SCUOLA DI DOTTORATO

UNIVERSITÀ DEGLI STUDI DI MILANO-BICOCCA

Department of Medicine and Surgery

PhD program in Translational and

Molecular Medicine

XXXI cycle

The role of the bone marrow microenvironment in aplastic anaemia and acute myeloid leukaemia: from pathogenesis to chemoresistance

Surname: MICHELOZZI   Name: ILARIA MARINA

Registration number: 726367

Tutor: Dr. MARTA SERAFINI

Coordinator: Prof. ANDREA BIONDI

ACADEMIC YEAR 2017/2018

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Human aplastic anaemia-derived mesenchymal stromal cells form functional haematopoietic stem cell niche *in vivo*

Chapter 1

General introduction

1.1 Haematopoietic stem cell (HSC) niche

The series of events through which mature blood cells are generated from haematopoietic stem cells (HSC), self-renewing and multipotent cells, is termed haematopoiesis and it takes place in the bone marrow (BM) since the fifth month of gestation.[1]

Within the BM, a haematopoietic and stromal compartment are present and are spatially and functionally interconnected forming the HSC niche, firstly supposed by Schofield in 1978.[2] The HSC niche represents the site in which HSC reside and in which BM microenvironment accomplishes specific tasks as physical support of haematopoietic cells and preservation and regulation of HSC characteristics and functionality (self-renewal, quiescence, expansion, differentiation and migration).[3]

Conventionally, two niches are distinguished in the BM: osteoblastic or endosteal niche and vascular niche (Fig. 1). The first one is hypoxic, localised in proximity to the bone surface and involved in HSC quiescence preservation; the second one provides a more oxygenated milieu, is localised near sinusoids in the centre of BM and favours HSC proliferation, differentiation and trafficking.[3] However, recent discoveries, made possible by the improvement of technologies, redefined the concept of HSC niche abolishing the strict physical compartmentalisation of the BM.[4] Nowadays, BM is considered as a unique wide area in which different cells could contribute simultaneously to HSC fate,[4] with numerous and specific niches according to the haematopoietic subpopulation involved (e.g.,

macrophages are associated with erythropoiesis and osteoblasts [OB] with B lymphopoiesis).[5–7]
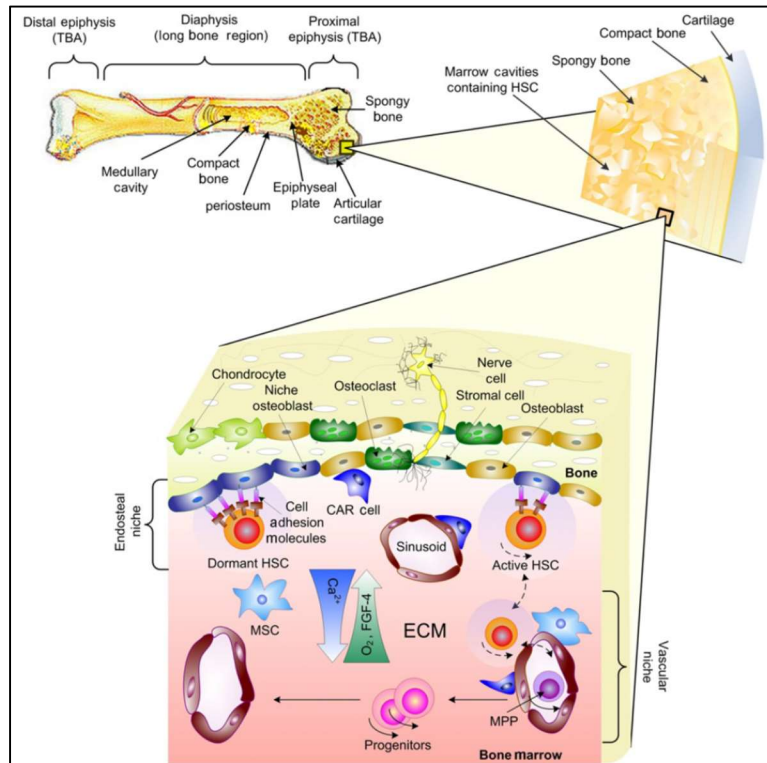
Fig. 1. Architecture of the HSC niche in murine BM, modified from.[3]

1.1.1   <u>Osteoblastic niche</u>

It is mainly formed by OB and osteoclasts[3] and it is enriched also in regulatory T cells (Tregs).[8]

OB were the first cells described involved in HSC control among cellular components of the BM.[9] They were reported to sustain the clonogenicity and the growth of progenitors and HSC *in vitro*[10] and to cause variations in HSC niche and HSC pool size proportionally to their numerical alteration, eventually leading to an enhanced extramedullary

haematopoiesis.[11–14] Moreover, they favoured allogeneic HSC engraftment when coinjected.[15]

In vivo imaging experiments revealed that cell localisation within the BM depends on the level of commitment: long-term HSC (LT-HSC) were detected especially nearer to the endosteum, whereas more mature progenitors were visualised in farther sites.[16] In particular, another study showed that LT-HSC were associated with spindle-shaped N-cadherin-positive OB (SNO) near the bone surface.[11] However, it seems that OB are not strictly involved in HSC regulation as only a minority of HSC was found near the endosteum[17,18] and B progenitors, rather than HSC, were the first to be depleted in genetically modified mice lacking OB.[7]

Osteoclasts accomplish two different tasks within the niche: they favour HSC homing and preservation, augmenting the availability of Ca^{2+} after bone resorption,[19] and the entrance into the bloodstream of progenitors by enzyme release (e.g., cathepsin K, which cleaves niche factors such as stromal cell-derived factor 1 [SDF-1, also known as chemokine CXC motif ligand [CXCL]12] and stem cell factor [SCF, also named Kit ligand]).[20]

Tregs, enriched in the endosteal surface near haematopoietic stem and progenitor cells (HSPC), were essential in transplants for the maintenance of allogeneic HSPC avoiding their immune-mediated destruction.[8]

Osteoblastic niche is regulated by several factors. Wnt and Notch signalling are considered among the main signalling pathways which control HSC, specifically HSC self-renewal. For example, N-cadherin, very late antigen-4 (VLA-4), CXCL12 and osteopontin (OPN) favour

HSC adhesion and maintenance within the niche.[3] Quiescence is promoted by angiopoietin-1 (Ang-1), thrombopoietin (TPO), OPN and N-cadherin.[21] OB release SCF and various growth factors and interleukins, as for example, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-1, IL-6 and transforming growth factor-beta (TGF-β).[3]

1.1.2 <u>Vascular niche</u>

OB are not the only ones able to support haematopoiesis as during foetal life haematopoiesis occurs in organs in which OB are not present.[3] Indeed, endothelial cells could sustain $CD34^+$ cell growth and myeloid and megakaryocytic differentiation *in vitro*.[22] Moreover, the greatest amount of HSC (60%), identified with signalling lymphocyte activation molecule (SLAM) and other markers ($CD150^+CD48^-CD41^-Lin^-$), occupied perisinusoidal position and only the lowest amount (14%) occupied periendosteal position.[17] Thus, a vascular niche has been proposed. It mainly comprises endothelial cells and perivascular cells.[5] Endothelial cells regulate HSC homing (e.g., via vascular cell adhesion molecule-1 [VCAM-1], intracellular adhesion molecule-1 [ICAM-1], E- and P-selectins),[3] HSPC self-renewal (e.g., via bone morphogenetic proteins [BMP-2, -4], insulin-like growth factor binding protein [IGFBP]-2, Kit ligand, CXCL12, Notch ligands) and multi-lineage differentiation (e.g., via IL-6, -8, G-CSF, GM-CSF, IL-1 and tumour necrosis factor [TNF]).[23] Moreover, they can suppress haematopoiesis through TGF-β1, dickkopf-related proteins, inhibitors of Wnt signalling, and Noggin, BMP antagonist.[23]

Perivascular mesenchymal stromal cells (MSC) (defined as CD146^{+} stromal progenitors, CXCL12-abundant reticular [CAR] cells, Nestin^{+} MSC and Leptin receptor^{+} [LepR^{+}] cells) are in contact with blood vessels and HSC and are the major producers of CXCL12.[5] CD146^{+} MSC express genes involved in HSC control, as for example *CXCL12*, and produce Ang-1.[24] CAR cells are defined by the great amount of CXCL12 released, can give rise to adipocytes and OB and control HSC expansion.[21] They can be also identified in the endosteal region.[4] Nestin^{+} cells are involved in HSC homing and maintenance through the expression, for example, of *Cxcl12*, *Vcam1*, *Il7* and *Angpt1*. They display a trilineage differentiation potential and the ability to generate bone and to support haematopoiesis *in vivo*.[25] *Scf* is mainly expressed by LepR^{+} cells within the BM.[5] LepR^{+} cells are associated with HSC preservation within the niche.[9] Nestin[+], LepR[+] and CAR cells are largely similar and quite coinciding.[26] MSC and their role within the niche will be further described in chapter 1.2.

The conventional concept of a proliferative sinusoidal-based vascular niche has recently changed. First of all, another vascular niche, based on arterioles favouring HSC quiescent status, has been suggested;[27] secondly, quiescent HSC have been identified also near sinusoids.[28]

The movement of HSC or progenitors within the BM, from osteoblastic to vascular niche, could be regulated physiologically by increased levels of oxygen, of fibroblast growth factor-4 (FGF-4) and of CXCL12. During stress conditions, HSC activation and migration to the vascular niche is due to the formation of soluble Kit ligand by matrix metalloproteinase-9 (MMP-9) upon CXCL12 and vascular endothelial growth factor (VEGF) stimulation.[29]

1.1.3 Other cellular components

Adipocytes negatively affect BM haematopoiesis. Indeed, it was reported an inverse correlation between adipocyte frequency in BM and HSPC presence and functionality. Moreover, genetically- or drug-mediated suppression of adipogenesis allowed better HSC repopulating properties. Their negative role is exerted by the production of neuropilin-1, lipocalin-2, adiponectin and TNF-α and of decreased levels of G-CSF and GM-CSF. However, it seems that it aims at safeguarding HSC.[30]

Macrophages keep HSPC in BM as their depletion *in vivo* caused a destruction of HSC osteoblastic niche with a decline of OB number, reduction of HSC-related cytokine gene expression and HSPC mobilisation.[31] Specifically, BM CD169^{+} macrophages were involved in HSPC maintenance within the Nestin^{+} niche.[32] Macrophages could also counteract the oxidative stress in HSC via prostaglandin E2 (PGE2) secretion.[9]

HSC are often detected near megakaryocytes, which preserve HSC quiescence via CXCL4, TGF-β1 and TPO release. Under stress conditions, as after irradiation, megakaryocytes promote OB growth contributing to niche reshaping.[9]

HSC and HSPC mobilisation is controlled by circadian variations in CXCL12 expression mediated by macrophages, through aged neutrophil removal, and by sympathetic nervous system, through norepinephrine signalling.[9]

Sympathetic nervous fibres mainly colocalise with arterioles,[33] highly present near the bone.[27] Non-myelinating Schwann cells are associated with HSC quiescence transforming TGF-β into its active form.[34]

1.1.4 HSC niche regulation

Besides the cellular players discussed above, HSC niche is also regulated by other factors as oxygen gradient, transcription factors and extracellular matrix.[3]

HSC were mainly detected in the hypoxic areas of the BM, which preserve their stemness[35] also counteracting oxidative stress.[19]

Hypoxia characterises BM regardless of BM highly vascularised nature. It is not homogeneously distributed in the BM and central areas near sinusoids are the least oxygenated (1.3% of oxygen tension),[36] in contrast to previous data.

Some genes, transcription factors and pathways control HSC self-renewal (e.g., Sox-17, HoxB4 and BMP, Notch, Wnt and Sonic hedgehog signalling) and cell survival and expansion (e.g., *Bcl2*, EGR-1).[3]

Extracellular matrix proteins (laminin, fibronectin, collagen I and IV, haemonectin, thrombospondin and proteoglycans) are essential for architectural stability of the niche, cell adhesion, induction of lots of signallings and they also regulate the accessibility of cytokines and growth factors.[3] Moreover, they can influence HSC and lineage commitment by modulating mechanical forces.[4] Niche reshaping and HSC trafficking are also driven by metalloproteinases (especially MMP-9) and their inhibitors, as tissue inhibitors of metalloproteinases-3 (TIMP-3), within the niche.[3]

Recently, it has been demonstrated that endosteal and vascular niches are integrated as blood vessels are present also in the endosteum, near OB,[16,37] and OB and endothelial cells influence each other. In fact, for example, OB can regulate vasculogenesis and blood vessel

permeability by producing Ang-1 and VEGF, which act on endothelial cells.[33] Moreover, same cells (endothelial, reticular and stromal cells) and mechanisms of haematopoietic control (Notch signalling, Ang-1 and Annexin II) are involved in both niches.[3]

1.1.5   <u>HSC niche and haematological pathologies</u>

The microenvironment can be the initiator of haematological diseases (e.g., myelodysplastic syndromes [MDS], myeloproliferative neoplasia [MPN], leukaemia) as observed in genetically modified murine models.[19] For example, myeloproliferative-like disease arose consequently to deletion of mindbomb1 (*Mib1*) or retinoic acid receptor (*Rarg*) genes in BM microenvironment[38,39] or of retinoblastoma gene (*Rb*) concurrently in haematopoietic and non-haematopoietic BM compartment.[40] Moreover, altered microenvironment is also supposed to be one of the causes of donor cell leukaemia, which arises in donor cells upon BM transplantation.[19]

It is also possible that the malignancy alters the niche to sustain its own survival and progression. Indeed, for example, chronic myeloid leukaemia (CML) cells rendered the microenvironment unfavourable for HSC lowering the levels of CXCL12 within the niche[19] (also observed in acute lymphoblastic leukaemia [ALL]);[41] moreover, CML cells promoted their own proliferation and the vascularisation enhancing the levels of placental growth factor (PlGF).[19] In MPN, the aberrant generation of osteolineage cells, unable to sustain normal haematopoiesis but capable of creating the malignant inflammatory and fibrotic niche, was directed by neoplastic cells.[42] In another model of

MPN, malignant cells led to the death of Schwann cells, through the secretion of IL-1β, and, as a consequence, to the loss of Nestin[+] MSC.[43] ALL cells destroyed osteoblastic and vascular niches and, during the anti-tumour treatment, promoted the generation of a temporary preservative niche.[44]

The role of the microenvironment in aplastic anaemia (AA) and acute myeloid leukaemia (AML) pathogenesis will be deeply discussed in chapter 1.3 and 1.4, respectively.

1.1.6   HSC niche models

In vitro and *in vivo* modelling have been used to better understand normal HSC niche regulation and its pathological alterations.[45,46]

Dexter's long-term BM culture model was based on BM cells seeded on a stromal feeder.[47] It represents a milestone in *in vitro* 2D-studies of HSC niche,[45] and 2D-culture systems, based on either stromal feeder or exogenous cytokines or both, remain the simplest tool to represent BM microenvironment and to maintain HSC *in vitro*.[45,46] More sophisticated systems, as bioreactors and microfabricated culture platforms, are also available, but culture on 3D scaffold is the most suitable tool to better mirror the intricated cellular connections within the BM.[46]

Several approaches have been tested to maintain and expand HSC/HSPC *ex vivo* using small molecules that inhibit HSC/HSPC differentiation and/or promote their self-renewal, including Notch ligand,[48] nicotinamide[49] and StemRegenin1 (SR1).[50] Alternatively, mesenchymal stromal cells have been used with the same purpose.[51]

In AML context, the preservation of leukaemic stem cells (LSC), which will be deeper discussed in chapter 1.4, is the most challenging aspect of *in vitro* cultures.[52] Only two approaches are reported to maintain LSC *in vitro*: culture on MSC layer, associated with LSC preservation up to 6 weeks,[53] and, above all, the culture in a medium containing SR1, inhibitor of aryl-hydrocarbon receptor pathway, and UM729, a pyrimido-indole derivative, which avoid LSC spontaneous differentiation.[52]

Recently, a widespread interest in generating human BM niche into immunodeficient mice has been reported in literature. Generally, starting with human MSC and passing through endochondral ossification, a heterotopic niche, also called ossicle, is generated and can sustain murine or human normal and pathological haematopoiesis.[54–57] The supportive function to the human haematopoiesis is better provided by the heterotopic human ossicle, as compared to the orthotopic murine BM, for both normal and cancer cells (e.g., certain subtypes of AML, MPN as myelofibrosis which normally are difficult to engraft in murine BM).[54] Uniquely BM-MSC and cord-blood (CB)-MSC could generate ossicles,[55,58,59] specifically CD146$^+$ cells.[24,58]

Currently, the majority of *in vivo* models take advantage of scaffolds[54–56,60,61] and a disorganised BM architecture is achieved.[61] These models differ in terms of implant site (kidney capsules or subcutaneous site),[55] scaffold material (e.g., Matrigel[54,55,61] or gelatin[60] or collagen[56] scaffold or hydroxyapatite tricalcium phosphate carriers),[24] type of cells implanted (MSC alone or with endothelial progenitors, resulting in human vessels within the ossicles),[61] the use of osteoinductive

molecules,[54,60] timing (*ex vivo* culture on scaffold or transplantation *in vivo* of haematopoietic cells which could precede or follow MSC implant)[60] and site of human haematopoiesis transplantation (directly in the exogenous forming niche or intravenously).[54,60]

Recently, an innovative *in vivo* model of BM niche has been described. Unlike most models, it avoided the use of exogenous scaffolds and it consisted in the implantation *in vivo* of cartilaginous pellets derived from BM- or CB-MSC. The resulting ossicles exhibited an extraordinar similarity to the bone and BM structure without artificiality: the cortical bone surrounded the BM cavity formed by human stroma, murine sinusoids and murine or human haematopoietic cells (HSPC and mature cells of different lineages).[58,59] This system showed several advantages as the lower initial number of MSC required and the absence of scaffolds, which simplified the implant procedures and the post-explant analysis.[58]

A similar result was obtained by Reinisch et al., starting from a suspension of Matrigel-equivalent and BM-MSC.[55] Well-organised ossicles allowed the engraftment of normal human HSPC, primary AML, T- and B-cell ALL, acute promyelocytic leukaemia and myelofibrosis, preserving the initial subclonal organisation of AML samples.[54]

1.2 Bone marrow-mesenchymal stromal cells (BM-MSC)

MSC are multipotent cells able to self-renew and differentiate into mesodermal tissue generating OB, adipocytes and chondrocytes.[26] Firstly isolated from adult BM in the '70s by Friedenstein, they were defined as clonogenic, fibroblast-like cells.[62] Even if derivable from several sources (adipose tissue, peripheral blood [PB], CB, umbilical cord, skin, dental pulp, foetal tissues, amniotic fluid and chorionic villi of the placenta), BM is the mainly investigated and usable organ, in which MSC account for 0.001-0.01% of the total BM population.[63] To uniquely characterise human MSC, the International Society for Cellular Therapy (ISCT) established the following minimal criteria: adhesion to plastic, positivity for CD105, CD90, CD73 ($\geq$95%) and negativity for CD45, CD34, CD14 or CD11b, CD79α or CD19 and human leukocyte antigen (HLA)-DR ($\leq$2%) and capability to differentiate *in vitro* into OB, adipocytes and chondroblasts.[64] They also seem to be able to trans-differentiate in non-mesodermal lineages, but this property needs to be clarified.[65]

BM-MSC are a heterogeneous population formed by committed progenitors and few multipotent stem cells (CD146^{+}), able to self-renew and generate bone and BM *in vivo*. As CD146^{+} MSC give rise to OB and sinusoidal adventitial reticular cells, they are the most important players in the formation of the HSC niche.[24] According to their heterogeneity, BM-MSC population contains cells with different morphology (fibroblast-like cells, large flattened cells and star-shaped cells),[66] proliferation rate[65] and differentiation potential.[67]

1.2.1 BM-MSC role in haematopoiesis

BM-MSC are an indispensable element of the HSC niche and are involved in haematopoietic support, HSC trafficking and in immunoregulation. In malignant niche, however, they contribute to cancer growth and to drug resistance.[63]

Their ability to support haematopoiesis is confirmed by *in vitro* long-term marrow cultures and by *in vivo* studies of niche modelling, as reported in chapter 1.1. Furthermore, *in vivo* MSC transplantation revealed that MSC could give rise to the elements of the HSC niche (pericytes, myofibroblasts, stromal cells, osteocytes, OB and endothelial cells).[68] Finally, the faster haematopoietic reconstitution when MSC are cotransplanted with HSC is an additional evidence.[65]

HSC survival, self-renewal, growth, migration and lineage commitment are controlled by BM-MSC via direct contact or via growth factors, cytokines (e.g., IL-6, -7, -8, -11, -12, -14, -15, leukaemia inhibitory factor [LIF], macrophage colony-stimulating factor [M-CSF], SCF, CXCL12, Flt-3 ligand), adhesion molecules (ICAM, VCAM) and extracellular matrix molecules (fibronectin, collagen, laminin) important for HSC receptor binding.[65,69] Moreover, they indirectly regulate haematopoiesis through the differentiation into OB, adipocytes and reticular cells, as previously discussed.

MSC are poorly immunogenic, can interplay with innate and adaptive immune cells and, according to the presence or absence of inflammatory milieu, they can exhibit anti-inflammatory (MSC2 type) or pro-inflammatory features (MSC1 type), respectively. Their immunosuppressive effect is mediated by direct contact or by release of soluble factors (e.g., indoleamine 2,3-dioxygenase [IDO], TGF-β,

hepatocyte growth factor [HGF], IL-6, -10, PGE2, heme oxygenase-1 [HO-1], HLA-G5). It mainly consists in the inhibition of proliferation and functions of T, B and not activated natural killer (NK) cells and of monocyte differentiation into immature dendritic cells (DC); moreover, they promote tolerogenicity in DC and formation of M2 macrophages and Tregs.[26,70,71]

On the other hand, BM-MSC were found to increase leukaemic cell survival, growth and proliferation through PI3K/Akt/Bad signalling pathway, Notch signalling pathway, overexpression of anti-apoptotic and downregulation of pro-apoptotic genes or through release of soluble factors as growth factors and inflammatory cytokines.[63] In general, in tumorigenesis, they also promote angiogenesis via VEGF, IL-6, monocyte chemotactic protein-1 (MCP-1) and hypoxia inducible factor (HIF)-1α signalling and they favour tumour immune escape through their immunosuppressive properties.[72] However, MSC provoked an anti-proliferative response in several leukaemic cell lines by blocking their cell cycle at G0/G1 phase, probably in order to maintain their self-renewal capabilities.[63,73]

In addition, BM-MSC contribute to drug resistance, for example, by enhancing anti-apoptotic gene expression[63] or by releasing CXCL12, which stimulated CXCL12/CXC-chemokine receptor type 4 (CXCR4) signalling decreasing caspase 3 activity in CML cells cocultured with MSC and treated with imatinib.[74] Moreover, BM-MSC reduced the adenosine-5'-triphosphate (ATP) and guanosine-5'-triphosphate (GTP) exhaustion normally caused by forodesine and they enhanced RNA synthesis in chronic lymphocytic leukaemia (CLL) cells.[75] They also

replenished asparagine depleted by L-asparaginase (ASNase) protecting ALL cells.[76]

Furthermore, MSC seem to be implicated in the pathogenesis of several haematological disorders as multiple aberrations have been observed. For example, genetic alterations were found in MDS-, AML- and childhood B cell precursor ALL-MSC; functional abnormalities, as increased expression or altered localisation of adhesion molecules, were observed in CML- and MDS-MSC, respectively; AML-, MDS-, AA-MSC exhibited alterations in haematopoietic support and in immunoregulation, this latter also found in CML-MSC. Finally, biological defects as aberrant morphology, altered proliferation and differentiation capabilities were reported in AA-, MDS- and AML-MSC.[63,77,78]

However, the exact involvement of MSC in these diseases is not yet fully understood.

1.3   Aplastic anaemia

1.3.1   BM failure syndromes

BM failure syndromes comprise haematological disorders defined by impaired haematopoiesis and peripheral cytopenia (single lineage or pancytopenia), which is not caused by BM infiltration or peripheral destruction.[79,80] They can occur in children and adults and can be due to genetic mutations and to abnormalities in the immune system and in the BM microenvironment. They can be divided into inherited form, more frequent in childhood and different in the genetic mutations (e.g., Fanconi anaemia, dyskeratosis congenita, Shwachman-Diamond and Diamond-Blackfan syndrome, congenital sideroblastic anaemia, congenital dyserythropoietic anaemia and congenital neutropenia), and into acquired form, as MDS, paroxysmal nocturna haemoglobinuria (PNH), acquired aplastic anaemia (aAA) and acquired megakaryocytic thrombocytopenia (AMT). Moreover, BM failure could be a consequence also of chemicals, radiations, drugs, viruses or of nutrient deficit (vitamin B12 or folic acid) or excess (zinc).[80]

1.3.2   Definition, classification, incidence and therapy

AA is a BM failure syndrome with a hypocellular BM, with no signs of infiltration or fibrosis, resulting in peripheral pancytopenia.[1]

According to its aetiology, it can be distinguished into aAA, idiopathic (70-80% of cases) and consequent to exposure to chemicals (e.g., benzene, pesticides), drugs (direct toxicity, idiosyncratic reactions or activation of immune system), radiations, viruses (e.g., Epstein barr virus, hepatitis A and C, Parvovirus B19), or to post-transfusion graft-

versus-host disease (GVHD), pregnancy, thymoma and PNH.[81,82] Rare forms of AA are congenital and coincide with some inherited BM failure syndromes.[1]

Based on its severity, three forms are distinguished evaluating PB count and haematopoietic BM cellularity (BMC): moderate/non severe AA (MAA or NSAA, BMC <30% without severe pancytopenia and absolute neutrophil count [ANC] between 500 and $1.000/mm^3$ or non-criteria of the other forms), severe AA (SAA, BMC <30% and two of three following conditions: absolute reticulocyte count $<20.000/mm^3$, ANC $<500/mm^3$ or platelet count $<20.000/mm^3$) and very severe AA (VSAA, like SAA + ANC $<200/mm^3$).[81,82]

aAA incidence is about 2-3-fold lower in Europe and North America (about 2/million/year, without considering radio- and chemotherapy-related forms) than in East Asia; it is higher between 10-25 years and >60 years and it is sex-balanced.[1] Anaemia (e.g., asthenia), purpura and bleeding/haemorrhage are the most frequent symptoms in AA patients; infections occur to a lesser extent[83] and BM biopsy is the most important clinical examination for AA diagnosis.[1]

All patients are treated with supportive care. However, SAA and VSAA must be treated with HSC transplantation and immunosuppressive therapy (IST).[1] The main choice of treatment for severe aAA young patients (children and young adults) is matched sibling donor transplantation. IST, based on cyclosporine (CsA) and horse anti-thymocyte globulin (h-ATG), is recommended for more than 70% of patients lacking matched sibling donor.[83]

Clonal evolution could occur and it was observed in 15% of aAA paediatric patients at 10 years after IST: 40% developed PNH, whereas

60% developed MDS/AML.[81] Other drugs (e.g., eltrombopag and alemtuzumab) were tested with positive results as salvage therapies.[82]

1.3.3   Pathophysiology

Currently, aAA is referred to as an immune-mediated disease in which oligoclonal cytotoxic T cells, T helper type 1 (Th1), Th17 and pro-inflammatory environment impair HSPC, BM-MSC, angioblasts and endothelial progenitors.[78,83] However, not all patients are responsive to immunosuppressive therapy and the intrinsic defects of HSPC and BM-MSC may be partly responsible for aAA development.[83]

1.3.3.1 Immunity alterations

The immune-mediated origin of aAA is suggested by clinical indications (e.g., response to immunosuppressive treatment [reaching 80% of AA patients],[83] autologous haematological recovery in patients treated with immunosuppressive conditioning undergoing BM failure and graft failure of syngenic BM transplant in non-pre-conditioned patients).[1] Moreover, it is also suggested by *in vitro* experiments, which revealed in severe aAA patients in remission the presence of BM T cells with inhibitory effect on haematopoiesis.[84]

Both the innate and adaptive compartments of the immune system are altered and could be implicated in the disease. In aAA patients, neutrophils, monocytes and NK cells are reduced in number and NK cells are also impaired in function, whereas DC are augmented and with enhanced expression of costimulatory molecules (CD80, CD86).[78] The involvement of NK cells in AA is not clear as a correlation between NK number and paediatric aAA severity or treatment response was not

found.[85] However, these cells were detected at high levels in SAA BM,[86] and natural killer group 2, member D (NKG2D) expressing lymphocytes, including NK cells, were able to attack haematopoietic progenitors expressing NKG2D ligand aberrantly.[83] Moreover, a restricted number of aAA patients was found mutated in the perforin (*PRF1*) gene and NK cells were reduced in their cytolytic functions.[87]

T cells are the main players in aAA initiation and development[78] and they have been found at high levels in the BM of aAA patients near the remaining haematopoiesis.[88] The aberrant immune response is mainly mediated by Th1/Th17 and CD8$^+$ T cells (augmented in patients), whereas Tregs are lower in number and functionality.[78,83] Th1 cells cause apoptosis of HSPC, through the release of interferon-γ (IFN-γ) and TNF-α, and activation of cytotoxic CD8$^+$ T cells,[78,83] whereas Th17 cells seem to be involved in the initial stage of the disease, even if their expansion has been questioned.[83]

The exact causes of the abnormal activation of T cells are still unclear as no autoantigens have been discovered; however, autoantibodies (e.g., anti-moesin, anti-kinectin, anti-diazepam-binding inhibitor-related protein 1, anti-post-meiotic segregation increased 1, anti-heterogeneous nuclear ribonucleoprotein K and others) have been identified in the serum of aAA patients.[83] T cell activation could be linked to some polymorphisms in *HLA* and in cytokine genes;[83,89] to increased numbers of DC that induce Th1 polarisation and consequently CD8$^+$ T activation; to the reduced Tregs, NK cells and monocytes; to deregulated gene expression in T cells;[78] and to mutations in *PRF1*[87] and in *STAT3* in cytotoxic T cells.[90]

B lymphocytes exhibit a reduction in number and an impaired immunoglobulin production;[78] however, they express high levels of CD86 in SAA, which could contribute to the aberrant T cell response,[91] and they produce autoantibodies.

In aAA reduced levels of cytokines promoting haematopoietic proliferation and differentiation (IL-1, -3, -11) and of TGF-β were observed. Enhanced levels of IL-2, -8, -12, -15, -17, -23 and macrophage inflammatory protein-1α (MIP-1α), which in the majority negatively regulate haematopoiesis and have a role in T cell activation, were also reported.[78] Furthermore, IFN-γ and TNF-α were highly produced in aAA BM[92] and their intracellular levels were associated with patient outcome or response to treatment.[83] They are the most important cytokines involved in haematopoietic inhibition[78] by suppressing the clonogenicity of CD34$^+$ cells and by inducing CD34$^+$ cell apoptosis (via Fas-FasL and TRAIL pathway).[93–95]

1.3.3.2 HSPC alterations

Clinical and *in vitro* evidence suggests an involvement of HSPC in AA pathogenesis. Indeed, after immunosuppressive treatment, haematopoiesis was not fully recovered (e.g., diminished levels of granulocytes, platelets, megakaryocytes and haemoglobin).[96] Moreover, as compared to control, it was observed a decrease of the number of long-term culture-initiating cells (LTC-IC) among CD34$^+$ BM cells (about 7-fold lower)[97] and of their regenerative function.[98] Besides the decreased number of CD34$^+$ cells (resulting in a reduction of 68% as compared to control,[99] reaching more than 99% in SAA considering HSC),[82] HSPC show functional and qualitative alterations.

They exhibited regenerative alterations, even if cocultured on healthy stroma,[98] and impaired sensitivity to exogenous stimulation; this latter is possibly due to diminished levels of erythropoietin (EPO), TPO, G-CSF and GM-CSF receptors, which lead to proliferative and differentiative defects in HSPC.[78] At a transcriptional level, it was observed, for example, upregulation of genes involved in inhibition of proliferation and stress response and of pro-apoptotic genes, whereas genes stimulating cell growth and anti-apoptotic genes were downregulated as compared to control.[100] A similar transcriptional signature was observed in normal CD34+ cells treated with IFN-γ *in vitro*, confirming the role of the immune system in AA.[101] Moreover, higher levels of apoptotic CD34+ cells were found in AA BM samples as compared to normal ones.[102]

A reduction in telomere length was observed in approximately 30% of aAA patients, in granulocyte and mononuclear cells indicating a telomere shortening in HSC.[78,103] It was detected mainly in patients resistant to immunosuppressive treatment, it was related to genetic instability, enhanced probability of clonal evolution, of monosomy 7 and of relapse and decreased overall survival. It was thought to be a consequence of an excessive HSPC expansion; however, in a small proportion of patients, a genetic cause, represented by mutations in telomerase RNA component (*TERC*) and telomerase reverse transcriptase (*TERT*) genes, was discovered. These mutations determine reduction in HSC survival, proliferation and number.[83] Moreover, some aAA patients carry somatic mutations in genes related to myeloid neoplasms (e.g., *ASXL1*, *DNMT3A*, *TET2*, *BCOR*), which are associated with enhanced probability of MDS/AML development.[83]

However, the presence of a residual normal HSPC fraction within aAA BM has been hypothesised as after immunosuppressive treatment an autologous haematopoietic recovery is often observed.[78]

1.3.3.3 <u>BM microenvironment alterations</u>

BM-MSC could be involved into AA pathogenesis considering their role in immune modulation, haematopoietic regulation and their differentiative potential. However, few studies, reporting contradictory results, have been conducted on the characterisation of these cells derived from AA patients. The different results could be due to the heterogeneity of patient cohort analysed, in terms of age and severity of the disease.[83]

Some studies described altered AA-MSC, unable to generate adherent and confluent monolayer,[104] with abnormal morphology,[105] reduced proliferation[104–108] and clonogenic capability,[104,105] enhanced apoptosis[105] and impaired adipogenic and osteogenic differentiation potential.[106,107,109] AA-MSC exhibited lower capacity to support haematopoiesis *in vitro*[104] and impaired immunosuppressive properties[110,111] due to diminished release of TGF-β and PGE2.[111] Altered cytokine and growth factor profile was reported in patient BM stromal cells, as, for example, reduction of GM-CSF, G-CSF and IL-3 production in a small group of patients[112] and elevated expression of membrane-bound IL-15 (mIL-15), an important stimulator of T cells that could be involved in the persistence of autoimmune T cells; this latter underlines an involvement of BM stromal cells, precisely BM fibroblast-like stromal cells, in T cell recruitment and stimulation.[113]

On the contrary, other studies demonstrated that AA-MSC exhibited normal morphology,[106,107,114,115] normal or even higher clonogenicity,[109,110] enhanced proliferative capability than control,[109] a normal differentiation potential[114] or increased towards the adipogenic lineage,[115] unaltered immunosuppressive properties[114,116] and capability to sustain haematopoiesis.[110,114]

AA-MSC exhibited changes in the transcriptome profile[105] with, in general, downregulation of genes involved in cell cycle, cell division, proliferation (e.g., *FGF2*),[108] chemotaxis, haematopoietic cell lineage and upregulation of immune response-related genes, apoptotic and adipogenic genes (e.g., overexpression of *PPARG*).[117] Interestingly, *GATA2* downregulation was reported in AA-MSC and was also observed in normal MSC after treatment with IFN-γ *in vitro*.[117] Moreover, reduction of *CXCL12* expression was also reported.[118]

It is still unknown if alterations in MSC lead to deregulation in immune system or if they are consequences of autoreactive T cells.[110] The hypothesis of an altered BM microenvironment in AA is supported by the fact that stromal cell functional anomalies were reported *in vitro*, although only in few patients,[119] and their growth capability seemed to correlate with AA extent in time and patient survival.[120] Moreover, an AA patient, pre-treated with three unsuccessful allogeneic HSC transplantations, achieved haematological recovery after donor MSC coinjection.[121] However, in the BM of transplanted patients host BM-MSC are totally or almost totally present[121,122] and donor MSC are transient,[121] demonstrating that AA-MSC could sustain durable normal haematopoiesis upon transplantation.[122]

Recently, in AA BM a reduction of endosteal, vascular and perivascular cells[123] and in angiogenesis, with decreased microvessel density, VEGF levels[124] and angioblasts, has been observed.[78] Thus, endosteal and vascular niches seem to be altered and could be involved in the reduction of HSC pool; however, it is not clear if these defects could be cause or effect of the disease.[123]

1.3.4   <u>Animal models</u>

aAA animal models are classified into chemically- or drug-induced and immune-mediated models,[125] and they have been used to ameliorate the comprehension of AA pathogenesis and to test new therapeutic agents. aAA animal models reproduce human BM alterations showing hypoplastic, fatty and poorly vascularised BM with interstitial edema and atrophic stroma. However, unlike humans, in these models also thymus, spleen and lymph nodes are injured, resulting into atrophy and incapability of producing blood cells in extramedullary sites.[78]

Chemically- or drug-induced AA models, generated mainly using benzene, busulfan or chloramphenicol, do not mirror human pathological conditions: they show delayed, late and chronic AA (in busulfan-induced model), early haematopoietic recovery (in chloramphenicol-induced model) with in general, mild cytopenia and BM damages, associated with low rates of lethality.[126,127]

Immune-mediated models include those developed after viral infection and those induced by lymphocyte transplant.[125] The former exhibit AA features within 1-2 weeks (cytomegalovirus-induced AA is limited in time and mortality, lymphocytic choriomeningitis virus-induced AA is acute and related to high mortality [<20 days]). The latter are the most

investigated and used due to their higher similarities to human AA. In these models, donor lymph node cells are injected into host mice with a partial mismatch in minor and/or major histocompatibility loci, which may or may not undergo sublethal irradiation. As a result, recipient mice exhibit acute, severe AA within 3 weeks with no evidence of GVHD.[126]

However, these animal models are not suitable for understanding the role of AA BM microenvironment in the development of the disease underlining the necessity to create a human AA BM niche model.

