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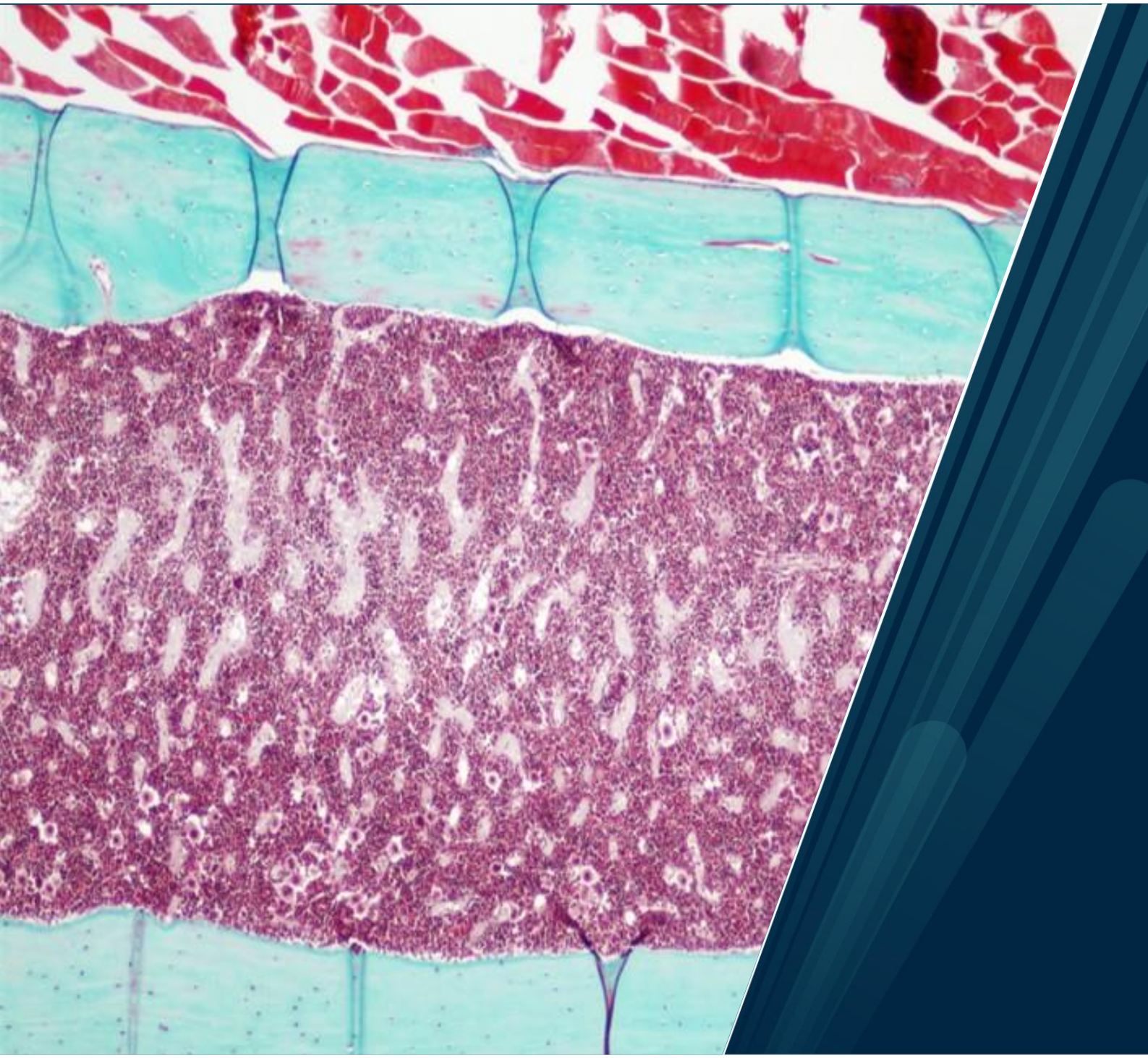
Alicia Villatoro

Stem Cells, Ageing and Cancer Group - Institute of Medical Biology - Faculty of Health Sciences

A dissertation for the degree of Philosophiae Doctor - May 2022



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Cover: Histological image of bone marrow from C57BL/6J wild type mouse stained with Masson´s trichrome, by Roisin Doohan (CNIC, Madrid). Property of Dr. Lorena Arranz (UiT – The Arctic University of Norway).

A Mamá y a la Tata

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ACKNOWLEDGEMENT / AGRADECIMIENTOS

(English / Inglés)

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Thank you very much to my mother, for all her strength, patience and support. She has been a symbol of effort and improvement in which to see myself reflected. Thank you for being side by side through the good times and the bad. Thank you for educating me and helping me grow in life. Thank you for protecting me and taking care of me. Thank you for loving me more than anyone. You are the best mom in the world. I will never forget you, always happy. You are the light at the end of the tunnel, that illuminates my life. I LOVE YOU MOMMY.

(Spanish / Español)

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PREFACE

The present work was conducted at the Institute of Medical Biology (IMB), Faculty of Health Sciences, UiT – Arctic University of Norway, at the Norwegian Center of Molecular Medicine (NCMM) and at the Departments of Biochemistry and of Comparative Medicine, University of Oslo (UiO), under the supervision of Dr. Lorena Arranz.

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ABREVIATIONS

5-FU: 5-fluorouracil

5-hmC: 5-hydroxymethylcytosine

5-mC: 5-methylcytosine

6-mA: 6-adenine methylation

ACTA2: actin α 2

AML: acute myeloid leukemia

ANGPT (Angpt): angiopoietin

Ara-C: cytarabine

AXCL: additional sex combs like

C/EBPα (Cebpa): CCAAT enhancer binding protein alpha

CAR cells: Cxcl12-abundant reticular cells

CCL3: chemokine ligand 3

CFU-C(s): colony forming units – cell(s)

CFU-F(s): colony forming units – fibroblast(s)

CH: clonal hematopoiesis (also known as CHIP)

CHIP: clonal hematopoiesis of indeterminate potential (also known as CH)

CLP(s): common lymphoid progenitor(s)

CML: chronic myeloid leukemia

CML-BP: CML blast phase

CML-CP: CML chronic phase

CMML: chronic myelomonocytic leukemia

CMP(s): common myeloid progenitor(s)

CpG: cytosine-phosphate-guanine

CSF: colony stimulating factor

CXCL12: C-X-C chemokine ligand

CXCR4: C-X-C chemokine receptor 4

DARC: Duffy antigen receptor for chemokines

DNMT(s): DNA methyltransferase(s)

ELISA: enzyme-linked immunosorbent assay

ESC(s): embryonic stem cell(s)

ET: essential thrombocythemia

EZH: Enhancer of zeste

FAB: French-American-British Organization

FABP4: fatty acid binding protein 4

FH: fumarate hydratase

Flk2: fetal liver kinase 2 (also known as Flt3)

Flt3: Fms related receptor tyrosine kinase 3 (also known as Flk2)

G-CSF: granulocyte colony stimulating factor

GFAP: glial fibrillary acidic protein

GFP (gfp): Green fluorescent protein

GLUT1: glucose transporter 1

GM-CSF: granulocyte macrophage colony stimulating factor

GMP(s): granulocyte macrophage progenitors(s)

GSH: reduced glutathione

Gy: gray

HDAC(s): histone deacetylase(s)

HiDAC: high dose of cytarabine

HIF: hypoxia-inducible factor

HSC(s): hematopoietic stem cell(s)

HSPC(s): hematopoietic stem progenitor cell(s)

IDH: isocitrate dehydrogenase(s)

IFN(s): interferon(s)

IKZF1: Ikaros family zinc finger protein 1

IL(s): interleukin(s)

IL-1R1(Il1r1): interleukin 1 receptor 1

IL1RAcP (Ilrap): interleukin 1 receptor accessory protein

IL-1RN (Il1rn): interleukin 1 receptor antagonist

Il2rgc: interleukin 2 receptor gamma chain gene

iPSC(s): inducible pluripotent stem cell(s)

IRAK: IL-1 receptor associated kinase

JMML: juvenile myelomonocytic leukemia

KAT(s): lysine acetyltransferase(s)

KITL (Kitl): KIT ligand (also known as SCF or steel factor)

KO: knockout

LDHA: lactate dehydrogenase A

LEPR (LepR): leptin receptor

LSC(s): leukemic stem cell(s)

LSK(s): lineage^{-} sca-1^{+} c-kit^{+} cell(s)

LT-HSC(s): long-term hematopoiesis stem cell(s)

MCAM: melanoma cell adhesion molecule (*also known as CD146)*

MDS: myelodysplastic syndrome(s)

MEP(s): megakaryocyte erythroid progenitor(s)

MITCH2: mitochondrial carrier homolog 2

MPN(s): myeloproliferative neoplasm(s)

MPP(s): multipotent progenitor(s)

MSC(s): mesenchymal stromal cell(s) (also known as MSPC(s))

MSPC(s): mesenchymal stem and progenitor cell(s) (also known as MSC(s))

MTA: material transfer agreement

MYH11: myosin heavy chain 11

Nes: nestin

NF-κB: nuclear factor kappa B

NG2: neural/glial antigen 2

NK: natural killer(s)

NO: nitric oxide

NSG: NOD-scid gamma

OXPHOS: oxidative phosphorylation

PDGFR: platelet-derived growth factor receptor

PGE_2: prostaglandin E_2

Ph chromosome: Philadelphia chromosome

PMF: primary myelofibrosis

polyI:polyC: poly-inosinic:poly-cytidylic acid

PPR(s): PTH-related protein receptor(s)

PRC: Polycomb repressive complex

PSC(s): pluripotent stem cell(s)

PTH: parathyroid hormone

PTPMT1: protein tyrosine phosphatase mitochondrial 1

Ptpn11: tyrosine-protein phosphatase non-receptor type 11

PV: polycythemia vera

qPCR: quantitative polymerase chain reaction

qRT-PCR: quantitative reverse transcription polymerase chain reaction

Rag2: recombination activating gene 2

Rho: rhodamine

RISP: Rieske iron-sulfur protein

RNA-Seq: RNA sequencing

ROS: reactive oxygen species

sAML: secondary AML

SCF: stem cell factor (also known as KITL and steel factor)

Scid: severe combined immune deficiency

SDF-1: stromal-derived factor-1 (also known as CXCL12)

SDH: succinate dehydrogenase

SHP-1: Src homology region 2 domain-containing phosphatase 1

SIRT1: sirtuin 1

SLAM(s): signalling lymphocyte activation molecule(s)

SOPF: specific opportunistic pathogen free

SPF: specific pathogen free

ST-HSC(s): sort-term hematopoiesis stem cell(s)

TCA: tricarboxylic acid cycle

TET: ten-eleven translocation

TF(s): transcription factor(s)

TGF-β: transforming growth factor β

TIM3: T-cell immunoglobulin mucin 3

TNF: tumor necrosis factor

T_{reg}: regulatory T cell(s)

VCAM1: vascular cell adhesion molecule 1 (also known as CD106)

VEGF: vascular endothelial growth factor

vWF: von Willebrand factor

WHO: World Health Organization

αKG: α-ketoglutarate

α-SMA: α-smooth muscle actin protein

NOMENCLATURE

Species	Gene	Protein
Human	Name in uppercase and italics	Name in uppercase, not italics
Mouse	First letter in uppercase and name in italics	Name in uppercase, not italics (Exception mouse IL-1rn)

LIST OF PAPERS

Paper I

Interleukin-1β as emerging therapeutic target in hematological malignancies and potentially in their complications.

Arranz L., Arriero M.D.M. and Villatoro A.

Blood Reviews. September 2017; 31: 306-317. doi.org/10.1016/j.blre.2017.05.001.

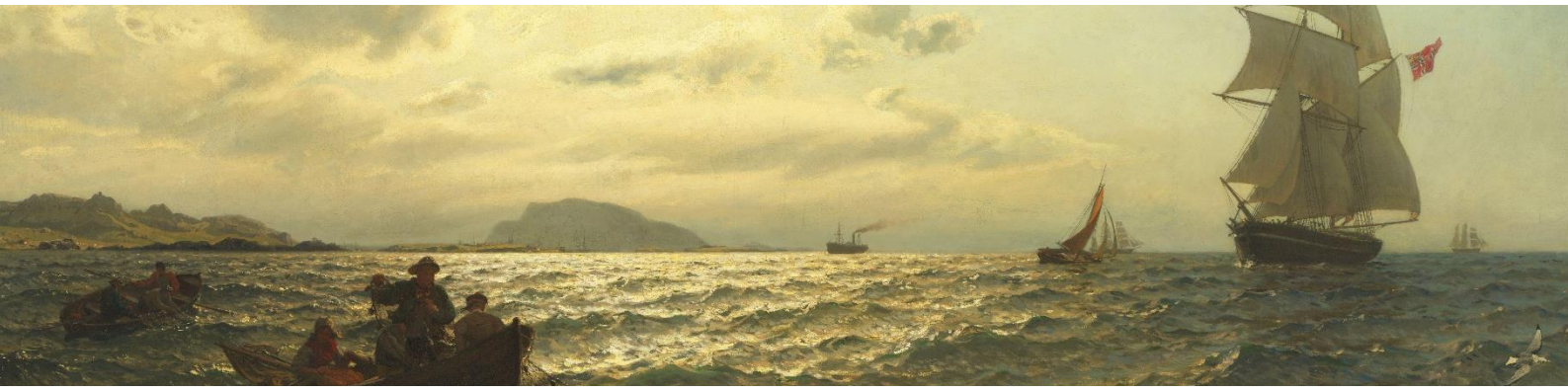
Paper II

Endogenous IL-1 receptor antagonist represses healthy and malignant myeloproliferation.

Villatoro A., Cuminetti V., Bernal A., Cossío I., Rubio A., Benguría A., Torroja C., Ferré M., Konieczny J., You X., Utnes P., Tello A., Vázquez E., Fenton C.G., Paulssen R.H., Zhang J., Sánchez-Cabo F., Dopazo A., Vik A., Anderssen E., Hidalgo A. and Arranz L.

Under review in *Nature Communications*. August 2021.

Introduction



Adapted from *Fresh breeze on the Norwegian coast*. Hans Gude. 1876. Photo: Børre Høstland / Nasjonalmuseet.

1. Hematopoietic stem cells (HSCs) and their surrounding microenvironment

Throughout the life of an organism, it is necessary to replace or regenerate damaged cells, however, this ability is limited to a small population of cells in adult organisms. The cells with this ability are called stem cells [2]. Stem cells are defined by their unique functional properties. They have low differentiation profile but great differentiation potential into the cell lineages of the tissue or tissues that they originate, they are quiescent, *i.e.* mainly kept in a resting status, and they are able to self-renew giving rise to at least one daughter cell that is identical to the stem cell [2-4].

According to their differentiation potential, stem cells are classified as totipotent, pluripotent or multipotent cells. Totipotent cells have the highest differentiation potential and can divide and differentiate into all cells of the whole organism. The zygote is a totipotent stem cell that undergoes cell division to form the morula, formed by several totipotent stem cells, which divides to form the blastocyst [5]. The cells located in the inner mass of the blastocyst are pluripotent stem cells (PSCs). PSCs differentiate into the three primary germ cell layers; ectoderm, mesoderm and endoderm, and are essential for organogenesis [2,5,6]. Another source of PSCs is umbilical cord blood. Isolation of PSCs from umbilical cord blood represents a scientific breakthrough in regenerative medicine where they are used to produce hematopoietic, epithelial, endothelial, and neural tissues both *in vitro* and *in vivo* [7]. PSCs can also be obtained in the lab by genetic engineering of tissue-specific cells, such as fibroblasts, into cells that behave like PSCs and can then be differentiated into other types of tissue-specific cells. These engineered cells are known as inducible PSCs (iPSCs). The use of iPSCs has become a critical tool in research on developmental biology, models of human diseases, regenerative medicine and drug discovery. Based on their developmental stage, stem cells are classified as embryonic stem cells (ESCs) or adult stem cells. In this context, PSCs are ESCs [2,8]. Finally, multipotent stem cells have a narrower differentiation potential and they can differentiate into discrete fully differentiated cells and specific lineages. Adult stem cells, also called somatic stem cells, belong to this group. Hematopoietic stem cells (HSCs), neural stem cells, muscle stem cells and skin stem cells are examples of adult stem cells. They are tissue-specific and important for the maintenance of the steady-state cell composition of the tissue and tissue regeneration after damage [2,3,6].

HSCs are one of the best characterized multipotent stem cells responsible for replenishing all blood cells in our body. The hematopoietic system supplies the organism with >100 billion mature blood cells every day. To carry out this process, HSCs self-renew, proliferate and differentiate to give rise to highly specialized blood lineages in a process called hematopoiesis. Loss of HSC self-renewal leads to depletion of the HSC pool and bone marrow failure syndromes, whereas an increase of HSC self-renewal, proliferation and/or differentiation leads to malignancies of different severity. Thus, to maintain hematopoietic homeostasis, the process of hematopoiesis is strictly regulated [1].

In adults, HSCs mainly reside in the bone marrow, in specialized niches, referred to as the HSC niche(s). At steady-state, most HSCs are quiescent within the niche and serve as a reservoir for blood cells. Cellular intrinsic factors, including genetic/epigenetic mechanisms and metabolites, and extrinsic signals emanating from the HSC niche, like growth factors and inflammatory cytokines, regulate HSC activity [9-17]. The interactions between HSCs and the HSC niche are insufficiently described. Alterations in both intrinsic and extrinsic factors derived from the HSC niche can originate and/or contribute to progression of hematological diseases [9-17]. The future development of selective and effective therapeutic strategies against multitude of human blood diseases will require improved understanding of HSCs and their context.

Of note, HSCs are the basis for one of the most successful cell therapies applied in the clinic, the bone marrow transplantation. The bone marrow transplant is a crucial therapeutic procedure in the treatment of several hematological diseases and beyond, like solid tumors (*i.e.* neuroblastoma) and metabolic disorders (*i.e.* primary amyloidosis). It involves the replacement of the individual hematopoietic and immune systems by the transplanted HSCs. Increasing the number and/or the quality of donor HSCs, including their engraftment, will require better understanding of the complexity of these cells and their surrounding bone marrow HSC microenvironment [18-24].

All of the above highlights the need for additional studies aimed at providing further insights into HSCs and the signals that control their behavior.

1.1. Hematopoiesis

Hematopoiesis is a continuous process of blood production in which HSCs give rise to all blood cell types followed by the egress of these cells into the bloodstream, to exert their function systemically [25,26]. At the beginning of human embryogenesis, hematopoiesis takes place in the yolk sac and the aorta-gonad-mesonephros, and between the second and sixth-seventh month of gestation, it occurs in liver, spleen and thymus. Later on, throughout adult life, blood formation is mainly restricted to the bone marrow [27].

HSCs form a heterogeneous and complex population of cells at the top of the hematopoietic hierarchy and are functionally defined by their ability to sustain multi-lineage engraftment over time, which may be monitored experimentally in serial transplantation in pre-conditioned mice [28]. Even though HSCs have great potential to proliferate, in adult organisms the number of HSCs remains relatively constant. This is because, in homeostasis, while most HSCs remain dormant within the HSC pool, a few self-renewing HSCs undergo symmetric or asymmetric divisions, and give rise to two HSCs or two different daughter cells: one will become a differentiated cell and the other will maintain the HSC pool, respectively [29-34].

Using mice as experimental model, combinations of cell surface markers have been used to define HSCs with different self-renewal capacities, which led to the subdivision of the HSC pool into long-term HSCs (LT-HSCs) and short-term HSCs (ST-HSCs). Traditionally, HSCs, presumably LT-HSCs, were identified as Lin^- $Sca\text{-}1^+$ $c\text{-}Kit^+$ (LSK) CD90 $(Thy1.1)^{low}$ or LSK $CD34^-$ cells [35-39]. However, only about 25% of these cells maintain long-term multilineage reconstitution [35-37,40]. In 2005, the group of Morrison proposed the use of Signaling Lymphocyte Activation Molecule (SLAM) family markers to identify an enriched HSC population with long-term reconstitution capacity defined as LSK $CD150^+$ $CD48^-$. 47% of these cells engraft and give rise to long-term multilineage reconstitution, which makes these markers more accurate for the identification of LT-HSCs [41]. Acquisition of CD135 (also known as Fms related receptor tyrosine kinase 3 (Flt3) or fetal liver kinase 2 (Flk2)) expression within the LSK compartment is accompanied by loss of self-renewal capacity. LSK cells lacking expression of CD135 contain all LT-HSCs and ST-HSCs [42]. Thus, common combinations of markers used to define LT-HSCs as of today are LSK $CD150^+$ $CD48^-$ and LSK $CD34^-$ $CD135^-$. ST-HSCs, which have multilineage reconstitution ability of up to 4 months in the first transplantation, reside in the LSK compartment expressing CD34 and lacking CD135 [37,42,43].

In the stepwise hierarchical HSC differentiation model, HSCs lose their multipotency and self-renewal capacity as differentiation progresses. LT-HSCs differentiate into ST-HSCs, with less self-renewal capacity, and ST-HSCs evolve into multipotent progenitors (MPPs) which are defined as LSK $CD34^{+}$ $CD135^{+}$. MPPs are devoid of self-renewal potential and only exhibit short-term reconstitution ability. MPPs generate lineage-committed progenitor cells with no self-renewal capacity that differentiate into mature cells [1,13] **(Figure 1A)**. LT-HSCs, ST-HSCs and MPPs make up the hematopoietic stem progenitor cell (HSPC) compartment.

Recently, a further subdivision of the HSPC compartment was proposed including HSCs, MPP1, MPP2, MPP3 and MPP4, and later addition of MPP5 and MPP6. According to this subdivision, the HSC compartment comprises HSC (defined as LSK $CD34^{-}$ $CD48^{-}$ $CD150^{+}$ $CD135^{-}$) and MPP1 (defined as LSK $CD34^{+}$ $CD48^{-}$ $CD150^{+}$ $CD135^{-}$) populations, while the MPP compartment comprises MPP2-MPP4 populations [30,44]. MPP1 are more similar to the previously defined ST-HSCs and have multi-lineage reconstitution ability, whereas MPP subsets are more committed and/or generate skewed lineages. Thus, while MPP2 (defined as LSK $CD34^{+}$ $CD48^{+}$ $CD150^{+}$ $CD135^{-}$) is a multipotent compartment, MPP3 (defined as LSK $CD34^{+}$ $CD48^{+}$ $CD150^{-}$ $CD135^{-}$) have mainly myeloid – biased output and MPP4 (defined as LSK $CD34^{+}$ $CD48^{+}$ $CD150^{-}$ $CD135^{+}$) have a preference for differentiating into lymphoid cells [44] **(Figure 1B)**. Two additional MPP5 (defined as LSK $CD34^{+}$ $CD48^{-}$ $CD150^{-}$ $CD135^{-}$) and MPP6 (defined as LSK $CD34^{+}$ $CD48^{-}$ $CD150^{-}$ $CD135^{-}$) subsets have been characterized, which are functionally located between HSCs and MPP2-MPP4 [45]. MPP5 are able to generate MPP1-MPP4 but not HSCs and produce both myeloid and lymphoid lineages. MPP6 represents a subset with longer term multilineage potential compared with MPP5, comparable to HSCs [45] **(Figure 1B)**. An alternative subclassification of MPPs with no use of CD34 as surface marker found that MPP2 is an erythroid/megakaryocyte-biased MPP subset [46] **(Figure 1C)**.

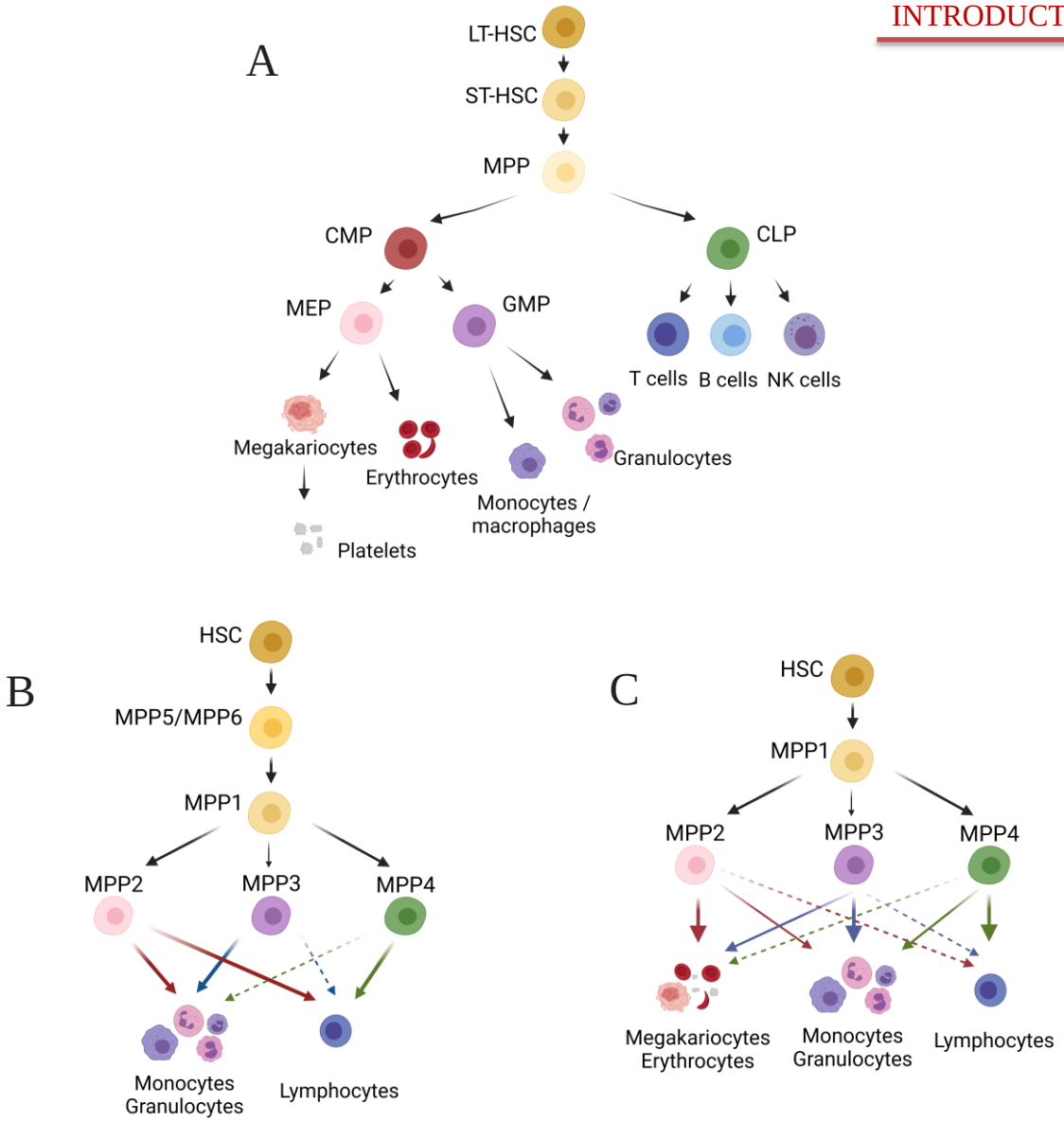


Figure 1: Suggested models of hematopoiesis. (A) Classical tree-like hierarchical hematopoietic stem cell (HSC) differentiation model. Long-term HSCs (LT-HSCs), with high self-renewal capacity and low differentiation profile, sit at the top of the hierarchy and, as differentiation progresses, give rise to short-term HSCs (ST-HSCs), with less self-renewal capacity. ST-HSCs evolve into multipotent progenitors (MPPs), which differentiate into common myeloid progenitors (CMPs) or common lymphoid progenitors (CLPs). CMPs will evolve into megakaryocyte erythroid progenitors (MEPs) and/or granulocyte macrophage progenitors (GMPs). MEPs ultimately give rise to erythrocytes and megakaryocytes that produce platelets, and GMPs give rise to granulocytes and monocytes/macrophages. On the other branch, CLPs ultimately differentiate into B and T lymphocytes and natural killer (NK) cells. **(B-C) Tree-like hierarchical HSC differentiation model with subdivision of the MPP compartment.** (B) The HSC compartment comprises HSCs, with long-term self-renewal, MPP6 with slower dynamics but long-term self-renewal, and MPP5 and MPP1, with short-term self-renewal. The MPP compartment is divided in different MPP subtypes, MPP2 to MPP4. MPP2 similarly differentiate into myeloid or lymphoid cells whereas MPP3 and MPP4 differentiation is skewed towards production of myeloid or lymphoid lineage, respectively [44,45]. (C) Alternative subclassification of MPPs with no use of CD34 marker. Here, MPP2-MPP4 subsets are skewed towards production of erythroid/megakaryocyte, myeloid or lymphoid

lineage, respectively [46]. (B-C) Biased-MPPs are able to give rise to cell subsets that are not their major progeny, depending on the organism requirements, as indicated by discontinued arrows. Progenitors are not represented for simplification.

These hierarchical models are commonly used to classify HSCs into subgroups with comparable functions and simplify studies of the hematopoietic system. However, hematopoiesis has been shown to be more flexible than traditionally described, and biased MPPs are able to give rise to cell lineages that are not their major progeny, depending on the organism requirements [1,44,46,47]. Further, the dogma of HSC ability to self-renew as long as a low lineage commitment is kept, with lineage commitment occurring in later stages while the self-renewal capacity is lost, has persisted for decades. However, recent findings from Yamamoto and colleagues showed megakaryocyte-restricted progenitors with long-term repopulating activity [48]. Similarly, a HSC subset primed for platelet-specific gene expression was discovered [49], with biased short- and long-term reconstitution for platelets. Platelet-primed HSCs self-renew, and frequently have a long-term myeloid lineage bias but can give rise to lymphoid-biased HSCs too. These findings suggest that loss of self-renewal capacity is not an essential request for lineage commitment, and that lineage commitment can arise from HSCs without going through stable multi- and bipotent intermediate stages. In fact, it is currently accepted that HSCs do not mature by jumping from one intermediate state to another, but gradually acquire lineage-specific fates in a continuous process in which, committed progenitors should be considered as transient stages [1,48,50]. Single-cell transplantation assays have further evidenced the large variability in the self-renewal capacity and bias towards the production of specific blood lineages among murine HSCs [1,51,52] **(Figure 2).** This new concept of hematopoiesis is rapidly developing and being applied in research thanks to breakthrough technologies based on single-cell analysis.

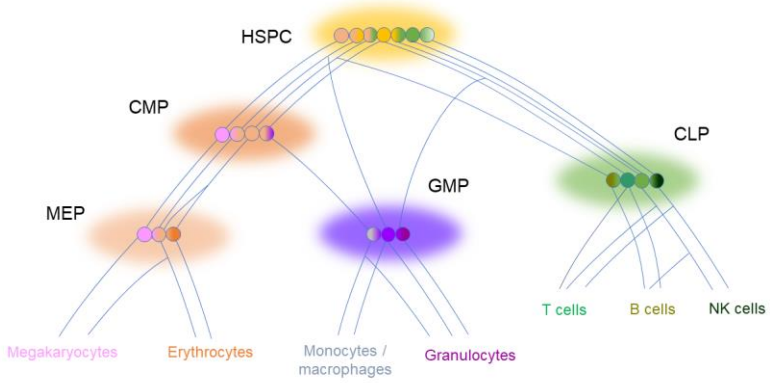


Figure 2: Continuous hematopoietic stem cell (HSC) differentiation model. Accepted model of hematopoiesis that considers that individual HSCs gradually acquire lineage biases without passing through hierarchically organized progenitors and acknowledges differentiation bias at the HSC level without losing self-renewal capacity [1].

In humans, surface markers present on HSPCs are similar but not identical to those on mouse HSPCs. As opposed to mice, human $CD34^+$ cells are enriched in HSCs [53]. However, human HSCs have also been found in bone marrow and cord blood – derived $CD34^-$ cells [54-56]. Human $CD34^+$ cells comprise HSCs and progenitors and can provide long-term and multilineage hematopoietic reconstitution [53]. Among $CD34^+$ cells, $CD34^+$ $CD38^-$ cells were identified as human HSPCs, while $CD34^+$ $CD38^+$ cells are lineage – restricted progenitors [57,58]. Within the HSPC subset, HSCs are identified by the cell surface marker profile Lin^- $CD34^+$ $CD38^-$ $CD45RA^-$ $CD90^+$. In human cord blood, lack of CD90 expression is concomitant with loss of self-renewal capacity, and therefore human MPPs are defined as Lin^- $CD34^+$ $CD38^-$ $CD45RA^-$ $CD90^-$ [59-61]. McKenzie and colleagues showed that cord blood HSCs with long-term self-renewal capacity, as measured by serial transplantations, are enriched in a Lin^- $CD34^+$ $CD38^-$ population with low uptake of the mitochondria dye rhodamine 123 (Rho). Rho is a fluorescent dye that binds to respiring mitochondria, reflecting the active metabolic state of the cell; and it is also a substrate of P-glycoprotein, an efflux pump responsible for multidrug resistance in tumor cells. Using a purification method based on Rho uptake, these authors were able to isolate a population of quiescent and long-term HSCs identified as Lin^- $CD34^+$ $CD38^-$ Rho^{low} cells [62]. Using cord blood, by tracking the expression of several adhesion molecules in HSC – enriched subsets, the group of John Dick revealed that HSC activity is restricted to $CD49f^+$ cells, so that human HSCs are identified as Lin^- $CD34^+$ $CD38^-$ $CD45RA^-$ $CD90^+$ $CD49f^+$, while MPPs are Lin^- $CD34^+$ $CD38^-$ $CD45RA^-$ $CD90^-$ $CD49f^-$ cells [63].

The study of human HSPCs *in vivo* is mainly approached by use of mouse models. In these models, human HSPCs are transplanted into recipients to study their fate and function. Xenograft models enable human HSPC transplantation and engraftment, but their greatest limitation is a mouse BM niche to support human HSPC engraftment and sustain hematopoiesis [64]. To prevent human graft rejection and allow its engraftment, *in vivo* models are immunodeficient, and usually recipients need to be preconditioned by irradiation or treatment with chemotherapy drugs prior to transplantation [65]. Immunodeficient *NOD-scid gamma* (NSG) mice, which carry the severe combined immune deficiency spontaneous mutation (*Prkdc*^{scid} or s*cid*) and the interleukin 2 receptor gamma null allele (*Il2rg*$^{-/-}$) resulting in lack of lymphocytes and functional natural killer production, have traditionally been used for transplantation of human cells. Despite the preconditioning of these mice, human cell engraftment is usually low [65]. Development of mice expressing human cytokines and growth factors has improved human donor cell engraftment. For example, humanized NSG-SGM3 mice expressing

supraphysiological levels of human interleukin 3 (IL-3), granulocyte macrophage colony stimulating factor (GM-CSF) and stem cell factor (SCF) have shown better myeloid engraftment compared to standard NSG immunodeficient mice [66-69]. However, some reports show that the overexpression of these human cytokines induces human HSC exhaustion in these mice [64,66,67]. Preconditioning is also harmful for the HSC niche in the recipients and impairs the function of the transplanted HSCs [70,71]. Thus, new animal models where no preconditioning is needed are being engineered. Of note, mutations of *Kit* in NSG mice or in BALB/c *Rag2*$^{-/-}$ and *Il2rg*$^{-/-}$ expressing a transgene of human signal regulatory protein alpha (SIRPa) that negatively regulates phagocytosis, have allowed to develop such new mouse models for human cell transplantation that open the BM niche to the human cells through genetic preconditioning [65,72].

The functional numbers of mouse or human HSPCs are evaluated *in vitro* by the generation of colonies (colony forming unit – cells (CFU-Cs)) [73], whereas primitive HSCs are studied by CFU-C replating in long-term culture-initiating cell (LTC-IC) assays [74-76] and by their ability *in vivo* to reconstitute the entire hematopoietic system after serial transplantations [77,78]. In serial transplantation assays, donor whole BM or HSCs, or a single HSC, are engrafted into a primary host and subsequently isolated and engrafted into a secondary and even a tertiary host [77,78]. This is considered the "gold standard" to assess HSC stemness capacity.

This section conveys the complexity of blood formation. HSCs and hematopoiesis must be strictly regulated by coordinated mechanisms that guarantee the correct functioning of the blood system at any given time and circumstance. These mechanisms are intrinsic or extrinsic, and they are the focus of current great scientific endeavour.

1.2. Intrinsic factors that define HSC identity

HSC fate and function are tightly regulated by cell-intrinsic factors such as transcription factors (TFs), epigenetic remodelers and metabolism, and their dysregulation can result in hematological diseases [17,79-81].

1.2.1. Transcription factors (TFs)

HSC self-renewal, proliferation and differentiation are regulated at the transcriptional level. Expression and repression of different gene sets determine HSC fate and functionality, which is mediated, in part, by TFs and their underlying signaling network. TFs such as nuclear factor kappa B (NF-κB) [82,83], GATA1/2/3 [84-86], PU.1 (encoded by the gene *SPI1*) [87-89], CCAAT enhancer binding protein alpha (C/EBPα; encoded by *CEBPA*) [90,91], Ikaros family zinc finger

proteins [92,93] and RUNX1 [94-97] are known to play critical roles in HSC fate and function, among others [79].

TFs modulate hematopoiesis by directing lineage-specificity, HSC self-renewal and proliferation, as well as HSC maintenance. Usually, one specific TF will be involved in the regulation of several HSC functions to coordinate a cellular response. For instance, C/EBPα promotes myeloid priming of HSCs and progenitor cells [90,91,98,99] and is critical for neutrophil differentiation [100]. C/EBPα also protects HSCs from apoptosis and helps them maintain quiescence [91]. NF-κB is a major player in the development of the myeloid lineage and in promoting survival of HSCs [82]. PU.1 restricts granulopoiesis, as PU.1 deficient mice show increased granulocyte production [89], and restricts HSC proliferation during IL-1β-induced inflammation [87,88]. GATA-1 is required for erythroid and megakaryocytic commitment, and reduces HSC self-renewal [84,101-103], and Ikaros family of TFs prime HSCs to the lymphoid lineage [92] and are crucial regulators of self-renewal in LT-HSCs [93]. GATA-2 and GATA-3 regulate the maintenance of HSCs by restricting their entry into cycle [85,86]. GATA-3 is also a lineage specific TF that impacts eosinophils and T cells [104]. *Runx1* loss affects HSC proliferation [94] and results in exhaustion of the HSPC pool [95]. RUNX1 is also required for megakaryocyte maturation and lymphoid cell differentiation [96,97]. Thus, activation or repression of one or another TF modulate hematopoiesis in an orchestrated fashion.

Given the crucial roles of TFs in hematopoiesis, direct mutations or secondary alterations in genes encoding C/EBPα [105,106], RUNX1 [107,108], NF-κB [82,109] and different members of the GATA family [110-112], among others, are associated with the development of hematological malignancies.

1.2.2. Epigenetic remodeling

Modulation of gene expression through epigenetic remodelers regulates HSC fate and function. DNA methylation and histone modifications promote alterations in genome accessibility, thus modifying gene expression without direct changes in DNA sequence and thereby regulating HSCs and their progeny.

DNA methylation involves the binding of a methyl group at the carbon-5 position of a cytosine base, primarily in cytosine-phosphate-guanine (CpG) dinucleotides, producing 5-methylcytosine (5-mC) [113,114]. DNA methylation is associated with stable transcription repression and is catalyzed by DNA methyltransferases (DNMTs) [114,115]. DNMTs are highly associated with lineage commitment during HSC differentiation and regulation of self-renewal

[116-123]. For example, *Dnmt3a* deletion in mice results in expansion of the HSC pool and block of myeloid differentiation [118-122] in favor of the erythroid lineage [122]. Mutations in *DNMT3A* in humans are associated with clonal hematopoiesis and myeloid malignancies, and it is one of the most mutated genes in acute myeloid leukemia (AML) [118,119,124-126].

Interestingly, isocitrate dehydrogenases 1 and 2 (IDH1 and IDH2), enzymes of the tricarboxylic acid cycle relevant in cellular metabolism, are also involved in the epigenetic regulation of HSPCs. IDH1 and IDH2 catalyze the reversible oxidative decarboxylation of isocitrate to α-ketoglutarate (αKG) in the cytoplasm and mitochondria, respectively, with concomitant reduction of $NADP^+$ to NADPH [127]. Mutant IDH enzymes catalyze the reduction of α-KG to the (R) enantiomer 2-hydroxyglutarate (2-HG), which is associated with DNA and histone hypermethylation, altered gene expression and blocked differentiation of HSPCs [128,129]. *Idh1* mutant mice develop myeloid dysplasia along with anemia and increased immature progenitors and erythroblasts. These data show the important role of IDH1 in normal erythropoiesis and associate its mutation to myeloid disorders [130]. Conversely, *Idh2* mutant mice develop myeloid and lymphoid neoplasms, with differentiation block and increased proliferation [131]. In humans, somatic mutations of both *IDH1* and *IDH2* have been found in patients of AML, secondary AML (sAML), myeloproliferative neoplasms (MPNs) and myelodysplastic syndromes (MDS) [132,133]. 2-HG is a competitive inhibitor of various α-KG-dependent dioxygenases that include histone demethylases and the ten-eleven translocation (TET) family of 5-mC hydroxylases [134].

To reverse genome methylation, 5-mC can be oxidized to 5-hydroxymethylcytosine (5-hmC) by enzymes of the TET family (TET1, TET2 and TET3), which results in hydroxymethylation that they can further oxidize eventually resulting in DNA demethylation [114,122,135]. Deletion of *Tet2* skews HSC differentiation into the myeloid lineage and expands the HSC compartment [136-139]. Further, deletion of both *Tet2* and *Tet3* in mice results in myeloid leukemia [140]. In humans, *TET2* is frequently mutated in myeloid neoplasms, AML and other hematopoietic malignancies [141].

DNA 6-adenine methylation (6-mA) is a recently discovered epigenetic modification in the human genome with only a few enzymes characterized to date to be involved in 6-mA methylation, like the writer N6AMT1 and the eraser ALKBH1, but more evidence is required to support presence and function [142,143]. 6-mA is abundant in RNA though, where it regulates mRNA post-transcriptionally by affecting splicing, export, stability, and translation [144-146]. Many 6-mA regulators, including both readers and erasers, are overexpressed in AML and they induce leukemogenesis through different pathways and downstream targets that affect leukemia

stem cell (LSC) self-renewal and maintenance, proliferation, apoptosis and chemoresistance [147].

Polycomb repressive complex (PRC) is a group of epigenetic regulators that play a crucial role in determining HSC fate and function. PRCs trigger transcriptional repression by catalyzing the monoubiquitylation of H2A (H2Aub1, PRC1) and the addition of up to three methyl groups to lysine 27 at histone H3 (H3K27me, PRC2) [148-151]. For example, accumulated evidence shows that enhancer of zeste (EZH1 and EZH2) proteins, components of PRC2, participate in hematopoiesis and HSC fate both during development and adult life [152-154]. In mice, EZH2 is essential for fetal development given that *Ezh2* deficient embryos die of anemia due to insufficient expansion of HSPCs and defective erythropoiesis in fetal liver [152]. In adults, *Ezh2* has low expression and is involved in lymphoid lineage priming [152,153]. In contrast, the number of HSCs in the fetal liver of BMI-1-knockout (KO) mice was normal but markedly reduced in postnatal animals, indicating that PRC protein BMI-1 is essential for the self-renewal of adult HSCs [155]. Conversely, enforced expression of *Bmi1* promotes self-renewal [156]. The polycomb group of proteins additional sex combs like 1 and 2 (ASXL1 and ASXL2) are also required for proper hematopoiesis and play an important role in HSC fate and self-renewal [157-159]. In humans, mutations in *EZH2*, *ASXL1* are common in myeloid neoplasms [160,161]. *BMI1* mutations have been found in early T cell precursors in acute lymphoblastic leukemia but its overexpression has been reported to contribute to leukemogenesis and poor prognosis in AML, myelodysplastic syndromes, chronic myeloid leukemia (CML) and other hematological malignancies [162,163].

Histone acetylation, addition of an acetyl group to lysine residues of histones, correlates with chromatin accessibility and active gene expression, and is mediated by lysine acetyltransferases (KATs). For example, deletion of *Kat6a* in mice results in quick loss of HSCs together with a reduction of their quiescence and transplantation ability whereas it extends self-renewal in early myeloid progenitors, and it is essential for the balanced development of myeloid, erythroid and B lineage cell progenitors [164]. In human AML, KAT6A is highly associated with the generation of fusion genes by chromosomal translocation [165]. The level of histone acetylation is also regulated by histone deacetylases (HDACs), including HDAC1 and HDAC2 among others, and other deacetylases like sirtuin (SIRT) enzymes. Double but not single deletion of *Hdac1* and *Hdac2* promotes hematopoietic failure [166,167]. No mutations in *HDACs* have been detected in human AML, but HDACs are recruited to specific gene promoters by oncogenic fusion proteins, such as PML-RARα, PLZF-RARα, or AML1-ETO, mediating aberrant gene silencing

that contributes to leukemogenesis [168,169]. Deletion of *Sirt1* induces defects in HSC maintenance and differentiation, resulting in reduced size of the lymphoid compartment and anemia [170,171]. In humans, *SIRT1* is overactivated in various hematopoietic malignancies including CML, AML and lymphoma [172-175]. For example, activation of *SIRT1* by BCR-ABL promotes transformation of HSCs and CML progression [172].

1.2.3. Metabolism

HSC bioenergetics and metabolism contribute to determining HSC identity [81,176,177]. Cellular metabolism is mediated, in part, by mitochondrial respiration. HSCs exhibit lower basal and maximal respiration rates than progenitor cells, however, mitochondrial respiration seems to be essential for HSC function and maintenance [178-180]. For example, inactivation of the mitochondrial complex III subunit Rieske iron-sulfur protein (RISP) in adult HSCs results in loss of HSC quiescence concomitant with severe pancytopenia and lethality [179]. Further, loss of mitochondrial carrier homolog 2 (MTCH2), a transporter found in the inner layer of mitochondria that is involved in mitochondrial depolarization, increases oxidative phosphorylation and reactive oxygen species (ROS) which cause entry of HSCs into cycle and compromises their self-renewal capacity [181]. Conversely, HSCs utilize oxidative phosphorylation during differentiation. Disrupting oxidative phosphorylation upon loss of protein tyrosine phosphatase mitochondrial 1 (PTPMT1), a lipid phosphatase involved in regulating the integrity of the mitochondrial membrane, increases the HSC pool and blocks differentiation resulting in hematopoietic failure [182].

LSCs in AML share metabolic characteristics with healthy HSCs like the low mitochondrial activity, but they are particularly dependent on low but intact oxidative phosphorylation due to their higher energy demands [183]. Oxidative phosphorylation in chemotherapy-resistant leukemia cells is fueled by mitochondrial fatty acid oxidation [184] and inhibition of fatty acid oxidation in mouse models of human AML seems to have therapeutic value [185]. More recently, LSCs have also been evidenced to be highly dependent on amino acid metabolism for oxidative phosphorylation and survival. Targeting amino acid uptake with the BCL-2 inhibitor venetoclax with azacitidine decreased oxidative phosphorylation resulting in LSC killing in AML patients [186].

Metabolism plays an important role in HSC maintenance under hypoxic conditions. HSCs reside in hypoxic niches, where they are quiescent [187-190]. Hypoxia requires a metabolic adaptation of the cells and thus HSCs rely on glycolysis rather than oxidative phosphorylation

for their energy production. Glycolysis is less effective than oxidative phosphorylation in generating ATP, but it reduces oxidative stress and ROS production [191,192]. Hypoxic conditions prevent HSC differentiation and maintain quiescence and self-renewal through induction of a glycolytic phenotype. Cellular responses to hypoxia are predominantly mediated by the hypoxia-inducible factor (HIF)-1 and HIF-2, which facilitate the transcription of hypoxia-responsive genes [187-190]. In HSCs, hypoxia induces HIF-1α stabilization, which activates transcription of genes important for quiescence [188,189,193,194] and stimulate glycolysis for the maintenance of the HSC pool [195]. In mice, *Hif1a* deletion in the hematopoietic system results in progressive loss of HSCs upon serial transplantation, indicating that *Hif-1α* is required for HSC maintenance and self-renewal [193]. However, more recent studies show that deletion of *Hif1a* or *Hif2a* alone or together had no impact on HSC numbers, steady-state hematopoiesis or reconstitution upon transplantation [196,197]. Further research is required to clarify the role of HIF family of factors in HSC function. Interestingly, oncometabolites activate a HIF-orchestrated "pseudohypoxic" response that is apparent for example in succinate dehydrogenase (SDH)- and fumarate hydratase (FH)-deficient tumors and has been associated to tumorigenesis [198,199]. However, in mice, deletion of *Hif1a* accelerated development of *Meis1/Hoxa9* AML and increased propagation of AML induced by *Aml1-Eto9a* [200], suggesting that *Hif1α* is dispensable for AML LSC maintenance [201]. Further, deletion of *Hif2a* accelerated LSC generation and shortened development of *Mll-AF9* AML, which was potentiated by *Hif1a* codeletion. The propagation of established LSCs had similar latencies in all experimental groups. Together, these data indicate that HIF-1α and HIF-2α may in fact synergize to suppress AML development and they may not be required for LSC maintenance.

In conclusion, cell-intrinsic mechanisms, including regulation of gene expression and cellular bioenergetics, are essential in determining the fate and function of HSCs and their alteration is involved in hematological malignancies. Under normal circumstances, these cell-intrinsic mechanisms are integrated with the *in vivo* microenvironment for a fine-tuned regulation of HSC self-renewal and differentiation.

1.3. At home: HSC niche

The HSC niche is the HSC surrounding microenvironment involved in the maintenance and regulation of HSC biology and function [9-13,202]. It is composed by various cell types, including hematopoietic and non-hematopoietic cells, and their interactions with the HSC, including membrane-bound and soluble factors released by these cells **(Figure 3)**. To carry out its

mission, the HSC niche is dynamic and its composition changes based on the organism needs [9-13,202].

