proliferate intensely and produce all mature blood cell types required. The formation of a leukemic stem cell (LSC) in myeloid leukemia may result from mutations in cells in different stages of the hematopoietic hierarchy. (a) In chronic phase CML patients, the presence of BCR/ABL1 in all blood lineages suggests that the LSC is derived from an HSC or an early MPP with multilineage differentiation potential (HSC cell-of-origin).<sup>27–29</sup> BCR/ABL1 is necessary and sufficient for the malignant phenotype, no further genetic lesions are required for chronic phase CML. (b) In contrast, in blast crisis CML and AML patients, LSCs exhibited immunophenotypes of LRPs, such as lymphoid-primed MPPs (LMPPs)<sup>31</sup> or GMPs.<sup>30,31</sup> This supports the concept that other more differentiated cells can give rise to LSCs after re-acquisition of self-renewal (progenitor cell-of-origin). (c) In addition, the recent demonstration that some AML LSCs even express low amounts of lineage markers<sup>32</sup> raised the question whether more differentiated hematopoietic cells may serve as cell-of-origin for LSCs as well. (d) In a 'pre-leukemic' disease phase, genetically unstable, self-renewing LSCs clonally expand, facilitating the acquisition of further mutations and (e) the development of different leukemic clones. B, B cell; CML, chronic myeloid leukemia; E, erythrocyte; G, granulocyte; NK, natural killer cell; M, monocyte; MEP, megakaryocyte-erythrocyte progenitor; P, platelet; T, T cell

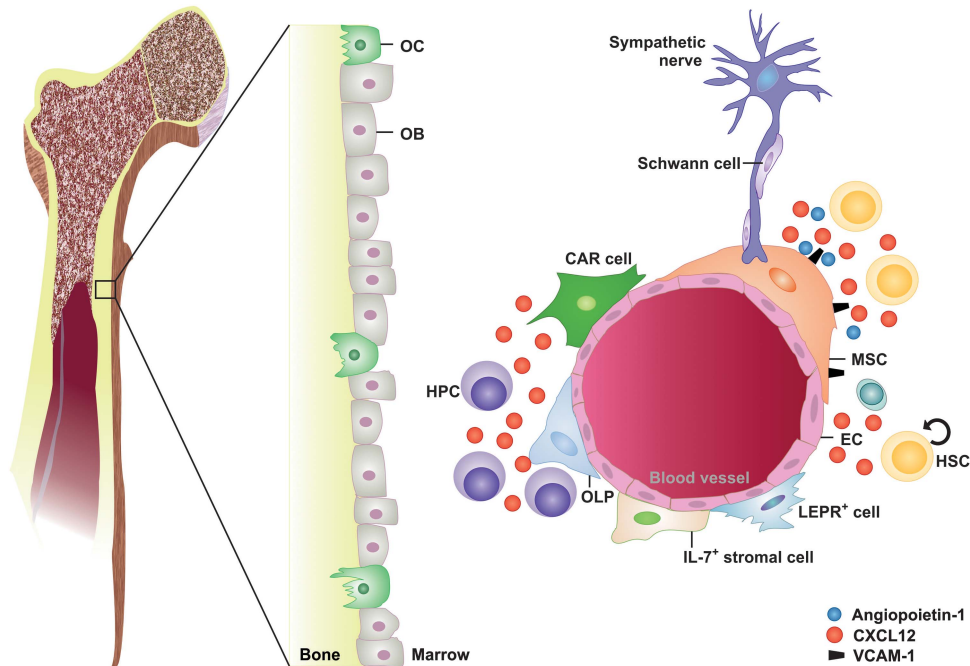
cells failed to induce leukemias.<sup>1,26</sup> Furthermore, in CML, the break point cluster region/Abelson murine leukemia viral oncogene homolog 1 (BCR/ABL1) oncogene can be detected in several hematopoietic lineages, indicating that the cell-of-origin is an HSC with multilineage differentiation potential.<sup>27–29</sup> In addition, DNA (cytosine-5)-methyltransferase 3A mutations have been found in HSCs, progenitor and mature cells of AML patients, but without coincident nucleophosmin 1 mutations present only in AML blasts.<sup>25</sup> This indicates that AML evolves from pre-leukemic HSCs. However, the hypothesis that HSCs represent the cell-of-origin and undergo oncogenic transformation has been challenged by studies demonstrating that in some leukemias, more mature progenitor cell types or even cells expressing lineage markers can serve as leukemia-initiating cells giving rise to LSCs (Figure 1).<sup>30–32</sup> This raised the question of how—besides the oncogenic events that are required for leukemia development—these leukemia-initiating cells re-acquire self-renewal capability to fully establish their LSC functions.<sup>23</sup> In addition, these findings nurtured the alternative hypothesis of tumorigenesis, the stochastic model, claiming that every cancer cell has the ability to self-renew and to recapitulate the disease phenotype given that it enters a permissive environment, an event which has a very low probability.<sup>2</sup>

Thus, although the cell-of-origin may not be identified for all types of leukemia yet, these cells have to exhibit the essential

stem cell characteristics of self-renewal and indefinite proliferative potential to give rise to LSCs that initiate and maintain the disease.

