Flt3 Ligand: Role in Control of Hematopoietic and Immune Functions of the Bone Marrow

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The concerted action of cytokines secreted locally in the bone marrow controls the maintenance, expansion, and differentiation of hematopoietic stem cells (HSCs), whereas aberrant cytokine signaling contributes to leukemic transformation. Potent effects of flt3 ligand on HSCs and the development of the immune system have generated much interest in the clinical application of this cytokine in stem cell transplantation and cancer immunotherapy.

Bone marrow provides a microenvironment in which hematopoietic stem cells (HSCs) can maintain their quiescence, undergo self-renewing self-divisions to expand their pool, and differentiate to fulfill their responsibility for life-long multilineage hematopoiesis. Several cytokines have been identified that contribute to the maintenance, expansion, and differentiation of HSCs. The important early-acting hematopoietic cytokines, flt3 ligand (FL) and stem cell factor (SCF), are expressed as transmembrane molecules or remain attached to extracellular matrix components and can be converted to soluble factors by proteolytic processing or shedding, thus serving the need for local control of HSC stimulation. This review focuses on FL, a cytokine that acts through the tyrosine kinase receptor flt3 and has pleiotropic and potent effects on the development of HSCs and the immune system.

Flt3 receptor signaling in HSCs

Flt3 receptor. FL belongs to a small family of hematopoietic cytokines, including SCF and macrophage colony-stimulating factor (M-CSF), that are specific for class III tyrosine kinase receptors (11). The receptor for FL, isolated independently by two groups and termed fms-like tyrosine kinase 3 (flt3) or fetal liver kinase-2 (flk-2), is structurally related to M-CSF and SCF receptors c-*fms* and c-*kit*, respectively (15, 18). The flt3 and c-*kit* are expressed predominantly on primitive hematopoietic progenitors, an indication that signaling through these receptors is important in the early stages of hematopoiesis. Whereas c-*kit* is also present on more differentiated cells of the basophilic lineage, flt3 expression is restricted to CD34$^+$ cells lacking the lineage-specific markers. Within the stem cell pool, expression of flt3 is heterogeneous, found on cells with long- and short-term marrow-reconstituting activity, and overlapping with that of c-*kit*. Mice with targeted disruption of flt3 develop into healthy adults but possess specific deficiencies in B lymphoid development and the multilineage repopulating function of earliest progenitors (13). Crossing flt3 knockout mice to white spotted locus mice with spontaneous mutations in c-*kit* resulted in a further decrease in hematopoietic cell numbers (in particular of the lymphoid lineages), reduction of bone marrow cellularity, and early death of the animals, indicating that flt3 and c-*kit* signaling have additive functions in hematopoietic development.

The flt3, c-*kit*, and c-*fms* receptors share 9% sequence identity in the extracellular domains and as much as 57% in their intracellular kinase domain. FL-mediated triggering of flt3 induces a receptor autophosphorylation at tyrosine residues, creating the docking sites for the signal-transducing effector molecules. The downstream signaling cascade involves the tyrosine phosphorylation and activation of multiple cytoplasmic molecules, including Ras GTPase, mitogen-activated protein kinase, phospholipase C-γ, and the p85 subunit of phosphatidylinositol 3'-kinase. Unlike other tyrosine kinase receptor ligands, FL stimulation selectively activates the signal transducer and activator Stat5a through a Janus kinase-independent mechanism (reviewed in Ref. 6).

FL. Cloning of the ligand was accomplished in 1993–1994 by two groups (8, 9). FL is a cell surface transmembrane protein type I that can be proteolytically processed and released as a soluble protein. Alternative splicing contributes to generation of a soluble FL isoform by a stop codon inserted into the reading frame of the sixth exon. In contrast to the restricted distribution of flt3 receptors, FL mRNA is ubiquitously expressed in hematopoietic and nonhematopoietic tissues. Despite the widespread expression of FL mRNA, the FL protein has only been found in stromal fibroblasts present in the bone marrow microenvironment and T lymphocytes (Fig. 1). Both the membrane-bound and soluble isoforms of FL are biologically active and stimulate the tyrosine kinase activity of flt3. Mice lacking FL as a result of targeted gene disruption are viable but have more severe defects than flt3 receptor knockouts, including reduced cellularity in the hematopoietic organs, reduced numbers of myeloid and lymphoid progenitors in the bone marrow, and a marked deficiency of natural killer (NK) cells and dendritic cells (DCs) in lymph nodes, spleen, and thymus (16). A central role played by FL in the proliferation, survival, and differentiation of early hematopoietic precursor cells is underscored by a broad range of hematopoietic activities mediated by this cytokine in vitro and in vivo (Fig. 2).