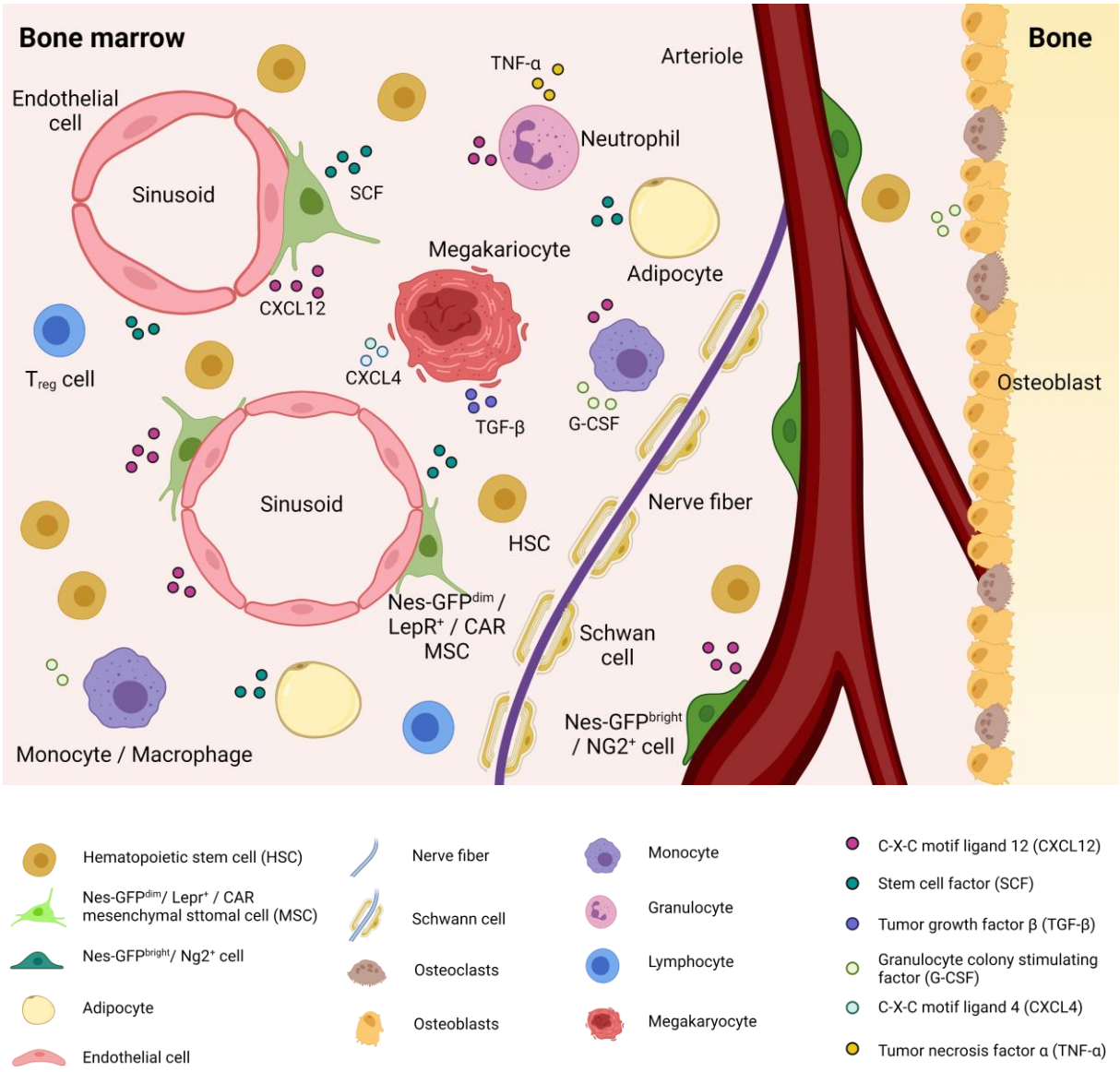


Figure 3: The hematopoietic stem cell (HSC) niche. Simplified illustration of the HSC niche that is the specialized microenvironment where HSCs reside, which contributes to HSC function and maintenance. The HSC niche is formed by cells of hematopoietic and non-hematopoietic origin. The nature of the communications between HSCs and their niche is bidirectional. The illustration depicts Nestin-GFP^{dim} (Nes-GFP^{dim}) mesenchymal stromal cells (MSCs), which overlap with Leptin receptor-expressing (LepR^{+}) MSCs and CXCL12-abundant reticular (CAR) cells in sinusoids, and Nestin-GFP^{bright} (Nes-GFP^{bright}) MSCs which overlap with NG2-expressing (NG2^{+}) cells in arterioles. Other relevant components of the HSC niche are hematopoietic cells such as megakaryocytes, monocyte/macrophages, neutrophils and regulatory T cells (T_{reg}), and non-hematopoietic cells, including endothelial cells, nerve fibers and Schwann cells, osteoblasts, osteoclasts and adipocytes. HSCs are regulated through direct contact or soluble factors that mainly include CXCL12 and SCF, but also G-CSF, TGF-β, CXCL4 and TNF-α, among others.

The complexity of the HSC pool raised the possibility of the existence of specialized niches for different HSC populations. The use of SLAM markers allowed to locate most HSCs (≈ 85%) adjacent to perivascular areas, specifically close to sinusoidal endothelial cells in bone marrow and spleen. This gave rise to the notion of a *perivascular niche* or, specifically, a *sinusoidal niche* [41,203,204]. Although most HSCs reside near sinusoids, a low number of quiescent HSCs has been associated with arteriolar structures, forming the *arteriolar niche* [205-208]. In addition, previous studies tracing labeled HSPCs after transplantation or myeloablation also found HSCs near the endosteum (*endosteal niche*) [209-211], suggesting a possible endosteal localization of HSC niches after being subjected to a challenge or stress stimuli, but its function under homeostasis is still under debate as of today.

Importantly, as the niche is a regulatory unit of HSC activity, its alterations impact HSC number and function, which can originate or contribute to the progression of hematological malignancies [9-13,212-214].

1.3.1. Cellular components of the HSC niche

In the HSC niche, hematopoietic and non-hematopoietic cells surround HSCs and regulate their function and maintenance through direct cell-to-cell interactions or the secretion of soluble factors such as cytokines, chemokines and growth factors [9-13] **(Figure 3)**.

Among hematopoietic cells, megakaryocytes, monocytes and macrophages, neutrophils and lymphocytes have shown to participate in the regulation of the niche. Additionally, non-hematopoietic cells such as mesenchymal stromal cells (MSCs), endothelial cells, nerve fibers and Schwann cells, osteoblasts and adipocytes also regulate HSC fate and function [9-13].

- ***Hematopoietic cells with roles as HSC regulators***

Hematopoietic progeny mainly regulates HSCs by altering their quiescence, differentiation and localization in the niche.

Megakaryocytes directly regulate HSC quiescence thorough secretion of several factors as C-X-C chemokine ligand 4 (CXCL4), transforming growth factor β (TGF-β) and Src homology region 2 domain-containing phosphatase 1 (SHP-1) [215-217]. A recent study confirmed the contribution of megakaryocytes to the quiescence of a subset of HSCs expressing von Willebrand factor (vWF) [218], which differentiate into platelets and myeloid cells upon transplantation [49]. Megakaryocyte depletion expands vWF^+ HSCs, and, after transplantation, reduces long-term self-renewal capacity and biased differentiation of these vWF^+ HSCs [218].

Macrophages and *monocytes* influence HSCs by multiple mechanisms. Macrophages retain HSC indirectly in the bone marrow by regulating endosteal osteoblastic cells and MSCs [219,220]. This is performed through the expression of oncostantin M, which induces C-X-C chemokine ligand 12 (*Cxcl12*) expression in MSCs that binds to C-X-C chemokine receptor 4 (CXCR4) and retains HSCs in the bone marrow and supports their survival [221]. Macrophages and monocytes are also the major producers of granulocyte colony stimulating factor (G-CSF), which promotes HSC differentiation into granulocytes [222-225]. G-CSF treatment is a powerful means that induces mobilization of HSCs and increases the number of phenotypic HSCs in the bone marrow, but results in a loss of HSC repopulating and self-renewal activity [222,226]. Monocytes-macrophages expressing α-smooth muscle actin protein (α-SMA) and the cyclooxygenase COX-2, located adjacent to HSCs, prevent HSC exhaustion, by limiting their production of ROS and enhancing stromal cell expression of the chemokine CXCL12, through prostaglandin E_2 (PGE_2) release [227]. LT-HSC dormancy is also maintained by interaction of CD82, expressed in HSCs, with Duffy antigen receptor for chemokines (DARC) – expressing macrophages [228].

HSC function is also modulated by *neutrophils*. Aged $CD62L^{low}$ $CXCR4^{high}$ neutrophil clearance by phagocytic macrophages within the bone marrow promotes circadian egress of hematopoietic progenitors into the bloodstream through reduction in CXCL12 abundant reticular cells (CAR cells) in the niche and CXCL12 [229]. After irradiation, neutrophils promote endothelial recovery via production of tumor necrosis factor α (TNF-α), which in turn improves HSC engraftment [230].

Regulatory T cells (T_{reg}) provide immune privilege for transplanted HSCs, enabling them to escape from allogeneic rejection, through a mechanism that at least partially involves production of adenosine. Adenosine generated via the CD39 cell surface ectoenzyme on niche T_{reg} protects HSCs from oxidative stress and maintains HSC quiescence. [231,232].

- ***Mesenchymal stromal cells as main HSC regulators***

Among the different components of the HSC niche, MSCs (also known as mesenchymal stem and progenitor cells (MSPCs)) are essential HSC niche regulators. MSCs form a heterogeneous and dynamic population with self-renewal capacity and ability to differentiate into osteoblasts, chondrocytes, adipocytes and other stromal cells like fibroblasts [233]. *In vitro*, these cells are able to generate adherent fibroblastic colonies when seeded at clonal density (colony forming unit – fibroblasts (CFU-Fs)) or non-adherent mesenspheres that self-renew and expand in serial

transplantations [234-236]. *In vivo*, MSCs have the ability to regenerate bone, cartilage and adipocytes [236-239]. MSCs localize close to perivascular areas, where HSCs are also preferentially distributed [203,236]. MSCs are the major producers of SCF and CXCL12, key regulators of HSCs that support HSC survival, self-renewal and maintenance in the bone marrow [206,236,240-243]. MSCs are also an important source of growth factors and pro-inflammatory/anti-inflammatory cytokines, which are involved, directly or indirectly, in the functional regulation of HSCs [244,245].

Still today, MSC characterization and refined identification is difficult due to the lack of a consensus unique combination of cell surface markers, differences and limitations among lineage tracing models and differences resulting from cell isolation techniques, among others [246-248].

MSCs are sufficient to generate a functional bone marrow microenvironment at ectopic sites both in humanized mouse models and in mouse models. Human $CD45^-$ $CD146^+$ $CD105^+$ MSCs, which contain all CFU-F activity from bone marrow, upon subcutaneous transplantation into immunodeficient mice, can establish a heterotopic niche with bone formation, appearance of sinusoidal system of mouse endothelial cells and human adventitial cells, and host HSCs [237]. Mouse $CD45^-$ $Tie2^-$ $CD90^-$ $CD51^+$ $CD105^+$ MSCs from fetal bones, upon sub-renal transplantation, are also able to form an ectopic bone cavity through endochondral ossification, populated by host vasculature and LT-HSCs [249].

A variety of markers are enriched on the surface of MSCs and have helped their characterization. Characterization of MSCs is based on the negative expression of leukocyte common antigen CD45, platelet endothelial cell adhesion molecule CD31 and erythroid cell marker Ter119, and the positive expression of some specific surface or intracellular markers [236,240,250-252]. For example, murine $CD45^-$ $Ter119^-$ $Sca\text{-}1^+$ PDGFR (platelet-derived growth factor receptor) α^+ cells are a population of rare MSCs, able to generate colonies at high frequency and differentiate into osteoblasts and adipocytes after *in vivo* transplantation. These MSCs are quiescent and reside in perivascular areas [250]. Additional markers enriched in MSCs are vascular cell adhesion molecule 1 (VCAM1; also known as CD106), endoglin (also known as CD105), actin α 2 (ACTA2), myosin heavy chain 11 (MYH11) and CD63 [248,253].

Due to the elusive nature of MSCs, mouse models are frequently used to help their study. MSCs subsets traced this way show only partial overlap [205,239,242,252,254,255]. Green fluorescent protein (GFP) reporter knocked into the endogenous locus of *Cxcl12* allowed the identification of CAR cells as a population of MSCs main source of CXCL12 within the bone marrow [240,241]. CAR

cells are near sinusoids and the endosteum [241,247,256], and are also major producers of SCF, another essential HSC niche regulator involved in maintenance of HSC self-renewal [240,241]. Depletion of CAR cells impairs adipogenesis and osteogenesis, and the production of SCF and CXCL12. This has an impact on HSCs, reducing their numbers, impeding their proper proliferation and biasing their differentiation into the myeloid lineage [240]. Recently, combined single-cell and spatial transcriptomics have revealed Adipo-CAR (characterized by expressing adipocyte lineage genes) and Osteo-CAR (characterized by expressing osteocytes lineage genes) cell subsets that differentially localize to sinusoidal and arteriolar surfaces, respectively, and express additional cytokines like interleukin 7 (*Il7*) and colony stimulating factor 1 (*Csf1*) [257]. CAR cells do not express CD45 or CD31, and display expression of the cell-surface protein VCAM1, CD44, CD51, PDGFRα and PDGFRβ [240].

Stromal cells positive for nestin protein, an intermediate filament highly expressed in neural stem cells [258-260], were identified as MSCs located around blood vessels and closely associated with HSCs. Nestin$^+$ cells contain all CFU-F activity and are able to differentiate into bone, cartilage and adipocytes *in vitro*, and to self-renew in serial transplantations and give rise to osteoblasts, osteocytes and chondrocytes *in vivo* after long-term lineage tracing (8 months) [236]. Depletion of these cells or deletion of their expression of *Cxcl12* or *Scf* reduces the number of HSPCs in the bone marrow [206,236]. Nestin$^+$ cells are also close to sympathetic nerves that regulate their expression of *Cxcl12*, which turned out relevant in different contexts like healthy HSC circadian egress or mutant HSC expansion in MPNs among others [212,236,261,262]. Nestin$^+$ cells are identified by expression of GFP under the endogenous Nestin promoter (Nes-GFP$^+$ cells) and based on the fluorescence intensity of Nes-GFP, nestin$^+$ cells can be further classified as Nes-GFP^{bright} and Nes-GFP^{dim} [205]. Nes-GFP^{bright} cells are a rare population of periarteriolar cells associated with more quiescent HSCs and enriched in neural/glial antigen 2 (NG2; NG2$^+$ cells). Nes-GFP^{dim} cells are more abundant and associated with sinusoids. Nes-GFP^{dim} cells show a lower clonogenic potential compared to Nes-GFP^{bright} cells, however, due to their higher frequency, the overall clonogenic capacity around sinusoids is higher [205,206,246]. These data suggest the existence of different niches that regulate distinct HSC pools.

MSCs are also identified based on the expression of the adipo-osteogenesis regulator leptin receptor (LEPR, LepR$^+$ cells) [242,255]. LepR$^+$ cells are also an important source of CXCL12 and SCF [241,243]. LepR$^+$ cells overlap by 90% with CAR cells in a young adult bone marrow, and have perivascular location and mesenchymal potential similar to CAR cells [239,241,242,252]. LepR$^+$ cells are also preferentially labeled as Nes-GFP^{dim} cells, associated with perisinusoidal areas

[205]. Deletion of *Scf* in LepR$^+$ cells depletes HSPCs, while deletion of *Cxcl12* promotes HSPC mobilization into the blood circulation [242,243]. However, a more recent study showed that selective deletion of *Cxcl12* in the arteriolar NG2$^+$ population, enriched in Nes-GFP^{bright} cells, but not deletion of *Cxcl12* in sinusoidal LepR$^+$ cells, depletes HSCs and alters their location in the bone marrow. In contrast, deletion of *Scf* in LepR$^+$ cells, but not in NG2$^+$ cells, reduces HSC number in the bone marrow [206]. The heterogeneity and complexity of MSCs require further investigation using refined mouse tools for identification of the distinct MSC subsets and their biological roles within the different HSC niches.

Like mouse MSCs, characterization of human MSCs is based on the negative expression of CD45, CD31 and Ter119, and the positive expression of specific surface markers [237,254,263]. Some of these surface markers are common to human and mice, like endoglin [264], VCAM [265] and CD63 [237]. However, the most broadly used human surface markers differ from mouse markers, such as human melanoma cell adhesion molecule (MCAM; also known as CD146) [237,263] and CD271 [266], among others [267]. In humans, CD45$^-$ Ter119$^-$ CD31$^-$ PDGFRα$^+$ CD51$^+$ cells were identified as MSCs, which express HSC maintenance genes and contain CFU-F activity. They form self-renewing mesenspheres that support expansion of hematopoietic progenitors, able to engraft in immunodeficient mice *in vivo* [254]. Similarly, human CD45$^-$ CD31$^-$ CD71$^-$ CD146$^+$ CD105$^+$ nestin$^+$ MSCs form mesenspheres *in vitro* that differentiate in mesenchymal lineages and promote expansion of cord blood CD34$^+$ cells through secreted soluble factors. Cultured CD34$^+$ HSPCs in the presence of these MSCs were serially transplantable into immunodeficient mice with increased long-term engraftment than CD34$^+$ cells cultured alone [263]. Like murine MSCs, the lack of standard combinations of specific human MSC markers and the great heterogeneity among different human MSC subpopulations has hindered the characterization of the identity and function of these cells *in vivo*, which requires further investigation [267,268].

- ***Other important non-hematopoietic regulators of HSCs***
    - *Endothelial cells*

Endothelial cells shape blood vessels and are the barrier between the HSC niche and the blood circulation. HSCs have preference for perivascular areas. As previously indicated, HSCs have traditionally been associated to sinusoids [41,203,204], but recent studies suggest that arterioles maintain the most quiescent HSCs [205,206,208]. These two types of blood vessels have distinct functions on HSCs and while less permeable arterioles maintain HSCs in a low ROS state, the

more permeable sinusoids promote their activation and are the site for trafficking to and from the bone marrow [207].

Endothelial cells exert their function on HSCs by cell-to-cell contacts and through a variety of released factors. Cell-to-cell contacts are mediated by adhesion molecules such as vascular endothelial growth factor (VEGF) and E-selectin [269-271]. Among the soluble factors secreted by endothelial cells with regulatory HSC function, SCF and CXCL12 stand out. Deletion of *Scf* or *Cxcl12* from endothelial cells depletes HSCs in bone marrow, indicative of the important role of endothelial cells in HSC regulation [242,243].

- *Nerve fibers and non-myelinating Schwann cells*

Sympathetic nerve fibers in the bone marrow play an important role in HSC mobilization. Pharmacological and genetic inhibition of adrenergic signals impairs G-CSF – induced HSPC mobilization [261]. Further, circadian adrenergic signals, through β3-adrenergic receptors located in MSCs, lead to a rapid decrease in CXCL12 release and egress of HSPCs into the bloodstream [262]. Glial fibrillary acidic protein $(GFAP)^+$ non-myelinating Schwann cells, which ensheath peripheral nerves, maintain HSC quiescence through the activation of TGF-β [272].

The nervous system has additionally been demonstrated to critically contribute to hematopoietic regeneration after chemotherapy or irradiation – induced toxicity. Administration of neuroprotective or neuroregenerative drugs protects or rescues the sympathetic nervous system, respectively, and promotes better hematopoietic recovery after stressors of that kind [273,274].

- *Osteoblasts*

Some HSCs locate in the endosteum in contact with osteoblasts [209,210], but the role of osteoblasts as HSC regulators remains controversial. Administration of parathyroid hormone (PTH), regulator of bone turnover, and constitutive activation of PTH and PTH-related protein receptors (PPRs) in osteoblastic cells increases the number of osteoblasts with high levels of the Notch ligand jagged 1 and promotes HSC expansion with evidence of Notch1 activation [209]. Deletion of the key HSC niche factors *Scf* and *Cxcl12* in osteoblasts has no effect on HSC number and function, suggesting that osteoblasts do not directly maintain HSCs, at least not via CXCL12 or SCF [242,243,261,275]. Depletion of osteoblasts leads to loss of B lymphocytes prior to depletion of HSCs [276]. The endosteal niche contains progenitors of the lymphoid lineage [243], though recent data locate common lymphoid progenitors and pro-B cells near MSCs and

sinusoids as well [277]. Osteoblasts are stromal cells with strong myelopoietic supportive capacity, which is mediated by G-CSF release [278-280]. Further studies are needed to dissect the roles of osteoblasts in HSC fate and hematopoiesis.

- *Adipocytes*

In a pioneering study, accelerated engraftment following irradiation was found using pharmacological and genetic approaches of reduced adipogenesis [281]. No efforts were taken to elucidate the interactions involved in these effects and those approaches seem to have major short-comings and affect endothelial cells in the vascular niche [185]. More recently, bone marrow adipocytes together with $LepR^+$ MSCs were demonstrated as the major sources of SCF after irradiation and chemotherapy, essential for hematopoietic recovery. Deletion of *Scf* in adipocytes inhibits hematopoietic regeneration after irradiation or 5-fluorouracil (5-FU) challenge, leading to depletion of HSCs and reduced mouse survival [282]. Although adipocytes are positive modulators of hematopoiesis under challenge, adipocytes accumulate in the bone marrow during aging and obesity and replace the HSC pool and their progeny. Thereby, red marrow, enriched in hematopoietic cells, is progressively replaced by yellow fatty marrow, enriched in adipocytes [185]. Accumulation of adipocytes during aging and obesity negatively regulates HSCs and bone healing [281,283].

1.3.2. Soluble factors of the HSC niche

The communication between the cellular components of the HSC niche and HSCs is mediated through several mechanisms, including a variety of adhesion molecules and soluble factors [12,284,285]. The following section will focus on the main growth factors and cytokines released from the cellular components of the HSC niche that regulate HSC fate and function and sustain hematopoiesis **(Figure 3)**.

- ***Stem cell factor (SCF)***

SCF is essential for HSC maintenance [206,242,286-288]. SCF, also known as KIT ligand (KITL) or steel factor, is mainly expressed by MSCs and endothelial cells [206,242] followed by other cellular components of the HSC niche like adipocytes [282]. SCF exerts its function by binding to c-KIT [289-291], expressed on HSPCs [292]. SCF promotes HSC survival by preventing apoptosis [293] and supports HSC self-renewal [294].

In addition to the soluble form, SCF can be bound to the membrane [290,291]. SCF membrane-bound form is an adhesion molecule expressed by stromal cells that plays an important role in

HSC maintenance. *Sl/Sl*^{d} mutant mice, which express the soluble form of SCF but not the membrane-bound form, show depletion of the HSC pool [286,295]. In chimera mice transplanted with wild type and *Sl/Sl*^{d} stromal cells into the spleen, only wild type stromal cells support normal hematopoiesis in their proximity, indicating that SCF membrane-bound form is essential for adequate HSC support [296].

- ***C-X-C chemokine ligand 12 (CXCL12)***

CXCL12, or stromal-derived factor-1 (SDF-1), is a member of a large family of chemotactic cytokines that is essential in regulation of HSC function. CXCL12 is primarily expressed by MSCs, but it is expressed by endothelial cells as well [241,243,261]. Other HSC niche cells, like osteoblasts, are rare sources of this chemokine [243]. CXCL12 signals through CXCR4, expressed on HSCs [297], and contributes to retain HSCs within the bone marrow and to maintain their quiescence [262,298]. Pioneering experiments deleting *Cxcl12* globally, *in vivo*, resulted in perinatal mortality, and reduced numbers of B cell and myeloid progenitors in bone marrow, indicative for a role of CXCL12 in progenitor survival [299]. Conversely, deletion of *Cxcr4*, using *Mx1-Cre* conditional model, resulted in severe reduction of HSC number in the bone marrow but no impairment of mature progenitors, attributed to an exit from quiescence of the most primitive HSCs [241]. Treatment of human CD34^{+} HSPCs with CXCR4 antibodies prevented their engraftment and reduced their repopulating capacity in immunodeficient mice [300]. Together, these studies demonstrate the important contribution of CXCL12/CXCR4 signaling to maintain the HSC pool and its adequate functioning.

- ***Pro-inflammatory cytokines***

Recent studies suggest that inflammation is a major event that controls HSC fate and function [301-303]. Pro-inflammatory cytokines that are the primary mediators during inflammation such as G-CSF, TNF, interferons (IFNs) and ILs can directly act on HSCs and regulate their fate and function, thereby affecting not only immediate responses of differentiated cells but long-term blood cell biology [304,305].

Pro-inflammatory cytokines modulate HSC self-renewal, proliferation and differentiation. Chronic administration of IL-1β induces HSC differentiation into the myeloid lineage and represses stem cell gene expression programs in a C/EBPα – dependent mechanism [88,99]. IL-1β administration also limits HSC proliferation in a PU.1 – dependent manner [87]. In contrast, IFN-γ induces HSC differentiation into the myeloid lineage but activates long-term HSCs to

proliferate [306,307]. IFN-α stimulates the proliferation of dormant HSCs [308,309]. Deletion of IL-6 impairs HSC self-renewal and proliferation capacity, and mutant embryos with deleted *gp130*, which transduces IL-6 family of cytokines signal, have reduced HSPCs in the liver [310,311]. In contrast, TNF negatively modulates self-renewal. Deletion of TNF receptors *Tnfrsf1*a or *Tnfrsf1b* increases HSC long-term repopulating capacity, whereas short-term treatment with TNF-α suppresses HSC cycling and decreases HSC long-term repopulating activity [312].

Mobilization of HSCs and their progeny is also regulated by pro-inflammatory cytokines. G-CSF promotes granulocyte differentiation and enforces mobilization of granulocyte into the bloodstream [224,313,314]. Importantly, G-CSF also induces HSC mobilization by similar mechanisms that disrupt CXCL12/CXCR4 signaling [222-225]. G-CSF is actually used in the clinic together with plerixafor (AMD3100) for mobilizing HSCs to bloodstream and, this combination, together with chemotherapy is under study for the treatment of relapsed or refractory AML patients (NCT01435343 and NCT00943943) [315,316].

In **conclusion**, the HSC niche is a complex unit composed of a variety of cells that are interconnected, either by cell-to-cell interactions or by soluble factors that act *in situ* or in a paracrine, or sometimes endocrine, fashion. The HSC niche in the bone marrow is the residence of HSCs, and the site of production of all blood and immune cells of our body. Alterations in HSCs and their niche originate and allow progression of hematological malignancies. Therefore, it is important to elucidate all the elements of the HSC niche as well as their roles, which will help complete the characterization of the abnormalities predisposing, causing and/or helping progression of hematological malignancies. This will ultimately allow to conceive new and efficient therapeutic approaches for these diseases.

2. The black hole: acute myeloid leukemia (AML)

AML is a heterogeneous clonal malignancy characterized by abnormal proliferation and differentiation of HSCs and their progenitors [317,318]. During the course of AML, aberrant HSCs and progenitors gain self-renewal and lose their normal ability to differentiate [319]. HSCs initiate an uncontrolled proliferation, with accumulation of immature and poorly differentiated myeloid cells, called blasts, mainly in the bone marrow and blood circulation [317-319]. AML is one of the

most common leukemias, accounting for 32% of all adult leukemia cases, 1.1 % of all new cancer cases and 1.9 % of all cancer deaths [320]. According to the 2016 World Health Organization (WHO) revision, AML is diagnosed when at least 20% blasts are accumulated in the bone marrow or peripheral blood after a histopathology smear, identified by morphology and manual counting [321]. With an average age at diagnosis of 68 years, 5-year relative survival in patients with AML is approximately 29.5% due to the high rate of relapse. Most AML patients die from the disease [320].

Traditionally, AML subtypes were classified according to the type of blasts accumulated and their level of maturity. An example is the French-American-British (FAB) classification of AML, based on clinical, morphological and immunophenotypic criteria **(Table 1),** which is frequently used in clinic. AML frequently originates as result of genetic abnormalities in more than 97% of patients [322-324]. Thus, recently, the WHO established a new classification system for AML, incorporating new genetic approaches. This new classification that considers molecular features is replacing the old FAB system [321].

Table 1: The French-American-British (FAB) classification of AML

FAB subtype	Name
M0	Undifferentiated acute myeloblastic leukemia
M1	Acute myeloblastic leukemia with minimal maturation
M2	Acute myeloblastic leukemia with maturation
M3	Acute promyelocytic leukemia
M4	Acute myelomonocytic leukemia
M4 eos	Acute myelomonocytic leukemia with eosinophilia
M5	Acute monocytic leukemia
M6	Acute erythroid leukemia
M7	Acute megakaryoblastic leukemia

AML patients have one or more clonal somatic abnormalities on mutational profiling, involving TFs, tumor suppressors and epigenetic regulators, and less often, chromosomal abnormalities such as chromosomal translocations [322,324]. The most common mutations observed in AML involve mutations in *FLT3*, *NPM1*, *DNMT3A*, *N/KRAS*, *TET2*, *CEBPA*, *RUNX1*, *IDH1/2* and *TP53* genes [125,322,323,325] **(Figure 4)**. Mutations are grouped in three categories according to their function. Class I mutations are the most common mutations in AML and include genes that confer survival and proliferation advantage to leukemic cells over healthy HSCs activating signal transduction pathways [125,322]. Mutations in *FLT3*, *RAS* or *KIT* genes belong to class I mutations [125,322,325,326]. Class II mutations affect TF genes involved in differentiation, and include mutations in *NMP1*, *CEBPA*, *RUNX1* and *MLL* genes, as well as chromosomal rearrangements, such as *RUNX1-ETO* and *MLL-AF*9 translocations [322,325,326]. Finally, class III mutations affect epigenetic regulators of differentiation and proliferation, such as mutations in *DNMT3A*, *TET2*, *ASXL1*, *IDH1/2* and *EZH2* genes [125,322,325,326].

Due to complexity, severity and acuteness of the final stages of AML, when patients are usually diagnosed, most patients receive a standardized treatment. The standard treatment for AML patients consists of induction chemotherapy followed by consolidation therapy and/or allogeneic HSC transplantation. Induction chemotherapy usually consists of 7 cycles of cytarabine (Ara-C) followed by 3 cycles of an anthracycline such as daunorubicin (also known as daunomycin) or idarubicin. For consolidation therapy, patients usually receive several cycles of high dose of cytarabine (HiDAC). Allogeneic transplantation is the most successful AML therapy recommended for younger patients at high risk of relapse. However, this option holds certain risks, including graft-versus-host disease that can be lethal. AML treatment needs urgent improvement and a variety of new targeted therapies are currently in preclinical and clinical development, including protein kinase inhibitors, antibodies, epigenetic modulators, inhibitors of mitochondrial activity and immunotherapy, among others [327,328].

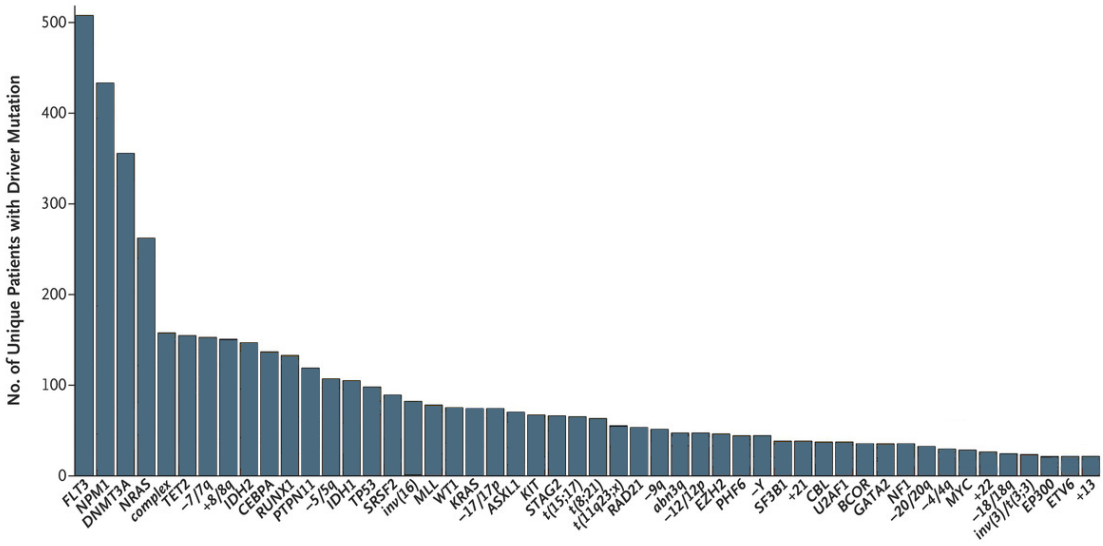


Figure 4: Mutational landscape in acute myeloid leukemia (AML). Mutational events in 1540 patients with AML. Most frequent mutations include *FLT3*, *NPM1*, *DNMT3A* and *NRAS* mutations. Adapted from Papaemmanuil *et al*., 2016. Reproduced with permission from Papaemmanuil *et al*., 2016, Copyright Massachusetts Medical Society.

2.1. The beginning: leukemic stem cells as the cells of origin of malignancy

Cancer stem cells are the origin of several types of human neoplasms, including leukemia [109,319,329-331]. In 1994, Lapidot and colleagues identified a population of human AML cells able to initiate and maintain malignancy in immunocompromised mice. These malignant HSCs were designated as leukemia initiating cells or LSCs [332].

LSCs, like their normal counterparts, characterize by their self-renewal ability, which sustains their survival over time, and by their capacity to engraft in immunodeficient mice [333]. Most AML samples engraft in immunocompromised mice [334-336] and engraftment levels correlate directly with LSC frequency and poor patient outcome [337-339]. LSC self-renewal can be studied in serial transplantations in these mouse models [319].

Like HSCs, LSCs are mainly enriched in the $CD34^+$ $CD38^-$ fraction in samples of leukemia patients [332,340]. In recent years, there has been significant scientific endeavor to identify specific cell surface markers to distinguish LSCs and thereby help their purification and selective targeting. Currently, $CD34^+$ $CD38^-$ $CD123^+$ immunophenotyping is highly standardized to quantify LSCs [341-344]. Additional surface markers such as T-cell immunoglobulin mucin 3 (TIM3), CD96, CD47, CD244, CD33 and C-type lectin-like molecule-1, among others, have

been reported as enriched in LSCs [345-350]. However, despite the number of markers identified to enrich for LSCs, there is no universal combination of surface markers that is not fully expressed in healthy HSCs and/or in the total bulk of leukemic cells [109,329]. In addition, most of these markers have not been studied at relapse, so their stability over time is unknown [329]. Thus, it is necessary to continue searching for new markers that will allow to distinguish and target LSCs.

Knowing the origin of LSCs may be essential for their eradication and for the prevention of transformation. In 2014, Slush and colleagues identified a population of HSCs, progenitors and mature cells from the blood of AML patients that contained recurrent *DNMT3A* mutations at high allele frequency, but without coincident *NPM1* mutations present in AML blasts. Therefore, *DNMT3A* mutations were thought to arise early in the course of AML, leading to clonal expansion of the affected HSCs, identified as pre-leukemic HSCs, from which AML evolved [351]. Since then, the presence of pre-leukemic mutations in HSCs leading to clonal expansion has been demonstrated to precede the development of blood malignancies and point to the origin of LSCs at these pre-leukemic cells that arise from genetic abnormalities in healthy HSCs [351-353]. However, functional AML LSCs have also found within the $CD34^-$ fraction and expressing lineage markers, CD38 or CD45RA [354,355], suggesting that these cells are not restricted to the immature $CD34^+$ $CD38^-$ fraction [109]. Combination of genetic and functional analyses demonstrated that LSCs with immunophenotype different to $CD34^+$ $CD38^-$ arise from more differentiated progenitors that acquired self-renewal capacity, rather than from primitive HSCs that acquired expression of markers of mature cells [335,356-358]. These findings show the complexity of the cellular origin of AML.

Therapy resistance and relapse after remission represent the bottleneck in curing leukemia and heavily rely on the presence of LSCs. Combined genetic and functional analysis of purified subpopulations and xenografts from paired diagnosis/relapse samples showed that relapse in AML may originate from rare LSCs or from larger subclones of committed leukemia cells that retain stemness transcriptional signatures [359]. Further, relapse originates from residual clones present at diagnosis and/or from clones and subclones of founder clones that survived initial therapy [359,360]. The clones can also arise as a consequence of the therapy, in particular chemotherapy [361]. This clonal selection and diversification have been postulated to drive the progression to more aggressive forms of the disease and lead to the fatal outcomes [329].

Both LSCs and HSCs reside in the bone marrow and compete for the same niches [109,362]. LSCs seem to be able to modify the HSC niche to their advantage, in a way that signals from the bone

marrow microenvironment then support their self-renewal and dormancy over those of HSCs. Thereby, dormant LSCs are better protected from apoptotic stimulus, like chemotherapy [109,363-365]. Recent preclinical data show that strategies promoting LSC release from their niche force them into cycle and make them sensitive to chemotherapy. Some of those strategies are being pursued in clinical trials aimed at eliminating LSCs and eradicating leukemia [109,364].

In this scenario, in view of the importance of LSCs in the onset and progression of leukemia, identification and full characterization of these cells in their microenvironment is an active area of research, aimed at developing new and efficient therapeutic approaches based on LSC eradication.

2.2. AML as a result of pre-leukemic mutations or previous myeloid malignancy

Somatic mutations accumulate in cells throughout life and most of them, generally, have no effect on cell fitness. However, mutations in certain genes confer proliferative advantage to a single stem cell and its progeny, and they may cause the expansion of a specific cell lineage over time. This process is known as clonal hematopoiesis (CH) or clonal hematopoiesis of indeterminate potential (CHIP). CHIP is common during aging and it is considered a pre-leukemic state that may evolve into a leukemic process, *de novo* AML, or not [366-370]. CHIP results from the acquisition of somatic mutations in leukemia – associated genes at a variant allele frequency of at least 2%, without evidence of hematological malignancy [368]. These early or pre-leukemic mutations enhance the self-renewal capacity of HSPCs and impair their differentiation [371,372], leading to expansion of the pre-leukemic clone [351,353]. The most frequent early mutations include mutations in *TP53* and epigenetic regulators such as *DNMT3A*, *TET2*, *IDH1/2* and *ASXL1*. These mutations are risk factors for leukemia development [351,353,373-376]. Over time, additional late mutations promote HSPC proliferation and full differentiation blockage leading to leukemogenesis. The most common late mutations occur in *FLT3* and *N/KRAS* genes [125,377] **(Figure 5)**. CHIP may also result from neutral drift, where all stem cells have the same proliferative potential, but some die over time, allowing the remaining better fitted stem cells to proliferate and expand an identical clonal population [378].

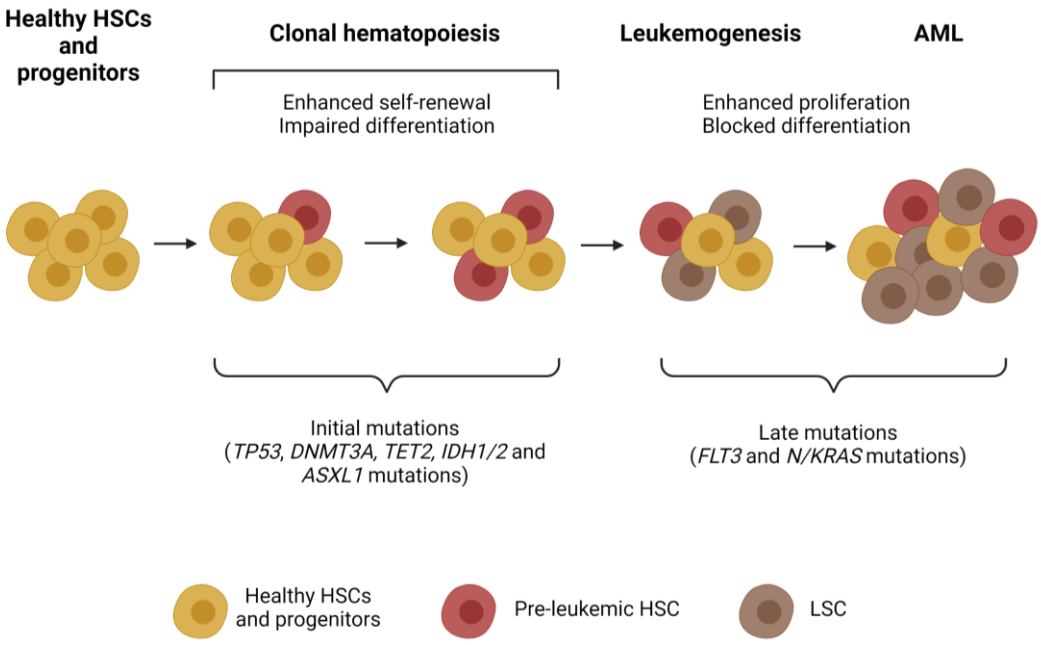


Figure 5: Clonal hematopoiesis and its potential evolution to acute myeloid leukemia (AML). Acquisition of initial mutations in certain genes in hematopoietic stem and progenitor cells (HSPCs) enhances their self-renewal capacity causing their asymptomatic expansion and bias of hematopoiesis towards specific cell lineages, *i.e.* clonal hematopoiesis. Over time, additional mutations in the pre-leukemic HSPCs cause differentiation block and rapid proliferation, thereby evolving into an acute leukemic process, *i.e.* AML.

AML arises as a *de novo* malignancy most frequently, but AML may also develop as a secondary process from other hematological disorders, such as MDS and MPNs, and as consequence of prior therapy. Although the complexity of the clonal evolution at the stem cell level to give rise to sAML is not well understood, sAML has been reported to be different, both morphologically and cytogenetically, from *de novo* AML, and virtually always fatal [379-381].

MDS are heterogeneous HSC clonal disorders characterized by aberrant and ineffective hematopoiesis that usually results in blood dysplasia and cytopenia in one or more cell lineages (commonly erythropenia, leukopenia and/or thrombocytopenia) [321]. In some MDS patients, cytopenia may occur along with an excess of aberrant clonal myeloid progenitors, or blasts. Approximately 20 to 30% MDS patients progress to AML or a disease that resembles AML. Regarding MDS genetic landscape, mutations in genes encoding proteins involved in pre-mRNA splicing (*SF3B1*, *SRSF2* and *U2AF1*) and epigenetic regulation (*DNMT3A*, *TET2*, *ASXL1* and *EZH2*), among others, are often observed [382-387]. Mutations in genes involved in

cellular proliferation (*RAS* and *FLT3*) and in TFs (*RUNX1*, *GATA2* and *CEBPA*) are associated with the progression of MDS to sAML [382,386,388-390].

MPNs are a rare group of HSC clonal disorders characterized by increased proliferation of erythroid, megakaryocytic and/or myeloid lineage, which retain full differentiation. Classical MPNs are negative for Philadelphia (Ph) chromosome and mainly include essential thrombocythemia (ET), polycythemia vera (PV) and primary myelofibrosis (PMF) [321,391,392]. One of the main causes of mortality in patients with MPNs is leukemic transformation [393-395]. Progression to sAML occurs in approximately 10 to 20% of PMF patients in the 10 first years after diagnosis [395], followed by PV patients, with a risk of 2.3% at 10 years [396]. AML progression in ET patients is rare [391]. Typical mutations in classical MPNs occur in *JAK2*, *CARL* and *MPL* genes but, approximately one third of patients with MPNs have additional mutations in epigenetic regulators [397-401]. Mutations in *ASXL1*, *IDH1*, *IDH2*, *EZH2* and *SRSF2*, among others, are associated with a higher risk of leukemic transformation and shorter overall survival in MPN patients [402-407]. High risk of leukemic transformation has also been associated with the so-called triple-negative molecular status (no mutation in *JAK2*, *CALR*, and *MPL*) in PMF patients [394,408].

Ph positive MPNs refer to CML. Ph chromosome is formed after the reciprocal translocation between the long arms of chromosome 9 and chromosome 22 (t(9:22)) resulting in the formation of *BCR–ABL1* fusion oncogene. CML is characterized by an excessive accumulation of apparently normal differentiated myeloid cells in the bone marrow and peripheral blood due to increased proliferation and differentiation of HSCs [409-411]. Most CML patients are diagnosed in an early phase, known as chronic phase (CML-CP), however, additional genetic mutations, mainly in *TP53*, epigenetic regulators and TFs such as *RUNX1*, make the disease progress to a more advanced stage, the blast phase (CML-BP) [412,413]. Blast phase is defined by presence of ≥ 30% blasts in peripheral blood or bone marrow, presence of clusters of blasts in bone marrow or presence of extramedullary hematopoiesis with immature cells [414,415]. During the blast phase, approximately 50% of CML cases transform into a disease that resembles AML [412,416,417].

Chronic myelomonocytic leukemia (CMML) is another myeloid neoplasm with a high risk of leukemic transformation. 30% of CMML cases progress to sAML. CMML is a hematologic malignancy classified as MDS/MPNs and characterized by sustained peripheral blood monocytosis. Gene mutations originating CMML frequently involve epigenetic regulators (*TET2* ~60%), modulators of chromatin (*ASXL1* ~40%), spliceosome components (*SRSF2*

~50%), TFs (*RUNX1* ~15%) and signaling pathways (*RAS* ~30%, *CBL* ~15%) [418]. Recent studies suggest that *Nras* rapidly and efficiently induces biased myelopoiesis and/or CMML – like disease in *in vivo* models, indicating that the *NRAS* oncogene may function as initiating event to induce leukemia [419-422].

Several authors have demonstrated induction of sAML by cytotoxic treatments, irradiation or chemotherapy, aimed at treating other cancers like breast cancer or lymphoma, also known as therapy – related AML (tAML) [361,423,424]. A platinum – based drug signature is present in patients with AML secondary to treatment with platinum – based drugs, with numbers of mutations associated to these drugs of around 1000. Thus, exposure to platinum induces chemotherapy mutations in healthy blood cells and coincides with the clonal expansion, originating the sAML, which also begin after the cytotoxic treatment [361]. The footprint of chemotherapies with different mutagenic mechanisms allows understanding of tAML development and evolution [361]. Other cytotoxic agents associated with the development of tAML include alkylating agents, topoisomerase-II-inhibitors and antimetabolites [423]. This approach also allows to distinguish when pre-existing clones of hematopoietic cells carrying a mutation that is advantageous to the cells in the presence of chemotherapy, are favored due to the selective constraint posed by the drug [361]. In general, tAML patients, like other sAML patients, show lower overall survival and relapse-free survival than patients of *de novo* AML due to increased probability of adverse cytogenetics [425-427].

All the above shows the complex genetic landscapes in the origin of AML, which differs from one patient to another highlighting the need for personalized medicine in this field.

2.3. AML and the HSC niche

Acquisition and/or accumulation of mutations are found in most patients and are thought to be the most frequent route for leukemogenesis, primarily responsible for the onset and development of leukemia. However, extrinsic factors, such as alterations in the HSC niche, influence disease development and progression **(Figure 6)**. In this context, the use of genetically modified mouse models has allowed to determine the important role played by the HSC niche in hematopoietic malignancies, having the ability to not only alter progression but also to potentially originate disease [11,428,429].

2.3.1. Cellular HSC niche components contribute to AML

In MPNs, *JAK2*^{V617F} mutant cells damage sympathetic fibers and Schwann cells through the secretion of IL-1β, which results in apoptosis of nestin^{+} MSCs in the bone marrow. Reduced number of MSCs allows expansion of mutant HSCs and accelerates MPN progression. Administration of β3-adrenergic agonists compensates the sympathetic nervous function on MSCs and restores their number, slowing down the progression of the disease [212]. This study supports the concept that malignant HSCs modify the HSC niche to their advantage. Later, it was also reported that AML development damages the sympathetic nervous fibers around arterioles in the bone marrow of *Mll-AF9* AML mice, and chemical denervation promotes AML expansion. Treatment with agonists of stromal β2 adrenergic receptors reduced disease phenotype but it also had a different cell-autonomous action, enhancing *in vitro* proliferation of *Mll-AF9* cells. In parallel to denervation, AML leads to the expansion of nestin^{+} MSCs, primed towards osteoprogenitors but blocked in their differentiation into fully mature bone – forming osteoblasts, accompanied by reduction in the number of HSC – supporting NG2^{+} periarteriolar niche cells [430] **(Figure 6A)**. Preserved nestin^{+} MSCs in *Mll-AF9* AML contribute to leukemogenesis and chemotherapy resistance by enhancing oxidative phosphorylation, tricarboxylic acid cycle activity, and glutathione – mediated antioxidant defense in AML blasts. Thus, MSCs undergo selective changes in presence of MPN or AML cells, which contribute to disease in either case. Glutathione depletion (using buthionine sulfoximine) in addition to chemotherapy improves mouse survival compared to chemotherapy alone [363] **(Figure 6B)**. Taken together, these results suggest that AML LSCs modify the HSC niche to their own advantage and that MSCs, which support function and maintenance of healthy HSCs under physiological conditions, can be exploited by malignant LSCs to support mutant cells and contribute to disease.

The remodeling of vascular structures allows the growth and dissemination of cancer cells, providing routes for metastasis. Enhanced vascular permeability and hypoxia are observed in AML, which contributes to malignant cell egress into blood circulation and disease progression in AML patient – derived xenografts [431]. According to transcriptomic analyses in endothelial cells, this is mediated by the high levels of nitric oxide (NO) both in patient – derived xenografts and biopsies. Conversely, inhibition of NO production reduces vascular permeability and improves response to chemotherapy [431]. Using a *Mll-AF9* – driven experimental AML model, endosteal AML cells were found to remodel the vasculature of the bone marrow, such that, while the vessels in the endosteum are reduced, the central area of the bone marrow remains

vascularized [432]. Degradation of the endosteal vasculature is mediated by pro-inflammatory and anti-angiogenetic cytokines, TNF and CXCL2 respectively, and correlates with the loss of healthy HSCs, stromal cells and osteoblasts. Preservation of endosteal endothelium, using small molecules (deferoxamine) or genetic approaches (*Fbxw7*$^{i\Delta EC}$ mutant mice, which after tamoxifen administration show increased activation of Notch signaling in endothelial cells, thereby increasing the number of endosteal vessels and arterioles), recues the number of healthy HSCs and increases the response to chemotherapy [432] **(Figure 6C)**.

Selective activation of PTH receptors in osteoblastic cells attenuates *BCR-ABL1* CML but enhances *Mll-AF9* AML in transplanted models potentially through opposing effects of increased TGF-β1 on CML or AML LSCs. PTH treatment also reduces leukemia burden and LSC numbers in CML mice and reduces engraftment in xenografts of CML patient samples [433]. This study suggests a specific contribution of osteoblasts in the leukemic process that may be different for CML and AML, and could be used as a therapeutic strategy to reduce LSCs in CML. Strikingly, activating mutation of β-catenin in osteoblasts changes the differentiation potential of myeloid and lymphoid cells and leads to AML development with common chromosomal aberrations and cell autonomous progression [434]. This was a pioneering study proving the concept that a lesion in the HSC niche has the potential to originate AML in *in vivo* models. β-catenin promotes expression of Notch ligand jagged 1 in osteoblasts, and the activation of Notch signaling in HSPCs induces the transformation. Genetic and pharmacological inhibition of the Notch pathway attenuates AML in mice, suggesting therapeutic value of these events. Proving the relevance for human disease, β-catenin signaling is increased in osteoblastic cells in 38% of patients with MDS and AML, which is accompanied by increased Notch signaling in hematopoietic cells [434] **(Figure 6D)**.