1.4   Acute myeloid leukaemia (AML)

1.4.1 Definition and incidence

AML is a heterogeneous clonal disorder characterised by myeloid differentiation arrest and aberrant proliferation of myeloblasts, which accumulate in BM, PB and rarely in other tissues.[128] As a potential consequence of the decreased generation of normal haematopoietic cells,[128] patients exhibit anaemia, fatigue, bleeding and higher susceptibility to infections; lymphadenopathy and organomegaly are infrequent.[129]

About 80% of cases of acute leukaemia in adults are represented by AML, whereas AML represents the second most frequent leukaemia in children (15-20%).[128] With an average age of about 68 years,[130] the incidence is lower in patients younger than 65 years old (approximately 1.3 cases per 100,000) than in older patients (approximately 12.2 cases per 100,000)[128] and it is higher in men as compared to women (3 *vs* 2).[131]

1.4.2 Classification

AML subtype classification developed during years, from the first French-American-British (FAB) classification, defined by morphologic and cytochemical features (Tab. 1), to the latest World Health Organisation (WHO) classification which integrates morphologic, immunophenotypic, genetic and clinical aspects (Tab. 2).[129]

Subclass	Description
M0	Acute non-differentiated leukemia – immature blast cells with minimal differentiation
M1	Acute myeloblastic leukemia without maturation – immature blast cells without signs of myeloid differentiation
M2	Acute myeloblastic leukemia with granulocytic maturation
M3	Promyelocytic or acute promyelocytic leukemia (APL)
M4	Acute myelomonocytic leukemia
M4eo	Myelomonocytic leukemia with bone marrow eosinophilia
M5	M5a – acute monocytic leukemia without maturation M5b – acute monocytic leukemia with partial maturation
M6	Acute erythromyelosis
M7	Acute megakaryoblastic leukemia

Tab. 1. FAB classification.[132]

Types	Genetic abnormalites
AML with recurrent genetic abnormalities	AML with t(8;21)(q22;q22); 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 ML 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 AML with BCR-ABL1 (provisional entity) AML with mutated NPM1 AML with biallelic mutations of CEBPA AML with mutated RUNX1 (provisional entity)
AML with myelodysplasia-related changes Therapy-related myeloid neoplasms AML, not otherwise categorised	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 ML associated with Down syndrome
Abbreviations: AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; ML, myeloid leukemia	

Tab. 2. WHO classification, modified from.[129]

In WHO classification, two additional categories are included: myeloid neoplasms with germ line predisposition and acute leukaemias of ambiguous lineage.[133]

1.4.3 Diagnosis

AML diagnosis is based on blast count in BM or PB (≥20%) and on myeloperoxidase activity test, immunophenotypic and morphologic examination, necessary to confirm blast myeloid lineage.[129] Moreover,

cytogenetic, molecular cytogenetic and molecular analyses are fundamental in the detection of chromosomal aberrations and AML peculiar mutations.[133] AML is diagnosed even if the blast count is not ≥20% in case of extramedullary infiltration or t(8;21), t(15;17), inv(16) or t(16;16) chromosomal rearrangements.[129,133]

1.4.4 Pathogenesis

Exposure to ionising radiation, benzene and chemotherapy drugs combined with polymorphisms in genes encoding for detoxifying enzymes, such as NAD(P)H quinone oxidoreductase 1 or cytochrome P450, is a risk factor for the development of AML.[131] AML can also arise from MDS, chronic myeloproliferative disorders[130] and AA, as reported before. In certain cases, AML could be classified among familial forms of myeloid neoplasms that develop in patients with germ line mutations in genes as *ANKRD26*, *CEBPA*, *DDX41*, *ETV6*, *GATA2*, *RUNX1*, *SRP72*, *TERC*, *TERT* and *TP53*.[134] Moreover, various congenital syndromes, as for example Down syndrome and some inherited BM failure syndromes, are associated with augmented risk of developing AML.[130] Nonetheless, the greater proportion of AML cases arises *de novo*.[129]

In the following pages, we will analyse AML at a molecular and cellular level, focusing on the role of LSC and BM microenvironment in AML pathogenesis and chemoresistance.

1.4.4.1 Molecular signature

According to the two-hit model proposed by Gilliland et al., two different types of mutations are necessary to induce leukaemia:[135] a

constitutive activation of pro-survival and proliferative pathways promoting haematopoietic cell growth (class I mutations, e.g., in *K/NRAS*, *FLT3*, *KIT*, *TP53*) and chromosomal aberrations or point mutations in transcription factor genes, which lead to myeloid differentiation arrest (class II mutations, e.g., in *CEBPA*, *NPM1*, *RUNX1*, t(8;21), t(15;17) and inv(16)).[129,135] Recently, a third class of mutations in epigenetic regulator genes (e.g., *DNMT3A*, *TET2*, *IDH1* and *2*) involved in leukaemogenesis has been proposed.[129]

Chromosomal abnormalities are widely harboured in both *de novo* AML (50-60%) and secondary MDS/AML patients (80-95%). In the former, they are mostly represented by structural alterations (about 40%).[136] AML structural aberrations mainly consist in t(8;21), t(15;17), inv(16), t(9;21), t(9;11), del5, del7.[137]

However, mutations in genes usually related to leukaemia were absent in more than 25% of AML patients, and normal karyotype was observed in approximately 50% of AML cases.[137]

Genes frequently mutated in *de novo* AML patients were divided into the following nine categories as reported in a study by The Cancer Genome Atlas Research Network:[138] transcription-factor fusions (18% of cases, e.g., *PML-RARA*, *MYH11-CBFB*, *RUNX1-RUNX1T1*), nucleophosmin (27%, *NPM1*), tumour-suppressor genes (16%, e.g., *TP53*), DNA methylation-related genes (44%, e.g., *DNMT3A*, *TET2*, *IDH1* and *2*), signalling genes (59%, e.g., *K/NRAS*, *FLT3*, *KIT*), chromatin modifiers (30%, e.g., *ASXL1*, *EZH2*), myeloid transcription factor (22%, e.g., *RUNX1*, *CEBPA*), cohesin-complex (13%, e.g., *STAG2*, *RAD21*) and spliceosome-complex (14%, e.g., *SRSF2*, *SF3B1*, *U2AF1*) genes. Mutations could be associated with specific AML FAB,

they increase in number during aging[137] and their acquisition follows a specific order: mutations in epigenetic regulators occur first in pre-leukaemic cells and *NPM1*, *FLT3* and *RAS* mutations occur subsequently in leukaemogenesis.[139]

1.4.4.2 <u>LSC</u>

The hierarchical structure of normal haematopoiesis is mirrored in AML, as a rare proportion of cells, LSC, is responsible for AML initiation, development through the formation of more differentiated blasts, relapse and non-response to therapy.[128]

In vivo xenotransplantation models demonstrated the presence of LSC within human primary AML samples.[140] LSC, denominated SCID leukaemia-initiating cells (SL-IC), were firstly identified as an infrequent population, $CD34^+CD38^-$, with peculiar proliferative, differentiative and self-renewal properties, confirming their involvement in AML initiation and development *in vivo*.[141,142]

LSC are heterogeneous according to their phenotype, origin and self-renewal capability, as they can be classified in short-term, long-term and quiescent long-term cells.[143]

In AML patients' samples, LSC number is variable, ranging from 1 in 1.6×10^3 to 1 in 1.1×10^6 of cells, and LSC were found enriched in $CD34^+CD38^-$ cell population (93% of samples), even if they were also present in $CD34^+CD38^+$ (62%) and in $CD34^-$ fractions ($CD34^-CD38^+$ 8%, $CD34^-CD38^-$ 21%) as indicated by *in vivo* experiments of sorted populations.[144] However, it is widely accepted that $CD34^+CD38^-$ compartment is the most representative, as described below. Indeed, it specifically exhibited downregulation of genes involved in DNA repair,

cell cycle and signal transduction and overexpression of genes involved in stemness (e.g., *MLL*, *VEGFB*, *JAG2*, *IGF1R*), when compared to CD34$^+$CD38$^+$.[145] Moreover, only this compartment exhibited elevated mRNA levels of efflux pump genes (*MDR1*, *BCRP1*), involved in drug resistance, in most of refractory AML patients and not in responders.[146] Furthermore, its proportion was related to patient outcome, survival, relapse and minimal residual disease (MRD) frequency.[147,148]

HSC or more differentiated progenitors could give rise to LSC.[143,144,149] Indeed, in AML-M1, -M4, -M5 the transformation occurs in HSC, whereas in AML-M3 *PML-RARA* mutation occurs in more differentiated progenitors.[142]

Similarly to HSC, LSC exhibit self-renewal, drug resistance, quiescence, repopulating ability and surface marker pattern (CD34$^+$CD38$^-$CD71$^-$HLA-DR$^-$).[128] However, it is possible to discriminate between LSC and HSC according to the presence of LSC-specific markers (Tab. 3), to the expression and activity of aldehyde dehydrogenase (ALDH) (intermediate expression in LSC *vs* high activity reported in HSC), even if this feature is not fully confirmed,[150] and to the distinct molecular profile. Indeed, LSC showed alterations in several pathways, as for example adherens junction, control of actin cytoskeleton and metabolic ones,[151] enhanced survival, proliferation and self-renewal and impaired differentiation.[149] These latter anomalies are due to fusion genes, downregulation of tumour suppressor-associated pathways or deletion of tumour suppressor genes, as *PTEN*, constitutive activation of NF-kB, PI3K/Akt/mTOR, JAK/STAT signalling, deregulation of Wnt/β-catenin, Notch and Hedgehog signalling, overexpression of the anti-apoptotic genes *BCL2* and

BCL2L1 (encoding for Bcl-X$_L$) and dysregulated levels of microRNA (e.g., miR-9 and elevated levels of miR-126).[128,149,151,152]

Marker	Identified as	Expression			
		Normal	In AML (%)	HSC	CD34+ CD38− LSC
IL1RAP	IL1R3	T cells	79	−	+
CLL-1	CLEC12A, MICL, DCAL-2	Myeloid cells	70	−	+
TIM-3	T-cell Ig Mucin 3	Activated T cells, NK cells	91	−	+
CD2	SRBC, LFA2, T11	T cells, NK cells	87	−	+
CD7	GP40, TP41, LEU-9	T cells	43	−	+
CD11b	Integrin alpha M, Mac-1	Myeloid cells	55	−	+
CD22	BL-CAM, Siglec-2	B cells	51	−	+
CD25	IL2RA, TAC	Activated B and T cells	25	−	+
CD33	P67, Siglec-3	Myeloid cells, NK cells	82	+	++
CD44	Adhesion molecule	Ubiquitously	100	+	++
CD45RA	Tyrosine phosphatase receptor type C	T cells, myeloid cells	65	−	+
CD47	Integrin-associated protein (IAP)	Ubiquitously	100	+	++
CD56	N-CAM, MSK39	NK cells, activated T cells	32	−	+
CD96	TACTILE	Activated T cells	33	−	+
CD99	MIC2, single-chain type-1 glycoprotein	Myeloid cells	83	−	+
CD123	IL3R	Myeloid cells	82	+	++

Tab. 3. Specific LSC markers, modified from.[150]

Pre-leukaemic HSC carry only certain AML lesions, mainly in DNA methylation, chromatin modification and topology-related genes, resulting in altered epigenetic regulator functions. They are unable to initiate AML and further proliferative alterations (e.g., mutations in *RAS* and *FLT3* genes), in these cells or in their progeny, are necessary to lead to leukaemia. Moreover, they were still detectable in the BM of some patients in remission and showed repopulation potential, resistance to chemotherapy and a probable role in relapse.[139]

1.4.4.3 BM microenvironment

BM in AML patients at diagnosis is characterised by enhanced microvessel density, neuropathy, reduced frequency of CD146$^+$ progenitors and OB number as compared to normal BM.[153–156] It is

important to underline the role of aging, as most of these alterations increase with age and an aged microenvironment may potentially be involved in haematopoietic dysfunctions. Indeed, aged MSC show impaired functional properties resulting in a decreased capability to sustain haematopoiesis.[153] Moreover, aged mice exhibited, after t(8;21) HSC injection, higher levels of pre-leukaemic stem cells in BM and of immature myeloid cells in PB as compared to younger mice.[157] However, the impact of BM microenvironment alterations on AML pathogenesis remains controversial, as BM niche can be remodelled because of AML development and can cause AML by itself, as discussed below.

BM microenvironment exerts an important role in the maintenance of leukaemic cells *in vitro* and *in vivo*. Indeed, adipocytes (through fatty acids)[158] and endothelial cells (e.g., through GM-CSF, G-CSF, IL-6, after VEGF stimulation, and adhesion molecules)[156] could sustain AML cell expansion and survival. OB could boost AML cell growth (through IL-1β and GM-CSF) and angiogenesis (promoting AML blast production of IL-8).[159] The maintenance of AML cells *in vivo* for more than one year, through serial transplantation, and the persistence of leukaemic cells, after chemotherapy, near vascular endothelium and endosteum, confirmed that the BM microenvironment supports LSC self-renewal, differentiation and protects LSC and cancer cells from drugs.[160,161]

AML-MSC were capable of promoting leukaemic cell quiescence and of protecting them from drugs *in vitro*,[154] for example by stimulating Notch signalling.[162] The protection exerted by stromal cells was usually related to upregulation of Bcl-2 and of the gene encoding for $Bcl-X_L$[163]

and to activation of c-Myc pathway in AML cells;[164] however, one study noted that stromal cells rescued leukaemic cells from apoptosis in a Bcl-2 independent manner.[165] Pathways involved in AML drug resistance are activated by soluble factors or by direct cell interaction (through CD44, VLA-4 and CXCR4).[166] Recently, a novel mechanism of chemoresistance has been discovered *in vitro* and confirmed *in vivo*: BM stromal cells provide mitochondria to AML cells and to LSC through direct exchange enhancing leukaemic cell energy availability and survival. This happens in physiologic conditions and is augmented throughout drug treatment.[167]

Furthermore, like in HSC, hypoxia is involved in LSC maintenance as HIF-1α stimulates CXCR4/CXCL12 signalling and HIF-2α exerts a protective role in AML cells, when overexpressed, and favours AML engraftment *in vivo*.[166]

<u>BM niche remodelling by AML</u>

The localisation within the niche is similar between leukaemic cells/LSC and HSC.[160,161] It leads to competition of cancerous cells with haematopoietic progenitors, disruption of normal BM microenvironment and generation of a leukaemic niche more suitable for neoplastic cell growth and inhospitable to normal CD34^{+} cells.[41]

As in the case of HSC, BM niche provides pro-survival and proliferative signals to LSC and AML cells and it represents a "sanctuary" for chemoresistance.[166] However, as compared to HSC, LSC dependency on BM microenvironment is lower for signallings that prevent excessive HSC proliferation and control myeloid differentiation, as Notch and TGF-β signalling, and higher for CD44-

based anchoring. Moreover, LSC depend, for example, on adhesion and pro-survival signalling activated by CXCL12/CXCR4 and integrin/OPN and on self-renewal and survival signalling mediated by Wnt/β-catenin and PI3K/Akt pathways.[166,168]

Furthermore, AML cells continuously release VEGF promoting their own survival and enhancing angiogenesis.[156]

Alterations of AML microenvironment due to AML cells were confirmed in studies characterising niche components *in vitro* and *in vivo*.

AML-MSC exhibited altered morphology,[169–171] decreased expression of CD146,[172] lower or absent clonogenic capability,[154,169] decreased proliferation,[154,169,170] higher senescence,[154] decreased or delayed osteogenic potential[169,172,173] and increased adipogenic capability.[172] Moreover, they showed impaired ability to sustain normal haematopoiesis *in vitro* and *in vivo*,[154,169,171] confirmed by downregulation of Kit ligand gene and overexpression of Jagged-1.[169] It seems that leukaemic cells are responsible for these alterations; indeed, normal MSC cultured in an AML-conditioned medium exhibited aberrant morphology and reduced growth and osteogenic potential, whereas a restored functionality of AML-MSC of patients in complete remission was observed.[169] According to other studies, AML-MSC did not show morphological,[172,174] proliferative,[173] differentiative[171] or functional alterations in sustaining normal or malignant haematopoiesis[175] or exhibited low adipogenic potential[176] compared to control.

Evidences suggest that leukaemic cells reprogram MSC at a transcriptional and epigenetic level, increasing the expression of pro-

survival genes (e.g., overexpression of *GSKA*, *STAT1*, *STAT5*, *CDKN1A* and *CDK4*), reducing osteogenesis (e.g., downregulation of *BMP4*, *SPP1*, low *OSX* and *OC* mRNA and upregulation of *IGFBP5* and overexpression of *PITX2*) and HSC support (e.g., downregulation of *BMP4*, *SPP1*) and upregulating *VEGFA, VEGFB, CXCL12* and pro-inflammatory cytokine-related genes (e.g., *IL8*, *IL1B*, *CCL2* in coculture with TF-1, an AML cell line).[166,169,173,177]

In vivo experiments evidenced in BM of leukaemic mice a defective bone turnover with a diminished generation of bone tissue, due to reduced and dysfunctional OB, and a temporary expansion of osteoclasts without variations in bone resorption.[178] Moreover, low OB frequency was reported to be essential for AML progression as a proper amount of functional OB seemed to decrease tumour load and augmented mice survival. On the contrary, osteoclasts did not influence AML propagation.[155]

In vivo, AML cells determined nerve fibre disruption and expanded better in denervated mice as compared to controls.[179]

In order to promote their growth, AML cells generate an immunosuppressive microenvironment (with impaired T and NK cell activity, expanded Tregs and induction of monocytic M2-like differentiation). This can be achieved through multiple mechanisms as, for example, the expression of IDO, arginase, programmed death ligand-1 (PD-L1) and release of nitric oxide and immunosuppressive cytokines.[180] Moreover, the deregulated immune system cooperates with AML cells in altering the functionality of MSC as CD4+ T cells derived from AML patients hampered BM-MSC proliferation through the release of miR-10a.[170]

AML cells determine niche remodelling via direct cell contact and via soluble factor and exosome secretion. Exosomes were reported to modify stromal cell and HSPC gene and protein expression, protein release and functionality (expansion, angiogenetic properties and migration) leading to the suppression of normal haematopoiesis.[181,182]

Additionally, metabolic requests in leukaemic niche were found deregulated as indicated by the high dependency on arginase to generate an immunosuppressive milieu[166] and by glutamine and adipogenesis addiction of AML blasts. Indeed, AML cells promoted lipolysis into adipocytes and increased fatty acid transport by enhancing *FABP4* expression, in order to provide energetic substrates for their own expansion.[158] AML glutamine addiction will be discussed in chapter 1.6.

<u>BM niche alteration develops AML</u>

Several evidences demonstrate the involvement of the microenvironment in AML initiation.

Mice carrying *Dicer1* deletion in immature cells committed to generate OB exhibited myelodysplastic and occasionally AML characteristics, even when transplanted with wild type (wt) BM cells. However, no clinical manifestations were observed when mutated haematopoietic cells were injected into wt recipients.[183] Likewise, OB carrying activating mutation of β-catenin affected HSC differentiation and generated AML *in vivo*, through a β-catenin/FoxO1 complex which induced Notch signalling in HSC.[184,185]

Additionally, the microenvironment, represented by different mouse strains and by different *in vitro* growth conditions, was reported to drive

the lineage commitment of *MLL-AF9*-transduced CB CD34^{+} cells into AML, ALL or biphenotypic leukaemic cells.[186] Moreover, AML-MSC exhibited chromosomal structural and numerical variations different[136,174] or similar[174] to those identified in AML cells, indicating increased genetic instability in AML-MSC. MSC aberrations correlated with unfavourable risk-related alterations in haematopoietic cells and, as a consequence, with poor patient outcome.[136] Further studies are needed to evaluate the role of these aberrations in MSC in larger patient cohorts.

In conclusion, AML could be initiated by genetic alterations in both haematopoietic and microenvironment compartments. A deeper comprehension of the function of the BM niche in leukaemogenesis is essential for developing new therapeutic drugs directed against both compartments.

The knowledge of AML pathogenesis could take advantage of humanised xenograft models, discussed in the next chapter.

1.4.5   **AML therapy**

1.4.5.1 **Prognostic classification factors**

Prognostic factors can contribute to the decision of the treatment and are classified into patient-related factors (e.g., age, comorbidity) and AML-related factors (e.g., white blood cells [WBC], previous treatment toxicity or MDS and, moreover, genetic lesions). The first are indices of treatment-related mortality, which has significantly reduced in last years, and the second are indices of therapy resistance.[134] The European

LeukemiaNet (ELN) recommendations distinguish into three different prognostic risk groups of AML according to genetic aberrations (Tab. 4).

Risk category	Genetic abnormality
Favorable	t(8;21)(q22;q22.1); *RUNX1-RUNX1T1*
	inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
	Mutated *NPM1* without *FLT3*-ITD or with *FLT3*-ITD[low.]
	Biallelic mutated *CEBPA*
Intermediate	Mutated *NPM1* and *FLT3*-ITD[high]
	Wild-type *NPM1* without *FLT3*-ITD or with *FLT3*-ITD[low] (without adverse-risk genetic lesions)
	t(9;11)(p21.3;q23.3); *MLLT3-KMT2A*
	Cytogenetic abnormalities not classified as favorable or adverse
Adverse	t(6;9)(p23;q34.1); *DEK-NUP214*
	t(v;11q23.3); *KMT2A* rearranged
	t(9;22)(q34.1;q11.2); *BCR-ABL1*
	inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2,MECOM(EVI1)*
	−5 or del(5q); −7; −17/abn(17p)
	Complex karyotype, monosomal karyotype
	Wild-type *NPM1* and *FLT3*-ITD[high]
	Mutated *RUNX1*
	Mutated *ASXL1*
	Mutated *TP53*

Tab. 4. ELN risk categories, modified from.[133]

Recently, the frequency of $CD34^+CD38^-$ cells resulted to be associated with patient prognosis and its evaluation, in combination with the monitoring of MRD after treatment, represents a superior index for patient risk stratification.[150]

1.4.5.2 <u>Treatment</u>

AML treatment is based on an induction phase, to achieve complete remission (CR) (BM blasts <5%; no circulating blasts and blasts exhibiting Auer rods [needle-shaped bodies observed in the cytoplasm of myeloblasts]; no extramedullary AML; ANC $\geq 1x10^9$/L; platelet

count $\geq 100 \times 10^9$/L), and a consolidation phase, to protract CR avoiding relapse.[129,133]

With the induction phase, in which cytarabine is continuously infused for 7 days and anthracycline is administered for 3 days, CR rates of 60-80% for adult patients <60 years of age and of 40-60% in patients $\geq$60 years of age are obtained.[133]

The consolidation therapy consists of intensive chemotherapy and haematopoietic cell transplantation. Intensive chemotherapy (2-4 cycles of intermediate-dose cytarabine) is usually recommended to favorable/intermediate-risk patients[133] and it leads to CR of 60-70% in favorable-risk related adult patients $\leq$60 years of age.[134] However, CR is achieved only in 10-15% of intermediate-risk patients >60 years of age.[134] Patients which cannot undergo intensive therapy can be treated with supportive care (e.g., hydroxyurea), low-dose cytarabine, hypomethylating agents or with new experimental drugs.[134] Patients with intermediate or high risk profile or resistant to primary treatment are eligible to undergo haematopoietic cell transplantation. Healthy younger patients could be conditioned with myeloablative regimens, whereas reduced-intensity conditioning is preferable for elderly or younger patients with poor health status.[133]

The majority of AML patients relapse within 3 years after diagnosis,[134] and after 3 years of remission the probability of relapse is <10%.[131]

Patients with refractory or relapsed AML can be treated with salvage therapy including intermediate-dose cytarabine plus or not anthracycline, allogeneic transplantation, mitoxantrone-based therapy or fludarabine, cytarabine, G-CSF and idarubicin treatment.[133]

Nowadays, 35-40% of adult patients ≤60 years old and 5-15% of patients >60 years old recover from AML. However, the survival of elderly patients, ineligible to consolidation chemotherapy, is really poor (median, 5–10 months).[134] Relapse causes unsustained remission and occurs in more than 50% of patients; the overall 5 year survival is achieved in a small amount of young (40-45%) and elderly patients (<10%).[128]

The increasing understanding of AML pathogenesis leads to the generation of new drugs which can selectively address LSC, biological processes deregulated in LSC and AML blasts and their interactions with the BM niche in order to improve therapeutic responses.

The new agents are currently tested in preclinical and clinical phase studies, firstly for the treatment of relapsed or refractory AML or elderly patients, ineligible for current chemotherapy.[133]

They comprise novel chemotherapeutic drugs, targeted molecular inhibitors, cell cycle regulators, pro-apoptotic agents, epigenetic modulators, metabolic therapies, immunotherapies and therapies targeting the BM microenvironment.[187,188]

New chemotherapeutic agents include CPX-351 (a liposomal preparation of cytarabine and daunorubicin), recently approved as a first-line treatment for aged patients with secondary AML, vosaroxin (a topoisomerase II inhibitor) which, unlike anthracyclines, is not cardiotoxic and sapacitabine (a nucleoside analogue).[187]

Numerous FLT3 inhibitors have been developed and are divided into two generations which differ in terms of specificity, potency and toxicity. Among them, midostaurin (PKC412, a first-generation FLT3 inhibitor), along with conventional chemotherapy, has been recently

approved for treating adult, newly diagnosed, *FLT3*-mutated AML patients.[187]

In addition to FLT3 inhibitors, targeted therapy comprises Bcl-2 inhibitor (venetoclax ABT-199), aurora B kinase inhibitor (barasertib AZD1152) and polo-like kinase 1 (PLK1) inhibitors (volasertib BI6727 and rigosertib ON 01910.Na). These kinases are involved in the control of mitosis and are highly expressed in AML.[187]

Emerging epigenetic regulators include SGI-110 (guadecitabine), a second-generation of hypomethylating agents with a prolonged half-life due to resistance to cytidine deaminase, and different bromodomain and extraterminal (BET) inhibitors which interfere with the transcription of oncogenes.[187]

New metabolic therapies target glutamine metabolism (as inhibitors of isocitrate dehydrogenase [IDH]1-2, enzymes mutated in a subgroup of patients leading to the formation of [D]-2-hydroxygluratate [2-HG] which alters DNA methylation; CB-839, a glutaminase inhibitor; and ASNase from *Erwinia chrysanthemi*, deeply described in chapter 1.6) and tryptophan catabolism, as indoximod (IDO inhibitor).[187]

Immunotherapy aims at increasing the immune system's response against neoplastic cells potentiating antigen recognition (through peptide or DC vaccination), T cell activation (through, for example, immune checkpoint inhibitors targeting cytotoxic T lymphocyte-associated antigen 4 [CTLA-4]) and AML cell identification and induction of death. These latter could be achieved through several ways based on monoclonal antibodies or genetically manipulated immune cells. Antibodies recognising CD33 and CD123 markers could be conjugated with chemotherapeutic drugs (as Gemtuzumab Ozogamicin

[GO] or SGN-CD33A) or modified in the Fc region to increase antibody-dependent cellular cytotoxicity. Moreover, bispecific monoclonal antibody constructs (bispecific T cell engager [BiTE] antibodies and dual affinity retargeting [DART] molecules), which bind to cytotoxic T cells and to leukaemic antigen, have been produced to facilitate the colocalisation of T and AML cells and therefore the killing of AML blasts. Genetically manipulated cells consist of chimeric antigen receptor (CAR) T cells, CAR NK cells and CAR cytokine-induced killer (CIK) cells.[187]

Besides CTLA-4, programmed cell death protein 1 (PD-1), which binds to its ligand (PD-L1), is another immune checkpoint involved in cancer cell immune evasion. In general, immune checkpoint inhibitors counteract the leukaemic immunological escape and, in particular, anti-PD-1 and anti-PD-L1 antibodies improve the activity of cytotoxic lymphocytes.[187]

Due to the complexity of the disease, combination trials, mixing immunotherapies with the other classes of drugs able to elicit immune activation (e.g., chemotherapy and epigenetic modulators), are the new challenge to increase therapeutic responses.[187]

Therapeutic approaches targeting the BM microenvironment can be divided into three groups. The first interferes with the connections between the niche and the neoplastic cells (e.g., E-selectin, which drives leukaemic cells into BM and protects them from drugs, and CXCR4/CXCL12 axis) and includes E-selectin inhibitor (GMI-1271), CXCR4 inhibitors (plerixafor [AMD3100] and BL-8040) and CX-01, which binds to CXCL12. The second acts on pathways in leukaemic cells regulated by environment stimulation (e.g., growth arrest-specific

6 [Gas6]/Axl pathway, which controls blast growth, migration and survival, targeted by BGb324, Axl inhibitor). The third can take advantage of specificities of the BM niche (e.g., hypoxia) to drive and activate drugs (e.g., TH-302, a prodrug activated by hypoxia).[188]

1.5 Normal and AML haematopoiesis xenograft models

We refer to humanised xenograft models as immunodeficient mice engrafted with human normal or malignant haematopoietic cells. They have improved our knowledge of human normal and malignant haematopoiesis, allowing studies on HSC and LSC, and they have provided useful models for haematopoietic diseases.[189] However, human haematopoiesis is not completely reproduced (problems with the maturation of B and T cells,[189] lack of erythrocytes and platelets and scarce human myeloid cells)[190] and it shows a time-limited reconstitution.[189] The impairment of human haematopoietic cell function could be due to the lack of human lymphoid tissues and of human HLA molecules and to mouse environment.[189] For this latter reason, humanised niche models implanted in xenograft mice were generated, as discussed in chapter 1.1. In addition, the engraftment of some AML subtypes as t(8;21) AML and of some haematological diseases (e.g., MDS, MPN, mature B and T-cell lymphomas) is still problematic in mouse xenograft models.[189,191]

1.5.1 Normal haematopoiesis xenograft models

Engraftment depends on several factors,[189] such as haematopoietic cell source (CD34$^+$ cells: foetal liver, CB, adult BM or G-CSF-mobilised PB), which determines the amount of cells to be transplanted according to their HSC frequency,[189] the route of injection (in adults intrafemoral injection is preferable for fast engraftment to intravenous one,[192] which

is preferable for higher engraftment to intraperitoneal one),[193] the age (newborn mice tolerate lower doses of irradiation as compared to adult ones[193] and, among severe combined immunodeficient [SCID] mice, they are more permissive recipients lacking NK cells),[194] sex (at limiting doses, NOD-SCID γ [NSG] females are more permissive than males),[195] conditioning regimens (briefly described in a specific paragraph at the end of the chapter) and mouse strain.

The mouse must be permissive with defective adaptive and innate immune response, to avoid rejection of human cells, and must exhibit a supportive BM environment.[189]

Through years, many strains of immunodeficient mice arose but in this chapter we will focus on some specific mouse models.

Nude mice (incapable of generating mature T cells because of the spontaneous mutation in the *Foxn1* gene, *Foxn1^nu^*, in homozygosis) were unable to support engraftment of human haematopoietic cells for persistence of murine humoral and innate immunity.[193,196] Thus, the first experiments reporting engraftments of human haematopoiesis in immunodeficient mouse models were reported in CB17-SCID mice (mice without mature T and B cells due to *scid* mutation, which causes deficient V(D)J recombination).[193] However, engraftment level was very low, limited in time and in localisation and without an efficient human immune system formation.[190,193] Human haematopoietic cells appeared/augmented in level when human cytokines (mast cell growth factor [MGF], PIXY321 [fusion of IL-3 and GM-CSF] and EPO) were administered to mice.[197] In this model, human engraftment is hampered by the elevated presence of murine innate immunity, in particular NK cells, and by the natural formation of mouse T and B cells with age

(leakiness).[193] With the term leakiness, we refer to the incomplete penetrance of the *scid* mutation.[198] Thus, casual productive V(D)J rearrangement can happen and generate a small number of T and B clones which undergo expansion.[193,199]

In SCID-beige mice (harbouring *scid* mutation and *beige* mutation; this latter determines alterations of cytotoxic T cells and macrophages and reduction of NK cell activity)[200] poor human engraftment was obtained. Indeed, they exhibited lower levels of human T cells as compared to non-obese diabetic (NOD)-SCID mice (in spleen, 17.5% *vs* 76.9%) 30 days after PB lymphocyte transplantation,[201] and inferior levels of hCD45$^+$ cells as compared to NSG mice (0.28±0.19% *vs* 28.41±29.60% in PB and 0.29±0.17% *vs* 56.9±22.30% in BM) 6 weeks after HSC-enriched transplantation.[202] In this latter case, NSG mice exhibited hCD11b$^+$, hCD11c$^+$, human NK and B cells in BM, whereas SCID-beige showed scarce human cells not sufficient for a comparable phenotypic analysis and only hCD11b$^+$ cells (0.22±0.13%) were reported in BM. In further experiments, 6 weeks after HSC-enriched transplantation, the BM of SCID-beige mice was reported to contain also hCD11c$^+$ (0.55±0.06%) and human NK (0.18±0.05%) besides hCD11b$^+$ cells (1.92±0.31%).[202]

NOD-SCID mice (combining *scid* mutation to NOD background; this latter results in defects in macrophages, DC, NK cells and deficiency in complement) represented a second improvement in xenotransplants.[193,196] They are more permissive for human haematopoiesis[193] and engraftment can be obtained with low cell doses (2x10^4 CD34$^+$ cells).[194] However, the limited lifespan (36 weeks median survival)[196] and the persistent activity of innate immunity,

which hinders the engraftment of the human lymphoid cells and the reconstitution of human red blood cells and platelets (this latter specifically due to macrophages), are the main disadvantages associated with this strain.[193,203,204]

The third progress derived from the generation of NSG mice (NOD/LtSz-*scid Il2rg*$^{-/-}$, NOD background, *scid* mutation and *null* mutation in the gene interleukin 2 receptor gamma chain [*Il2rg*]), which result in further immunosuppression without NK cell development.[193,196] In comparison to other strains, more consistent engraftment and at higher levels, with fewer HSC needed, was observed in the immunodeficient mice *Il2rg*-/-.[193] NSG mice have been widely used in short- and long-term experiments for their lifespan (>89 weeks), absence of SCID mice leakiness and enhanced efficiency of HSC engraftment (human CD45$^+$ cells: 6-fold higher than NOD-SCID mice in BM, about 10-fold in spleen), showing development of human myeloid, NK, plasmacytoid DC, T and B cells.[196,205] Nevertheless, the persistence of macrophages in 6-10 weeks old NSG mice causes the same problems already discussed in NOD-SCID.[203,204]

However, in NOD-SCID and NSG mice the complete reproduction of human haematopoiesis is not yet achieved because of the low levels or lack of mature and well-working myeloid, NK, B and T cells and because of the prevalence of B cell differentiation as compared to T cell one. The absence of cross-reactivity of mouse cytokines probably determines these disadvantages. Therefore, mice expressing human cytokines have been developed,[189] and we focus on NSG-SGM3 (NSGS) mice.