## The HSC Niche

Many of the functional characteristics of HSCs and LSCs are driven by their surrounding microenvironment in the BM, the so-called HSC niche (Figure 2). The HSC niche has been initially defined as microenvironment that retains HSCs in their localization, avoids differentiation and ensures their stem cell phenotype.<sup>33</sup> The functional and anatomical definition as well as the cellular composition of the HSC niche have been highly debated during the last decade. First, osteoblastic lineage cells have been described as critical components of the HSC niche (Figure 2). Bone-forming osteoblasts (OBs) that are located at the endosteal surface of the bone cavities and on trabeculae co-localized with HSCs and regulated the HSC pool size *in vivo*.<sup>34,35</sup> Furthermore, endosteal osteoclasts influence HSC maintenance and retention in the BM.<sup>36,37</sup> Later, Kiel and co-workers demonstrated that primitive HSCs defined by SLAM markers (CD150<sup>+</sup>, CD48<sup>−</sup>) preferentially localize in close proximity to sinusoidal endothelial cells (ECs) but not OBs, identifying sinusoidal blood vessels as HSC niche<sup>9</sup> (Figure 2). Clinical observations confirm an important role for ECs in the formation of the HSC niche, as HSCs



**Figure 2** The hematopoietic stem cell niche. Various cell types including osteoclasts (OCs), osteoblasts (OBs), osteolineage progenitor cells (OLPs), endothelial cells (ECs), mesenchymal stem/stromal cells (MSCs), specialized CXCL12-abundant reticular (CAR) cells and leptin receptor (LEPR)-positive cells contribute to the structure of the BM microenvironment. In addition, this microenvironment is innervated by sympathetic nerves fibers ensheathed by nonmyelinating Schwann cells. Hematopoietic stem cells (HSCs) are located in the perivascular region of sinusoids and arterioles in close proximity to MSCs and ECs that regulate HSC maintenance and differentiation through soluble factors such as CXCL12 and angiopoietin-1 or cell contact-dependent signals such as vascular cell adhesion molecule-1 (VCAM1). HPC, hematopoietic progenitor cell

depend on an intact vasculature for recovery after myeloablation or BM transplantation.<sup>38,39</sup> More recently, perivascular nestin<sup>+</sup> mesenchymal stem cells (MSCs) have been defined as central components of the HSC niche that regulate HSCs via the expression of stem cell factor, CXCL12, angiopoietin-1 and vascular cell adhesion molecule-1 (VCAM-1).<sup>12</sup> In addition, it was demonstrated that  $\beta$ -adrenergic signals from the sympathetic nervous system mobilize HSCs<sup>13</sup> and regulate circadian HSC egress.<sup>40</sup>

These data suggested that at least two distinct HSC niches exist, an endosteal and a vascular niche (Figure 2). According to this hypothesis, the endosteal niche keeps HSCs quiescent and regulates their migration to the vascular niche, where differentiation occurs according to the organism's demand.<sup>22,41</sup> However, recent studies challenged the existence of an endosteal niche and a role of OBs in the maintenance of HSC quiescence. Transforming growth factor- $\beta$  (TGF- $\beta$ ) is a niche factor that controls HSC dormancy.<sup>42</sup> Nonmyelinating Schwann cells that are located around the blood vessels in the BM induce HSC dormancy by secreting TGF- $\beta$ , suggesting that HSC quiescence is maintained in the vascular niche.<sup>43</sup> Recently, these findings have been further elaborated by conditional deletion of CXCL12 in different cell types of the HSC niche.<sup>10,44</sup> HSC maintenance and self-renewal in the BM was primarily regulated by CXCL12 secreted from immature mesenchymal stem and progenitor cells and to a lesser extent from ECs. In contrast, CXCL12 secreted from OBs was dispensable for HSC function. In addition, retention of HSPCs in the BM was

mediated by perivascular sinusoidal stromal cells, including CXCL12-abundant reticular cells<sup>45</sup> and osteolineage progenitors that express leptin receptor and osterix.<sup>10,44</sup> Immunofluorescence imaging together with computational modeling revealed that quiescent HSCs associate with small arterioles in the endosteal BM. A rare subtype of pericytes (NG2<sup>+</sup> pericytes) ensheathed these arterioles and mediated HSC quiescence.<sup>46</sup>

In summary, there is strong evidence that HSCs reside in the perivascular region of the BM and that MSCs, ECs and pericytes regulate HSC maintenance and differentiation through soluble factors and cell contact-dependent signals<sup>8,10,44</sup> (Figure 2).

### The LSC Niche

Although LSCs harbor genetic abnormalities that result in increased proliferation and resistance to apoptosis, they still depend on similar interactions with niche cells as described for HSCs. Therefore, during leukemogenesis, LSCs 'hijack' the niche and the signaling molecules from normal HSCs. Analogous to normal HSCs, transplanted leukemia cells preferentially migrate to CXCL12-expressing vascular niches.<sup>47</sup> Moreover, the CXCL12 receptor C-X-C motif chemokine receptor 4 (CXCR4) regulates the migration of human AML cells in xenotransplant models.<sup>48</sup> Signaling via CXCR4 leads to the upregulation of pro-survival signals and quiescence, both contributing to chemotherapy resistance. Importantly, CXCR4 is highly expressed on different types of



human leukemia, including acute lymphoblastic leukemia and AML<sup>49,50</sup> and high CXCR4 expression on leukemia blasts correlates with poor outcome.<sup>51</sup> Blocking CXCR4 *in vitro* using peptides resulted in reduced chemotaxis toward CXCL12-expressing cells and an inactivation of pro-survival signals. As a consequence, blocking CXCR4 increased the susceptibility of AML cells to chemotherapy.<sup>52</sup> Similarly, preclinical studies demonstrate that the CXCL12-CXCR4 inhibitor plerixafor and novel monoclonal antibodies (mAbs) blocking CXCR4 increase the chemosensitivity of leukemia cells *in vitro* and *in vivo*.<sup>53,54</sup> In contrast to AML blasts, CML cells have a decreased expression of CXCR4 and therefore an attenuated chemotaxis toward CXCL12. Treatment with imatinib restores CXCR4 expression and CXCL12-mediated pro-survival signals. Thereby, imatinib may contribute to CXCL12/CXCR4 signaling-mediated resistance of the few remaining LSCs in CML.<sup>55</sup>