Biological activities of FL in vitro. Numerous in vitro studies have demonstrated that FL stimulates the growth of hematopoietic progenitors from the bone marrow, peripheral blood, and cord blood. FL is usually not efficient when used as a sin-
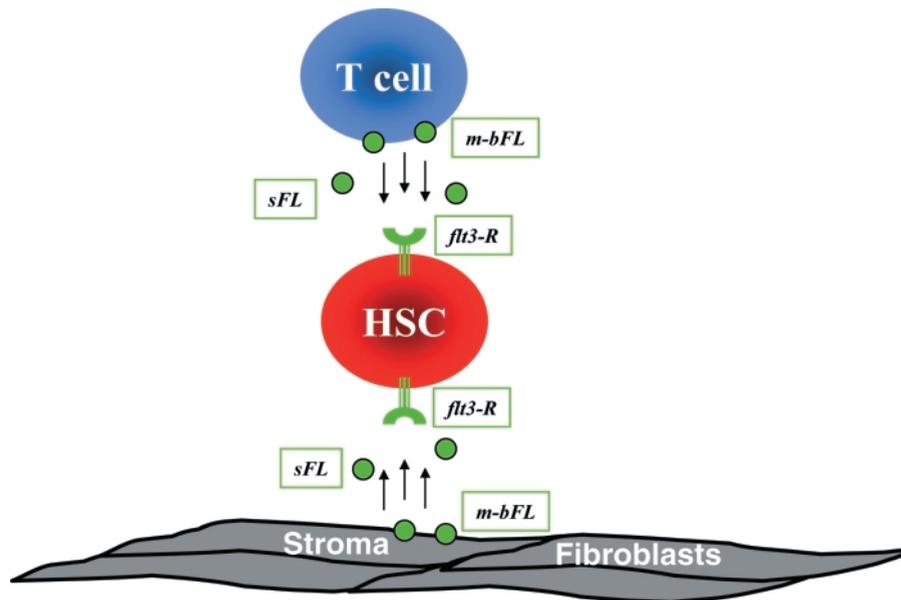
FIGURE 1. Flt3 ligand (FL)-mediated interactions in the bone marrow microenvironment. HCSC, hematopoietic stem cells; m-bFL, membrane-bound FL; sFL, soluble FL; flt3-R, flt3 receptor.

gle cytokine, but in synergy with other hematopoietic growth factors and interleukins it exerts pleiotropic effects on precursors of myeloid and lymphoid lineages. In this respect, FL and SCF share many functional properties, augmenting the responses of multipotent and lineage-committed progenitor cells to a variety of cytokines. A combination of FL with SCF and thrombopoietin induces several-thousandfold expansion of cord blood CD34+ progenitors in stroma-free cultures. In combination with myeloid growth factors, granulocyte-macrophage and granulocyte colony-stimulating factor (GM-CSF and G-CSF), or M-CSF, FL enhances the number of myeloid colonies generated from the committed colony-forming units and more primitive long term culture-initiating cells. FL also synergizes with the interleukins IL-7, IL-3, and IL-11 to stimulate B lymphopoiesis in vitro, with IL-12 in the presence of thymic stroma to promote T cell development, and with IL-15 to drive the development of NK cells. Recent studies have demonstrated that FL belongs to the cytokine network that regulates the development of DCs. FL has been shown to increase the output of myeloid-type DCs, termed DC1, generated from CD34+ human bone marrow or cord blood progenitors cultured in the presence of GM-CSF, IL-4, and TNF-α. Also used as a single cytokine, FL stimulates the development of human lymphoid type DC2 precursors. In contrast to SCF, FL has no effect on erythroid differentiation, whereas SCF has no effect on DCs, indicating that despite many overlapping activities and similarities in structure distribution of flt3 and c-*kit* receptors, the two early-acting growth factors play nonredundant roles in hematopoiesis (11).