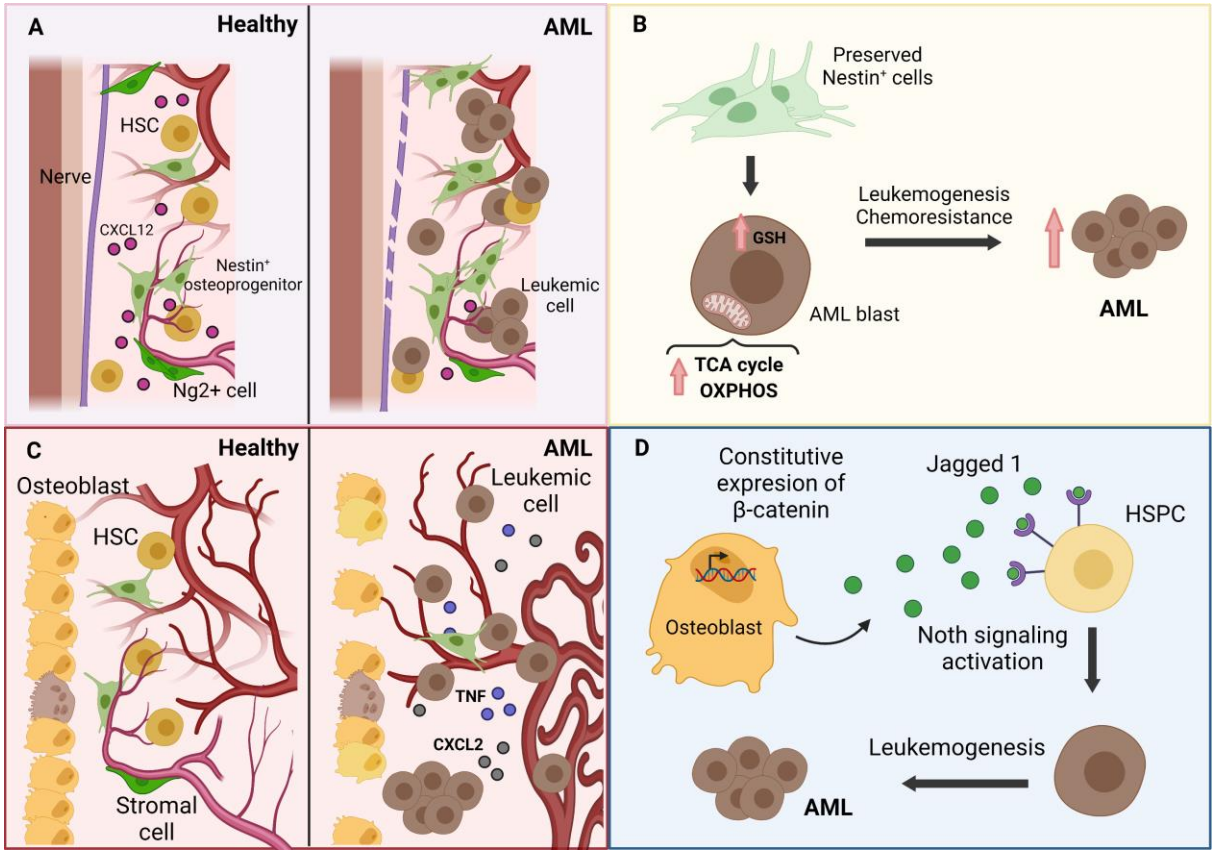


Figure 6: The hematopoietic stem cell (HSC) niche in mouse models of acute myeloid leukemia (AML). Simplified illustrations depicting different scenarios on the influence of the HSC niche in AML progression and origin. (A) AML promotes sympathetic nervous fiber damage along with expansion of nestin^{+} osteoprogenitors and reduction in NG2^{+} periarteriolar cells, which contribute to AML progression in a positive feedback loop [430]. Adapted from Hanoun *et al*., 2014. (B) Preserved nestin^{+} cells in AML enhance oxidative phosphorylation (OXPHOS), tricarboxylic acid cycle (TCA) activity, and glutathione – mediated antioxidant defense (GSH: reduced glutathione) in AML blasts that contribute to disease progression and chemotherapy resistance [363]. Adapted from Forte *et al*., 2020. (C) Vascular remodeling in AML involves reduction of the vasculature of the endosteum mediated partially by TNF and CXCL2 produced by AML cells. This process correlates with the loss of healthy HSCs, stromal cells and osteoblasts in this area [432]. Adapted from Duarte *et al*., 2018. (D) Constitutive expression of β-catenin promotes expression of Notch ligand jagged 1 in osteoblasts, which activates Notch signaling in hematopoietic stem and progenitor cells (HSPCs) and induces leukemic transformation [434]. Adapted from Kode *et al*., 2014.

About 50-70% of the bone marrow in human adults is made up of adipocytes, and AML patients have increased number of small adipocytes whose volume and number correlate with poor prognosis [185,435]. LSCs and/or blasts in AML could transform the BM adipocytic niche to fuel them with fatty acids to meet the high energy demands required to sustain their high proliferation rate [185,436]. Shafat and colleagues demonstrated that the interplay between bone marrow adipocytes and AML blasts contributes to leukemia progression. *In vitro*, AML blasts

alter adipocyte metabolism by inducing phosphorylation of hormone-sensitive lipase and consequently activating lipolysis, which then enables the transfer of fatty acids from adipocytes to AML blasts. The transfer is mediated by fatty acid binding protein 4 (FABP4). *FABP4* expression was upregulated in adipocytes and AML blasts in coculture, as well as in AML cells from bone marrow, and FABP4 in both compartments is essential to transport adipocyte – derived fatty acids to AML cells. The exogenous fatty acids are then used as a substrate for β-oxidation, generating the energy required for leukemic growth and proliferation. *In vivo*, NSG mice have increased survival when engrafted with AML blasts knockdown for carnitine palmitoyltransferase 1A (*CPT1A*), which transports fatty acyl chains from cytosol to mitochondria, a process essential for fatty acid oxidation [436]. Further, *in vivo* knockdown of *FABP4* in Hoxa9/Meis1 AML blasts reduces fatty acid uptake by these cells and improves survival of Hoxa9/Meis1 AML mouse model [436]. FABP4 has been linked to aggressive AML in obesity through enhanced DNMT1 – dependent DNA methylation, which can be targeted therapeutically [437,438]. Further research is required to determine the mechanisms of transformation of the adipocytic niche by AML cells.

2.3.2. Soluble factors derived from the HSC niche contribute to AML

Mutant HSCs, blasts and the altered niche deregulate soluble factor secretion to favor disease progression [11,109,212,213,430]. In AML patients, while reduced levels of CXCL12 were shown in the bone marrow extracellular fluid and supernatant of cultured AML MSCs isolated by plastic adherence, increased endogenous expression and intracellular protein levels of CXCL12 were observed in these cells, suggesting a defect in secretion rather than expression [439]. However, in mice, PDGFRα$^+$ CD51$^+$ MSCs from *Mll-AF9* AML mice express the HSC supportive genes *Cxcl12* and *Scf*, as well as *Vcam1* and *Angpt1* (angiopoietin 1, an agonist of the *Tie2* receptor, expressed on bone marrow endothelial cells and HSCs [211]), at lower levels than healthy MSCs [430]. Reduced levels of these niche factors, especially CXCL12 and SCF, have been hypothesized to contribute to the impaired maintenance of healthy HSCs, in favor of AML [109]. In fact, AML blasts secrete SCF that sequester transplanted healthy human CD34$^+$ cells in malignant niches, in xenografts, causing decline in their numbers and function [440].

Another recent study using single-cell RNA sequencing (RNA-seq) to define a cellular taxonomy of the mouse bone marrow stroma in health and disease found a cell type-specific deregulation of HSC niche factors in AML mice, especially *Cxcl12* and *Scf*. *Cxcl12* and *Scf* were downregulated in AML LepR$^+$ MSCs, subsets of bone marrow endothelial cells and early

osteolineage cells. LepR$^+$ MSCs in leukemia further downregulated expression of *Angpt1*, *Il7* (involved in lymphoid differentiation) [441,442], *Csf1* (involved in myeloid differentiation) [443], and *Vcam1* (regulator of HSC homing and retention in the bone) [444]; and upregulated *Angpt2* (antagonist of *Angpt1*) [445]. Conversely, *Cxcl12*, *Scf*, and *Angpt1* were upregulated in a subset of fibroblasts [248].

As mentioned above, AML LSCs are retained in the HSC niche through cell-to-cell contacts and soluble factors, and this is a way hypothesized to help them stay protected from toxic stimuli [109]. This is mediated, in part, by CXCL12 signaling through CXCR4 [221] as disruption of the axis mobilizes LSCs from their niche into the bloodstream and forces them into cycle, sensitizing them to chemotherapy [109,223,241,446,447].

Myeloid neoplasms, including AML, are associated with inflammatory events and overproduction of pro-inflammatory cytokines, notably IL-1β [448,449], IL-6 [450] and TNF-α [451]. In a recent study, Carey and colleagues showed that various cytokines like GM-CSF, IL-3, M-CSF, G-CSF and TNF-α increased AML cell growth in culture, however, the most robust effects were obtained when AML cells were cultured in the presence of IL-1α or IL-1β. IL-1 promoted the growth and survival of leukemic blasts, leading to aberrant dose-dependent cell expansion in 67% of human AML samples while suppressing the growth of normal progenitors [448]. High expression of both *IL1B* and *CXCL2* predicts poor prognosis in cytogenetically normal AML, but no differences were found in the prognostic characteristics of IL-1β-sensitive and -nonsensitive AML patients [110,448]. Regarding the latter, the authors concluded that most AML patient samples show cellular expansion in the presence of IL-1 irrespective of mutational status and other features, suggesting that targeting this shared mechanism may be applicable across heterogeneous AML subtypes [448].

As discussed in the previous section, IL-1β produced by mutant cells and HSCs in a model of MPN expressing the human *JAK2*^{V617F} mutation, induces neuroglial damage in the bone marrow [212]. IL-1β is a pathogenic event that together with the neuroglial damage leads to MSC apoptosis and, as consequence, a reduction in CXCL12 which then allows the expansion of the mutant cells [212]. Both treatment with IL-1 receptor antagonist (IL-1RN) and β_3-adrenergic agonists showed therapeutic value *in vivo* [212]. Later, a tyrosine-protein phosphatase non-receptor type 11 (Ptpn11)-activating mutation in MSCs and progenitors, as well as in osteoprogenitors, was demonstrated to originate juvenile myelomonocytic leukemia (JMML). *Ptpn11*-activating mutation in the bone marrow stroma results in increased secretion of the chemokine ligand 3 (CCL3) that recruits monocytes to the bone marrow. These monocytes produce IL-1β that in

turn hyperactivates HSCs leading to JMML. Treatment with CLL3 receptor antagonists reverses JMML originated by *Ptpn11* mutation in the bone marrow microenvironment [213].

Some preliminary reports indicate a potential participation of the bone marrow stromal compartment in inflammation during leukemia in patients. sAML MSCs with MDS – related changes, isolated by adhesion to plastic, showed significant increase in *IL6* expression, whereas *de novo* AML MSCs showed stimulation of the expression of a variety of factors *VEGFA*, *CXCL12*, *RPGE2*, *IDO*, *IL1B*, *IL6* and *IL32* followed by a decrease in *IL10* expression in culture [452]. Increased levels of pro-inflammatory cytokines, such as IL-1β, IL-6, and IL-32, together with reduced levels of anti-inflammatory cytokines, such as IL-10, produced by AML MSCs may indeed contribute to the inflammatory events in AML. While normal *IL6* expression was observed in MDS MSCs, high expression was seen in sAML MSCs [452]. Of note, high IL-6 expression predicts poor survival in MDS, but it is also predictive of inferior leukemia-free survival [453]. The role of MSCs and their secreted factors in hematological malignancies is poorly understood and further studies are needed.

Together, these studies highlight the important role played by the deregulation of the HSC niche, including cellular components and soluble factors, in the development and progression of myeloid neoplasms and particularly AML, where our knowledge is still at its infancy. Further studies are needed for a complete understanding of the processes that lead to leukemic transformation, either *de novo* or from chronic phases to AML, and elucidation of the orchestrated roles of the HSC niche components that allow and support this transformation. Better understanding of these pathways stands out as a promising approach to tackle leukemia and other hematological malignancies.

Aims of the study

Adapted from *Street in Røros in winter*. Harald Sohlberg. 1903. Photo: Jacques Lathion / Nasjonalmuseet.

Chronic administration of IL-1β drives HSC differentiation into the myeloid lineage, and enhanced IL-1β and a range of components of the signaling pathway are involved in the development and progression of hematological malignancies.

The general aim of the present work was **to determine the role of IL-1β endogenous repressor cytokine IL-1RN in both healthy and malignant hematopoiesis and explore its value in the treatment of AML**.

The general aim was divided into the following specific aims to provide a complete picture of the biology of IL-1RN in hematopoiesis:

I. **Compile relevant scientific contributions on the role of IL-1β in hematological malignancies, the FDA – approved therapeutic strategies for IL-1 blockade and the therapeutic potential of IL-1 targeting.** (Paper I).

II. **Explore the status of IL-1RN in primary samples from AML patients and its value to predict disease progression in publicly available bigger data sets.** (Paper II).

III. **Test a novel therapeutic intervention against AML by *in vivo* treatment of AML xenografts with anakinra** (recombinant form of the human IL-1RN), and compare this treatment to IL-1β blockade with the human monoclonal antibody canakinumab that is being tested in a Phase II clinical trial for the treatment of low or intermediate risk MDS and CML (NTC04239157). (Paper II).

IV. **Investigate the role of IL-1RN in steady-state hematopoiesis by the characterization of the bone marrow hematopoietic and stromal compartments in IL-1rn knockout strain**. (Paper II).

V. **Study the status and functional role of IL-1rn *in vivo* in a mouse model of pre-leukemic myelopoiesis driven by the oncogene *Nras*^{G12D}.** For this purpose, a conditional and inducible *in vivo* model was used, *i.e. Mx1-Cre Nras*^{G12D}. To study the functional role of IL-1rn in pre-leukemic myelopoiesis, we studied the disease development under deletion of IL-1rn from the hematopoietic or stromal compartments. Conversely, pre-leukemic mice were treated with anakinra. (Paper II).

Methodological considerations

Adapted from *Far, far away Soria Moria Palace shimmered like gold*. Theodor Kittelsen. 1900. Photo: Børre Høstland / Nasjonalmuseet.

This chapter provides an overview and discussion of the main methods used in the papers of this thesis. A more detailed description of all methods and reagents can be found in our research article.

PAPER I – Review

Interleukin-1β as emerging therapeutic target in hematological malignancies and potentially in their complications. Blood Reviews 2017; 31: 306-317.

Systematic literature review

The systematic search of literature was conducted to compile all existing relevant knowledge on the role of IL-1β in normal and malignant hematopoiesis, as well as the therapeutic potential of IL-1β targeting. Several databases were used for literature search, including PubMed, Embase and ScienceDirect. We considered publications with both positive and negative results. After identification of all existing literature on the topic, each primary study was critically evaluated to prevent potential bias arising from them.

PAPER II – Research Article

Endogenous IL-1 receptor antagonist represses healthy and malignant myeloproliferation. Nature Communications. Under Review.

Mouse models

IL-1rn$^{-/-}$ mice (B6.129S-*Il1rn*^{tm1Dih}; IL-1rn-KO mice) have an insertion of a neomycin resistance cassette into exon III of the *Il1rn* gene that disrupts the sequence encoding IL-1rn protein. After lipopolysaccharide challenge, no *Il1rn* mRNA is detected by Northern blot analysis of lung tissue in homozygous IL-1rn-KO mice, as opposed to its increased levels in heterozygous and wild-type mice for the *Il1rn* gene [454]. IL-1rn-KO mice have decreased body mass, produce small litters, and are more susceptible to develop lethal endotoxemia [454], spinal abnormalities [455], autoimmune arthritis [456], psoriasis – like cutaneous diseases [457] and arterial inflammation [458]. Granulocytes derived from the bone marrow of IL-1rn-KO mice enhance Pten-loss – induced cellular senescence, when they are adoptively transferred to *PTEN*$^{-/-}$ mice [459]. We used these

mice to determine the functional role of IL-1rn in normal and pre-leukemic hematopoiesis. *IL-1rn*$^{-/-}$ mice are currently maintained in a pure C57BL/6J genetic background.

C57BL/6J mice are the most common inbred mouse strain used in research, being the most common background for many genetically modified mouse models, including *IL-1rn*$^{-/-}$ and *Mx1-Cre Nras*^{G12D} mice. This strain is long-lived and not very susceptible to generate tumors over time. However, it is prone to develop diet-induced obesity, type 2 diabetes and atherosclerosis [460-463]. We used these mice as wild-type controls for IL-1rn-KO mice and for transplantation.

Mx1-Cre Nras^{G12D} mice have an insertion of a Cre – dependent conditional allele *Nras*^{G12D} (*Lox-STOP-Lox – Nras*^{G12D}) and of a *Mx1-Cre* transgene. Both are used in heterozygosis to model human disease. *Nras*^{G12D} mutation is expressed after the cleavage of the inhibitory *Lox-STOP-Lox* cassette by Cre enzyme. *Mx1-Cre* efficiently cleavages *Lox-STOP-Lox* cassette selectively in hematopoietic cells, but also in additional cells that activate the *Mx1* promoter including those of the osteoblastic lineage in the bone marrow [251]. Thus, *Nras*^{G12D} mutation will also be expressed by these stromal cells. *Mx1-Cre* system is inducible and its transcriptional activation requires administration of IFN-α, IFN-β, or synthetic double-stranded RNA like poly-inosinic:poly-cytidylic acid (polyI:polyC), which activates Toll-like receptors and IFN signaling. This, however, can affect HSCs and mouse phenotype, particularly shortly after induction [308,464-466]. To prevent potential confounding results derived from IFN signaling, littermates containing the *Lox-STOP-Lox – Nras*^{G12D} allele but not the *Mx1-Cre* transgene were injected with polyI:polyC and used as controls. Single allelic endogenous expression of *Nras*^{G12D} mutation in mice results in a myeloproliferative disorder characterized by elevated white blood cell counts, splenomegaly, and myeloid infiltration of bone marrow and spleen [420], defined as MPN, CMML [467] or JMML [468]. We used these mice to study the functional role of IL-1rn in pre-leukemic myelopoiesis.

NSG-SGM3 mice (NSGS mice) are NSG mice, deficient in mature lymphocytes and natural killer cells [469], which additionally express supraphysiological human IL-3, GM-CSF and SCF. NSG-SGM3 mice allow superior engraftment from normal CD34^{+} progenitors and primary AML samples than NSG mice [64,68,69,470]. However, the transgenic overexpression of human cytokines has also been reported to cause HSC exhaustion in these mice, with expansion of myelopoiesis and suppression of other lineages like erythropoiesis and B-lymphopoiesis [64,66]. This model was used for AML cell transplantation and was suitable to study the effect on human myelopoiesis of IL-1RN and IL-1β monoclonal antibody treatments *in vivo*.

Nes-gfp mice have the *gfp* transgene inserted between the promoter and the intron sequence of the *Nes* (nestin) gene. The *Nes* gene encodes an intermediate filament protein expressed in neural stem cells and progenitors, but also cells with progenitor and/or regulatory functions in additional tissues like bone marrow [471]. This mouse model confirms *Nes* expression indirectly through the visualization of the GFP reporter protein [472]. We used this strain to study a subset of bone marrow MSCs, identified as stromal Nes-GFP$^+$ cells [236].

Transplantation assays

Bone marrow transplantation is broadly used in research, especially in hematology, to study HSC fate and function and the HSC niche, model hematological diseases and do research on improvement of transplantation settings [78,473,474]. In this procedure, recipients are preconditioned (myeloablated) to enable HSC engraftment, and then transplanted usually by intravenous injection of isolated cells [475]. For myeloablation, whole body irradiation was performed with 2 doses of 4.5 gray (Gy) separated by 3 hours to reduce radiation sickness [475,476]. Lethal whole body irradiation kills proliferating cells without significantly damaging other tissues, but certain degree of damage has been reported to the bone marrow microenvironment [477]. Cell depletion and released factors from the HSC niche after irradiation guide transplanted cells to the bone marrow, and help their engraftment and proliferation [475,478]. NSG-SGM3 mice were whole body irradiated with a sublethal irradiation dose of 2 Gy, given that higher doses of radiation can be lethal for immunosuppressed mice. This low level of irradiation does not induce bone marrow depletion but increases the release of factors from the HSC niche and prevents innate immune rejection, allowing the engraftment of transplanted cells and their proliferation [470,479]. Older mice are more likely to develop radiation sickness and graft-versus-host disease than younger mice [475,480], therefore irradiation was performed in adult animals aged 7 to 12 weeks. We used this technique to study the role of IL-1rn in pre-leukemic myelopoiesis as well as the potential of its boosting and IL-1β blockade as therapy against pre-leukemic myelopoiesis and AML.

Flow cytometry

Flow cytometry is a powerful technique that allows single cell analysis, providing qualitative and quantitative information of multiple parameters. Flow cytometers use lasers as light sources to generate scattered and fluorescent light signals, which are then read by detectors. This technique is used from clinical testing to basic research to measure cell size, cytoplasmic

complexity, DNA or RNA content, and a broad range of extracellular and intracellular proteins [481,482]. Flow cytometry is widely used in hematology, mainly for immunophenotyping of whole blood, bone marrow and other hematopoietic tissues [483-485]. Using flow cytometry, we characterized the hematopoietic and stromal compartments of the mouse bone marrow. Analysis of leukocytes in peripheral blood using flow cytometry allowed to monitor disease progression in mice that underwent treatment and/or transplantation. Apoptosis, proliferation and quantification of cells expressing membrane IL-1R1 and intracellular phosphorylated NF-κB were also assessed using flow cytometry. Laser voltage and manual compensation settings were optimized using unstained and single staining samples as well as compensation beads to ensure accurate measurements of fluorochromes. Staining with IgG2b isotype was performed to determine unspecific antibody binding as opposed to specific phospho-NF-κB p65 antibody binding. Flow cytometry misses the information of local interactions *in situ*. Unlike other techniques, such as immunofluorescence, flow cytometry provides quantitative data and allows accurate characterization of small subsets of cells including subsets of HSPCs, which is why we opted for this technique for most analyses.

Assessment of gene expression – RNA isolation and qRT-PCR

Real time quantitative reverse transcription polymerase chain reaction (qRT-PCR) is a robust technique used for the assessment of gene expression. We measured the expression of genes involved in IL-1β signaling, MSC and HSC regulation, myeloid differentiation, and myeloid neoplasms, in hematopoietic and/or stromal cells, using qRT-PCR. Considering the limited number of cells, we performed mRNA isolation using magnetic beads (Dynabeads mRNA DIRECT kit), which allows to isolate mRNA from few cells (less than 5 000 cells) obtaining pure, intact and full-length gene transcripts [486]. Then, we reverse transcribed total mRNA into cDNA which was used as template for PCR, using SyBR green dye. For amplification of our target genes, specific and sensitive primers were designed, which were tested before use. These primers were designed with a GC content between 40 and 60% to promote stability but prevent primer-dimer formation. The melting temperature of the primers was kept between 65°C and 75°C, and similar in the primer pair *i.e.* 5°C of each other maximum. In general, the primers were designed to amplify short cDNA fragments containing around 100bp and to bind regions contained in two different exons with one intron in between to prevent unspecific amplification of potential traces of genomic DNA in the original RNA sample. Primer specificity was analyzed using melting curve analysis. The expression level of each gene was calculated by

interpolation from a standard curve. Endogenous housekeeping control genes were used to normalize all sample values. Final expression levels of human genes were expressed relative to IL1RN expression.

Cytokine analysis

Assessment of cytokines was performed using multiplex immunoassay. Multiplex immunoassay is based in Luminex technology and uses a magnetic bead and fluorescence – based detection system able to identify a large variety of target proteins in small sample volumes with high sensitivity [487]. Multiplex immunoassay is more sensitive than enzyme-linked immunosorbent assay (ELISA) and allows the simultaneous detection of multiple targets in a single reaction [488]. Due to the limited amounts of cells and/or sample volume, and considering the advantages of the multiplex immunoassay, we chose this technique in our study. Given the lack of mouse IL-1rn antibodies in the multiplex immunoassay system, mouse IL-1rn was quantified using a different bead – based fluorescence immunoassay (Mouse IL-1rn AimPlex - Biosciences), followed by flow cytometry. Cytokines were measured in bone marrow extracellular fluid from mice and in human circulating $CD34^+$ progenitors.

RNA-sequencing (RNA-seq)

RNA-seq is a powerful technique to characterize the total cellular content of RNAs, and it is useful to detect alternative spliced transcripts, post-transcriptional modifications, mutations, gene fusions and gene expression. RNA-seq is sensitive and accurate for measuring high-throughput gene expression and has eliminated many challenges of microarrays, such as cross-hybridization artifacts and poor quantification of low and high expressed genes [489-491]. For RNA-seq, total RNA from small numbers of sorted bone marrow hematopoietic and stromal cells was isolated using Arcturus Picopure RNA isolation kit. This RNA isolation kit allows to isolate high quality total RNA from fewer than ten cells, even from a single cell [492]. RNA was amplified, fragmented and libraries prepared before RNA-seq was performed using Illumina platform, which provides information of sequences of up to about two hundred nucleotides with low sequencing error rate and high coverage to detect transcripts expressed at low levels [491].

Summary of the results

Adapted from *September*. Erik Werenskiold. 1883. Photo: Børre Høstland / Nasjonalmuseet.

PAPER I – Review

Interleukin-1β as emerging therapeutic target in hematological malignancies and potentially in their complications. Blood Reviews 2017; 31: 306-317.

IL-1β is a pleiotropic inflammatory cytokine that exerts multiple roles in both physiological and pathological conditions. It is produced by a variety of cell subsets, and drives a wide range of inflammatory responses in numerous target cells. Enhanced IL-1β signaling drives HSC differentiation into the myeloid lineage and is actively involved in the development and progression of hematological diseases. Preclinical animal models together with clinical data have contributed to decipher some of the cellular and molecular mechanisms of IL-1β – induced pathogenesis and the value of their therapeutic targeting in several types of hematological malignancies, including MPNs, CML, JMML, AML and lymphoid malignancies. Here, we also suggest a potential contribution of IL-1β to complications of these malignancies, including bone morbidity, pain and autoimmune diseases, which requires further investigation. Hence, drugs that target IL-1 may be helpful to improve outcome or reduce morbidity in patients. Currently, these drugs include IL-1RN, soluble receptors, antibodies, and IL-1 traps among others. Some of these agents are FDA – approved, and used safely and efficiently in the clinic as therapy against autoimmune diseases like rheumatoid arthritis. Anakinra is the recombinant form of the human IL-1RN, which reduces IL-1α and IL-1β activity through competitive binding to IL-1R1 but has a short half-life of hours. Canakinumab is a human monoclonal antibody against IL-1β specifically, but with a half-life of weeks it does not allow to quickly combat and control infectious processes where inflammation is essential. Further studies and drug development will allow to determine the potential of IL-1 and IL-1β targeting as therapy in hematological malignancies and their related complications.

PAPER II – Research Article

Endogenous IL-1 receptor antagonist represses healthy and malignant myeloproliferation. Nature Communications. Under Review.

In view of the roles of enhanced IL-1β signaling in healthy hematopoiesis and leukemia, we investigated the role of its natural repressor cytokine IL-1RN. We found that low IL-1RN is a prognostic marker in AML patients, associated with poor survival. Low IL-1RN is frequent in AML patients, particularly in those within lower maturation/differentiation profiles as defined by the FAB classification of AML (M0-M3). Boost of IL-1RN using anakinra reduced the numbers of leukemic cells and $CD34^+$ progenitors in xenografts from AML patients, indicative of the therapeutic potential of IL-1RN. IL-1β inhibition with the monoclonal antibody canakinumab further confirmed the therapeutic potential of IL-1β blockade against AML in xenografts. *In vivo* genetic deletion of IL-1rn induced HSC differentiation into the myeloid lineage and hampered B cell development via transcriptional activation of IL-1β-induced myeloid differentiation pathways dependent on NF-κB activation. These data support that HSC differentiation is controlled by balanced IL-1β/IL-1rn levels under steady-state and IL-1rn represses myeloproliferation under healthy conditions. We further found low IL-1rn in an experimental model of pre-leukemic myelopoiesis driven by the oncogene $Nras^{G12D}$ in a conditional and inducible fashion. Loss of IL-1β repression through IL-1rn genetic deletion promoted myeloproliferation, with participation of both bone marrow hematopoietic and stromal compartments. Conversely, treatment with anakinra protected against $Nras^{G12D}$ – driven pre-leukemic myelopoiesis and improved disease progression. Our data suggest that deregulation of the anti-inflammatory cytokine IL-1RN leads to loss of repression of IL-1β and may underlie pre-leukemic lesion and AML progression. We further provide a new rationale for IL-1β blockade therapeutic potential in AML and a new means through administration of exogenous IL-1RN anakinra.

Discussion

Adapted from *The scream*. Edvard Munch. 1893. Photo: Børre Høstland / Nasjonalmuseet.

PAPER I - Review

Interleukin-1β as emerging therapeutic target in hematological malignancies and potentially in their complications. Blood Reviews 2017; 31: 306-317.

This chapter focusses on the discussion of the most relevant novel findings on the role of IL-1β in healthy hematopoiesis and hematological malignancies, and the therapeutic potential of its targeting as a cure for AML, published after 2017.

1. IL-1β role in healthy hematopoiesis

The study of HSC function in the context of inflammation has been the focus on remarkable scientific endeavor during the past recent years. New animal models and sophisticated techniques have helped elucidate the important role of inflammation in HSC self-renewal, survival, proliferation and differentiation [87,88,226,301-303,305,308,312]. Chronic IL-1β administration causes rapid HSC differentiation bias into myeloid cells and repression of stem cell gene signatures [88,99]. Conversely, Cebpa-KO HSPCs are resistant to the myeloid differentiation induction of chronic IL-1β, and competitively expand [99]. The effects of IL-1 are concomitant to PU.1-dependent repression of protein synthesis and cell cycle in LT-HSCs, and IL-1 exposure triggers expansion, aberrant protein synthesis and cell cycle activity in PU.1-deficient HSCs [87]. Thus, both C/EBPα and PU.1 seem to be key TFs that mediate IL-1β signaling and biological effects on HSCs.

IL-1β – induced HSC activation and myeloid differentiation is mediated by IL-1R1 given that *Il1r1*$^{-/-}$ mice do not respond to IL-1β treatment [88]. However, *in vivo* deletion of *Il1r1* does not affect blood production or HSPC function [88,493,494]. Similarly, deletion of IL1 receptor accessory protein (IL-1RAP), required for IL-1β signaling through IL-1R1, has no effect on hematopoiesis *in vivo* [495]. In turn, administration of mouse recombinant IL-1RN, which binds to IL-1R1 and blocks IL-1 signaling, inhibits hematopoiesis in pathogen-free wild type mice [496]. These data are seemly contradictory and require further investigation. As of today though, it is widely accepted that IL-1β has no role under steady-state hematopoiesis and IL-1β – induced myeloid differentiation occurs only under challenge such as infection [497].

Whereas myeloid recovery is significantly delayed in 5-FU – injected *Il1r1*$^{-/-}$ mice, long-term treatment with IL-1 leads to almost complete exhaustion of HSCs after transplantation,

suggesting that adequate IL-1 cytokine levels are required for correct HSC function and responses under challenge [88]. Conversely, pretreatment with mouse recombinant Il-1rn protects bone marrow hematopoietic cells from 5-FU-induced cytotoxicity and reduces mouse lethality [496], further suggesting that adequate balanced levels of IL-1 to IL-1rn are required for correct HSC function under challenge.

In conclusion, IL-1β heavily influences HSC fate and function, but its physiological role and regulation in steady-state hematopoiesis requires further research.

2. IL-1β role in malignant hematopoiesis

Hematological neoplasms are associated with inflammatory events and excess of a variety of pro-inflammatory cytokines, like IL-1β [448-451,498,499]. Several studies demonstrate the important role of IL-1β in myeloid malignancies, specifically in AML [110,448,449]. AML is related to inflammation and overproduction of pro-inflammatory cytokines, notably IL-1β [448,449], IL-6 [450] and TNF-α [451]. Carey and colleagues conducted an *ex vivo* experiment where they cultured primary samples from 69 AML patients in presence of different cytokines [448]. Several pro-inflammatory cytokines increased AML cell growth, including G-CSF, M-CSF, GM-CSF, IL-3 and TNF-α. The most robust effect was obtained when the cells were cultured with IL-1α and IL-1β. IL-1 promoted remarkable expansion of leukemic blasts in 67% of AML samples, and suppressed the growth of normal progenitors. These effects were mediated, at least in part, by enhanced p38MAPK phosphorylation. IL-1 – sensitive AML samples showed upregulation of *IL1B*, *IL1R1* and *IL1RAP*, although AML $CD34^+$ progenitors displayed high expression of *IL1R1* only. Knockdown of *IL1R1* attenuates cell growth *ex vivo* using shRNA in primary AML cells, and deletion of *Il1r1 in vivo* slows down disease progression using a mouse model of AML after transplantation of *Aml1-Eto9a/Nras*^{G12D} – transduced bone marrow cells from wild-type and *Il1r1*$^{-/-}$ mice into wild-type recipients [448]. These data reveal the key role of IL-1β/IL-1R1 signaling in AML progression. Katsumura and colleagues observed upregulation of *IL1B* gene expression in AML cells as well [110]. These authors described a regulatory circuit triggered by RAS through p38 and ERK signaling that promotes phosphorylation and activation of the HSC regulator GATA-2 in AML. The end result is the transcription of different chemokines and cytokines that are GATA-2 targets, including *IL1B* and *CXCL2*. In turn, IL-1β and CXCL2 activate the axis p38/ERK and GATA-2 – mediated transcription in a positive feedback loop. High expression of *GATA2* correlates with increased expression of *IL1B* and *CXCL2* in AML

M5, and high expression of these genes predicts poor prognosis of cytogenetically normal AML [110].

Interestingly, as discussed in our Review [449], studies showed that $CD34^+$ $CD38^-$ AML cells, enriched in LSCs, have lower *IL1B* expression levels compared to $CD34^+$ $CD38^+$ AML progenitors and healthy $CD34^+$ cells [332]. Lower levels of IL-1β protein were also found in the plasma of mainly M0-M2 AML patients compared to healthy controls [500]. These seemingly contradictory results may be attributed to AML subtype and molecular heterogeneity, biological sample and cell subset considered, and stage of the disease. However, the contributions highlighted above and described thoroughly in our previous compilation [449] demonstrate the key role played by IL-1β in AML, which is widely accepted as of today. In turn, the endogenous regulation, or potential dysregulation, of IL-1β in the context of AML and other myeloid neoplasms remains an open question that should be subject of scientific endeavor.

3. IL-1β targeting as a potential tool against leukemia

Blockade of IL-1β and its signaling pathway shows therapeutic value in preclinical models of hematological malignancies [212,448,449,495,501-503]. For example, inhibition of p38, involved in IL-1β signaling and AML progression, inhibits growth of primary AML cells and cell lines and enhances normal hematopoiesis *ex vivo* [448]. IL-1 receptor associated kinase (IRAK) inhibition slows down disease progression and increases survival of *Mll-AF9* mice. This is performed through the stabilization of wild type MLL protein, which then outcompetes chimeric MLL that is displaced from some of its target genes [501]. However, the signaling mediators downstream of IL-1R1 are common to other pathways independent of IL-1β and thus their targeting is expected to have broader effects potentially collateral. Therapeutic interventions with improved selectivity to IL-1R1/IL-1β are expected to have better therapeutic windows. Targeting of IL-1RAP via RNA interference or antibodies, reduces growth of human primary AML cells and cell lines by induction of differentiation and apoptosis, thereby inhibiting pathogenesis in xenografts of AML cell lines [495]. Similar results were obtained for leukemia pathogenesis in a genetic model of AML by transplantation of wild-type or *Il1rap* deleted bone marrow HSPCs transduced with *Mll-AF9* [495]. *In vivo* administration of anti-IL-1RAP antibodies in mice transplanted with primary chronic and blast phase CML cells resulted in therapeutic effects, with reductions in $CD34^+$ $CD38^-$ $IL\text{-}1RAP^+$ CML stem cells [502].

Several studies in mouse models showed the potential value of human IL-1RN anakinra, the recombinant form of the natural repressor of IL-1, in the treatment of MPNs and CML [212,503]. In *JAK2*^{V617F} MPN mice, anakinra treatment reduced signs of disease, recovered MSC number *in vivo* and prevented apoptosis of Schwann cells cocultured with mutant HSCs *ex vivo* [212]. Pretreatment with anakinra in combination with the tyrosine kinase inhibitor nilotinib to CML cells, previous to transplantation into immunodeficient mice, showed greater inhibition of CML LSCs compared to nilotinib alone *in vivo* [503]. Secondary transplantation of bone marrow cells from *SCL-tTA/BCR-ABL* – transplanted mice treated with this drug combination, associated with reduced fraction of CML cell engraftment, reduced leukemia development and improved survival. Mechanistically, *ex vivo*, treatment of human Lin^- $CD34^+$ $CD38^-$ $CD90^+$ CML cells with the combination anakinra plus nilotinib inhibited NF-κB target gene and cytokine gene expression [503].

In the clinic, many studies demonstrated the efficiency of IL-1β blockade as cure in different inflammatory diseases [449,504,505]. However, its therapeutic value in patients with AML and other myeloid neoplasms needs further investigation. Decoy and soluble receptors (rilonacept, Arcalyst®), monoclonal antibodies (canakinumab, Ilaris®) and the recombinant form of IL-1RN (anakinra, Kineret®), reduce IL-1α and/or IL-1β activity but differ in their pharmacokinetics, and are approved for the treatment of different inflammatory and autoimmune diseases [449]. Currently, canakinumab is being tested in a phase II trial for the treatment of low- and intermediate-risk MDS and CML (NTC04239157). However, canakinumab was associated with increased risk of fatal infection or sepsis despite exclusion of patients with chronic or recurrent infection in the Cantos trial, aimed at preventing adverse cardiac events [506,507]. Thus, the ability of anakinra to allow quick discontinuation of IL-1 inhibition in case of infection seems to be advantageous.

In summary, the therapeutic potential of IL-1β targeting and anakinra in particular in the treatment of myeloid neoplasms seems to be remarkable and needs further investigation.

PAPER II – Research Article

Endogenous IL-1 receptor antagonist represses healthy and malignant myeloproliferation. Nature Communications. Under Review.

Enhanced IL-1β signaling is associated with different myeloid neoplasms, including AML, and its blockade shows therapeutic potential in the clinic for the treatment of inflammatory and autoimmune diseases [449]. Nonetheless, little is known about the potential contribution of its endogenous repressor IL-1RN to healthy and/or malignant hematopoiesis. In this section, the main findings of our research on the role of IL-1RN during normal hematopoiesis and AML are discussed.

1. Unbalanced IL-1RN in human AML and its therapeutic potential

First, we observed a decrease of *IL1RN* expression in circulating $CD34^+$ progenitor cells from AML patients compared to $CD34^+$ cells from healthy controls, and particularly in M0-M3 AML subtypes according to the FAB classification. This IL-1RN deregulation in the different AML subtypes was confirmed after data reanalysis of a bigger publicly available cohort of AML patients that used purified AML blasts [508-510]. These findings are consistent with a previous study showing that circulating AML blasts from M4-M5 AML subtypes exhibit increased IL-1RN release in culture supernatants compared to M1-M2 AML blasts [511]. Further, we observed that reduced expression of *IL1RN* in AML blasts was associated with lower survival rate within a cohort of 426 AML patients [508-510]. In a cohort of 19 matched-pair diagnosis-relapsed AML patients [512], AML blasts at relapse displayed reduced expression of *IL1RN* compared to blasts at time of diagnosis. Those patients with higher expression of *IL1RN* have a better prognosis and higher relapse-free probability. Thus, *IL1RN* expression level is low in AML patients and can be used as a prognostic marker for disease progression.

Low IL-1RN in AML was accompanied by enhanced IL-1β signaling pathway. We observed increased gene expression and protein levels of IL-1β over IL-1RN in circulating $CD34^+$ progenitors from AML patients versus healthy controls. These data confirm increased IL-1β in AML, as previously described [110,448]. We also suggested, for the first time, that high IL-1β in AML may result from the reduction of its natural repressor IL-1RN, in addition to the enhancement of IL-1β signaling [110,448,495,513]. Further genetic and epigenetic mechanistic studies

to understand the cause for low *IL1RN* in human AML are underway in the lab. These studies will include methylation in CpG sites, as this epigenetic modification was previously involved in low *IL1RN* expression in AML blasts from relapsed versus diagnosis paired AML patient samples [512]. NF-κB may be activated by a variety of signals, but unbalanced IL-1RN in AML was associated with higher numbers of $CD34^+$ activated through NF-κB, consistent with IL-1β signaling activation. This is in agreement with previous observations of NF-κB activation in human $CD34^+$ $CD38^-$ LSCs [109,514,515]. Additional TFs previously involved in IL-1β signaling, such as PU.1 [87,88] and C/EBPα [99] should be evaluated in the future. In our study, IL-1β treatment promoted human AML cell expansion in peripheral blood and bone marrow of immunodeficient NSG-SGM3 mice transplanted with $CD34^+$ progenitors derived from AML patients, confirming the contribution of IL-1β signaling in human AML pathogenesis as previously described [110,448,449,495,513]. Our data also point to $CD34^+$ progenitors as important drivers of inflammation in human AML, but the role of other cell types remains to be clarified. In particular, the lab is currently investigating the potential role for low IL-1RN anti-inflammatory properties of bone marrow MSCs in human AML. It will be interesting to address the role of MSCs in this process.

Pre-clinical studies have underscored the value of IL-1 inhibition in the treatment of a variety of myeloid malignancies [212,213,448,449,501-503]. Our findings showed that anakinra, recombinant form of human IL-1RN, and canakinumab, monoclonal anti – IL-1β antibody, reduced leukemic burden in a similar way in NSG-SGM3 mice transplanted with $CD34^+$ AML progenitors. Thus, IL-1RN boost or IL-1β blockade stand out as possible therapeutic strategies for AML patients based on their unbalanced IL-1RN. Pharmacologically, canakinumab provides advantage over anakinra considering their half-lives of 26 days and 4-6 hours, respectively [449,516-518]. Canakinumab was recently tested in trial for treatment of patients with atherosclerotic disease. It was effective preventing adverse cardiac events, but was associated with deaths attributed to infections and sepsis [506,507]. Thus, anakinra may offer a better physiological response and allows to quickly discontinue IL-1 inhibition in case this is needed and life-threatening in the treatment of inflammatory diseases and leukemia. New strategies are being developed to extend the half-life of anakinra. For example, IL-1RN loaded into biodegradable polymers showed prolonged anti-inflammatory effect after 4 weeks of subcutaneous administration and successfully alleviated the symptoms of type 2 diabetes mellitus in rats [519]. Rilonacept, with extended circulation half-life *in vivo* of 8.6 days [520], should be considered in further work. In the future, if current genetic studies allow for the selection of

patients with best chances of success with anakinra, the lab is planning a clinical trial to evaluate the therapeutic potential of IL-1RN in combination with conventional chemotherapy to treat AML.

Administration of MSCs was more effective inhibiting bleomycin – mediated inflammation and fibrosis than administration of recombinant IL-1RN in mice, and MSC transcriptome identified *Il1rn* as a potential mediator of this effect [521]. Thus, administration of MSCs could be considered as cell therapy for treatment of IL-1β – induced inflammation and pathogenesis during AML and other hematological neoplasms. The lab is currently investigating the human MSC subset responsible for IL-1RN secretion, as this is unknown as of today. Treatment based on the use of IL-1RN secreting – MSCs as alternative to IL-1RN administration would overcome challenges derived from the short half-life of IL-1RN, given that MSCs would reach the bone marrow and produce this cytokine *in situ*. However, this strategy would not allow to regulate the dose of IL-1RN delivered and would pose additional practical challenges derived from allogeneic transplantation of MSCs.

In conclusion, IL-1RN is downregulated in AML and its higher expression correlates with better prognosis and outcome. Conversely, IL-1RN boost stands out as a potential therapy for AML patients in xenograft models based on their unbalanced IL-1RN.

2. Role of IL-1RN in steady-state hematopoiesis

2.1. Role of IL-1rn in the hematopoietic compartment of the bone marrow in absence of infection or injury

Cytokines determine HSC lineage choice [522]. In particular, chronic administration of IL-1β enhances myeloid differentiation of HSCs [88]. In our study, we observed that IL-1rn deletion using IL-1rn-KO mice, in the absence of infection or injury, biases the differentiation of HSCs towards the myeloid lineage and hampers B cell differentiation, with reduction in numbers of HSCs ($CD135^{-}$ $CD34^{-}$ $CD48^{-}$ $CD150^{+}$ LSK) and expansion of the MPP2 subset in IL-1rn-KO versus wild type mice. While no changes were observed in the CLP and CMP subsets, myelopoiesis occurs at the expense of further reduction in the numbers of MEP and GMP. These data suggest complex effects of IL-1rn deletion in the hematopoietic hierarchy that are selective of the progenitor cell type considered and require additional investigation. This excess myeloid lineage expansion is reminiscent of early hematological disease, consistent with the

observation upon long-term administration of high IL-1β doses [88], and indicates that IL-1rn is required under physiological conditions to prevent abnormally expanded myelopoiesis.

In cell expressing IL-1R1, endogenous IL-1RN binds and blocks IL-1α and IL-1β signaling, thereby preventing the activation of their own expression in a positive feedback loop [523-525]. Further, IL-1R1 signaling is required for IL-1β – induced myeloid differentiation induction, given that *Il1r1*$^{-/-}$ HSCs do not show exacerbated myeloid differentiation in the presence of IL-1β [88]. *In vivo*, deletion of IL-1rn enhanced IL-1β levels in bone marrow and the number of cells with membrane expression of IL-1R1 production, while other pro-inflammatory cytokines did not change. This suggested participation of enhanced IL-1β signaling through IL-1R1 mediating the myeloid biased differentiation in IL-1rn-KO mice. The mechanistic connection was performed by *in vivo* treatment with a monoclonal antibody against IL-1β, which reversed biased myelopoiesis in IL-1rn-KO mice to a similar extent than *in vivo* treatment with anakinra. IL-1β – induced myelopoiesis of HSPCs in IL-1rn-KO mice was reversed by adding anakinra in *ex vivo* cultures as well. These data confirmed that IL-1β is the main effector of the enhanced myelopoiesis in absence of IL-1rn. Although unlikely, *in vivo* treatment with a monoclonal antibody against IL-1α is underway and will reveal the functional contribution of this cytokine, if any.

In our study, only IL-1rn-KO mice were treated with anakinra. A previous study showed that administration of IL-1RN to wild type mice inhibits normal hematopoiesis [496]. This is seemingly contradicting to the absence of hematopoietic phenotype in IL-1r1-KO and IL-1rap-KO mice [88,493-495]. An important source of variation among studies on this pathway is the microbiological status of experimental mice in different animal facilities. Opportunistic agents in specific pathogen free (SPF) – housed mice may serve as immunogenic triggers inducing subtle changes in basal inflammatory levels and affect the balance of IL-1β to IL-1rn. We carefully controlled for this issue and our mice were maintained under specific opportunistic pathogen free (SOPF) environments.

To better understand the underlying mechanisms to the biased myelopoiesis, RNA-seq of HSPCs was conducted. These studies suggested that the abnormal myelopoiesis in IL-1rn-KO mice is mediated mainly through transcriptional activation of IL-1β – induced myeloid differentiation pathways dependent on NF-κB activation in HSPCs, consistent with our findings in AML patients. The mechanistic link was confirmed by treatment with bortezomib, proteasome inhibitor that prevents NF-κB activation. *In vivo* treatment with bortezomib reverted the myeloid bias of progenitors from IL-1rn-KO mice. Studies of the impact of

additional gene programs previously involved in IL-1β signaling like C/EBPα [99] and PU.1 [87,88] are underway. In fact, *Cebpa*, *Spi1* (PU.1) and *Csf2rb* are all increased in expression in LT-HSCs and ST-HSCs from IL-1rn-KO versus wild type mice, as previously reported upon chronic IL-1β administration to wild type mice [87,88,99]. The RNA-seq data of HSPCs showed that LT-HSCs and ST-HSCs are the most affected population after deletion of *Il1rn*. *Il1b* is higher expressed in LT-HSCs and ST-HSCs upon deletion of *Il1rn*, but not in monocytes and granulocytes, main producers of IL-1β. Further, *Il1r1* expression is higher in HSPCs versus myeloid cells. These data suggest that enhanced IL-1β in IL-1rn-KO mice results from increased number of myeloid cells and enhanced *Il1b* expression in HSPCs. HSPCs show sustained engagement of an IL-1β – positive feedback loop through IL-1R1 in IL-1rn-KO mice, leading to abnormally expanded myelopoiesis.

Together, these data demonstrate that IL-1β and IL-1rn coexist in a fine-tuned balance where IL-1rn represses IL-1β signaling and prevents IL-1β – induced HSPC differentiation into the myeloid lineage, under physiological conditions. Deletion of IL-1rn induces differentiation of HSPCs towards the myeloid lineage through enhanced IL-1β signaling dependent on NF-κB activation, phenotype that resembles early myeloproliferative disorder features.

2.2. Role of IL-1n in the stromal compartment of the bone marrow in absence of infection or injury

MSCs play an important role in the regulation and maintenance of HSC number and function [236,240,242,243,262]. Enhanced IL-1β levels in the bone marrow of MPN mice initiate a series of pathogenic events that result in apoptosis of MSCs, which contributes to disease progression [212]. In our study, we observed that deletion of IL-1rn, in absence of infection or injury, lowers the numbers of MSCs, their HSC-regulatory activity at the transcriptomic level and causes incipient reticulin fibrosis in the bone marrow. We chose $CD45^{-}$ $CD31^{-}$ $Ter119^{-}$ $CD63^{+}$ cells ($CD63^{+}$ cells) as a representative population of bone marrow MSCs for most of our experiments because CD63 is a marker enriched in Nes-GFP^{+} MSCs [212]. A recent study suggests that $CD63^{+}$ stromal cells represent a subpopulation of trabecular MSCs [253]. However, reanalysis of single-cell transcriptional data of mouse bone marrow stromal populations from the same study showed that *Cd63* expression largely overlaps with expression of *Lepr*, *Cxcl12*, *Scf* and other genes previously reported to be expressed by MSCs. Moreover, *Cd63* expression highly overlaps with expression of *Il1rn*, and our qRT-PCR analyses revealed that $CD63^{+}$ cells are great producers of IL-1rn.