Similar to the role of quiescence induction in HSCs, TGF- $\beta$  has been reported to induce quiescent G<sub>0</sub> state of the cell cycle in AML cells. Blocking TGF- $\beta$  by mAbs increased proliferation and susceptibility to cytarabine.<sup>56</sup> In addition, TGF- $\beta$  is a crucial regulator of protein kinase B (AKT/PKB) activation and controls forkhead-box protein O3a localization, thereby maintaining CML LSCs.<sup>57</sup> Interestingly, it was shown that the effect of TGF- $\beta$  on LSCs varies in different subtypes of leukemia. For instance, OB-specific activation of the parathyroid hormone receptor and subsequent secretion of TGF- $\beta$  reduced LSCs in CML but increased LSC numbers in MLL/AF9-induced AML in mouse transplantation models.<sup>58</sup>

Niche cells not only interact with HSPCs via soluble factors but also via direct cell–cell interactions. For example, CD44, a transmembrane glycoprotein that exists in differently spliced isoforms mediates adhesion of LSCs through cell–cell and cell–extracellular matrix interactions by binding to hyaluronan that is concentrated at the endosteal region of the BM niche. In addition to its function as an adhesion molecule, CD44 also transduces intracellular signals that are involved in the regulation of cell proliferation and differentiation. Blocking CD44 *in vivo* prevented the migration of human AML and murine CML LSCs to the stem cell-supportive microenvironment in the BM and led to their eradication.<sup>59,60</sup>

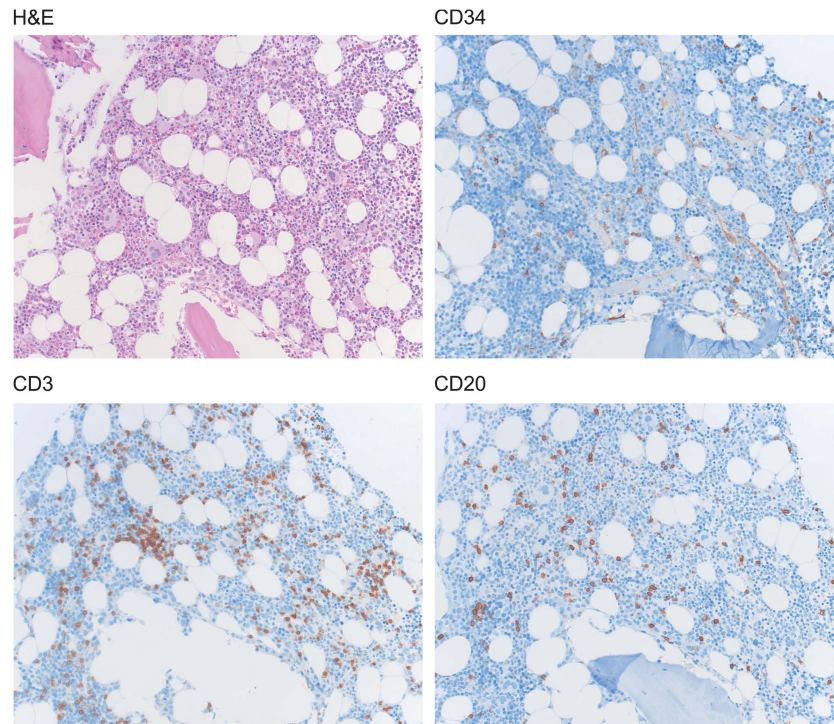
Taken together, there is strong evidence that LSCs at least partially depend on similar signals from the microenvironment as HSCs do. These requirements may differ in the distinct subtypes of myeloid leukemia. In addition, accumulating evidence suggests that molecular changes in the BM niche actually contribute to leukemia development. For example, Walkley *et al.* reported that a dysfunction of the tumor suppressor retinoblastoma protein or of the retinoic acid receptor in the BM microenvironment results in myeloproliferative disease.<sup>61,62</sup> Similarly, conditional knock-down of DICER1, a gene that regulates microRNAs, in osteoblastic precursors results in leukemia predisposition.<sup>50</sup> More recently, Kode and colleagues showed that a single activating  $\beta$ -catenin mutation in OBs is sufficient to alter the differentiation of myeloid progenitors, leading to AML with common chromosomal aberrations and cell-autonomous progression.<sup>63</sup>