Biological activities of FL in vivo. Results of in vivo studies in animals and humans support the notion that FL plays an important role in hematopoiesis. Administration of FL into mice results in expansion of HSCs and significant stimulation of hematopoiesis leading to bone marrow hyperplasia, splenomegaly, and enlargement of lymph nodes and liver. These effects are transient, and hematopoietic values return to

normal upon cessation of FL treatment (1). A strong synergistic effect of FL and G-CSF on the mobilization of hematopoietic progenitors to the peripheral circulation has been seen in mice and primates. Administration of FL to mice and rabbits subjected to lethal doses of irradiation protected HSCs and allowed rapid hematopoietic recovery (7). The most remarkable in vivo effects of FL have been observed within the DC compartment. Large numbers of DCs are generated in murine spleen, bone marrow, and the peripheral circulation, and these effects of FL are far more potent than those following treatment with other DC growth-promoting cytokines such as GM-CSF, IL-4, or G-CSF. Remarkable expansion has also been achieved in healthy human volunteers receiving FL for 10–14 days, leading to the mobilization of both myeloid- and lymphoid-derived DC1 and DC2 precursors by 48- and 13-fold, respectively (18).
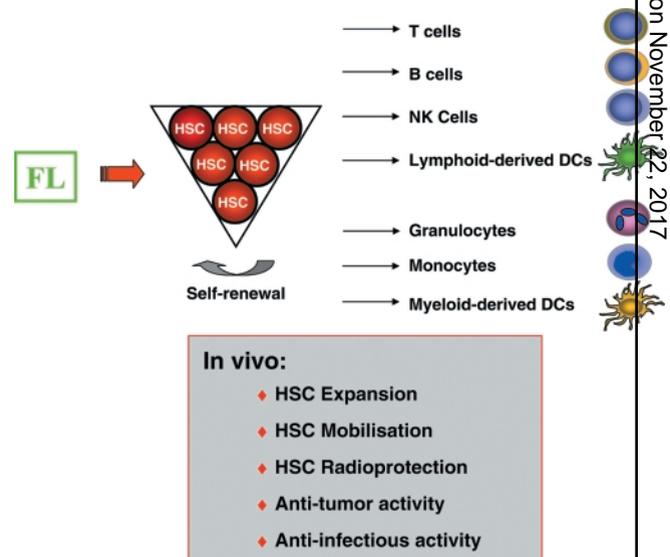

FIGURE 2. Biological activities of FL in vitro and in vivo. NK, natural killer; DC, dendritic cell.

Together, these results suggest that FL is a key regulator of the development of bone marrow-derived DCs in vivo. Moreover, successful treatment of mouse fibrosarcomas, human leukocyte antigen class I-deficient mesotheliomas, and other experimental tumors by FL injections revealed a potent antitumor activity of this cytokine. The regression of tumor growth is dependent on the FL-expanded DCs that generate specific antitumor T cell responses and directly trigger and activate the cytolytic activity of NK cells (4, 12). These results, together with a marked reduction in numbers of DCs and NK cells in mice lacking FL expression, indicate that FL is important for the development and function of the immune system.

FL and steady-state hematopoiesis

Hematopoiesis is one of the most rapidly regenerating systems. The vast majority of mature blood cells have a limited life span. For example, granulocytes remain functionally active for a few hours and erythrocytes for a few weeks before undergoing apoptotic death. To supply the normal steady-state demands, $\sim 4 \times 10^{11}$ new blood cells must be produced every day to replace cells lost to natural decay. HSCs, representing only a fraction (estimated as 1 in 10^5-10^6 cells) of bone marrow cells, sustain the life-long hematopoietic demands through a complex regulatory program within the bone marrow microenvironment. Intracellular communication via cytokines, secreted in either membrane-bound or soluble forms, is of primary importance for maintaining the balance between HSC self-renewal and their commitment toward differentiated progeny. Under steady-state conditions, the regulation is supported by a tightly balanced network of cytokines with growth-promoting functions, such as FL and SCF, and growth-inhibitory properties, such as transforming growth factor-β. Therefore, elucidating the mechanisms that regulate expression and release of hematopoietic cytokines is pivotal for understanding the process of hematopoiesis.