MSCs from IL-1rn-KO mice showed increased membrane expression of IL-1R1, suggesting that enhanced levels of IL-1β in IL-1rn-KO mice activate IL-1β signaling in stromal cells. Further, *in vivo* administration of anakinra or anti – IL-1β monoclonal antibody rescued the number of $CD63^+$ MSCs, confirming that the reduced numbers of $CD63^+$ cells in IL-1rn-KO mice is induced by IL-1β.

MSCs from IL-1rn-KO mice showed increased expression of *Il1b* versus wild type mice, which contributes to enhanced IL-1β in IL-1rn-KO mice. Taken together, $CD63^+$ MSCs show sustained engagement of an IL-1β – positive feedback loop through IL-1R1, like HSPCs.

In summary, deletion of IL-1rn results in IL-1β – induced damage to the stromal compartment of the bone marrow, with contribution of $CD63^+$ MSCs to the inflammatory environment in IL-1rn-KO mice through enhanced *Il1b* expression.

Currently, to better understand the selective contribution of the hematopoietic and stromal compartment of the bone marrow to the phenotype induced by IL-1rn global deletion, the lab has obtained the *IL-1rn*$^{fl/fl}$ mouse model under material transfer agreement (MTA) from Dr. Cem Gabay (University Hospital of Geneva). *IL-1rn*$^{fl/fl}$ mice have been intercrossed with inducible and conditional Cre systems including *Mx1-Cre* and *Nes-Cre*^{ERT2} mice, which will allow targeting of IL-1rn selectively in hematopoietic cells, osteoblastic cells and $nestin^+$ MSCs [210,236,419,420,526].

3. Role of IL-1RN in pre-leukemic myelopoiesis

3.1. Presence of IL-1rn deficiency in a mouse model of pre-leukemic myelopoiesis

Preclinical models of myeloid neoplasms, AML and MDS showed the important role of enhanced IL-1β and other members of its signaling pathway in disease pathogenesis [212,213,448,495,501,527-529]. The knowledge about IL-1rn in this context is little or none. To further study the potential presence and contribution of IL-1rn deficiency to malignant myelopoiesis and the molecular mechanisms that mediate the process, we used the *Mx1-Cre Nras*^{G12D} mouse model. Among *RAS* mutations, *NRAS* mutations are frequent in human AML, affecting about 10-20% of AML patients [125,530]. In mice, single allelic expression of the *Nras*^{G12D} mutation results in a chronic myeloproliferative disorder [420,421,467,531], and according to our records in the lab only about 20% of the mice develop AML in the old age. This makes the model suitable to

study the development of leukemia in a stepwise manner. Further, the choice of the *Mx1-Cre Nras^{G12D}* mouse model was based on the fact that oncogenic RAS potentiates IL-1β signaling in different types of cancer [532,533].

Our study showed that the inducible *Nras^{G12D}* mutation leads to abnormally expanded myelopoiesis in mice, which is concomitant with reduced levels of IL-1rn and increased levels of IL-1β in the bone marrow of *Nras^{G12D}* mice compared to controls. Other pro-inflammatory cytokines were unchanged. Thus, unbalanced IL-1β over IL-1rn levels in *Nras^{G12D}* mice may induce inflammation in this mouse model. Granulocytes and monocytes from *Nras^{G12D}* mice showed no increase of *Il1b* expression or reduction of *Il1rn* compared to controls. However, HSPCs from *Nras^{G12D}* mice, including LT-HSCs, ST-HSCs and MPPs, displayed increased *Il1b* expression, suggesting that HSPCs may be drivers of inflammation in *Nras^{G12D}* mice. Thus, the high IL-1β levels in the bone marrow of diseased mice seem to be result of a gene regulation event in HSPCs together with expansion of IL-1β – producing hematopoietic cell types, *i.e.* HSPCs, granulocytes and monocytes, in *Nras^{G12D}* mice. Reduction of *Il1rn* expression was only observed in MPPs. High *Il1b* and low *Il1rn* expression in MPPs coincided with the selective expansion of MPPs across the three HSPC subsets studied in *Nras^{G12D}* mice. The selective regulation of IL-1rn and IL-1β expression across hematopoietic cells in *Nras^{G12D}* mice is notable and its underlying mechanisms should be subject of future investigation.

To better understand the specific contribution of HSPCs to the IL-1β – induced inflammatory environment in the bone marrow of *Nras^{G12D}* mice, RNA-seq was performed in HSPC subsets. In agreement with our observations in AML patients and IL-1rn-KO mice, low IL-1rn associated to *Nras^{G12D}* – driven myelopoiesis through increased NF-κB activation in HSPCs, given that HSPCs from *Nras^{G12D}* mice exhibited increased NF-κB TF calculated activity. The causal association was made when the myeloid bias of progenitors from *Nras^{G12D}* mice could be improved *in vivo* by treatment with bortezomib. Conversely, previous data showed that dominant negative Ras (DN-RasN17) reduces IL-1β – dependent NF-κB activity [532].

The data indicated damage to the stromal compartment in *Nras^{G12D}* mice. Reduced numbers of CD63^{+} MSCs, as consequence of higher apoptosis, along with reduced expression of *Il1rn* further contribute to unbalance the levels of IL-1rn in the bone marrow of *Nras^{G12D}* mice compared to controls.

In summary, low IL-1rn is present and contributes to inflammation in *Nras^{G12D}* – driven pre-leukemic myelopoiesis. Low IL-1rn in *Nras^{G12D}* mice can be explained on the basis of the

increased apoptosis of MSCs together with reduced *Il1rn* expression in MPPs and mainly in MSCs.

3.2. Contribution of IL-1rn deficiency from the hematopoietic or the stromal compartment to pre-leukemic myelopoiesis in a mouse model

Through the generation of chimera models transplanting equal amounts of bone marrow cells from IL-1rn-KO and wild type (1:1), *Nras*^{G12D} and wild type (1:1) or IL-1rn-KO and *Nras*^{G12D} (1:1) mice into wild type recipients, we were able to demonstrate that IL-1rn deletion from hematopoietic cells synergizes with *Nras*^{G12D} hematopoietic cells to promote aberrant myelopoiesis. In turn, when transplanting *Nras*^{G12D} or control bone marrow cells into wild type or IL-1rn-KO recipients, we observed that IL-1rn deletion from the microenvironment also contributes to faster progression of *Nras*^{G12D} – driven myeloproliferative disorder. These data indicate that the IL-1rn deficiency from the hematopoietic or the stromal compartment contributes functionally to *Nras*^{G12D} – driven malignant myelopoiesis and may play an important role in the early stages of leukemia. Both compartments are functionally relevant for IL-1rn production to repress myelopoiesis under neoplastic hematopoiesis.

Currently, the lab has generated *Mx1-Cre Nras*^{G12D} *IL-1rn*$^{fl/fl}$ mice for a fine-tuned study of the contribution of the simultaneous presence in the same cells of IL-1rn deletion together with *Nras*^{G12D} mutation to drive malignancy. To confirm how broadly applicable the role of low IL-1rn in other mouse models of leukemia will be, we are also studying a highly aggressive mouse model of AML driven by MLL-AF9 [534]. It would be interesting to further extend these studies to additional mutational landscapes.

In agreement with our observations in primary AML xenografts, short-term *in vivo* treatment with anakinra ameliorated early signs of abnormal myelopoiesis in *Nras*^{G12D} mice. Current and future work from the lab will evaluate long-term treatments and additional mouse models.

To sum up, these data provide evidence of the contribution of low IL-1rn to aberrant myeloproliferation in the presence of the pre-leukemic lesion *Nras*^{G12D} in mice and of a novel mechanistic rationale for IL-1RN boost therapeutic potential in myeloid malignancies.

Concluding remarks

Adapted from *From Kaupanger in Song*. Knud Baade. 1833. Photo: Nasjonalmuseet.

Chronically enhanced IL-1β drives HSC differentiation into the myeloid lineage, and both enhanced IL-1β and a variety of downstream mediators of its signaling pathway show roles in hematological malignancies. Several FDA – approved strategies are available for IL-1 blockade. Despite their therapeutic potential, they have received little interest in the context of hematological malignancies as evidenced in our compilation of relevant scientific contributions. Currently, canakinumab is being tested in a Phase II clinical trial for the treatment of low or intermediate risk MDS and CML (NTC04239157).

The potential role of IL-1β endogenous repressor cytokine IL-1RN in both healthy and malignant hematopoiesis and its potential value as therapy against AML were unknown as of today. Through the series of experiments performed in this research using clinical, preclinical and molecular data, systematic characterizations of blood and bone marrow cell populations *in vivo* and model systems largely physiological and disease relevant, we are able to conclude that **IL-1 receptor antagonist represses healthy and malignant myeloproliferation and shows value in the treatment of AML**.

The completion of the specific aims of this research work further allows us to conclude the following:

I. **Low IL-1RN is a prognostic marker of poor survival in AML patients, it is frequent in AML patients, and it characterizes the lower maturation profiles according to the FAB classification of AML.** Low IL-1RN provides a new potential origin for high IL-1β in human AML.

II. **Both IL-1RN boost through anakinra or IL-1β blockade with canakinumab have similar therapeutic potential for AML patients in xenograft models.** Low IL-1RN provides a novel mechanistic rationale for the success of these strategies. We further depict a type of patient that may benefit from anti-IL-1β therapies over patients that present with high IL-1β resulting from primary lesions in its signaling pathway downstream of IL-1R1.

III. **Balanced IL-1rn and IL-1β play a critical role on steady-state HSPC function *in vivo*.** The IL-1rn-KO strain displays IL-1β – induced bias in HSPC differentiation towards the myeloid lineage and reduced B cell development, via NF-κB activation under steady-state conditions. This phenotype is reminiscent of pre-leukemic disease and is evident in the absence of injury or infection. The IL-1rn-KO strain displays IL-1β – induced damage to the stromal compartment of the bone marrow.

IV. **Low IL-1rn is a hallmark of an experimental *Nras*^{G12D} model of biased pre-leukemic myelopoiesis and further loss of IL-1β repression through IL-1rn genetic deletion promotes myeloproliferation, with contribution of both hematopoietic and stromal compartments of the bone marrow.** Conversely, treatment with anakinra reverts pre-leukemic myeloproliferation.

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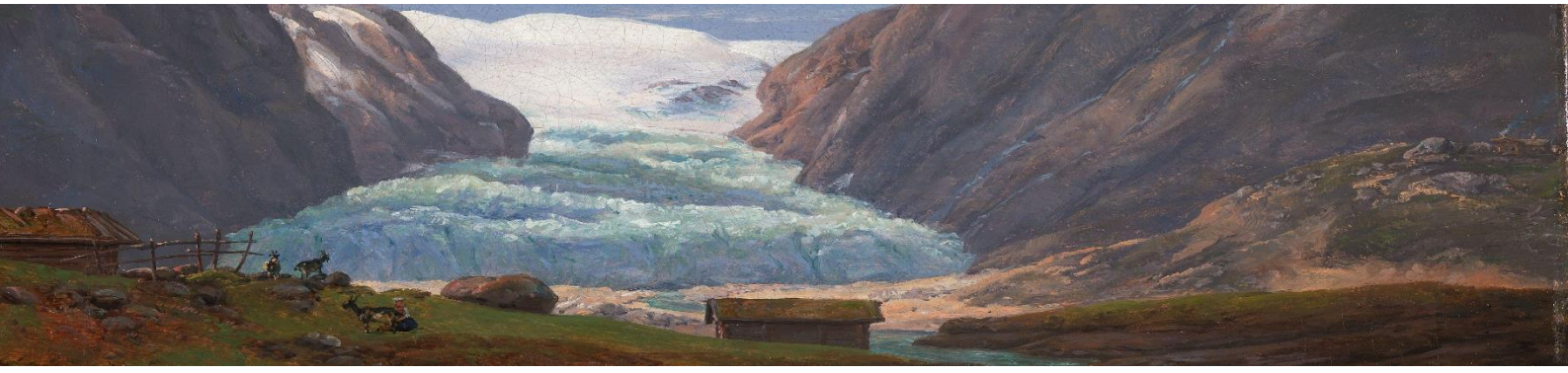

Adapted from *View of Nigardsbreen in Jostedalen*. Johan Christian Dahl. 1847. Photo: Jacques Lathion / Nasjonalmuseet.

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Papers

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PAPER I - Review

Interleukin-1β as emerging therapeutic target in hematological malignancies and potentially in their complications.

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Review

Interleukin-1β as emerging therapeutic target in hematological malignancies and potentially in their complications

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ABSTRACT

Interleukin-1β (IL-1β) is a pleiotropic cytokine that exerts multiple roles in both physiological and pathological conditions. It is produced by different cell subsets, and drives a wide range of inflammatory responses in numerous target cells. Enhanced IL-1β signaling is a common event in patients of hematological malignancies. Recent body of evidence obtained in preclinical models shows the pathogenic role of these alterations, and the promising therapeutic value of IL-1 targeting. In this review, we further highlight a potential contribution of IL-1β linking to complications and autoimmune disease that should be investigated in future studies. Hence, drugs that target IL-1 may be helpful to improve outcome or reduce morbidity in patients. Some of them are FDA-approved, and used efficiently against autoimmune diseases, like IL-1 receptor antagonist. In the clinic, however, this agent seems to have limited properties. Current improved drugs will allow to determine the true potential of IL-1 and IL-1β targeting as therapy in hematological malignancies and their related complications.

1. Introduction

Inflammation is a refined immune mechanism essential to fight against pathogens and tumor cells, and orchestrated by a variety of cells and mediators. When dysregulated, compiled data supports the hypothesis that chronic inflammation promotes cancer. This is particularly evident in hematological malignancies. Strikingly, a Swedish epidemiological study found that history of any infectious disease was associated with a 1.3-fold significantly increased risk of both acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS), even when infection had occurred 3 or more years before AML or MDS onset. By using population-based central registries, a total of 9219 patients with primary AML (diagnosed from January 1, 1965, through December 31, 2004) and 1662 patients with primary MDS (diagnosed from January 1, 1993, through December 31, 2004), as well as 36,389 and 6489 population-based controls, respectively, were included. Further, to minimise bias, patients diagnosed with another cancer before their AML or MDS were excluded. Men represented 52.8% of the patients with AML and 54.9% of MDS patients, and the median ages at diagnoses were 68 and 76 years for AML and MDS, respectively. Interestingly, although history of any infectious disease was associated to similar increased risk of both AML and MDS, fewer individual subgroups of infections were associated to MDS. A broad range of infections were associated to AML including pneumonia, tuberculosis, intestinal infections, septicemia, hepatitis C, pyelonephritis, sinusitis, nasopharyngitis, upper respiratory tract infection, cytomegalovirus infection, and cellulitis [1]. One plausible explanation of these data is that chronic immune stimulation may act as trigger for AML and MDS development.

Chronic inflammation and autoimmune conditions have been consistently linked with increased risk of malignant lymphomas, with varying risk levels [2]. More recently, in patients of myeloproliferative neoplasms (MPN), chronic inflammation has been evidenced as potential initiating event and driver of clonal expansion that predisposes to second cancer [3–5]. Interestingly, another Swedish large population-based study found that patients with prior history of autoimmune disease had 20% increased risk of MPN development. In total, 11,039 MPN patients (diagnosed from 1958 to 2005) were included together with 43,550 matched controls. Men represented 48.4% of MPN patients, and the mean age at diagnosis was 67 years. A total of 288 (2.6%) MPN patients had a previous history of autoimmune disease. Higher risk of MPN was associated with prior thrombocytopenic purpura, Crohn's disease, polymyalgia rheumatic, giant cell arteritis, Reiter's syndrome and aplastic anemia [6]. High basal inflammatory status seems to promote mutagenesis through induction of chronic

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Table 1
IL-1 family member nomenclatures and main activity.

Family member	Alternative name	Function
IL-1α	IL-1F1	Inflammatory
IL-1β	IL-1F2	Inflammatory
IL-1Ra	IL-1F3	Anti-inflammatory (Receptor antagonist)
IL-18	IL-1F4	Inflammatory
IL-33	IL-1F11	Inflammatory
IL-36α	IL-1F6	Inflammatory
IL-36β	IL-1F7	Inflammatory
IL-36γ	IL-1F8	Inflammatory
IL-36Ra	IL-1F5	Anti-inflammatory (Receptor antagonist)
IL-37	IL-1F7	Anti-inflammatory
IL-38	IL-1F10	Anti-inflammatory (Receptor antagonist)

oxidative stress and subsequent DNA oxidative damage, and elicits epigenetic changes that further promote inflammation [3]. In addition, the MPN population has a significant inflammation-mediated comorbidity burden, ranging from second cancer to cardiovascular and thromboembolic disease, chronic kidney disease, autoimmune disease and osteopenia [7].

One of the cytokine families most related to innate immune responses and inflammation is the IL-1 family. It comprises 11 members (Table 1) with agonist activity, receptor antagonists and an anti-inflammatory cytokine, for a tight control of inflammatory responses [8]. IL-1α, IL-1β, IL-1 receptor antagonist (IL-1Ra) and IL-18 have been extensively studied *in vitro*, animal models of disease and humans [9]. Among these, IL-1β stands out as initiator of inflammatory processes, and blocking its activity in humans is currently applied in clinical treatments. This review presents the pathogenic role of dysregulated IL-1β in patients of hematological malignancies, its promising therapeutic value in preclinical models, and its potential contribution linking to second disease and complications based on lessons learned from other systemic inflammatory diseases.

2. Physiological characteristics of IL-1β and role in the hematopoietic system

IL-1β is mainly produced by myeloid cells [10,11]. It is synthesized as an inactive form (Fig. 1A), pro-IL-1β that is activated intracellularly by caspase 1 [8,11]. Under normal conditions, IL-1β is secreted in low levels, and its expression and/or caspase 1-mediated activation increases under disease [12,13]. In autoinflammatory diseases, high IL-1β tissue levels are usually accompanied by an increase in blood levels given that monocytes release more processed IL-1β [9,14–17]. Secreted IL-1β binds to its IL-1 receptor 1 (IL-1R1) and triggers a signaling cascade that controls gene expression of multiple transcription factors, growth factors and other interleukins involved in hematological function (Fig. 1B) [10]. Thereby, IL-1β plays an important role in innate and adaptive immune cellular responses. It stimulates maturation of T cells and enhances proliferation of B cells [18–20]. Further, IL-1β promotes expression of inflammatory molecules such as cyclooxygenase type 2, type 2 phospholipase A, prostaglandin E2, platelet activating factor and nitric oxide [9], among others.

Importantly, IL-1β modulates hematopoietic stem cell (HSC) function. In preclinical models, it promotes HSC differentiation biased into the myeloid linage, in part through activation of PU-1 signaling (Fig. 2A) [21]. While acute IL-1β exposure contributes to HSC regeneration after myeloablation and transplantation [21,22], chronic exposure promotes uncontrolled HSC division and eventual exhaustion of the HSC pool [21]. Several studies have shown neutrophilia, leukocytosis and thrombocytosis following IL-1β treatment [12,23]. In contrast, inhibition of IL-1β signaling using IL-1Ra, which competitively binds to IL-1R1 and prevents binding of the cytokine (Fig. 1B) [24], reduces colony formation *ex vivo* [25,26]. *In vivo*, IL-1Ra suppresses cell cycle in bone marrow HSC, and reduces numbers of leukocytes and platelets [26]. Thus, preclinical models show that fine-tuned IL-1β levels play a physiological role in hematopoiesis, and suggest that their dysregulation may participate in hematological diseases [10,21,27].

3. IL-1β in clinical and preclinical models of hematological malignancies: emerging therapeutic implications

3.1. MPN

MPN are a group of clonal HSC disorders characterized by increased proliferation of at least one of the following lineages; eythroid, megakaryocytic and myeloid, and retaining full differentiation [28]. Underlying chronic inflammation has been suggested to contribute to disease initiation and/or progression [3]. Classical Philadelphia chromosome negative (negative for *BCR-ABL* gene fusion) MPN includes mainly essential thrombocythemia (ET), polycythemia vera (PV) and primary myelofibrosis (PMF) (Table 2 [29]). Most frequent *BCR-ABL* negative MPN are associated with Janus kinase 2 (*JAK2*), calreticulin and myeloproliferative leukemia virus oncogene (*MPL*) mutations, among others [28,30].

MPN patients show increased levels of inflammatory cytokines in serum [31,32], and gene expression profiling and functional annotation analysis confirms deregulation of inflammatory and immune system genes [33]. Pro-inflammatory cytokines have traditionally been related to initiation and progression of bone marrow myelofibrosis at advanced stages of disease [34]. Unlike PV or ET patients [35–37], PMF patients show high levels of IL-1β together with other pro-inflammatory cytokines and growth factors in plasma [35,37]. If high IL-1β levels are present in PV patients, those are correlated to fibrotic transformation, poor prognosis and lower survival [37].

Mastocytosis is a less common form of myeloid neoplasm characterized by mast cell expansion in bone marrow and other organs [27]. It has been separated from other MPNs in the 2016 revision to the WHO classification of myeloid neoplasms and acute leukemia due to its unique clinical and pathological characteristics, ranging from indolent cutaneous disease to aggressive systemic disease (Table 2 [29]). Aggressive phenotypes of mastocytosis are related to up-regulation of IL-1β in mast cells [38].

Our recent work has shed light on the pathogenic role of IL-1β in preclinical models of MPN. Using a transgenic mouse model that expresses the human mutant JAK2-V617F under the endogenous promoter of *Jak2* in an inducible way, we showed that IL-1β produced at early stages of disease, at least in part by mutant HSCs, induces damage of the neuroglial components in the bone marrow. Reduced sympathetic regulation together with IL-1β stimulation results in mesenchymal stem cell (MSC) apoptosis that then allows expansion of mutant HSCs (Fig. 2B) [39]. The pathogenic role of IL-1β was uncovered by administration of IL-1Ra, which ameliorates hallmarks of disease, recovers MSC numbers *in vivo* and prevents apoptosis of glial cells *ex vivo* (Table 3) [39]. These data suggest that targeting IL-1β may have clinical implications to improve treatment of MPN patients.

3.2. Chronic myeloid leukemia (CML)

BCR-ABL or Philadelphia positive CML is classified as an MPN disorder, but it is usually considered as a separate entity because of its unique features and responses to treatment (Table 2 [29]) [28]. CML is a biphasic disease characterized by excessive expansion of the granulocytic lineage during the initial chronic phase. Acquisition of additional genetic and/or epigenetic abnormalities causes the progression to blast phase, which characterizes by a block of cell differentiation that results in presence of 30% or more myeloid or lymphoid blast cells in peripheral blood or bone marrow, or presence of extramedullary infiltrates of blast cells [40].

High levels of IL-1β are associated with poor prognosis in CML

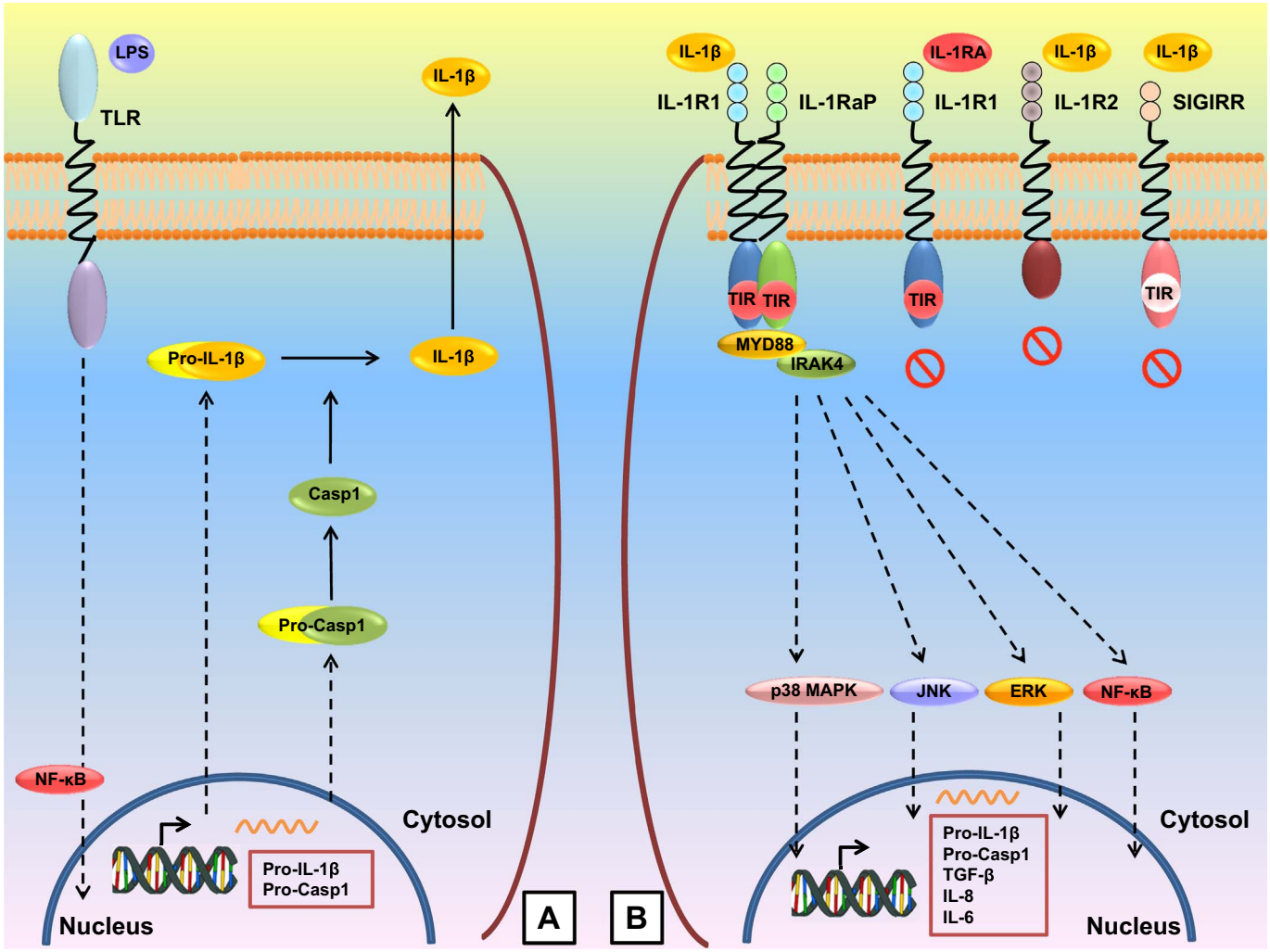

Fig. 1. Interleukin-1β initiates inflammation and controls essential cell responses. A) Several external inflammatory stimuli that signal through TLR, activate a cascade of events that culminate in activation of the transcription factor NF-κB. Following NF-κB activation, IL-1β is synthesized as its inactive form, pro-IL-1β, which is activated by cleavage through caspase 1. Pro-caspase 1 is synthesized and activated in response to similar stimuli. B) Secreted IL-1β binds to its IL-1R1 and triggers a signaling cascade, which involves p38 MAPK, JNK, ERK and NF-κB activation that control gene expression of multiple transcription factors, growth factors and interleukins involved in cell functional activation, survival responses and cell fate. Under normal conditions, IL-1β signaling is negatively regulated through IL-1Ra, IL-1R2 and SIGIRR. LPS, lipopolysaccharide; TLR, toll-like receptors; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; IL, interleukin; Casp1, caspase 1; IL-1Ra, IL-1 receptor antagonist; IL-1R, IL-1 receptor; IL-1RAP, IL-1 receptor accessory protein; TIR, toll-IL-1 receptor; SIGIRR; single immunoglobulin and TIR domain containing; MYD88, myeloid differentiation primary response 88; IRAK4, IL-1 receptor associated kinase 4; MAPK, mitogen-activated protein kinases; JNK, c-Jun N-terminal kinases; ERK, extracellular signal-regulated kinases; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; TGF-β, transforming growth factor β.

[41,42]. Increased IL-1β is seen in advanced blast phase as compared to chronic phase and healthy controls, and correlates with blast expansion in bone marrow and peripheral blood, poor prognosis and shorter survival in patients [41,43]. IL-1β stimulates proliferation of mutant long-term HSC *ex vivo*, at concentrations comparable to those observed in CML bone marrow [44], and helps promote colony growth of mutant hematopoietic progenitors [45]. Use of IL-1Ra or soluble IL-1 receptor suppressed this effect, suggesting that IL-1β could confer a proliferative advantage to leukemic stem cells (LSC) [45]. Interestingly, IL-1 receptor accessory protein (IL-1RAP) that is a required component of the IL-1R complex (Fig. 1B), is highly expressed in *BCR-ABL*+ CML cells [46]. In particular, IL-1RAP is up-regulated in CD34+ and CD34+ CD38- cells from CML patients compared to controls, and its expression increases with disease progression [47]. Further, anti-IL1RAP antibody targets CML CD34+ CD38- cells *via* antibody-dependent cell-mediated cytotoxicity [46]. Hence, alterations in several components of the pathway leading to strengthened IL-1 signaling may contribute to disease.

CML patients may display relapses through mechanisms dependent on *BCR-ABL* [48,49] or through additional mutations, like those in genes promoting HSC survival or multidrug resistance [50–52]. Importantly, IL-1β contributes to resistance to BCR-ABL tyrosine kinase inhibitor imatinib in CML cells, where it increases cell survival and decreases apoptosis rate through cyclooxygenase 2 [53]. Interferon (IFN) family members, alternative treatment against CML, have anti-inflammatory effects and inhibit IL-1β [54–57]. Higher levels of IL-1β were seen in IFN-α-resistant CML patients as compared to sensitive patients and healthy controls, and IL-1β stimulates colony growth in IFN-α-sensitive CML cells [45].

In mouse models of disease, IL-1Ra in combination with nilotinib, drug with greater power and selectivity for BCR-ABL than imatinib [58,59], reduces numbers of leukemic cells in blood and bone marrow, and the self-renewal potential of leukemic stem cells (LSC). This correlates with extended survival after completion of treatment compared to mice treated with nilotinib alone (Table 3) [60]. *In vitro*, this combination significantly reduced human CML progenitor cell growth, including CD34+ CD38+ and CD34+ CD38- cells [61]. Then, blockade of IL-1 signaling together with BCR-ABL tyrosine kinase inhibition may pave the way to more efficient therapies against CML in patients.

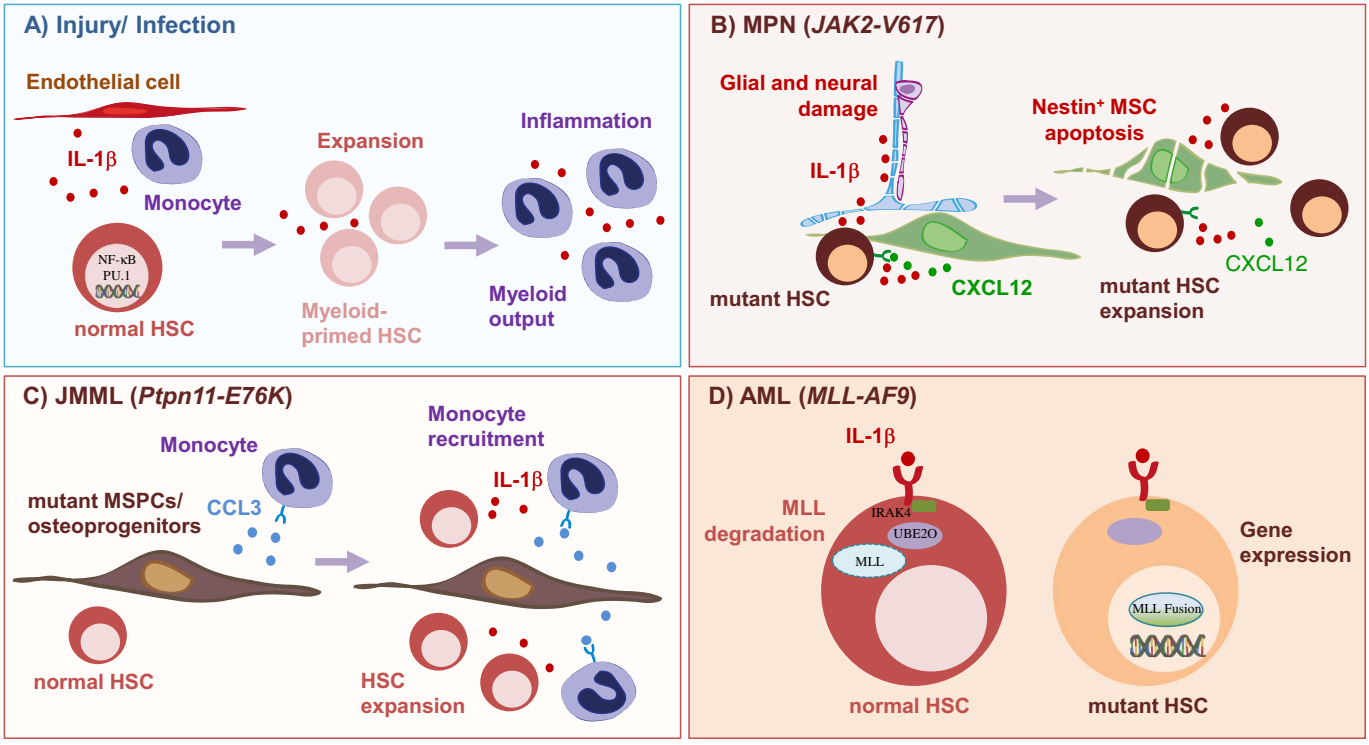

Fig. 2. Pathological mechanisms of IL-1β on hematopoietic stem cell function identified in mouse models. A) Upon injury or infection, IL-1 is produced at high levels in the bone marrow by monocytes and endothelial cells, among others. IL-1 drives myeloid differentiation through activation of the NF-κB pathway and a PU.1-dependent myeloid gene program that results in HSC expansion, biased differentiation into myeloid progenitors and ultimately myeloid cells [21]. B) In a mouse model of MPN that expresses the human mutant JAK2-V617F, IL-1β is produced at early stages of disease by mutant HSCs, and induces damage of the neuroglial components in the bone marrow. Reduced neural regulation together with enhanced IL-1β results in mesenchymal stem cell apoptosis that then allows expansion of mutant HSCs [39]. C) In a mouse model of JMML that results from *Ptpn11* activating mutation in MSCs and progenitor cells, and in osteoprogenitors, increased levels of CCL3 recruits monocytes to the bone marrow. These produce IL-1β that promotes HSC expansion [65]. D) In normal HSCs, IL-1β signaling through IL-1R1 drives UBE2O phosphorylation mediated by IRAK4. This increases UBE2O interaction with MLL and its degradation. In contrast, in a mouse model of AML that results from expression of MLL-AF9 fusion protein, MLL chimeras are resistant to degradation driven by IL-1β [86]. IL-1β, interleukin-1β; HSC, hematopoietic stem cell; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PU.1, purine-rich nucleic acid binding protein 1; MPN, myeloproliferative neoplasm; JAK2, Janus kinase 2; MSC, mesenchymal stem cell; CXCL12, C-X-C motif chemokine ligand 12; JMML, juvenile myelomonocytic leukemia; Ptpn11, tyrosine-protein phosphatase non-receptor type 11; MSPCs, mesenchymal stem and progenitor cells; CCL3, C-C motif chemokine ligand 3; AML, acute myeloid leukemia; MLL, mixed-lineage leukemia gene; IRAK4, interleukin-1 receptor associated kinase 4; UBE2O, ubiquitin conjugating enzyme E2 O.

3.3. Juvenile myelomonocytic leukemia (JMML)

JMML is a childhood MDS/MPN (Table 2 [29]) that may arise as consequence of germline activating mutations of the protein tyrosine phosphatase SHP2, encoded by the gene *PTPN11* [62,63]. Interestingly, both cell-autonomous [64] as well as *Ptpn11* activating mutations in the bone marrow microenvironment [65] promote development and progression of JMML. In mouse models of disease, *Ptpn11* activating mutations in MSCs and progenitor cells as well as in osteoprogenitors cause increased secretion of the chemokine (C-C motif) ligand 3 (CCL3) or macrophage inflammatory protein 1α (MIP-1α). CCL3 recruits monocytes to the bone marrow microenvironment where HSCs reside. Recruited monocytes produce IL-1β, and this in turn hyperactivates HSCs leading to JMML (Fig. 2C). Interestingly, treatment with CLL3 receptor antagonists reverses JMML originated by the mutated microenvironment (Table 3) [65]. However, it remains to be seen how broadly applicable this mechanism will be, given that no human cases were examined for presence of *PTPN11* activating mutations particularly in the bone marrow microenvironment. Further, from 4 patients examined positive for *PTPN11* activating mutations and with Noonan syndrome, which predisposes to JMML, MSCs and progenitor cells showed in culture varying levels of CLL3.

3.4. AML

AML is a heterogeneous disease characterized by aberrant myeloid lineage proliferation and differentiation, and at least one clonal somatic abnormality on mutational profiling in the majority of the patients (> 97%) [66] (Table 2 [29]). IL-1β is produced by human AML blasts, where its expression relates to poor patient prognosis [67,68]. Both endogenous and exogenous IL-1β promote blast proliferation, by induction of growth factors and other cytokines like granulocyte-macrophage colony stimulating factor [69–74]. Poorer patient prognosis and lower survival is observed in those patients with higher proliferative response to exogenous IL-1β [75]. Further, IL-1β direct inhibition or indirect inhibition targeting IL-1RAP, blocks colony formation and proliferation of AML cells [76,77]. Endogenous IL-1 has also been related to apoptosis resistance in human AML, and addition of recombinant human IL-1 in culture enhances cell survival through pathways like phosphoinositide-3 kinase and ceramidase [78]. In addition, IL-1β secreted by human AML blasts, stimulates expression of adhesion molecules that promote their recruitment by epithelial cells [79], effect that may be relevant for tissue infiltration and metastasis.

In spite of these studies, the role of IL-1β in human AML remains controversial. According to Su et al. [80], lower levels of IL-1β are present in the plasma of AML patients compared to healthy controls. Further, CD34+ CD38- progenitors, enriched within the LSC subset, down-regulate IL-1β expression through epigenetic mechanisms, compared with more mature CD34+ CD38+ AML progenitors and normal CD34+ cells [81]. Forced expression of IL-1β stimulates cell cycle and apoptosis in CD34+ CD38- AML progenitors, by down-regulation of cyclin-dependent kinase inhibitor 1 ($p21^{waf1}$) and antiapoptotic proteins respectively. Similarly, over-expression of IL-1β in CD34+ CD38- cells, reduces engraftment and reconstitution after transplantation into

Table 2
2016 World Health Organization classification of myeloid neoplasms and acute leukemia.

WHO classification of myeloid neoplasm and acute leukemia classification
Myeloproliferative neoplasms (MPN)
Chronic myeloid leukemia (CML), *BCR-ABL1*$^{+}$
Chronic neutrophilic leukemia (CNL)
Polycythemia vera (PV)
Primary myelofibrosis (PMF)
PMF, prefibrotic/early stage
PMF, overt fibrotic stage
Essential thrombocythemia (ET)
Chronic eosinophilic leukemia, not otherwise specified (NOS)
MPN, unclassifiable
Mastocytosis
Myeloid/lymphoid neoplasms with eosinophilia and rearrangement of *PDGFRA*, *PDGFRB*, or *FGFR1*, or with *PCM1-JAK2*
Myeloid/lymphoid neoplasms with *PDGFRA* rearrangement
Myeloid/lymphoid neoplasms with *PDGFRB* rearrangement
Myeloid/lymphoid neoplasms with *FGFR1* rearrangement
Provisional entity: Myeloid/lymphoid neoplasms with PCM1-JAK2
Myelodysplastic/myeloproliferative neoplasms (MDS/MPN)
Chronic myelomonocytic leukemia (CMML)
Atypical chronic myeloid leukemia (aCML), *BCR-ABL1*$^{-}$
Juvenile myelomonocytic leukemia (JMML)
MDS/MPN with ring sideroblasts and thrombocytosis (MDS/MPN-RS-T)
MDS/MPN, unclassifiable
Myelodysplastic syndromes (MDS)
MDS with single lineage dysplasia
MDS with ring sideroblasts (MDS-RS)
MDS-RS and single lineage dysplasia
MDS-RS and multilineage dysplasia
MDS with multilineage dysplasia
MDS with excess blasts
MDS with isolated del(5q)
MDS, unclassifiable
Provisional entity: Refractory cytopenia of childhood
Myeloid neoplasms with germ line predisposition
Acute myeloid leukemia (AML) and related neoplasms
AML with recurrent genetic abnormalities
AML with t(8;21)(q22;q22.1);*RUNX1-RUNX1T1*
AML with inv.(16)(p13.1q22) or t(16;16)(p13.1;q22);*CBFB-MYH11*
APL with *PML-RARA*
AML with t(9;11)(p21.3;q23.3);*MLLT3-KMT2A*
AML with t(6;9)(p23;q34.1);*DEK-NUP214*
AML with inv.(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2);*GATA2, MECOM*
AML (megakaryoblastic) with t(1;22)(p13.3;q13.3);*RBM15-MKL1*
Provisional entity: AML with BCR-ABL1
AML with mutated *NPM1*
AML with biallelic mutations of *CEBPA*
Provisional entity: AML with mutated RUNX1
AML with myelodysplasia-related changes
Therapy-related myeloid neoplasms
AML, NOS
AML with minimal differentiation
AML without maturation
AML with maturation
Acute myelomonocytic leukemia
Acute monoblastic/monocytic leukemia
Pure erythroid leukemia
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferations related to Down syndrome
Transient abnormal myelopoiesis (TAM)
Myeloid leukemia associated with Down syndrome
Blastic plasmacytoid dendritic cell neoplasm
Acute leukemias of ambiguous lineage
Acute undifferentiated leukemia
Mixed phenotype acute leukemia (MPAL) with t(9;22)(q34.1;q11.2); *BCR-ABL1*
MPAL with t(v;11q23.3); *KMT2A* rearranged
MPAL, B/myeloid, NOS
MPAL, T/myeloid, NOS
B-lymphoblastic leukemia/lymphoma
B-lymphoblastic leukemia/lymphoma, NOS
B-lymphoblastic leukemia/lymphoma with recurrent genetic abnormalities
B-lymphoblastic leukemia/lymphoma with t(9;22)(q34.1;q11.2);*BCR-ABL1*
B-lymphoblastic leukemia/lymphoma with t(v;11q23.3);*KMT2A* rearranged
B-lymphoblastic leukemia/lymphoma with t(12;21)(p13.2;q22.1); *ETV6-RUNX1*
B-lymphoblastic leukemia/lymphoma with hyperdiploidy
B-lymphoblastic leukemia/lymphoma with hypodiploidy
B-lymphoblastic leukemia/lymphoma with t(5;14)(q31.1;q32.3) *IL3-IGH*
B-lymphoblastic leukemia/lymphoma with t(1;19)(q23;p13.3);*TCF3-PBX1*
Provisional entity: B-lymphoblastic leukemia/lymphoma, BCR-ABL1–like
Provisional entity: B-lymphoblastic leukemia/lymphoma with iAMP21
T-lymphoblastic leukemia/lymphoma
Provisional entity: Early T-cell precursor lymphoblastic leukemia
Provisional entity: Natural killer (NK) cell lymphoblastic leukemia/lymphoma

immunodeficient mice [82]. Interestingly, in the same study, the authors showed that low dose IL-1β exposure stimulates colony formation in AML cells, while high doses promote the opposite effect [82]. This highlights the importance of balanced levels of IL-1β in AML, where future studies are required aiming at understanding the specific role played by IL-1β.

It is important to note that IL-1β may be produced by certain subsets of non-hematopoietic cells, like some stromal components of the hematopoietic stem cell niche that supports HSC function. In normal conditions, IL-1β at levels similar to those found in human serum, stimulates MSC proliferation *in vitro* and their capacity to maintain hematopoietic progenitor cells [83]. Bone marrow stromal cells from healthy controls co-cultured with different leukemia cell lines, show an up-regulation of IL-1β [84]. However, MSC from AML patients show lower expression of IL-1β at the time of diagnosis, previous to bone marrow transplantation and at least 6 months after the transplant, compared to healthy controls [85].

Recently, preclinical models of AML have pinpointed IL-1 as a potential therapeutic strategy. Using mixed-lineage leukemia (*MLL*)-rearranged leukemia models, Liang et al. showed that IL-1 negatively regulates the stability of wild-type but not chimeric MLL protein, resulting in improved stability of the latter (Fig. 2D) [86]. Strikingly, pharmacological inhibition of this signaling pathway using IL-1 receptor-associated kinase (IRAK) inhibitors (Fig. 1B), remarkably delays disease progression and improves survival in *MLL-AF9+* murine leukemia (Table 3) [86]. Future studies are required to extend these promising mouse studies to primary human samples.

3.5. Lymphoid malignancies

A role for IL-1β has been suggested in lymphoid malignancies. Chronic lymphocytic leukemia (CLL) is a malignancy of mature clonal B lymphocytes that accumulate in the blood, bone marrow and other lymphoid tissues [87]. The specific single nucleotide polymorphism *IL1B-511*T, when presented in homozygosis, correlates with low risk of CLL. Interestingly, a different single nucleotide polymorphism in *IL1B* gene (*IL1B-511*C) together with *IL6-174*C, both in homozygosis, increase to 11-fold the risk of CLL, compared to 4.5 fold increase with *IL6-174*C alone [88]. This points to an association between IL-1β and IL-6 in CLL development. Low levels of IL-1β and high levels of IL-6 are found in the plasma of patients [88,89]. However, previous work showed that IL-1β induces differentiation and activation of leukemic cells in CLL patients [90]. Besides, MSCs from acute lymphocytic leukemia (ALL) patients show increased IL-1β expression at diagnosis [85]. Thus, future work should elucidate the potential participation of IL-1β in lymphoid malignancies.

Table 3
Summary of treatments targeting IL-1 pathway efficient in mouse models of hematological malignancies.

Hematological malignancy	Preclinical model	Drug	Mechanism	Reference
MPN	*JAK2-V617F*	IL-1Ra	IL-1Ra is a competitive inhibitor of α and IL-1β signaling that binds to IL-1R1	[39]
CML	*BCR-ABL*	IL-1Ra and Imatinib	impeding its interaction with the cytokines	[60]
JMML	*Ptpn11-E76K* in MSPCs	CCR1a or CCR5a	Antagonists that block CCL3 binding to CCR1 or CCR5, respectively	[65]
AML	*MLL-AF*9	IRAK1/4 or IRAK4 inhibitors	Inhibition of IL-1R-associated kinases that impedes signaling downstream IL-1R1	[86]

4. Additional roles for IL-1β in pathophysiology: lessons from systemic inflammatory diseases

4.1. Bone

IL-1 is a pleiotropic cytokine that exerts numerous roles in other systems like bone, where it contributes to the fine-tuned balance between bone resorption and formation that maintains its homeostasis. In particular, IL-1 enhances the expression of extracellular matrix enzymes, like collagenases that facilitate destruction of articular cartilage [91,92]. Further, IL-1 induces differentiation of bone-resorbing osteoclasts from mononuclear precursors, and has stimulating effects on osteoclasts and resorption *via* TNF ligand superfamily member 11 (RANKL) [93]. It also induces vasodilatation, promotes attraction of granulocytes, and enhances expression of prostaglandins, events that further help bone resorption [94].

The typical manifestation of accelerated bone remodeling is osteoporosis. Osteoporosis characterizes by bone thinning, damage in its architecture and reduced mechanical strength due to diminished mineral density. This is accompanied by high fracture risk [95]. It is most frequent in postmenopausal women, so loss of bone mineral density was traditionally attributed to estrogen loss [96]. More recently, estrogens were suggested to have only minor effects [95], and inflammatory cytokines like IL-1 were pointed out [96]. In women who had undergone surgical menopause, increase in IL-1 secretion by peripheral blood mononuclear cells associates with significant loss in bone mineral density [97]. Administration of IL-1Ra improves bone mineral density in ovariectomized rats, uncovering the therapeutic value of targeting IL-1 against bone loss [98].

Osteoporosis and fractures are frequent in patients of systemic inflammatory diseases, like rheumatoid arthritis [96], disease that primarily affects synovial joints. In rheumatoid arthritis, high levels of pro-inflammatory cytokines promote osteoclast differentiation and bone degradation, resulting in osteoporosis [99]. High levels of IL-1 were found in the synovial membrane and fluid of patients [100,101], while experimental models showed a major role for IL-1 in cartilage and bone degradation [92,102,103]. This disease was the first where IL-1 antagonism was tested and proved for clinical use. Use of IL-1 inhibitors was supported by severe arthritis development in IL-1Ra deficient mice [104].

In osteoarthritis, IL-1 promotes cartilage degradation [105]. While IL-1Ra prevents cartilage degeneration in animal models and improves clinical outcomes in patients [106,107], intra-articular gene transfer of IL-1Ra showed improved results in experimental models [108]. Recently, a method was developed to produce an autologous conditioned serum rich in IL-1Ra that seems to be an option as supplementary therapy in patients [108,109].

4.2. Pain

IL-1 induces hyperalgesia that is increased sensitivity to pain, through damage to nociceptors or peripheral nerves. Hyperalgesia may affect primary afferent fibers for mechanical stimuli, resulting in a highly disabling symptom [110]. IL-1 activates nociceptors directly causing activation of intracellular signaling cascades, and indirectly *via* production of kinins and prostanoids [111].

In certain chronic inflammatory diseases, like osteoarthritis, pain is one of the most prominent symptoms. Studies have related IL-1 levels with pain perception and radiographic knee lesions in patients [112]. Inflammatory stimuli, and in particular IL-1, start the cascade of events that cause disease and drive pain in parallel [113,114]. Treatments that reduce cartilage degeneration, reduce pain as well [106,107].

5. Clinical complications derived from hematological malignancies

5.1. Bone morbidity

Increased inflammatory cytokines in MPN patients relate to myelofibrosis at advanced stages of disease [34]. Fibrosis typically derives in osteosclerotic lesions, particularly in PMF. PMF is a severe form of MPN characterized by hematopoietic failure and osteosclerosis, which originates as result of growth and thickening of bone trabeculae, and new bone formation in abnormal budding plaques [115]. PMF patients show high levels of IL-1β in plasma [35,37], and high IL-1β levels in PV patients predispose to fibrotic transformation [37]. Histomorphometric measurements in 75 PMF patients showed elevated bone mineral density compared to other forms of MPN, and correlation between amount of bone and degree of fibrosis [116]. Surprisingly, a more recent study using non-invasive methods in 18 patients with MF and healthy controls matched for age, sex, and height, showed that bone mineral density, geometry and microarchitecture in MF patients were not significantly different [117]. Several reasons may underlie differences in results, including sample size or disease stage. Hence, future work will be required for a better understanding of the bone disease and a potential link to IL-1β in PMF patients.