NSGS mice mix the characteristics of the NSG with the constitutive synthesis of human IL-3, GM-CSF and SCF. They show durable engraftment of myeloid and lymphoid lineages, with higher numbers of human T cells (T helper, cytotoxic cells and Tregs), B cells, myeloid progenitors and CD33^{+} cells, DC, mast cells and HSC as compared to NSG mice, and increased human myelopoiesis and differentiation. However, they do not fully support self-renewal of human normal HSC and exhibit inhibition of human erythropoiesis and decreased human B-lymphopoiesis.[196]

1.5.2   AML xenograft models

Human AML engraftment into immunodeficient mice depends on mouse strain (NSGS and NSG are the most efficient models),[206,207] routes of injection (intravenous is preferable to intraperitoneal injection in NOD-SCID[208] and SCID,[209] not in NSG mice),[207] conditioning regimen (irradiation enhances the engraftment levels in SCID mice transplanted intravenously,[209] but not in NSG)[207] and sex of mice (NSG males exhibit enhanced frequency of human acute leukaemic cells [B-, T-cell ALL and AML] in PB than females, mean: 27.3% *vs* 15.7%).[210] However, the cell dose and source (BM or PB) seemed not to be linked to engraftment.[210,211]

Only the 40-50% of AML samples result in consistent engraftment in humanised mouse models,[189] probably because of the lack of human supportive niche and human growth factors, of murine innate immunity persistence and of peculiarities of AML samples.[206]

A connection between FAB subtypes and engraftment was not reported in NSG mice,[212] but it was in NOD-SCID. Indeed, in NOD-SCID mice,

greater engraftment was associated with AML-M0, -M1 and -M4, minor with -M4eo, -M5 and -M7;[208] as compared to other subtypes, AML-M3 and -M2 engrafted less,[213] whereas AML-M0 exhibited superior engraftment than AML-M2, -M4 and -M5.[214]

Furthermore, unfavourable characteristics of AML samples (e.g., high WBC, *FLT3* mutations, elevated frequency of $CD34^+$ cells, non-response to treatment) influenced positively the engraftment in NOD-SCID and NSG mice,[208,212,214] although some parameters (e.g., WBC, relapse and *FLT3-ITD* mutation) were questioned.[208,211]

The xenotransplantation of AML cells began in nude mice resulting in confined myelosarcomas with limited or absent presence in BM.[198] The first promising results were obtained in SCID mice showing a correlation between AML subtypes and infiltration and preservation of the karyotype and phenotype of the original blasts transplanted.[141,215] The initial studies to identify LSC were performed in SCID mice;[141] however, in these recipients, secondary transplants or transplants of limited number of AML-M4 cells were impossible,[142] just AML-M5 was serially passaged.[215]

NOD-SCID mice became the best recipients for AML studies[193] as increased engraftment levels were achieved using lower cell doses[198] and the original features of transplanted cells, as morphology, infiltration, gene expression and genetic aberrations, were unaltered or almost unaltered.[142,208,211] However, an enhanced expression of genes related to granulocytic differentiation[208] and the generation of $CD34^+CD38^+$ leukaemic cells from $CD34^+CD38^-$ transplanted cells suggested *in vivo* differentiation.[142] The engraftment in BM was higher than in spleen and PB.[213] Enhanced engraftment levels were obtained

transplanting secondary AML than primary AML (median, 73.3% *vs* 8.94%).[214] It seemed that the engraftment was not potentiated after *ex vivo* cultivation of AML cells before transplant[208] or after the administration of human cytokines to recipient mice.[213] In secondary transplants, the engraftment was not increased as compared to primary ones[208] and, in primary transplants, the quantity of NOD/SL-IC was retained for at least 3-4 weeks.[213]

As in NOD-SCID mice, in NSG the engraftment was major in BM than PB or spleen[210] and with *FLT3* mutated samples.[212] However, differently from NOD-SCID mice, the percentage of NSG engrafted with AML was higher (e.g., primary AML: about 85% in NSG *vs* about 27% in NOD-SCID mice) and the engraftment was more robust and rapid after acute leukaemia (ALL, AML) transplant.[207] In secondary transplants, the engraftment was generally augmented as compared to primary ones with an increment of the leukaemic cells (up to 44-fold). Subsequent passages showed the maintenance of SL-IC population and of the original properties of the samples.[212] Moreover, little amount of AML blasts was necessary to determine engraftment in NSG (even 0.1-1×10^6).[210] Variations of the original AML phenotype were restricted to CD44, CD38 and CD34 markers.[216]

Recently, it has been demonstrated that NSGS, compared for example to NSG or NOD-SCID mice, favoured the engraftment of pre-leukaemic cells and of AML samples which did not engraft in NSG, resulting in major BM and PB engraftment of AML cells and faster fatal development of the pathology; they also preserve LSC compartment in secondary transplants.[206] NSGS mice represent useful tools to simulate induction therapy after AML transplantation, contributing to the study

of the susceptibility of leukaemic cells to current therapies, and to test new drugs on LSC resistant to previous treatment.[217] Furthermore, deeper analysis of AML clonal organisation was performed in NSG and NSGS mice: the recipients could influence the subclonal expansion of the AML cells transplanted modifying the original subclonal structure of the sample. Moreover, in NSG the expression of the marker CD34 in leukaemic cells was lower as compared to NSGS mice.[218]

1.5.3   Conditioning regimens

The conditioning procedure is another factor which plays a crucial role in allowing enhanced human engraftment in immunodeficient mice. In particular, in SCID mice the permanence of cells of the innate immunity not perturbed by the *scid* mutation, such as macrophages, NK cells and granulocytes, is responsible for the low levels of human engraftment. The conditioning before transplant led to improvement in the reconstitution not only generating space for human cells into murine BM (myeloablation) but also suppressing residual immune function to counteract rejection of human graft (immunosuppression).[219]

The most popular conditioning regimen is sublethal irradiation (up to 4 Gy because SCID mice are x-ray-sensitive) alone or with the coadministration of antibodies, which inhibit residual immune activity or recurrent mouse haematopoietic cells, or the provision of immunosuppressive and alkylating drugs, as cyclophosphamide and busulfan, currently used in clinic for BM transplantation.

There are several strategies to eliminate persistent murine cells of the innate immunity. For example, NK cells can be depleted using anti-asialo-GM1 antibody in SCID mice[198] or anti-CD122 antibody in

SCID[201] and in NOD-SCID mice.[189] Macrophages can be eliminated by administration of clodronate.[201,203]

ACK2, an antibody directed against c-Kit, can be used to deplete murine HSC with the aim to create space for human HSC avoiding the recurrence of recipient haematopoiesis.[220]

In NSG mice, busulfan was found to affect body weight and blood cell count to a lesser extent as compared to irradiation. However, animals treated with busulfan and transplanted with AML cell lines showed diminished overall survival as compared to the irradiated ones, although the levels of engraftment of almost all cell lines were similar between the two groups of mice.[216]

Moreover, conditioning regimens including administration of human cytokines can help human haematopoietic cell reconstitution. Indeed, an improvement in the level and composition of human engraftment in SCID mice was observed when animals were irradiated and administered with human cytokines (PIXY321, MGF, EPO).[197]

The combination of irradiation and human cytokines (PIXY321 with MGF or with SCF) was also used to improve/obtain the engraftment of AML cells in SCID and NOD-SCID mice.[141,142]

In the following subparagraph, we will focus on fludarabine, as in this work of thesis we investigated its use in the conditioning procedure of SCID-beige mice.

1.5.3.1 **Fludarabine**

Fludarabine (9-β-D-arabinosyl-2-fluoroadenine 5'-monophosphate or F-ara-AMP) is a nucleoside analogue characterised by water solubility,

for the 5' phosphate group, and deamination resistance.[221,222] It acts as a prodrug because of the presence of negative charge at physiological pH, which prevents its entrance into the cells.[221]

To obtain the pharmacologically active form (F-ara-ATP), fludarabine has to be dephosphorylated to F-ara-A, probably by 5' nucleotidase, in plasma. Subsequently, through nucleoside transporters, F-ara-A enters the cells where it has to undergo several phosphorylations mediated by deoxycytidine kinase and other kinases (Fig. 2).[221] The incorporation into nucleic acids of the resulting F-ara-ATP determines the suppression of DNA and RNA synthesis and probably DNA repair by interfering with ribonucleotide reductase, DNA polymerases, DNA ligase I and DNA primase; possibly F-ara-ATP interferes with RNA polymerase II, resulting also in reduced protein synthesis.[221,223] Its inhibitory effect could be due to physical interaction with these enzymes, as reported for DNA ligase I in which F-ara-ATP obstructs the AMP-binding site of the enzyme. The inhibitory effect could be as well achieved indirectly through the incorporation of F-ara-ATP into the 3' terminus of the nucleic acid as reported for DNA ligase I and polymerases.[223,224] As a consequence, cells are led to apoptosis.[221]
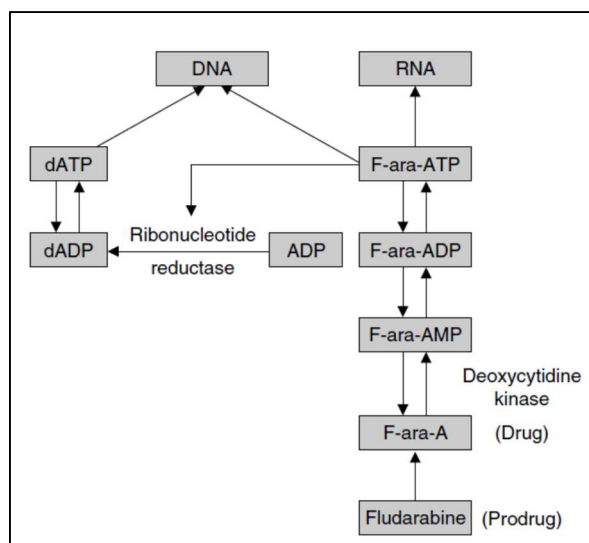
Fig. 2. Fludarabine metabolism and mechanisms of action.[221]

Moreover, other properties have been associated with this drug as reported below.

It has been demonstrated that fludarabine is a suppressor of signal transducer and activator of transcription 1 (STAT1).[225] As STAT1 inhibits osteoclastogenesis and OB differentiation,[226] fludarabine has been reported to act on the BM microenvironment enhancing bone tissue generation in a heterotopic ossification model[226] and restoring in part osteoclastogenesis *in vitro*.[227] Moreover, its effect on STAT1, not related to its incorporation into DNA,[225] can explain its immunosuppressive properties.[228] Indeed, Frank et al., noticed that fludarabine, in normal quiescent or activated lymphocytes, caused a specific inhibition of STAT1 activation and, therefore, of the transcription of STAT1-related genes.[225]

Immunosuppression could also be due to fludarabine inhibition of NF-kB pathway.[229]

Clinically, fludarabine has been reported to be active in many haematological disorders but ineffective in the treatment of solid tumours.[230] It has been a milestone in the treatment of CLL patients[230] and it is used as a first-line therapy in combination with other drugs, cyclophosphamide and rituximab.[130]

Fludarabine positive effects are reported also in patients not previously treated, not responding and relapsed with low grade non-Hodgkin's lymphomas, in mantle-cell lymphoma and in Waldenström macroglobulinemia, as a single agent or administered with other drugs.[230] In AML, the combination of fludarabine, cytarabine, G-CSF and idarubicin is used as a salvage therapy in relapsed and refractory patients.[133]

Additionally, it has been exploited in conditioning regimens preceding allogeneic stem cell transplantation in several haematological disorders, alone or with, for example, busulfan, cytarabine, cyclophosphamide or low-dose total body irradiation (TBI).[230] Its use in combination resulted in minimal toxicity, acute GVHD, transplant/treatment-related death[231–234] and in prolonged remission.[235]

The main side effects related to fludarabine are myelosuppression and enhanced risk of infections.[230]

1.5.3.1.1 Fludarabine in transplantation models

Mice are metabolically different from humans, with faster F-ara-A plasma clearance (half-live of 1.6 hours in mice *vs* about 9 hours in humans) and 10-fold lower activity of deoxycytidine kinase in BM.[223] As a consequence, mice can tolerate 10 to 30 times higher doses than human.[236]

In BM transplantation context, preclinical studies were few and mainly oriented to test fludarabine effects in GVHD mouse models, showing increased survival with reduced GVHD (due to $CD4^+CD44^{low}$ T cell depletion) and retained graft-versus-leukaemia effect.[236–238] So far, no one has investigated if fludarabine could potentiate the haematopoietic engraftment in a mouse model.

1.6   L-Asparaginase (ASNase)

ASNase is an amidase enzyme with asparaginase activity and, depending on its source, with glutaminase activity at different degree. Indeed, catalysing a deamidation reaction, it produces ammonia and L-aspartic or L-glutamic acid starting from L-asparagine (Asn) or L-glutamine (Gln), respectively.[239]

Since the revelation of its anti-lymphoma properties, which dates to the '60s, its therapeutic use has been explored, and microbes became the most suitable and available source for its extensive trade.[240]

Nowadays, the approved clinical formulations *E. coli* ASNase and *Erwinia* ASNase are obtained from *Escherichia coli* and *Erwinia chrysanthemi* bacteria. Another formulation, PEG-ASNase, a pegylated variant of *E. coli* ASNase, is currently employed and it was created to minimise immunogenicity and to maximise the half-life of the drug.[239]

E. coli and *Erwinia* ASNase are very resembling in structure (four indistinguishable subunits, all presenting a catalytic site)[239] but their amino acidic sequence correspondence is only of 46.5%.[241] Thus, sensitivity to proteases[241] and immunogenicity[242] distinguish the two formulations.

Moreover, even if the mechanism of action and almost all harmful effects are the same in *E. coli* and *Erwinia* ASNase, the proportion between the two activities, asparaginase and glutaminase, and pharmacokinetics are distinct.[240,243] Indeed, the affinity for Asn is superior in *E. coli* ASNase (*E. coli* ASNase Km 1.15×10^{-5} M *vs Erwinia* ASNase Km $5.8\text{-}8.0 \times 10^{-5}$ M), whereas the glutaminase activity is increased in *Erwinia* ASNase (*Erwinia* ASNase Km $1.7\text{-}6.7 \times 10^{-3}$ M

and Kcat Gln 65-72 s^{-1} *vs E. coli* ASNase Km 3.5-6.25x10^{-3} M and Kcat Gln 0.33 s^{-1}).[239,243]

Comparing the three formulations in terms of half-life, duration of Asn exhaustion and generation of anti-ASNase antibodies, *Erwinia* ASNase exhibits the lowest half-life and period of Asn deprivation, whereas PEG-ASNase shows the best characteristics (Tab. 5).

Types	Elimination half-life (IM administration)	Asparagine depletion (days after dose)	Anti-asparaginase antibody positive (% patients)
Native *E. coli* asparaginase	26–30 h	14–23	45–75
Pegaspargase	5.5–7 days	26–34	5–18
Asparaginase *Erwinia chrysanthemi*	16 h	7–15	30–50
Dosage for native *E. coli* asparaginase and asparaginase *Erwinia chrysanthemi* was 25,000 IU/m^2, and dosage for pegaspargase was 2,500 IU/m^2			

Tab. 5. Pharmacologic properties of distinct ASNase formulations.[243]

1.6.1 **Mechanism of action**

Asn and Gln are nonessential amino acids but their exhaustion, mediated by ASNase, initiates the amino acid stress response (AAR) via general control nonderepressible 2 (GCN2) and hinders mammalian target of rapamycin (mTOR) pathway, which modulate cellular proliferation and protein synthesis according to amino acidic, nutritive and energetic supply.[239]

Moreover, in leukaemic cells ASNase suppressed c-Myc.[244–246]

Starting from the stimulation of GCN2 and subsequent phosphorylation of the eukaryotic initiation factor 2 (eIF2α), AAR via GCN2 slows down protein synthesis to save energy. Concurrently, through activating transcription factor 4 (ATF4) for example, AAR via GCN2 promotes the expression of pro-survival (e.g., *ASNS* gene, which encodes for asparagine synthetase) or apoptotic genes if the stress persists.

Furthermore, ASNase causes a potentiation of the glutamine synthetase (GS) activity.[239]

Gln exhaustion determines also the suppression of the mammalian target of rapamycin complex 1 (mTORC1) avoiding leucine uptake and the lysosomal placement of mTORC1.[246]

Traditionally, the most important activity of ASNase was thought to be the asparaginase one, whereas glutaminase one was associated with harmful effects.[239] Asn can be derived from the diet or be synthesised by cells through a transamidation reaction catalysed by ASNS enzyme: starting from aspartate and Gln, glutamate and Asn are obtained.[240,243]

The anti-neoplastic effect of ASNase was associated with the exhaustion of Asn serum levels, which determines the death of malignant cells, as ALL cells, subordinated to exogenous Asn for their expansion and viability. This dependence is due to their incapability of producing Asn *de novo* because of ASNS extremely low levels or absence.[240,243] However, glutaminase activity was found to be fundamental to reach anti-tumour response in neoplastic cells expressing ASNS protein, since this protein gives them the ability to generate Asn by themselves.[247] Moreover, ALL cells were reported to be more subordinated to Gln than Asn for cell survival.[248]

In addition, several studies underlined the necessity of having both activities to obtain or potentiate ASNase efficacy on the majority of leukaemic cell lines and on primary ALL cells.[249,250] Furthermore, *Erwinia* ASNase showed superior effects on leukaemic cell lines and on primary AML and ALL cells as compared to *E. coli* or to glutaminase-free mutant ASNase.[246,250]

1.6.2   <u>Glutamine metabolism and tumours</u>

Gln is the most represented within amino acids in humans.[251] Gln can be derived from the diet or be produced *de novo* by GS, even if certain tumours rely only on extracellular Gln due to low expression of GS.[252,253] Neoplastic cells can obtain Gln also through macropinocytosis and extracellular vesicles.[253]

Aerobic glycolysis in tumour cells fulfils their accelerated expansion (Warburg effect). However, in some types of cancer the tricarboxylic acid (TCA) cycle is unaltered because of huge utilisation of Gln.[251] Alterations in oncogenes and tumour suppressor genes which guide Gln metabolism (e.g., *MYC*, *TP53*, *RAS*) are linked to Gln tumour dependence.[251]

Gln, through glutaminolysis, furnishes cells with components of macromolecules and energy "nourishing" the TCA cycle with α-ketoglutarate. Indeed, it is processed by glutaminases obtaining glutamate, and, subsequently, by glutamate dehydrogenase or aminotransferases obtaining α-ketoglutarate. Besides being essential for cell expansion, Gln is also involved into autophagy prevention (through mTOR pathway, inhibition of GCN2 stimulation and through its anti-oxidative derivatives) and in redox homeostasis (via glutathione [GSH] and nicotinamide adenine dinucleotide phosphate [NADPH] formation).[252] Gln was also reported to preserve cancer stem cells through the generation of GSH which lowers oxidative stress that impairs β-catenin pathway.[254]

Impairment of Gln metabolism reported anti-neoplastic effects, for example, in haematological malignancies (myeloma, lymphoma,

leukaemia) and in brain, pancreatic, breast and non-small lung cancer cells.[251]

In general, AML cells were characterised by Gln addiction and by elevated levels of glutaminase mRNA and protein expression, encoded by *GLS1* gene, in particular *GAC* isoform, and of glutamate dehydrogenase 1 (*GLUD1*) at a transcriptional level.[255,256] *GLS1* and *GLUD1* expression level could be related to specific AML subgroups.[256] Reducing Gln availability in AML cells, through different approaches (including suppression of glutaminase, of Gln import and modulation of Gln levels in culture medium), collectively impaired energetic metabolism (TCA cycle, characterised by impoverishment of Gln derivatives, and oxidative phosphorylation) and cell expansion, provoked apoptosis and myeloid and monocytic differentiation.[246,255–257] This latter was reported in *IDH1-2*-mutated AML cells and in U937, respectively.[256,257]

1.6.3   ASNase in AML

Few studies have been conducted in this context *in vitro*. As compared to ALL cells, AML blasts exhibited superior *ASNS* expression at mRNA level[258] and in paediatric context they were less responsive to ASNase, except for AML-M1[259] and AML-M5.[260] The susceptibility to ASNase of paediatric AML-M1, -M4, -M5, probably due to the non-expression of ASNS protein, was greater than that of AML-M2 and -M3 subtypes.[259] Appreciable response to ASNase has been described in AML cells with monosomy of chromosome 7 because of the monoallelic status of *ASNS* which maps to 7q21.3,[261] and it could be achieved in primary *IDH1-2*-mutated AML cells. Indeed, these latter

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cells were reported to respond to glutaminase inhibitor bis-2-[5-(phenylacetamide)-1,3,4-thiadiazol-2-yl]ethyl sulfide (BPTES) due to their addition to Gln.[262]

It was shown that AML cell survival was related to Gln rather than Asn and, therefore, the major cytotoxic effects of ASNase on AML cells were associated with glutaminase activity, which caused the hindrance of mTORC1 and of protein synthesis, apoptosis and autophagy.[246]

1.6.4   ASNase resistance

Several mechanisms of resistance to ASNase have been identified and could be mediated by genetic, immunological, enzymatic, environmental and catabolic factors as discussed below.

Genes involved in ASNase resistance in paediatric ALL cells included genes related to protein metabolism, for example, ribosomal protein genes (e.g., *RPL3*, *RPL4*, *RPL5*, *RPL6*, and *RPL11*),[263] and apoptotic genes (*BCL2L13*, *HRK*, and *TNF*).[264] Additionally, *ASNS* gene expression, the most investigated, was approximately 3-fold increased in resistant primary ALL cells,[263] augmented in resistant ALL cell lines,[265] and *ASNS* suppression rendered AML cell lines more responsive to ASNase.[261] However, the proposal for *ASNS* mRNA as a predictive determinant of resistance has been questioned.[266,267] Recently, the enhanced levels of GS protein upon ASNase treatment were suggested to hamper ASNase efficiency in some cases of AML.[246] ASNase activity can be impaired by anti-bacterial ASNase antibodies *in vivo*[242] and by lysosomal cysteine proteases, cathepsin B (CTSB) and asparaginyl endopeptidase (AEP).[241] Anti-ASNase antibodies are generated following several administrations of the drug, are related to

poor response and outcome and a therapeutic change of ASNase type is required to overcome resistance,[242] as further explained in 1.6.5. paragraph.

CTSB degraded both *E. coli* and *Erwinia* ASNase. AEP specifically recognised at first the amino acid residue N24 on *E. coli* ASNase altering structurally and functionally the enzyme[241] and creating antigenic portions.[242] In an ALL paediatric patient, a germ line mutation in *CTSB* gene, which lowered the proteolytic activity of the enzyme extending ASNase half-life, was detected.[268] Moreover, enhanced mRNA content and activity of CTSB were reported in PB mononuclear cells (PBMC) of AML paediatric patients, and CTSB was proposed as a prognostic determinant.[269]

The involvement of the microenvironment, specifically BM-MSC, adipocytes and macrophages, in ASNase resistance has been recently suggested.

BM-MSC represented an additional provider of Asn for ALL cells by their elevated expression of *ASNS* (at a gene level, about 20 times more than ALL cells), which was potentiated by ALL-produced IGFBP7 in an insulin/insulin-like growth factor (IGF)-related way, and hampered ASNase cytotoxicity.[76,270]

Adipocytes negatively affected *E. coli* ASNase toxicity against ALL cells providing Gln and *Erwinia* ASNase could counteract this effect *in vitro*. Also *in vivo*, in obese leukaemic mice, the therapeutic effect of *E. coli* and PEG-ASNase was reduced as compared to non-obese mice.[248]

The persistence of *E. coli* ASNase in blood and, potentially in BM microenvironment, was hindered by splenic, liver and BM phagocytic

cells, mainly macrophages, which removed ASNase from circulation and, through CTSB, catalysed its elimination.[271]

Moreover, ASNase causes an autophagic response through the hindrance of mTORC1, as reported in ALL[244] and in AML,[246] and through the decrease of Akt/mTOR and the intensification of Erk signalling, as observed in K562, a CML cell line.[272] Autophagy could represent a protective cellular response against ASNase cytotoxicity. Indeed, autophagy is a cellular mechanism leading to protection against apoptosis caused by nutrient deprivation. The blockage of autophagy, in addition to ASNase treatment, potentiates ASNase effectiveness against leukaemic cell lines.[246,272,273]

Autophagy can execute its protective role by favouring the replenishment of amino acids depleted by ASNase[244,272] or by the removal of damaged mitochondria, which determines the lowering of reactive oxygen species (ROS) levels,[273] upon ASNase treatment. Thus, therapeutic approaches made of ASNase and autophagic inhibitors have been proposed.[246,272,273]

1.6.5   ASNase in clinic

In ALL, polytherapy comprising ASNase from *E. coli* or PEG-ASNase represents the treatment of choice; *Erwinia* ASNase is used to counteract hypersensitive reactions against *E. coli* ASNases and in general represents the second/third-line therapy.[274] ASNase application has been reported also in patients with different haematological tumours, as for example, Hodgkin's and non-Hodgkin's lymphomas, multiple myeloma, myelosarcoma and in NK/T-cell lymphoma.[239] In

general, ASNase was not effective in the treatment of lots of solid tumours.[275]

In AML, few but encouraging results followed the administration of ASNase mainly in relapsed or non-responder patients.[243] Initially, ASNase alone resulted in CR in about 10% of cases[275] but, when combined with other pre-existing treatments, superior results were achieved.[276] Indeed, it enhanced the achievement of CR in adults and overall survival in adults and in children when combined with high-dose cytarabine.[276,277] Moreover, ASNase use as a rescue therapy was proposed in aged patients, with high-dose cytarabine and mitoxantrone, or in children, with methotrexate.[278,279] ASNase-including induction regimen was effective as a first-choice treatment in an AML patient which refused blood transfusions.[280] As in ALL, *E. coli* ASNases were mainly used,[275,276,279,280] whereas *Erwinia* ASNase was employed in case of *E. coli* ASNase allergic reactions.[279] Recently, promising results have been obtained in a phase I clinical trial testing *Erwinia* ASNase as a single agent in non-responder or relapsed adult AML patients.[281]

The main side effects of ASNase, which negatively influence its clinical exploitation, are hypersensitivity, hepatotoxicity, pancreatitis, immunosuppression, neurotoxicity and coagulation problems.[240] Alternative sources, engineered, modified and encapsulated forms of ASNase are under investigation to alter its enzymatic activity and potentiate its half-life, to ameliorate storage and proteolytic stability and to counteract antibody generation and harmful effects.[239]

1.7   Scope of the thesis

The purposes of this PhD thesis project were to study the role of the bone marrow (BM) microenvironment in the pathogenesis and in the chemoresistance of two haematological disorders, aplastic anaemia (AA) and acute myeloid leukaemia (AML).

The project has developed as follows:

- The first part of the project aimed at characterising BM mesenchymal stromal cells (MSC) derived from AA paediatric patients in comparison with healthy donor MSC. We have examined in particular their capability to generate *in vivo* a functional and architecturally normal BM niche.
- The second part of the project had the purpose of evaluating the cytotoxicity of L-asparaginase (ASNase) against AML cells. Specifically, we focused our attention on the effect of the drug on leukaemic stem cell (LSC)-enriched compartments and on intrinsic (possible clearance of ASNase mediated by a lysosomal cysteine protease present in blasts) and extrinsic (mediated by monocytes/macrophages and MSC in the BM) mechanisms of drug resistance.
- The third part of the project aimed at exploring the possibility of improving the engraftment of human normal and, especially, malignant haematopoiesis in SCID-beige mice in order to create an affordable model of AML. Specifically, we acted at the

conditioning regimen level combining irradiation and fludarabine.

References

1. Bosi, A., De Stefano, V., Di Raimondo, F. & La Nasa, G. *Manuale di malattie del sangue*. (Elsevier Srl, 2012).

2. Schofield, R. The relationship between the spleen colony-forming cell and the haemopoietic stem cell. *Blood Cells* **4,** 7–25 (1978).

3. Wasnik, S., Tiwari, A., Kirkland, M. A. & Pande, G. Osteohematopoietic stem cell niches in bone marrow. *Int. Rev. Cell Mol. Biol.* **298,** 95–133 (2012).

4. Wang, L. D. & Wagers, A. J. Dynamic niches in the origination and differentiation of haematopoietic stem cells. *Nat. Rev. Mol. Cell Biol.* **12,** 643–55 (2011).

5. Morrison, S. J. & Scadden, D. T. The bone marrow niche for haematopoietic stem cells. *Nature* **505,** 327–34 (2014).

6. Chow, A. *et al.* CD169+ macrophages provide a niche promoting erythropoiesis under homeostasis and stress. *Nat. Med.* **19,** 429–36 (2013).

7. Zhu, J. *et al.* Osteoblasts support B-lymphocyte commitment and differentiation from hematopoietic stem cells. *Blood* **109,** 3706–12 (2007).

8. Fujisaki, J. *et al.* In vivo imaging of Treg cells providing immune privilege to the haematopoietic stem-cell niche. *Nature* **474,** 216–9 (2011).

9. Sánchez-Aguilera, A. & Méndez-Ferrer, S. The hematopoietic stem-cell niche in health and leukemia. *Cell. Mol. Life Sci.* **74,** 579–90 (2017).

10. Taichman, R. S., Reilly, M. J. & Emerson, S. G. Human osteoblasts support human hematopoietic progenitor cells in vitro bone marrow cultures. *Blood* **87,** 518–24 (1996).

11. Zhang, J. *et al.* Identification of the haematopoietic stem cell niche and control of the niche size. *Nature* **425,** 836–41 (2003).

12. Calvi, L. M. *et al.* Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature* **425,** 841–6 (2003).

13. Visnjic, D. *et al.* Hematopoiesis is severely altered in mice with an induced osteoblast deficiency. *Blood* **103,** 3258–64 (2004).

14. Deguchi, K. *et al.* Excessive extramedullary hematopoiesis in Cbfa1-deficient mice with a congenital lack of bone marrow. *Biochem. Biophys. Res.*

Commun. **255,** 352–9 (1999).

15.   El-Badri, N. S., Wang, B. Y., Cherry & Good, R. A. Osteoblasts promote engraftment of allogeneic hematopoietic stem cells. *Exp. Hematol.* **26,** 110–6 (1998).

16.   Lo Celso, C. *et al.* Live-animal tracking of individual haematopoietic stem/progenitor cells in their niche. *Nature* **457,** 92–6 (2009).

17.   Kiel, M. J. *et al.* SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell* **121,** 1109–21 (2005).

18.   Kiel, M. J., Radice, G. L. & Morrison, S. J. Lack of evidence that hematopoietic stem cells depend on N-cadherin-mediated adhesion to osteoblasts for their maintenance. *Cell Stem Cell* **1,** 204–17 (2007).

19.   Hoggatt, J., Kfoury, Y. & Scadden, D. T. Hematopoietic stem cell niche in health and disease. *Annu. Rev. Pathol.* **11,** 555–81 (2016).

20.   Kollet, O. *et al.* Osteoclasts degrade endosteal components and promote mobilization of hematopoietic progenitor cells. *Nat. Med.* **12,** 657–64 (2006).

21.   Bydlowski, S. P., Levy, D., Ruiz, J. M. L. & Pereira, J. Hematopoietic stem cell niche: role in normal and malignant hematopoiesis. *Stem cell biology in normal life and diseases* (2013). Available at: https://www.intechopen.com/books/stem-cell-biology-in-normal-life-and-diseases/hematopoietic-stem-cell-niche-role-in-normal-and-malignant-hematopoiesis.

22.   Rafii, S. *et al.* Human bone marrow microvascular endothelial cells support long-term proliferation and differentiation of myeloid and megakaryocytic progenitors. *Blood* **86,** 3353–63 (1995).

23.   Rafii, S., Butler, J. M. & Ding, B. S. Angiocrine functions of organ-specific endothelial cells. *Nature* **529,** 316–25 (2016).

24.   Sacchetti, B. *et al.* Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. *Cell* **131,** 324–36 (2007).

25.   Méndez-Ferrer, S. *et al.* Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* **466,** 829–34 (2010).

26.   Frenette, P. S., Pinho, S., Lucas, D. & Scheiermann, C. Mesenchymal stem cell: keystone of the hematopoietic stem cell niche and a stepping-stone for

regenerative medicine. *Annu. Rev. Immunol.* **31,** 285–316 (2013).

27.   Kunisaki, Y. *et al.* Arteriolar niches maintain haematopoietic stem cell quiescence. *Nature* **502,** 637–43 (2013).

28.   Acar, M. *et al.* Deep imaging of bone marrow shows non-dividing stem cells are mainly perisinusoidal. *Nature* **526,** 126–30 (2015).

29.   Yin, T. & Li, L. The stem cell niches in bone. *J. Clin. Invest.* **116,** 1195–201 (2006).

30.   Naveiras, O. *et al.* Bone-marrow adipocytes as negative regulators of the haematopoietic microenvironment. *Nature* **460,** 259–63 (2009).

31.   Winkler, I. G. *et al.* Bone marrow macrophages maintain hematopoietic stem cell (HSC) niches and their depletion mobilizes HSCs. *Blood* **116,** 4815–28 (2010).

32.   Chow, A. *et al.* Bone marrow CD169+ macrophages promote the retention of hematopoietic stem and progenitor cells in the mesenchymal stem cell niche. *J. Exp. Med.* **208,** 261–71 (2011).

33.   Purton, L. E. & Scadden, D. T. The hematopoietic stem cell niche. *StemBook* Cambridge (MA) (2008). Available at: http://www.stembook.org.

34.   Yamazaki, S. *et al.* Nonmyelinating Schwann cells maintain hematopoietic stem cell hibernation in the bone marrow niche. *Cell* **147,** 1146–58 (2011).

35.   Parmar, K., Mauch, P., Vergilio, J. A., Sackstein, R. & Down, J. D. Distribution of hematopoietic stem cells in the bone marrow according to regional hypoxia. *Proc. Natl. Acad. Sci. U. S. A.* **104,** 5431–6 (2007).

36.   Spencer, J. A. *et al.* Direct measurement of local oxygen concentration in the bone marrow of live animals. *Nature* **508,** 269–73 (2014).

37.   Xie, Y. *et al.* Detection of functional haematopoietic stem cell niche using real-time imaging. *Nature* **457,** 97–101 (2009).

38.   Kim, Y. W. *et al.* Defective Notch activation in microenvironment leads to myeloproliferative disease. *Blood* **112,** 4628–38 (2008).

39.   Walkley, C. R. *et al.* A microenvironment-induced myeloproliferative syndrome caused by retinoic acid receptor gamma deficiency. *Cell* **129,** 1097–110 (2007).

40.   Walkley, C. R., Shea, J. M., Sims, N. A., Purton, L. E. & Orkin, S. H. Rb regulates interactions between hematopoietic stem cells and their bone

marrow microenvironment. *Cell* **129,** 1081–95 (2007).

41.   Colmone, A. *et al.* Leukemic cells create bone marrow niches that disrupt the behavior of normal hematopoietic progenitor cells. *Science* **322,** 1861–5 (2008).

42.   Schepers, K. *et al.* Myeloproliferative neoplasia remodels the endosteal bone marrow niche into a self-reinforcing leukemic niche. *Cell Stem Cell* **13,** 285–99 (2013).

43.   Arranz, L. *et al.* Neuropathy of haematopoietic stem cell niche is essential for myeloproliferative neoplasms. *Nature* **512,** 78–81 (2014).

44.   Duan, C. W. *et al.* Leukemia propagating cells rebuild an evolving niche in response to therapy. *Cancer Cell* **25,** 778–93 (2014).

45.   Dhami, S. P. S., Kappala, S. S., Thompson, A. & Szegezdi, E. Three-dimensional ex vivo co-culture models of the leukaemic bone marrow niche for functional drug testing. *Drug Discov. Today* **21,** 1464–71 (2016).

46.   Di Maggio, N. *et al.* Toward modeling the bone marrow niche using scaffold-based 3D culture systems. *Biomaterials* **32,** 321–9 (2011).

47.   Dexter, T. M., Moore, M. A. & Sheridan, A. P. Maintenance of hemopoietic stem cells and production of differentiated progeny in allogeneic and semiallogeneic bone marrow chimeras in vitro. *J. Exp. Med.* **145,** 1612–6 (1977).

48.   Delaney, C. *et al.* Notch-mediated expansion of human cord blood progenitor cells capable of rapid myeloid reconstitution. *Nat. Med.* **16,** 232–6 (2010).

49.   Horwitz, M. E. *et al.* Umbilical cord blood expansion with nicotinamide provides long-term multilineage engraftment. *J. Clin. Invest.* **124,** 3121–8 (2014).