Mechanisms responsible for cytokine production differ widely among individual growth factors (Fig. 3). SCF represents an example of a constitutively produced cytokine whose serum concentration reaches remarkably high levels of 3.0 ng/ml even under steady-state conditions; at present, there is no evidence for the existence of control mechanisms that can up- or downregulate SCF expression. Thrombopoietin is also produced constitutively, but its serum concentration correlates inversely with platelet counts, due to uptake of the cytokine by its target cells, primarily megakaryocytes and platelets. In contrast, the supply of FL for hematopoiesis in humans is tightly regulated by a process specific for this cytokine. This regulation is based on intracellular retention of preformed FL and its release from intracellular stores, depending on the status of the stem cell compartment (2). During steady-state hematopoiesis, FL is expressed constitutively but little of the cytokine is released by cells. Consequently, it is undetectable or very sparse on the cell surface, and FL levels in the circulation are <100 pg/ml. Instead, preformed FL can be detected in the cytoplasm of bone marrow stroma fibroblasts, T cells, B cells, and CD34+ progenitors. In fibroblasts and T cells, intracellular FL colocalizes with proteins resident in the Golgi and trans-Golgi compartments. This mechanism of intracellular retention of FL argues for the existence of a negative regulator controlling the availability of the cytokine during steady-state hematopoiesis (Fig. 4). The nature of this regulator remains unknown.

FL and hematopoietic deficiency

In vivo, release of FL may be triggered by stem cell deficiency in the bone marrow. In the case of blood disorders that affect the stem cell compartment (Fanconi anemia and acquired aplastic anemia), serum levels of FL are highly elevated up to 10 ng/ml (10, 20). A similar increase in both membrane-bound and soluble FL is observed during transient aplasia caused by cytoreductive chemotherapy used as a preparation for stem cell transplantation (SCT). FL levels do not increase in diseases affecting single blood lineages, in autoimmune diseases, or in response to graft-vs.-host disease (GvHD) after transplantation. Conversely, high FL levels in multilineage bone marrow failure do not normalize upon single lineage correction, for example, in response to red blood cell transfusions or administration of G-
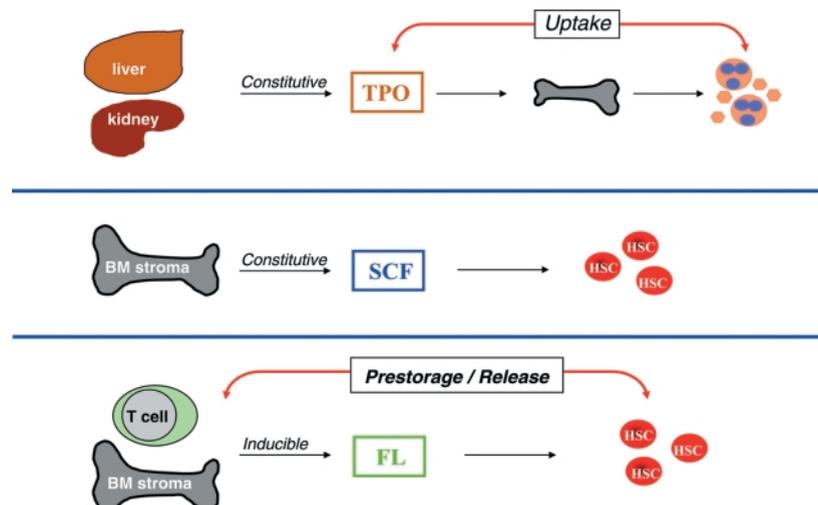
FIGURE 3. Mechanism of expression of early-acting hematopoietic cytokines. TPO, thrombopoietin; SCF, stem cell factor; BM, bone marrow.
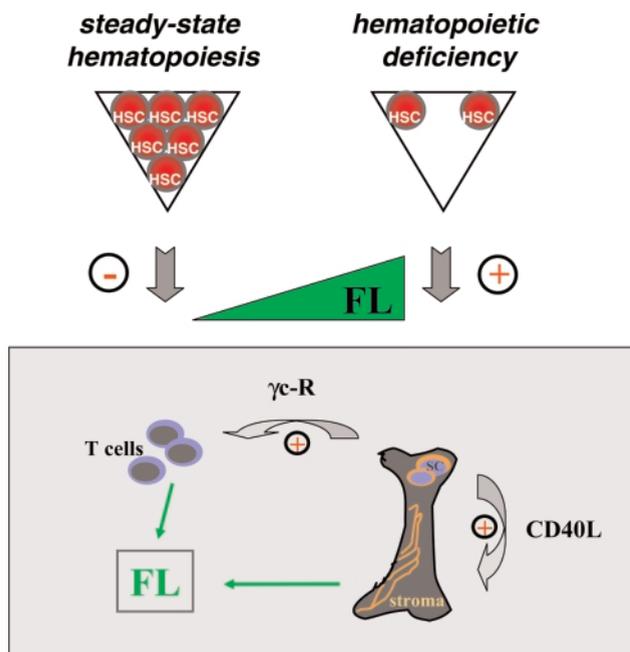
FIGURE 4. Expression of FL during steady-state hematopoiesis and hematopoietic deficiency. γc-R, common γ-chain of IL-2 receptor; CD40L, CD40 ligand.