In MPN and CML patients, epidemiological studies have concluded increased risk of osteoporosis. For instance, a Danish study reported increased risk of fractures among MPN patients [118]. This study compared fracture risk among 7595 MPN patients and a cohort of 338,974 members of the general population. The fracture rates were consistently higher at several anatomic locations including femur, humerus, and distal forearm. The 10-year hip fracture risk was 7% in ET patients and 9% in PV patients, with a risk of 5% among matched controls. Interestingly, the same study showed risk of hip fracture 2.7-fold higher in CML patients than in the general population [118]. CML patients were stratified according to presence or absence of tyrosine kinase inhibitor treatment. Treatment turns CML into a more chronic condition with longer life expectancy, and reduces the need for allogeneic bone marrow transplantation [119]. However, it does not influence the fracture risk in CML patients [118]. In another study performed on 36 CML patients, skeletal lesions were examined by x-ray. Lesions were positive in 16% of the cases, and included osteoporosis, osteolytic and osteoblastic lesions, and chloromas, *i.e.* myeloid sarcomas outside of the bone marrow [120]. Further, osteoporosis and vertebral fracture are frequent in patients with systemic mastocytosis with respectively 31 and 17% in a cohort of 75 patients [121]. Nevertheless, the direct contribution of chronic inflammation and IL-1 to bone loss specifically in myeloid leukemias remains unknown, and should be subject of future investigation (Table 4).

Table 4
Summary of clinical data in hematological malignancies where both increased IL-1β and bone morbidity are present in patients.

Hematological malignancy	Increased IL-1β	Bone morbidity
PMF	[35,37]	[37,115,116]
PV	[37]	[118]
Mastocytosis	[38]	[121]
CML	[41–43,46,47]	[118,120]
ALL	[85]	[122–124]

Bone morbidity seems to be present in other types of hematological malignancies like ALL (Table 4). ALL is the most common leukemia in childhood, and induces significant effects on the skeleton of children and adolescents that show, at the moment of diagnosis, lower bone density than their healthy counterparts [122]. Low bone turnover status explains through reduced bone formation but normal resorption markers [123]. Further, ALL patients have increased fracture rate compared to healthy controls [124], and fracture risk is higher in ALL survivors after the end of the treatment [125,126]. However, little is known about the molecular mechanisms driving bone complications in ALL patients.

5.2. Hematopoietic malignancies and pain

Interestingly, the most important hematopoietic disease-related pain affects bone, and it was traditionally related to osteolytic lesions and infiltration of bone marrow with malignant cells. In the context of hematopoietic disorders, pain may be correlated to disease and its complications, or to diagnostic procedures and treatments [127]. When pain is present at disease onset, treatment with chemotherapeutic agents or other therapies usually drive pain relief. This is frequent in ALL patients [128].

Our recent work may provide hints linking pathogenesis and pain in hematopoietic malignancies. Particularly in experimental models of MPN, we showed that mutant cells produce IL-1β that damages the neuroglial components in the bone marrow at early stages of the disorder. Schwann cells, that cover and protect the integrity of the peripheral neural fiber, are rapidly reduced in the disease bone marrow. Sympathetic fibers are subsequently injured, in both disease mice and humans, which may contribute to bone pain reported in MPN patients [129]. Reduction in sympathetic regulation together with IL-1β stimulation results eventually in expansion of mutant cells, that is ameliorated by treatment with IL-1Ra *in vivo* [39]. Hence, IL-1 may be pathogenic factor and pain driver in MPN, and represents a good candidate for clinical interventions.

Additionally, both ALL and AML survivors may experience chronic pain due to complications associated to hematopoietic cell transplantation [130]. Pain origin after transplantation seems to relate to injury to mucosal tissues induced by the conditioning regimen, like chemotherapy [131]. In mouse models and clinical settings, cisplatin, that is a common chemotherapy, induces sensory neuropathy [132,133]. Further, experimental models demonstrated cisplatin-induced bone marrow nerve injury that impairs hematopoietic regeneration and could thereby compromise success of the transplant [132]. To date, the molecular mechanisms driving neural damage after chemotherapy have not been thoroughly defined.

6. Hematopoietic malignancies and autoimmune diseases

The connection between autoimmune diseases and hematopoietic malignancies goes beyond common bone affectation and pain. Actually, a number of epidemiological studies show higher risk of hematopoietic malignancies in patients with autoimmune diseases compared to the general population, with further increase after cytotoxic treatment [134]. Interestingly, autoimmune disease patients with secondary acute leukemia usually develop AML rather than ALL [135]. History of any autoimmune disease has been associated with increased risk of AML and MDS [1,136]. In particular, AML risk is significantly associated with rheumatoid arthritis, systemic lupus erythematosus, polymyalgia rheumatica, autoimmune hemolytic anemia, systemic vasculitis, pernicious anemia, and inflammatory bowel disease like ulcerative colitis and Crohn's disease [134,136,137]. Additionally, systemic mastocytosis is related to higher prevalence of inflammatory joint diseases like spondyloarthritis and rheumatoid arthritis [138,139]. Interestingly, the clinical appearance of non-Hodgkin's lymphoma and systemic lupus erythematosus is similar, making them difficult to distinguish at early stages. This raises the possibility that systemic lupus erythematosus may be a paraneoplastic syndrome and appears on the grounds of the hematopoietic malignancy [140]. Conversely, a hematopoietic disorder may precede the autoimmune disease, and for instance early manifestation of an occult malignancy may be fast development of rheumatoid arthritis-like syndromes [141].

Additionally, increased risk of AML is associated to an autoimmune disease of the central nervous system: multiple sclerosis. Multiple sclerosis develops as consequence of autoimmune demyelination of the central nervous system leading to progressive disability. Immunomodulatory drugs like IFN-β are used as first-line therapy, and non-responsive patients are treated with strong immunosuppressive and cytotoxic drugs like mitoxantrone [142]. Multiple sclerosis patients treated with mitoxantrone are at particularly high risk of developing AML. However, not all patients exposed to this drug develop AML, whereas others do without mitoxantrone treatment [143,144].

The factors predisposing to AML in autoimmune diseases are currently subject of extensive research. Defective immune system and, as previously mentioned, immunosuppressive therapies seem to be risk factors that allow tumor progression [145,146]. Mutations in certain genes are shared by both autoimmune diseases and cancer, including the tumor suppressor *p53*, the death receptor *Fas*, and the signaling pathway phosphatidylinositol 3-kinase/protein kinase B/mammalian Target Of Rapamycin, among others [147–151]. Further, inflammation is a common event within both pathogenic processes. Inflammation enhances tumor progression through complex inflammatory signaling cascades that involve NF-κB activation, related to both leukemia and autoimmune diseases like rheumatoid arthritis [152–154]. Importantly, it is well-described that activated NF-κB induces transcription of proIL-1β.

As discussed in the previous sections, IL-1 and specifically IL-1β plays a pathogenic role in a variety of hematopoietic malignancies, particularly those involving the myeloid lineage. This statement holds true for a wide range of systemic inflammatory and autoimmune diseases [155]. In both hematopoietic malignancies and autoimmune diseases, there is a link to bone and pain complications. Hence, it is reasonable to hypothesize that IL-1 may underlie morbidity and may as well provide a link between hematopoietic malignancies and autoimmune diseases. Future work is required to validate this hypothesis. If IL-1 participates in pathogenesis, complications and second disease in both hematopoietic malignancies and autoimmune diseases, fine-tuned management of IL-1 levels would have utility in numerous disorders and substantially improve quality of life in patients.

7. FDA-approved therapeutic strategies for IL-1 blockade

Extensive clinical research is being performed with a variety of agents that reduce IL-1 activity. Currently, these drugs include IL-1Ra, soluble receptors, antibodies, and IL-1 traps among others. Some of these drugs are being actively pursued at Phases I to III in clinical trials to treat a broad spectrum of diseases [155–158]. In spite of their therapeutic potential, so far few studies have evaluated their effects against different types of cancer and hematological malignancies. One example of the latter is MABp1, naturally occurring monoclonal antibody that neutralizes IL-1α. In 2012, a Phase I clinical study was

completed with patients of advanced hematological malignancies (NCT01260545), but its results are not published yet. One prevailing presumption for this little interest is that IL-1 blockade may be contraindicated for patients as it may further promote cancer-related immunosuppression [158]. However, this theory may be misinterpreted [159], given that IL-1 neutralization reduces the inflammation that contributes to cancer-related immunosuppression [160]. Future studies are required to further clarify this perspective. As of today, the following therapeutic opportunities targeting IL-1 are FDA-approved: anakinra, rilanocept and canakinumab.

7.1. Anakinra (Kineret)

Anakinra is the recombinant form of the naturally occurring IL-1Ra, it exerts its function blocking the IL-1 receptor and thus reduces the activity of both IL-1α and IL-1β. Anakinra was FDA-approved in 2001 to treat rheumatoid arthritis, and since then it has been proved as an efficient and safe therapy in a variety of diseases [156]. It is currently being tested in numerous clinical trials. A Phase II study in patients with smoldering or indolent multiple myeloma (NCT00635154), who were at risk of progression to active myeloma, tested the ability of anakinra to delay or prevent active myeloma. Between November 19, 2002, and May 24, 2007, 47 patients were enrolled in the study and treated with anakinra [161]. In 25 (53%) of the patients, low-dose dexamethasone was administered in addition. Treatment with anakinra alone lead to a minor response in 3 patients, a partial response in 5 patients and a minor response after addition of dexamethasone in 4 patients. In those who responded, anakinra decreased high-sensitivity C-reactive protein (hs-CRP) levels and myeloma proliferative rate, which correlated with prolonged chronic disease state and improved progression-free survival [161]. A Phase I/II clinical study (NCT02492750) is currently recruiting participants for treatment of early stage multiple myeloma patients with lenalidomide and dexamethasone with or without anakinra.

Intravenous administration is preferred considering its safety even at blood levels 100-fold higher than those achieved following subcutaneous injection [156]. In addition, one important limitation of anakinra is its relatively short half-life of 4 to 6 h [155], leading to drop in blood levels within hours after injection [156]. Evidence from preclinical models of disease indicates that the therapeutic effectiveness of IL-1Ra is crucially dependent on optimal level of dosing for continuous saturation of IL-1 receptors [162]. Hence, anakinra may not allow adequate evaluation of the efficiency of anti-IL-1 treatments, given that partial reactivation of inflammation may occur during 24-hour dosage.

7.2. Rilanocept (Arcalyst)

Rilonacept, also known as IL-1 trap, is a soluble decoy receptor comprising the human IL-1 receptor 1 (extracellular domain and accessory protein) and the Fc portion of human IgG1 [163]. This recombinant fusion protein neutralizes both free IL-α and IL-β with high affinity [155]. Rilonacept was approved by FDA in 2008 for the treatment of cryopyrin-associated periodic syndromes, a group of diseases caused by inherited mutations on the genes *CIAS*1 or *NLRP*3, encoding cryopyrin or NALP3, respectively, which result in spontaneous assembly of the inflammasome with caspase 1 over-activation and IL-1β secretion [164]. A number of clinical trials are currently being developed to use rilanocept against a variety of diseases, including type 1 diabetes (NCT00962026), atherosclerosis (NCT00417417), hepatitis (NCT01903798) and chronic kidney disease (NCT01663103). However, none of these studies involve patients of hematological malignancies.

In addition to its high affinity binding IL-α and IL-β, rilonacept has been proved as a safe and well-tolerated therapy [155,165,166]. When compared to anakinra, rilonacept shows an extended circulation half-life *in vivo* of 8.6 days, and thus it is administered in patients as a weekly subcutaneous injection [166]. Further, both *in vitro* and *in vivo* studies showed that IL-1 trap is more efficient than IL-1Ra [165,167]. In mice, IL-1 trap injected subcutaneously 24 h prior to IL-1β injection was able to fully block IL-1-induced inflammation. This single dose of IL-1 trap also blocked the effect of a second IL-1β injection, 24 h later. In contrast, IL-1Ra did not inhibit IL-1-induced inflammatory response at a dose 15-fold higher than that of IL-1 trap [167]. Thus, on-going and future clinical trials with IL-1 trap should help us determine accurately the promising therapeutic value of IL-1 blockade in multiple diseases including hematological malignancies.

7.3. Canakinumab (Ilaris)

The most recent approach is canakinumab, a monoclonal antibody that specifically neutralizes human IL-1β and was produced in a transgenic mouse strain. It binds to human IL-1β with high affinity and specificity, and the complex formed with the cytokine is unable to attach to the receptor, thereby blocking IL-1β dependent signaling [168,169]. Canakinumab was FDA-approved in 2009 for the treatment of cryopyrin-associated periodic syndromes [170]. Currently, numerous clinical trials are being performed, to treat a broad spectrum of diseases like osteoarthritis (NCT01160822), chronic obstructive pulmonary disease (NCT00581945), type 2 diabetes (NCT00605475), atherosclerosis (NCT00995930) and rheumatoid arthritis (NCT00504595, NCT00424346). So far, however, no clinical trial has been registered that considers patients of hematological malignancies.

Its high affinity and specificity have been proven both *in vitro* and *in vivo*. While it does not interfere with IL-1α signaling [170], canakinumab fully blocks IL-β-induced inflammation and cartilage destruction in mouse models of arthritis [168,171]. In patients, it can be administered intravenously or subcutaneously. The maximum blood concentration is found after 7 days of a single subcutaneous dose, and its half-life is 26 days [168,170,172]. This is a substantial advantage over anakinra and rilonacept, given that canakinumab is administered bimonthly, as opposed to the weekly or daily injections with rilonacept or anakinra, respectively [173]. It is well tolerated in patients, and no severe adverse effects have been reported. Its use is approved in children [170]. IL-1β neutralization will allow to see if IL-1β is indeed a crucial mediator of numerous diseases, and its targeting may serve as therapy or coadjuvant treatment in hematological malignancies and their related complications.

8. Summary and future directions

IL-1 is a pleiotropic cytokine that exerts numerous roles in both physiological and pathological conditions. It is produced by a variety of cells, and elicits a wide range of inflammatory responses in a number of cell subsets. Dysregulated IL-1 seems to be essential in many human diseases, including hematopoietic malignancies and autoimmune diseases, their complications, and may be their connection. Hence, drugs that target IL-1 may be helpful in numerous inflammatory conditions and have shown promising therapeutic value in experimental models of hematological malignancies. Currently, these drugs include IL-1Ra, soluble receptors, antibodies, and IL-1 traps among others. Some of these agents are FDA-approved, and used safely and efficiently as therapy against autoimmune diseases like rheumatoid arthritis.

In the clinical setting, however, IL-1Ra seems to be limited by its biological and pharmacokinetic properties [155]. Likely, this has prevented the full potential of IL-1 targeting to be tested in patients. IL-1 trap is more efficient and has extended half-life *in vivo* [165,166]. The drug rilonacept is a fusion protein comprising the human IL-1 receptor 1 (extracellular domain and accessory protein) and the Fc portion of human IgG1 [163]. Further, the monoclonal antibody canakinumab neutralizes IL-1β specifically, and has even more prolonged half-life [168,170,172]. These next generation of drugs with improved chemical, pharmacological and biological properties, should

allow us to determine accurately the promising therapeutic value of IL-1 and in particular IL-1β in multiple hematological malignancies and their related complications.

Practice points

- High IL-1β signaling is present in patients of hematological malignancies, in particular those with myeloid component.
- Recent preclinical studies demonstrate the pathogenic role of IL-1β in hematological malignancies.
- Targeting of IL-1β pathway shows great therapeutic potential in mouse models.
- IL-1β is responsible for bone degeneration and pain in systemic inflammatory diseases.
- IL-1β is a good candidate promoting morbidity in patients, by linking to bone complications, pain and second disease.

Research agenda

- Clinical trials with patients of hematological diseases, combining specific treatments with targeting of IL-1 pathway.
- Test rilanocept and canakinumab in mouse models of hematological malignancies.
- Further development of drugs with improved properties and test in preclinical mouse models.
- Use of mouse models of hematological malignancies to investigate the causative role of IL-1β in complications and autoimmune disease.

Conflict of interest

The authors declare no competing financial interests.

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PAPER II - Research paper

Endogenous IL-1 receptor antagonist represses healthy and malignant myeloproliferation.

Endogenous IL-1 receptor antagonist represses healthy and malignant myeloproliferation

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Competing interests

The authors declare no competing financial interests.

Abstract

Enhanced interleukin-1β (IL-1β) signaling drives hematopoietic stem cell (HSC) differentiation into the myeloid lineage and contributes to hematological malignancies. Here we explored the role of the endogenous repressor cytokine, IL-1 receptor antagonist (IL-1rn), in both healthy and abnormal hematopoiesis. We find that low IL-1RN is a prognostic marker of reduced survival for acute myeloid leukemia (AML) patients, and is frequent in patients, particularly in those within lower maturation/differentiation profiles as defined by the French-American-British (FAB) classification of AML (M0-M3). Treatments with IL-1RN and the human IL-1β monoclonal antibody canakinumab reduce the expansion of human leukemic cells, including $CD34^+$ progenitors, in xenografts from AML patients. *In vivo* genetic deletion of IL-1rn induces HSC differentiation into the myeloid lineage and hampers B cell development via transcriptional activation of IL-1β-induced myeloid differentiation pathways dependent on NF-κB activation. Low IL-1rn is present in an experimental model of pre-leukemic myelopoiesis, and loss of IL-1β repression through IL-1rn genetic deletion promotes myeloproliferation, which relies on the bone marrow hematopoietic and stromal compartments. Conversely, IL-1rn protects against pre-leukemic myelopoiesis. Our data support that HSC differentiation is controlled by balanced IL-1β/IL-1rn levels under steady-state, and suggest that loss of repression rather than enhanced stimulation of IL-1β signaling may underlie pre-leukemic lesion and AML progression.

Significance

HSPC differentiation is controlled by balanced IL-1β/IL-1rn under steady-state. Low IL-1rn contributes to biased IL-1β-induced myelopoiesis in healthy conditions and pre-leukemic disease, is a prognostic marker for AML patients, and provides a new rationale for IL-1β blockade therapeutic potential.

Introduction

IL-1β stands out as initiator of inflammation, and blocking its activity in humans is applied in clinical treatments against diseases with inflammatory component [1]. When dysregulated, chronic inflammation through autoimmune conditions and infections is linked to increased risk of hematological malignancies [1]. Chronic IL-1β administration biases differentiation of normal HSC to the myeloid lineage and reduces their self-renewal properties through a cell-autonomous effect [2]. However, IL-1 receptor 1 knockout (IL-1r1-KO) mice have unaffected blood production and normal stem and progenitor bone marrow (BM) compartments, suggesting that IL-1β – induced myeloid priming of HSC occurs under conditions of injury or infection only and tonic IL-1 signaling has none or small basal hematopoietic effects [2-4]. In turn, enhanced IL-1β and other members of its signaling pathway, including IL-1RAP, MyD88, IRAK1, IRAK4 and TRAF6, have been involved in AML and/or myelodysplastic syndrome (MDS) [4-10]. Furthermore, altered inflammation affects cells from the BM microenvironment, and thereby contributes to malignant hematopoiesis [6]. In this regard, we reported a causal association between high IL-1β – induced damage to the HSC microenvironment and onset of myeloproliferative neoplasms (MPNs) [11].

Hence, IL-1β blockade shows promising therapeutic value in experimental models of MPN, chronic myeloid leukemia (CML), juvenile myelomonocytic leukemia and AML [8,11-13]. Currently, IL-1β blockade with the human monoclonal antibody (mAb) Canakinumab is being tested in a Phase II clinical trial for the treatment of low or intermediate risk MDS and CML (NTC04239157). Another interesting IL-1 blocking agent is the FDA-approved anakinra, the recombinant form of the naturally occurring IL-1 receptor antagonist (IL-1RN), which competes with IL-1 for IL-1R1. Anakinra is currently used as therapy against autoimmune diseases [1] and it showed therapeutic value in experimental models of MPNs and CML [11,13]. However, little is known about the participation of the endogenous IL-1 repressor IL-1RN in healthy and/or malignant hematopoiesis. IL-1RN production follows after IL-1 by roughly the same cell types of hematopoietic and non-hematopoietic origin [14]. In the BM, these include cells from the BM microenvironment, like BM Nestin^{+} mesenchymal stromal cells (MSC) [11] that represent a niche component that controls HSC function [11,15-18]. Despite these intriguing precedents, it is unclear to what extent repression of the IL-1-signaling pathway by endogenous IL-1RN may influence HSC behaviour in healthy and diseased hematopoiesis.

Here, we find that low IL-1RN has prognostic value of poor survival in AML patients, it is a common event in AML patients, and it characterizes the lower maturation/differentiation

profiles according to the French-American-British (FAB) classification of AML (M0-M3). Still, IL-1RN boost or IL-1β blockade have therapeutic potential for AML patients as previously suggested, based on their low IL-1RN. To study the effect of low IL-1RN in the hematopoietic system, we used the IL-1rn-KO strain, which displays IL-1β-induced bias in HSC differentiation towards the myeloid lineage whilst hampering B cell development, via NF-κB activation under steady-state conditions. This phenotype is reminiscent of pre-leukemic disease and becomes apparent in the absence of injury or infection. Thus, we demonstrate a critical role for balanced IL-1rn and IL-1β on steady-state HSC function *in vivo*. Low IL-1rn is present in an experimental *Nras*^{G12D} model of biased pre-leukemic myelopoiesis and further loss of IL-1β repression through IL-1rn genetic deletion promotes myeloproliferation, which relies on the BM hematopoietic and stromal compartments. Conversely, treatment with exogenous IL-1rn reverts pre-leukemic myeloproliferation. Our data support that loss of repression of IL-1β through low IL-1RN may originate and worsen hematopoietic disease, and predicts poor survival in AML patients.

Material and Methods

Humans

Written informed consent was obtained in accordance with the Norwegian legislation and the Declaration of Helsinki. Human studies were approved by the Regional Committee for Medical Research Ethics North Norway (REC North 2015/1082). The diagnosis of AML and MDS were established according to the revised criteria of the World Health Organization. Cytogenetic risk group for AML patients was established according to Dohner and colleagues [19]. Risk group for MDS patients was determined according to the revised prognostic scoring of Greenberg and colleagues [20]. Patient group was formed by 24 AML patients and 2 MDS patients; 8 women (30.8%) and 18 men (69.2%) aged 68 years (range 52-94). All samples were collected at diagnosis time. Control group was formed by 37 healthy volunteers; 22 women (59.5%) and 15 men (40.5%) aged 42 years (range 23-79). Individual characteristics of patients and controls are shown in Supplementary Table S1. Subjects did not provide the full set of data.

Mice

Age and gender matched *Il1rn*$^{-/-}$ [21], B6.SJL (CD45.1^{+}), C57BL/6J, immunodeficient *NOD Scid Gamma* (NSG) mice expressing human *IL3*, *GM-CSF* and *SCF* (NSG-SGM3) [22,23] (The Jackson Laboratory), *Nes-gfp* [17] and *Mx1-Cre Nras*^{G12D} [24,25] were used in experiments. All experiments were conducted with the ethical approval of the Norwegian Food and Safety Authority. Experimental animals were housed under specific pathogen free environment at the Animal Facility of the UiT – The Arctic University of Norway or the University of Oslo.

Transplantation assays

BM transplantation was performed through the tail vein after myeloablation. Low dose irradiation was used in xenografts.

In vivo pharmacological treatments

In vivo treatments were human IL-1β (Peprotech), human IL-1RN (Anakinra – Kineret® Sobi), mouse IL-1β mAb (1400.24.17, Thermo Fisher and Novus Biologicals) and IgG1 kappa isotype control (P3.6.2.8.1, Thermo Fisher), human IL-1β mAb (Canakinumab – Ilaris® Novartis) and bortezomib (Alfa Aesar – Fisher Scientific).

Mouse hematopoietic and stromal cell fraction extraction

Blood samples, bones and spleens were processed as previously described [11].

Human CD34^{+} cell enrichment

Peripheral blood mononuclear cells (PBMC) and BM nucleated cells from AML patients, and PBMC from healthy controls were obtained by density gradient centrifugation (Lympholyte, Cedarlane), and were used for CD34^{+} cell enrichment using immune magnetic technology (Stem Cell Technologies).

Fluorescence-activated cell sorting (FACS)

Antibodies used are listed in Supplementary Table S2. Immunophenotype of hematopoietic stem progenitor cells (HSPC) was defined as linage-negative (lin^-), Sca-1^+ and c-kit^+; LSK. LSK were further defined as long-term hematopoietic stem cells (LT-HSC; LSK $CD34^-Flt3^-$), short-term HSC (ST-HSC; LSK $CD34^+Flt3^-$), and multipotent progenitors (MPP; LSK $CD34^+Flt3^+$). Detailed FACS analysis of the five stem and progenitor cell subsets corresponding to HSC and MPP1-MPP4 was performed as previously described [26,27]. Lineage-negative hematopoietic progenitor subsets were defined as committed common lymphoid progenitors (CLP; c-kit^{low}Sca-$1^{low}CD127^+$), common myeloid progenitors (CMP; c-kit^+Sca-1^- (LK) $CD34^+FcR\gamma^-$), megakaryocyte erythroid progenitors (MEP; LK $CD34^-FcR\gamma^-$), and granulocyte-monocyte progenitors (GMP; LK $CD34^+FcR\gamma^+$).

Phospho-flow

Human $CD34^+$ cells were fixed, permeabilized and stained with Alexa Fluor 647 mouse anti-NF-κB p65 (pS529) or correspondent isotype control according to the supplier. Antibodies used are listed in Supplementary Table S2.

Proliferation and apoptosis assays

Proliferation was analyzed after 5-Bromo-2-Deoxyuridine (BrdU) (BD Biosciences) injection *in vivo*. Analysis of apoptotic cells was performed as previously described (10). Antibodies used are listed in Supplementary Table S2.

Cell culture

Colony-forming unit cell (CFU-C) assay was performed as previously described [28]. For *ex vivo* differentiation, $4x10^5$ lineage-negative cells/mL were cultured in 1.3mL of Stem Span medium (Stem Cell Technologies), supplemented with 20ng/mL thrombopoietin, 125ng/mL stem cell factor and 50ng/mL Flt3-ligand (Peprotech), in low-adherence 6-well plates (Stem Cell Technologies) for 5 days. Only suspensions with >65% lineage-negative cells were used. IL-1RN at 1000ng/mL or saline solution was added twice per day every 12h. Fresh medium was added on day 3. Cells were harvested on day 5, and checked for differentiation by staining with

rat anti-mouse CD11b (M1/70) Alexa Fluor 647 and flow cytometry. Samples were studied in triplicate. Antibodies used are listed in Supplementary Table S2.

Cytokine analyses

BM extracellular fluid (BMEF) was obtained as previously described and used for cytokine analyses [11]. Multiplex immunoassays were performed using Bio-Plex 200 System (Bio-Rad). IL-1rn in mouse samples was analyzed with Mouse IL-1rn AimPlex bead-based immunoassay (Aimplex Biosciences).

Immunofluorescence and histology

For immunofluorescence, femoral bones were processed as previously described [11], and 15µm cryostat longitudinal sections were used. Antibodies are listed in Supplementary Table S2. At least 2 different sections and a total of 8 images at magnification 400X per animal were quantified using ImageJ. For histological studies, femoral bones were used as previously described [11]. For quantification, one section of the entire femur at 40X was used per mouse and analyzed with ImageJ.

RNA isolation and quantitative reverse transcription PCR

RNA isolation, reverse transcription and gene expression analysis were performed as previously described [11]. The expression level of each gene was calculated by interpolation from a standard curve. All values were normalized with mouse *Gapdh* or *B2m*, or human *B2M* as endogenous housekeeping genes. qRT-PCR mRNA expression of *IL1RN* relative to *B2M* in immune magnetically enriched $CD34^+$ circulating progenitors from healthy volunteers was compared to AML patients classified according to FAB categories; M0-M3 versus M4-M5. The two MDS patients in the cohort were considered together with the AML M4-M5 group based on the presence but altered distributions of BM differentiated monocytic lineage cells in MDS patients. Primers used are listed in Supplementary Table S3.

RNA sequencing and bioinformatic data analysis

RNA sequencing (RNA-Seq) data from FACS-sorted LT-HSC, ST-HSC, MPP and $CD63^+$ MSC obtained from the BM of *Il1rn*$^{-/-}$ and C57BL/6J wild-type (WT) female mice aged 20-25 weeks have accession number GSE126428. RNA-Seq data from FACS-sorted LT-HSC, ST-HSC and MPP obtained from the BM of *Nras*^{G12D} female mice and control littermates 6 weeks after poly-inosine:poly-cytosine (polyI:polyC) induction, aged 34 weeks, have accession number GSE126625. RNA amplification, RNA-Seq library production and RNA-Seq analysis were performed at Eurofins Genomics (Germany). RNA-Seq data from FACS-sorted *Nes*-GFP^+ cells obtained from the BM of 28 week-old *Nes-gfp* male mice 4 weeks after the transplant with BM cells from previously polyI:polyC induced control or *Nras*^{G12D} mice, have accession number GSE157038. RNA amplification and RNA-Seq library production were performed at the Genomics Support Center Tromsø (GSCT, UiT – The Arctic University of Norway).

For RNA-Seq data analysis, the Bioconductor DESeq2 package (https://bioconductor.org/packages/release/bioc/html/DESeq2.html) was used to generate lists of differentially expressed transcripts, Venn diagrams, volcano plots and principal component analysis (PCA). In the gene set enrichment analysis (GSEA), the gseGO function from the clusterProfiler package was used to enrich for biological processes. The gseaplot2 function of the Bioconductor enrichplot package was used to draw the GSEA plots of the significantly enriched gene sets. NF-κB transcription factor activities were derived from the main component using PCA [29]. The complete NF-κB gene list (n=1832) is detailed in Supplementary Table S4.

Further details are provided in Supplementary Methods.

Results

Low IL-1RN predicts AML progression in patients

To study the expression of IL-1RN in human AML, we reanalyzed publicly available arrays from purified AML blasts in a cohort of 381 AML patients (GSE14468) [30-32] and found that low *IL1RN* was associated with reduced survival rate (Fig. 1A). We identified the lower maturation/differentiation M0-M3 patients according to FAB as those patients with low *IL1RN* expression, versus M4-M5 (Fig. 1B). M0-M3 patients displayed high *IL1B* to *IL1RN* ratio (Supplementary Fig. S1A). In a cohort of 19 matched-pair diagnosis-relapsed AML patients (dbGaP accession phs001027) [33], we correlated low *IL1RN* expression in RNA-Seq data from AML blasts with poorer prognosis (HR=0.56, p=0.0312, 95% CI=0.33-0.95) (Supplementary Fig. S1B), and reduced expression of *IL1RN* at relapse versus diagnosis (Supplementary Fig. S1C).

We studied the potential contribution of $CD34^+$ progenitors to unbalanced IL-1RN in AML in a cell-intrinsic fashion. Expression levels of *IL1RN* were reduced in circulating $CD34^+$ progenitors from patients versus healthy controls (Fig. 1C), which was particularly evident in the more undifferentiated FAB categories of AML (M0-M3) (Fig. 1D). Gene expression ratios of all *IL1B*, *CASP1* and *IRAK1*, to *IL1RN* were higher in AML patients (Fig. 1E; Supplementary Fig. S1D), suggesting reduced IL-1RN associates with activation of IL-1β pathway. This was confirmed at the protein level with higher ratio of IL-1β to IL-1RN in $CD34^+$ progenitors from AML patients versus healthy donors (Fig. 1F).

As a surrogate of IL-1β signaling pathway activation, we studied NF-κB activation by phospho-flow in $CD34^+$ progenitors from AML patients and healthy controls. The percentage of PB $CD34^+$ progenitors activated through NF-κB activation was higher in AML patients (Fig. 1G). To confirm the functional role of increased IL-1β signaling in human AML pathogenesis, immunodeficient NSG-SGM3 mice were transplanted with $CD34^+$ progenitors isolated from the BM of AML patients, and treated *in vivo* with either human IL-1β or vehicle. IL-1β treatment promoted expansion of human AML cells in PB and BM of NSG-SGM3 mice (Fig. 1H and I). We compared the therapeutic potential for AML patients in NSG-SGM3 xenografts of boosting IL-1RN and blocking IL-1β. Short-term treatments with IL-1RN and the human IL-1β mAb canakinumab were efficient in reducing the expansion of human leukemic cells, *i.e.* myeloid cells, to similar extents in the PB of NSG-SGM3 mice transplanted with $CD34^+$ progenitors isolated from the BM of AML patient samples (Fig. 1J). Long-term treatment with

IL-1RN was followed up and confirmed reduced numbers of human leukemic cells, *i.e.* $CD45^+$ leukocytes, $CD33^+$ myeloid blasts and $CD34^+$ progenitors in the BM of IL-1RN-treated NSG-SGM3 mice (Fig. 1K). Thus, IL-1RN boost has therapeutic potential for AML patients.

Deletion of IL-1rn induces myelopoiesis in the absence of immunogenic stimulus

To determine the role of IL-1rn deficiency in hematopoiesis, we characterized the IL-1rn-KO mouse strain, which has not been used before in hematopoiesis. Adult IL-1rn-KO mice had higher cellularity in BM, and increased circulating numbers of neutrophils with no abnormalities in spleen (Fig. 2A; Supplementary Fig. S2A and S2B). Relative numbers, fractions and proliferation of LSK were similar between WT and IL-1rn-KO mice (Supplementary Fig. S2C). FACS analysis of the five stem and progenitor cell subsets corresponding to HSC and MPP1-MPP4 [26,27] revealed reduction of HSC and increase of MPP2 in the BM of IL-1rn-KO mice (Fig. 2B; Supplementary Fig. S2D). Absolute numbers of CLP and CMP were unchanged, whereas MEP and GMP were reduced in the BM of IL-1rn-KO versus WT mice (Supplementary Fig. S2E). Numbers of colonies formed *ex vivo* by HSPC were higher in IL-1rn-KO mice (Fig. 2C). Analysis of IL-1rn-KO mice also revealed increased apoptosis in total BM cells (Supplementary Fig. S2F) but not in HSPC (Supplementary Fig. S2G and S2H). We found impact of IL-1rn abrogation in the differentiation bias of hematopoiesis towards the myeloid lineage with significant impairment of B lymphoid development (Fig. 2D; Supplementary Fig. S2I). These results indicate that IL-1rn represses HSPC differentiation under steady-state conditions.

Production of IL-1β in the BM was described under conditions of injury or infection [2]. Our data showed presence of IL-1β in healthy BM and higher IL-1β BM levels in IL-1rn-KO versus WT mice induced by the absence of IL-1rn, under steady-state conditions (Fig. 2E). There were no changes in other BM cytokines (Supplementary Fig. S2J). We then studied whether IL-1rn genetic deletion resulted in globally enhanced IL-1R1. IL-1R1 – expressing cells in the BM were rare but increased in frequency in IL-1rn-KO mice (Fig. 2F). The myeloid bias of progenitors from IL-1rn-KO mice could be prevented *in vitro* (Supplementary Fig. S2K) and *in vivo* by treatment with IL-1RN (Fig. 2G and H) or mAb against IL-1β (Fig. 2I and J). Thus, under steady-state conditions, IL-1rn prevents IL-1β-driven HSPC differentiation into the myeloid lineage at the expense of lymphoid development.

Both IL-1rn-KO LT-HSC and ST-HSC upregulated the expression of *Il1b* gene, whilst *Il1r1* was expressed by HSPC and showed no changes in IL-1rn-KO mice (Fig. 2K). This

suggested engagement of IL-1β-positive feedback loop for IL-1β expression [34] in HSPC in the absence of any immunogenic trigger, due to IL-1rn loss. Myeloid cells, selectively granulocytes, were expanded in the BM of IL-1rn-KO mice (Fig. 2D; Supplementary Fig. S2I) but, unlike HSPC and similarly to monocytes, they showed no engagement of IL-1β-positive feedback loop through IL-1R1 (Fig. 2L and M). Compared to WT HSPC, mRNA levels of *Il1rn* were 170- and 20-fold higher in WT granulocytes and monocytes, respectively (Fig. 2N).

To identify specific changes in HSPC driven by IL-1rn loss, we next performed gene expression profiling by RNA-Seq of WT and IL-1rn-KO LT-HSC, ST-HSC and MPP. We identified impact of IL-1rn abrogation on the transcriptional programs of the LT-HSC and the ST-HSC compartments, with 1435 and 2618 differentially expressed genes, respectively (adjusted $p<0.05$). The effect on MPP was smaller, with 186 genes differentially expressed (Supplementary Fig. S3A). Gene set enrichment analysis (GSEA) for LT-HSC revealed coordinated changes in a variety of genes associated with the immune system, the immune response and leukocyte differentiation (Supplementary Fig. S3B and S3C). GSEA of ST-HSC dysregulated genes identified changes in similar gene programs (Supplementary Fig. S3D and S3E). These changes were related to increased NF-κB transcription factor calculated activity (Fig. 2O), and activation of myeloid differentiation genes including the transcription factor PU.1 (*Spi1*) and some of its target genes like *Cebpa* and *Csf2rb* (Fig. 2P) in IL-1rn-KO HSPC compared to WT HSPC, particularly in LT-HSC and ST-HSC. Various RNA-Seq hits involving genes related to myeloid differentiation (*Spi1*, *Csf2rb*) and genes previously found abnormally expressed in myeloid neoplasias (*Axl*, *Stat3*, *Tlr1*) were confirmed by qRT-PCR (Supplementary Fig. S3F-H) [35-37]. The myeloid bias of progenitors from IL-1rn-KO mice could be reverted *in vivo* by treatment with bortezomib, a proteasome inhibitor that prevents NF-κB activation (Fig. 2Q). Taken together, these results suggest that IL-1β and IL-1rn control myeloid output mainly through the LT-HSC and ST-HSC compartments and transcriptional control of myeloid differentiation pathways dependent on NF-κB activation, with contribution of these cells to *Il1b* production in the absence of immunogenic stimuli. IL-1rn-KO mice develop an IL-1β-induced phenotype reminiscent of early hematopoietic disease.

Deletion of IL-1rn causes damage to the BM stroma in the absence of immunogenic stimulus

Adult IL-1rn-KO mice showed incipient reticulin fibrosis despite non-detectable changes in collagen deposits in the BM (Fig. 3A; Supplementary Fig. S4A). BM MSC numbers, identified

as stromal ($CD45^{-}CD31^{-}Ter119^{-}$) cells positive for CD63 [38], CD105 or CD105 Vcam-1 [39], and *Nestin* expression [17,40], which partially overlap [17,38-40], were reduced in IL-1rn-KO mice (Fig. 3B; Supplementary Fig. S4B and S4C). Reanalysis of publicly available single-cell transcriptional data of mouse BM stromal populations [38] showed that *Cd63* expression largely overlaps with expression of *Lepr*, *Cxcl12*, *Kitl* and *Il1rn* (Supplementary Fig. S4D). Therefore, BM $CD63^{+}$ MSC were chosen for most follow-up studies. Reduced numbers of BM $CD63^{+}$ cells coincided with increased levels of membrane IL-1R1 (Fig. 3C), suggesting potential IL-1β contribution. IL-1β-induced damage to the stromal compartment was confirmed by *in vivo* treatment with IL-1RN or mAb against IL-1β, which rescued numbers of BM $CD63^{+}$ MSC in IL-1rn-KO mice (Fig. 3D and E).

To better understand BM MSC alterations, genome-wide expression was profiled by RNA-Seq in sorted $CD45^{-}CD31^{-}Ter119^{-}CD63^{+}$ cells. Some of the most downregulated genes in BM MSC derived from IL-1rn-KO mice included MSC- (*Lepr*, *Adipoq*) and HSC regulatory- (*Cxcl12*, *Vcam1*, *Angpt1*) genes (Fig. 3F). These changes were confirmed by qRT-PCR (Fig. 3G). qRT-PCR data also revealed engagement of IL-1β-positive feedback loop in BM $CD63^{+}$ MSC from IL-1rn-KO mice through increased *Il1b* expression compared to WT BM $CD63^{+}$ MSC and that these cells produce high levels of *Il1rn* in the BM of adult WT mice (Fig. 3G); 2.8-fold higher than WT granulocytes (Fig. 2N). Taken together, absence of IL-1rn leads to IL-1β-induced damage to the stromal compartment of the BM, with contribution of BM $CD63^{+}$ MSC to *Il1b* production.

Low IL-1rn is present in a mouse model of pre-leukemic myelopoiesis

The reduction of IL-1RN in AML patients and the effect of IL-1rn loss in HSPC suggest a participation in hematological diseases. To address this possibility, we used the *Mx1-Cre* $Nras^{G12D}$ mouse model of aberrant pre-leukemic myelopoiesis [24,41]. As previously described, pre-leukemic $Nras^{G12D}$ HSPC outcompete WT cells (Supplementary Fig. S5A) [41]. Primary $Nras^{G12D}$ mice display a mild hematopoietic phenotype, characterized as chronic myelomonocytic leukemia [42] with increased circulating white blood cells, particularly myeloid cells. In the BM, numbers of c-kit^{+} progenitors and LSK HSPC were higher in $Nras^{G12D}$ mice, with abnormal relative amounts of all LSK subsets (Supplementary Fig. S5B-S5E) [24]. Detailed analysis revealed reduction of HSC and MPP1, and increase of MPP4 in the BM of $Nras^{G12D}$ mice (Supplementary Fig. S5F). More committed CLP and GMP progenitors were expanded in the BM of $Nras^{G12D}$ versus control mice (Supplementary Fig. S5G).

RAS transduces IL-1β signaling through MyD88, and oncogenic RAS results in IL-1β amplification through persistent activation of the autocrine feedback loop in other types of cancer [43]. IL-1β levels in *Nras*^{G12D} BM were increased, while levels of IL-1rn were reduced in the BM of diseased mice (Fig. 4A and B). At this stage, BM hypercellularity was prominent, with expansion of cell subsets previously described to produce IL1-β including neutrophils, monocytes and LSK cells (Fig. 4C). Expression of *Il1b* was not induced in granulocytes or monocytes from the BM of *Nras*^{G12D} mice compared to healthy controls, and these hematopoietic cells did not show reduced expression of *Il1rn* either (Fig. 4D). In contrast, all HSPC subsets expressed higher levels of *Il1b* measured by qRT-PCR, and *Il1rn* was reduced in *Nras*^{G12D} versus control MPP only (Fig. 4E). To better understand the specific contribution of HSPC to the IL-1β-induced inflammatory environment of the BM in *Nras*^{G12D} mice, gene expression profiling was performed by RNA-Seq in LT-HSC, ST-HSC and MPP. The transcriptional programs of both LT-HSC and MPP were influenced by expression of *Nras*^{G12D}, with 894 and 651 differentially expressed genes, respectively. The effect of *Nras*^{G12D} expression on ST-HSC was smaller, with 340 genes differentially expressed (Supplementary Fig. S5H). PCA showed coherent clustering of replicates and revealed that LT-HSC are most influenced by *Nras*^{G12D} expression (Supplementary Fig. S5I). All *Nras*^{G12D} HSPC subsets exhibited increased NF-κB transcription factor calculated activity (Fig. 4F). The myeloid bias of progenitors from *Nras*^{G12D} mice could be improved *in vivo* by treatment with bortezomib (Fig. 4G). Taken together, the biased myelopoiesis in *Nras*^{G12D} mice was dependent on NF-κB activation in HSPC, and HSPC contributed to loss of repression of IL-1β pathway through increased expression of *Il1b*, and reduced expression of *Il1rn* in the case of MPP.

We then studied the stromal compartment and found reduction in BM CD63^{+} MSC numbers (Fig. 4H) together with increased apoptotic rates in *Nras*^{G12D} mice (Fig. 4I). BM CD63^{+} MSC were sorted and qRT-PCR analyses revealed no changes for *Il1b* and reduced expression of *Il1rn* in BM MSC from diseased mice (Fig. 4J). Reduced numbers of Nestin^{+} MSC and activation of apoptosis at the transcriptomic level was confirmed using *Nes-gfp* mice that had previously received *Nras*^{G12D} BM, compared to recipients of control BM (Supplementary Fig. S5J and S5K). Hence, damage to the microenvironment is present in the *Nras*^{G12D} mouse model of pre-leukemic myelopoiesis, and MSC contribute to the low IL-1rn in BM.

Deletion of IL-1rn from the hematopoietic or stromal compartments promotes pre-leukemic myelopoiesis, whereas exogenous IL-1rn protects against it

To address for the role of IL-1rn in hematopoietic cells under pre-leukemic myelopoiesis, we generated mixed-chimera systems using WT and IL-1rn-KO mice; WT and *Nras*^{G12D} mice; or IL-1rn-KO and *Nras*^{G12D} mice, as donors of BM to WT recipients (Fig. 5A). Deletion of IL-1rn in hematopoietic cells exerted a synergistic effect with presence of *Nras*^{G12D} in neighbouring hematopoietic cells to promote myeloid output (Fig. 5B). B cells showed no changes in presence of *Nras*^{G12D} irrespective of WT or IL-1rn deficient competitor cells (Fig. 5C).

To investigate the role of IL-1rn deficiency in the stroma under pre-leukemic myelopoiesis, we then performed transplants using WT and IL-1rn-KO mice as recipients of BM from induced disease-free *Nras*^{G12D} control and *Mx1-Cre Nras*^{G12D} mice (Fig. 5D). Numbers of circulating myeloid cells, particularly monocytes, were higher in IL-1rn-KO mice transplanted with diseased *Nras*^{G12D} cells as compared to WT recipients (Fig. 5E). B cells were expanded only in IL-1rn-KO mice transplanted with diseased *Nras*^{G12D} cells (Fig. 5F). These results suggest that loss of IL-1β repression through IL-1rn deletion within either the BM hematopoietic or stromal compartment may play a role in pre-leukemic disease. The data further demonstrate that the reduced numbers of BM MSC and their *Il1rn* expression in induced *Mx1-Cre Nras*^{G12D} mice (Fig. 4H and J) contributes to *Nras*^{G12D} disease.

Conversely, short-term *in vivo* treatment of induced *Mx1-Cre Nras*^{G12D} mice with IL-1RN (Fig. 5G) ameliorated early signs of abnormal myelopoiesis by means of reduced circulating myeloid cells, particularly monocytes (Fig. 5H), with no detected changes in B cells (Fig. 5I). Thus, the low IL-1rn conditions contribute to the biased myelopoiesis in *Nras*^{G12D} mice. These data uncover the protective effect of the endogenous IL-1 repressor IL-1rn against pre-leukemic myelopoiesis.

Discussion

Despite the emerging role of IL-1β signaling in the pathogenesis of hematological diseases [1,4-12], little is known about the potential contribution of its endogenous counteracting cytokine IL-1RN to healthy and/or malignant hematopoiesis. Here, we find that low expression of *IL1RN* in purified blasts correlated with negative prognosis in a publicly available big cohort of 381 AML patients at diagnosis time [30-32], as well as in matched-pair diagnosis-relapsed AML patients [33]. We provide translational data in primary CD34^{+} progenitors from AML patients showing, for the first time, IL-1RN deregulation in newly diagnosed AML patients, particularly

those with lower differentiation profiles. This was confirmed in a publicly available cohort of AML patients that used purified AML blasts [30-32]. These data are of particular interest considering AML heterogeneity, as it indicates that low IL-1RN is a common event in AML that may contribute to disease aggravation and poor outcome. Future studies aimed at uncovering the underlying molecular mechanisms for IL-1RN reduction in AML will be relevant, and should include methylation in CpG sites as it was previously involved in relapsed versus diagnosis paired AML samples [33].

NF-κB may be activated by multiple signals but in patients, unbalanced IL-1RN in AML $CD34^+$ progenitors associated with higher numbers of cells activated through NF-κB. This is consistent with previous observations of NF-κB activation in human $CD34^+CD38^-$ leukemic stem cells [44]. Chronic IL-1β treatment of NSG-SGM3 mice transplanted with $CD34^+$ progenitors isolated from the BM of AML patients promoted AML cell expansion, in agreement with previous results [4]. Conversely, boosting IL-1RN improved signs of disease in the $Nras^{G12D}$ – driven myeloid pre-leukemia mouse model and in mouse xenografts from AML patients. IL-1RN boost or IL-1β blockade through treatment with canakinumab, currently under Phase II trial for the treatment of low and intermediate risk MDS and CML (NTC04239157), reduced to similar extent the expansion of human myeloid cells, in NSG-SGM3 mice. Pharmacologically, canakinumab provides advantage over anakinra considering their half-lives of 26 days and 4-6h, respectively [1]. Anakinra provides the advantage of potentially better regulated physiological responses. Efforts towards prolonging its half-life by fusion protein technology and/or development of biodegradable polymers to increase its steady-state sustained release are underway [45].

Functionally, loss of IL-1rn *in vivo* biases HSC differentiation into the myeloid lineage and induces excess myeloid lineage expansion reminiscent of early hematological disease, through IL-1R1 and IL-1β overactivation in HSC, in the absence of injury or infection. These findings are consistent with previous observations upon long-term administration of high IL-1β doses [2], but further demonstrate that IL-1rn represses HSC differentiation into the myeloid lineage driven by IL-1β under steady-state conditions. Our mouse studies showed that abnormal myelopoiesis after IL-1rn deletion is mediated mainly through transcriptional activation of IL-1β-induced myeloid differentiation pathways dependent on NF-κB activation in HSPC, consistent with our findings in AML patients. Our results suggest that these effects are mediated mainly through the LT-HSC and ST-HSC compartments, with selective reduction in HSC and increase in MPP2 numbers and colonies *ex vivo*. The myeloid lineage was expanded at the further expense of reduced numbers of MEPs and GMPs, whereas lymphoid lineage repression

in the absence of IL-1rn did not involve active coordinated transcriptomic repression in LT-HSC or ST-HSC, or changes in CLP numbers. This suggests complex effects of IL-1rn deletion in the hematopoietic hierarchy that require additional investigation.

The expanded myeloid compartment in IL-1rn-KO mice may contribute to higher levels of IL-1β in the BM, as previously suggested under IL-1β administration [2]. Unlike myeloid cells, LT-HSC and ST-HSC showed sustained engagement of an IL-1β-positive feedback loop through IL-1R1, suggesting that HSPC are active modulators of inflammation rather than passive receivers. Together, our data suggest that LT-HSC and ST-HSC play a key role in the chronic inflammatory process driven by IL-1rn deletion through IL-1β production and sustained supply of effector inflammatory cells, in the absence of immunogenic triggers. Selective regulatory mechanisms of *Il1b* expression and responsiveness in HSC, MPP, progenitors and fully differentiated myeloid cells, should be subject of future work.