50.   Wagner, J. E. Jr. *et al.* Phase I/II trial of StemRegenin-1 expanded umbilical cord blood hematopoietic stem cells supports testing as a stand-alone graft. *Cell Stem Cell* **18,** 144–55 (2016).

51.   de Lima, M. *et al.* Cord-blood engraftment with ex vivo mesenchymal-cell coculture. *N. Engl. J. Med.* **367,** 2305–15 (2012).

52.   Pabst, C. *et al.* Identification of small molecules that support human leukemia stem cell activity ex vivo. *Nat. Methods* **11,** 436–42 (2014).

53.   Ito, S. *et al.* Long term maintenance of myeloid leukemic stem cells cultured

with unrelated human mesenchymal stromal cells. *Stem Cell Res.* **14,** 95–104 (2015).

54. Reinisch, A. *et al.* A humanized bone marrow ossicle xenotransplantation model enables improved engraftment of healthy and leukemic human hematopoietic cells. *Nat. Med.* **22,** 812–21 (2016).

55. Reinisch, A. *et al.* Epigenetic and in vivo comparison of diverse MSC sources reveals an endochondral signature for human hematopoietic niche formation. *Blood* **125,** 249–60 (2015).

56. Scotti, C. *et al.* Engineering of a functional bone organ through endochondral ossification. *Proc. Natl. Acad. Sci. U. S. A.* **110,** 3997–4002 (2013).

57. Scotti, C. *et al.* Recapitulation of endochondral bone formation using human adult mesenchymal stem cells as a paradigm for developmental engineering. *Proc. Natl. Acad. Sci. U. S. A.* **107,** 7251–6 (2010).

58. Serafini, M. *et al.* Establishment of bone marrow and hematopoietic niches in vivo by reversion of chondrocyte differentiation of human bone marrow stromal cells. *Stem Cell Res.* **12,** 659–72 (2014).

59. Pievani, A. *et al.* Human umbilical cord blood-borne fibroblasts contain marrow niche precursors that form a bone/marrow organoid in vivo. *Development* **144,** 1035–44 (2017).

60. Abarrategi, A. *et al.* Versatile humanized niche model enables study of normal and malignant human hematopoiesis. *J. Clin. Invest.* **127,** 543–8 (2017).

61. Chen, Y. *et al.* Human extramedullary bone marrow in mice: a novel in vivo model of genetically controlled hematopoietic microenvironment. *Blood* **119,** 4971–80 (2012).

62. Friedenstein, A. J., Chailakhyan, R. K., Latsinik, N. V., Panasyuk, A. F. & Keiliss-Borok, I. V. Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo. *Transplantation* **17,** 331–40 (1974).

63. Wong, R. S. & Cheong, S. K. Role of mesenchymal stem cells in leukaemia: Dr. Jekyll or Mr. Hyde? *Clin. Exp. Med.* **14,** 235–48 (2014).

64. Dominici, M. *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* **8,** 315–7 (2006).

65. Dazzi, F., Ramasamy, R., Glennie, S., Jones, S. P. & Roberts, I. The role of mesenchymal stem cells in haemopoiesis. *Blood Rev.* **20,** 161–71 (2006).

66. Muraglia, A., Cancedda, R. & Quarto, R. Clonal mesenchymal progenitors from human bone marrow differentiate in vitro according to a hierarchical model. *J. Cell Sci.* **113,** 1161–6 (2000).

67. Pittenger, M. F. *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science* **284,** 143–7 (1999).

68. Muguruma, Y. *et al.* Reconstitution of the functional human hematopoietic microenvironment derived from human mesenchymal stem cells in the murine bone marrow compartment. *Blood* **107,** 1878–87 (2006).

69. Deans, R. J. & Moseley, A. B. Mesenchymal stem cells: biology and potential clinical uses. *Exp. Hematol.* **28,** 875–84 (2000).

70. Bernardo, M. E. & Fibbe, W. E. Mesenchymal stromal cells and hematopoietic stem cell transplantation. *Immunol. Lett.* **168,** 215–21 (2015).

71. Uccelli, A., Moretta, L. & Pistoia, V. Mesenchymal stem cells in health and disease. *Nat. Rev. Immunol.* **8,** 726–36 (2008).

72. Houthuijzen, J. M., Daenen, L. G., Roodhart, J. M. & Voest, E. E. The role of mesenchymal stem cells in anti-cancer drug resistance and tumour progression. *Br. J. Cancer* **106,** 1901–6 (2012).

73. Ramasamy, R. *et al.* Mesenchymal stem cells inhibit proliferation and apoptosis of tumor cells: impact on in vivo tumor growth. *Leukemia* **21,** 304–10 (2007).

74. Vianello, F. *et al.* Bone marrow mesenchymal stromal cells non-selectively protect chronic myeloid leukemia cells from imatinib-induced apoptosis via the CXCR4/CXCL12 axis. *Haematologica* **95,** 1081–9 (2010).

75. Balakrishnan, K. *et al.* Influence of bone marrow stromal microenvironment on forodesine-induced responses in CLL primary cells. *Blood* **116,** 1083–91 (2010).

76. Iwamoto, S., Mihara, K., Downing, J. R., Pui, C. H. & Campana, D. Mesenchymal cells regulate the response of acute lymphoblastic leukemia cells to asparaginase. *J. Clin. Invest.* **117,** 1049–57 (2007).

77. Kastrinaki, M. C. *et al.* Mesenchymal stem cells in immune-mediated bone marrow failure syndromes. *Clin. Dev. Immunol.* **2013,** 265608 (2013).

78.   Li, J. P., Zheng, C. L. & Han, Z. C. Abnormal immunity and stem/progenitor cells in acquired aplastic anemia. *Crit. Rev. Oncol. Hematol.* **75,** 79–93 (2010).

79.   Gordon-Smith, T. Bone marrow failure. *Medicine* **32,** 54–7 (2004).

80.   Zhang, L. Inherited and acquired bone marrow failure syndromes: in the era of deep gene sequencing. *J. Leuk.* **4,** e119 (2016).

81.   Gruppo di lavoro "insufficienze midollari". Raccomandazioni diagnostico-terapeutiche sulle aplasie midollari acquisite in età pediatrica. (2014). Available at: http://www.aieop.org/web/wp-content/uploads/2017/05/LG-AIEOP-2014-sulle-Anemie-Aplastiche-Acquisite-rev.-2.pdf

82.   Boddu, P. C. & Kadia, T. M. Updates on the pathophysiology and treatment of aplastic anemia: a comprehensive review. *Expert Rev. Hematol.* **10,** 433–48 (2017).

83.   Zeng, Y. & Katsanis, E. The complex pathophysiology of acquired aplastic anaemia. *Clin. Exp. Immunol.* **180,** 361–70 (2015).

84.   Bacigalupo, A. *et al.* Immune suppression of hematopoiesis in aplastic anemia: activity of T-gamma lymphocytes. *J. Immunol.* **125,** 1449–53 (1980).

85.   Sutton, K. S., Shereck, E. B., Nemecek, E. R. & Kurre, P. Immune markers of disease severity and treatment response in pediatric acquired aplastic anemia. *Pediatr. Blood Cancer* **60,** 455–60 (2013).

86.   Maciejewski, J. P., Hibbs, J. R., Anderson, S., Katevas, P. & Young, N. S. Bone marrow and peripheral blood lymphocyte phenotype in patients with bone marrow failure. *Exp. Hematol.* **22,** 1102–10 (1994).

87.   Solomou, E. E. *et al.* Perforin gene mutations in patients with acquired aplastic anemia. *Blood* **109,** 5234–7 (2007).

88.   Melenhorst, J. J. *et al.* T cells selectively infiltrate bone marrow areas with residual haemopoiesis of patients with acquired aplastic anaemia. *Br. J. Haematol.* **99,** 517–9 (1997).

89.   Young, N. S., Scheinberg, P. & Calado, R. T. Aplastic anemia. *Curr. Opin. Hematol.* **15,** 162–8 (2008).

90.   Jerez, A. *et al.* STAT3 mutations indicate the presence of subclinical T-cell clones in a subset of aplastic anemia and myelodysplastic syndrome patients. *Blood* **122,** 2453–9 (2013).

91.   Tu, M. F. *et al.* Study on the peripheral blood dendritic cells subtypes and the expression of co-stimulating molecules on dendritic cells and B cells in severe aplastic anemia patients. *Zhonghua Xue Ye Xue Za Zhi* **27,** 611–5 (2006).

92.   Dubey, S., Shukla, P. & Nityanand, S. Expression of interferon-gamma and tumor necrosis factor-alpha in bone marrow T cells and their levels in bone marrow plasma in patients with aplastic anemia. *Ann. Hematol.* **84,** 572–7 (2005).

93.   Selleri, C., Sato, T., Anderson, S., Young, N. S. & Maciejewski, J. P. Interferon-gamma and tumor necrosis factor-alpha suppress both early and late stages of hematopoiesis and induce programmed cell death. *J. Cell. Physiol.* **165,** 538–46 (1995).

94.   Maciejewski, J., Selleri, C., Anderson, S. & Young, N. S. Fas antigen expression on CD34+ human marrow cells is induced by interferon gamma and tumor necrosis factor alpha and potentiates cytokine-mediated hematopoietic suppression in vitro. *Blood* **85,** 3183–90 (1995).

95.   Kakagianni, T. *et al.* A probable role for trail-induced apoptosis in the pathogenesis of marrow failure. Implications from an in vitro model and from marrow of aplastic anemia patients. *Leuk. Res.* **30,** 713–21 (2006).

96.   Tichelli, A. *et al.* Morphology in patients with severe aplastic anemia treated with antilymphocyte globulin. *Blood* **80,** 337–45 (1992).

97.   Rizzo, S. *et al.* Stem cell defect in aplastic anemia: reduced long term culture-initiating cells (LTC-IC) in CD34+ cells isolated from aplastic anemia patient bone marrow. *Hematol. J.* **3,** 230–6 (2002).

98.   Marsh, J. C., Chang, J., Testa, N. G., Hows, J. M. & Dexter, T. M. The hematopoietic defect in aplastic anemia assessed by long-term marrow culture. *Blood* **76,** 1748–57 (1990).

99.   Scopes, J., Bagnara, M., Gordon-Smith, E. C., Ball, S. E. & Gibson, F. M. Haemopoietic progenitor cells are reduced in aplastic anaemia. *Br. J. Haematol.* **86,** 427–30 (1994).

100.   Zeng, W. *et al.* Gene expression profiling in CD34 cells to identify differences between aplastic anemia patients and healthy volunteers. *Blood* **103,** 325–32 (2004).

101.   Zeng, W. *et al.* Interferon-gamma-induced gene expression in CD34 cells:

identification of pathologic cytokine-specific signature profiles. *Blood* **107,** 167–75 (2006).

102. Philpott, N. J., Scopes, J., Marsh, J. C., Gordon-Smith, E. C. & Gibson, F. M. Increased apoptosis in aplastic anemia bone marrow progenitor cells: possible pathophysiologic significance. *Exp. Hematol.* **23,** 1642–8 (1995).

103. Ball, S. E. *et al.* Progressive telomere shortening in aplastic anemia. *Blood* **91,** 3582–92 (1998).

104. Hamzic, E., Whiting, K., Gordon Smith, E. & Pettengell, R. Characterization of bone marrow mesenchymal stromal cells in aplastic anaemia. *Br. J. Haematol.* **169,** 804–13 (2015).

105. Li, J. *et al.* Differential gene expression profile associated with the abnormality of bone marrow mesenchymal stem cells in aplastic anemia. *PLoS One* **7,** e47764 (2012).

106. El-Mahgoub, E. R., Ahmed, E., Afifi, R. A., Kamal, M. A. & Mousa, S. M. Mesenchymal stem cells from pediatric patients with aplastic anemia: isolation, characterization, adipogenic, and osteogenic differentiation. *Fetal Pediatr. Pathol.* **33,** 9–15 (2014).

107. Chao, Y. H., Peng, C. T., Harn, H. J., Chan, C. K. & Wu, K. H. Poor potential of proliferation and differentiation in bone marrow mesenchymal stem cells derived from children with severe aplastic anemia. *Ann. Hematol.* **89,** 715–23 (2010).

108. Jiang, S. y. *et al.* Low expression of basic fibroblastic growth factor in mesenchymal stem cells and bone marrow of children with aplastic anemia. *Pediatr. Hematol. Oncol.* **31,** 11–9 (2014).

109. Shipounova, I. N. *et al.* Alterations in hematopoietic microenvironment in patients with aplastic anemia. *Clin. Transl. Sci.* **2,** 67–74 (2009).

110. Bacigalupo, A. *et al.* T-cell suppression mediated by mesenchymal stem cells is deficient in patients with severe aplastic anemia. *Exp. Hematol.* **33,** 819–27 (2005).

111. Li, J. *et al.* Impaired immunomodulatory ability of bone marrow mesenchymal stem cells on CD4(+) T cells in aplastic anemia. *Results Immunol.* **2,** 142–7 (2012).

112. Young, N. S. & Maciejewski, J. The pathophysiology of acquired aplastic

anemia. *N. Engl. J. Med.* **336,** 1365–72 (1997).

113.   Wenxin, L. *et al.* Expression of membrane-bound IL-15 by bone marrow fibroblast-like stromal cells in aplastic anemia. *Int. Immunol.* **17,** 429–37 (2005).

114.   Bueno, C. *et al.* Bone marrow mesenchymal stem cells from patients with aplastic anemia maintain functional and immune properties and do not contribute to the pathogenesis of the disease. *Haematologica* **99,** 1168–75 (2014).

115.   Tripathy, N. K., Singh, S. P. & Nityanand, S. Enhanced adipogenicity of bone marrow mesenchymal stem cells in aplastic anemia. *Stem Cells Int.* **2014,** 276862 (2014).

116.   Xu, Y. *et al.* Immunosuppressive activity of mesenchymal stem cells is not decreased in children with aplastic anemia. *Int. J. Hematol.* **89,** 126–7 (2009).

117.   Xu, Y. *et al.* Downregulation of GATA-2 and overexpression of adipogenic gene-PPARgamma in mesenchymal stem cells from patients with aplastic anemia. *Exp. Hematol.* **37,** 1393–9 (2009).

118.   Chao, Y. H. *et al.* Downregulated CXCL12 expression in mesenchymal stem cells associated with severe aplastic anemia in children. *Ann. Hematol.* **94,** 13–22 (2015).

119.   Hotta, T. *et al.* Functional changes in marrow stromal cells in aplastic anaemia. *Acta Haematol.* **74,** 65–9 (1985).

120.   Holmberg, L. A., Seidel, K., Leisenring, W. & Torok-Storb, B. Aplastic anemia: analysis of stromal cell function in long-term marrow cultures. *Blood* **84,** 3685–90 (1994).

121.   Jaganathan, B. G. *et al.* Effects of MSC co-injection on the reconstitution of aplastic anemia patient following hematopoietic stem cell transplantation. *Leukemia* **24,** 1791–5 (2010).

122.   Stute, N. *et al.* Human mesenchymal stem cells are not of donor origin in patients with severe aplastic anemia who underwent sex-mismatched allogeneic bone marrow transplant. *J. Hematother. Stem Cell Res.* **11,** 977–84 (2002).

123.   Wu, L. *et al.* Impairment of hematopoietic stem cell niches in patients with aplastic anemia. *Int. J. Hematol.* **102,** 645–53 (2015).

124. Füreder, W. *et al.* Evaluation of angiogenesis and vascular endothelial growth factor expression in the bone marrow of patients with aplastic anemia. *Am. J. Pathol.* **168,** 123–30 (2006).

125. Chen, J. Animal models for acquired bone marrow failure syndromes. *Clin. Med. Res.* **3,** 102–8 (2005).

126. Weston, W., Gupta, V., Adkins, R. & Jurecic, R. New therapeutic approaches for protecting hematopoietic stem cells in aplastic anemia. *Immunol. Res.* **57,** 34–43 (2013).

127. Scheinberg, P. & Chen, J. Aplastic anemia: what have we learned from animal models and from the clinic. *Semin. Hematol.* **50,** 156–64 (2013).

128. Siveen, K. S., Uddin, S. & Mohammad, R. M. Targeting acute myeloid leukemia stem cell signaling by natural products. *Mol. Cancer* **16,** 13 (2017).

129. De Kouchkovsky, I. & Abdul-Hay, M. 'Acute myeloid leukemia: a comprehensive review and 2016 update'. *Blood Cancer J.* **6,** e441 (2016).

130. American Cancer Society. (2018). Available at: http://www.cancer.org/cancer/.

131. Estey, E. & Döhner, H. Acute myeloid leukaemia. *Lancet* **368,** 1894–907 (2006).

132. Rulina, A. V., Spirin, P. V. & Prassolov, V. S. Activated leukemic oncogenes AML1-ETO and c-kit: role in development of acute myeloid leukemia and current approaches for their inhibition. *Biochemistry (Mosc).* **75,** 1650–66 (2010).

133. Döhner, H. *et al.* Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. *Blood* **129,** 424–47 (2017).

134. Döhner, H., Weisfdorf, D. J. & Bloomfield, C. D. Acute myeloid leukemia. *N. Engl. J. Med.* **373,** 1136–52 (2015).

135. Kelly, L. M. & Gilliland, D. G. Genetics of myeloid leukemias. *Annu. Rev. Genomics Hum. Genet.* **3,** 179–98 (2002).

136. Blau, O. *et al.* Mesenchymal stromal cells of myelodysplastic syndrome and acute myeloid leukemia patients have distinct genetic abnormalities compared with leukemic blasts. *Blood* **118,** 5583–92 (2011).

137. Welch, J. S. *et al.* The origin and evolution of mutations in acute myeloid

leukemia. *Cell* **150,** 264–78 (2012).

138. Cancer Genome Atlas Research Network *et al.* Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. *N. Engl. J. Med.* **368,** 2059–74 (2013).

139. Corces-Zimmerman, M. R., Hong, W. J., Weissman, I. L., Medeiros, B. C. & Majeti, R. Preleukemic mutations in human acute myeloid leukemia affect epigenetic regulators and persist in remission. *Proc. Natl. Acad. Sci. U. S. A.* **111,** 2548–53 (2014).

140. Snauwaert, S., Vandekerckhove, B. & Kerre, T. Can immunotherapy specifically target acute myeloid leukemic stem cells? *Oncoimmunology* **2,** e22943 (2013).

141. Lapidot, T. *et al.* A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* **367,** 645–8 (1994).

142. Bonnet, D. & Dick, J. E. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat. Med.* **3,** 730–7 (1997).

143. Hope, K. J., Jin, L. & Dick, J. E. Acute myeloid leukemia originates from a hierarchy of leukemic stem cell classes that differ in self-renewal capacity. *Nat. Immunol.* **5,** 738–43 (2004).

144. Eppert, K. *et al.* Stem cell gene expression programs influence clinical outcome in human leukemia. *Nat. Med.* **17,** 1086–93 (2011).

145. Gal, H. *et al.* Gene expression profiles of AML derived stem cells; similarity to hematopoietic stem cells. *Leukemia* **20,** 2147–54 (2006).

146. Ho, M. M., Hogge, D. E. & Ling, V. MDR1 and BCRP1 expression in leukemic progenitors correlates with chemotherapy response in acute myeloid leukemia. *Exp. Hematol.* **36,** 433–42 (2008).

147. van Rhenen, A. *et al.* High stem cell frequency in acute myeloid leukemia at diagnosis predicts high minimal residual disease and poor survival. *Clin. Cancer Res.* **11,** 6520–7 (2005).

148. Terwijn, M. *et al.* Leukemic stem cell frequency: a strong biomarker for clinical outcome in acute myeloid leukemia. *PLoS One* **9,** e107587 (2014).

149. Passegué, E., Jamieson, C. H., Ailles, L. E. & Weissman, I. L. Normal and leukemic hematopoiesis: are leukemias a stem cell disorder or a reacquisition

of stem cell characteristics? *Proc. Natl. Acad. Sci. U. S. A.* **100 Suppl1,** 11842–9 (2003).

150. Hanekamp, D., Cloos, J. & Schuurhuis, G. J. Leukemic stem cells: identification and clinical application. *Int. J. Hematol.* **105,** 549–57 (2017).

151. Majeti, R. *et al.* Dysregulated gene expression networks in human acute myelogenous leukemia stem cells. *Proc. Natl. Acad. Sci. U. S. A.* **106,** 3396–401 (2009).

152. Konopleva, M. *et al.* The anti-apoptotic genes Bcl-X(L) and Bcl-2 are over-expressed and contribute to chemoresistance of non-proliferating leukaemic CD34+ cells. *Br. J. Haematol.* **118,** 521–34 (2002).

153. Medyouf, H. The microenvironment in human myeloid malignancies: emerging concepts and therapeutic implications. *Blood* **129,** 1617–26 (2017).

154. Kim, J. A. *et al.* Microenvironmental remodeling as a parameter and prognostic factor of heterogeneous leukemogenesis in acute myelogenous leukemia. *Cancer Res.* **75,** 2222–31 (2015).

155. Krevvata, M. *et al.* Inhibition of leukemia cell engraftment and disease progression in mice by osteoblasts. *Blood* **124,** 2834–46 (2014).

156. Mohammadi Najafabadi, M., Shamsasenjan, K. & Akbarzadehalaleh, P. Angiogenesis status in patients with acute myeloid leukemia: from diagnosis to post-hematopoietic stem cell transplantation. *Int. J. Organ Transplant. Med.* **8,** 57–67 (2017).

157. Vas, V., Wandhoff, C., Dörr, K., Niebel, A. & Geiger, H. Contribution of an aged microenvironment to aging-associated myeloproliferative disease. *PLoS One* **7,** e31523 (2012).

158. Shafat, M. S. *et al.* Leukemic blasts program bone marrow adipocytes to generate a protumoral microenvironment. *Blood* **129,** 1320–32 (2017).

159. Bruserud, Ø., Ryningen, A., Wergeland, L., Glenjen, N. I. & Gjertsen, B. T. Osteoblasts increase proliferation and release of pro-angiogenic interleukin 8 by native human acute myelogenous leukemia blasts. *Haematologica* **89,** 391–402 (2004).

160. Ninomiya, M. *et al.* Homing, proliferation and survival sites of human leukemia cells in vivo in immunodeficient mice. *Leukemia* **21,** 136–42 (2007).

161. Ishikawa, F. *et al.* Chemotherapy-resistant human AML stem cells home to

and engraft within the bone-marrow endosteal region. *Nat. Biotechnol.* **25,** 1315–21 (2007).

162. Takam Kamga, P. *et al.* Notch signalling drives bone marrow stromal cell-mediated chemoresistance in acute myeloid leukemia. *Oncotarget* **7,** 21713–27 (2016).

163. Konopleva, M. *et al.* Stromal cells prevent apoptosis of AML cells by up-regulation of anti-apoptotic proteins. *Leukemia* **16,** 1713–24 (2002).

164. Xia, B. *et al.* c-Myc plays part in drug resistance mediated by bone marrow stromal cells in acute myeloid leukemia. *Leuk. Res.* **39,** 92–9 (2015).

165. Garrido, S. M., Appelbaum, F. R., Willman, C. L. & Banker, D. E. Acute myeloid leukemia cells are protected from spontaneous and drug-induced apoptosis by direct contact with a human bone marrow stromal cell line (HS-5). *Exp. Hematol.* **29,** 448–57 (2001).

166. Zhou, H. S., Carter, B. Z. & Andreeff, M. Bone marrow niche-mediated survival of leukemia stem cells in acute myeloid leukemia: Yin and Yang. *Cancer Biol. Med.* **13,** 248–59 (2016).

167. Moschoi, R. *et al.* Protective mitochondrial transfer from bone marrow stromal cells to acute myeloid leukemic cells during chemotherapy. *Blood* **128,** 253–64 (2016).

168. Schepers, K., Campbell, T. B. & Passegué, E. Normal and leukemic stem cell niches: insights and therapeutic opportunities. *Cell Stem Cell* **16,** 254–67 (2015).

169. Geyh, S. *et al.* Functional inhibition of mesenchymal stromal cells in acute myeloid leukemia. *Leukemia* **30,** 683–91 (2016).

170. Yu, Z., Li, D. & Ju, X. L. CD4+ T cells from patients with acute myeloid leukemia inhibit the proliferation of bone marrow-derived mesenchymal stem cells by secretion of miR-10a. *J. Cancer Res. Clin. Oncol.* **142,** 733–40 (2016).

171. Chandran, P. *et al.* Mesenchymal stromal cells from patients with acute myeloid leukemia have altered capacity to expand differentiated hematopoietic progenitors. *Leuk. Res.* **39,** 486–93 (2015).

172. Le, Y. *et al.* Adipogenic mesenchymal stromal cells from bone marrow and their hematopoietic supportive role: towards understanding the permissive

marrow microenvironment in acute myeloid leukemia. *Stem Cell Rev.* **12,** 235–44 (2016).

173. Binato, R., de Almeida Oliveira, N. C., Du Rocher, B. & Abdelhay, E. The molecular signature of AML mesenchymal stromal cells reveals candidate genes related to the leukemogenic process. *Cancer Lett.* **369,** 134–43 (2015).

174. Huang, J. C. *et al.* Mesenchymal stromal cells derived from acute myeloid leukemia bone marrow exhibit aberrant cytogenetics and cytokine elaboration. *Blood Cancer J.* **5,** e302 (2015).

175. Desbourdes, L. *et al.* Alteration analysis of bone marrow mesenchymal stromal cells from de novo acute myeloid leukemia patients at diagnosis. *Stem Cells Dev.* **26,** 709–22 (2017).

176. Battula, V. L. *et al.* Connective tissue growth factor regulates adipocyte differentiation of mesenchymal stromal cells and facilitates leukemia bone marrow engraftment. *Blood* **122,** 357–66 (2013).

177. Civini, S. *et al.* Leukemia cells induce changes in human bone marrow stromal cells. *J. Transl. Med.* **11,** 298 (2013).

178. Frisch, B. J. *et al.* Functional inhibition of osteoblastic cells in an in vivo mouse model of myeloid leukemia. *Blood* **119,** 540–50 (2012).

179. Hanoun, M. *et al.* Acute myelogenous leukemia-induced sympathetic neuropathy promotes malignancy in an altered hematopoietic stem cell niche. *Cell Stem Cell* **15,** 365–75 (2014).

180. Isidori, A. *et al.* The role of the immunosuppressive microenvironment in acute myeloid leukemia development and treatment. *Expert Rev. Hematol.* **7,** 807–18 (2014).

181. Huan, J. *et al.* RNA trafficking by acute myelogenous leukemia exosomes. *Cancer Res.* **73,** 918–29 (2013).

182. Huan, J. *et al.* Coordinate regulation of residual bone marrow function by paracrine trafficking of AML exosomes. *Leukemia* **29,** 2285–95 (2015).

183. Raaijmakers, M. H. *et al.* Bone progenitor dysfunction induces myelodysplasia and secondary leukaemia. *Nature* **464,** 852–7 (2010).

184. Kode, A. *et al.* Leukaemogenesis induced by an activating β-catenin mutation in osteoblasts. *Nature* **506,** 240–4 (2014).

185. Kode, A. *et al.* FoxO1-dependent induction of acute myeloid leukemia by

osteoblasts in mice. *Leukemia* **30,** 1–13 (2016).

186. Wei, J. *et al.* Microenvironment determines lineage fate in a human model of MLL-AF9 leukemia. *Cancer Cell* **13,** 483–95 (2008).

187. Stahl, M., Lu, B. Y., Kim, T. K. & Zeidan, A. M. Novel therapies for acute myeloid leukemia: are we finally breaking the deadlock? *Target. Oncol.* **12,** 413–47 (2017).

188. Karantanou, C., Godavarthy, P. S. & Krause, D. S. Targeting the bone marrow microenvironment in acute leukemia. *Leuk. Lymphoma* 1–11 (2018).

189. Theocharides, A. P., Rongvaux, A., Fritsch, K., Flavell, R. A. & Manz, M. G. Humanized hemato-lymphoid system mice. *Haematologica* **101,** 5–19 (2016).

190. Manz, M. G. & Di Santo, J. P. Renaissance for mouse models of human hematopoiesis and immunobiology. *Nat. Immunol.* **10,** 1039–42 (2009).

191. Goyama, S., Wunderlich, M. & Mulloy, J. C. Xenograft models for normal and malignant stem cells. *Blood* **125,** 2630–40 (2015).

192. Mazurier, F., Doedens, M., Gan, O. I. & Dick, J. E. Rapid myeloerythroid repopulation after intrafemoral transplantation of NOD-SCID mice reveals a new class of human stem cells. *Nat. Med.* **9,** 959–63 (2003).

193. Shultz, L. D., Ishikawa, F. & Greiner, D. L. Humanized mice in translational biomedical research. *Nat. Rev. Immunol.* **7,** 118–30 (2007).

194. Lapidot, T., Fajerman, Y. & Kollet, O. Immune-deficient SCID and NOD/SCID mice models as functional assays for studying normal and malignant human hematopoiesis. *J. Mol. Med. (Berl).* **75,** 664–73 (1997).

195. McDermott, S. P., Eppert, K., Lechman, E. R., Doedens, M. & Dick, J. E. Comparison of human cord blood engraftment between immunocompromised mouse strains. *Blood* **116,** 193–200 (2010).

196. The Jackson Laboratory. (2018). Available at: http://www.jax.org/.

197. Lapidot, T. *et al.* Cytokine stimulation of multilineage hematopoiesis from immature human cells engrafted in SCID mice. *Science* **255,** 1137–41 (1992).

198. McCormack, E., Bruserud, O. & Gjertsen, B. T. Animal models of acute myelogenous leukaemia – development, application and future perspectives. *Leukemia* **19,** 687–706 (2005).

199. Carroll, A. M., Hardy, R. R. & Bosma, M. J. Occurrence of mature B (IgM+,

B220+) and T (CD3+) lymphocytes in scid mice. *J. Immunol.* **143,** 1087–93 (1989).

200.   Taconic Bioscience. (2018). Available at: http://www.taconic.com/mouse-model/scid-beige.

201.   Berney, T. *et al.* Patterns of engraftment in different strains of immunodeficient mice reconstituted with human peripheral blood lymphocytes. *Transplantation* **72,** 133–40 (2001).

202.   Kirkiles-Smith, N. C. *et al.* Development of a humanized mouse model to study the role of macrophages in allograft injury. *Transplantation* **87,** 189–97 (2009).

203.   Hu, Z., Van Rooijen, N. & Yang, Y. G. Macrophages prevent human red blood cell reconstitution in immunodeficient mice. *Blood* **118,** 5938–46 (2011).

204.   Hu, Z. & Yang, Y. G. Full reconstitution of human platelets in humanized mice after macrophage depletion. *Blood* **120,** 1713–6 (2012).

205.   Shultz, L. D. *et al.* Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. *J. Immunol.* **174,** 6477–89 (2005).

206.   Wunderlich, M. *et al.* AML xenograft efficiency is significantly improved in NOD/SCID-IL2RG mice constitutively expressing human SCF, GM-CSF and IL-3. *Leukemia* **24,** 1785–8 (2010).

207.   Agliano, A. *et al.* Human acute leukemia cells injected in NOD/LtSz-scid/IL-2Rgamma null mice generate a faster and more efficient disease compared to other NOD/scid-related strains. *Int. J. Cancer* **123,** 2222–7 (2008).

208.   Lumkul, R. *et al.* Human AML cells in NOD/SCID mice: engraftment potential and gene expression. *Leukemia* **16,** 1818–26 (2002).

209.   Terpstra, W. *et al.* Conditions for engraftment of human acute myeloid leukemia (AML) in SCID mice. *Leukemia* **9,** 1573–7 (1995).

210.   Woiterski, J. *et al.* Engraftment of low numbers of pediatric acute lymphoid and myeloid leukemias into NOD/SCID/IL2Rcγnull mice reflects individual leukemogenecity and highly correlates with clinical outcome. *Int. J. Cancer* **133,** 1547–56 (2013).

211.   Pearce, D. J. *et al.* AML engraftment in the NOD/SCID assay reflects the

outcome of AML: implications for our understanding of the heterogeneity of AML. *Blood* **107,** 1166–73 (2006).

212. Sanchez, P. V. *et al.* A robust xenotransplantation model for acute myeloid leukemia. *Leukemia* **23,** 2109–17 (2009).

213. Ailles, L. E., Gerhard, B., Kawagoe, H. & Hogge, D. E. Growth characteristics of acute myelogenous leukemia progenitors that initiate malignant hematopoiesis in nonobese diabetic/severe combined immunodeficient mice. *Blood* **94,** 1761–72 (1999).

214. Rombouts, W. J., Martens, A. C. & Ploemacher, R. E. Identification of variables determining the engraftment potential of human acute myeloid leukemia in the immunodeficient NOD/SCID human chimera model. *Leukemia* **14,** 889–97 (2000).

215. Cesano, A. *et al.* The severe combined immunodeficient (SCID) mouse as a model for human myeloid leukemias. *Oncogene* **7,** 827–36 (1992).

216. Saland, E. *et al.* A robust and rapid xenograft model to assess efficacy of chemotherapeutic agents for human acute myeloid leukemia. *Blood Cancer J.* **5,** e297 (2015).

217. Wunderlich, M. *et al.* AML cells are differentially sensitive to chemotherapy treatment in a human xenograft model. *Blood* **121,** e90-7 (2013).

218. Klco, J. M. *et al.* Functional heterogeneity of genetically defined subclones in acute myeloid leukemia. *Cancer Cell* **25,** 379–92 (2014).

219. Zhang, B., Duan, Z. & Zhao, Y. Mouse models with human immunity and their application in biomedical research. *J. Cell. Mol. Med.* **13,** 1043–58 (2009).

220. Tanner, A., Taylor, S. E., Decottignies, W. & Berges, B. K. Humanized mice as a model to study human hematopoietic stem cell transplantation. *Stem Cells Dev.* **23,** 76–82 (2014).

221. Gandhi, V. & Plunkett, W. Cellular and clinical pharmacology of fludarabine. *Clin. Pharmacokinet.* **41,** 93–103 (2002).

222. Hood, M. A. & Finley, R. S. Fludarabine: a review. *DICP* **25,** 518–24 (1991).

223. Plunkett, W. & Saunders, P. P. Metabolism and action of purine nucleoside analogs. *Pharmacol. Ther.* **49,** 239–68 (1991).

224. Yang, S. W., Huang, P., Plunkett, W., Becker, F. F. & Chan, J. Y. Dual mode

of inhibition of purified DNA ligase I from human cells by 9-beta-D-arabinofuranosyl-2-fluoroadenine triphosphate. *J. Biol. Chem.* **267,** 2345–9 (1992).

225. Frank, D. A., Mahajan, S. & Ritz, J. Fludarabine-induced immunosuppression is associated with inhibition of STAT1 signaling. *Nat. Med.* **5,** 444–7 (1999).

226. Tajima, K. *et al.* Inhibition of STAT1 accelerates bone fracture healing. *J. Orthop. Res.* **28,** 937–41 (2010).

227. Furukawa, M. *et al.* IL-27 abrogates receptor activator of NF-kappa B ligand-mediated osteoclastogenesis of human granulocyte-macrophage colony-forming unit cells through STAT1-dependent inhibition of c-Fos. *J. Immunol.* **183,** 2397–406 (2009).

228. Terenzi, A. *et al.* Efficacy of fludarabine as an immunosuppressor for bone marrow transplantation conditioning: preliminary results. *Transplant. Proc.* **28,** 3101 (1996).

229. Nishioka, C., Ikezoe, T., Togitani, K. & Yokoyama, A. Fludarabine induces growth arrest and apoptosis of cytokine- or alloantigen-stimulated peripheral blood mononuclear cells, and decreases production of Th1 cytokines via inhibition of nuclear factor kappaB. *Bone Marrow Transplant.* **41,** 303–9 (2008).

230. Montillo, M., Ricci, F. & Tedeschi, A. Role of fludarabine in hematological malignancies. *Expert Rev. Anticancer Ther.* **6,** 1141–61 (2006).

231. Rambaldi, A. *et al.* Busulfan plus cyclophosphamide versus busulfan plus fludarabine as a preparative regimen for allogeneic haemopoietic stem-cell transplantation in patients with acute myeloid leukaemia: an open-label, multicentre, randomised, phase 3 trial. *Lancet Oncol.* **16,** 1525–36 (2015).

232. Andersson, B. S. *et al.* Once daily i.v. busulfan and fludarabine (i.v. Bu-Flu) compares favorably with i.v. busulfan and cyclophosphamide (i.v. BuCy2) as pretransplant conditioning therapy in AML/MDS. *Biol. Blood Marrow Transplant.* **14,** 672–84 (2008).