## Immune Cells Contributing to the Niche

Although recent evidence documents that MSCs and ECs are fundamental regulators of HSC maintenance and quiescence, many other cell types that are present in the BM contribute to the microenvironment. These include adipocytes, fibroblasts and immune cells (Figure 3). Besides its main function as a hematopoietic organ, the BM serves as a primary and secondary lymphoid organ, hosting various mature immune cells including T and B cells, plasma cells, dendritic cells (DCs), neutrophils and macrophages (reviewed in Mercier *et al.*,<sup>64</sup> Figure 4). These immune cells provide an ‘immune niche’ that is involved in the regulation of HSC homeostasis and emergency hematopoiesis.<sup>17,64</sup> Lymphocytes represent a major fraction of total BM mononuclear cells, are widely distributed throughout the BM parenchyma and are occasionally organized as small lymphoid aggregates, typically consisting of mature CD3<sup>+</sup> T cells<sup>64</sup> (Figure 3). Clinical and experimental approaches investigating engraftment after BM transplantation suggested a fundamental role of CD4<sup>+</sup> T cells in hematopoiesis.<sup>65,66</sup> T-cell-depleted allogeneic BM failed to engraft in a majority of the patients. In addition, HSC maintenance and successful long-term reconstitution depends on the expression of major histocompatibility complex class II, implying a role for CD4<sup>+</sup> T cells in maintaining HSC function.<sup>67,68</sup> The mouse BM contains ~1.5% of CD4<sup>+</sup> T cells.<sup>15</sup> Most of these cells have an activated memory phenotype and a diverse V $\beta$  T cell receptor repertoire.<sup>69,70</sup> Observations in mice lacking the common gamma chain ( $\gamma_c^{-/-}$ ) indicated that hematopoiesis-promoting cytokines such as IL-3 and GM-CSF secreted by activated T cells in the BM modulate normal hematopoiesis.<sup>71</sup> In addition, adoptive transfer of CD4<sup>+</sup> T cells but not CD8<sup>+</sup> T cells restored defective myeloid differentiation in T-cell-deficient mice, suggesting that especially antigen-activated CD4<sup>+</sup> T cells maintain basal hematopoiesis in the BM.<sup>16</sup>

T<sub>regs</sub> represent one-third of all CD4<sup>+</sup> T cells in the BM.<sup>69,70</sup> This proportion is substantially higher than in lymph nodes and spleen, where the frequency of T<sub>regs</sub> is approximately 5–10%. Depletion experiments and co-transfer of BM with or without T<sub>regs</sub> indicated that T<sub>regs</sub> suppress colony formation and myeloid differentiation of HSPCs.<sup>72</sup> High-resolution *in vivo* imaging demonstrated that T<sub>regs</sub> colocalize with HSPCs in the endosteum. Furthermore, T<sub>regs</sub> provide an immune-privileged niche in the BM, protecting HSPCs from immune destruction.<sup>73</sup>

In addition to cells of the adaptive immune system, mononuclear phagocytes contribute to the regulation of HSCs in the BM. Depletion of mononuclear phagocytes using clodronate liposomes increased the number of circulating HSCs.<sup>74–76</sup> CD169<sup>+</sup> macrophages in the BM secrete soluble factors that stimulate nestin<sup>+</sup> MSCs to express HSC retention factors, such as CXCL12, angiopoietin-1 and VCAM-1. In addition, the same macrophages are involved in steady-state and stress-induced erythropoiesis.<sup>77</sup> Moreover, a rare population of monocytes and macrophages expressing high levels of  $\alpha$ -smooth muscle actin and cyclooxygenase 2 induce prostaglandin E<sub>2</sub> production and upregulation of CXCL12 on nestin<sup>+</sup> MSCs.<sup>78</sup>



**Figure 3** Differentiated immune cells in the bone marrow (BM). Representative example of a healthy human BM stained for hematoxylin/eosin (HE), for CD34<sup>+</sup> HSPCs, CD3<sup>+</sup> T cells and CD20<sup>+</sup> B cells

Furthermore, macrophages have been identified as central regulators of HSC egress from the BM after phagocytosis of aged neutrophils<sup>79</sup> (Figure 4).

### The BM Microenvironment During Immune Activation

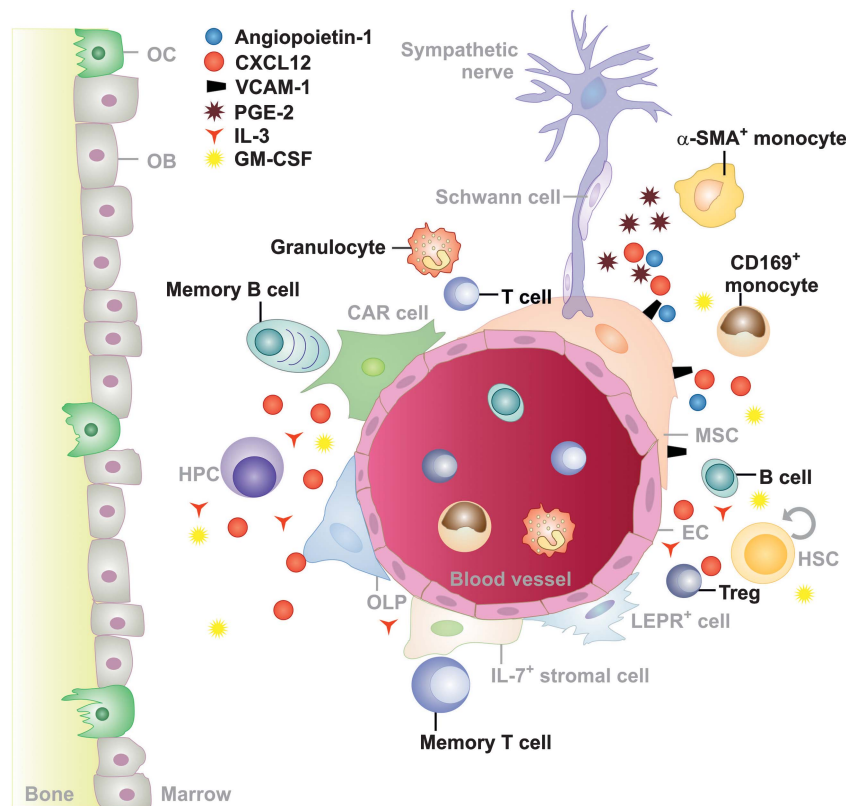
The BM not only assures the continuous supply of different blood lineages during homeostasis, it also responds to the organism's increased demands during stress situations, such as infections or chemotherapy. Many of the mechanisms that regulate HSPCs during demand-adapted hematopoiesis may also regulate LSCs and leukemic progenitor cell function (Table 1, Figure 5).