CSF elevating the granulocyte count. This suggests that the increase in FL levels reflects a compensatory response whose aim is to restore the HSC compartment. Since FL levels return to normal upon hematopoietic recovery after engraftment of stem cells or successful immunosuppressive treatment, the serum concentration of this cytokine may serve as a surrogate marker of HSC content in the bone marrow. SCF serum levels are not dependent on hematopoietic function and are not altered in aplastic anemia or in response to chemotherapy. The nature of inductive signals capable of upregulating FL is only partly understood. In vitro, IL-2, IL-7, and other cytokines using a common γ-chain of the IL-2 receptor enhance expression and release of FL from T cells, whereas CD40 ligand induces a release of FL from stromal fibroblasts (3). Whether the same pathways are responsible for upregulation of FL during bone marrow failure in vivo is not known (Fig. 4). The rapid and massive increase in FL levels, which is restricted to pathological conditions of a multilineage hematopoietic deficiency and is unique among hematopoietic cytokines, provides an important argument that FL plays a nonredundant role in the control of early hematopoiesis in vivo.

FL and leukemic hematopoiesis

There are no known spontaneous mutations associated with the FL locus. However, mutations in flt3 receptor have been detected in ~30% of patients with acute myelogenous leukemia (AML), which represents the highest rate of alterations associated with a single gene in this disease. A small number of patients with acute lymphoblastic leukemia (ALL) and myelodysplastic syndrome (MDS) also carry flt3 mutations, which are, however, not present in patients with chronic myelogenous leukemia (CML). The most frequent are the internal tandem duplications (ITDs) in the juxtamembrane domain of

flt3 detected in all subtypes of AML (17). ITDs are always in frame but vary in length in individual patients. The second type of flt3 mutation in human leukemias are point mutations occurring in the so-called activation loop of the intracellular tyrosine kinase domain. The most common is a substitution affecting the aspartic acid residue at position 835, found in AML as well as MDS and ALL patients. Both ITDs in the juxtamembrane domain and point mutations in the tyrosine kinase domain result in constitutive activation of the receptor, presumably due to unfolding of the active tyrosine kinase site allowing the access of ATP and substrates. Retrospective analysis of clinical data revealed that flt3 mutations confer a poor prognosis, especially in younger patients. Cell surface flt3 expression was found in the majority of samples from AML, CML in blast crisis, and B cell ALL but not in T cell ALL. FL alone or in combination with other growth factors induces, to a variable extent, a proliferative response of leukemiac blasts, suggesting that in some cases autocrine stimulation may play a role in the outgrowth of malignant clones. Together, these results provide an argument that inhibition of flt3 signaling may be an effective approach to treatment of leukemias. A number of inhibitors targeting the flt3 receptor have been developed recently and are currently undergoing clinical trials (19).