233. Khouri, I. F. *et al.* Nonablative allogeneic hematopoietic transplantation as adoptive immunotherapy for indolent lymphoma: low incidence of toxicity, acute graft-versus-host disease, and treatment-related mortality. *Blood* **98,** 3595–9 (2001).

234.   Aversa, F. *et al.* Treatment of high-risk acute leukemia with T-cell-depleted stem cells from related donors with one fully mismatched HLA haplotype. *N. Engl. J. Med.* **339,** 1186–93 (1998).

235.   Niederwieser, D. *et al.* Low-dose total body irradiation (TBI) and fludarabine followed by hematopoietic cell transplantation (HCT) from HLA-matched or mismatched unrelated donors and postgrafting immunosuppression with cyclosporine and mycophenolate mofetil (MMF) can induce durable complete chimerism and sustained remissions in patients with hematological diseases. *Blood* **101,** 1620–9 (2003).

236.   Luznik, L., Jalla, S., Engstrom, L. W., Iannone, R. & Fuchs, E. J. Durable engraftment of major histocompatibility complex-incompatible cells after nonmyeloablative conditioning with fludarabine, low-dose total body irradiation, and posttransplantation cyclophosphamide. *Blood* **98,** 3456–64 (2001).

237.   Giver, C. R. *et al.* Ex vivo fludarabine exposure inhibits graft-versus-host activity of allogeneic T cells while preserving graft-versus-leukemia effects. *Biol. Blood Marrow Transplant.* **9,** 616–32 (2003).

238.   Weiss, L., Abdul-Hai, A., Or, R., Amir, G. & Polliack, A. Fludarabine in combination with cyclophosphamide decreases incidence of GVHD and maintains effective graft-versus-leukemia effect after allogeneic stem cell transplantation in murine lymphocytic leukemia. *Bone Marrow Transplant.* **31,** 11–5 (2003).

239.   Covini, D. *et al.* Expanding targets for a metabolic therapy of cancer: L-asparaginase. *Recent Pat. Anticancer Drug Discov.* **7,** 4–13 (2012).

240.   Narta, U. K., Kanwar, S. S. & Azmi, W. Pharmacological and clinical evaluation of L-asparaginase in the treatment of leukemia. *Crit. Rev. Oncol. Hematol.* **61,** 208–21 (2007).

241.   Patel, N. *et al.* A dyad of lymphoblastic lysosomal cysteine proteases degrades the antileukemic drug L-asparaginase. *J. Clin. Invest.* **119,** 1964–73 (2009).

242.   Avramis, V. I. Asparaginases: biochemical pharmacology and modes of drug resistance. *Anticancer Res.* **32,** 2423–37 (2012).

243.   Emadi, A., Zokaee, H. & Sausville, E. A. Asparaginase in the treatment of non-ALL hematologic malignancies. *Cancer Chemother. Pharmacol.* **73,**

875–83 (2014).

244. Hermanova, I. *et al.* Pharmacological inhibition of fatty-acid oxidation synergistically enhances the effect of l-asparaginase in childhood ALL cells. *Leukemia* **30,** 209–18 (2016).

245. Song, J. H. *et al.* L-Asparaginase-mediated downregulation of c-Myc promotes $1,25(OH)_2$ D_3 -induced myeloid differentiation in acute myeloid leukemia cells. *Int. J. Cancer* **140,** 2364–74 (2017).

246. Willems, L. *et al.* Inhibiting glutamine uptake represents an attractive new strategy for treating acute myeloid leukemia. *Blood* **122,** 3521–32 (2013).

247. Chan, W. K. *et al.* The glutaminase activity of L-asparaginase is not required for anticancer activity against ASNS-negative cells. *Blood* **123,** 3596–606 (2014).

248. Ehsanipour, E. A. *et al.* Adipocytes cause leukemia cell resistance to L-asparaginase via release of glutamine. *Cancer Res.* **73,** 2998–3006 (2013).

249. Offman, M. N. *et al.* Rational engineering of L-asparaginase reveals importance of dual activity for cancer cell toxicity. *Blood* **117,** 1614–21 (2011).

250. Parmentier, J. H. *et al.* Glutaminase activity determines cytotoxicity of L-asparaginases on most leukemia cell lines. *Leuk. Res.* **39,** 757–62 (2015).

251. Chen, L. & Cui, H. Targeting glutamine induces apoptosis: a cancer therapy approach. *Int. J. Mol. Sci.* **16,** 22830–55 (2015).

252. Altman, B. J., Stine, Z. E. & Dang, C. V. From Krebs to clinic: glutamine metabolism to cancer therapy. *Nat. Rev. Cancer* **16,** 619–34 (2016).

253. Cluntun, A. A., Lukey, M. J., Cerione, R. A. & Locasale, J. W. Glutamine metabolism in cancer: understanding the heterogeneity. *Trends Cancer* **3,** 169–80 (2017).

254. Liao, J. *et al.* Regulation of stem-like cancer cells by glutamine through β-catenin pathway mediated by redox signaling. *Mol. Cancer* **16,** 51 (2017).

255. Jacque, N. *et al.* Targeting glutaminolysis has antileukemic activity in acute myeloid leukemia and synergizes with BCL-2 inhibition. *Blood* **126,** 1346–56 (2015).

256. Matre, P. *et al.* Inhibiting glutaminase in acute myeloid leukemia: metabolic dependency of selected AML subtypes. *Oncotarget* **7,** 79722–35 (2016).

257. Spittler, A. *et al.* Low glutamine concentrations induce phenotypical and functional differentiation of U937 myelomonocytic cells. *J. Nutr.* **127,** 2151–7 (1997).

258. Leslie, M., Case, M. C., Hall, A. G. & Coulthard, S. A. Expression levels of asparagine synthetase in blasts from children and adults with acute lymphoblastic leukaemia. *Br. J. Haematol.* **132,** 740–2 (2006).

259. Okada, S. *et al.* In vitro efficacy of l-asparaginase in childhood acute myeloid leukaemia. *Br. J. Haematol.* **123,** 802–9 (2003).

260. Zwaan, C. M. *et al.* Cellular drug resistance profiles in childhood acute myeloid leukemia: differences between FAB types and comparison with acute lymphoblastic leukemia. *Blood* **96,** 2879–86 (2000).

261. Bertuccio, S. N. *et al.* Identification of a cytogenetic and molecular subgroup of acute myeloid leukemias showing sensitivity to L-Asparaginase. *Oncotarget* **8,** 109915–23 (2017).

262. Emadi, A. *et al.* Inhibition of glutaminase selectively suppresses the growth of primary acute myeloid leukemia cells with IDH mutations. *Exp. Hematol.* **42,** 247–51 (2014).

263. Holleman, A. *et al.* Gene-expression patterns in drug-resistant acute lymphoblastic leukemia cells and response to treatment. *N. Engl. J. Med.* **351,** 533–42 (2004).

264. Holleman, A. *et al.* The expression of 70 apoptosis genes in relation to lineage, genetic subtype, cellular drug resistance, and outcome in childhood acute lymphoblastic leukemia. *Blood* **107,** 769–76 (2006).

265. Hutson, R. G. *et al.* Amino acid control of asparaginase synthetase: relation to asparaginase resistance in human leukemia cells. *Am. J. Physiol.* **272,** C1691-9 (1997).

266. Stams, W. A. *et al.* Sensitivity to L-asparaginase is not associated with expression levels of asparagine synthetase in t(12;21)+ pediatric ALL. *Blood* **101,** 2743–7 (2003).

267. Appel, I. M. *et al.* Up-regulation of asparagine synthetase expression is not linked to the clinical response to L-asparaginase in pediatric acute lymphoblastic leukemia. *Blood* **107,** 4244–9 (2006).

268. van der Meer, L. T. *et al.* A germ line mutation in cathepsin B points toward

a role in asparaginase pharmacokinetics. *Blood* **124,** 3027–9 (2014).

269. Jain, M., Bakhshi, S., Shukla, A. A. & Chauhan, S. S. Cathepsins B and L in peripheral blood mononuclear cells of pediatric acute myeloid leukemia: potential poor prognostic markers. *Ann. Hematol.* **89,** 1223–32 (2010).

270. Laranjeira, A. B. *et al.* IGFBP7 participates in the reciprocal interaction between acute lymphoblastic leukemia and BM stromal cells and in leukemia resistance to asparaginase. *Leukemia* **26,** 1001–11 (2012).

271. van der Meer, L. T. *et al.* In vivo imaging of antileukemic drug asparaginase reveals a rapid macrophage-mediated clearance from the bone marrow. *J. Nucl. Med.* **58,** 214–20 (2017).

272. Song, P. *et al.* Asparaginase induces apoptosis and cytoprotective autophagy in chronic myeloid leukemia cells. *Oncotarget* **6,** 3861–73 (2015).

273. Takahashi, H. *et al.* Autophagy is required for cell survival under L-asparaginase-induced metabolic stress in acute lymphoblastic leukemia cells. *Oncogene* **36,** 4267–76 (2017).

274. Pieters, R. *et al.* L-asparaginase treatment in acute lymphoblastic leukemia: a focus on Erwinia asparaginase. *Cancer* **117,** 238–49 (2011).

275. Clarkson, B. *et al.* Clinical results of treatment with E. coli L-asparaginase in adults with leukemia, lymphoma, and solid tumors. *Cancer* **25,** 279–305 (1970).

276. Capizzi, R. L. *et al.* Synergy between high-dose cytarabine and asparaginase in the treatment of adults with refractory and relapsed acute myelogenous leukemia--a Cancer and Leukemia Group B Study. *J. Clin. Oncol.* **6,** 499–508 (1988).

277. Wells, R. J. *et al.* Impact of high-dose cytarabine and asparaginase intensification on childhood acute myeloid leukemia: a report from the Childrens Cancer Group. *J. Clin. Oncol.* **11,** 538–45 (1993).

278. Ahmed, T. *et al.* High dose cytarabine, mitoxantrone and l-asparaginase (HAMA) salvage for relapsed or refractory acute myeloid leukemia (AML) in the elderly. *Leuk. Res.* **39,** 945–9 (2015).

279. Buaboonnam, J. *et al.* Sequential administration of methotrexate and asparaginase in relapsed or refractory pediatric acute myeloid leukemia. *Pediatr. Blood Cancer* **60,** 1161–4 (2013).

280. Emadi, A., Bade, N. A., Stevenson, B. & Singh, Z. Minimally-myelosuppressive asparaginase-containing induction regimen for treatment of a Jehovah's witness with mutant IDH1/NPM1/NRAS acute myeloid leukemia. *Pharmaceuticals (Basel)* **9,** 12 (2016).

281. Emadi, A. *et al.* Asparaginase Erwinia chrysanthemi effectively depletes plasma glutamine in adult patients with relapsed/refractory acute myeloid leukemia. *Cancer Chemother. Pharmacol.* **81,** 217–22 (2018).

Chapter 2

Human aplastic anaemia-derived mesenchymal stromal cells form functional haematopoietic stem cell niche *in vivo*

Ilaria M. Michelozzi[1*], Alice Pievani[1*], Fabio Pagni[2], Laura Antolini[3], Marta Verna[4], Paola Corti[4], Attilio Rovelli[4], Mara Riminucci[5], Francesco Dazzi[6], Andrea Biondi[4], Marta Serafini[1]

[1]Department of Paediatrics, Dulbecco Telethon Institute at Centro Ricerca Tettamanti University of Milano-Bicocca, [2]Department of Pathology University of Milano-Bicocca, [3]Centro di Biostatistica per l'epidemiologia clinica, Department of Health Sciences University of Milano-Bicocca, [4]Department of Paediatrics, University of Milano-Bicocca, San Gerardo Hospital/Fondazione MBBM, Monza, [5]Department of Molecular Medicine, Sapienza University of Rome, Rome, Italy and [6]Department of Haemato-Oncology, Rayne Institute, King's College, London, UK.
E-mail: serafinim72@gmail.com

[*]I.M.M. and A.P. contributed equally to this work.

Aplastic anaemia (AA) is characterised by pancytopenia resulting from a marked reduction in haematopoietic stem and progenitor cells.[1] Despite considerable progress, the mechanisms involved in the pathophysiology of AA remain largely unknown.[2]

In most cases, the underlying mechanism is immune-mediated, in fact immunosuppressive therapies and/or allogeneic haematopoietic stem cell transplantation (HSCT) produce a high rate of clinical remissions.[3] Nevertheless, there are data suggesting an abnormal bone marrow (BM) microenvironment unable to support normal haematopoiesis. Indeed, a proportion of patients does not respond to HSCT.[4] Some studies have reported that AA-derived mesenchymal stromal cells (AA-MSC) display decreased proliferation and clonogenic potential, aberrant morphology, altered transcriptome profile, impaired adipogenic and osteogenic differentiation.[5–8] In contrast, other studies reported that AA-MSC display normal morphology and differentiation properties.[9] Moreover, data from *in vitro* investigations involving patient-derived stromal cells and their ability to support homeostasis of $CD34^+$ cells have been similarly controversial. Whilst the capacity of AA-MSC to sustain haematopoiesis in a coculture *in vitro* assay was reduced in some studies, others observed a normal ability of patients' cells to support the haematopoietic progenitor function.[8,9]

The great limitation to these studies is that they have been conducted *in vitro*. Therefore, *in vivo* models would be tremendously useful to better understand the effective role of the stromal compartment in AA.

We recently described an *in vivo* system that accurately reproduces a miniature bone organ, including cortical bone, marrow cavity, donor-derived marrow stroma, host-derived sinusoidal circulation and host-

derived haematopoietic tissue, based on implant of chondroid pellets in immunodeficient mice.[10] We have used this approach to reproduce the AA microenvironment and test its ability to support haematopoiesis *in vivo* (for methods see Data S1).

We compared MSC isolated from two cohorts of paediatric AA patients and healthy donors (HD-MSC), homogeneous in terms of age and passages in culture. All AA patients had low blood cell counts in all three blood lineages (Tab. SI) and aplastic BM (cellularity ≤5%).

MSC preparations could be established from all AA patients. The colony-forming efficiency was decreased in BM mononuclear cells of AA in comparison with healthy donors (P=0.02) (Fig. 1B).[7,8] However, we did not observe any difference in the morphology and phenotypic profiles of AA-MSC compared to HD-MSC (Fig. 1A, C). Although it has been reported that AA-MSC proliferate significantly slower than their normal counterparts,[7] we found no differences in the number of cumulative population doublings (CPDs) between AA- and HD-MSC (Fig. 1D). Osteogenic and adipogenic differentiation was achieved with similar efficiency in normal and patients' MSC (Fig. 1E, F, top). Osteogenic differentiation was confirmed by a comparable increase of gene expression associated with bone differentiation (Fig. 1E, bottom). Likewise, the expression of adipogenesis-related genes was similar in both groups (Fig. 1F, bottom).

Equivalent trilineage differentiation capacity between AA- and HD-MSC was completed comparing the morphology and histology of differentiated cartilage pellets (Fig. 1G, top). Gene expression studies confirmed no significant differences in the transcript levels of chondrogenic genes (Fig. 1G, bottom). Finally, the gene expression of

haematopoiesis-related factors (DKK1, IL-6, TGF-β1) constitutively secreted by MSC was unaffected in AA-MSC (Fig. 1H).
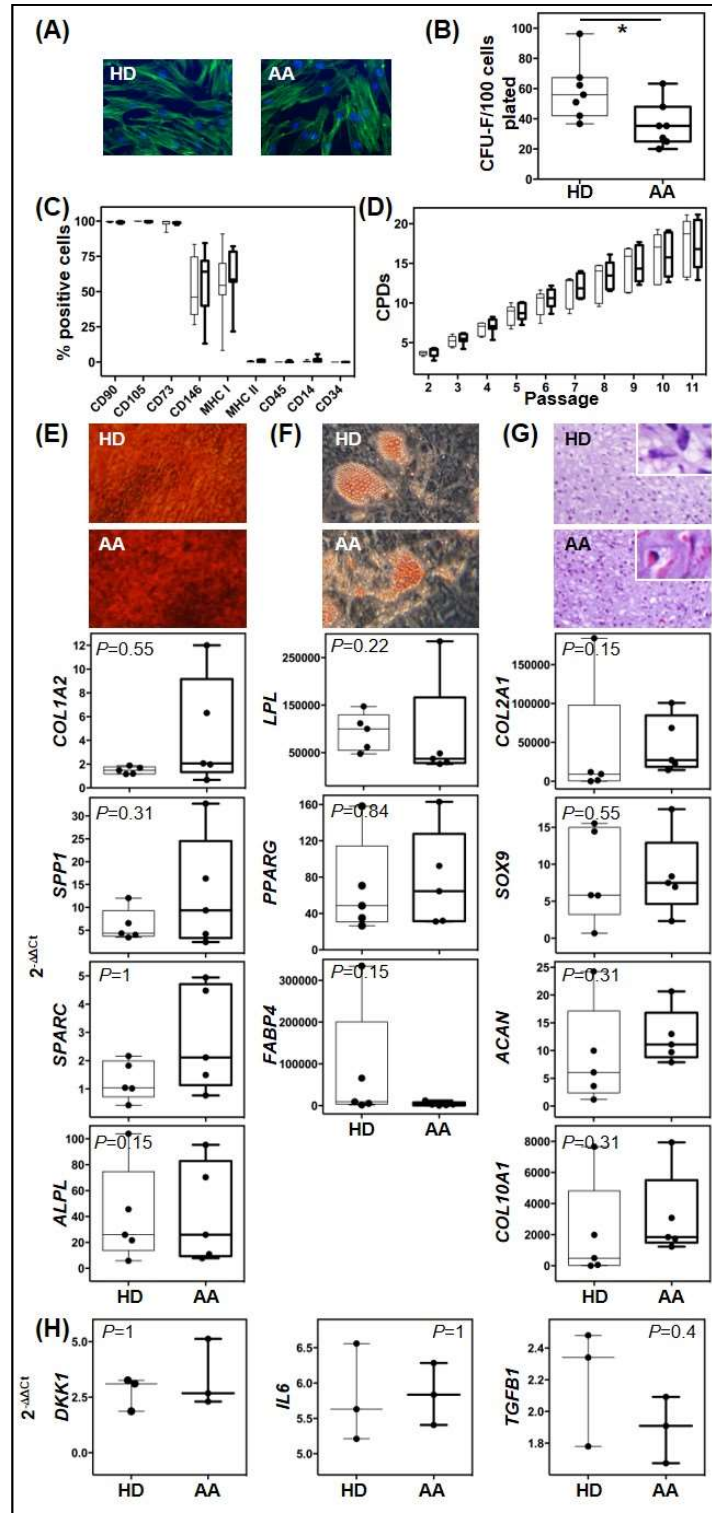
Fig. 1. In vitro characterisation of AA-MSC.

*(A) Morphology of AA- (right) and HD- (left) MSC assessed at passage 3 of culture by actin filament staining with phalloidin. Magnification 40x. (B) Clonogenic capacity (colony-forming unit-fibroblast; CFU-F). *P<0.05, with Wilcoxon test, 2 sides. (C) Comparative surface antigenic profiling of MSC derived from AA patients (thick line) and HD donors (thin line) analysed by flow cytometry. (HD n=7; AA n=7). (D) Expansion curve of AA-MSC and HD-MSC. (HD, thin line, n=7; AA, thick line, n=7). (E) Osteogenic differentiation of AA-MSC and HD-MSC detected by Alizarin Red S staining. Magnification 20x (top). qPCR for the osteogenesis-related genes. (HD n=5; AA n=5) (bottom). (F) Adipogenic differentiation of AA-MSC and HD-MSC demonstrated by Oil red O staining. Magnification 20x (top). qPCR for the adipogenesis-related genes. (HD n=5; AA n=5) (bottom). (G) Presence of chondrocytes within lacunae in haematoxylin and eosin stained sections revealing chondrogenic differentiation of AA-MSC and HD-MSC. Magnification 4x and inset, magnification 20x (top). qPCR for the chondrogenesis-related genes. (HD n=5; AA n=5) (bottom). (H) Expression of haematopoiesis-related genes DKK1, IL6 and TGFB1 by AA-MSC and HD-MSC. Median, and min/max values are shown. (HD n=3; AA n=3). AA, aplastic anaemia; HD, healthy donor; MSC, mesenchymal stromal cells; CPDs, cumulative population doublings; qPCR, quantitative real-time polymerase chain reaction.*

We then tested the ability of AA-MSC to form a haemopoietic niche *in vivo*. To do this we used our recently described approach that mimics the development of a miniature bone organ.[10] Cartilage pellets, differentiated *in vitro* from AA-MSC and HD-MSC, were implanted into SCID-beige mice. Ossicles were generated from both 3 AA patients (n=33 ossicles) and 5 healthy donors (n=40 ossicles). Eight weeks after

the implant, bone remodelling and formation of marrow occurred in patient-derived ossicles and normal controls. Immunohistochemistry analysis of the haematopoietic tissue in the intertrabecular space within the ossicles revealed the presence of murine macrophages (Iba1), myeloid cells (myeloperoxidase), megakaryocytes (von Willebrand factor), red blood cells (TER-119) and osteoclasts (TRAP-positive cells), in similar proportions in normal and patient-derived sections (Fig. 2A).

Accordingly, the same number of cells belonging to the erythroid, myeloid and megakaryocytic lineages were found within the affected ossicles and their counterparts from normal BM (Fig. 2B). The presence of clonogenic haematopoietic progenitors was evaluated in the ossicle-derived marrows by enumerating colony-forming cells in a methylcellulose assay and did not differ between patients and controls (Fig. 2C).

Also the human stromal compartment of the ossicles from AA-MSC and HD-MSC shared similar features with comparable amount and disposition of adipose marrow and similar degree of iron storages (Fig. 2D). The number of osteoblasts lining the osseous trabeculae, the extent of BM interstitial fibrosis and reticulin deposition were almost identical between the ossicles derived from the two sources (Fig. 2D).
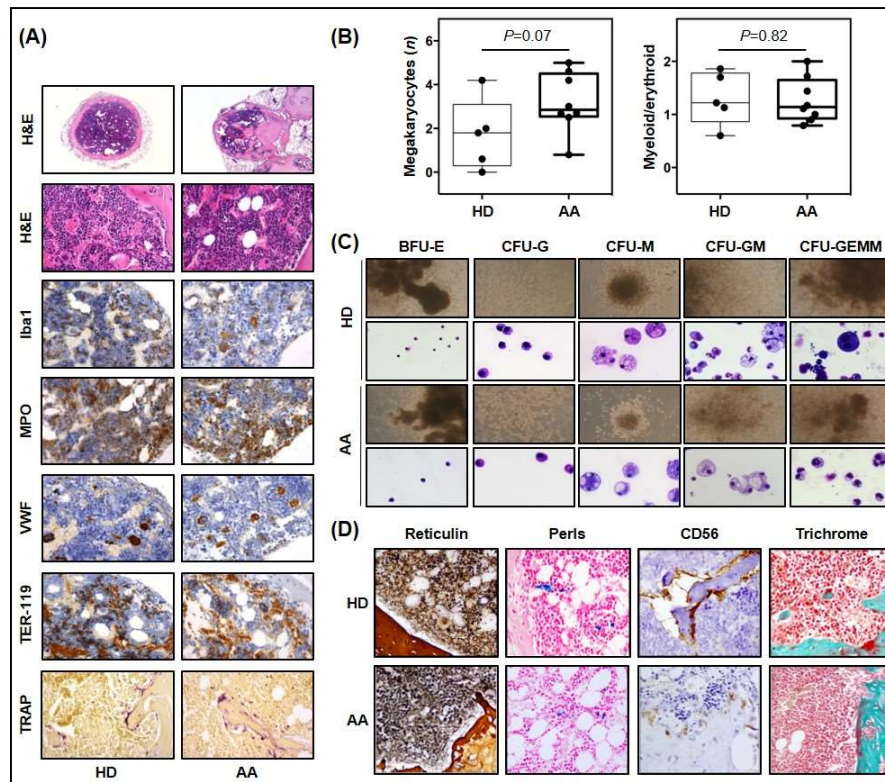
***Fig. 2. Evaluation of the haematopoietic and stromal compartment in AA-
and HD-ossicles obtained after the in vivo implant of chondroid pellets
derived from MSC.***

*AA- and HD-MSC were cultured for 3 weeks as micro-masses in chondrogenic
differentiation medium, then cartilaginous pellets were implanted
subcutaneously into SCID-beige mice to generate heterotopic ossicles. After
8 weeks in vivo, ossicles were harvested and fixed for histology and
immunohistology or digested in collagenase for haematopoietic cell isolation.
(A) Histology of AA- (right) and HD- (left) ossicles and evaluation of the
presence of monocytes/macrophages (Iba1), myeloid cells (myeloperoxidase,
MPO), megakaryocytes (von Willebrand factor, VWF), erythroid cells (TER-
119) and osteoclasts (TRAP staining). Magnification 50x and 400x. (B)
Quantification of megakaryocyte number and myeloid/erythroid ratio in AA-
and HD-derived ossicles. 3-10 fields for each sample were scored. Two-sided*

Wilcoxon test was used to calculate P-values. (C) Morphology of haematopoietic colonies and cells formed by heterotopic AA- and HD-ossicle-derived cells in methylcellulose at day 14. (D) Analysis of stromal compartment in ossicles derived from AA-MSC (bottom row) and HD-MSC (top row). Evaluation of reticulin fiber deposition (Gomori's silver staining), iron storages (Perls' staining), collagen deposition (Masson's trichrome staining) and osteoblasts (immunolabelling for CD56). Magnification 20x. AA, aplastic anaemia; HD, healthy donor; MSC, mesenchymal stromal cells; H&E, haematoxylin and eosin; BFU-E, burst-forming unit-erythroid; CFU-G, colony-forming unit-granulocyte; CFU-M, colony-forming unit-macrophage; CFU-GM, colony-forming unit-granulocyte, macrophage; CFU-GEMM, colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte.

So far no study has addressed the competence of AA-MSC to establish a functional BM niche *in vivo*. Using our *in vivo* model, we have been able to show that AA-MSC generate normal ossicles characterised by cortical bone, marrow cavity, donor-derived marrow stroma, and host-derived haematopoietic tissue. We detected a normal proportion of haematopoietic clonogenic progenitors, as well as committed haematopoietic cells, including erythroid, myeloid and megakaryocytic lineages. In addition, all the elements evaluated within the stromal compartment appeared normal.

Our study randomly selected patients who had responded to immunosuppressive treatment - including HSCT - and therefore our conclusions should be exclusively limited to those patients. However, it is possible that, in patients in whom such a therapeutic approach has failed, the causative role of the haematopoietic microenvironment in the

disease pathogenesis could be more important and that functional abnormalities could be identified. Further studies are warranted to clarify this issue, and our model could be a versatile and reproducible tool to dissect the role of microenvironment in the pathophysiology of AA.

Acknowledgments

Authorship contribution

I.M.M. and A.P. performed and analysed most experiments and contributed to the writing of the paper; F.P. and M.R. performed and supervised some experiments and contributed to the writing of the paper; L.A. performed statistical analysis; M.V., P.C. and A.R. provided patient samples and contributed to the writing of the paper; F.D. and A.B. interpreted the data and edited the manuscript; and M.S. designed, and supervised all experiments and wrote the paper. All authors approved the final manuscript.

Conflict of interest

The authors declare no competing financial interests.

Data S1. Methods

Cell isolation and culture

We obtained MSC from fresh or frozen BM biopsies or aspirate samples
of AA patients and from the washouts of discarded BM collection bags
and filters used for BM transplantation, as previously described.[11] We
isolated MSC from 8 newly diagnosed AA patients (hereafter named
AA-MSC) and 7 age-matched healthy donors (hereafter named HD-
MSC), after informed consent according to institutionally approved
protocols. Clinical and biological information is provided in Tab. SI.

Morphology of MSC

Cellular morphology was analysed with phalloidin (Alexa Fluor 488,
Invitrogen) staining after cellular fixation with 3.7% paraformaldehyde
and membrane permeabilisation with 0.01% Triton X100 in PBS.
Images were acquired by fluorescence confocal microscope (Eclipse
E800).

Colony-forming unit-fibroblast assays

Fibroblast colony-forming unit (CFU-F) formation was assessed by
plating 1×10^2 MSC, harvested from passage 0, into Petri dishes
(Corning Incorporated, NY) in basal growth medium. After 2 weeks of
culture, the dishes were washed twice with PBS and the cells were fixed
with methanol and stained with Giemsa solution (Merck KGaA,
Darmstadt, Germany). The clonogenic efficiency was calculated as the
number of colonies per 1×10^2 initially seeded cells.

Proliferation kinetics

The population doublings (PD) were calculated for each MSC sample using the following equation:

$PD_n = PD_{n-1} + [\log(C_1/C_0)]/\log 2$, wherein C_0=cell number initially seeded and C_1=cell number harvested.

Results were expressed as cumulative PD from P1 to P11.

Immunophenotype

MSC at passage 3 were stained with phycoerythrin-conjugated or fluorescein isothiocyanate-conjugated monoclonal antibodies specific for CD14 (clone 61D3; eBioscience, San Diego, CA), CD34 (clone 581; BD Biosciences, San Jose, CA), CD45 (clone HI30; BD Biosciences), CD90 (clone 5E10; eBioscience), CD73 (clone AD2; BD Biosciences), CD105 (clone SN6; eBioscience), CD146 (clone P1H12; BD Biosciences), class I-HLA (clone G46-2.6; BD Biosciences), and class II-HLA (clone G46-6; BD Biosciences).

Isotype antibodies were used as control. Flow cytometric analysis was performed with a FACSCanto cytometer (BD Biosciences), and data were analysed using the FACSDiva software (BD Biosciences).

Differentiation capability

The osteogenic differentiation capability of MSC was assessed at P3 by incubating cells (6×10^4 cells/cm^2) with osteogenic induction medium consisting of DMEM-low glucose (Invitrogen), supplemented with 10% FBS (Biosera), 100 nmol/L dexamethasone (Invitrogen), 10 mM β-glycerophosphate (Invitrogen), and 0.05 mM 2-phosphate-ascorbic acid (Invitrogen).

Adipogenic differentiation was evaluated at P3 by incubating cells $(2 \times 10^5$ cells/cm^2) with adipogenic induction medium consisting of DMEM-high glucose (Invitrogen) supplemented with 10% FBS (Biosera), 1 µM dexamethasone (Sigma-Aldrich), 1 µM indomethacin (Invitrogen), 500 µM 3-isobutyl-1-methylxantine (Sigma-Aldrich), and 10 µg/mL human recombinant insulin (Sigma-Aldrich).

Both osteogenic and adipogenic cultures were maintained for 21 days in differentiation medium before evaluating differentiation by staining and by quantitative real-time polymerase chain reaction (qPCR) for the assessment of lineage-specific genes.

To detect osteogenic differentiation, cells at the end of the differentiation were stained for calcium deposition with Alizarin Red S (Sigma-Aldrich). The transcript levels for osteopontin *(SPP1)*, osteonectin *(SPARC)*, alkaline phosphatase *(ALPL)*, and type I collagen alpha 2 chain *(COL1A2)* were analysed at day 0 and day 21 of culture.

Adipogenic differentiation was evaluated through the staining of fat droplets with Oil Red O (Sigma-Aldrich). At days 0 and 21, the transcript levels for the fatty acid binding protein 4 (*FABP4*), lipoprotein lipase (*LPL*), and peroxisome proliferator-activated receptor gamma (*PPARG*) were analysed.

Chondrogenic differentiation was obtained and detected as described.[10] Briefly, MSC were cultured for 3 weeks as micro-masses in 15 mL conical tubes $(3 \times 10^5$ cells/tube) in chondrogenic differentiation medium consisting of DMEM-high glucose (Invitrogen), supplemented with ITS^{TM} Premix (BD Biosciences), 1 mM pyruvate (Sigma-Aldrich), 50 µg/mL 2-phosphate-ascorbic acid (Fluka, Sigma-Aldrich), 100 nM

dexamethasone (Sigma-Aldrich), and 10 ng/mL transforming growth factor (TGF)-β1 (R and D Systems, Minneapolis, MN).

For histology, the resulting tissues were fixed in 4% formaldehyde in PBS, embedded in paraffin and stained with haematoxylin and eosin (Bio-Optica).

For the assessment of chondrogenic gene expression profile, on days 0 and 21 after induction, transcript levels for type II collagen (*COL2A1*), type X collagen (*COL10A1*), SRY-box 9 (*SOX9*), and aggrecan (*ACAN*) were analysed.

RNA isolation and qPCR

Total RNA was extracted with the use of TRIZOL reagent (Invitrogen), following the manufacturer's protocol. 1 μg of RNA was then reverse-transcribed with the use of a SuperScript II Reverse Transcriptase kit (Invitrogen) in the presence of random hexamers. Quantitative real-time PCR assays were performed on an ABI 7900 Real-Time PCR system thermal cycler with the qPCR Mastermix (Applied Biosystems-Invitrogen). All TaqMan gene expression assays were provided by Applied Biosystems (Tab. 2S). We used glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as endogenous control. The level of each target gene was normalised to *GAPDH* levels and expressed relative to the day 0 of each group (ΔΔCt method).

The expression of factors implicated in the regulation of haematopoietic stem cells such as transforming growth factor beta 1 (*TGFB1*), interleukin 6 (*IL6*), and Dickkopf-related protein1 (*DKK1*) was quantified on normal and patient-derived MSC.

Histology and immunohistology

Ossicles obtained from chondroid pellets implanted subcutaneously for 8 weeks into SCID-beige mice were harvested, fixed in 4% formaldehyde in PBS, decalcified in 10% EDTA and processed for paraffin embedding. 3-4 μm thick sections were stained with haematoxylin and eosin, or with antibodies specific for murine haematopoietic compartment or human stromal component.

For the murine haematopoietic lineages, we used antibodies anti-TER-119 (clone TER-119, BD Biosciences), Iba1 (#019-19741, Wako Chemicals USA, Inc., Richmond, VA), Von Willebrand factor (#A0082, Dako, Glostrup, Denmark) and myeloperoxidase (#A0398, Dako). Tartrate resistant acid phosphatase (TRAP) staining (Sigma-Aldrich) was performed to evaluate the presence of osteoclasts.

For the human stromal compartment, we used a monoclonal mouse anti-human CD56 (NCAM, Dako). Briefly, after endogenous peroxidase blockage and antigen retrieval, slides were incubated with the primary antibody. Multiple washes were performed and then the secondary antibody conjugated with peroxidase was applied. Finally, the specimens were washed in PBS and developed in 3,3'-diaminobenzidine (DAB). Masson's trichrome (Bio-Optica, Milano, IT), Gomori's silver (Bio-Optica) and Perls' staining (Bio-Optica) were performed to appreciate respectively collagen, reticulin deposition and iron storages.

Brightfield light microscopy images were obtained using light microscopes (Leica DM 2500 or Olympus BX 41).

Haematopoietic colony-forming efficiency (h-CFE) assay

The h-CFE assay was performed in semi-solid medium as previously described.[10] Briefly, harvested heterotopic ossicles were digested twice with 100 U/mL type II collagenase (Gibco, Grand Island, NY), and filtered to obtain single cell suspensions (mean of cells collected per ossicle: 2.54×10^5 cells). 8×10^4 cells were resuspended in 1 mL of MethoCult GF M3434 (StemCell Technologies, Vancouver, BC, CA), plated in 35 mm low-adherence plastic dishes (Nunc, Rochester, NY), and incubated at $37\,°C$ and 5% CO_2 for 14 days. Haematopoietic colonies were identified by morphology on an inverted microscope. The nature of individual colonies was confirmed by plucking colonies, cytospinning the cells on glass slides, and staining with May-Grunwald Giemsa (Merck KGaA).

Statistical analysis

Continuous data were described by boxplots where upper horizontal line represents 75th percentile, lower horizontal line represents 25th percentile, horizontal bar within the box represents median, and vertical lines out the box represent minimum and maximum. Hypothesis test was performed by two-sided nonparametric Wilcoxon test at an overall 5% significance level.

Supplementary tables

Tab. SI. Clinical/biological characteristics of AA patients.