During an infection, antigenic stimulation drives clonal expansion of naïve and memory lymphocytes to meet the increased demand for T and B cells.<sup>80</sup> In contrast, granulocytes are short-lived and do not have the capacity to undergo clonal expansion. Consequently, they must be continuously produced and recruited from the BM. Therefore, the BM must be capable of recognizing the increased demand for myeloid cells during an infection and immediately react with enhanced production, differentiation and mobilization of granulocytes and monocytes. The importance of this so-called 'emergency myelopoiesis' is best documented in bacterial infections. HSPCs sense the increased demand for myeloid cells by systemic and local danger and inflammatory signals. These include the recognition of conserved microbial products via Toll-like receptor (TLR) activation and of soluble factors such as type I and type II interferons (IFNs).<sup>81,82</sup> TLR signaling has been shown to induce cell cycling and myeloid differentiation in a MyD88-dependent manner in murine HSCs and in human CD34<sup>+</sup> progenitor cells.<sup>82,83</sup> Although some TLRs interact

with endogenous ligands, no effect of TLR signaling on LSCs has been reported so far.

HSPCs express cytokine receptors and respond to inflammatory signals produced by mature immune cells, such as IFNs.<sup>17,84,85</sup> IFNs are crucially involved in host protection against various infections and emerged as a major pathway of HSC regulation.<sup>86</sup> Type I IFNs (IFN $\alpha$  and  $\beta$ ) are synthesized by various cell types, especially plasmacytoid DCs, in response to viral infection and prevent viral replication and thereby viral spread.<sup>87</sup> IFN $\alpha$  stimulates dormant HSCs to enter the cell cycle in a signal transducer and activator of transcription 1 (STAT-1)-dependent way.<sup>88</sup> In addition, IFN regulatory factor 2, a transcriptional repressor of IFN $\alpha$  signaling, preserves quiescence and multilineage reconstitution capacity of HSCs.<sup>89</sup> Thus, acute IFN $\alpha$  production stimulates HSCs to proliferate during viral infection. In contrast, chronic and excessive signaling through this pathway leads to HSC exhaustion.<sup>88–90</sup> A clinically relevant effect of IFN $\alpha$  on CML cells has been well documented. Before the era of the BCR/ABL1-targeting tyrosine kinase inhibitors, IFN $\alpha$  was a standard treatment in CML.<sup>91</sup> Clinical and experimental data suggest that IFN $\alpha$  can actually target CML LSCs. The mechanism of action of IFN $\alpha$  in CML is complex, including direct modulation of gene expression in LSCs, induction of apoptosis, anti-proliferative signals and immunomodulatory effects.<sup>91</sup> In addition, it has been proposed that IFN $\alpha$ , similar to its effect on HSCs, may induce proliferation of LSCs and render them more susceptible to chemotherapy.<sup>92</sup>

IFN $\gamma$ , a type II IFN, is secreted by activated innate and adaptive immune cells, mainly by macrophages and activated T cells. Similar to type I IFNs, IFN $\gamma$  has activating and suppressive effects on hematopoiesis, probably depending



**Figure 4** The 'immune niche'. The BM microenvironment hosts various mature immune cell types including T and B cells, dendritic cells, neutrophils and macrophages. These immune cells contribute to the BM microenvironment ('immune niche') and regulate hematopoietic stem cells (HSCs) during steady-state and emergency hematopoiesis directly by secretion of hematopoiesis-promoting cytokines such as IL-3 and GM-CSF.<sup>16</sup> In addition, immune cells can indirectly regulate HSPCs through signaling via mesenchymal stem/stromal cells (MSCs), for example, by prostaglandin E<sub>2</sub> that increases the expression of CXCL12, angiopoietin-1 and vascular cell adhesion molecule-1 (VCAM-1) in MSCs.<sup>77</sup>  $\alpha$ -SMA, monocytes/macrophages expressing high levels of  $\alpha$ -smooth muscle actin; CAR cell, CXCL12-abundant reticular cell; EC, endothelial cell; HPC, hematopoietic progenitor cell; OB, osteoblast; OC, osteoclast; OLP, osteolineage progenitor cell; Treg, CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> regulatory T cell

on the timing, duration and amount of secretion. Initially, IFN $\gamma$  was described as a suppressor of hematopoiesis. This was based on experiments indicating that IFN $\gamma$  induces differentiation and apoptosis of human and murine HSCs and reduces their colony formation capacity *in vitro*.<sup>93,94</sup> In analogy, infection of perforin-deficient mice with lymphocytic choriomeningitis virus (LCMV) induced lethal pancytopenia because of the persistence of the virus, leading to prolonged and increased secretion of tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) and IFN $\gamma$  by cytotoxic effector CD8<sup>+</sup> T cells (CTLs).<sup>95</sup> In contrast, accumulating evidence from more physiologic infection models indicates that IFN $\gamma$  induces an expansion of HSCs and myeloid progenitors and modulates the production of mature myeloid cells.<sup>96–101</sup> Baldridge *et al.* documented that IFN $\gamma$  directly increases HSC proliferation through IFN $\gamma$  receptor 1-STAT-1 signaling during infection with *Mycobacterium avium*.<sup>96</sup> In contrast, we recently demonstrated that IFN $\gamma$  secreted by activated CTLs during acute LCMV-infection stimulates the expansion of early MPPs and downstream myeloid precursors in the BM.<sup>102</sup> Interestingly, IFN $\gamma$  did not act directly on hematopoietic cells, but stimulated MSCs in the BM to secrete IL-6, which induced proliferation of MPPs and myeloid differentiation. This resulted in elevated myeloid cell counts in the circulation and an increased number of inflammatory monocytes in