IL-1rn deletion induced alterations to the BM microenvironment, including reduced MSC numbers and their HSC-regulatory activity at the transcriptomic level, which were caused by IL-1β. This is in accordance with our previous observations in the IL-1β inflammatory conditions driven by JAK2-V617F^{+} hematopoietic cells [11]. In view of the overlap with other relevant MSC markers and high expression of *Il1rn*, we used BM CD63^{+} MSC as a surrogate for BM MSC. Normal BM MSC are able to switch between their pro- and anti-inflammatory phenotypes, to initiate inflammation at early stages of damage sensing and to promote its resolution at advanced stages, respectively [46]. Our data suggest that BM CD63^{+} MSC contribute to inflammation with *Il1b* expression in the absence of *Il1rn* with no need of injury or infection sensing.

In further support of our data in human AML, we provide multiple evidence demonstrating unbalanced IL-1rn in aberrant myelopoiesis in mouse. We used the *Nras*^{G12D} model of pre-leukemic myelopoiesis based on IL-1β amplification mediated by oncogenic RAS described in other types of cancer [43]. This paradigm was true for *Nras*^{G12D} HSPC but no other major producers of IL-1β within the differentiated cell compartment, *i.e.* granulocytes and monocytes. Whereas MPP were resistant to IL-1β-positive feedback loop in IL-1rn deficient mice, they expressed higher levels of *Il1b* in the presence of oncogenic NRAS, comparable to LT-HSC and ST-HSC. Thus, the high IL-1β levels in the BM of diseased mice were result of a HSPC-selective gene regulation event together with expansion of IL-1β-producing hematopoietic cell types, *i.e.* HSPC, granulocytes and monocytes, in *Nras*^{G12D} disease. Of note, MPP were the most expanded HSPC subset in *Nras*^{G12D} mice and this was coincident with their abnormally high *Il1b* expression, and selective downregulation of *Il1rn* across all hematopoietic

cell types studied, including LT-HSC and ST-HSC. Thus, both human AML $CD34^+$ cells and mouse pre-leukemic MPP participate to unbalanced IL-1RN. Like IL-1β, selective regulation of IL-1rn production across hematopoietic cells was apparent and its underlying mechanism should be subject of future work. Congruent with our findings in AML patients and IL-1rn-KO mice, low IL-1rn contributed to the biased myelopoiesis in *Nras*^{G12D} mice through pathways dependent on NF-κB activation in HSPC. Our data showed that BM $CD63^+$ MSC contributed to the lower levels of IL-1rn in the BM of *Nras*^{G12D} mice by both their increased apoptotic rates resulting in reduced cell numbers, and selective downregulated expression of *Il1rn*. Absence of IL-1rn from either the hematopoietic or the stromal compartments induced faster progression of *Nras*^{G12D} – driven pre-malignant hematopoiesis. Thus, both compartments are relevant for IL-1rn production to repress myelopoiesis under neoplastic hematopoiesis. Our data are consistent with the idea that inflammation damages the BM microenvironment, which then establishes abnormal communication with HSPC and thereby plays a role in abnormal hematopoiesis [6,11]. We further suggest that this abnormal communication comprises deficient control of IL-1β-induced inflammation by BM MSC through their disrupted switch towards IL-1rn anti-inflammatory phenotype in the presence of a pre-leukemic lesion. The study of the potential role for IL-1RN anti-inflammatory properties of BM MSC in human AML will be of interest.

Collectively, our data support that endogenous IL-1RN represses myelopoiesis in HSPC under steady-state and protects from neoplasia, whereas low IL-1RN is a prognostic marker for reduced survival in AML patients, it contributes to myeloproliferation in the presence of a pre-leukemic lesion and provides a novel mechanistic rationale for IL-1RN boost or IL-1β blockade therapeutic potential in AML patients. We depict a new potential origin for high IL-1β in human AML, and a type of patient that may be particularly good responder to anti-IL-1β therapies over patients that present with high IL-1β resulting from primary lesions in its signaling pathway downstream of IL-1R1.

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Figure 1

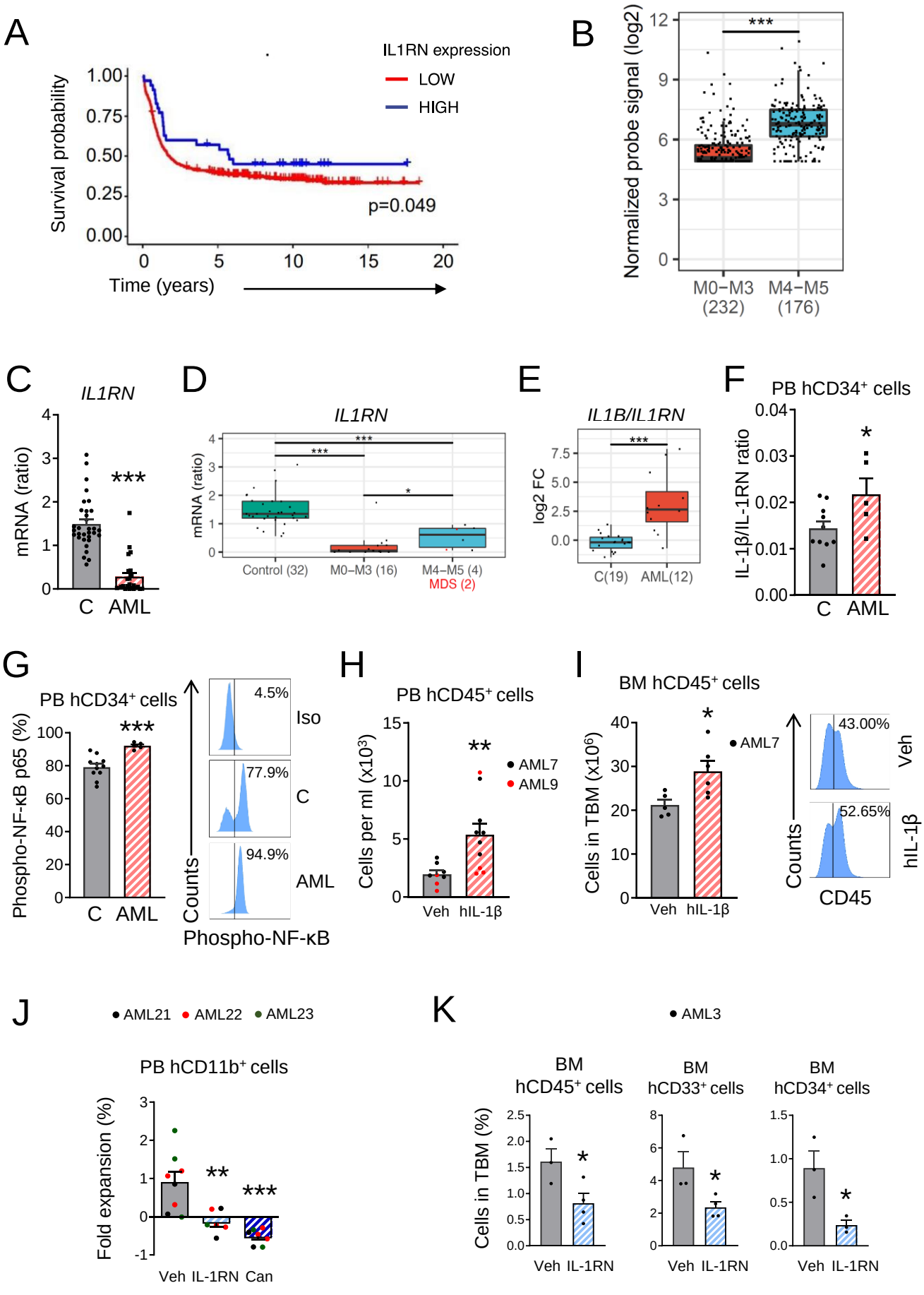

A
IL1RN expression
LOW
HIGH
Survival probability
1.00
0.75
0.50
0.25
0.00
p=0.049
0
5
10
15
20
Time (years)
B
Normalized probe signal (log2)
12
9
6
3
0
***
M0−M3
(232)
M4−M5
(176)
C
IL1RN
mRNA (ratio)
4
3
2
1
0
***
C
AML
D
IL1RN
mRNA (ratio)
***
***
*
Control (32)
M0−M3 (16)
M4−M5 (4)
MDS (2)
E
IL1B/IL1RN
log2 FC
7.5
5.0
2.5
0.0
***
C(19)
AML(12)
F
PB hCD34⁺ cells
IL-1β/IL-1RN ratio
0.04
0.03
0.02
0.01
0.00
*
C
AML
G
PB hCD34⁺ cells
Phospho-NF-κB p65 (%)
100
80
60
40
20
0
***
C
AML
4.5%
Iso
77.9%
C
94.9%
AML
Counts
Phospho-NF-κB
H
PB hCD45⁺ cells
Cells per ml (x10³)
15
10
5
0
**
AML7
AML9
Veh
hIL-1β
I
BM hCD45⁺ cells
Cells in TBM (x10⁶)
40
30
20
10
0
*
AML7
Veh
hIL-1β
43.00%
Veh
52.65%
hIL-1β
Counts
CD45
J
AML21
AML22
AML23
PB hCD11b⁺ cells
Fold expansion (%)
3
2
1
0
-1
**
***
Veh
IL-1RN
Can
K
AML3
BM
hCD45⁺ cells
BM
hCD33⁺ cells
BM
hCD34⁺ cells
Cells in TBM (%)
2.5
2.0
1.5
1.0
0.5
0.0
8
6
4
2
0
1.5
1.0
0.5
0.0
*
*
*
Veh
IL-1RN
Veh
IL-1RN
Veh
IL-1RN

Fig. 1. Low IL-1RN predicts AML progression in patients. (A-B) Analyses of publicly available gene expression profiling through arrays of purified AML blasts from bone marrow (BM) or peripheral blood (PB) obtained from AML patients at diagnosis time (GSE14468). (A) AML patient survival data (n=381) analyzed as a function of *IL1RN* expression expressed as normalized probe signal (log_2) using Cox proportional hazard regression adjusted for age and stratified by cytogenetic risk. p value indicates likelihood ratio test. (B) mRNA expression of *IL1RN* as normalized probe signal (log_2) comparing the French-American-British (FAB) classification of AML subtypes M0-M3 (n=224) versus M4-M5 (n=171). (C) qRT-PCR mRNA expression of *IL1RN* relative to *B2M* in immune magnetically enriched $CD34^+$ circulating progenitors from healthy volunteers (C; n=32) and patients (AML, n=21; MDS, n=2). (D) qRT-PCR mRNA expression of *IL1RN* relative to *B2M* in immune magnetically enriched $CD34^+$ circulating progenitors from healthy volunteers (n=32) and AML patients classified according to FAB categories (n=20; AML M0-M3, n=16; AML M4-M5, n=4). MDS patients were considered together with the AML M4-M5 group (MDS, n=2). (E) qRT-PCR mRNA expression of *IL1B* relative to *B2M*, and expressed as log_2 fold change (FC) versus mean value of the ratio *IL1RN* relative to *B2M*, in immune magnetically enriched $CD34^+$ circulating progenitors from healthy volunteers (n=19) and AML patients (n=12). (F) IL-1β versus IL-1RN protein levels in immune magnetically enriched $CD34^+$ circulating progenitors from healthy volunteers (n=10) and AML patients (n=5). (G) Percentage of singlet cells positive for phosphorylated NF-κB analyzed by fluorescence-activated cell sorting (FACS) in healthy volunteers (n=11) and AML patients (n=5), and representative FACS histograms (Iso; IgG2b Isotype negative control). (B-G) Numbers of patients in parenthesis. (H-K) NSG-SGM3 mice were transplanted with BM cells or immune magnetically enriched $CD34^+$ progenitors from the BM of AML patients, used for *in vivo* treatments and analyzed by FACS. (H-I) *In vivo* treatment with IL-1β or vehicle. (H) Treatment started 4-7 weeks after transplant and continued for 4 weeks (AML9, n=3-4) and 16 weeks (AML7, n=5-6). Number of human $CD45^+$ hematopoietic cells per mL of PB in transplanted NSG-SGM3 mice. (I) Representative FACS histograms and number of cells in total BM (TBM) of human $CD45^+$ hematopoietic cells in NSG-SGM3 mice transplanted with AML7 and treated for 16 weeks with vehicle (n=5) or IL-1β (n=6). (J-K) *In vivo* IL-1RN boost or IL-1β blockade versus vehicle. (J) *In vivo* treatment with IL-1RN (n=6), canakinumab (n=8) or vehicle (n=8) started 6 weeks after the transplant and continued for 6 weeks (AML21, n=2; AML22, n=3; AML23, n=1-3). Expansion of human leukemic cells represented as percentage of change versus time 0 (6 weeks post-transplant), of human $CD11b^+$ hematopoietic cells in PB of NSG-SGM3 mice. (K) *In vivo* treatment with IL-1RN (n=4) or

vehicle (n=3) started 6 weeks after the transplant and continued for 36 weeks. Frequency of human $CD45^+$, $CD33^+$ and $CD34^+$ hematopoietic cells in TBM of NSG-SGM3 mice transplanted with AML3 patient sample. Data are individual data points, and means ± S.E.M. (B, D, E) For box and whisker plots, horizontal line indicates median, and box top and bottom are upper and lower quartiles. Whiskers extend to most extreme point within 1.5 times the interquartile range of the box. ***$p<0.001$, **$p<0.01$, *$p<0.05$.

Figure 2

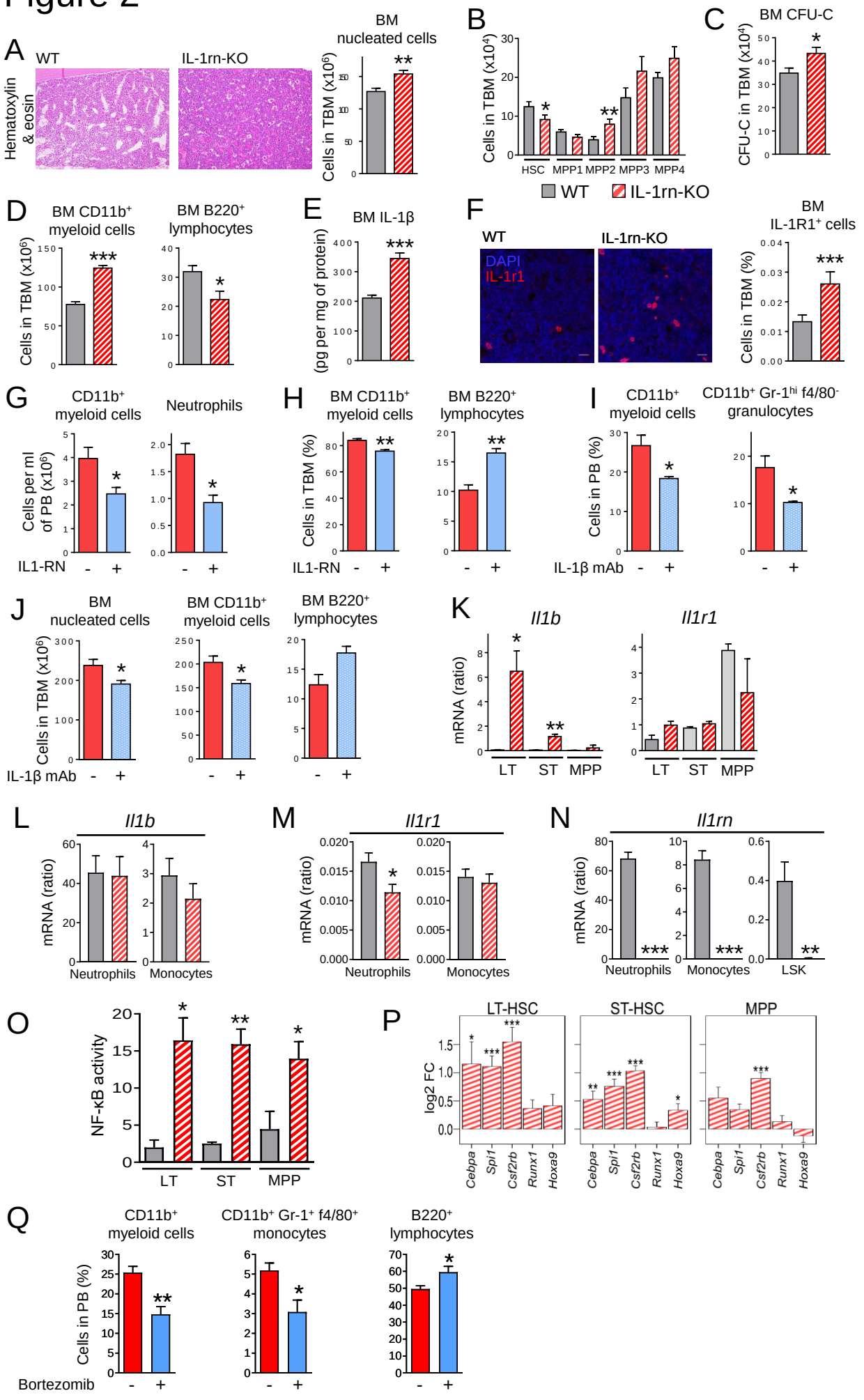

A
WT
IL-1rn-KO
Hematoxylin & eosin
BM nucleated cells
Cells in TBM (x10⁶)
B
Cells in TBM (x10⁴)
HSC
MPP1
MPP2
MPP3
MPP4
WT
IL-1rn-KO
C
BM CFU-C
CFU-C in TBM (x10⁴)
D
BM CD11b⁺ myeloid cells
BM B220⁺ lymphocytes
Cells in TBM (x10⁶)
E
BM IL-1β
(pg per mg of protein)
F
WT
IL-1rn-KO
DAPI
IL-1r1
BM IL-1R1⁺ cells
Cells in TBM (%)
G
CD11b⁺ myeloid cells
Neutrophils
Cells per ml of PB (x10⁶)
IL1-RN
H
BM CD11b⁺ myeloid cells
BM B220⁺ lymphocytes
Cells in TBM (%)
IL1-RN
I
CD11b⁺ myeloid cells
CD11b⁺ Gr-1hi f4/80- granulocytes
Cells in PB (%)
IL-1β mAb
J
BM nucleated cells
BM CD11b⁺ myeloid cells
BM B220⁺ lymphocytes
Cells in TBM (x10⁶)
IL-1β mAb
K
Il1b
Il1r1
mRNA (ratio)
LT
ST
MPP
L
Il1b
mRNA (ratio)
Neutrophils
Monocytes
M
Il1r1
mRNA (ratio)
Neutrophils
Monocytes
N
Il1rn
mRNA (ratio)
Neutrophils
Monocytes
LSK
O
NF-κB activity
LT
ST
MPP
P
LT-HSC
ST-HSC
MPP
log2 FC
Cebpa
Spi1
Csf2rb
Runx1
Hoxa9
Q
CD11b⁺ myeloid cells
CD11b⁺ Gr-1⁺ f4/80⁺ monocytes
B220⁺ lymphocytes
Cells in PB (%)
Bortezomib

Fig. 2. Deletion of IL-1rn triggers IL-1β-induced myelopoiesis in the absence of immunogenic stimulus. (A) Representative hematoxylin and eosin stainings of femoral bone marrow (BM) sections (magnification, x100) from C57BL/6J wild-type (WT; n=8) and IL-1rn knockout (IL-1rn-KO; n=10) mice and total BM (TBM) nucleated cells (n=16-23). (B) TBM number of lin$^-$c-kit$^+$Sca-1$^+$ (LSK) cell subsets: LSK CD34$^-$Flt3$^-$CD48$^-$CD150$^+$, hematopoietic stem cells (HSC); LSK CD34$^+$Flt3$^-$CD48$^-$CD150$^+$, multipotent progenitors 1 (MPP1); LSK CD34$^+$Flt3$^-$CD48$^+$CD150$^+$, MPP2; LSK CD34$^+$Flt3$^-$CD48$^+$CD150$^-$, MPP3; LSK CD34$^+$Flt3$^+$CD48$^+$CD150$^-$, MPP4, analyzed by fluorescence-activated cell sorting (FACS) (n=9). (C) BM colony-forming unit cells (CFU-C) (n=6-10). (D) TBM number of CD11b$^+$ myeloid cells and B220$^+$ lymphocytes analyzed by FACS (n=7-8). (E) IL-1β content in BM extracellular fluid (n=9-12). (F) Representative IL-1 receptor 1 (IL-1R1) stainings (red) of BM sections; nuclei were counterstained with DAPI (blue); scale bar, 20 μm, and quantification of the frequency of TBM IL-1R1-expressing cells (n=7). (G-H) IL-1rn-KO mice were treated with vehicle (n=5) or IL-1RN (n=4). (G) Number of CD11b$^+$ myeloid cells analyzed by FACS and number of neutrophils measured with hematological counter, per mL of peripheral blood (PB) in IL-1rn-KO mice treated for 4 weeks with vehicle or IL-1RN. (H) Frequency of CD11b$^+$ myeloid cells and B220$^+$ lymphocytes analyzed by FACS in TBM of IL-1rn-KO mice treated for 10 weeks with vehicle or IL-1RN. (I-J) IL-1rn-KO mice were treated with IgG1 kappa isotype or mouse IL-1β monoclonal antibody (IL-1β mAb) (n=5). (I) Frequency of CD11b$^+$ myeloid cells and CD11b$^+$Gr-1^{hi}f4/80$^-$ granulocytes analyzed by FACS in PB of IL-1rn-KO mice treated with IgG1 kappa isotype or IL-1β mAb for 16 weeks. (J) TBM nucleated cells, CD11b$^+$ myeloid cells and B220$^+$ lymphocytes analyzed by FACS in IL-1rn-KO mice treated with IgG1 kappa isotype or IL-1β mAb for 18 weeks. (K) qRT-PCR mRNA expression of *Il1b* and *Il1r1* relative to *Gapdh* in FACS-sorted LSK subsets: LSK CD34$^-$Flt3$^-$, long-term HSC (LT-HSC); LSK CD34$^+$Flt3$^-$, short-term HSC (ST-HSC); LSK CD34$^+$Flt3$^+$ MPP, from WT and IL-1rn-KO mice (n=3). (L-N) qRT-PCR mRNA expression of (L) *Il1b*, (M) *Il1r1* and (N) *Il1rn* relative to *Gapdh* in FACS-sorted CD11b$^+$Gr-1^{hi}f4/80$^-$ granulocytes (n=7-8), CD11b$^+$Gr-1$^+$f4/80$^+$ monocytes (n=7-11) and LSK cells (n=4) from WT and IL-1rn-KO mice. (O-P) RNA sequencing in FACS-sorted LT-HSC, ST-HSC and MPP from IL-1rn-KO versus WT mice (n=3). (O) NF-κB transcription factor activity calculated based on NF-κB target gene expression levels. Data are means ± S.E.M of the main principal component analysis (PCA) component. (P) Expression of myeloid differentiation genes. (Q) Frequency of CD11b$^+$ myeloid cells, CD11b$^+$Gr-1$^+$f4/80$^+$ monocytes and B220$^+$ lymphocytes analyzed by FACS in PB of IL-

1rn-KO mice treated with vehicle (n=7) or bortezomib (n=6), 12 weeks after the start of the treatment. Data are means ± S.E.M. ***p<0.001, **p<0.01, *p<0.05.

Figure 3

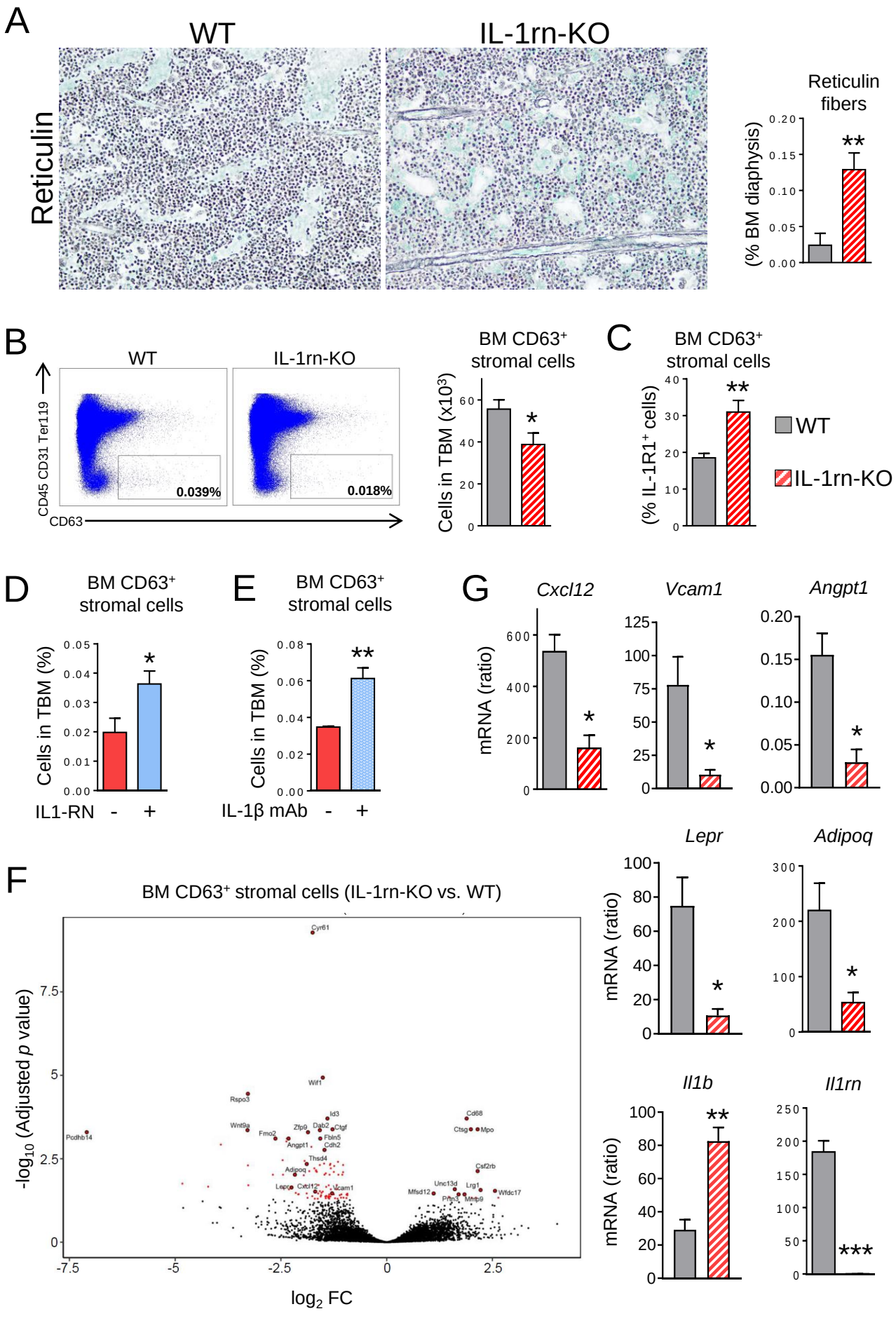

A
WT
IL-1rn-KO
Reticulin
Reticulin fibers
(% BM diaphysis)
0.20
0.15
0.10
0.05
0.00
**
B
WT
IL-1rn-KO
CD45 CD31 Ter119
CD63
0.039%
0.018%
BM CD63+ stromal cells
Cells in TBM (x10^3)
60
40
20
0
*
C
BM CD63+ stromal cells
(% IL-1R1+ cells)
40
30
20
10
0
**
WT
IL-1rn-KO
D
BM CD63+ stromal cells
Cells in TBM (%)
0.05
0.04
0.03
0.02
0.01
0.00
*
IL1-RN - +
E
BM CD63+ stromal cells
Cells in TBM (%)
0.08
0.06
0.04
0.02
0.00
**
IL-1β mAb - +
G
Cxcl12
Vcam1
Angpt1
mRNA (ratio)
600
400
200
0
125
100
75
50
25
0
0.20
0.15
0.10
0.05
0.00
*
Lepr
Adipoq
100
80
60
40
20
0
300
200
100
0
*
F
BM CD63+ stromal cells (IL-1rn-KO vs. WT)
-log10 (Adjusted p value)
7.5
5
2.5
0
-7.5
-5
-2.5
0
2.5
log2 FC
Cyr61
Wif1
Rspo3
Id3
Wnt9a
Fmo2
Zfp9
Dab2
Ctgf
Fbln5
Angpt1
Cdh2
Pcdhb14
Thsd4
Adipoq
Lepr
Cxcl12
Vcam1
Cd68
Ctsg
Mpo
Csf2rb
Unc13d
Lrg1
Mfsd12
Wfdc17
Il1b
Il1rn
100
80
60
40
20
0
**
250
200
150
100
50
0
***

Fig. 3. Deletion of IL-1rn causes IL-1β-induced damage to the bone marrow stroma in the absence of immunogenic stimulus. (A) Representative Gordon and Sweet's stainings of femoral bone marrow (BM) sections (magnification, x200) from C57BL/6J wild-type (WT; n=6) and IL-1rn knockout (IL-1rn-KO; n=6) mice, and quantification of reticulin fibers (arrows) in BM diaphysis. (B) Representative fluorescence-activated cell sorting (FACS) analysis and total BM (TBM) number of immunophenotypically defined $CD45^{-}CD31^{-}Ter119^{-}CD63^{+}$ stromal cells (n=11-15). (C) Frequency of TBM $CD45^{-}CD31^{-}Ter119^{-}CD63^{+}$ stromal cells expressing IL-1r1 (n=12) analyzed by FACS. (D) Frequency of TBM $CD45^{-}CD31^{-}Ter119^{-}CD63^{+}$ stromal cells analyzed by FACS in IL-1rn-KO mice treated for 10 weeks with vehicle (n=5) or IL-1RN (n=6). (E) Frequency of TBM $CD45^{-}CD31^{-}Ter119^{-}CD63^{+}$ stromal cells analyzed by FACS in IL-1rn-KO mice treated for 18 weeks with IgG1 kappa isotype or mouse IL-1β monoclonal antibody (IL-1β mAb) (n=5). (F) RNA sequencing (RNA-Seq) in FACS-sorted BM $CD45^{-}CD31^{-}Ter119^{-}CD63^{+}$ stromal cells (n=3). Volcano plot shows differentially expressed genes (red dots) and depicts genes of interest. Adjusted $p<0.01$; fold change (FC), $-0.5>\log_2 FC>0.5$. (G) qRT-PCR mRNA expression of selected RNA-Seq hits (*Cxcl12*, *Vcam1*, *Angpt1*, *Lepr*, *Adipoq*), *Il1b* and *Ilrn* relative to *Gapdh* in independent biological samples (n=3; *Lepr*, n=10-11). Data are means ± S.E.M. ***p<0.001, **p<0.01, *p<0.05.

Figure 4

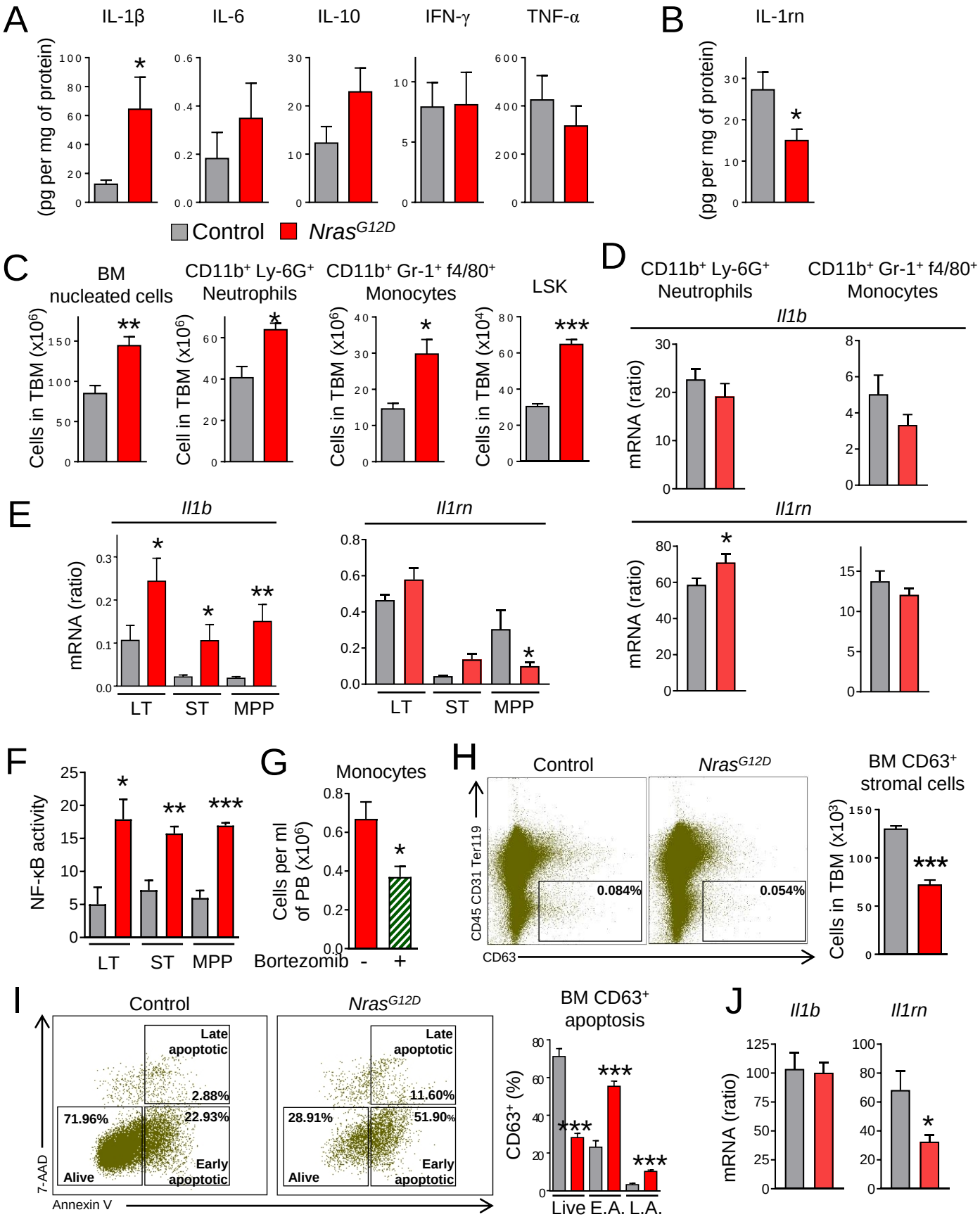
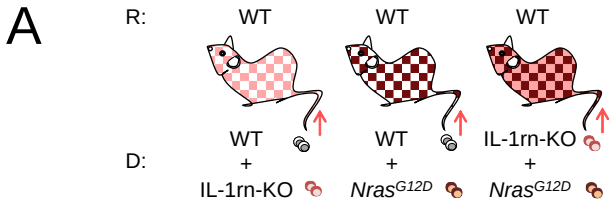
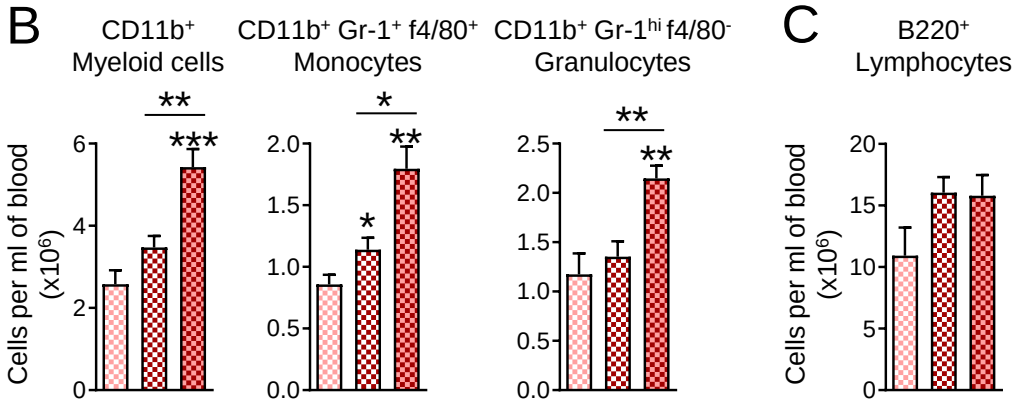
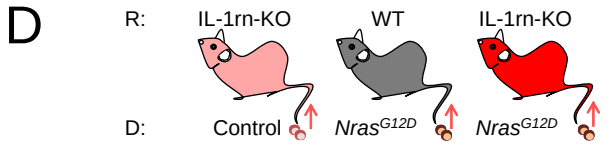

A
IL-1β
IL-6
IL-10
IFN-γ
TNF-α
(pg per mg of protein)
Control
Nras^G12D
B
IL-1rn
(pg per mg of protein)
C
BM nucleated cells
CD11b+ Ly-6G+ Neutrophils
CD11b+ Gr-1+ f4/80+ Monocytes
LSK
Cells in TBM (x10^6)
Cell in TBM (x10^6)
Cells in TBM (x10^4)
D
CD11b+ Ly-6G+ Neutrophils
CD11b+ Gr-1+ f4/80+ Monocytes
Il1b
Il1rn
mRNA (ratio)
E
Il1b
Il1rn
mRNA (ratio)
LT
ST
MPP
F
NF-κB activity
LT
ST
MPP
G
Monocytes
Cells per ml of PB (x10^6)
Bortezomib - +
H
Control
Nras^G12D
CD45 CD31 Ter119
CD63
0.084%
0.054%
BM CD63+ stromal cells
Cells in TBM (x10^3)
I
Control
Nras^G12D
Late apoptotic
2.88%
71.96%
22.93%
Early apoptotic
Alive
11.60%
28.91%
51.90%
7-AAD
Annexin V
BM CD63+ apoptosis
CD63+ (%)
Live E.A. L.A.
J
Il1b
Il1rn
mRNA (ratio)

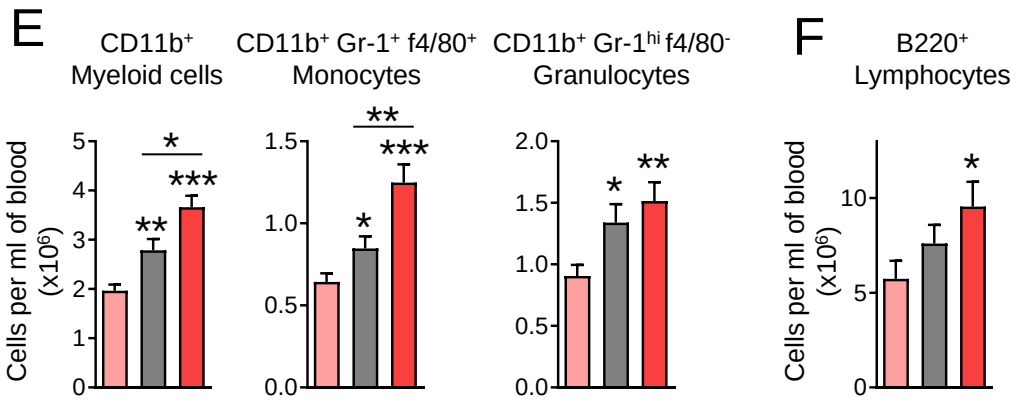
Fig. 4. Low IL-1rn is present in a mouse model of pre-leukemic myelopoiesis. (A) Cytokine levels in bone marrow (BM) extracellular fluid (BMEF) from control (*Mx1-Cre*$^{-}$ *Nras*^{G12D}, n=4-6) and *Nras*^{G12D} (*Mx1-Cre*$^{+}$ *Nras*^{G12D}, n=3-5) mice, 20 weeks after poly-inosine:poly-cytidylic acid (polyI:polyC) induction. (B) IL-1rn levels in BMEF from control (n=19) and *Nras*^{G12D} (n=18) mice, 10-30 weeks after polyI:polyC. (C) Numbers of cells in total BM (TBM), and TBM number of CD11b^{+}Ly6G^{+} neutrophils, CD11b^{+}Gr-1^{+}f4/80^{+} monocytes and lin^{-}c-kit^{+}Sca-1^{+} (LSK) cells analyzed by fluorescence-activated cell sorting (FACS), from control and *Nras*^{G12D} (n=3) mice, 28-32 weeks after polyI:polyC. (D) qRT-PCR mRNA expression of *Il1b* and *Il1rn* relative to *Gapdh* in FACS-sorted CD11b^{+}Gr-1^{hi}f4/80^{-} granulocytes (n=9-16) and CD11b^{+}Gr-1^{+}f4/80^{+} monocytes (n=11-17), from control and *Nras*^{G12D} mice, 12-30 weeks after polyI:polyC. (E) qRT-PCR mRNA expression of *Il1b* and *Il1rn* relative to *Gapdh* in FACS-sorted LSK subsets: LSK CD34^{-}Flt3^{-}, long-term hematopoietic stem cells (LT-HSC); LSK CD34^{+}Flt3^{-}, short-term HSC (ST-HSC); LSK CD34^{+}Flt3^{+}, multipotent progenitors (MPP) (n=4-8), from control and *Nras*^{G12D} mice, 11-12 weeks after polyI:polyC. (F) NF-κB transcription factor activity calculated based on NF-κB target gene expression levels from RNA sequencing data of FACS-sorted LT-HSC, ST-HSC and MPP, from control and *Nras*^{G12D} mice, 11-12 weeks after polyI:polyC (n=3). Data are means ± S.E.M of the main principal component analysis component. (G) Number of monocytes per mL of peripheral blood (PB) measured with hematological counter in *Nras*^{G12D} mice treated with vehicle (n=3) or bortezomib (n=4), 8 weeks after the first bortezomib injection and 52 weeks after polyI:polyC. (H-J) Study of the BM stromal compartment. (H) Representative FACS analysis and TBM number of CD45^{-}CD31^{-}Ter119^{-}CD63^{+} stromal cells from control and *Nras*^{G12D} (n=3) mice, 28-32 weeks after polyI:polyC. (I) Representative FACS analysis and frequencies of live, early apoptotic (EA) and late apoptotic (LA) cells within the CD45^{-}CD31^{-}Ter119^{-}CD63^{+} stromal compartment in TBM from control (n=6) and *Nras*^{G12D} (n=5) mice, 36 weeks after polyI:polyC. (J) qRT-PCR mRNA expression of *Il1b* and *Ilrn* relative to *Gapdh* in FACS-sorted CD45^{-}CD31^{-}Ter119^{-}CD63^{+} stromal cells from control (n=16-20) and *Nras*^{G12D} (n=19-24) mice, 12-30 weeks after polyI:polyC. Data are means ± S.E.M. ***p<0.001, **p<0.01, *p<0.05.

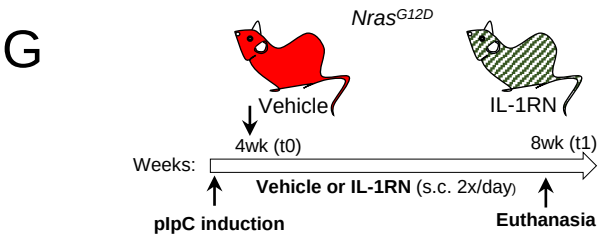
Figure 5


A
R:
WT
WT
WT
D:
WT + IL-1rn-KO
WT + NrasG12D
IL-1rn-KO + NrasG12D


B
CD11b+ Myeloid cells
CD11b+ Gr-1+ f4/80+ Monocytes
CD11b+ Gr-1hi f4/80- Granulocytes
Cells per ml of blood (x10⁶)
C
B220+ Lymphocytes
Cells per ml of blood (x10⁶)


D
R:
IL-1rn-KO
WT
IL-1rn-KO
D:
Control
NrasG12D
NrasG12D


E
CD11b+ Myeloid cells
CD11b+ Gr-1+ f4/80+ Monocytes
CD11b+ Gr-1hi f4/80- Granulocytes
Cells per ml of blood (x10⁶)
F
B220+ Lymphocytes
Cells per ml of blood (x10⁶)


G
NrasG12D
Vehicle
IL-1RN
4wk (t0)
8wk (t1)
Weeks:
Vehicle or IL-1RN (s.c. 2x/day)
pIpC induction
Euthanasia

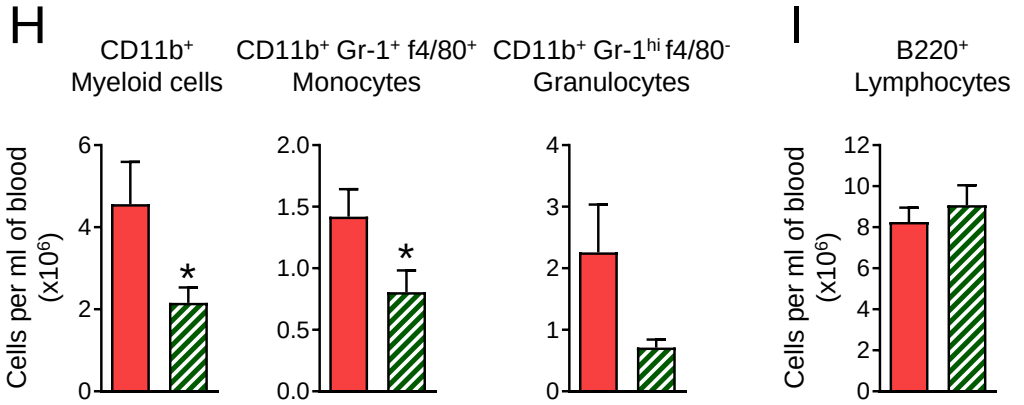

H
CD11b+ Myeloid cells
CD11b+ Gr-1+ f4/80+ Monocytes
CD11b+ Gr-1hi f4/80- Granulocytes
Cells per ml of blood (x10⁶)
I
B220+ Lymphocytes
Cells per ml of blood (x10⁶)

Fig. 5. Deletion of IL-1rn from the hematopoietic or stromal compartments promotes pre-leukemic myelopoiesis, and it is therapeutically targetable. (A-C) C57BL/6J wild-type (WT) mice were used as recipients in competitive transplants (1:1) of bone marrow (BM) nucleated cells from WT and IL-1rn knockout (IL-1rn-KO) mice (n=5), WT and *Nras*^{G12D} mice (n=5) or IL-1rn-KO and *Nras*^{G12D} mice (n=6), and analyzed in peripheral blood (PB) 32 weeks after the transplant by fluorescence-activated cell sorting (FACS). (A) Illustration of the experimental design. R: recipient. D: donor cells. (B) Number of CD11b^{+} myeloid cells, CD11b^{+}Gr-1^{+}f4/80^{+} monocytes and CD11b^{+}Gr-1^{hi}f4/80^{-} granulocytes per mL of PB. (C) Number of B220^{+} lymphocytes per mL of PB. (D-F) C57BL/6J WT mice were used as recipients of BM nucleated cells from *Nras*^{G12D} mice (n=20-21), and IL-1rn-KO mice were used as recipients of BM nucleated cell of control (n=19) and *Nras*^{G12D} mice (n=19), and analyzed by FACS in PB 4-8 weeks after the transplant. (D) Illustration of the experimental design. (E) Number of CD11b^{+} myeloid cells, CD11b^{+}Gr-1^{+}f4/80^{+} monocytes and CD11b^{+}Gr-1^{hi}f4/80^{-} granulocytes per mL of PB. (F) Number of B220^{+} lymphocytes per mL of PB. (G-I) *Nras*^{G12D} mice were induced with poly-inosine:poly-cytidylic acid (polyI:polyC) and 4 weeks after, treated with vehicle (n=9) or IL-1RN (n=8) for 4 weeks and analyzed by FACS in PB. (G) Illustration of the experimental design. (H) Number of CD11b^{+} myeloid cells, CD11b^{+}Gr-1^{+}f4/80^{+} monocytes and CD11b^{+}Gr-1^{hi}f4/80^{-} granulocytes per mL of PB. (I) Number of B220^{+} lymphocytes per mL of PB. Data are means ± S.E.M. ***p<0.001, **p<0.01, *p<0.05.

Figure S1

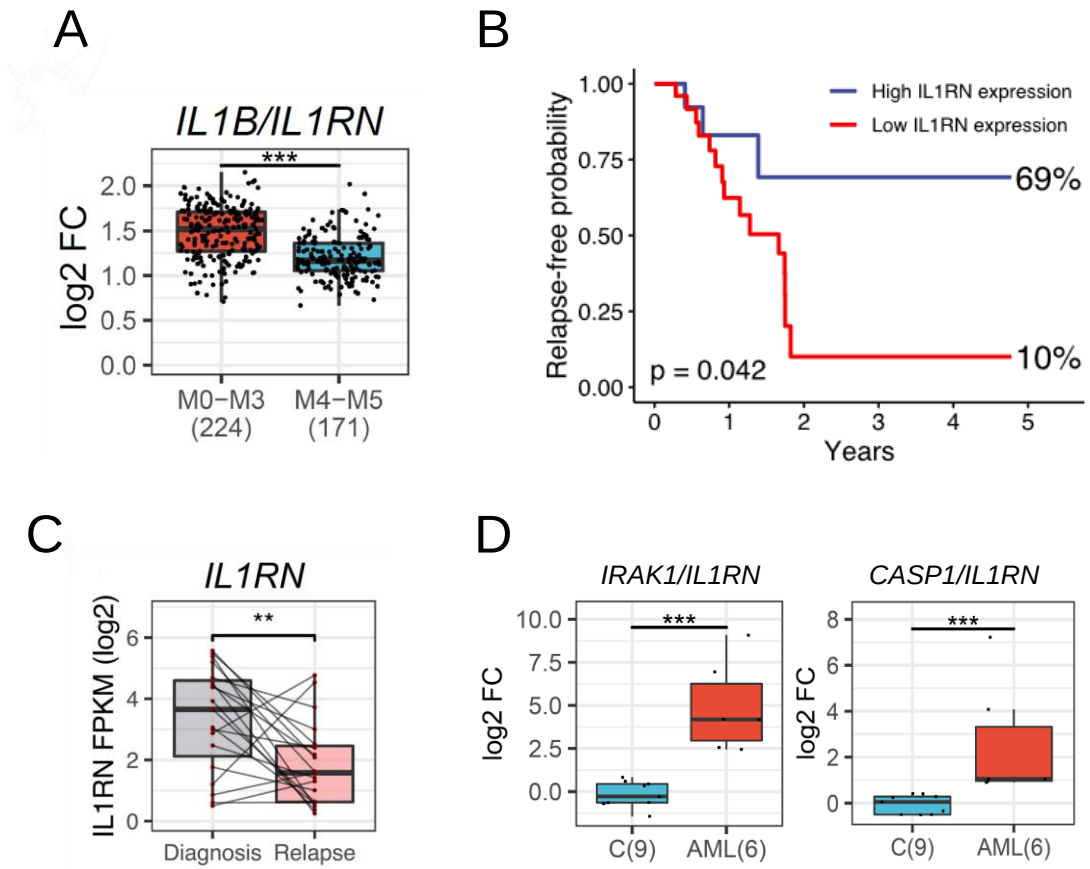

A
IL1B/IL1RN
***
log2 FC
2.0
1.5
1.0
0.5
0.0
M0−M3
(224)
M4−M5
(171)
B
Relapse-free probability
1.00
0.75
0.50
0.25
0.00
High IL1RN expression
Low IL1RN expression
69%
10%
p = 0.042
0
1
2
3
4
5
Years
C
IL1RN
**
IL1RN FPKM (log2)
6
4
2
0
Diagnosis
Relapse
D
IRAK1/IL1RN
***
log2 FC
10.0
7.5
5.0
2.5
0.0
C(9)
AML(6)
CASP1/IL1RN
***
log2 FC
8
6
4
2
0
C(9)
AML(6)

Figure S1 (Related to Figure 1). Low IL-1RN predicts AML progression in patients. (A) Analyses of publicly available gene expression profiling through arrays of purified AML blasts from bone marrow (BM) or peripheral blood (PB) obtained from AML patients at diagnosis time (GSE14468). mRNA expression of *IL1B* as normalized probe signal (log2) expressed as log2 fold change (FC) versus mean value of mRNA expression of *IL1RN* as normalized probe signal, comparing the French-American-British (FAB) classification of AML subtypes M0-M3 (n=224) versus M4-M5 (n=171). (B-C) Analyses of publicly available RNA sequencing of enriched AML blasts from BM obtained from 19 matched-pair AML patients (dbGaP accession phs001027). (B) AML patient relapse-free probability using Kaplan-Meier survival analysis, with expression above or below mean *IL1RN* expression. p value indicates log-rank test. (C) Expression of *IL1RN* (log2 FPKM). (D) qRT-PCR mRNA expression of *CASP1* and *IRAK1* relative to *B2M*, and expressed as $\log_2$ fold change (FC) versus mean value of the ratio *IL1RN* relative to *B2M*, in immune magnetically enriched $CD34^+$ circulating progenitors from healthy controls (C; n=9) and AML patients (n=6). (A, D) Numbers of patients in parenthesis. (A, C, D) For box and whisker plots, horizontal line indicates median, and box top and bottom are upper and lower quartiles. Whiskers extend to most extreme point within 1.5 times the interquartile range of the box. Individual data points are shown. ***p<0.001, **p<0.01.