Patient code	Age (years)	Sex	Disease severity	OMB cellularity	Neutrophils (x10^9/ L)	Hb (g / dL)	PLT (x10^9/ L)	Post sample collection therapy	Cellular source
AA1	11	M	NSAA	5%	1.12	7.80	3	HSCT FD	BM (thawed)
AA2	15	M	SAA	<5%	0.44	9.50	8	IST 1° cycle	BM (thawed)
AA3	12	F	SAA	<5%	0.38	8.40	4	IST 1° cycle HSCT MUD	OMB
AA4	7	M	SAA	<5%	0.24	4.40	10	IST 1° cycle IST 2° cycle ANDRIOL	BM (thawed)
AA5	10	F	VSAA	<5%	0.18	5.60	6	HSCT FD	BM (fresh)
AA6	13	M	SAA	<5%	1.28	8.20	3	IST 1° cycle HSCT MUD	BM (thawed)
AA7	18	M	NSAA	5%	2.00	8.30	7	HSCT FD	BM (fresh)
AA8	15	F	VSAA	5%	0.18	8.40	14	IST 1° cycle	BM (fresh)

Abbreviations: OMB, osteo-medullary biopsy; Hb, haemoglobin; PLT, platelets; NSAA, non severe aplastic anaemia; SAA, severe aplastic anaemia; VSAA, very severe aplastic anaemia; HSCT FD, haematopoietic stem cell transplantation from family donor; HSCT MUD, haematopoietic stem cell transplantation from matched unrelated donor; IST, immunosuppressive therapy; BM, bone marrow.

Tab. S2. qPCR primers.

Protein	Gene symbol	Primer for qPCR (TaqMan assay no.)
Glyceraldehyde 3-phosphate dehydrogenase	*GAPDH*	4352934E
Type II collagen (COLII)	*COL2A1*	Hs01060345_m1
Type X collagen (COLX)	*COL10A1*	Hs00166657_m1
SRY-box containing gene 9 (SOX9)	*SOX9*	Hs00165814_m1
Aggrecan (AGGRECAN)	*ACAN*	Hs00202971_m1
Type I collagen (COLI)	*COL1A2*	Hs01028970_m1
Alkaline phosphatase (ALPL)	*ALPL*	Hs01029144_m1
Osteonectin (OTN)	*SPARC*	Hs00234160_m1
Osteopontin (OPN)	*SPP1*	Hs00959010_m1
Fatty acid binding protein 4 (FABP4)	*FABP4*	Hs01086177_m1
Lipoprotein lipase (LPL)	*LPL*	Hs00173425_m1
Peroxisome proliferator-activated receptor gamma (PPARG)	*PPARG*	Hs01115513_m1
Dickkopf-related protein 1 (DKK1)	*DKK1*	Hs00183740_m1
Transforming growth factor beta 1 (TGFB1)	*TGFB1*	Hs00998133_m1
Interleukin 6 (IL-6)	*IL6*	Hs00174131_m1

References

1. Young, N. S., Calado, R. T. & Scheinberg, P. Current concepts in the pathophysiology and treatment of aplastic anemia. *Blood* **108,** 2509–19 (2006).

2. Zeng, Y. & Katsanis, E. The complex pathophysiology of acquired aplastic anaemia. *Clin. Exp. Immunol.* **180,** 361–70 (2015).

3. Scheinberg, P. & Young, N. S. How I treat acquired aplastic anemia. *Blood* **120,** 1185–96 (2012).

4. Chatterjee, S. *et al.* Alteration in marrow stromal microenvironment and apoptosis mechanisms involved in aplastic anemia: an animal model to study the possible disease pathology. *Stem Cells Int.* **2010,** 932354 (2010).

5. Xu, Y. *et al.* Downregulation of GATA-2 and overexpression of adipogenic gene-PPARgamma in mesenchymal stem cells from patients with aplastic anemia. *Exp. Hematol.* **37,** 1393–9 (2009).

6. Chao, Y. H., Peng, C. T., Harn, H. J., Chan, C. K. & Wu, K. H. Poor potential of proliferation and differentiation in bone marrow mesenchymal stem cells derived from children with severe aplastic anemia. *Ann. Hematol.* **89,** 715–23 (2010).

7. Li, J. *et al.* Differential gene expression profile associated with the abnormality of bone marrow mesenchymal stem cells in aplastic anemia. *PLoS One* **7,** e47764 (2012).

8. Hamzic, E., Whiting, K., Gordon Smith, E. & Pettengell, R. Characterization of bone marrow mesenchymal stromal cells in aplastic anaemia. *Br. J. Haematol.* **169,** 804–13 (2015).

9. Bueno, C. *et al.* Bone marrow mesenchymal stem cells from patients with aplastic anemia maintain functional and immune properties and do not contribute to the pathogenesis of the disease. *Haematologica* **99,** 1168–75 (2014).

10. Serafini, M. *et al.* Establishment of bone marrow and hematopoietic niches in vivo by reversion of chondrocyte differentiation of human bone marrow stromal cells. *Stem Cell Res.* **12,** 659–72 (2014).

11.   Gatto, F. *et al.* Hurler disease bone marrow stromal cells exhibit altered ability to support osteoclast formation. *Stem Cells Dev.* **21,** 1466–77 (2012).

Chapter 3

Response to L-asparaginase is regulated by the acute myeloid leukaemia niche

Michelozzi I.M.[1], Granata V.[1], Maggi M.[2], De Ponti G.[1], Alberti G.[1], Tomasoni C.[1], Antolini L.[3], Scotti C.[2], Gambacorti-Passerini C.[4], Gentner B.[5], Dazzi F.[6], Biondi A.[1,7], Coliva T.[7], Rizzari C.[7], Pievani A.[1§], Serafini M.[1§]

[1]M. Tettamanti Research Centre, Department of Paediatrics, University of Milano-Bicocca, Monza, Italy. [2]Department of Molecular Medicine, Unit of Immunology and General Pathology, University of Pavia, Pavia, Italy. [3]Centro di Biostatistica per l'epidemiologia clinica, Department of Health Sciences, University of Milano-Bicocca, Monza, Italy. [4]Department of Haematology, San Gerardo Hospital, University of Milano-Bicocca, Monza, Italy. [5]San Raffaele Telethon Institute for Gene Therapy (SR-TIGET), San Raffaele Scientific Institute, Milan, Italy. [6]Department of Haemato-Oncology, Rayne Institute, King's College London, London, UK. [7]Department of Paediatrics, Fondazione MBBM/San Gerardo Hospital, Monza, Italy.
E-mail: serafinim72@gmail.com

[§]These authors are equal last authors.

Manuscript in preparation

Abstract

Eradicating the malignant stem cell is the ultimate challenge in the treatment of leukaemia. Leukaemic stem cells (LSC) hijack the normal haemopoietic niche in which they are largely protected from cytotoxic drugs. The anti-leukaemic effect of L-asparaginase (ASNase) has been extensively investigated in acute lymphoblastic leukaemia, but only partially in acute myeloid leukaemia (AML). We investigated the susceptibility of AML-LSC-enriched compartments to ASNase as well as the role of the two major cell types that constitute the bone marrow (BM) microenvironment, e.g., mesenchymal stromal cells (MSC) and macrophages. Whilst ASNase was effective on $CD34^+CD38^+$ and $CD34^+CD38^-$ LSC-enriched fractions, sparing healthy cells, MSC and macrophages could partially counteract the effect of the drug against the unfractionated and LSC-enriched populations. Indeed, the production of cathepsin B, a lysosomal cysteine protease, by BM macrophages and by a specific AML subtype could induce the degradation of ASNase. Our work demonstrates that while MSC and macrophages may provide a protective niche for AML cells, ASNase has a cytotoxic effect on AML blasts and, importantly, LSC-enriched subpopulations.

Introduction

Acute myeloid leukaemia (AML), a heterogeneous blood cancer, represents the most frequently diagnosed leukaemia in adults (25%) and it accounts for 15-20% in children. Despite continuous amelioration in the comprehension of AML pathogenesis and in AML diagnosis,

patients are still subject to high rate of relapse and poor overall survival.[1]

Biologically, AML cells could be represented as a hierarchy at the top of which there are leukaemic stem cells (LSC).[2] LSC are a heterogeneous group of cells, with stemness properties, which are responsible for initiating and maintaining the disease giving rise to more differentiated blasts.[2–4] Moreover, LSC refractoriness to conventional chemotherapies determines AML relapse.[1,5] This is due to their peculiar characteristics (e.g., quiescence and expression of efflux pumps)[1] and to the protection by the bone marrow (BM) microenvironment.[6]

Indeed, the BM microenvironment is modified by leukaemic cells generating a malignant niche able to support neoplastic cells and unable to maintain normal haematopoiesis.[5,7] Furthermore, it provides a protective milieu for LSC and cancer cells against pharmacological therapies.[5,8]

Undeniably, stromal cells in the BM niche contribute to establish a sanctuary in which LSC can acquire a drug-resistant phenotype and thereby evade chemotherapy-induced death. In particular, mesenchymal stromal cells (MSC) can favour AML blast and LSC survival upon chemotherapy through several mechanisms, including release of factors, e.g., CXCL12/CXCR4 and VCAM-1/VLA-4 axis, modification of leukaemic metabolism, and enhancement of the expression of c-myc.[6] In addition, BM contains various mature immune cell types, such as T and B cells, dendritic cells and macrophages, that contribute to the haematopoietic stem cell (HSC) niche.[9] In malignant

conditions, it is well documented that these different types of cells may provide a protective environment for leukaemic cells.

According to the proved importance of LSC in AML pathogenesis, therapeutic approaches aiming at targeting LSC are necessary to eradicate these cells preventing their further evolution and consequent AML relapse.[10]

L-Asparaginase (ASNase) is a deamidating enzyme that catalyses the hydrolysis of L-asparagine (Asn) and L-glutamine (Gln) causing Asn depletion in blood and in BM,[11,12] Gln reduction[12] and leukaemic cell death due to the incapacity of most blasts to *de novo* synthesise sufficient quantities of these amino acids.[13,14] Among the clinically approved formulations, ASNase derived from *Erwinia chrysanthemi* exhibits 10-fold higher glutaminase activity as compared to ASNase derived from *Escherichia coli*.[15]

Although ASNase has been widely exploited in the treatment of acute lymphoblastic leukaemia (ALL) since 1960s,[16] in the context of AML it has been partially investigated both *in vitro* and in clinical trials.[17] It has been recently demonstrated that AML cells are addicted in particular to glutamine for their energetic and biosynthetic metabolism[14,18,19] and, consequently, *Erwinia* ASNase exhibited greater cytotoxicity on AML cells as compared to *E. coli* ASNase.[14]

Despite the limited number of studies and the evidence of a major effectiveness of ASNase on ALL than AML blasts,[20,21] some specific subtypes and a subgroup of AML were reported to be more susceptible to ASNase as compared to others.[20–22]

Resistance to ASNase has been suggested to occur in ALL due to the asparagine and glutamine secreted by MSC and adipocytes surrounding

blasts in BM.[23,24] A further mechanism proposed as cause of therapy failure is the inactivation of ASNase mediated by cellular lysosomal cysteine proteases, such as cathepsin B (CTSB) and asparaginyl endopeptidase (AEP).

Microenvironment cells such as macrophages can produce CTSB and contribute to ASNase turnover *in vivo* in mice.[25] CTSB and AEP are overexpressed also by ALL blasts themselves, in particular by high-risk subsets of Philadelphia positive and iAMP21 leukaemia.[26,27]

In this study, we aimed at investigating the effects of ASNase within the AML niche focusing on the role of different players of the microenvironment, e.g., LSC, MSC and macrophages, in susceptibility to ASNase. Herein, we demonstrated that, while MSC and macrophages could contribute to provide a protective microenvironment to AML cells, ASNase exerts an effect on LSC-enriched subpopulations, as well as AML leukaemic blasts.

Materials and methods

Patient samples

Peripheral blood (PB) or bone marrow (BM) samples of 36 AML patients at diagnosis were collected after having obtained informed consent. Mononuclear cells were isolated using a Ficoll-Paque[TM] Plus (GE Healthcare, Little Chalfont, Buckinghamshire, UK) density gradient separation and used fresh or after cryopreservation for cytotoxicity experiments. The study was approved by the Ethics Committee of San Gerardo Hospital-Monza (LMA ASNASE 2900).

Clinical and molecular patients' details are reported in Supplementary Tab. S1.

Reagents and compounds

We tested two formulations of L-asparaginase (ASNase): *E. coli* ASNase (Kidrolase®) and *E. chrysanthemi* ASNase (*Erwinia* ASNase, Erwinase®) (Jazz Pharmaceuticals, Dublin, Ireland).

StemRegenin1 (SR1) and UM729 (StemCell[TM] Technologies, Vancouver, BC, CA) were used at a final concentration of 250 nM and 1 μM, respectively.

ASNase cytotoxicity

To determine the half maximal inhibitory concentration (IC50) of each ASNase formulation on AML cell lines, 4×10^4 cells were seeded in 96-well plates in complete culture medium with different concentrations of *E. coli* (0.1-300 IU/mL) and *Erwinia* (0.0001-100 IU/mL) ASNase. After 48 hours of treatment, we determined the cell count (cells/μL). IC50 was calculated using CompuSyn Software (www.combosyn.com).

For primary AML samples, 2×10^5 cells were plated in 96-well plates in complete Advanced RPMI 1640 medium with or without 1 IU/mL of *Erwinia* ASNase and cell viability was evaluated by flow cytometry after 48 hours (Supplementary methods). These experiments were also conducted in LSC supportive culture conditions using complete medium supplemented with SR1 and UM729.[28]

For MSC, healthy donor (HD)- and AML-MSC (P5-P6) were seeded at $1.7 \text{-} 2 \times 10^4$ cells/well in 96-well plates in complete RPMI 1640 medium

and, when confluent (in 1-2 days), were treated with *Erwinia* ASNase at different concentrations (0.1-1-3 IU/mL). After 48 hours, cells were trypsinised and their viability was evaluated by flow cytometry. All experiments were performed in triplicate.

qPCR

qPCR assays were used to determine *ASNS* expression in HD- and AML-MSC, and *CTSB* expression in full healthy BM and AML BM (samples with >70% blast cell content), and in CD14$^+$ and respective CD14$^-$ BM fractions purified from healthy donors using MIDIMACS immunoaffinity columns (Miltenyi Biotec, Bergisch Gladbach, Germany). Full details are provided in Supplementary methods.

Statistical analysis

Data were analysed using GraphPad Prism 7 (GraphPad Software, LA Jolla, CA, USA). If not otherwise specified, statistically significant differences between experimental groups were determined using Wilcoxon matched-pairs signed rank test. P-values: *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$.

Results

ASNase affects leukaemic clonogenic cells and leukaemic stem cells within patient-derived AML cells

In order to define the half maximal inhibitory concentration (IC50) of ASNase on AML, we first tested the inhibitory effect on cell proliferation of two different formulations of ASNase (*E. coli* and *Erwinia* ASNase) on AML cell lines (THP-1, KG-1 and HL-60) and on 697 ALL cell line, used as a control. As shown in Fig. 1A, the IC50 of *Erwinia* ASNase was lower as compared to *E. coli* ASNase for each cell line tested (*Erwinia vs E. coli* ASNase IC50 values: 697, 0.12 *vs* 0.26 IU/mL; THP-1, 2.89 *vs* 12.75 IU/mL; KG-1, 0.13 *vs* 0.65 IU/mL; HL-60, 0.11 *vs* 0.91 IU/mL). Indeed, the concentration at which cell proliferation was inhibited by 50% was higher for *E. coli* ASNase as compared to *Erwinia* ASNase (*E. coli* ASNase IC50/*Erwinia* ASNase IC50: 697, 2.17-fold; THP-1, 4.41-fold; KG-1, 5-fold; HL-60, 8.27-fold).

The superior effectiveness of *Erwinia* ASNase was observed in all AML cell lines tested also in terms of induction of apoptosis, evaluated at IC50 doses of treatment (data not shown). Indeed, in THP-1, KG-1 and HL-60, *Erwinia* ASNase, used at doses approximately 4-, 5- and 8-fold lower than *E. coli* ASNase respectively, was able to induce apoptosis at comparable levels to that of the latter one (P=0.8571, P>0.9999, P=0.6286).

Then, we evaluated the effects of *Erwinia* ASNase on primary AML samples representative of various leukaemia subtypes according to French-American-British (FAB) classification (Supplementary Tab.

S1). Within specimens, we distinguished between blast and non-blast populations according to the side scatter combined with the CD45 expression. Interestingly, ASNase caused a significant decrease in the number of live cells (P<0.0001) as such as an increase in the percentage of apoptotic cells (P<0.0001) within AML blasts. Instead, the number of normal lymphocytes exposed to ASNase was minimally reduced (P=0.0286) as such as the percentage of apoptosis in non-blast cells after treatment was slightly increased compared to untreated control (P=0.0112) (Fig. 1B). However, the median of differences between data obtained from treated and untreated conditions was 169.50- and 19.03-fold lower in non-blasts as compared to blasts for proliferation and apoptosis, respectively.

Next, with the aim of understanding the ability of ASNase to target the AML tumour-initiating cells, we decided to investigate the effects of the drug on LSC-enriched subpopulations within AML samples, identified according to the expression of CD34 and CD38 markers.[3] Notably, the two $CD34^+CD38^+$ and $CD34^+CD38^-$ populations were susceptible to ASNase, showing a significant cytotoxic effect on both these subpopulations (P<0.0001) (Fig. 1C, left), with levels comparable to those obtained with the bulk population (P=0.4282 calculated by Friedman test) (Fig. 1C, right).

Moreover, to evaluate the cytotoxic effect of ASNase on leukaemic clonogenic cells within primary AML specimens, colony growth was determined by colony-forming unit (CFU) assay in the presence or in the absence of ASNase. The exposure of the cells to ASNase (0.01 IU/mL) was able to significantly reduce the clonogenic potential of AML cells as compared to untreated controls (P=0.0001) (Fig. 1D).

Notably, colonies' formation was completely blocked by higher drug concentrations (0.1-1 IU/mL) (data not shown).

To deeply investigate the specific effect of ASNase on LSC, we decided to modify the culture conditions of primary AML specimens by adding the small molecules SR1 and UM729 which have been previously described for their capability to maintain the survival and stemness of AML-LSC in culture.[28]

As shown in Supplementary Fig. S1, the two compounds acted on $CD34^+CD38^-$ fraction enhancing significantly their viability as compared to a control population incubated without small molecules (P=0.0244).

Of note, also in these LSC supportive culture conditions, $CD34^+CD38^+$ and $CD34^+CD38^-$ subpopulations displayed high sensitivity to ASNase (P=0.0015 and P=0.0005, respectively). Similarly, the drug effect was maintained on the bulk population (P=0.0005) (Fig. 1E).

Furthermore, we observed a concomitant reduction of miR-126, a regulator involved in governing LSC self-renewal and quiescence,[29] for four of five AML samples treated with ASNase (Fig. 1F).
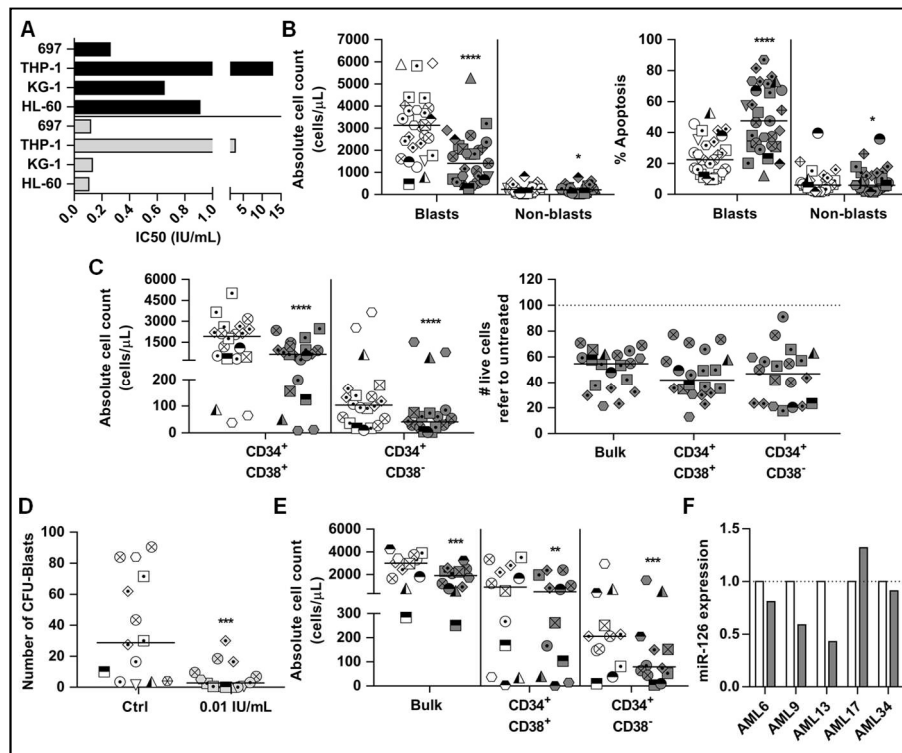
Fig. 1. ASNase cytotoxicity on AML cell lines and on primary AML samples.
(A) IC50 values of E. coli (black) and Erwinia (grey) ASNase obtained with 697, THP-1, KG-1 and HL-60 cell lines evaluating the reduction of cell number after 48 hours of treatment. (B) Cell viability was measured by flow cytometry in primary AML samples after incubation for 48 hours without (white) or with ASNase (1 IU/mL) (grey). We evaluated within blast and non-blast populations the absolute cell count (cells/μL) (on the left) and the percentage of apoptotic cells (on the right). Each symbol represents an individual AML patient (mean of biological triplicates). Bar indicates the median for each group. 29 independent experiments performed on 17 different patients are shown. (C) The effect of the drug on cell viability was analysed also in primitive CD34+CD38+ and CD34+CD38- fractions of 9 primary AML samples. We evaluated the absolute cell count (cells/μL) (on the left) and the number of live cells in treated samples referred to the relative vehicle control

(on the right). 20 independent experiments are shown. The dotted horizontal line represents the control. (D) The effect of the drug on the clonogenic potential of 9 primary AML samples was assayed after 14 days of culture on methylcellulose without (white) or with 0.01 IU/mL of ASNase (grey). Numbers of CFU-Blasts (mean of biological duplicates) counted in 14 independent experiments are shown. (E) AML samples were incubated without (white) or with (1 IU/mL) ASNase (grey) in the presence of SR1 and UM729. The absolute cell count (cells/µL) was evaluated in the bulk population, and in the $CD34^+CD38^+$ and $CD34^+CD38^-$ fractions. 12 independent experiments performed on 10 different patients are shown. (F) Relative miR-126 expression levels evaluated by quantitative ddPCR in AML samples after being incubated for 48 hours without (white) or with (1 IU/mL) ASNase (grey) in the presence of SR1 and UM729. 5 independent experiments performed on 5 different patients are shown.

Patients' symbols:

◯, AML1; △, AML2; □, AML3; ◇, AML4; ▽, AML5; ⊙, AML6; ⊡, AML7, ◈, AML8; ⊗, AML9; ⊠, AML10; ⊕, AML11; ⊙, AML12; ◯, AML13; ▲, AML14; ▭, AML15; ◆, AML16; ◓, AML17; ⊛, AML18; ◓, AML34.

MSC show a protective role against ASNase cytotoxicity within AML niche

In order to elucidate whether the BM microenvironment could exert an effect against the action of ASNase on AML blasts, AML cells were maintained in culture in the presence of normal or patient-derived MSC layer and treated with ASNase. The effect due to the coculture with MSC has been determined comparing the number of live cells and the proportion of apoptotic cells in AML blast cultures treated with the drug

in the presence or not of MSC and normalised to respective untreated control.

MSC from both healthy and AML BM were poorly sensitive to ASNase (Supplementary Fig. S2).

We showed that MSC derived from healthy donors (HD-MSC) were able to counteract ASNase cytotoxicity on AML cells, significantly increasing the number of live cells (P=0.0010) and decreasing the percentage of apoptotic cells (P=0.0005) upon treatment if compared with those obtained in plastic cultures (Fig. 2A).

Similarly, the presence of HD-MSC significantly enhanced the viability of CD34$^+$CD38$^+$ and CD34$^+$CD38$^-$ fractions (P=0.0078 and P=0.0039, respectively) upon ASNase treatment (Fig. 2B), demonstrating that MSC protect also these primitive populations from the drug cytotoxicity.

Considering that several microenvironment features could be modified by the disease, we subsequently performed additional experiments using cocultures of primary AML samples and AML-MSC derived from the same patient. Also in this autologous setting, AML-MSC significantly enhanced the number of leukaemic live cells (P=0.0078) and reduced the percentage of leukaemic apoptotic cells (P=0.0391) in treated samples (Fig. 2C).

Similarly, the presence of AML-MSC significantly decreased ASNase cytotoxicity against the CD34$^+$CD38$^+$ subpopulation (P=0.0078) and showed an effect on the CD34$^+$CD38$^-$ where we found a positive trend in the majority of the performed experiments approaching significance (P=0.0547) (Fig. 2D). Furthermore, AML-MSC showed an asparagine synthetase (*ASNS*) expression comparable to normal MSC samples

(P=0.0952), demonstrating that the protective capacity of patient-derived MSC may be dependent on the release of asparagine within the AML microenvironment (Fig. 2E).
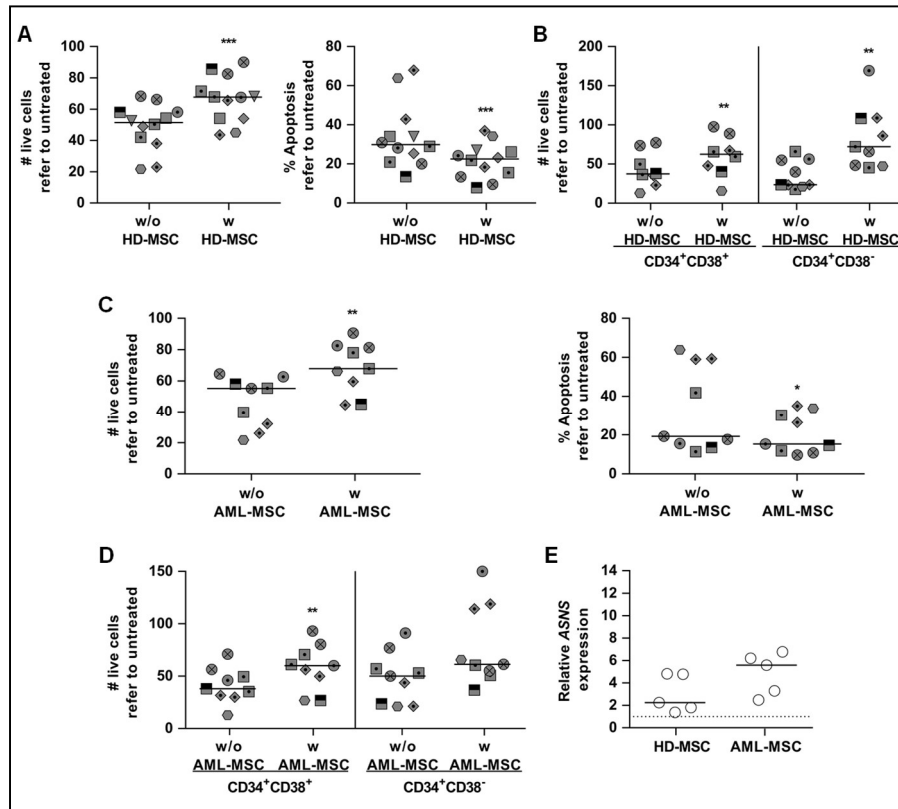

Fig. 2. Protective role of MSC against ASNase cytotoxicity.

(A) Primary AML samples were cultured without (w/o HD-MSC) or with (w HD-MSC) HD-MSC in the presence of ASNase (1 IU/mL) for 48 hours. The number of live cells (left) and the percentage of apoptosis (right) normalised to untreated control in the presence or in the absence of HD-MSC are represented. Each symbol represents an individual AML patient (mean of biological triplicates). Bar indicates the median for each group. 12 independent experiments performed on 9 different AML patients and 2 HD-MSC lines are shown. (B) Analysis of ASNase effect in the presence or in the

absence of HD-MSC on number of live cells (referred to control) in primitive CD34$^+$CD38$^+$ and CD34$^+$CD38$^-$ AML fractions. 9 independent experiments performed on 6 different AML patients are shown. (C) Primary AML samples were cultured without (w/o AML-MSC) or with (w AML-MSC) AML-MSC in the presence of ASNase (1 IU/mL) for 48 hours. The number of live cells (left) and the percentage of apoptosis (right) normalised to untreated control in the presence or in the absence of AML-MSC are represented. 9 independent experiments performed on autologous coculture of blasts and MSC from 6 different AML patients are shown. (D) Analysis of ASNase effect in the presence or in the absence of AML-MSC on number of live cells (referred to control) in primitive CD34$^+$CD38$^+$ and CD34$^+$CD38$^-$ AML fractions. 9 independent experiments performed on 6 different AML patients are shown. (E) Expression of ASNS in HD- vs AML-MSC. The expression is showed as fold change, calculated as 2$^{-\Delta\Delta Ct}$ using the 697 cell line as the reference (2$^{-\Delta\Delta Ct}$ =1, dotted horizontal line). 10 different MSC donors (5 HD-MSC and 5 AML-MSC) were analysed. P-value was calculated using Mann Whitney test.

Patients' symbols:
□, AML3; ◇, AML4; ▽, AML5; ⊙, AML6; ⊡, AML7; ◈, AML8; ⊗, AML9; ◌, AML13; ▰, AML15.

CTSB, involved in ASNase degradation, is expressed especially by BM monocytic cells and AML-M5 blasts

A further mechanism of ASNase resistance to consider is the drug clearance mediated by cellular lysosomal cysteine proteases, especially CTSB.[25,45] For this reason, we investigated the expression of *CTSB* by cells of BM microenvironment and by AML blasts themselves.

We found that in the normal BM, *CTSB* was expressed primarily by CD14$^+$ monocytic cells. Indeed, the median 2$^{-\Delta\Delta Ct}$ value of *CTSB*

mRNA of CD14$^+$ samples (26.13) was observed to be higher as compared to the median $2^{-\Delta\Delta Ct}$ value of *CTSB* mRNA in CD14$^-$ samples (6.08; P<0.0001; Fig. 3A).

AML BM exhibited an average of 3.4-fold higher *CTSB* mRNA as compared to normal BM. When examining relative expression levels among the 27 AML patients, *CTSB* was upregulated by 4-12-fold in 9 out of 27 (33%) patients in comparison to controls. Among these *CTSB* overexpressing patients, 8 (89%) were of FAB M5 subtype.

The relative expression of *CTSB* mRNA was elevated in 8 out of 12 (67%) FAB M5 AML samples, with a median $2^{-\Delta\Delta Ct}$ value of 30.34. Instead, *CTSB* is constitutively expressed at lower levels by FAB M0/1 (median $2^{-\Delta\Delta Ct}$ value of 4.79; P *vs* AML-M5=0.0037), FAB M2 (median $2^{-\Delta\Delta Ct}$ value of 4.82; P *vs* AML-M5=0.0044) and FAB M4 (median $2^{-\Delta\Delta Ct}$ value of 4.21; P *vs* AML-M5=0.0044) AML samples (Fig. 3B).
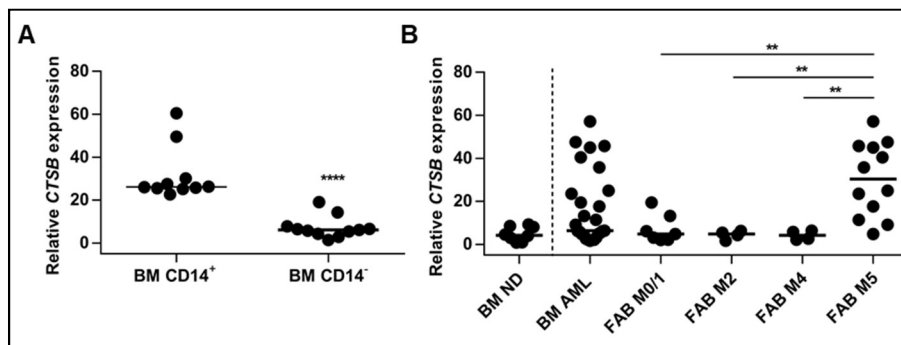

Fig. 3. CTSB overexpression in BM CD14$^+$ monocytic cells and FAB M5 subset of AML.

(A) CTSB expression by CD14$^+$ and CD14$^-$ cells isolated from healthy donor BM. The expression was analysed by qPCR and is showed as fold change, calculated as $2^{-\Delta\Delta Ct}$ using the REH cell line as the reference ($2^{-\Delta\Delta Ct}$=1). Ten CD14$^+$ samples (average purity 85%) and 11 CD14$^-$ samples (average purity

84%) from 11 different donors were analysed. (B) CTSB expression by AML
BM. 27 AML BM (with >70% of blast cell content) and 8 HD BM samples
were analysed. Within AML patients, 7 are FAB M0/1, 4 are FAB M2, 4 are
FAB M4, and 12 are FAB M5.

Discussion

In the last few years it has been clarified that in order to be curative, any
AML therapy needs to be effective against the cells that propagate and
sustain the disease, the so called LSC. In particular, relapse of AML is
thought to occur because of the failure of chemotherapy to eradicate
LSC, which are biologically distinct from more mature leukaemic blasts
and may not be responsive to conventional chemotherapeutic
regimens.[10] By analogy to normal HSC, LSC are thought to reside in
specific BM niches that regulate their self-renewal, quiescence, and
sensitivity to chemoradiation therapy. Indeed, the malignant niche is
composed by several different players, which may interfere with the
efficacy of a therapeutic treatment. Thus, eliminating the malignant
LSC within the BM microenvironment is the ultimate challenge in the
treatment of AML.[6]

As a consequence, to assess the potential of new compounds, it is
pivotal to investigate their toxicity on leukaemia and progenitor cell
populations in relation with other cell types contained within the
leukaemic niche.

Here, we report the susceptibility of AML-LSC and progenitors to
ASNase as well as the role of two cell types that constitute the BM
microenvironment, e.g., MSC and macrophages. Whilst ASNase was

effective on CD34$^+$CD38$^+$ and CD34$^+$CD38$^-$ LSC-enriched fractions, sparing healthy haematopoietic cells, MSC and macrophages could partially counteract the effect of the drug against blasts and distinct LSC-enriched populations.

The anti-leukaemic effect of ASNase has been extensively investigated in ALL therapy,[30,31] but only partially in AML.[32–34] Its anti-neoplastic properties are caused by its depletion of extracellular asparagine and glutamine, creating a state of amino acid deficiency and subsequent inhibition of protein synthesis in neoplastic cells. Despite its efficacy in ALL, ASNase has been used only occasionally to treat other haematological malignancies and solid tumours, these latter with scarce results.[17,35] Previous *in vitro* studies have observed varied response to ASNase in AML, showing that specific FAB subtypes and cytogenetic and molecular subgroups are more sensitive.[20–22] ASNase acts firstly by inhibiting the proliferation of leukaemic cells and, subsequently, by inducing their apoptosis.[36] For this reason, we analysed the drug effect in terms of absolute number of live cells and percentage of apoptotic cells. The number of live cells could be influenced by the cytotoxic effect, but we cannot exclude also a cytostatic effect of the drug.

Regardless of patients' age, we noticed that ASNase, used at clinically attainable dose, was able to reduce the number and to induce apoptosis of AML blasts, while minimally affecting healthy haematopoietic cells. For the limited number of samples collected we are not able to make any assumption about FAB-related drug sensitivity.

Effects of ASNase on AML primitive cell fractions have not been examined so far. Firstly, we analysed the effect of the drug on clonogenic capacity, as an *in vitro* measure of the self-renewal in AML cells. Treatment with ASNase reduced the clonogenicity of primary AML specimens.

Next, we investigated the susceptibility of AML-LSC to ASNase. We considered the CD34^{+}CD38^{-} and CD34^{+}CD38^{+} LSC-enriched compartments since LSC are not characterised by a unique phenotype and both compartments were reported to contain the most of the LSC.[3] Notably, we showed that ASNase could reduce the number of live cells within CD34^{+}CD38^{+} and CD34^{+}CD38^{-} fractions in a proportion similar to the non-LSC bulk blast population.

Recently, two small molecules, StemRegenin1 (SR1) and UM729 that support human LSC activity *ex vivo* have been identified. Their use in culture systems preserves AML-LSC by inhibiting their spontaneous differentiation *in vitro*.[28] SR1 is an antagonist of aryl hydrocarbon receptor, a transcription factor involved in HSC expansion and differentiation,[37] whereas UM729 does not act on the same pathway but it showed an additive effect with SR1.[28] In particular, these two compounds were shown to support the maintenance of CD34^{+}CD38^{-} and CD34^{+}CD38^{+} subpopulations, especially CD34^{+}CD38^{-}, preserving the functionality of AML-LSC as proved by their engraftment capacity.[28] Therefore, using these improved culture conditions, we were able to further prove the effect of ASNase on AML compartments containing LSC and progenitor cells. In addition, the concomitant reduction of miR-126, a regulator implicated in governing the stemness

state of human LSC,[29] offered an additional evidence of the effect of ASNase on LSC frequency.