secondary lymphoid organs that contributed to pathogen clearance. Therefore, IFN $\gamma$  has an important role in the demand-adapted response to infections and probably regulates HSPC proliferation via direct and indirect mechanisms. However, IFN $\gamma$  may have comparable effects on LSCs. We found that CTL-secreted IFN $\gamma$  induces proliferation of LSCs and leukemia progression in a murine CML model (Figure 5).<sup>103</sup> Interestingly, this effect was dependent on the amount of secreted IFN $\gamma$ . If adoptively transferred, activated leukemia-specific CTLs were re-stimulated *in vivo* by large amounts of antigen, CTL-secreted IFN $\gamma$  induced LSC proliferation and expansion. The quantity of secreted IFN $\gamma$  correlated with the leukemia load and with the antigen expression pattern that is, leukemia-specific expression *versus* expression in, healthy, non-malignant tissue. Similarly, IFN $\gamma$  increased the colony formation capacity of lin<sup>–</sup>CD34<sup>+</sup> stem/progenitor cells from CML patients *in vitro*.<sup>103</sup>

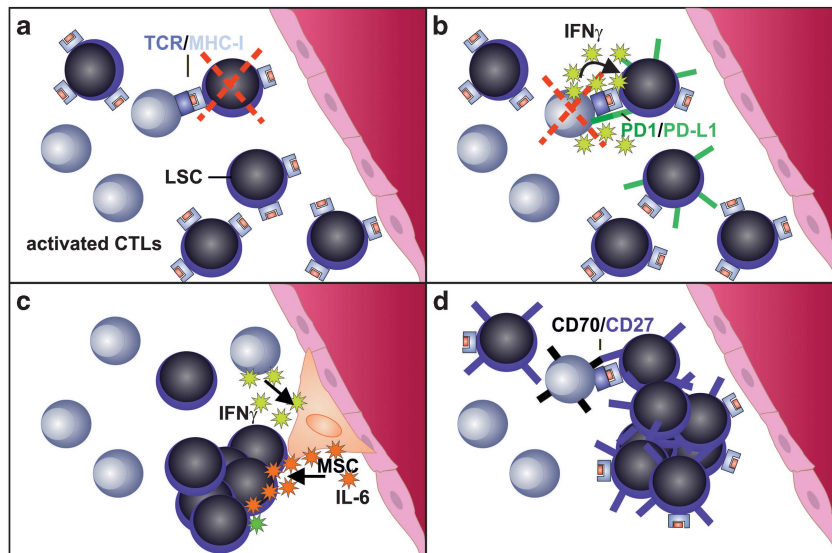
TNF $\alpha$  is another major pro-inflammatory cytokine that is released by activated macrophages, natural killer cells and T cells. Similar to IFN $\gamma$ , TNF $\alpha$  has been shown to suppress the colony formation capacity of human lin<sup>–</sup>CD34<sup>+</sup> stem/progenitor cells and of murine HSCs *in vitro* as well as their ability to reconstitute recipient mice.<sup>93,104</sup> Rezzoug *et al.* documented contradictory results in that TNF $\alpha$  promoted



**Table 1** Shared molecular pathways in the regulation of HPSCs during infection and LSCs in leukemia

Effector molecule	Expression of receptor	Effect in demand-adapted myelopoiesis	Effect in leukemia
IFN $\alpha$	HSCs, MPPs LSCs	Dormant HSCs enter the cell cycle <sup>88,89</sup> Permanent signaling leads to exhaustion of HSCs <sup>88–90</sup>	Modulation of gene expression (CML) <sup>143</sup> Pro-apoptotic and anti-proliferative signals (CML) <sup>144</sup> Adhesion to microenvironment (CML) <sup>145</sup> Downregulation of BCR/ABL (CML) <sup>146</sup> Immunomodulation (CML) <sup>147</sup>
IFN $\gamma$	HSCs, MPPs, CMPs, MEPs, GMPs LSCs	Apoptosis of HSCs <i>in vitro</i> <sup>94</sup> Proliferation of HSCs and MPPs <i>in vivo</i> <sup>96,102</sup> Increased myeloid differentiation <i>in vivo</i> Permanent signaling leads to suppression of HSCs <sup>95</sup>	Pro-apoptotic effects (CML) Proliferation of CD34 <sup>+</sup> cells (CML) Proliferation of LSCs and leukemia progression <i>in vivo</i> (CML)
TNF $\alpha$	HSCs LSCs	Reduced colony formation <i>in vitro</i> and reconstitution <i>in vivo</i> <sup>104,146</sup> Increased colony formation capacity <i>in vitro</i> <sup>105</sup> Suppression of HSC proliferation <i>in vivo</i> <sup>106</sup>	promotes NF- $\kappa$ B activity, LSC survival and expansion (CML, AML) <sup>110,111</sup>
IL-1 $\beta$	HSCs LSCs	Increased granulocyte numbers in BM <sup>148</sup>	Inhibits self-renewal capacity of LSCs (AML) <sup>149</sup>
IL-6	MPPs Leukemia MPPs	Reduced erythropoiesis <sup>115</sup> Increased myelopoiesis <sup>102,115</sup>	Directs myeloid differentiation and sustains leukemia development (CML) <sup>116</sup>
CD70-CD27	HSCs, MPPs, CMPs, GMPs LSCs	Negative feedback signal to leukocyte differentiation <sup>122</sup>	Increased proliferation of LSCs and leukemia progression in CML <sup>123</sup> and AML (unpublished results)