Figure S2 (I)

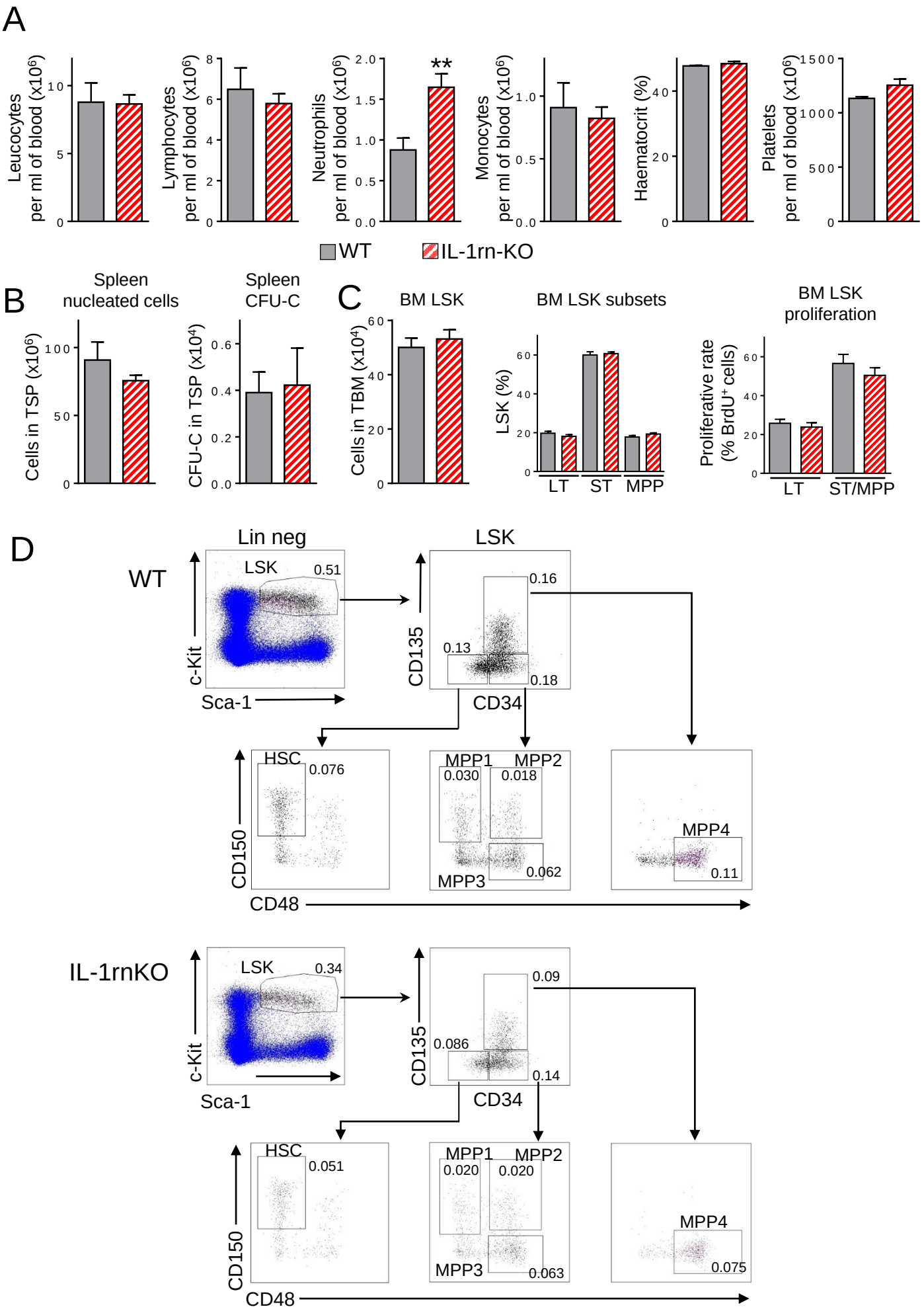

A
Leucocytes per ml of blood (x10⁶)
Lymphocytes per ml of blood (x10⁶)
Neutrophils per ml of blood (x10⁶)
**
Monocytes per ml of blood (x10⁶)
Haematocrit (%)
Platelets per ml of blood (x10⁶)
WT
IL-1rn-KO
B
Spleen nucleated cells
Cells in TSP (x10⁶)
Spleen CFU-C
CFU-C in TSP (x10⁴)
C
BM LSK
Cells in TBM (x10⁴)
BM LSK subsets
LSK (%)
LT
ST
MPP
BM LSK proliferation
Proliferative rate (% BrdU⁺ cells)
LT
ST/MPP
D
WT
Lin neg
LSK
0.51
c-Kit
Sca-1
LSK
0.16
0.13
0.18
CD135
CD34
HSC
0.076
MPP1
MPP2
0.030
0.018
0.062
MPP3
MPP4
0.11
CD150
CD48
IL-1rnKO
LSK
0.34
c-Kit
Sca-1
0.09
0.086
0.14
CD135
CD34
HSC
0.051
MPP1
MPP2
0.020
0.020
0.063
MPP3
MPP4
0.075
CD150
CD48

Figure S2 (II)

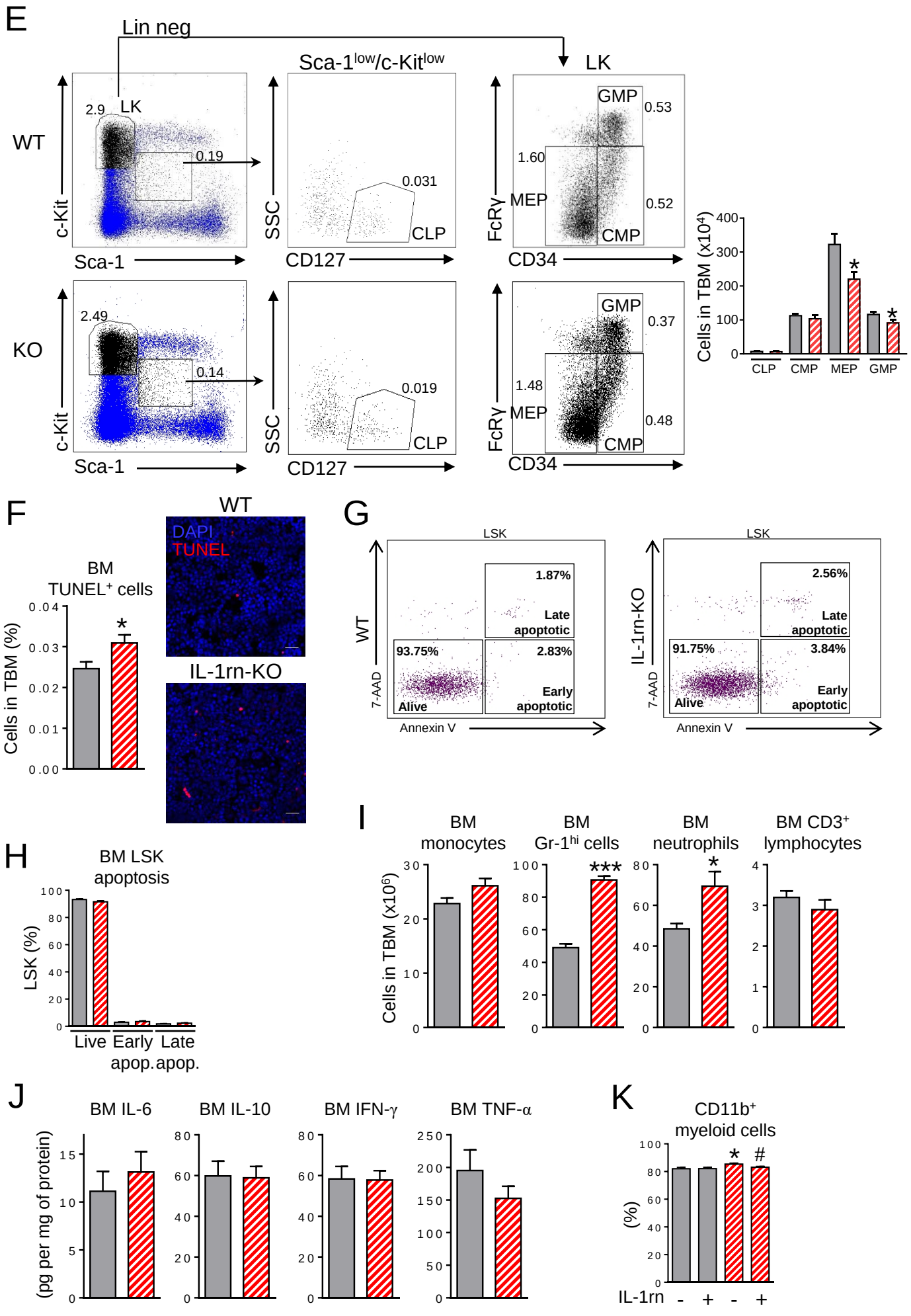

E
Lin neg
Sca-1low/c-Kitlow
LK
WT
KO
c-Kit
Sca-1
SSC
CD127
FcRγ
CD34
2.9
LK
0.19
0.031
CLP
GMP
0.53
1.60
MEP
0.52
CMP
2.49
0.14
0.019
0.37
1.48
0.48
Cells in TBM (x10⁴)
CLP
CMP
MEP
GMP
F
WT
DAPI
TUNEL
IL-1rn-KO
BM
TUNEL⁺ cells
Cells in TBM (%)
G
LSK
WT
IL-1rn-KO
7-AAD
Annexin V
1.87%
Late apoptotic
93.75%
2.83%
Alive
Early apoptotic
2.56%
91.75%
3.84%
H
BM LSK apoptosis
LSK (%)
Live
Early apop.
Late apop.
I
BM monocytes
BM Gr-1hi cells
BM neutrophils
BM CD3⁺ lymphocytes
Cells in TBM (x10⁶)
J
BM IL-6
BM IL-10
BM IFN-γ
BM TNF-α
(pg per mg of protein)
K
CD11b⁺ myeloid cells
(%)
IL-1rn - + - +

Figure S2 (Related to Figure 2). Deletion of IL-1rn triggers IL-1β-induced myelopoiesis in the absence of immunogenic stimulus. (A) Hematological counts per mL of peripheral blood (PB) from C57BL/6J wild-type (WT; n=6) and IL-1rn knockout (IL-1rn-KO; n=10) mice. (B) Total number of spleen nucleated cells (TSP) and SP colony-forming unit cells (CFU-C) from WT (n=3) and IL-1rn-KO (n=5) mice. (C) Total bone marrow number (TBM) of $lin^-c\text{-}kit^+Sca\text{-}1^+$ (LSK) cells (n=10-11), frequencies of BM LSK subsets (LSK $CD34^-Flt3^-$, long-term hematopoietic stem cells (LT-HSC); LSK $CD34^+Flt3^-$, short-term HSC (ST-HSC); LSK $CD34^+Flt3^+$, multipotent progenitors (MPP)) (n=9-11), and proliferative rate by bromodeoxyuridine (BrdU) incorporation *in vivo* over 24h within BM LSK subsets $CD34^-$ (LT-HSC) and $CD34^+$ (ST-HSC/MPP) (n=5-7), analyzed by fluorescence-activated cell sorting (FACS). (D) Representative FACS analysis of BM hematopoietic stem and progenitor cell subsets corresponding to HSC (LSK $CD34^-Flt3^-CD48^-CD150^+$), MPP1 (LSK $CD34^+Flt3^-CD48^-CD150^+$), MPP2 (LSK $CD34^+Flt3^-CD48^+CD150^+$), MPP3 (LSK $CD34^+Flt3^-CD48^+CD150^-$) and MPP4 (LSK $CD34^+Flt3^+CD48^+CD150^-$) from WT and IL-1rn-KO mice. (E) Representative FACS analysis and TBM number of common lymphoid progenitors (CLP, $lin^-c\text{-}Kit^{low}Sca\text{-}1^{low}CD127^+$), common myeloid progenitors (CMP, $lin^-c\text{-}Kit^+Sca\text{-}1^-$ (LK) $CD34^+FcR\gamma^-$), megakaryocyte erythroid progenitors (MEP, LK $CD34^-FcR\gamma^-$) and granulocyte-monocyte progenitors (GMP, LK $CD34^+FcR\gamma^+$) from WT and IL-1rn-KO mice (n=9). (F) Apoptotic rate of TBM nucleated cells (n=6), and representative TUNEL staining (red) of BM sections; nuclei were counterstained with DAPI (blue). Scale bar, 20 μm. (G) Representative FACS analysis to identify the fraction of live, early apoptotic and late apoptotic cells within the LSK compartment in the BM from WT and IL-1rn-KO mice. (H) Apoptotic rate within the BM LSK compartment (n=15-20). (I) TBM number of $CD11b^+Gr\text{-}1^+f4/80^+$ monocytes, $CD11b^+Gr\text{-}1^{hi}f4/80^-$ granulocytes, $CD11b^+Ly6G^+$ neutrophils and $CD3^+$ lymphocytes analyzed by FACS from WT (n=5-6) and IL-1rn-KO (n=6-7) mice. (J) IL-6, IL-10, IFN-γ and TNF-α protein level in BM extracellular fluid from WT (n=9) and IL-1rn-KO mice (n=12). (K) Frequency of $CD11b^+$ myeloid cells differentiated *ex vivo* from lineage-negative progenitors enriched from the BM of WT (n=8) and IL-1rn-KO (n=5-6) mice (± IL1RN) analyzed by FACS. Data are means ± S.E.M. ***p<0.001, **p<0.01, *p<0.05 (versus WT); #p<0.05 (versus vehicle of the same genotype).

Figure S3

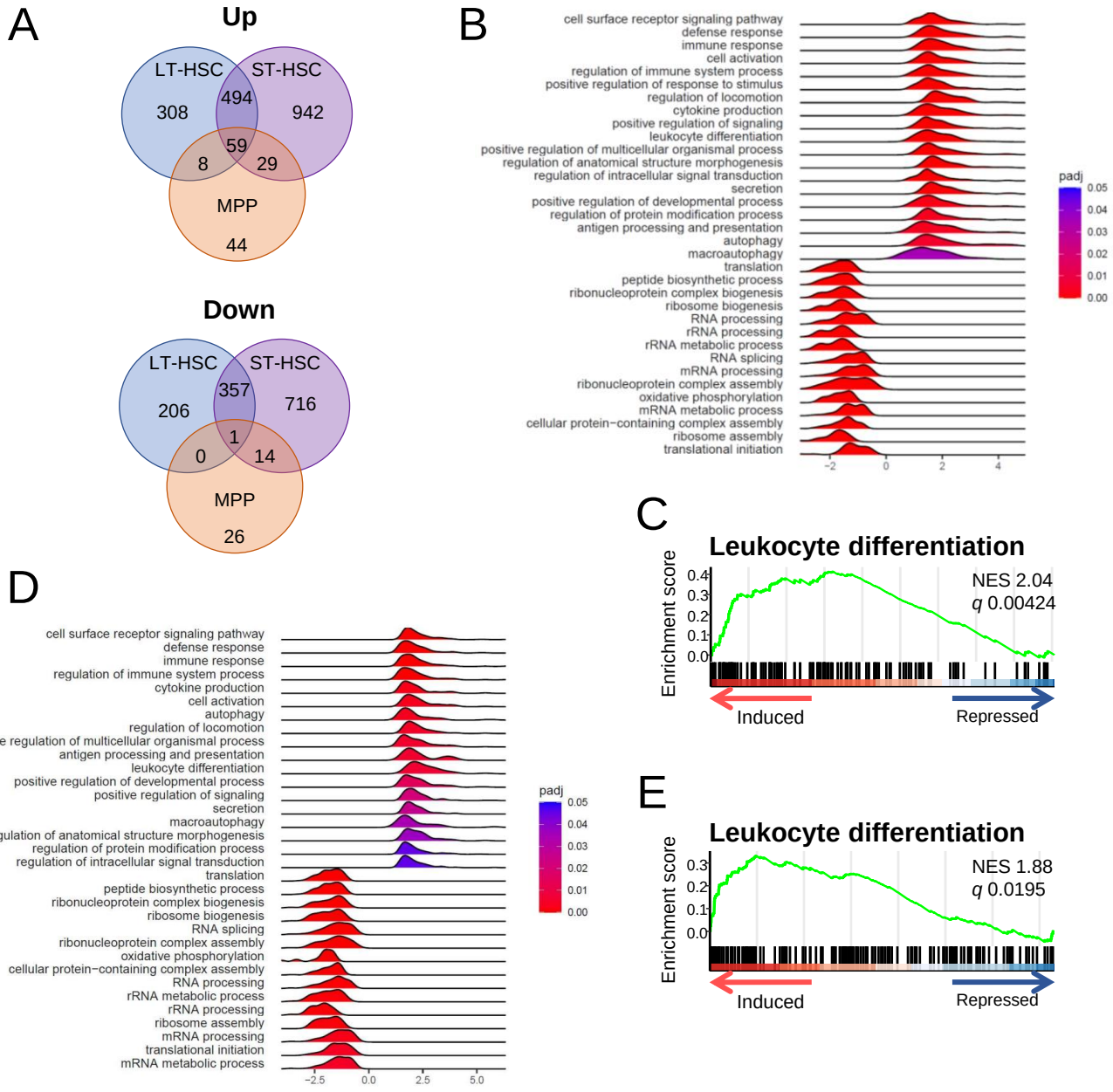

A
Up
LT-HSC
ST-HSC
MPP
308
494
942
59
8
29
44
Down
LT-HSC
ST-HSC
MPP
206
357
716
1
0
14
26
B
cell surface receptor signaling pathway
defense response
immune response
cell activation
regulation of immune system process
positive regulation of response to stimulus
regulation of locomotion
cytokine production
positive regulation of signaling
leukocyte differentiation
positive regulation of multicellular organismal process
regulation of anatomical structure morphogenesis
regulation of intracellular signal transduction
secretion
positive regulation of developmental process
regulation of protein modification process
antigen processing and presentation
autophagy
macroautophagy
translation
peptide biosynthetic process
ribonucleoprotein complex biogenesis
ribosome biogenesis
RNA processing
rRNA processing
rRNA metabolic process
RNA splicing
mRNA processing
ribonucleoprotein complex assembly
oxidative phosphorylation
mRNA metabolic process
cellular protein−containing complex assembly
ribosome assembly
translational initiation
padj
0.05
0.04
0.03
0.02
0.01
0.00
−2
0
2
4
C
Leukocyte differentiation
Enrichment score
NES 2.04
q 0.00424
Induced
Repressed
D
cell surface receptor signaling pathway
defense response
immune response
regulation of immune system process
cytokine production
cell activation
autophagy
regulation of locomotion
positive regulation of multicellular organismal process
antigen processing and presentation
leukocyte differentiation
positive regulation of developmental process
positive regulation of signaling
secretion
macroautophagy
regulation of anatomical structure morphogenesis
regulation of protein modification process
regulation of intracellular signal transduction
translation
peptide biosynthetic process
ribonucleoprotein complex biogenesis
ribosome biogenesis
RNA splicing
ribonucleoprotein complex assembly
oxidative phosphorylation
cellular protein−containing complex assembly
RNA processing
rRNA metabolic process
rRNA processing
ribosome assembly
mRNA processing
translational initiation
mRNA metabolic process
padj
0.05
0.04
0.03
0.02
0.01
0.00
−2.5
0.0
2.5
5.0
E
Leukocyte differentiation
Enrichment score
NES 1.88
q 0.0195
Induced
Repressed
F
Spi1
Csf2rb
Axl
Stat3
mRNA (ratio)
G
Spi1
Csf2rb
Axl
Cd93
mRNA (ratio)
H
Spi1
Csf2rb
Axl
Tlr1
mRNA (ratio)

Figure S3 (Related to Figure 2). RNA sequencing from hematopoietic stem and progenitor cells in C57BL/6J wild-type and IL-1rn knock-out mice. (A-E) Bone marrow $lin^{-}c\text{-}kit^{+}Sca\text{-}1^{+}$ (LSK) subsets; LSK $CD34^{-}Flt3^{-}$, long-term hematopoietic stem cells (LT-HSC); LSK $CD34^{+}Flt3^{-}$, short-term HSC (ST-HSC); LSK $CD34^{+}Flt3^{+}$, multipotent progenitors (MPP), from C57BL/6J wild type (WT) and IL-1rn knockout (IL-1rn-KO) mice (n=3) were isolated by fluorescence-activated cell sorting (FACS) and analyzed for RNA sequencing (RNA-Seq). (A) Venn diagrams show number of up- and down- regulated genes in LT-HSC, ST-HSC and MPP from IL-1rn-KO mice versus WT, and overlap of genes shared by two or all LSK subsets. (B-E) Gene Ontology biological processes and kernel density plots showing differences in core enriched gene sets in (B) LT-HSC and (D) ST-HSC, selected within gene set enrichment analysis enriched categories common to both LT-HSC and ST-HSC. Enrichment plot of coordinated changes of genes previously reported to be induced or repressed in leukocyte differentiation in (C) LT-HSC and (E) ST-HSC. (F-H) qRT-PCR validation of selected RNA-Seq hits relative to Gapdh in independent biological samples (n=3), in (F) LT-HSC, (G) ST-HSC and (H) MPP. Data are means ± S.E.M. $^{**}p < 0.01$, $^{*}p < 0.05$.

Figure S4

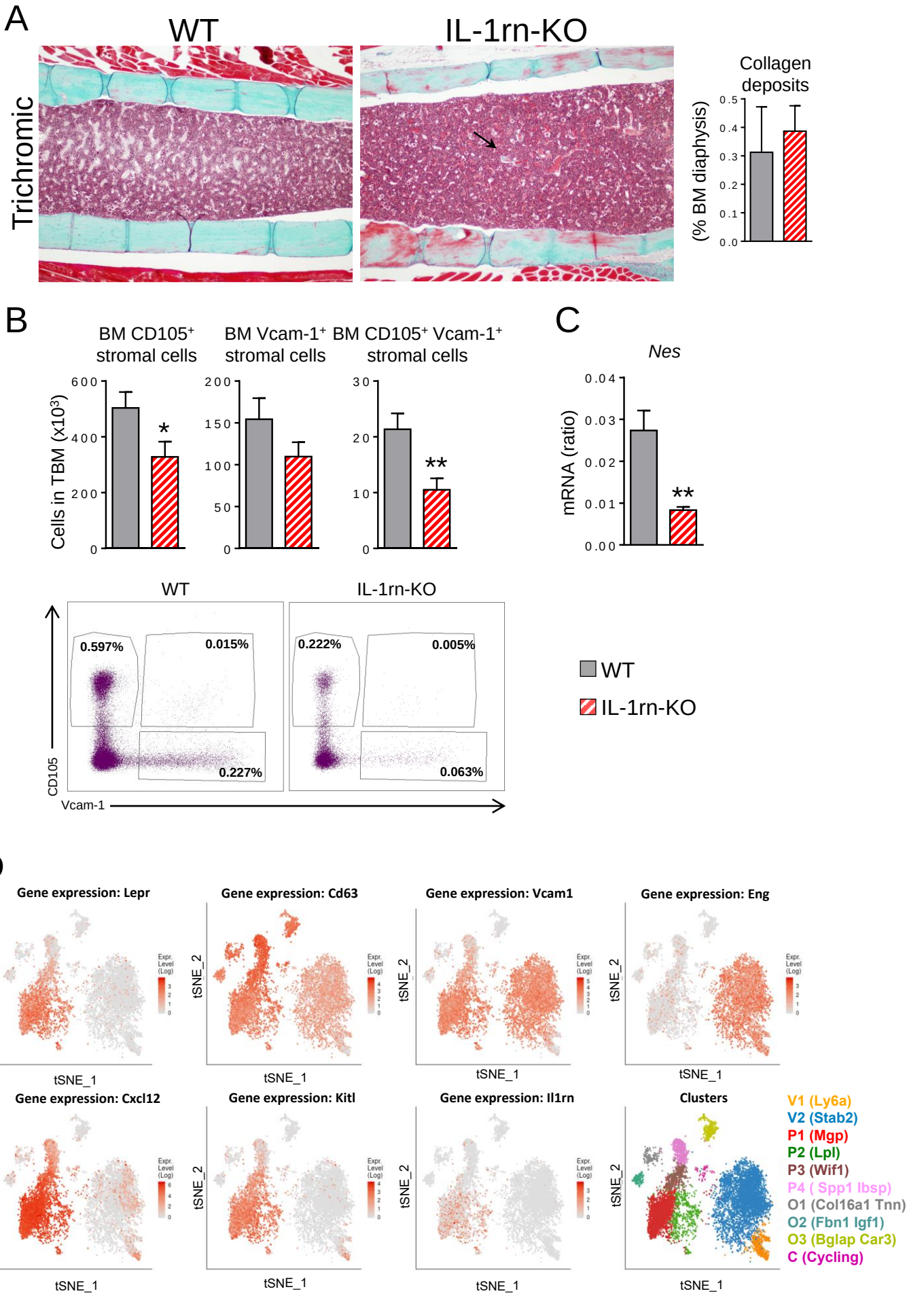

A
WT
IL-1rn-KO
Trichromic
Collagen deposits
(% BM diaphysis)
0.5
0.4
0.3
0.2
0.1
0.0
B
BM CD105+ stromal cells
BM Vcam-1+ stromal cells
BM CD105+ Vcam-1+ stromal cells
Cells in TBM (x10³)
600
400
200
0
200
150
100
50
0
30
20
10
0
*
**
WT
IL-1rn-KO
0.597%
0.015%
0.227%
0.222%
0.005%
0.063%
CD105
Vcam-1
C
Nes
mRNA (ratio)
0.04
0.03
0.02
0.01
0.00
**
WT
IL-1rn-KO
D
Gene expression: Lepr
Gene expression: Cd63
Gene expression: Vcam1
Gene expression: Eng
Gene expression: Cxcl12
Gene expression: Kitl
Gene expression: Il1rn
Clusters
tSNE_1
tSNE_2
Expr. Level (Log)
V1 (Ly6a)
V2 (Stab2)
P1 (Mgp)
P2 (Lpl)
P3 (Wif1)
P4 (Spp1 Ibsp)
O1 (Col16a1 Tnn)
O2 (Fbn1 Igf1)
O3 (Bglap Car3)
C (Cycling)

Figure S4 (Related to Figure 3). Deletion of IL-1rn causes IL-1β-induced damage to the bone marrow stroma in the absence of immunogenic stimulus. (A) Representative Masson's trichrome staining of femoral bone marrow (BM) sections (magnification, x40), and quantification of collagen deposits (arrow) in BM diaphysis from C57BL/6J wild-type (WT) and IL-1rn knockout (IL-1rn-KO) mice (n=6). (B) Total BM (TBM) number and representative fluorescence-activated cell sorting (FACS) analysis of $CD45^{-}CD31^{-}Ter119^{-}CD105^{+}$, $CD45^{-}CD31^{-}Ter119^{-}Vcam\text{-}1^{+}$ and $CD45^{-}CD31^{-}Ter119^{-}CD105^{+}Vcam\text{-}1^{+}$ stromal cells from WT (n=11) and IL-1rn-KO (n=12-15) mice. (C) qRT-PCR mRNA expression of *Nes* relative to *Gapdh* in BM nucleated cells (n=8). (D) t-distributed stochastic neighbor embedding plots of expression of selected genes from publicly available single-cell transcriptional analysis to characterize mouse BM mesenchymal stromal cells (GSE108892). Data are means ± S.E.M. $^{**}p<0.01$, $^{*}p<0.05$.

Figure S5

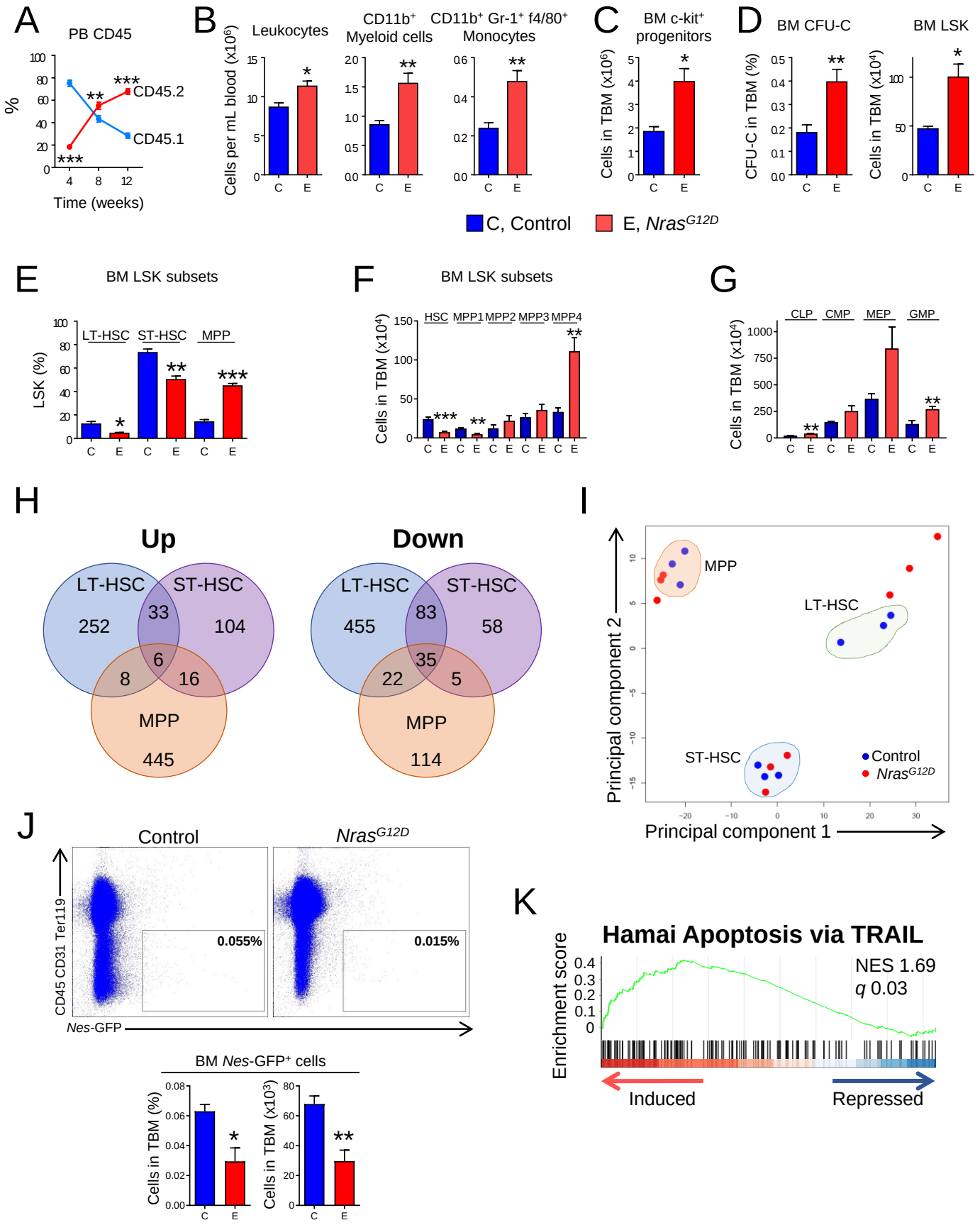

A
PB CD45
%
CD45.2
CD45.1
Time (weeks)
B
Leukocytes
Cells per mL blood (x10⁶)
CD11b⁺ Myeloid cells
CD11b⁺ Gr-1⁺ f4/80⁺ Monocytes
C
BM c-kit⁺ progenitors
Cells in TBM (x10⁶)
D
BM CFU-C
CFU-C in TBM (%)
BM LSK
Cells in TBM (x10⁴)
C, Control
E, NrasG12D
E
BM LSK subsets
LSK (%)
LT-HSC
ST-HSC
MPP
F
BM LSK subsets
Cells in TBM (x10⁴)
HSC
MPP1
MPP2
MPP3
MPP4
G
CLP
CMP
MEP
GMP
Cells in TBM (x10⁴)
H
Up
LT-HSC
ST-HSC
MPP
252
104
445
33
6
8
16
Down
LT-HSC
ST-HSC
MPP
455
58
114
83
35
22
5
I
MPP
LT-HSC
ST-HSC
Control
NrasG12D
Principal component 2
Principal component 1
J
Control
NrasG12D
CD45 CD31 Ter119
Nes-GFP
0.055%
0.015%
BM Nes-GFP⁺ cells
Cells in TBM (%)
Cells in TBM (x10³)
K
Hamai Apoptosis via TRAIL
Enrichment score
NES 1.69
q 0.03
Induced
Repressed

Figure S5 (Related to Figure 4). Unbalanced IL-1rn in *Nras^{G12D}* – driven pre-leukemic myelopoiesis. (A) Competitive repopulation assay. CD45.2^{+} *Nras^{G12D}* bone marrow (BM) cells were transplanted into CD45.1^{+} B6.SJL (n = 15) together with competitor healthy BM cells (1:1) isogenic to the recipient. Peripheral blood (PB) chimerism was assessed by fluorescence-activated cell sorting (FACS) analysis for 12 weeks. (B-G) Analysis of hematopoiesis in *Nras^{G12D}* (*Mx1-Cre^{+} Nras^{G12D}*) and control (*Mx1-Cre^{-} Nras^{G12D}*) mice (B-E) 20 weeks and (F-G) 44 weeks after polyinosine-polycytidylic acid (polyI:polyC) induction. (B) Number of circulating leukocytes measured with hematological counter, and CD11b^{+} myeloid cells and CD11b^{+} Gr-1^{+} f4/80^{+} monocytes measured by FACS in PB (n = 17). (C) Total BM (TBM) number of lin^{-} c-kit^{+} Sca-1^{-} (LK) progenitors (n = 3). (D) BM frequency of colony-forming unit cells (CFU-C; left) (n = 5-6) and total number of lin^{-} c-kit^{+} Sca-1^{+} (LSK) cells (right) (n = 3). (E) Frequencies of LSK subsets within the BM; LSK CD34^{-} Flt3^{-} long-term hematopoietic stem cells (LT-HSC), LSK CD34^{+} Flt3^{-} short-term HSC (ST-HSC) and LSK CD34^{+} Flt3^{+} multipotent progenitors (MPP) (n = 3). (F) TBM number of hematopoietic stem and progenitor cell subsets corresponding to HSC (LSK CD34^{-} Flt3^{-} CD48^{-} CD150^{+}), MPP1 (LSK CD34^{+} Flt3^{-} CD48^{-} CD150^{+}), MPP2 (LSK CD34^{+} Flt3^{-} CD48^{+} CD150^{+}), MPP3 (LSK CD34^{+} Flt3^{-} CD48^{+} CD150^{-}) and MPP4 (LSK CD34^{+} Flt3^{+} CD48^{+} CD150^{-}) (n = 5-6). (G) TBM number of common lymphoid progenitors (CLP, lin^{-} c-Kit^{low} Sca-1^{low} CD127^{+}), common myeloid progenitors (CMP, lin^{-} c-Kit^{+} Sca-1^{-} (LK) CD34^{+} FcRγ^{-}), megakaryocyte erythroid progenitors (MEP, LK CD34^{-} FcRγ^{-}) and granulocyte-monocyte progenitors (GMP, LK CD34^{+} FcRγ^{+}) (n = 5-6). (H-I) RNA-Seq in LT-HSC, ST-HSC and MPP (n = 3) isolated by FACS from *Nras^{G12D}* mice versus controls, 6 weeks after polyI:polyC induction. (H) Venn diagrams show number of (left) up- and (right) down- regulated genes in LT-HSC, ST-HSC and MPP from *Nras^{G12D}* mice versus controls, and overlap of genes shared by two or all LSK subsets. (I) Principal component analysis of RNA-Seq data. (J-K) *Nes-gfp* mice were used as recipients of BM nucleated cells from *Nras^{G12D}* and control mice, and analyzed in BM 4 weeks after the transplant. (J) Representative FACS analysis (up) and TBM number (down) of Nes-GFP^{+} BM stromal cells (n = 4). (K) Nes-GFP^{+} BM stromal cells (n = 2) mice were isolated by FACS and analyzed for RNA-Seq. Enrichment plot of coordinated changes of genes previously reported to be induced or repressed in apoptosis (E-MEXP-247). Data are means ± S.E.M. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

DATA PATIENTS / CONTROLS – Table S1

Table S1.1:

Patient id	Diagnosis	Gender	Age	Induction treatment curative int vs not	Treatment regime first	Classification	Remission	Relapse	Dead	Ot d	Blast flow (%)
1	AML	Female	67	1	7+3	M1	0	0	1	89	30
3	AML	Female	74	1	7+3	M1-M2	1	1	1	416	30
5	AML	Male	63	1	7+3	M0-M2	1	0	0	1569	20
6	AML	Male	66	1	7+3	M0-M2	1	1	1	1359	30
7	AML	Male	65	1	7+3	M1-M2	1	0	1	272	70
9	AML	Female	61	0	Cytarabine	M1	1	1	1	440	70
10	AML	Male	52	1	7+3	M0-M2	1	0	1	33	50
11	AML	Male	65	1	7+3	M4-M5	1	0	1	51	30
12	AML	Male	64	1	7+3	M0-M2	1	0	0	846	20
13	AML	Female	52	1	7+3	M1-M2	1	0	0	918	50
14	AML	Male	52	1	7+3	M5a	1	1	0	872	90
15	AML	Male	71	0	no	M2	0	0	1	7	30
16	MDS	Male	52	1	7+3	MDS-EB 2	1	1	0	813	4
17	AML	Female	94	0	Tioguanine	M0-M1	0	0	1	63	30
18	AML	Female	76	0	Decitabine	Unknown	0	0	0	580	30
19	AML	Male	73	1	7+3	M0-M1	1	0	0	562	35
20	AML	Male	74	0	Azacitidine	M0-M2	1	1	1	373	25

Patient id	Diagnosis	Gender	Age	Induction treatment curative int vs not	Treatment regime first	Classification	Remission	Relapse	Dead	Ot d	Blast flow (%)
21	AML	Male	73	1	Venetoclax + Azacitidine	M0-M2	1	1	0	488	25
22	AML	Female	59	1	7+3	M5	1	0	0	371	90
23	AML	Male	63	1	7+3	M0-M2	1	0	0	422	70
24	MDS	Male	68	0	Azacitidine	MDS-EB 2	1	1	0	361	16
26	AML	Male	70	1	7+3	M1-M2	1	0	0	190	20
27	AML	Female	72	0	Azacitidine	M0-M2	0	0	0	188	20
28	AML	Male	69	1	7+3	M5	1	0	0	98	90

Table S1.2:

Patient id	Risk	Karyotype	FISH	Translocations	Mutated genes
1	Adverse	47,XX,t(17)(q10),+mar(4)/47.XX.+21[2]/46.XX[14]	Monosomy 7 in 15% cells. Loss of one CBFB signal in 17% cells. Loss of TP53 (17p13.1) in 23% cells.		CSF3R|ASXL1|U2AF1|RUNX1
3	Adverse	Failure	Normal		
5	Favorable	45,X,-Y,t(8;21)(q22;q22)[8]		t(8;21): RUNX1-RUNX1T1	
6	Intermediate	46, XY[20]		Normal	
7	Adverse	46,XY,?i(17)(q10)[11]/46,XY[9]	Loss of TP53 signal in 30% of the cells	Normal	
9	Favorable	46,XX[20]		Normal	
10	Favorable	46 ,XY inv(16)(p13.1;q22)[14]/46,XY[6]		inv(16): CBFB-MYH11	
11	Favorable	46,XY[20]		Normal	
12	Adverse	45, XY,-7[5]/46,XY[14]		Normal	
13	Favorable	45, X,-X, t(8;21)(q22;q22)[20]		t(8;21): RUNX1-RUNX1T1	
14	Favorable	46,XY[20]		Normal	
15	Adverse	44,XY,add(5)(q11.2),add(6)(p22),add(7)(q11.2),-18,-18,add(21)q22.3)[10]			
16	High	46,XY[20]		Normal	
17	Unknown	Unknown	Unknown	Not done	

Patient id	Risk	Karyotype	FISH	Translocations	Mutated genes
18	Adverse	46,XX,add(3)(q29),del(5)(q15),del(7)(p14.1),-7,add(17)(q21),+mar[cp20]		Normal	
19	Favorable	46,XY,t(8;21)(q22;q22)[10]		t(8;21): RUNX1-RUNX1T1	
20	Intermediate	46,XY,+(7)(p11.1q11.1),r(7)(p11.1q11.1).r(7)(p22q22),-21[7)/45,XY,r(7)(p22q22),-21[2]/47,XY,+(7)(p11.1q11.1),r(7)(p22q22)x2,-21[1]	7q31 D7S486 /CEP7: two clones with loss of 7q31 42% and 12%, MECOM (EVI) normal.	Normal	
21	Intermediate	46,XY[20]		Normal	DNMT3A|IDH2|SRSF2
22	Adverse	46,XX[20]		Normal	
23	Intermediate	47,XY,+8[19]/46,XY[1]	KMT2A normal		
24	Very high	41-44,XY,-5,add(8)(q24.3),-12,add(12)(p13),-13,add(17)(p11.2),+mar[cp7]/45,X,-Y[2]/46,XY[1]		Normal	
26	Intermediate	46,XY[20]	7q31 D7S486 /CEP7 normal	Normal	SRSF2|RUNX1
27	Adverse	46,XX,del(5)(q31q33)[8]/46,XX[12]	KMT2A normal	Normal	DNMT3A|IDH2|BCORL1|RUNX1|KRAS
28	Favorable	46, XY		Normal	DNMT3A|NPM1|KRAS|NRAS

Table S1.3:

Patient id	TruSight myeloid panel (% of reads)	FLT3 ITD	NPM1	CEBPA	FLT3 Asp835 / Ile836	Used in mouse xenograft
1	CSF3R (64,5%): NM_156039.3:c.2326C>T; p.(Gln776*) ASXL1 (51,2%): NM_015338.5:c.2420delT; p.(Pro808Leufs*10) U2AF1 (45,8%): NM_001025203.1 c.467G>A; p.(Arg156His) RUNX1 (38,6%): NM_001754.4:c.508G>A; p.(Gly170Arg) RUNX1 (11,8%): NM_001754.4 c.412G>A; p(Glu138Lys)	0	0			
3		1	0			1
5		0	0			
6		0	0			
7		0	0			1
9		1	1			1
10		0	0			
11		0	1		0	
12		0	0		0	
13		0	0		0	
14		0	1			
15		9	9		9	
16						
17		9	9		9	
18		0	0	0	0	
19		0	0	0	0	

Patient id	TruSight myeloid panel (% of reads)	FLT3 ITD	NPM1	CEBPA	FLT3 Asp835 / Ile836	Used in mouse xenograft
20		0	0	0	0	
21	DNMT3A (29%): NM_175629.2:c.2645G>A; NP_783328.1:p.Arg882His IDH2 (9,9%): NM_002168.2:c.419G>A; NP.002159.2:p.Arg140Gln SRSF2 (7%): NM_003016.4:c.284C>G; NP_003007.2:p.Pro95Arg	0	0	0	0	1
22		1	0	0	0	1
23		0	0		0	1
24		0	0		0	
26	SRSF2 (7.3%): NM_003016.4:c.284C>T; NP_003007.2:p.Pro95Leu RUNX1 (6%): NM_001754.4:c.619del; NP_001745.2:p.Arg207GlyfsTer4	0	0	0		
27	DNMT3A (87.5%):: NM_175629. 2:c.2226dup; NP_783328.1:p.Pro743AlafsTer6 IDH2 (41.7%): NM_002168.2:c.419G>A; NP_002159.2:p.Arg140GIn BCORL1 (41%): NM_021946.4:c.3845_3848dup; NP_068765.3:p.GLn1283HisfsTer35 BCORL1 (39.4%): NM_021946.4:c.3409A>G; NP_068765.3:p.Lys1137Glu RUNX1 (37.6%): NM_001754.4:c.344dup; NP_001745.2:p.Lys117GlnfsTer21 KRAS: (13.9 %): NM_033360.2:c.34G>A; NP_203524.1:p.Gly12Ser	0	0			
28	DNMT3A (49.5%): NM_175629.2: c.2645G>A; NP_783328.1:p.Arg882His NPM1 (37.4%): NM 002520.6: c.860_ 863dup; NP_002511.1:p. Trp288CysfsTer12 KRAS (36%): NM_033360.2:c.38G>A; NP_203524.1:p.Gly13Asp NRAS (5%): NM_ 002524.4:c.183A>C; NP_O02515.1:p.Gln61His	0	1			

Table S1.4:

Patient id	Diagnosis	Gender	Age
C1	Control	Male	52
C3	Control	Male	30
C4	Control	Female	27
C5	Control	Male	43
C8	Control	Female	27
C9	Control	Male	27
C10	Control	Female	44
C11	Control	Male	23
C12	Control	Female	36
C13	Control	Male	41
C14	Control	Male	53
C15	Control	Male	28
C16	Control	Female	24
C17	Control	Female	30
C18	Control	Female	41
C20	Control	Female	24
C21	Control	Male	36
C22	Control	Male	29
C23	Control	Female	48
C24	Control	Female	65
C25	Control	Male	34
C27	Control	Female	42
C28	Control	Female	47

Patient id	Diagnosis	Gender	Age
C29	Control	Female	59
C30	Control	Female	38
C31	Control	Female	37
C32	Control	Female	58
C33	Control	Female	48
C34	Control	Female	47
C35	Control	Female	56
C36	Control	Female	65
C39	Control	Female	45
C40	Control	Female	46
C42	Control	Male	26
C43	Control	Male	28
C46	Control	Male	79
C47	Control	Male	62

*Column Descriptions - Table S1

Column name	Description	Values
Patient id	Patient ID	
Diagnosis	Final diagnosis. AML: Acute Myeloid Leukemia. MDS-EB2: Myelodysplastic syndrome with excess blasts-2	
Gender	Gender	
Age	Age at diagnosis of AML patients or at time of sample collection of control subjects	
Induction treatment curative int vs not	Induction treatment intended to cure or not	0=No. 1=Yes.
Treatment regime first	7+3; 7 days cytarabine, 3 days of anthracycline	
Classification	AML: French-American-British classification. MDS: IPSS-R.	
Remission	Binary remission classification	0=No remission/Censored. 1=Remission.
Relapse	Binary relapse classification	0=No relapse/Censored. 1=Relapse.
Dead	Binary survival classification	0=Alive/Censored. 1=Dead.
Ot d	Observation period (days) from date of diagnosis to death or last follow up. Study end date: Oct 15th 2020	
Blast flow (%)	Percentage of blasts in peripheral blood obtained by flow cytometry	
Risk	Risk for AML: 2017 European Leukemia Net (ELN). Risk for MDS: Revised International Prognostic Scoring System (IPSS-R).	

Column name	Description	Values
Karyotype	Karyotype identification using G-banding	
FISH	Fluorescent in-situ hybridization data	
Translocations	Screening of 28 chromosomal translocations involved in leukemogenesis. See translocation screening info below for more information.	
Mutated genes	Summary of mutated genes found in TruSight Myeloid Sequencing Panel.	
TruSight myeloid panel (% of reads)	TruSight Myeloid Sequencing Panel. See box below for list of genes sequenced. Numbers in parenthesis correspond to percent of reads with mutation	
FLT3 ITD	Genomic PCR results for Internal Tandem Duplication (84 bp) in FLT3 exon 14 and 15	0=Normal/Wild-type. 1=Mutated. 9=Unknown.
NPM1	Genomic PCR results for NPM1 4 bp insertion in exon 12	0=Normal/Wild-type. 1=Mutated. 9=Unknown.
CEBPA	Sanger sequencing results for CEBPA mutation	0=Normal/Wild-type. 1=Mutated. 9=Unknown.
FLT3 Asp835/Ile836	Genomic PCR results for FLT3 Asp835/IIe836 in exon 20	0=Normal/Wild-type. 1=Mutated. 9=Unknown.
Used in mouse xenograft	Whether or not used in mouse xenograft	1=Yes. Blank=No.

*Translocation screening info - Table S1

RNA was purified using PAXgene Bone Marrow RNA Kit (Qiagen).