Clinical applications of FL

Potential uses of FL in the clinic exploit both the effects on the HSC compartment and the development of DCs and NK cells. In vitro and in vivo preclinical studies have suggested a number of potential clinical benefits of FL in SCT and cancer immunotherapy.

SCT. Mobilization of HSCs to the circulation is based on administration of cytokines, which influence the adhesion properties of stem and progenitor cells and induce the transfer of these cells from the bone marrow to the periphery. G-CSF is a commonly used agent that efficiently mobilizes large numbers of CD34+ cells for harvesting and use in autologous as well as allogeneic SCT. FL acts synergistically with G-CSF with respect to progenitors but differs from G-CSF with respect to DCs. Whereas G-CSF increases only the lymphoid DC2 precursors, injections of FL mobilize both DC subsets, with a preferential expansion of DC1. The effect on DCs argues for a possible benefit of including FL in the mobilization protocol. Expansion of donor-derived DCs in the graft may have particular implications for functional immune recovery in the early posttransplantation period. Furthermore, taking into account that reconstitution of a diversified T cell repertoire does not start for at least 6 mo and is impaired with age, postgrafting administration of FL to boost immune recovery may also be considered for selected transplant recipients.

Interactions between DCs and T cells and a more recently established cross-talk between DCs and NK cells underlie the main immunologic responses that are required for induction of tolerance to prevent GvHD and for antitumor defense to prevent disease relapses after SCT. In animal models, FL was reported to induce tolerance and reduce GvHD by expanding host DCs. Numbers of circulating DCs in patients with acute GvHD are usually below normal values, and there is so far no

evidence that high FL and consequently high DC1 levels may aggravate the risk of GvHD. Furthermore, treatment with FL enhances protective immunity against pathogens in mice, possibly related to the efficient capturing of microbial antigens by blood DC1, and may be important for reducing the risk of opportunistic infections after SCT. Clinical application of FL to achieve higher than endogenous concentrations in patients who have undergone SCT could conceivably have multiple beneficial effects by accelerating hematopoietic and immune recovery and most importantly may enhance antitumor immunity without causing GvHD.

Cancer immunotherapy. DCs specialize in processing and presenting antigens and thus are central for the induction of antitumor immunity. In blood, DCs represent a rare cell population of <0.5% of mononuclear cells. The possibility of generating large numbers of DCs makes FL a promising tool for the development of cell-mediated immunotherapy strategies. Expansion of DCs in vitro from CD34^{+} progenitors or monocytes by using FL and other growth factors is feasible although time and cost intensive. Generation of DCs in vivo offers an alternative approach. Indeed, administration of FL to tumor-bearing mice results in the generation of an effective immunologic response to tumors by promoting DC-T cell and DC-NK cell interactions. Inhibition of tumor growth and even regression of preexisting tumors has been achieved. Taking advantage of the ability of FL to expand the DCs, numerous trials have been undertaken to immunize patients against their tumors by using DCs. Recently, patients with advanced colon and lung carcinomas were immunized with large numbers of autologous DCs collected after systemic administration of FL. In a phase I clinical trial, 2 out of 12 patients experienced dramatic tumor regression, and in 3 patients the disease was stabilized (5). Further efforts are underway to apply FL together with other clinically useful hematopoietic growth factors to develop DCs as vaccine adjuvants.

Genetic modification of HSCs is a promising approach to treatment of inherited and acquired disorders of the hematopoietic system, provided that the relevant gene can be stably expressed in cells with the ability to self-renew and differentiate to the affected hematopoietic lineages. The capacity of FL to maintain and even expand HSCs in culture facilitates the viral vector-mediated gene transfer to these cells. Likewise, the influence of FL on the development of DCs from HSCs opens the possibility of generating large numbers of genetically engineered DCs for cancer immunotherapy.

In summary, potent and pleiotropic effects of FL on the stem and immune cell compartments allow one to envisage a number of important clinical uses. Therapeutic application of FL, as of any immunomodulatory cytokine, raises complex and often controversial issues and thus requires understanding of the molecular mechanisms governing its action.

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