Abbreviations: AML, acute myeloid leukemia; BM, bone marrow; CML, chronic myeloid leukemia; CMP, common myeloid progenitor; GMP, granulocyte-macrophage progenitor; HSCs, hematopoietic stem cells; HPSCs, hematopoietic stem- and progenitor cells; IFN, interferon; IL, interleukin; LSCs, leukemic stem cells; MPPs, multipotent progenitors; MEPs, megakaryocyte-erythrocyte progenitors; NF- $\kappa$ B, nuclear factor  $\kappa$ B; TNF, tumor necrosis factor



**Figure 5** The interaction of activated cytotoxic effector CD8<sup>+</sup> T cells (CTLs) with CML leukemic stem cells (LSCs). In CML, the BM is infiltrated by activated leukemia antigen-specific CTLs. (a) LSCs express MHC class I and present peptides derived from leukemia antigens to specific CTLs. Specific CTLs can eliminate LSCs *in vitro* and donor-derived CTLs can eliminate LSCs after allogeneic HSC transplantation.<sup>103,141</sup> Whether autologous CTLs can eliminate LSCs *in vivo* is currently unclear. (b) LSCs express programmed death ligand 1 (PD-L1) that interacts with programmed death 1 (PD-1) on activated CTLs and leads to CTL inhibition and ultimately deletion. CTL-secreted IFN $\gamma$  further leads to upregulation of PD-L1 on LSCs and therefore protection of LSCs from CTL attack.<sup>103</sup> (c) LSCs express the IFN $\gamma$  receptor and can directly respond to IFN $\gamma$  resulting in proliferation of LSCs and leukemia progression.<sup>103</sup> In addition, IFN $\gamma$  activates mesenchymal stem/stromal cells (MSCs) to produce IL-6.<sup>102</sup> IL-6 secretion induces myeloid differentiation at the level of MPPs. In CML, IL-6 is secreted by BCR/ABL1-expressing leukemic cells, leading to a paracrine feedback loop.<sup>116</sup> (d) LSCs and leukemic progenitors express the TNFR molecule CD27. CD27 is ligated by CD70 expressed on activated CTLs.<sup>123</sup> CD27 signaling leads to activation of the Wnt pathway, proliferation of LSCs and leukemia progression

engraftment and colony formation of HSCs resulting in increased numbers of HSPCs.<sup>105</sup> A small fraction of CD8<sup>+</sup> T-cell receptor-negative cells in the BM was identified as a major source of TNF $\alpha$  in this process. Furthermore, mice

deficient of the p55 TNF receptor 1  $\alpha$  (TNFRSF1 $\alpha^{-/-}$ ) have increased numbers of HSPCs and an increased BM cellularity compared with wild-type mice and mice lacking the p75 TNF receptor 1  $\beta$  (TNFRSF1 $\beta^{-/-}$ ).<sup>106</sup> This increase in



HSPC numbers is accompanied with a decrease in HSC function. Competitive repopulation assays documented that  $TNFRSF1\alpha^{-/-}$  HSCs have an impaired self-renewal capacity. Therefore,  $TNF\alpha$  is a major regulator of baseline and demand-adapted hematopoiesis via signaling through the p55 subunit of the TNF receptor (TNFR). However, in analogy to IFNs, prolonged and excessive  $TNF\alpha$  signaling is associated with BM failure and myelodysplastic syndrome.<sup>107</sup> Early *in vitro* experiments using AML blasts demonstrated that  $TNF\alpha$  has the ability to either support or inhibit cell proliferation, depending on the growth factors present in the culture medium.<sup>108,109</sup> Very recently, two studies highlighted the importance of  $TNF\alpha$  for CML and AML stem cell survival and expansion.<sup>110,111</sup> Autocrine  $TNF\alpha$  production by LSCs increased nuclear factor  $\kappa B$  pathway activation and leukemia progression.

IL-6 was originally identified as a T-cell-derived cytokine that induces B-cell maturation into antibody-producing cells.<sup>112</sup> IL-6 is expressed by a variety of normal and transformed cells including T and B cells and has important functions in the regulation of immune responses, acute-phase reaction and hematopoiesis.<sup>113,114</sup> IL-6 regulates hematopoiesis in response to *Toxoplasma gondii* infection in mice. BM stromal fibroblast-derived IL-6 blocked erythroid development but expanded granulocyte-macrophage progenitors.<sup>115</sup> Similarly, as discussed above, LCMV infection increased IL-6 secretion by MSCs because of CTL-secreted  $IFN\gamma$ .<sup>102</sup> IL-6 then expanded MPPs and myeloid progenitors leading to increased numbers of myeloid cells in the circulation and in lymphoid organs. Quite comparable to its function during an acute infection, IL-6 directs CML MPPs toward myeloid lineage.<sup>116</sup> CML cells were the main source of IL-6 and BCR/ABL1 activity controlled IL-6 expression, establishing a feedback-loop that contributed to disease progression.