Threshold for detecting fusion transcripts was set to a Cq-value of 34.
28 leukemia causing translocations were examined using Hemavision 28Q (DNA Diagnostics):

Chromosomal translocation	Fusion genes	Leukemia
inv(16) (p13;q22)	CBFB-MYH11	AML/MDS
t(6;9) (p23;q34)	DEK-NUP214	AML/MDS
t(9;12) (q34;p13)	ETV6-ABL1	AML/MDS
t(12;22) (p13;q11)	ETV6-MN1	AML/MDS
t(5;12) (q33;p13)	ETV6-PDGFRB	AML/MDS
t(16;21) (p11;q22)	FUS-ERG	AML/MDS
t(3;5) (q25;q34)	NPM1-MLF1	AML/MDS
t(5;17) (q35;q21)	NPM1-RARA	AML/MDS
t(15;17) (q24;q21)	PML-RARA	AML/MDS
t(3;21) (q26;q22)	RUNX1-MECOM	AML/MDS
t(8;21) (q22;q22)	RUNX1-RUNX1T1	AML/MDS
t(9;9) (q34;q34)	SET-NUP214	AML/MDS
t(9,22) (q34;q11)	BCR-ABL1	ALL
t(12;21) (p13;q22)	ETV6-RUNX1	ALL
t(6;11) (q27;q23)	KMT2A-AFDN	ALL
t(4;11) (q21;q23)	KMT2A-AFF1	ALL
t(11;19) (q23;p13.1)	KMT2A-ELL	ALL
t(1;11) (p32;q23)	KMT2A-EPS15	ALL
t(X;11) (q13;q23)	KMT2A-FOXO4	ALL
t(11;19) (q23;p13.3)	KMT2A-MLLT1	ALL
t(10;11) (p12;q23)	KMT2A-MLLT10	ALL
t(1;11) (q21;q23)	KMT2A-MLLT11	ALL
t(9;11) (p22;q23)	KMT2A-MLLT3	ALL
t(11;17) (q23;q21)	KMT2A-MLLT6	ALL
del1(p32)	STIL-TAL1	ALL
t(17;19) (q22;p13)	TCF3-HLF	ALL
t(1;19) (q23;p13)	TCF3-PBX1	ALL
t(11;17) (q23;q21)	ZBTB16-RARA	ALL

Illumina TruSight Myeloid Sequencing Panel Gene List* - Table S1

ASXL1	CSF3R	IDH1	NOTCH1	SMC1A
ATRX	CUX1	IDH2	NPM1	SMC3
BCOR	DNMT3A	IKZF1	NRAS	SRSF2
BCORL1	ETV6/TEL	JAK2	PDGFRA	STAG2
BRAF	EZH2	JAK3	PHF6	TET2
CALR	FBXW7	KDM6A	PTEN	TP53
CBL	FLT3**	KIT	PTPN11	U2AF1
CBLB	GATA1	KRAS	RAD21	WT1
CBLC	GATA2	MLL	RUNX1	ZRSR2
CDKN2A	GNAS	MPL	SETBP1	

*Source: https://www.illumina.com/products/by-type/clinical-research-products/trusight-myeloid.html

** Does not detect FLT3-ITD

Supplementary Materials

Supplementary Tables

Table S2. Antibodies used in this study

Antibody	SOURCE	IDENTIFIER
Flow cytometry		
Alexa Fluor 647 rat anti-mouse CD11b	BD Biosciences	Cat# 557686; RRID AB_396796; cloneM1/70
PE rat anti-mouse Ly-6G/Ly-6C (Gr-1)	BD Biosciences	Cat# 553128; RRID AB_394644; clone RB6-8C5
FITC rat anti-mouse CD45R/B220	BD Biosciences	Cat# 553088; RRID AB_394618; clone RA3-6B2
PE-Cy5 hamster anti-mouse CD3e	BD Biosciences	Cat# 553065; RRID AB_394598; clone 145-2C11
PE rat anti-mouse Ly-6G	BD Biosciences	Cat# 551461; RRID AB_394208; clone 1A8
PE rat anti-mouse Ly6A/E (Sca-1)	BD Biosciences	Cat# 553336; RRID AB_394792; clone E13-161.7
FITC rat anti-mouse CD34	BD Biosciences	Cat# 553733; RRID AB_395017; clone RAM34
APC rat anti-mouse CD135 (Flt3)	BD Biosciences	Cat# 560718; RRID AB_1727425; clone A2F10.1
PE-Cy7 rat anti-mouse CD117 (c-Kit)	BD Biosciences	Cat# 558163; RRID AB_647250; clone 2B8
PE rat anti-mouse CD63	BD Biosciences	Cat# 564222; RRID AB_2738678; clone NVG-2
PE rat anti-mouse CD106 (VCAM)	BD Biosciences	Cat# 561613; RRID AB_10897990; clone 429(MVCAM.A)
Alexa Fluor 647 rat anti-mouse CD121a (IL-1R1)	BD Biosciences	Cat# 563629;RRID AB_2738332; clone 35F5
FITC mouse anti-human CD45	BD Biosciences	Cat# 345808; RRID AB_2732010; clone 2D1
PE mouse anti-human CD33	BD Biosciences	Cat# 555450; RRID AB 395843
FITC mouse anti-human CD34	BD Biosciences	Cat# 560942; RRID AB 10562559; clone 581
PE-Cy7 anti-mouse CD105 (Endoglin)	BioLegend	Cat# 120410; RRID AB_1027700; clone MJ7/18
Alexa Fluor 488 Rat anti-mouse F4/80	eBioscience	Cat# 53-4801-82; RRID AB_469915; clone BM8
Biotin rat anti-mouse CD31	BD Biosciences	Cat# 553371; RRID AB_394817; clone MEC13.3
Biotin rat anti-mouse TER-119/Erythroid cells	BD Biosciences	Cat# 553672; RRID AB_394985; clone TER-119
Biotin mouse anti-mouse CD45.2	BD Biosciences	Cat# 553771; RRID AB_395040; clone 104
APC-Cy7-conjugated streptavidin	BD Biosciences	Cat# 554063; RRID AB_10054651

BB700 rat anti-mouse Ly6A/E (Sca-1)	BD Biosciences	Cat# 742089, RRID AB_2871369, clone D7
PE rat anti-mouse CD135 (Flt3)	BD Biosciences	Cat# 553842, RRID AB_395079, clone A2F10.1
PE rat anti-mouse CD16/32 (FcRγ)	BD Biosciences	Cat# 567020, RRID AB_2870010, clone Ab93
PE-Cy5 rat anti-mouse CD150 (SLAM)	BioLegend	Cat# 115912, RRID AB_493598, clone TC15-12F12.2
APC hamster anti-mouse CD48	BD Biosciences	Cat# 562746, RRID AB_2737765, clone HM48-1
APC rat anti-mouse CD127 (IL-7R)	BD Biosciences	Cat# 564175, RRID AB_2732843; clone SB/199
Biotin Mouse Lineage Depletion Cocktail	BD Biosciences	Cat# 51-9000794
4',6-diamidino-2-phenylindole (DAPI)	Sigma-Aldrich Merck	Cat# D8417
7-Amino-Actinomycin D (7-AAD)	Thermo Fisher Scientific	Cat# A1310
Pacific Blue conjugated Annexin V antibody	Life technologies	Cat# A35122
APC mouse anti-BrdU	BD Biosciences	Cat# 552598; RRID AB_2861367; clone B44
PE-Cy7 mouse anti-mouse CD45.1	BD Biosciences	Cat# 560578; RRID:AB_1727488; clone A20
FITC rat anti-mouse CD45	BD Biosciences	Cat# 553080; RRID:AB_394610; clone 30-F11
Alexa Fluor 647 Mouse anti-NF-κB p65 (pS529)	BD Biosciences	Cat# 558422, RRID AB_647136, clone K10-895.12.50
Alexa Fluor 647 Mouse IgG2b, κ Isotype Control	BD Biosciences	Cat# 558713, RRID AB_1645618, clone 27-35
Immunofluorescence		
Goat polyclonal anti-mouse IL-1R1	R&D system	Cat# AF771; RRID AB_355587
Donkey polyclonal anti-goat IgG H&L (Cy3)	Abcam	Cat# ab6949; RRID AB_955018
Cy3-Streptavidin conjugate (ZyMAX^{TM} grade)	Invitrogen	Cat# 438323

Table S3. qRT-PCR primers used in this study

Gene	Organism	Forward primer	Reverse primer	Source
Il1b	Mouse	GAAATGCCACCTTTTGACAGTG	TGGATGCTCTCATCAGGACAG	This paper
Il1r1	Mouse	TTACCCGAGGTCCAGTGGTA	CCCCCGGAACGTATAGGACA	This paper
Il1rn	Mouse	GAGAAACAACCAGCTCATTGC	GGATGCCCAAGAACACACTATG	This paper
Cxcl12	Mouse	TGCATCAGTGACGGTAAACCA	TTCTTCAGCCGTGCAACAATC	This paper
Adipoq	Mouse	TGTTCCTCTTAATCCTGCCCA	CCAACCTGCACAAGTTCCCTT	This paper
Axl	Mouse	ATCACAGGTGCCAGAGGACT	CTGTCCATCTCGAAGCCACA	This paper
Stat3	Mouse	CATTGACCTGCCGATGTCCC	TCAAACGTGAGCGACTCAAAC	This paper
Tlr1	Mouse	CAGTTGGTGAAGAACTCAGGC	CCACATGGGTATAGGACGTTT	This paper
Spi1	Mouse	CCCTCCATCGGATGACTTG	ACATGGTGTGCGGAGAAATC	This paper
Csf2rb	Mouse	GGGCCAGTGTCTACACCCG	GTAAGCCATCTTCTGAGTTTCCCAATGC	This paper
Nes	Mouse	GTCAGATCGCTCAGATCCT	GTGTCTGCAAGCGAGAGTTC	This paper
Vcam1	Mouse	TGGAGGTCTACTCATTCCCTGA	GACAGGTCTCCCATGCACAA	This paper
Angpt1	Mouse	CTCGTCAGACATTCATCATCCAG	CACCTTCTTTAGTGCAAAGGCT	This paper
Lepr	Mouse	AGCTGCACTTAACCTGGCAT	AAAGCCGAGGCATTGTTTGG	This paper
B2m	Mouse	ACCGTCTACTGGGATCGAGA	TGCTATTTCTTTCTGCGTGCAT	This paper
Gapdh	Mouse	CTCCCACTCTTCCACCTTCG	GCCTCTCTTGCTCAGTGTCC	This paper
IL1B	Human	CTGTCCTGCGTGTTGAAAGA	TTGGGTAATTTTTGGGATCTACA	This paper
ILRN	Human	TGCAAGCCTTCAGAATCTGGG	TCCTTGCAAGTATCCAGCAACT	This paper
CASP1	Human	ACCAACTACAGAAGAGTTTGAGGA	ACATTATCTGGTGTGGAAGAGC	This paper
IRAK1	Human	TGTGCCGCTTCTACAAAGTGA	ACGATCAGGGCGGCGAAC	This paper
B2M	Human	AGTATGCCTGCCGTGTGAAC	TTCAAACCTCCATGATGCTGCT	This paper

Supplementary Methods

Mice

Phenotyping of *Il1rn*$^{-/-}$ mice versus C57BL/6J wild-type (WT) mice was performed in either females or males aged 10-52 weeks. *Cre* expression in *Mx1-Cre Nras*^{G12D} mice was induced by intraperitoneal (i.p.) injection of one or two doses (in two consecutive days) of 300μg of poly-inosine:poly-cytosine (polyI:polyC, Sigma-Aldrich). Phenotyping of *Mx1-Cre Nras*^{G12D} mice versus *Nras*^{G12D} control littermates was performed in either females or males aged 30-63 weeks, 10-44 weeks after polyI:polyC induction. Animals were used as donors at least 4 weeks after polyI:polyC induction and they displayed splenomegaly.

Transplantation assays

For most myeloablation experiments and unless otherwise indicated, 7-12 weeks old female mice were whole body irradiated with 9Gy (in 2 doses separated by 3h) using an X-RAY source (Rad Source's RS 2000). 4h after, mice were transplanted intravenously (i.v.) with 2x10^6 bone marrow (BM) nucleated cells.

In competitive repopulation assays, B6.SJL (CD45.1$^+$) were used as recipients of BM nucleated cells from previously induced *Mx1-Cre Nras*^{G12D} mice, mixed 1:1 with competitor BM nucleated cells isogenic to the recipient. Myeloablation in competitive repopulation assay using

B6.SJL ($CD45.1^+$) mice as recipients was performed through intraperitoneal (i.p.) injection of Busulfan (Busilvex) at 25mg/kg.

To study the role of *Il1rn* deletion in hematopoietic cells to $Nras^{G12D}$ oncogene-driven expanded myelopoiesis, C57BL/6J WT mice were transplanted with equal numbers of C57BL/6J WT and $Il1rn^{-/-}$ BM nucleated cells (1:1), C57BL/6J WT and $Nras^{G12D}$ BMNC (1:1), or $Il1rn^{-/-}$ and $Nras^{G12D}$ BM nucleated cells (1:1). To study the role of *Il1rn* deletion in the stroma to $Nras^{G12D}$ oncogene-driven expanded myelopoiesis, $Il1rn^{-/-}$ and C57BL/6J WT mice were transplanted with $Nras^{G12D}$ or control BM nucleated cells.

To cокirm the changes in BM mesenchymal stromal cells (MSC) from *Mx1-Cre* $Nras^{G12D}$ mice, *Nes-gfp* mice were transplanted with $Nras^{G12D}$ or control BM nucleated cells. *Nes-gfp* female mice were 28 weeks old at the time of transplantation.

Male or female NSG-SGM3 mice, were whole body irradiated with 2Gy and, 4h after irradiation, transplanted with nucleated (AML3) or $CD34^+$ (AML7, 9, 21, 22, 23) cells isolated from the BM of AML patients. The number of transplanted cells was $4x10^4$ – 10^6 per mouse. Animals showing human engraftment <0.1% in BM were excluded.

In vivo pharmacological treatments

In the NSG-SGM3 AML xenograft mouse model, treatments started 4-7 weeks post-transplant, when animals evidenced signs of engraftment. Human $CD34^+$ transplanted NSG-SGM3 mice were injected daily i.p. with 25μg/kg human IL-1β in 100μL of saline solution or saline solution alone, during 4 months (AML7); or were injected every other day using the same doses during 4 weeks (AML9). Human BM $CD34^+$ transplanted NSG-SGM3 mice were injected subcutaneously (s.c.) every 12h with 1600mg/kg of human IL-1RN in 300μL of anakinra (Kineret® Sobi) or saline solution for 6 weeks (AML21, 22, 23). To compare IL-1RN with IL-1β blockade, mice were injected twice i.p. with 22mg/kg of human IL-1β monoclonal antibody (mAb) (Canakinumab – Ilaris® Novartis), 3 weeks apart. Human BM nucleated cell transplanted NSG-SGM3 mice were injected s.c. every 12h with increasingly higher doses of human IL-1RN in 100μL of saline solution or saline solution alone, as follows: 20mg/kg for 18 weeks; 75mg/kg for 4 weeks; 150mg/kg for 7 weeks and 300mg/kg for 7 weeks, for a total of 9 months (AML3).

For IL-1RN treatment *in vivo*, $Il1rn^{-/-}$ mice (9-22 weeks) were injected s.c. every 12h with human IL-1RN in 100μL of saline solution or saline solution alone for 10 weeks. The dose used to rescue the damage in the stromal compartment was 75mg/kg per injection. To rescue the damage in the hematopoietic compartment, IL-1RN was injected at 300mg/kg per injection. For IL-1β mAb treatment *in vivo*, $Il1rn^{-/-}$ mice (9-21 weeks) were injected i.p. with 10mg/kg of mouse IL-1β mAb or IgG1 kappa isotype control in 250μL of PBS once per week for 10 weeks and twice per week for 8 weeks, for a total of 4.5 months. For bortezomib treatment, $Il1rn^{-/-}$ mice (17-31 weeks) were injected i.p. with 0.8mg/kg of bortezomib in 100μL of 0.5% DMSO in PBS or 0.5% DMSO in PBS solution alone once per week for 2 consecutive weeks, and received a last injection 3 weeks after. Animals were analyzed 12 weeks after the start of the treatment.

Treatments were initiated after animals evidenced signs of disease in the $Nras^{G12D}$ mouse model of expanded myelopoiesis. Primary mutant $Nras^{G12D}$ mice (12-16 weeks; 4 weeks after polyI:polyC induction) were injected s.c. every 12h with 600mg/kg of human IL-1RN in 100μL of anakinra or saline solution for 4 weeks. Primary mutant $Nras^{G12D}$ mice (59-62 weeks; 44 weeks after polyI:polyC induction) were injected i.p. with 0.8mg/kg of bortezomib in 100μL of 0.5% DMSO in PBS or 0.5% DMSO in PBS solution alone once, and received a second and

third injection, 4 and 5 weeks after. Animals were analyzed 8 weeks after the start of the treatment.

Fluorescence-activated cell sorting (FACS)

Detailed FACS analysis of the five stem and progenitor cell subsets corresponding to hematopoietic stem cells (HSC) and multipotent progenitors MPP1-MPP4 [26,27] was as follows. Long-term (LT-HSC) were further defined as HSC (LT-HSC $CD150^+CD48^-$). Short-term (ST-HSC) were further defined as MPP1 (ST-HSC $CD150^+CD48^-$), MPP2 (ST-HSC $CD150^+CD48^+$) and MPP3 (ST-HSC $CD150^-CD48^+$). MPP were further defined as MPP4 (MPP $CD150^-CD48^+$).

For cell sorting, HSC and progenitors were enriched in lineage-negative cells by immune magnetic depletion of differentiated cells. Cells were stained with the specific antibodies to identify LT-HSC, ST-HSC and MPP. LSK cells were sorted from total BM with no enrichment step. For MSC sorting, cells were identified as $CD45.2^-Ter119^-CD31^-CD63^+$. Granulocytes were identified as $CD11b^+Gr\text{-}1^{hi}f4/80^-$ and monocytes as $CD11b^+Gr\text{-}1^+f4/80^+$, for cell sortings.

Phospho-flow

Phospho-flow was used to measure NF-κB p65 phosphorylation. Frozen human $CD34^+$ cells enriched from peripheral blood mononuclear cells (PBMC) of AML patients and healthy controls were thawed, and DMSO and cell debris were washed with heat-inactivated fetal bovine serum (FBS, Gibco), PBS 2% FBS and PBS in consecutive centrifugations at room temperature (RT, 1500rpm, 5min). Cells were fixed with 100µL of 4% formaldehyde (Thermo Scientific) per 10^6 cells for 15min at RT, washed with PBS and permeabilized with pre-chilled 90% methanol for 10min at 4°C. Cells were washed twice with PBS 2% FBS and stained with Alexa Fluor 647 mouse anti-NF-κB p65 (pS529) or correspondent isotype control according to the supplier protocol. Antibodies used are listed in Supplementary Table S2.

Proliferation and apoptosis assays

To analyze proliferation, mice were injected i.p. with 2mg 5-Bromo-2-Deoxyuridine (BrdU) (BD Biosciences). Mice were euthanized 24h post-injection and BM nucleated cells were collected. Lineage-negative cells were enriched, and after surface antibody staining to identify $CD34^-$ LSK (LT-HSC) and $CD34^+$ LSK (ST-HSC/MPP) cells, these were co-stained with anti-BrdU APC and DAPI. Analysis of apoptotic cells was performed as previously described [11], using BM nucleated cells for the study of LSK, or enriched $CD45.2^-Ter119^-CD31^-$ stromal cells for $CD63^+$ MSC. Antibodies used are listed in Supplementary Table S2.

Cytokine analyses

BM extracellular fluid was isolated from one femur and one tibia of each mouse, and proteins were quantified (Pierce™ BCA Protein Assay Kit, Thermo Fisher). To measure the level of intracellular cytokines in human PB $CD34^+$ cells, total proteins were isolated with Trizol (Tri Reagent®, Sigma-Aldrich Merck) and quantified. Intracellular protein samples were normalized to a concentration of 200µg/mL and used for multiplex analysis. Results were expressed as pg per mg of protein. For human IL-1β, ratios versus IL-1RN were calculated for each individual.

RNA isolation and quantitative reverse transcription PCR

For human *IL1B*, *IRAK1*, and *CASP1* gene expression normalized to *B2M* was expressed as a ratio versus *IL1RN* normalized to *B2M* in each sample. Ratios in individual samples were then

expressed as a fold change (FC) versus the mean ratio of control samples, and the $\log_2$ of these values were calculated ($\log_2$FC).

RNA sequencing and bioinformatic data analysis

RNA-Seq data from FACS-sorted LT-HSC, ST-HSC, MPP and CD63$^+$ stromal cells obtained from the BM of *Il1rn*$^{-/-}$ and C57BL/6J WT female mice aged 20-25 weeks have accession number GSE126428. RNA-Seq data from FACS-sorted LT-HSC, ST-HSC and MPP obtained from the BM of *Nras*^{G12D} (induced *Mx1-Cre Nras*^{G12D}) female mice and control (induced *Nras*^{G12D}) littermates 6 weeks after polyI:polyC induction, aged 34 weeks, have accession number GSE126625. RNA amplification, RNA-Seq library production and RNA-Seq analysis were performed at Eurofins Genomics (Germany). RNA-Seq data from FACS-sorted *Nes*-GFP$^+$ cells obtained from the BM of 28 week-old *Nes-gfp* male mice 4 weeks after the transplant with BM cells from previously polyI:polyC induced control (induced *Nras*^{G12D}) or *Nras*^{G12D} (induced *Mx1-Cre Nras*^{G12D}) mice, have accession number GSE157038. RNA amplification and RNA-Seq library production were performed at the Genomics Support Center Tromsø (GSCT, UiT – The Arctic University of Norway).

Total RNA was isolated using the Arcturus Picopure RNA isolation kit (Arcturus – Thermo Fisher) from small numbers of FACS-sorted cells (6000 – 80000).

For accessions GSE126428 and GSE126625, RNA was amplified and prepared for RNA sequencing (RNA-Seq) using the SMART-Seq v4 Ultra Low Input RNA kit (Clontech). The RNA-Seq library was prepared with the Low Input Library Prep Kit v2 (Clontech) to construct index-tagged cDNA. Libraries were sequenced on the Illumina HiSeq 2500 (Illumina) following the standard sequencing protocol with the TruSeq SBS Kit v4 and the following software versions: HCS 2.2.58, RTA 1.18.64. RNA amplification, RNA-Seq library production and RNA-Seq analysis were performed at Eurofins Genomics (Germany). At least 3 biological replicates were used per experimental group. The quality, quantity and the size distribution of the Illumina libraries were determined using the Fragment Analyzer High Sensitivity NGS Fragment Analysis Kit (Advanced Analytical). Fastq files containing reads for each library were extracted and demultiplexed using bcl2fastq-1.8.4 pipeline. Raw read counts were created using featureCounts [47]. Genome assembly GCF_000001635.25 and the associated annotation (https://www.ncbi.nlm.nih.gov/assembly/GCF_000001635.25/) were used. Only reads overlapping coding sequences were counted. All reads mapping to the same gene were summed. Only reads with unique mapping positions and a mapping quality score of at least 1 were considered for read counting. Supplementary alignments were ignored for read counting. Paired-end reads that mapped to different chromosomes or with unexpected strandedness were ignored. Paired-end reads were counted as single fragments. Reads mapping to multiple genes were assigned to the gene with the largest number of overlapping bases. Forward and reverse fastq reads, raw Counts, normalized counts per million (CPM), metadata and Eurofins Genomics sequencing statistics are accessible at GEO archive.

For RNA-Seq data analysis, the Bioconductor DESeq2 package (https://bioconductor.org/packages/release/bioc/html/DESeq2.html) was used to generate lists of differentially expressed transcripts, Venn diagrams, volcano plots and principal component analysis (PCA). Gene symbols were derived using bitr function from the bioconductor clusterProfiler package (https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html) and the bioconductor org.Mm.eg.db annotation library from the supplied entrez identifiers (https://bioconductor.org/packages/release/data/annotation/html/org.Mm.eg.db.html) to ensure accepted gene symbol nomenclature. Raw counts were used as recommended by the DESeq2

package. Only genes with at least 1 CPM in at least half of the samples in any of the LT-HSC, ST-HSC, MPP or $CD63^+$ conditions were kept; a total of 16552 transcripts in the IL-1rn-KO project (GSE126428). Only genes with an average mean raw count total of 16 or greater in any of the LT-HSC, ST-HSC or MPP were kept; a total of 11881 in the $NRAS^{G12D}$ project (GSE126625). Differentially expressed transcripts were considered, with an adjusted $p<0.05$ for each of the LT-HSC, ST-HSC, MPP, and $CD63^+$ stromal cells versus their respective controls. The DESeq2 results for LT-HSC, ST-HSC, and MPP were used to generate the Venn diagrams.

The $CD63^+$ stromal cell volcano plot from GSE126428 shows genes with an absolute $-0.5>\log_2FC>0.5$. Genes in red for $CD63^+$ stromal cell volcano plot have an adjusted $p<0.05$. The top 10 most differentially expressed genes and various genes of interest are labeled in the diagram.

In the gene set enrichment analysis (GSEA), only gene sets with an adjusted $p<0.05$ were considered. The gseGO function from the clusterProfiler package was used to enrich for biological processes. Genelist was supplied as the Euclidian distance of $-\log_{10}$ adjusted p and $\log_2FC$. This ensured that genes ranked with higher significance and higher FC values were given more weight. Kernel density plots using core enriched genes from enriched gene sets were plotted using the bioconductor ggplot2 package. The gseaplot2 function of the bioconductor enrichplot package was used to draw the GSEA plots of the significantly enriched gene sets.

PCA in LT-HSC, ST-HSC, and MPP from the $NRAS^{G12D}$ project GSE126625 was done using the plotPCA function of the DESeq2 package. The top 5000 most variable genes after rlog normalization were used to create the PCA plot.

The method for quantifying activity levels of NF-κB targets in LT-HSC, ST-HSC and MPP from accessions GSE126428 and GSE126625 [48], was adapted from pathway level analysis of gene expression [29]. The method performs PCA on the subset of the gene expression matrix that only includes those genes that are members of the gene set of interest (i.e. NF-κB target genes from Synapse ID syn4956655 [49]). The sample scores on the first principal component capture the major pattern of variation for the gene set and are used as an estimate of overall gene set activity. Three modifications were made to the original microarray-based method to adapt it for RNA-Seq data. First, due to the high number of genes with very low counts present in cell lines, the step in the algorithm where gene expression is normalized by variance was removed. This reduces the risk of low count genes being overestimated by the normalization. Second, as activity levels are compared between different datasets, it is necessary to correct for the arbitrary choice of positive direction of PCA. As such, a check of the PCA loadings between datasets was included and the positive direction chosen to obtain the maximum consistency between the PCA loadings, or rotation matrixes, between datasets. Third, to simplify interpretation and plotting, the scale was adjusted by subtracting the minimum activity score for a dataset to obtain only positive activity values. Stem cell relevant NF-κB target genes were taken from the human cd34+_stem_cells_-_adult_bone_marrow_derived network found in Synapse ID syn4956655 [49]. Human target genes were converted to the mouse genome using biomaRt [50] before their use as a gene set to calculate NF-κB activities. Per sample transcription factor activities between groups were compared using a standard t-test. This NF-κB gene list (n=1832) is detailed in Supplementary Table S4.

For accession GSE157038, RNA was amplified with the NEBNext Single cell/Low input RNA libray kit v. 3.0 (NEB#E6420) following the protocol to the fragmentation step. cDNA was fragmented to target size of 150 base pairs with Covaris M220. Libraries were prepared with

TruSeq RNA library preparation kit v. 2.0 (RS#122-2001, Illumina) starting the protocol from "perform end repair" to final library. Libraries were sequenced with the NextSeq550 instrument (Illumina) at the GSCT (UiT – The Arctic University of Norway). Two biological replicates were used per experimental group. Fastq files were trimmed for adapters using flexbar as recommended by the NEBNext® Single Cell/Low Input cDNA kit workflow. Transcript quantification of fastq files were performed using Salmon [51] with the commands --numBootstraps 100 --seqBias --gcBias --validateMappings --minScoreFraction 0.2 --consensusSlack 0.1. Gene level estimation was performed with Sleuth [52]. Forward and reverse fastq reads, differential gene expression and normalized CPM from FACS-sorted *Nes*-GFP$^+$ (GSE157038) are accessible at GEO archive. GSEA was performed as previously described using curated gene sets from the Molecular Signatures Database.

Expression data using normalized signal intensities were downloaded from GSE14468 [30-32], and used for survival analysis of 381 AML patients, and violin plots of 395 AML patients comparing the FAB classification of AML subtypes M0-M3 versus M4-M5. An average of probes '212657_s_at', '212659_s_at', '216243_s_at' specific to the human gene IL1RN ENST00000409930 was used in the analysis of survival. Probe "216243_s_at" specific to the *IL1RN* transcript 1, NM_173842, was used in the analysis of expression. Survival data were gently provided by contributor author Dr. P. J. M. Valk (Department of Hematology, Erasmus University Medical Center, Rotterdam, The Netherlands). Patients with overall survival of less than 16 days, patients aged less than 18 years of age and patients of unknown age or unknown cytogenetic risk were excluded from the study. Survival curves were plotted for the entire population with the function ggsurvplot from the survminer R package, and the Cox proportional hazards regression model. *IL1RN* low and high expressing samples were determined in the global population based on continuous gene expression values and classified using an optimal cutoff based on the split with minimal p value, which had the most significant association with overall survival. The optimal cutoff was identified as 7.970175 (log_2 normalized signal) corresponding to the 91st percentile. Cox proportional hazards regression analysis was perfomed using log_2-transformed gene expression values. Likelihood ratio test was used to test significance. The R packages data.table, ggplot2, gridExtra, survival and survminer were used in the analysis.

Expression data using Reads Per Kilobase per Million mapped reads (RPKM) were downloaded from GSE83533 [34], and used for relapse-free probability analysis and violin plots of 19 matched-pair diagnosis-relapsed AML patients. Accompanying relapse-free time data were downloaded from the accession phs001027 from the database of Genotypes and Phenotypes (dbGaP) using the dbGaP File Selector and the SRA Run Selector. Relapse-free time data were plotted using the Kaplan-Meier method. *IL1RN* low and high expressing samples were classified using a mean cutoff. Cox proportional hazards regression analysis was perfomed using log_2-transformed counts, as previously described. Log rank test was used to test significances. The R packages data.table, ggsci, ggplot2, survival and survminer were used in the analysis.

Statistical analyses

Data are expressed as standard error of the mean (SEM), except in box and whisker plots. Here, horizontal line indicates median, and box top and bottom are upper and lower quartiles, respectively. Whiskers extend to most extreme point within 1.5 times the interquartile range of the box. Individual data points are shown in main human studies. Statistical analyses were performed using Prism 9 software (GraphPad) and the R software environment. Statistical significance was evaluated by unpaired two-tailed t-test or Mann-Whitney U test where appropriate. Statistical significance in *IL1RN* RNA-Seq data from dbGaP accession phs001027

(GSE83533) from paired diagnosis-relapse AML patient samples was assessed with paired two-tailed t-test. Age-adjusted and cytogenetic risk-stratified survival curves from GSE14468 were carried out using the Cox proportional hazards regression model. Likelihood ratio test was used to test significance. Relapse-free probability analysis from GSE83533 was carried out through the Kaplan-Meier method. Cox proportional hazards regression model followed by log rank test was used to test significances. A p value less than 0.05 was considered significant. Adjusted p values in RNA-Seq were derived from raw p values corrected for multiple testing using the Benjamini-Hochberg procedure.

Mice were randomized to treatment groups, without blinding. Animals that showed symptoms of disease or health issues unrelated to aberrant myelopoiesis were excluded from the study, i.e. obesity, tumor masses, etc. Criteria applied for mouse termination before the established end point were in accordance with the Norwegian Food and Safety Authority. Outliers in mouse studies were excluded using Grubbs or Dixon test where appropriate. For *in vivo* experiments, sample size was calculated based on estimation of the minimum number of animals required to obtain biologically meaningful results, and most experiments were performed at least in duplicate. Cohort size in xenografts was informed by the total number of available cells.

For human studies, all available samples were used. Outliers in human studies were not excluded.

Supplementary References

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Table S4

TF	Target
NFKB	Aagab
REL	Aagab
NFKB1	Aagab
RELA	Aagab
NFKB2	Aars2
RELA	Aars2
NFKB	Aars2
NFKB1	Aars2
REL	Aars2
REL	Abca1
RELA	Abca1
NFKB	Abca1
RELA	Abca7
NFKB	Abca7
NFKB	Abcb6
REL	Abcb6
RELA	Abcb6
REL	Abcb9
NFKB	Abcb9
NFKB	Abcf1
NFKB1	Abcf1
NFKB	Abcg1
NFKB1	Abcg1
REL	Abcg1
NFKB	Abhd17a
NFKB1	Abhd17a
NFKB	Abi3
NFKB	Abr
NFKB	Acap1
NFKB1	Acap1
NFKB2	Acap1
REL	Acap1
NFKB	Acot13
NFKB	Acox1
REL	Acp2
NFKB	Acp2
RELA	Acp2
NFKB	Acsf3
NFKB2	Acsf3
NFKB1	Acsf3
REL	Acsf3
RELA	Acsf3
NFKB	Actb

RELA Actr1a
NFKB Actr1a
RELA Actr5
NFKB Actr5
NFKB Adal
NFKB Adarb1
NFKB1 Adarb1
NFKB1 Adat3
NFKB Adat3
NFKB Adcy7
NFKB Adipor1
NFKB2 Adipor1
REL Adipor1
RELA Adipor1
NFKB1 Adipor1
NFKB Aebp2
NFKB2 Agfg1
NFKB1 Agfg1
NFKB Agfg1
NFKB Ago1
NFKB Ago3
NFKB1 Ago3
NFKB1 Agpat3
NFKB Agpat3
NFKB Ahcyl2
NFKB1 Ahnak
RELA Ahnak
NFKB2 Ahnak
NFKB Ahnak
NFKB Ahr
NFKB1 Ahr
NFKB Ahsa1
REL Ahsa1
NFKB1 Ahsa1
RELA Ahsa1
NFKB Ak1
NFKB Ak2
RELA Akap11
NFKB Akap11
REL Akap11
NFKB Akirin2
RELA Akirin2
NFKB1 Akirin2
NFKB2 Akirin2
NFKB2 Alas1
NFKB Alas1

RELA Alas1
NFKB1 Alas1
NFKB Aldh16a1
NFKB1 Aldh16a1
NFKB2 Aldh16a1
NFKB Aldh1b1
NFKB1 Aldh1b1
NFKB2 Aldh1b1
RELA Aldoc
NFKB2 Aldoc
NFKB Aldoc
NFKB Alg1
NFKB2 Alg1
NFKB1 Alg1
NFKB2 Alg12
NFKB Alg12
NFKB1 Alg12
RELA Alg6
NFKB Alg6
REL Alg6
NFKB Alkbh1
NFKB1 Alkbh6
NFKB Alkbh6
NFKB2 Alkbh6
NFKB2 Alox5
NFKB Alox5
NFKB1 Alox5
NFKB Alpk1
REL Angel1
NFKB Angel1
RELA Angel1
NFKB1 Angel1
NFKB Ankhd1
NFKB Ankhd1
NFKB Ankra2
NFKB1 Ankra2
NFKB1 Ankrd16
NFKB Ankrd16
RELA Ankrd39
NFKB Ankrd39
REL Ankrd39
NFKB Ankzf1
RELA Ankzf1
REL Ankzf1
NFKB Anln
NFKB1 Anln

NFKB2 Anln
NFKB Anp32e
NFKB1 Anp32e
NFKB Anxa4
RELA Anxa4
NFKB1 Anxa6
NFKB Anxa6
NFKB Aoah
NFKB1 Aoah
NFKB2 Aoah
NFKB Ap1ar
NFKB Ap1m1
NFKB1 Ap3d1
NFKB Ap3d1
NFKB Ap4m1
NFKB1 Apba3
NFKB Apba3
RELA Apba3
NFKB1 Aplf
NFKB2 Aplf
NFKB Aplf
RELA Aplf
REL Aplf
REL Appl1
RELA Appl1
NFKB Appl1
NFKB1 Aprt
REL Aprt
RELA Aprt
NFKB2 Aprt
NFKB Aprt
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NFKB Arfgef1
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NFKB2 Arhgef2
NFKB1 Arhgef40
NFKB2 Arhgef40
NFKB Arhgef40
RELA Arhgef40
NFKB1 Arhgef9
NFKB Arhgef9
NFKB Arid1a
RELA Arid1a
REL Arid1a
RELA Arid3a
NFKB Arid3a
RELA Arid5a
NFKB Arid5a
REL Arid5a
RELA Arl2bp
NFKB Arl2bp
REL Arl2bp
NFKB Arl3
RELA Arl3
NFKB Arl5b
NFKB1 Arl5b
NFKB Arl6ip4
NFKB Arl6ip5
REL Arl6ip5
RELA Arl6ip5
NFKB1 Arl6ip5
RELA Arl8b
NFKB Arl8b
NFKB Armc7
NFKB Armc9
RELA Armc9
NFKB1 Armc9
RELA Arrb1
NFKB Arrb1

RELA As3mt
NFKB As3mt
NFKB Asap1
NFKB Asb13
NFKB Ascc1
RELA Ascc2
NFKB Ascc2
REL Ascc2
NFKB Asf1b
NFKB1 Asf1b
NFKB2 Asf1b
NFKB Ash2l
NFKB1 Ash2l
RELA Asrgl1
NFKB1 Asrgl1
NFKB2 Asrgl1
NFKB Asrgl1
RELA Atad2b
NFKB Atad2b
NFKB1 Atad2b
NFKB Atat1
NFKB Atg13
NFKB Atg14
NFKB1 Atg14
NFKB2 Atg14
NFKB2 Atg16l2
NFKB Atg16l2
RELA Atg16l2
NFKB1 Atg16l2
REL Atg9a
RELA Atg9a
NFKB Atg9a
NFKB1 Atl2
RELA Atl2
NFKB Atl2
RELA Atp11c
REL Atp11c
NFKB Atp11c
NFKB1 Atp11c
NFKB Atp1a1
NFKB1 Atp1a1
REL Atp1a1
RELA Atp1a1
RELA Atp1a3
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NFKB1 Atp1a3
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NFKB1 Atp1b2
NFKB2 Atp1b2
REL Atp2b4
RELA Atp2b4
NFKB1 Atp2b4
NFKB2 Atp2b4
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NFKB Atp2c1
NFKB Atp6v0a2
NFKB Atp6v0b
REL Atp6v1c1
NFKB Atp6v1c1
RELA Atp6v1c1
REL Aurkb
NFKB2 Aurkb
NFKB1 Aurkb
NFKB Aurkb
NFKB B2m
RELA B2m
REL B2m
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NFKB2 B3gat3
RELA B3gat3
NFKB1 B3gat3
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NFKB B3gntl1
REL B3gntl1
NFKB B4galt2
REL B9d2
NFKB B9d2
RELA Bach1
NFKB Bach1
NFKB1 Bach2
NFKB2 Bach2
NFKB Bach2
NFKB Bag2

NFKB1 Bag4
NFKB Bag4
RELA Bag4
NFKB Batf
NFKB Batf3
NFKB Baz1b
NFKB1 Baz1b
NFKB2 Bbx
RELA Bbx
REL Bbx
NFKB Bbx
NFKB1 Bckdk
NFKB Bckdk
NFKB1 Bcl11a
NFKB Bcl11a
NFKB2 Bcl11a
NFKB Bcl7c
NFKB1 Bcl7c
RELA Bcor
NFKB1 Bcor
NFKB2 Bcor
NFKB Bcor
NFKB Bcs1l
RELA Bcs1l
REL Bcs1l
NFKB Bex4
NFKB2 Bglap
RELA Bglap
NFKB Bglap
RELA Bhlhe40
NFKB Bhlhe40
NFKB Bin1
RELA Bin1
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REL Bin1
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NFKB Bloc1s1
NFKB1 Bloc1s1
NFKB Bnip1
RELA Bnip1
REL Bnip1
RELA Brat1

NFKB Brat1
REL Brat1
NFKB1 Brat1
NFKB1 Brd8
NFKB Brd8
NFKB1 Brf2
NFKB Brf2
NFKB Brms1
NFKB Brox
NFKB Bscl2
NFKB2 Bscl2
RELA Bscl2
NFKB1 Bscl2
NFKB Bsg
RELA Bsg
NFKB Bst2
REL Btbd19
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NFKB2 Btbd19
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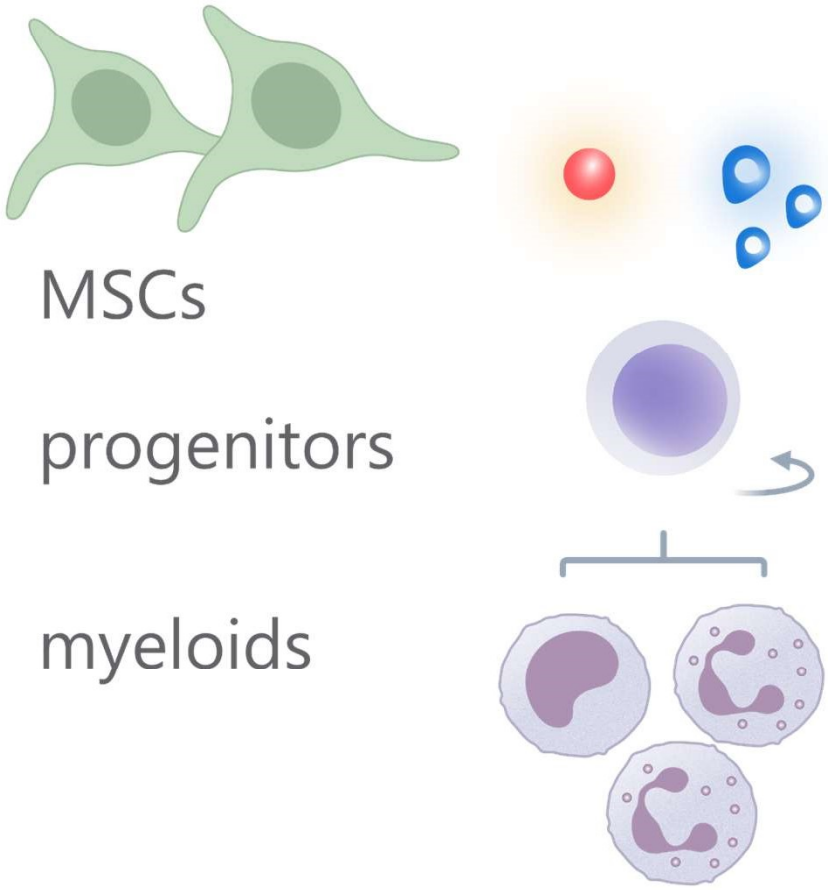
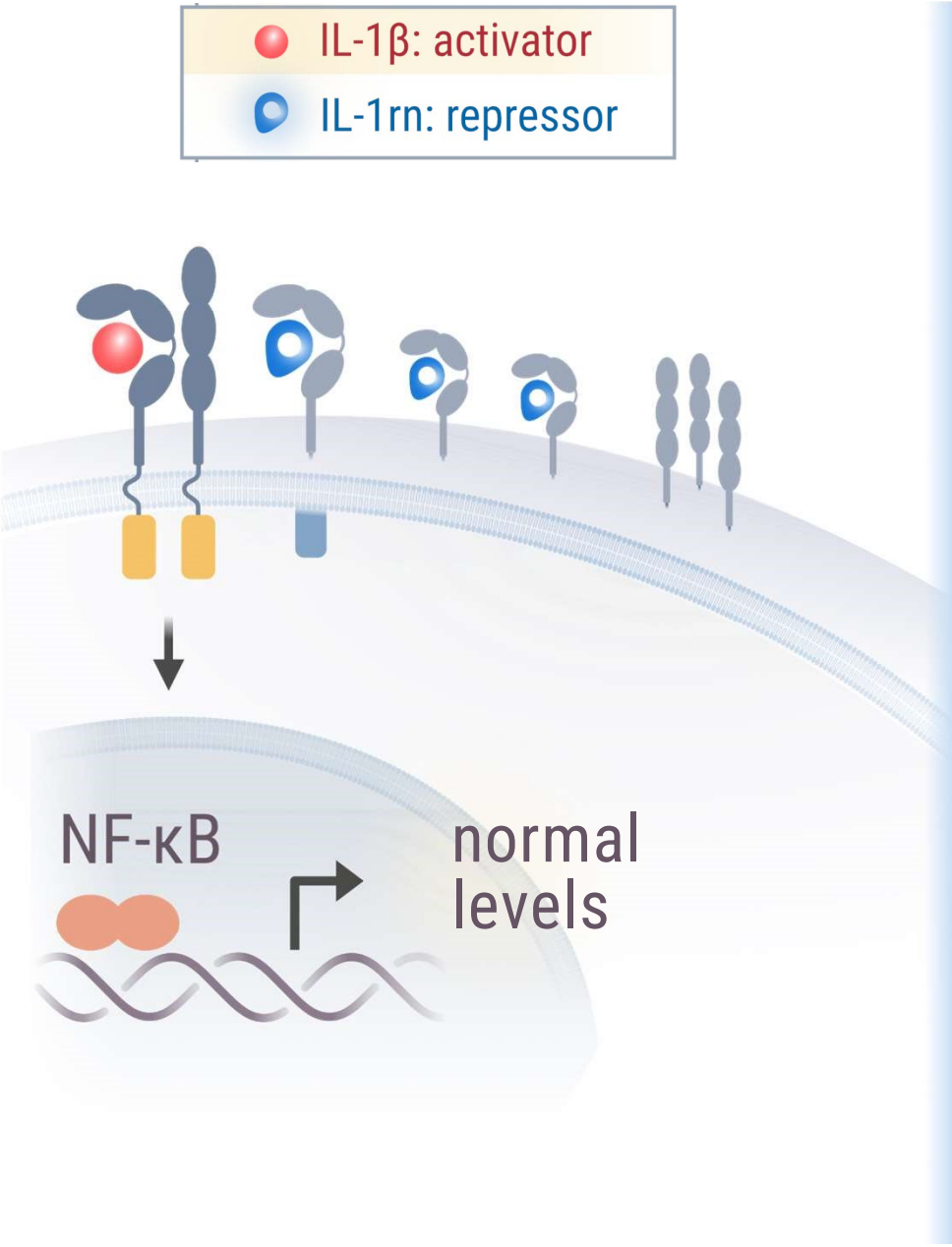
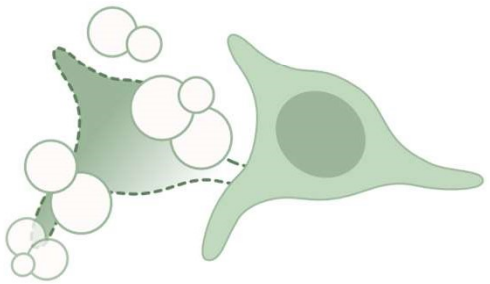
healthy


MSCs
progenitors
myeloids


IL-1β: activator
IL-1rn: repressor
NF-κB
normal
levels

Acute Myeloid Leukemia



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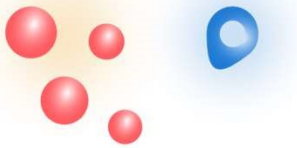
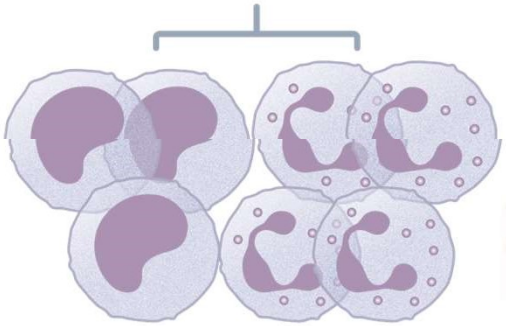
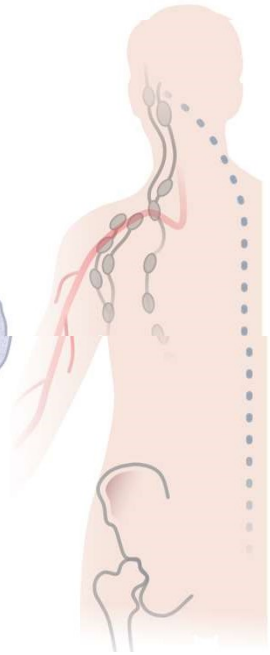
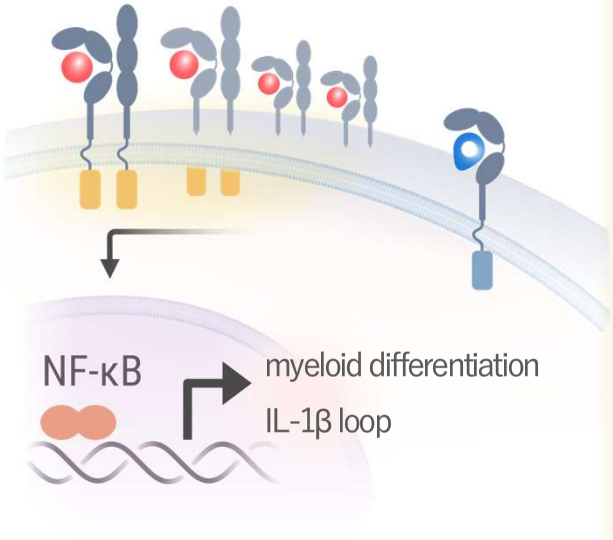


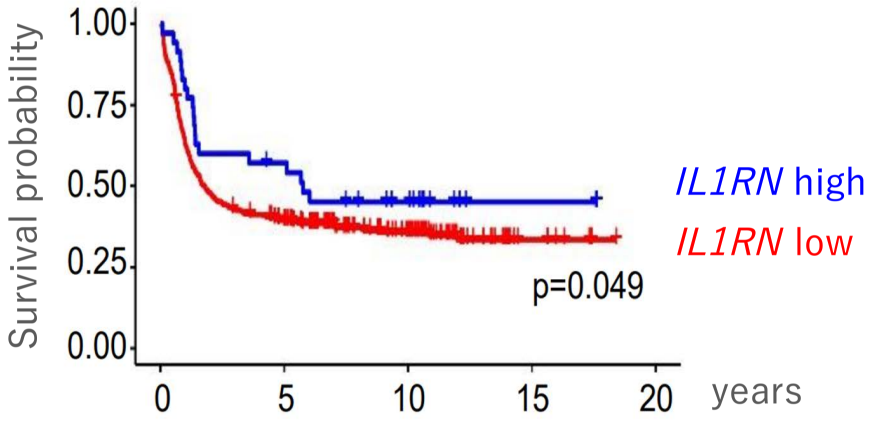
progenitors



myeloids







$IL1RN_{low}$: poorer survival

IL-1β: activator

IL-1rn: repressor