The capability of ASNase to act on cancer stem cells and to reduce their clonogenic potential has been observed in solid tumours and it was related to its glutaminase activity. Indeed, in the absence of Gln the levels of reactive oxygen species augmented through attenuation of glutathione synthesis, leading to the downregulation of β-catenin pathway and subsequently to the reduction of cancer stem cells.[38] As LSC show susceptibility to oxidative stress and to β-catenin pathway,[39] we can speculate that the effects observed in our work could be linked to the glutaminase activity of the drug.

AML-LSC have been involved in drug resistance and disease relapse and are responsible for minimal residual disease. The comprehension of the mechanisms underlying drug resistance that allow LSC survival is therefore critical to advance therapy. Extrinsic factors involved in chemoresistance arise from the protective effect of the microenvironment, which nurtures LSC survival.[10]

Concerning ASNase activity within microenvironment, Iwamoto et al., proposed that BM-MSC might support ALL blasts during ASNase treatment through local amino acid secretion. They demonstrated that coculture with MSC protected ALL cells from the cytotoxicity caused by ASNase, and this protective effect correlated with *ASNS* expression levels. Therefore, *ASNS* silencing decreases the protection, whereas enforced expression gives enhanced protection.[23] Laranjeira et al., showed that insulin-like growth factor-binding protein 7 (IGFBP7) released by leukaemic cells boosts asparagine synthesis by stromal

cells.[40] It is yet to be established whether this association is critical *in vivo*.

In accordance with these works, we found that MSC exert a protective role also in AML blasts against the cytotoxic effects of ASNase. Primary AML cells varied in their susceptibility to the protective effects of MSC, probably because of differences in the capacity of leukaemic cells to interact with the microenvironment.

Not only bulk AML cells, but especially the progenitor fractions $CD34^+CD38^+$ and $CD34^+CD38^-$, *bona fide* LSC, showed an increased viability upon ASNase treatment in the presence of MSC. This suggests that protective signals within the stromal microenvironment could maintain residual leukaemic cells, in particular LSC, relatively insensitive to ASNase therapy, potentially responsible for the recurrence of the disease.

We tested the protective activity of MSC using different BM specimens derived both from healthy donors and from AML patients. This approach eliminated the potential heterogeneity inherent in allogeneic human MSC. Furthermore, it is known that AML-MSC present alterations in their transcriptome supporting leukaemogenesis and chemoresistance due to the leukaemia-induced remodelling of the BM microenvironment.[41] Similarly to HD-MSC, also AML-MSC increased significantly the resistance to ASNase of $CD34^+CD38^+$ cells, whereas in $CD34^+CD38^-$ compartment we found a similar trend in the majority of experiments performed (7/9), approaching but not reaching statistical significance. We believe that this result could be explained by the limited number of samples analysed rather than by defects in blasts supportive capabilities of AML-MSC.

The role of the microenvironment in the regulation of the response to chemotherapy in AML is already known. Indeed, Matsunaga et al., found that the interaction between VLA-4 on AML cells and fibronectin on MSC was essential for the persistence of cytarabine-resistant disease and the VLA-4 expression is an adverse prognostic factor in patients with AML.[42] Moreover, Konopleva et al., observed that MSC increased the expression of anti-apoptotic proteins and augmented the resistance to cytarabine in AML cells.[43] In the case of ASNase, protection seems to be attributable to asparagine released by MSC in the microenvironment. We observed that *ASNS* gene expression levels in MSC were variable but similar between HD- and AML-derived MSC. The development of appropriate techniques to reduce the expression of asparagine synthetase by the BM-MSC could then improve the effect of ASNase therapy. Cytarabine, a first-line AML chemotherapeutic, has been reported to induce downregulation of *ASNS* transcription.[44] Thus, the combination of ASNase with other anti-cancer agents may enhance its anti-leukaemic action.

Furthermore, proteolytic inactivation of ASNase could have a potential role in the modulation of its effect within the malignant niche. Indeed, it has been previously reported that the lysosomal CTSB and AEP hydrolyse ASNase, resulting in inactivation and exposure of immune epitopes.[26] AEP and CTSB are expressed by lymphoblasts, in particular by Philadelphia positive (Ph+) and iAMP21 leukaemia cells, two high-risk cytogenetic subtypes.[26,27] Moreover, a germ line mutation in the gene encoding CTSB has been linked with a strongly prolonged ASNase turnover in a patient.[45]

Increased CTSB activity has been described in solid tumours, deriving not only from the tumour mass but also from the cells surrounding the tumour, with a role in cancer progression and metastasis.[46] In particular, tumour-associated macrophages have been identified as the primary source of high levels of cathepsin activity in pancreatic islet cancers, mammary tumours, and lung metastases.[47] Phase I and phase I-II clinical trials using ASNase were conducted in patients with solid tumours showing that a large portion of patients was not responsive to the treatment mainly because the active dose of the drug quickly decreased after administrations, probably due to proteolytic inactivation.[35]

In healthy human BM samples, we found that the expression of *CTSB* is attributable to monocytic CD14$^+$ cells. This is consistent with findings of *in vivo* ASNase distribution showing that the drug is rapidly cleared from the serum by BM-resident phagocytic cells.[25] Therefore, BM-resident macrophages may collaborate in the establishment of a protective niche for leukaemic cells by effectively removing ASNase from the BM through the release of CTSB. It is known that macrophages in tumour microenvironment can protect tumour cells from cell death induced by a range of additional chemotherapeutic drugs (e.g., taxol, etoposide and doxorubicin), via a cathepsin-dependent mechanism.[48]

Furthermore, myeloid blasts themselves can express *CTSB* and, possibly, be involved in ASNase degradation. Increased expression of CTSL and CTSB in AML patients seems to be associated with reduced overall survival.[49] Notably, we found that in primary AML, the majority of FAB M5 samples specifically overexpressed *CTSB*, showing a 5.3-

fold increase in mRNA levels compared to the other subtypes. Our study contains, in addition to FAB M5, only FAB M0/1, M2 and M4 specimens because numbers in the other FAB-type subgroups were too small for separate analysis. Although the above mentioned data had been assayed in unsorted AML BM samples, we included in the analysis only specimens with >70% of blast cell content, assuming that the results will remain roughly the same even in purified blast cells.

Given these data, some previous results need to be reconsidered. Indeed, there is a general agreement that FAB M5 blast cells are responsive to ASNase *in vitro*.[21] Zwaan et al., reported that FAB M5 is equally sensitive *in vitro* to L-asparaginase as ALL and this can be explained by the low level of asparagine synthetase in FAB M5.[20,50] Nevertheless, it should be considered that the *in vitro* response to ASNase could not reliably match with the *in vivo* clinical response because other factors, such as the levels of expression of proteases, e.g., CTSB, are likely to significantly modulate ASNase levels *in vivo* and then determine the drug efficacy in patients.

To improve the outcome and decrease morbidity in patients undergoing chemotherapy an option would be to use specific protease inhibitors in association with ASNase therapy.[51] Another option can be the generation of novel modified versions of ASNase. Indeed, several studies have shown that the structure of ASNase permits the introduction of modifications to resist proteolytic cleavage without impairment of enzymatic function.[52,53]

In conclusion, whilst ASNase was effective on AML bulk blasts and LSC-enriched fractions, sparing healthy haematopoietic cells, MSC,

macrophages and FAB M5 blasts themselves via CTSB-dependent mechanism may partially counteract the effect of the drug. Thus, our work highlights crucial aspects, which should be considered in the design of future clinical studies aimed at testing ASNase efficacy in AML patients.

Acknowledgements

Author contributions

I.M.M., V.G., and M.M. performed research and analysed the data; D.G., G.A. and C.T. performed research; L.A. performed statistical analysis; C.G. provided patient samples; T.C., and C.R. designed research, provided patient samples and contributed to the writing of the paper; C.S., B.G., F.D., and A.B. interpreted the data and edited the manuscript; A.P. and M.S. designed research, interpreted the data, and wrote the manuscript.

Conflict of interest

The authors declare no competing financial interests.

Supplementary methods

Cells

The human AML cell lines KG-1, THP-1 and HL-60 were obtained from ATCC and the human ALL cell line 697 was purchased from DSMZ. Cells were cultured according to manufacturer's recommendations in complete RPMI 1640 medium (EuroClone, Milan, Italy) or complete Advanced RPMI 1640 medium (Gibco[TM], Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10-20% of heat-inactivated foetal bovine serum (FBS) (Biosera, Ringmer, UK), 2 mM L-glutamine, 50 IU/mL penicillin and 50 μg/mL streptomycin (EuroClone).

MSC were isolated from BM aspirates of AML patients at diagnosis (AML-MSC) and of healthy donors (HD-MSC), as previously described.[54] Cells were grown in DMEM-low glucose (1 g/L; Gibco[TM], Thermo Fisher Scientific), supplemented with 10% FBS, 2 mM L-glutamine and antibiotics (50 IU/mL penicillin and 50 μg/mL streptomycin). MSC were not used for more than 7 passages.

Analysis of cell viability

For analysis of apoptosis, cells were stained with AnnexinV/7-AAD (Apoptosis/Necrosis Detection Kit, Enzo Life Sciences, Farmingdale, NY, USA). The percentage of AnnexinV[+]/7-AAD^{-/+} early and late apoptotic cells was evaluated by FACS analysis.

Apoptosis relative to untreated control has been calculated as reported.[55]

To evaluate the number of viable cells, counting beads (CountBright^{TM} absolute counting beads, Invitrogen^{TM}, Thermo Fisher Scientific) were added to samples before the acquisition and the absolute cell count (cells/μL) was calculated following manufacturer's protocol.

Primary AML samples were labelled with pacific orange-anti CD45 (clone HI30; Invitrogen, Thermo Fisher Scientific), phycoerythrin-cyanine^{TM} 7-anti CD34 (clone 8G12; BD Biosciences, Franklin Lakes, NJ, USA) and allophycocyanin-Alexa Fluor® 750-anti CD38 (clone LS198-4-3; Beckman Coulter Inc., Brea, CA, USA) to perform the analysis gating on the normal cells within the sample, the bulk blast population and the primary $CD34^+CD38^-$ and $CD34^+CD38^+$ subpopulations.

Experiments were performed on a FACSCanto^{TM} II (BD Biosciences) and analysed with FACSDiva^{TM} software v.6.1.3 (BD Biosciences).

<u>Coculture experiments</u>

HD-MSC (P4-P7) were seeded at 1.7-$2x10^4$ cells/well in 96-well plates. When confluent (in 1-2 days), primary AML samples ($2x10^5$ cells/well) were added to the culture in complete Advanced RPMI 1640 medium with or without 1 IU/mL of *Erwinia* ASNase. After 48 hours of treatment, the bottom of the wells was scraped and the harvested cells were passed through a 18-gauge needle, to eliminate MSC aggregates. Then, cell suspensions were analysed for viability by flow cytometry. We executed the same experiment coculturing primary AML samples with the autologous AML-MSC (P3-P6).

All experiments were performed in triplicate.

Clonogenic assay

1×10^4 of primary AML cells were resuspended with 1 mL of MethoCult^{TM} H4434 classic (StemCell^{TM} Technologies) in the presence or in the absence of 0.01 IU/mL of ASNase. The mixture was plated in 35 mm low-adherence plastic dishes (Thermo Scientific^{TM} Nunc^{TM}, Thermo Fisher Scientific) and maintained at 37 °C and 5% CO_2. After 14 days, colonies were counted on an inverted microscope. Experiments were performed in duplicate.

miR-126 expression

After 48 hours of incubation in the presence of SR1 and UM729 with or without ASNase, as previously described, primary AML cells were resuspended in TRIzol^{TM} reagent (Invitrogen^{TM}, Thermo Fisher Scientific) and frozen. Total RNA, including miRNA, was extracted from samples using miRNeasy Micro Kit (Qiagen, Hilden, Germany), following manufacturer's instructions. RNA concentration was measured using Quantus^{TM} Fluorometer. cDNA was synthetised using Universal cDNA synthesis kit II (Exiqon, Copenhagen, Denmark) following the company's guidelines for miRNA profiling. UniSP6 spike-in was included in each reaction as a retrotranscription and PCR plate-loading control. Digital droplet PCR (ddPCR) was performed using EvaGreen supermix (Bio-Rad, Hercules, CA, USA) and one of the following miRCURY LNA PCR primer sets (Exiqon): hsa-miR-126-3p (ID 204227), hsa-let-7a-5p (ID 205727), hsa-miR-16-5p (ID 205702), SNORD24 (ID 206999), SNORD48 (ID 203903), UniSP6 (ID 203956). Droplets were generated using Automated Droplet Generator (Bio-Rad). Recommended thermal cycling conditions for EvaGreen

assays were used, except for annealing step optimisation. Droplets were analysed using QX200 Droplet Reader (Bio-Rad) and QuantaSoft^TM. miR-126-3p levels were normalised by the geometric mean of let-7a-5p, miR-16-5p, SNORD24 and SNORD48.

qPCR

Total cellular RNA was isolated using TRIzol^TM reagent according to manufacturer's protocol. One μg of total RNA was reversely transcribed using the SuperScript II Reverse Trascriptase (Invitrogen^TM, Thermo Fisher Scientific).

qPCR experiments were performed using Light Cycler 480II with Universal Probe Master system (Roche Diagnostics, Rotkreuz, Switzerland).

ASNS and *CTSB* primers were designed through the Software Probe Finder (Roche Diagnostics) and are the following: h*ASNS*upl-left 5'-GATGAACTTACGCAGGGTTACA-3' and h*ASNS*upl-right 5'-CACTCTCCTCCTCGGCTTT-3'; h*CTSB*upl-left 5'-CAGCCACCCAGATGTAAGC-3' and h*CTSB*upl-right 5'-GCCGGATCCTAGATCCACTA-3'. As reference, housekeeping gene *ABL1* was used (h*ABL1*upl-left: 5'-AGGAATCCAGTATCTCAGACGAA-3' and h*ABL1*upl-right: 5'-GGAGGTCCTCGTCTTGGTG-3'). UPL probe number 2 or 30 and 57 were used in combination to detect *ASNS* and *CTSB* expression.

Three independent replicates were performed. qPCR data were calculated with the ΔΔCt method using as a reference the 697 and REH cell lines.
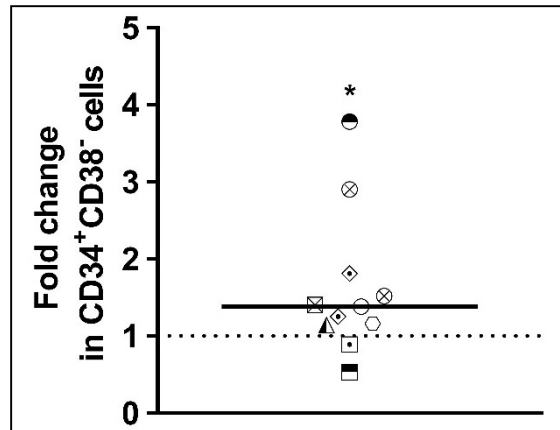
Supplementary figures

Fig. S1. Effect of SR1 and UM729 on maintenance of primary CD34$^+$CD38$^-$ AML cells in culture.

Primary AML samples were cultured with or without SR1 and UM729 for 48 hours. The effect of these two reagents on primary CD34$^+$CD38$^-$ subpopulation was expressed as fold change relative to the control cultured for 48 hours without the two compounds. Each symbol represents an individual AML patient (mean of biological triplicates): ⊙, AML6; ⊡, AML7; ◈, AML8; ⊗, AML9; ⊠, AML10; ○, AML13; ▲, AML14; ▬, AML15; ◓, AML17. 11 independent experiments performed on 9 different patients are shown. Bar indicates the median and the dotted horizontal line represents the control. P-value was calculated using Wilcoxon signed rank test.
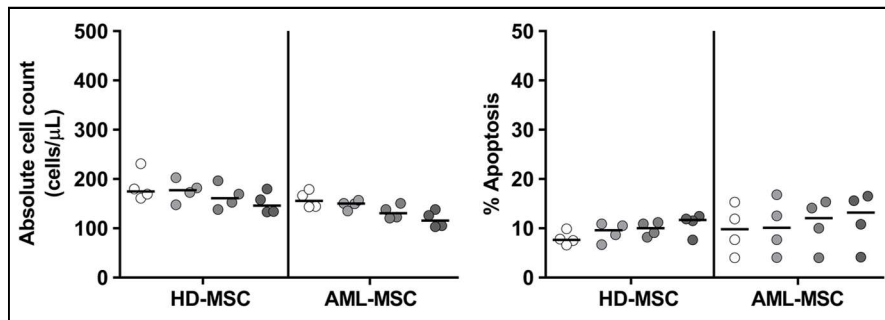
Fig. S2. ASNase cytotoxicity on HD- and AML-MSC.

HD- and AML-MSC were incubated for 48 hours in the absence (white) or in the presence of 0.1-1-3 IU/mL of ASNase (represented by darker shades of grey as the dose increases). The absolute cell count (cells/µL) (on the left) and the percentage of apoptotic cells (on the right) were evaluated by flow cytometry. 4 independent experiments were performed on 8 different MSC donors (4 HD and 4 AML). Each dot represents the mean of biological triplicates and bar indicates the median for each group.

Supplementary table

Tab. S1. Clinical and molecular patients' details.

Patient code	Age (y)	Sex	AML type	WBC 10³/uL	% Blasts in BM	% Blasts in PB	Cellular source	Molecular status	Karyotype
AML1	51	F	M5a, de novo	64	90	70	BM (fresh/thawed)	*FLT3-ITD, NPM1* mut	46,XX[25]
AML2	64	M	M0, de novo	33.03	90	90	BM (fresh/thawed)	none	48,XX,+8,+13[10]/46,XY[2]
AML3	30	M	M5a, de novo	72.61	90	94	BM (fresh/thawed)	none	46,XY,del(11)(q23)[20]
AML4	17	M	M1, de novo	12.58	60	70	BM (thawed)	none	46,XY[15]
AML5	13	M	M2, de novo	28.35	40	58	BM (thawed)	*FLT3-ITD*	46,XY[20]
AML6	8	F	M4, de novo	22.42	85	64	BM (thawed)	*FLT3-ITD, DEK-CAN –* t(6;9)	47,XX,+8[18]/47,idem,iso(13)(q11)[2]
AML7	9	F	M4, de novo	4.57	90	40	BM (thawed)	*MLL-ELL*	46,XX,t(11;19)(q23;p13)[18]/46,XX[2]
AML8	1	M	M5a, de novo	39.42	80	51	BM (thawed)	*MLL-AF10*	46,XY,t(10;11)(p12;q23),der(14)t(1;14)(q?21;q11)[20]
AML9	3	M	M2, de novo	14.28	80	42	BM (thawed)	*NUP98-NDS1 -* t(5;11)	46,XY[20]
AML10	71	F	M1/M2, de novo	N.A.	50	N.A.	PB (fresh)	none	N.A.
AML11	48	F	M0, de novo	31.46	>70	N.A.	BM (thawed)	*FLT3-ITD, NPM1* mut	46,XX[20]
AML12	3	M	M5a, de novo	378.92	N.A.	92	PB (fresh)	none	47,XY,+9,t(11;17)(q23;q12 or q21)[20]

Patient code	Age (y)	Sex	AML type	WBC 10³/uL	% Blasts in BM	% Blasts in PB	Cellular source	Molecular status	Karyotype
AML13	65	M	M0, de novo	49.86	90	N.A.	BM (fresh)/(thawed)	none	46,XY,del(5)(q13q31)[20]
AML14	48	M	M4, de novo	104.43	80	N.A.	BM (thawed)	*FLT3 D835, CBFB-MYH11*	46,XY,inv(16)(p13q22)[20]
AML15	70	F	M4, secondary	6.45	50	30	BM (thawed)	none	44~45,XX,?+X,t(1;22)(q12;q11),+1,del(5)(q13q34),?inv(7)(q14q22),tas(8;15)(q24;p13),-17,-21,del(22)(q11)der(22)t(1;22)(q12;q11),+mar[cp18]/45,XX,t(1;22)(q12;q11),+1,del(5)(q13q34),-7,der(17)(q11;q25),-21,del(22)(q11)der(22)t(1;22)(q12;q11)[7]
AML16	62	F	M1, de novo	1.91	70	5	BM (fresh)	*NPM1* mut	46,XX,del(13)(q14q22)[3]/46,XX[17]
AML17	30	F	M1/M2, de novo	23	94	90	BM (fresh)/(thawed)	*FLT3 D835, CEBPA* mut	46,XX[20]
AML18	58	M	M1/M2, de novo	130	95	88	BM (fresh)	*FLT3-ITD, NPM1* mut	46,XY,i(7)(q10)[9]/46,XY[12]
AML19	85	F	M4eo, secondary	42.90	80	65	BM (thawed)	N.A.	47,XX,del(7)(q22),+?22[20]
AML20	56	F	M4, de novo	33.81	80	N.A.	BM (thawed)	*FLT3-ITD, NPM1* mut	46,XX,del(9)(q21)[20]
AML21	14	M	M1, de novo	9.73	95	80	BM (thawed)	none	47,XY,+?13[11]/46,XY[9]
AML22	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
AML23	12	M	M1, de novo	56.24	95	77	PB (thawed)	*FLT3 D835, NPM1* mut	46,XY[25]
AML24	13	F	M2, de novo	42.40	80	71	BM (thawed)	*AML1-ETO, cKIT* mut	46,XX,t(3;7)(?q25;?q22),t(8;21)(q22;q22)[2]/46,idem,del(9)(q12q22)[18]

Patient code	Age (y)	Sex	AML type	WBC 10³/uL	% Blasts in BM	% Blasts in PB	Cellular source	Molecular status	Karyotype
AML25	12	F	M2, de novo	13.70	90	N.A.	BM (thawed)	*AML1-ETO*	46,XX,t(8;21)(q22;q22)[20]
AML26	39	M	M1, de novo	6.29	50	43	BM (thawed)	*NPM1* mut	46,XY[20]
AML27	13	M	M1, de novo	8.83	85	75	BM (thawed)	none	46,XY[19]
AML28	15	M	M5a, de novo	97.32	N.A.	93	PB (thawed)	none	46,XY,del(17)(p11.2)[3]/46,XY,del(9)(p21), del(17)(p11.2)[16]
AML29	9 months	F	M5b, de novo	353.69	N.A.	90	PB (thawed)	*MLL-AF6*	51,XX,+3,+6,t(6;11)(q27;q23),+7,+8,+12[20]
AML30	16	M	M5a, de novo	73.85	N.A.	89	PB (thawed)	*NPM1* mut	46,XY[20]
AML31	77	M	M5b, de novo	24.80	95	67	BM (thawed)	N.A.	47,XY,+8[20]
AML32	4	F	M5a, de novo	235.32	95	N.A.	BM (thawed)	N.A.	47-48,XX,del(2)(p12),del(5)(p12),?t(6;7)(q21;q32),t(9;?)(q34;?), -11,del(12)(p11),+19,+4markers[cp9]/46,XX[3]
AML33	1	F	M5a, de novo	372.79	N.A.	85	PB (thawed)	*MLL-AF10* – t(10;11)	46,XX[20]
AML34	12	M	M5a, de novo	1.73	90	25	BM (fresh)	*MLL-AF9* – t(9;11)	48,XXY,+21c[22]
AML35	41	F	M5b, de novo	85.17	90	N.A.	BM (fresh)	*FLT3-ITD*, *NPM1* mut	47,XX,+8[20]
AML36	66	M	M5b, de novo	83.96	90	N.A.	BM (fresh)	*FLT3-ITD*, *NPM1* mut	46,XY[25]

Abbreviations: WBC, white blood cells; BM, bone marrow; PB, peripheral blood; none, negative for mutations and translocations analysed; N.A., not analysed.

References

1. Siveen, K. S., Uddin, S. & Mohammad, R. M. Targeting acute myeloid leukemia stem cell signaling by natural products. *Mol. Cancer* **16,** 13 (2017).

2. Bonnet, D. & Dick, J. E. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat. Med.* **3,** 730–7 (1997).

3. Eppert, K. *et al.* Stem cell gene expression programs influence clinical outcome in human leukemia. *Nat. Med.* **17,** 1086–93 (2011).

4. Hope, K. J., Jin, L. & Dick, J. E. Acute myeloid leukemia originates from a hierarchy of leukemic stem cell classes that differ in self-renewal capacity. *Nat. Immunol.* **5,** 738–43 (2004).

5. Ishikawa, F. *et al.* Chemotherapy-resistant human AML stem cells home to and engraft within the bone-marrow endosteal region. *Nat. Biotechnol.* **25,** 1315–21 (2007).

6. Korn, C. & Méndez-Ferrer, S. Myeloid malignancies and the microenvironment. *Blood* **129,** 811–22 (2017).

7. Colmone, A. *et al.* Leukemic cells create bone marrow niches that disrupt the behavior of normal hematopoietic progenitor cells. *Science* **322,** 1861–5 (2008).

8. Ninomiya, M. *et al.* Homing, proliferation and survival sites of human leukemia cells in vivo in immunodeficient mice. *Leukemia* **21,** 136–42 (2007).

9. Riether, C., Schürch, C. M. & Ochsenbein, A. F. Regulation of hematopoietic and leukemic stem cells by the immune system. *Cell Death Differ.* **22,** 187–98 (2015).

10. Pollyea, D. A. & Jordan, C. T. Therapeutic targeting of acute myeloid leukemia stem cells. *Blood* **129,** 1627–35 (2017).

11. Tong, W. H. *et al.* No evidence of increased asparagine levels in the bone marrow of patients with acute lymphoblastic leukemia during asparaginase therapy. *Pediatr. Blood Cancer* **60,** 258–61 (2013).

12. Steiner, M. *et al.* Asparagine and aspartic acid concentrations in bone marrow versus peripheral blood during Berlin–Frankfurt–Münster-based induction

therapy for childhood acute lymphoblastic leukemia. *Leuk. Lymphoma* **53,** 1682–7 (2012).

13. Asselin, B. L. *et al.* In vitro and in vivo killing of acute lymphoblastic leukemia cells by L-asparaginase. *Cancer Res.* **49,** 4363–8 (1989).

14. Willems, L. *et al.* Inhibiting glutamine uptake represents an attractive new strategy for treating acute myeloid leukemia. *Blood* **122,** 3521–32 (2013).

15. Avramis, V. I. Asparaginases: biochemical pharmacology and modes of drug resistance. *Anticancer Res.* **32,** 2423–37 (2012).

16. Egler, R. A., Ahuja, S. P. & Matloub, Y. L-asparaginase in the treatment of patients with acute lymphoblastic leukemia. *J. Pharmacol. Pharmacother.* **7,** 62–71 (2016).

17. Emadi, A., Zokaee, H. & Sausville, E. A. Asparaginase in the treatment of non-ALL hematologic malignancies. *Cancer Chemother. Pharmacol.* **73,** 875–83 (2014).

18. Jacque, N. *et al.* Targeting glutaminolysis has antileukemic activity in acute myeloid leukemia and synergizes with BCL-2 inhibition. *Blood* **126,** 1346–56 (2015).

19. Matre, P. *et al.* Inhibiting glutaminase in acute myeloid leukemia: metabolic dependency of selected AML subtypes. *Oncotarget* **7,** 79722–35 (2016).

20. Zwaan, C. M. *et al.* Cellular drug resistance profiles in childhood acute myeloid leukemia: differences between FAB types and comparison with acute lymphoblastic leukemia. *Blood* **96,** 2879–86 (2000).

21. Okada, S. *et al.* In vitro efficacy of l-asparaginase in childhood acute myeloid leukaemia. *Br. J. Haematol.* **123,** 802–9 (2003).

22. Bertuccio, S. N. *et al.* Identification of a cytogenetic and molecular subgroup of acute myeloid leukemias showing sensitivity to L-Asparaginase. *Oncotarget* **8,** 109915–23 (2017).

23. Iwamoto, S., Mihara, K., Downing, J. R., Pui, C. H. & Campana, D. Mesenchymal cells regulate the response of acute lymphoblastic leukemia cells to asparaginase. *J. Clin. Invest.* **117,** 1049–57 (2007).

24. Ehsanipour, E. A. *et al.* Adipocytes cause leukemia cell resistance to L-asparaginase via release of glutamine. *Cancer Res.* **73,** 2998–3006 (2013).

25. van der Meer, L. T. *et al.* In vivo imaging of antileukemic drug asparaginase

reveals a rapid macrophage-mediated clearance from the bone marrow. *J. Nucl. Med.* **58,** 214–20 (2017).

26. Patel, N. *et al.* A dyad of lymphoblastic lysosomal cysteine proteases degrades the antileukemic drug L-asparaginase. *J. Clin. Invest.* **119,** 1964–73 (2009).

27. Strefford, J. C. *et al.* Complex genomic alterations and gene expression in acute lymphoblastic leukemia with intrachromosomal amplification of chromosome 21. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 8167–72 (2006).

28. Pabst, C. *et al.* Identification of small molecules that support human leukemia stem cell activity ex vivo. *Nat. Methods* **11,** 436–42 (2014).

29. Lechman, E. R. *et al.* miR-126 regulates distinct self-renewal outcomes in normal and malignant hematopoietic stem cells. *Cancer Cell* **29,** 214–28 (2016).

30. Pession, A. *et al.* Long-term results of a randomized trial on extended use of high dose L-asparaginase for standard risk childhood acute lymphoblastic leukemia. *J. Clin. Oncol.* **23,** 7161–7 (2005).

31. Faderl, S. *et al.* Augmented hyper-CVAD based on dose-intensified vincristine, dexamethasone, and asparaginase in adult acute lymphoblastic leukemia salvage therapy. *Clin. Lymphoma Myeloma Leuk.* **11,** 54–9 (2011).

32. Ahmed, T. *et al.* High dose cytarabine, mitoxantrone and l-asparaginase (HAMA) salvage for relapsed or refractory acute myeloid leukemia (AML) in the elderly. *Leuk. Res.* **39,** 945–9 (2015).

33. Buaboonnam, J. *et al.* Sequential administration of methotrexate and asparaginase in relapsed or refractory pediatric acute myeloid leukemia. *Pediatr. Blood Cancer* **60,** 1161–4 (2013).

34. Emadi, A. *et al.* Asparaginase Erwinia chrysanthemi effectively depletes plasma glutamine in adult patients with relapsed/refractory acute myeloid leukemia. *Cancer Chemother. Pharmacol.* **81,** 217–22 (2018).

35. Taylor, C. W., Dorr, R. T., Fanta, P., Hersh, E. M. & Salmon, S. E. A phase I and pharmacodynamic evaluation of polyethylene glycol-conjugated L-asparaginase in patients with advanced solid tumors. *Cancer Chemother. Pharmacol.* **47,** 83–8 (2001).

36. Ueno, T. *et al.* Cell cycle arrest and apoptosis of leukemia cells induced by L-asparaginase. *Leukemia* **11,** 1858–61 (1997).

37. Boitano, A. E. *et al.* Aryl hydrocarbon receptor antagonists promote the expansion of human hematopoietic stem cells. *Science* **329,** 1345–8 (2010).

38. Liao, J. *et al.* Regulation of stem-like cancer cells by glutamine through β-catenin pathway mediated by redox signaling. *Mol. Cancer* **16,** 51 (2017).

39. Wang, Y. *et al.* The Wnt/beta-catenin pathway is required for the development of leukemia stem cells in AML. *Science* **327,** 1650–3 (2010).

40. Laranjeira, A. B. *et al.* IGFBP7 participates in the reciprocal interaction between acute lymphoblastic leukemia and BM stromal cells and in leukemia resistance to asparaginase. *Leukemia* **26,** 1001–11 (2012).

41. Kim, J. A. *et al.* Microenvironmental remodeling as a parameter and prognostic factor of heterogeneous leukemogenesis in acute myelogenous leukemia. *Cancer Res.* **75,** 2222–31 (2015).

42. Matsunaga, T. *et al.* Interaction between leukemic-cell VLA-4 and stromal fibronectin is a decisive factor for minimal residual disease of acute myelogenous leukemia. *Nat. Med.* **9,** 1158–65 (2003).

43. Konopleva, M. *et al.* Stromal cells prevent apoptosis of AML cells by up-regulation of anti-apoptotic proteins. *Leukemia* **16,** 1713–24 (2002).

44. Takagaki, K. *et al.* cDNA microarray analysis of altered gene expression in Ara-C-treated leukemia cells. *Biochem. Biophys. Res. Commun.* **309,** 351–8 (2003).

45. van der Meer, L. T. *et al.* A germ line mutation in cathepsin B points toward a role in asparaginase pharmacokinetics. *Blood* **124,** 3027–9 (2014).

46. Rakashanda, S., Rana, F., Rafiq, S., Masood, A. & Amin, S. Role of proteases in cancer: a review. *Biotechnol. Mol. Biol. Rev.* **7,** 90–101 (2012).

47. Gocheva, V. *et al.* IL-4 induces cathepsin protease activity in tumor-associated macrophages to promote cancer growth and invasion. *Genes Dev.* **24,** 241–55 (2010).

48. Shree, T. *et al.* Macrophages and cathepsin proteases blunt chemotherapeutic response in breast cancer. *Genes Dev.* **25,** 2465–79 (2011).

49. Jain, M., Bakhshi, S., Shukla, A. A. & Chauhan, S. S. Cathepsins B and L in peripheral blood mononuclear cells of pediatric acute myeloid leukemia: potential poor prognostic markers. *Ann. Hematol.* **89,** 1223–32 (2010).

50. Dübbers, A. *et al.* Asparagine synthetase activity in paediatric acute

leukaemias: AML-M5 subtype shows lowest activity. *Br. J. Haematol.* **109,** 427–9 (2000).

51.   Olson, O. C. & Joyce, J. A. Cysteine cathepsin proteases: regulators of cancer progression and therapeutic response. *Nat. Rev. Cancer* **15,** 712–29 (2015).

52.   Offman, M. N. *et al.* Rational engineering of L-asparaginase reveals importance of dual activity for cancer cell toxicity. *Blood* **117,** 1614–21 (2011).

53.   Maggi, M. *et al.* A protease-resistant Escherichia coli asparaginase with outstanding stability and enhanced anti-leukaemic activity in vitro. *Sci. Rep.* **7,** 14479 (2017).

54.   Michelozzi, I. M. *et al.* Human aplastic anaemia-derived mesenchymal stromal cells form functional haematopoietic stem cell niche in vivo. *Br. J. Haematol.* **179,** 669–73 (2017).

55.   Podhorecka, M. *et al.* Resveratrol increases rate of apoptosis caused by purine analogues in malignant lymphocytes of chronic lymphocytic leukemia. *Ann. Hematol.* **90,** 173–83 (2011).

Chapter 4

Fludarabine as a cost-effective adjuvant to enhance engraftment of human normal and malignant haematopoiesis in immunodeficient mice

A. Pievani[1,2], **I. M. Michelozzi**[1], B. Rambaldi[1], V. Granata[1], A. Corsi[3], F. Dazzi[2], A. Biondi[1] and M. Serafini[1]

[1]M. Tettamanti Research Centre, Department of Paediatrics, University of Milano-Bicocca, Monza, 20900, Italy. [2]Department of Haemato-Oncology, Rayne Institute, King's College London, London, SE59NU, UK. [3]Department of Molecular Medicine, Sapienza University of Rome, Rome, 00161, Italy. E-mail: serafinim72@gmail.com

Abstract

There is still an unmet need for xenotransplantation models that efficiently recapitulate normal and malignant human haematopoiesis. Indeed, there are a number of strategies to generate humanised mice and specific protocols, including techniques to optimise the cytokine environment of recipient mice and drug alternatives or complementary to the standard conditioning regimens, that can be significantly modulated. Unfortunately, the high costs related to the use of sophisticated mouse models may limit the application of these models to studies that require an extensive experimental design. Here, using an affordable and convenient method, we demonstrate that the administration of fludarabine (Fludara^{TM}) promotes the extensive and rapid engraftment of human normal haematopoiesis in immunodeficient mice. Quantification of human CD45$^+$ cells in bone marrow revealed approximately a 10^2-fold increase in mice conditioned with irradiation plus fludarabine. Engrafted cells in the bone marrow included haematopoietic stem cells, as well as myeloid and lymphoid cells. Moreover, this model proved to be sufficient for robust reconstitution of malignant myeloid haematopoiesis, permitting primary acute myeloid leukaemia cells to engraft as early as 8 weeks after the transplant. Overall, these results present a novel and affordable model for engraftment of human normal and malignant haematopoiesis in immunodeficient mice.