Although the immune system mainly interacts with HSPCs via soluble factors, direct cell-cell interactions are also involved in the regulation of HSCs and LSCs. CD27, a member of the TNFR superfamily, is expressed on lymphocytes and on HSCs.<sup>117</sup> The cellular effects that are initiated after ligation of CD27 by its unique ligand CD70 have been extensively studied in lymphocytes. CD70-CD27 signaling leads to cell expansion, survival, memory formation and cytokine production.<sup>118–121</sup> CD27 signaling on HSPCs reduces colony formation *in vitro* and lymphocyte, mainly B cell, differentiation *in vivo*.<sup>122</sup> As CD70 is only expressed on lymphocytes and on subsets of DCs upon immune activation, CD27 signaling on HSPCs may represent an important regulatory mechanism during infection. We recently documented that CD27 is expressed on LSCs in CML<sup>123</sup> and AML (unpublished results). In contrast to its inhibitory effect on HSCs, CD27 signaling on LSCs increased LSC proliferation and colony formation. Thus, the activated immune system contributes to leukemia progression by CD27 signaling on LSCs and leukemic progenitors (Figure 5). CD27 signaling in LSCs activated the Wnt pathway via the TRAF2- and NCK-interacting kinase. As the Wnt pathway is crucial for CML and AML stem cells,<sup>124–126</sup> blocking CD27 signaling and reducing Wnt pathway activity resulted in a reduction of LSC numbers and delayed disease progression.

## Immune Responses to Leukemia

It is assumed that HSCs reside in an immune-privileged environment, supported by BM-resident  $T_{regs}$ . In addition, HSCs are resistant to most infectious pathogens with only few exceptions such as the human polyomavirus 2 (JC virus).<sup>85</sup> Therefore, during homeostasis and infection, HSPCs are probably not direct targets of CTLs or antibodies. This situation may be different in leukemia. Leukemic cells express antigens that are immunogenic and can be recognized by CTLs.<sup>127</sup> Some leukemia antigens originate directly from the oncogenic event and are therefore leukemia-specific, such as BCR/ABL1 in CML<sup>128–130</sup> and DEK/nucleoporin 214,<sup>129</sup> promyelocytic leukemia/retinoic acid receptor- $\alpha$ ,<sup>131,132</sup> fms-like tyrosine kinase 3—internal tandem duplication (FLT3-ITD)<sup>133,134</sup> and mutated nucleophosmin 1<sup>135</sup> in AML. However, apart from BCR/ABL1 and FLT3-ITD, these leukemia-specific antigens are only expressed in a minority of patients. In addition, the vast majority of the more than 200 known leukemia-specific chromosomal translocations does not give rise to antigenic proteins.<sup>127</sup> Other antigens are not leukemia specific but are overexpressed by leukemic cells (leukemia-associated antigens), such as Wilms tumor protein, proteinase 3, baculoviral IAP repeat-containing gene 5/survivin, telomerase reverse transcriptase and others. Leukemic cells including LSCs express the molecular repertoire to interact with T cells, that is major histocompatibility molecules and co-stimulatory ligands.<sup>103,136,137</sup> Clinical and experimental studies have documented immune responses to leukemia.<sup>3,127</sup> CTLs directed against leukemia antigens have been detected in chronic phase CML and in AML patients.<sup>18,138</sup> In an experimental model of CML, depletion of  $CD8^{+}$  T cells by mAbs led to rapid disease progression, documenting an important role of CTLs in the immunosurveillance of leukemia.<sup>139</sup> The role of  $CD4^{+}$  T cells in leukemia is less clear and  $CD4^{+}$  T cells have been shown to be dysfunctional *in vivo*.<sup>140</sup> Therefore, there is ample evidence that antigen-specific immune responses toward the leukemia are elicited. However, whether activated T cells can interact with and eliminate LSCs is under debate. It is reasonable to assume that similar to HSCs, LSCs are at least partially protected in an immune-suppressive environment because of the high frequency of  $T_{regs}$  in the BM. In addition, we found in a murine CML model that LSCs express the inhibitory molecule programmed death ligand 1 and that its expression is further upregulated in response to  $IFN\gamma$ . In contrast, leukemia-specific effector CTLs were able to eliminate LSCs *in vitro* and *in vivo* in a setting with minimal leukemia load.<sup>103</sup> Furthermore, allogeneic HSC transplantation can lead to cure of the leukemia, an effect that is mediated most likely by donor-derived CTLs that eliminate residual LSCs.<sup>141,142</sup> This indicates that human LSCs may also be targeted by donor-derived allo-reactive CTLs *in vivo*.

## Conclusions

Besides its main role as a hematopoietic organ, the BM executes functions of a primary and secondary lymphoid organ.<sup>64</sup> Memory  $CD4^{+}$  and  $CD8^{+}$  T cells and antibody-secreting plasma cells are maintained long-term in the BM by cytokines such as IL-7 and IL-15. Up to one-third of all  $CD4^{+}$  T cells in the BM are  $T_{regs}$ . During homeostasis, especially

CD4<sup>+</sup> T cells contribute to the cytokine milieu in the BM and influence quiescence, proliferation and differentiation of HSCs. During acute infections, the immune system regulates the expansion and differentiation of HSPCs by cell-cell interactions and by the secretion of cytokines. These feedback mechanisms evolved to ensure a concerted action of lymphoid and myeloid cells in response to an infection. Although these mechanisms are beneficial to fight an infection, they may be detrimental in the case of leukemia. LSCs share many characteristics with normal HSCs and inflammatory cytokines and signaling via cell contact-dependent receptors such as the TNFR CD27 may induce their expansion. Cure from leukemia implies the elimination of LSCs, and a better understanding of the 'immune niche' and its function in the BM microenvironment may help to develop specific therapies targeting leukemia at the level of the LSC.

### Conflict of Interest

The authors declare no conflict of interest.

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