Introduction

In the 2000s, various immunodeficient models were developed by combining the *Il2rg^{null}* gene with conventional *Prkdc^{scid}* and *Rag1/2^{null}*

mutations. These strains showed high levels of engraftment and differentiation of human haematopoietic progenitor cells, leading to remarkable advances in the development of human disease models.[1] Nevertheless, humanised mouse models are still under development, and various protocols have been established to improve human cell engraftment, in terms of rate, endurance, and function. Techniques to achieve higher levels of human cell engraftment at earlier time points include the identification of: 1) optimal sources of stem cells, 2) route of donor cell administration, 3) methods to modulate the cytokine environment of recipient mice, and 4) drug alternatives or complementary to the standard conditioning regimens. Furthermore, the identification of the factors responsible for a better engraftment of malignant human haematopoiesis, in particular, acute myeloid leukaemia (AML) samples derived from patients, would be highly desirable to improve the recapitulation of the disease.[2]

In recent years, fludarabine has been used as a single agent or in combination with other drugs in the conditioning regimen before allogeneic stem cell transplantation.[3–7] This nucleoside analogue is also well known for its immunosuppressive properties, independently of its incorporation into DNA, which results in leuko- and lymphopenia in patients.[8] Notably, it has been shown that the fludarabine-induced immunosuppression is associated with the inhibition of the cytokine-induced activation of STAT1 and STAT1-dependent gene transcription in normal resting or activated lymphocytes.[9] Fludarabine could have also a role within the bone/marrow microenvironment since it has been demonstrated that this drug significantly increases bone formation in a heterotopic ossification model and promotes osteoclastogenesis.[10,11]

Despite numerous clinical studies in human haematopoietic stem cell transplantation, there are inadequate studies on the cytotoxic activity of fludarabine in a limited number of animal models. Within the context of bone marrow (BM) transplantation, fludarabine has been mainly administered in graft-versus-host disease mouse models.[12–14]

In our study, we have investigated whether the addition of fludarabine to irradiation in the conditioning regimen of a xenotransplantation mouse model would make recipients more permissive for the engraftment of normal and malignant human cells.

Results

Fludarabine enables efficient human cell reconstitution

We decided to adopt the SCID-beige mouse model based on the fact that if mice are conditioned with a sublethal dose of irradiation, they exhibit low levels of human engraftment.[15] Fludarabine was injected intraperitoneally in mice that were previously irradiated with 250 cGy. Two days later, human cord blood (CB)-derived CD34$^+$ cells (hCD34$^+$) were injected intravenously (Fig. 1A). Mice irradiated with the same dose and transplanted with hCD34$^+$ cells derived from the same CB donor but not receiving fludarabine were used as controls.

We determined the toxicities of these two conditioning regimens by measuring survival, body weight and the blood counts. The addition of fludarabine did not significantly worsen the survival rate of the treated mice over 6 weeks compared to control mice (P=0.74), but caused a substantial reduction in body weight only at early points after

conditioning. Otherwise, all blood parameters were comparable (Fig. 1C, D).

The addition of fludarabine to irradiation resulted in a larger extent of human engraftment, when compared to the control group receiving only irradiation (Fig. 1B). Quantification of human CD45$^+$ (hCD45$^+$) cells in BM at 6 weeks after transplantation revealed a significant difference between the two groups both in proportion (median 58.3% in irr+fluda, range from 10.49 to 90.34%; 0.64% in irr, range from 0.11 to 4.60%; P=0.0011) and absolute numbers (median 7.4x10^6 in irr+fluda, range from 2.25 to 13.6x10^6; 0.06x10^6 in irr, range from 0.01 to 0.30x10^6; P=0.0015) (Fig. 1E). A similar difference in the levels of human cell engraftment was also observed in spleen and peripheral blood (PB), in which in the presence of fludarabine the increase was 17- and 96-fold higher, respectively. BM analysis showed human engraftment to be multilineage, consisting not only of myeloid (CD33$^+$) but also B lymphoid (CD19$^+$) cells (Fig. 1F). Fludarabine by itself was not sufficient to increase human engraftment, resulting in engraftment levels significantly lower than those obtained with the combination of fludarabine and irradiation (data not shown).

In order to understand whether the synergistic effect of fludarabine was the result of remodelling of the haematopoietic niche[10,11] or an immunosuppressive effect, we tested the approach in a syngeneic murine transplantation model, using donor-recipient pair congenic for a CD45 polymorphism (Fig. 1G). The addition of fludarabine did not change the engraftment levels obtained with irradiation only both at 4 and 6 weeks post-transplantation, also after administrating very few donor cells, thus suggesting that the fludarabine effect should be related

to its immunosuppressive effect rather than an activity on the BM microenvironment. Moreover, we proved that the capacity of murine splenocytes to proliferate after *in vitro* stimulation with concanavalin A was markedly inhibited by fludarabine (Supplementary Fig. S1).

Notably, the administration of fludarabine promoted an early engraftment of human haematopoiesis in transplanted mice, in which it was possible to observe a detectable percentage of human cells in BM since the second week after transplant (median 0.23% hCD45$^+$ cells, range from 0.11 to 1.78%) (Fig. 1H). The long-term reconstitution induced by fludarabine was durable, since we could still detect the presence of haematopoietic precursors CD34$^+$ 12 weeks after transplantation (Fig. 1I).
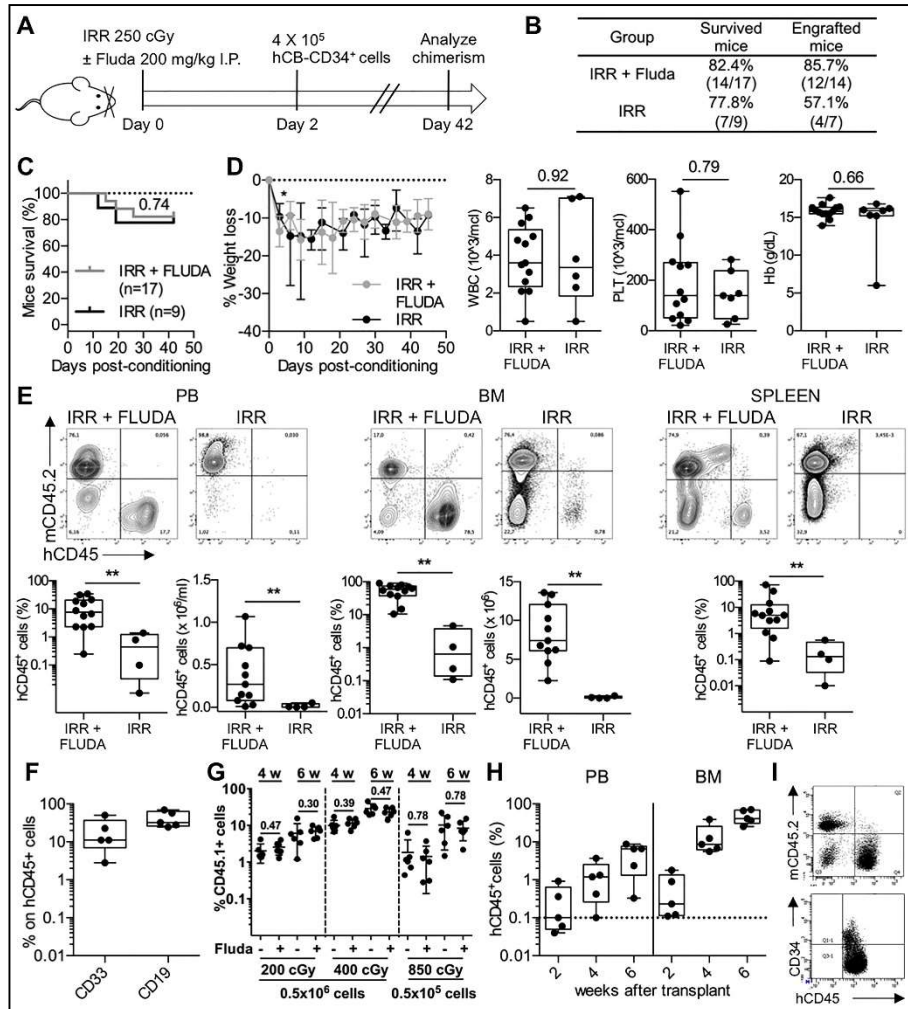
Fig. 1. Fludarabine enables efficient human cell reconstitution.

(A) Representation of experimental outline: irradiation (250 cGy) and 200 mg/kg fludarabine treatment of SCID-beige mice followed by transplantation of hCD34⁺ cells. Where not otherwise specified, mice were euthanised 6 weeks (day 42) after treatment to analyse chimerism in haematopoietic organs. Mice receiving only irradiation constitute the control group. (B) Percentages of engrafted mice that survived (defined by more than 0.1% hCD45 cells within BM) in the two experimental groups at the fixed end-point. 10 independent experiments (2-4 mice/experiment). (C) Kaplan-Meier curve of overall

survival of mice treated with irradiation+fludarabine (grey line) versus irradiation (black line). P-value by log-rank Mantel-Cox test. 10 independent experiments (2-4 mice/experiment). (D) SCID-beige mice receiving irr+fluda or irr were weighed over 6 weeks following the conditioning procedure (on left) and their blood was collected at sacrifice for testing white blood cells (WBC), platelets (PLT) and haemoglobin (Hb) (on right). Percent change in body weight is represented as mean and standard deviation. Blood parameters are represented by boxplot graph, showing the exact data values by black dots. P-values by Wilcoxon test. 10 independent experiments (2-4 mice/experiment). (E) Human engraftment in irr+fluda treated or irr SCID-beige mice. On the top, representative dot plots showing hCD45$^+$ cells in PB, BM and spleen 6 weeks after transplantation. On the bottom, proportion and absolute numbers of hCD45$^+$ cells in the same groups. 10 independent experiments (2-4 mice/experiment). (F) Relative proportion of myeloid (CD33$^+$) and B lymphoid (CD19$^+$) cells within the human graft in irr+fluda mice at 6 weeks. 2 independent experiments (2-3 mice/experiment). (G) Frequency of donor haematopoiesis in PB 4 and 6 weeks after transplantation of mice irradiated (200, 400 or 850 cGy), treated or not with fludarabine and injected with 0.5 or 0.05x10^6 BM cells of congenic mice. 2 independent experiments (3 mice/group in each experiment). (H) Levels of human chimerism analysed in PB and BM of recipient mice at 2, 4 and 6 weeks after conditioning with irr+fluda. 2 independent experiments (2-3 mice/experiment). (I) Long-term human engraftment (at 12 weeks) in irr+fluda treated mice and presence of hCD34$^+$ precursors.

Fludarabine promotes AML engraftment

We applied the conditioning regimen to evaluate the effect on the engraftment of acute myeloid leukaemia (AML) that is notoriously difficult to engraft in SCID models. Also in this case, the addition of

fludarabine to irradiation could favour the engraftment of the AML cell line KG-1. The presence of high percentages of AML cells within BM (median 78.91%, range from 66.24 to 97.59%) was accompanied by signs of distress, including weakness, weight loss, hunched back, loss of ambulation, laboured breathing and paralysis, and mice were humanely killed within 3 weeks when transplanted with $2x10^6$ KG-1 cells/mouse (Fig. 2A, B). Using the same conditioning strategy, it was possible to transplant lower numbers of cells ($5x10^5$ or $2x10^5$/mouse) obtaining dose-dependent sustained engraftment levels, with a longer overall survival (from 21 to 29 days) (Fig. 2C).
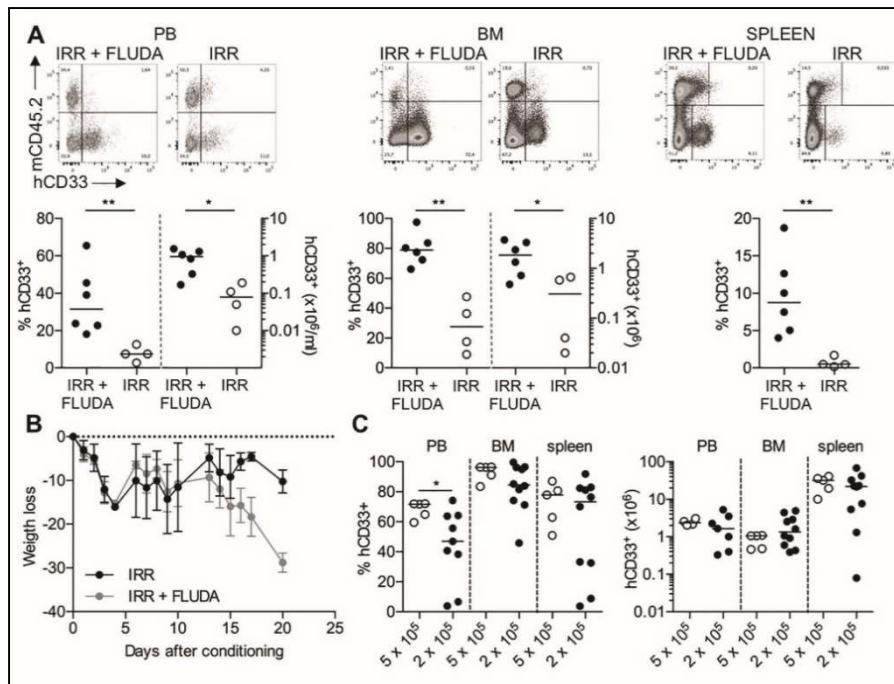
Fig. 2. Fludarabine promotes AML cell line engraftment.

(A) SCID-beige mice were intravenously injected with $2x10^6$ cells from the established human myeloid cell line KG-1 on day 2 post conditioning (irr+fluda or irr). Mice were euthanised for the engraftment assessment in PB, BM and spleen when they developed signs of overt leukaemia. Upper panels, exemplary data from one representative experiment; lower panels, presence (as percentages and numbers) of $hCD33^+$ cells in PB, BM and spleen of transplanted mice. 2 independent experiments (2-3 mice/group in each experiment). (B) Weight loss in SCID-beige mice conditioned with irr+fluda (grey line) or irr (black line) and transplanted with KG-1 cells. 2 independent experiments (2-3 mice/group in each experiment). (C) SCID-beige mice were treated with irr+fluda and injected with two different doses ($5x10^5$ or $2x10^5$ cells/mouse) of KG-1 cells. At euthanasia, the engraftment level in PB, BM and spleen was assessed by flow cytometry as a proportion (on left) or as absolute numbers (on right). 3 independent experiments (3-6 mice/group in each experiment).

Finally, we evaluated if this preconditioning strategy could sustain the engraftment of primary AML blasts derived from 8 patients with various genetic backgrounds, including *NPM1* mutated, *FLT3* mutated, *NPM1/FLT3* mutated and wild type (Fig. 3A). Strikingly, primary AML samples injected in fludarabine-preconditioned mice produced a detectable engraftment in 50% of transplanted AML cases, within the first 8 weeks after transplantation. Leukaemic cells infiltrated BM (range from 1.5 to 71.5% $hCD45^+hCD33^+$, evaluated at 8 weeks) and haematopoietic organs (spleen, range from 0.8 to 7.6% $hCD45^+hCD33^+$ and PB, range from 1.4 to 19.6% $hCD45^+hCD33^+$, evaluated at 14 weeks), the primary sites of clinical AML (Fig. 3B, C). Flow cytometry data correlated with immunohistochemistry analysis (Fig. 3C).
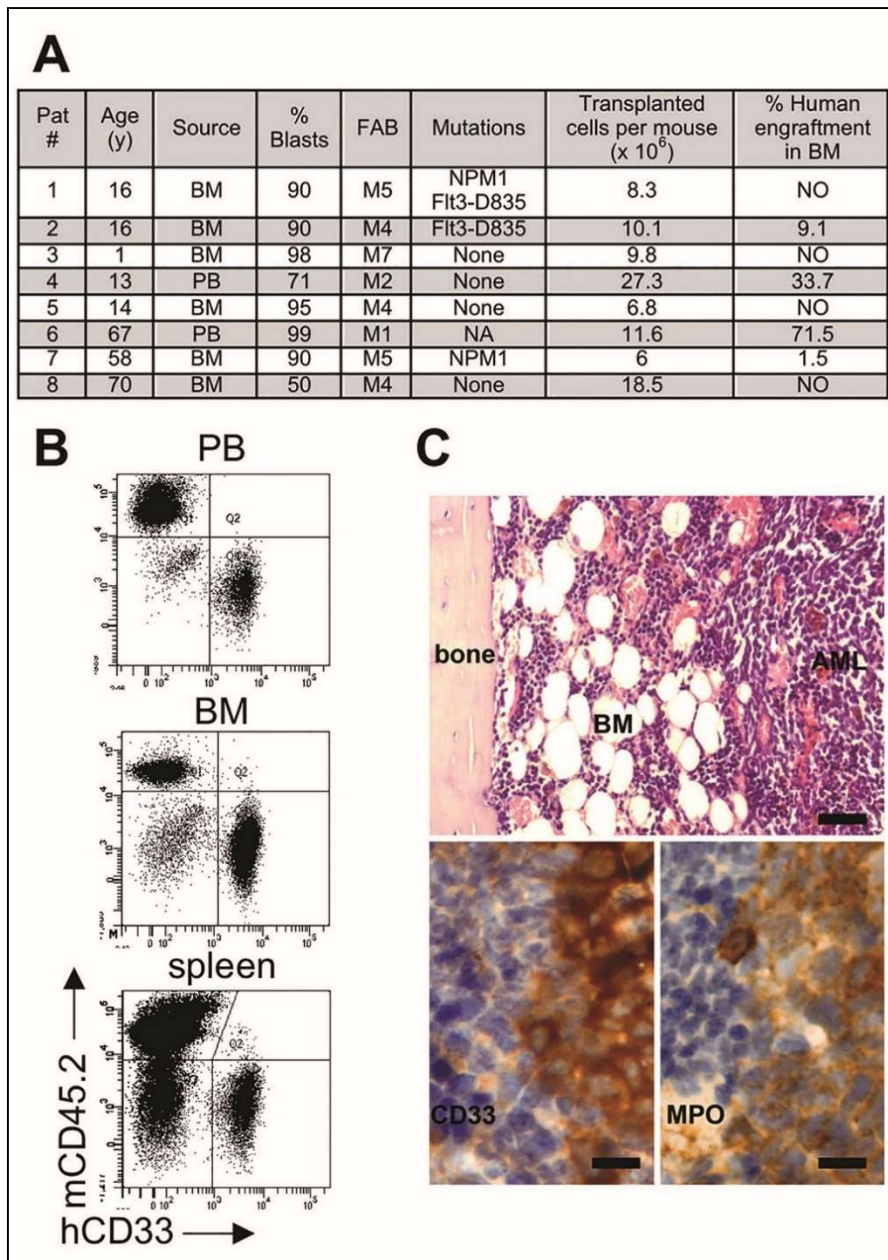
Fig. 3. Fludarabine permits engraftment of primary AML blasts.

(A) Clinical characteristics of AML patients (age, source and percentage of blasts, FAB classification, NPM1 and FLT3 mutational status) and engraftment details (transplanted cell dosage and % human engraftment

detected in BM at final analysis) following transplantation in SCID-beige mice conditioned with irr+fluda. NA, not analysed. 8 independent experiments. (B) Representative dot plots showing hCD33$^+$ blasts in PB, BM and spleen of SCID-beige mice previously treated with irr+fluda (patient #6). (C) Representative paraffin sections from the same mouse (patient #6) stained with haematoxylin and eosin (top panel) to show human blasts infiltrating the BM. The infiltrating blasts display a myeloid phenotype as proved by their immunoreactivity with anti-hCD33 and MPO antisera.

Bars: 100 μm in the top panel and 20 μm in the bottom panels.

Discussion

In recent years, fludarabine has been adopted as a single agent or in combination with other drugs for the treatment of various haemato-oncologic malignancies and has been shown to be particularly effective in indolent lymphoproliferative disorders, predominantly chronic lymphocytic leukaemia (CLL) and follicular lymphoma. Furthermore, combination regimens containing fludarabine have also been administered in the treatment of aggressive lymphomas and acute leukaemias. The inclusion of fludarabine in the conditioning regimen of allogeneic stem cell transplantation was initially proposed for its remarkable immunosuppressive activities.[8] Most recently, due to its synergistic cytotoxic activity against both myeloid and lymphoid malignancies when used in combination with alkylating agents or radiotherapy, it has become a standard of treatment in allogeneic stem cell transplantation.[4–7] Indeed, the use of fludarabine-containing regimens has modified the incidence and the degree of graft-versus-host disease (GVHD) in these patients.

In experimental BM transplantation, fludarabine has been mainly administered in graft-versus-host disease.[12–14] To date, no study has addressed the ability of fludarabine to favour the engraftment of normal and malignant haematopoiesis in a mouse model. Our data convincingly show that the administration of fludarabine in irradiated mice renders SCID-beige mice excellent recipients not only for normal human haematopoietic cells, but also for the leukaemic counterparts.

The *in vivo* kinetics of fludarabine suggest to wait at least 48 hours before infusing haematopoietic stem cells to enable the complete clearance of the drug and potential toxic effects on the graft.[3]

Therefore, in our hands fludarabine can be exploited to obtain a fast and durable human engraftment in low-care immunodeficient strains. The mechanism of this phenomenon could be ascribed to the fludarabine-induced immunosuppression associated with the cytotoxic potential against lymphocytes due to the inhibition of STAT1 signalling.[9] The fact that fludarabine does not enhance the engraftment level in a congenic model seems to support this hypothesis. The inhibition of mitogen-induced murine splenocyte proliferation by fludarabine supports the hypothesis that it may control the host lymphocyte leakage that interferes with the human graft.[16] SCID-beige mice are congenic mice that possess both genetic autosomal recessive mutations *scid* (*Prkdc^{scid}*) and *beige* (*Lyst^{bg}*). The *beige* mutation results in defective NK cells. The *scid* mutation results in deficiency in V(D)J recombination, producing severe lymphopenia but not absolute absence of T and B cells. Indeed, through the incomplete penetrance ("leakiness") of the *scid* mutation, occasional productive V(D)J rearrangement can occur and give rise to clonal expansion of limited T

and B cell clones.[16] A similar leaky phenotype is also described in SCID-beige mice.[17] Leaky SCID lymphocytes can respond to mitogens, are capable of producing cytokines and serum Ig, and may develop reaction to allogeneic tissue.[18] For instance, it is possible that the immunosuppressant drug fludarabine could affect the residual T and B lymphocytes in SCID-beige mice, that may represent a potential interference with graft acceptance.

In summary, we demonstrate that the administration of fludarabine promotes in SCID-beige mice an extensive and durable engraftment of normal human haematopoiesis that exceeds the levels currently achievable in other models. The engraftment comprised haematopoietic stem cells as well as myeloid and lymphoid cells and could be reproduced using cells sourced from myeloid malignancy. The disease phenotype observed in leukaemia-engrafted mice recapitulated the features of the human counterpart. We conclude that fludarabine treatment may represent a tool to maximise normal and malignant xenotransplantation in otherwise inefficient but relatively inexpensive immunocompromised recipients such as SCID-beige mice. The approach could be applied to other existing models of diseases (e.g., SCID models of rare genetic disorders) that remain in need of sufficiently informative levels of human engraftment. Overall, our results provide novel and affordable information for the engraftment of human normal and malignant haematopoiesis in immunodeficient mice.

Methods

Study approval

All animal experiments were performed under license approved by the Italian Ministry of Health and in accordance with Italian Cancer Research guidelines. The use of umbilical cord blood (UCB) and AML samples was approved by the Ethics Committee of San Gerardo Hospital-Monza and carried out in accordance with the Declaration of Helsinki. All samples were only processed from patients who had consented to the use of biological material for ethically approved research.

Cell lines and primary AML samples

Human AML cell line KG-1 (obtained from the ATCC) was maintained in culture, splitting every 2-3 days, in Advanced RPMI medium (Invitrogen) supplemented with 10% heat-inactivated foetal bovine serum (Biosera), 2 mM L-glutamine, 50 IU/mL of penicillin and 50 µg/mL of streptomycin (Lonza).

For primary acute myeloid leukaemia samples, PB or BM samples from adult or paediatric patients were collected at diagnosis. Samples were enriched for mononuclear cells by using a Ficoll-Paque gradient, and subsequently frozen in 10% dimethyl sulfoxide solution (Sigma-Aldrich). Details of patients' samples are provided in Fig. 3.

Xenotransplantation procedures

Animals were used in accordance with a protocol approved by the Italian Ministry of Health. Adult (10-12 weeks old) SCID-beige

(CB17.Cg-*Prkdc^scidLyst^bg-J*/Crl) mice purchased from Charles River Laboratories (Calco, Italy) were sublethally irradiated (250 cGy) and treated with 200 mg/kg fludarabine (Teva) by intraperitoneal administration, 48 hours before intravenous injection of human cells.

For normal reconstitution, CD34$^+$ progenitors from human CB were obtained by Ficoll-Paque Plus (GE Healthcare Europe) separation of the mononuclear fraction followed by immunomagnetic selection using the CD34 MicroBeads kit (Miltenyi Biotec). 10 independent hCD34$^+$ batches were used for transplantation experiments, with an average purity of 81% (range: 70-95.2%).

For leukaemic reconstitution, cultured AML cell line KG-1 or freshly thawed PB/BM samples from patients with AML were used for transplants.

Daily monitoring of mice for symptoms of disease (ruffled coat, hunched back, weakness and reduced motility) determined the time of euthanasia for injected animals with signs of distress.

Engraftment evaluation

Human engraftment (defined as more than 0.1% human cells in murine BM) was assessed in PB, BM and spleen at defined time points (in the case of normal haematopoietic cell transplant) or at signs of distress (in the case of AML cell line KG-1 transplant). Engraftment of human cells (in the case of primary patient-derived AML cells) was evaluated in BM at 8 weeks after transplantation (analysing femoral BM aspirates) and in spleen and PB at the time of euthanasia (14 weeks). Kaplan-Meier survival analysis and body weight assessment were performed on all animals.

For engraftment evaluation, 50 µL of PB were collected in heparin by tail bleeding, analysed with haematology counter (Coulter AcT Diff, Beckman Coulter), and lysed with ACK (Ammonium-Chloride-Potassium) lysing buffer (StemCell Technologies). BM (mixed from tibiae and femora) was collected by flushing long bones, while splenocytes were collected by smashing spleen on a 70 µm cell strainer (Greiner Bio-One). Single cell suspensions were counted in Bürker chamber with Turk solution and processed for analysis by flow cytometry.

Flow cytometry and histopathology

For flow cytometry analyses, fluorescent antibodies against murine CD45.2 (clone 104, eBioscience) and against human CD45 (clone HI30), CD33 (clone P67.6), CD34 (clone 8G12), CD19 (clone SJ25C1) (BD Biosciences), and CD45 (clone HI30, Invitrogen) were used. The analyses were performed on a FACS CantoII (BD Biosciences).

Humeri of fludarabine-treated SCID-beige mice transplanted with primary human AML blasts were fixed in 4% formaldehyde in phosphate buffer, decalcified in 10% EDTA (Sigma-Aldrich) and routinely processed for paraffin embedding. Serial 5 µm-thick sections were stained with haematoxylin-eosin and stained with anti-human CD33 (#NCL-L-CD33, clone PWS44, 1:100; Novocastra™) and myeloperoxidase (#NCL-MYELO, clone 59A5, 1:100, Novocastra™) antisera.

Murine BM transplantation

For murine BM transplantation experiments, 12 weeks old C57BL/6-CD45.2 mice were conditioned by irradiation alone or followed by injection of fludarabine and BM cells from congenic C57BL/6-CD45.1 mice were transplanted by a single intravenous injection within 48 hours from conditioning. In one experimental setting mice received a sublethal irradiation (200 or 400 cGy) with the infusion of $0.5x10^6$ donor cells/mouse and in the other one an irradiation of 850 cGy with $0.05x10^6$ donor cells/mouse.

For engraftment evaluation, 50 µL of PB were collected 4 and 6 weeks after transplant in heparin by tail bleeding, lysed with ACK buffer and stained with antibodies against murine CD45.1 (clone A20, eBioscience) and CD45.2 (clone 104, eBioscience).

Immunosuppressive assay

$5x10^5$ C57BL/6 murine splenocytes were labelled with PE-Cell Tracker (Thermo Fisher Scientific), plated in the presence or in the absence of fludarabine (2.5 µg/mL) for 24 hours, and then stimulated with ConA (3 µg/mL). Controls consisted of splenocytes plated without ConA. Proliferation was assessed by flow cytometry after 72 hours. Cell counts were determined by adding CountBright absolute counting beads (Molecular Probe) to the flow cytometric samples.

Statistical analysis

Unless otherwise stated, data are represented as median and range. Nonparametric Wilcoxon test for equality of the medians was used to

calculate P-values. Significance is represented as follows: *P<0.05, **P<0.01, ***P<0.001.

<u>Acknowledgements</u>

<u>Author contributions</u>

A.P. performed research, analysed the data and wrote the manuscript; I.M.M., B.R. and V.G. performed research; A.C. designed research and interpreted the data; F.D. and A.B. interpreted the data and edited the manuscript; M.S. designed research, interpreted the data, and wrote the manuscript.
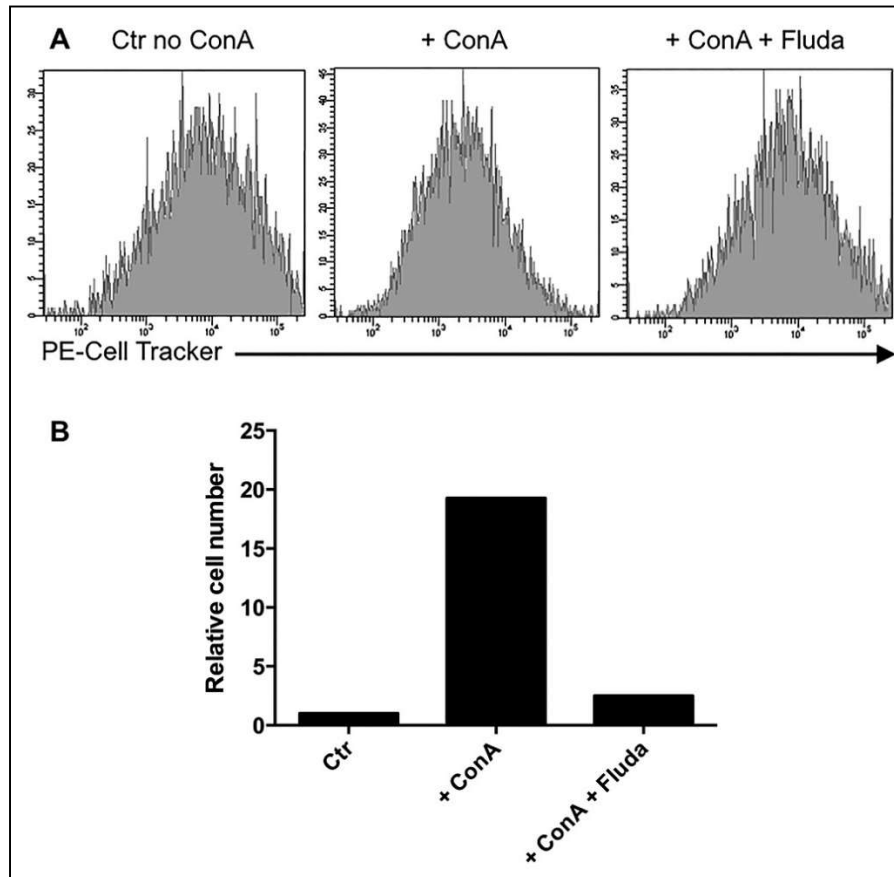
Supplementary information

Fig. S1. Treatment of C57BL/6 murine splenocytes with fludarabine leads to inhibition of mitogen-induced proliferation.

Splenocytes labelled with PE-Cell Tracker were treated or not with fludarabine (2.5 μg/mL) and cultured with or without concanavalin A (ConA) for 72 hours. Proliferation and cell number were determined by flow cytometry analysing the Cell Tracker dilution and using the single-platform method. (A) Representative histogram plots were shown. (B) Relative cell number was expressed in comparison to control (splenocytes not treated with fludarabine and not stimulated with ConA).

Competing interests

The authors declare no competing interests.

References

1.   Goyama, S., Wunderlich, M. & Mulloy, J. C. Xenograft models for normal and malignant stem cells. *Blood* **125**, 2630–40 (2015).

2.   McCormack, E., Bruserud, O. & Gjertsen, B. T. Animal models of acute myelogenous leukaemia – development, application and future perspectives. *Leukemia* **19**, 687–706 (2005).

3.   Aversa, F. *et al.* Treatment of high-risk acute leukemia with T-cell-depleted stem cells from related donors with one fully mismatched HLA haplotype. *N. Engl. J. Med.* **339**, 1186–93 (1998).

4.   Andersson, B. S. *et al.* Once daily i.v. busulfan and fludarabine (i.v. Bu-Flu) compares favorably with i.v. busulfan and cyclophosphamide (i.v. BuCy2) as pretransplant conditioning therapy in AML/MDS. *Biol. Blood Marrow Transplant.* **14**, 672–84 (2008).

5.   Niederwieser, D. *et al.* Low-dose total body irradiation (TBI) and fludarabine followed by hematopoietic cell transplantation (HCT) from HLA-matched or mismatched unrelated donors and postgrafting immunosuppression with cyclosporine and mycophenolate mofetil (MMF) can induce durable complete chimerism and sustained remissions in patients with hematological diseases. *Blood* **101**, 1620–9 (2003).

6.   Rambaldi, A. *et al.* Busulfan plus cyclophosphamide versus busulfan plus fludarabine as a preparative regimen for allogeneic haemopoietic stem-cell transplantation in patients with acute myeloid leukaemia: an open-label, multicentre, randomised, phase 3 trial. *Lancet Oncol.* **16**, 1525–36 (2015).

7.   Khouri, I. F. *et al.* Nonablative allogeneic hematopoietic transplantation as adoptive immunotherapy for indolent lymphoma: low incidence of toxicity, acute graft-versus-host disease, and treatment-related mortality. *Blood* **98**, 3595–9 (2001).

8. Terenzi, A. *et al.* Efficacy of fludarabine as an immunosuppressor for bone marrow transplantation conditioning: preliminary results. *Transplant. Proc.* **28**, 3101 (1996).

9. Frank, D. A., Mahajan, S. & Ritz, J. Fludarabine-induced immunosuppression is associated with inhibition of STAT1 signaling. *Nat. Med.* **5**, 444–7 (1999).

10. Tajima, K. *et al.* Inhibition of STAT1 accelerates bone fracture healing. *J. Orthop. Res.* **28**, 937–41 (2010).

11. Furukawa, M. *et al.* IL-27 abrogates receptor activator of NF-kappa B ligand-mediated osteoclastogenesis of human granulocyte-macrophage colony-forming unit cells through STAT1-dependent inhibition of c-Fos. *J. Immunol.* **183**, 2397–406 (2009).

12. Giver, C. R. *et al.* Ex vivo fludarabine exposure inhibits graft-versus-host activity of allogeneic T cells while preserving graft-versus-leukemia effects. *Biol. Blood Marrow Transplant.* **9**, 616–32 (2003).

13. Weiss, L., Abdul-Hai, A., Or, R., Amir, G. & Polliack, A. Fludarabine in combination with cyclophosphamide decreases incidence of GVHD and maintains effective graft-versus-leukemia effect after allogeneic stem cell transplantation in murine lymphocytic leukemia. *Bone Marrow Transplant.* **31**, 11–5 (2003).

14. Luznik, L., Jalla, S., Engstrom, L. W., Iannone, R. & Fuchs, E. J. Durable engraftment of major histocompatibility complex-incompatible cells after nonmyeloablative conditioning with fludarabine, low-dose total body irradiation, and posttransplantation cyclophosphamide. *Blood* **98**, 3456–64 (2001).

15. Kirkiles-Smith, N. C. *et al.* Development of a humanized mouse model to study the role of macrophages in allograft injury. *Transplantation* **87**, 189–97 (2009).

16. Carroll, A. M., Hardy, R. R. & Bosma, M. J. Occurrence of mature B (IgM+, B220+) and T (CD3+) lymphocytes in scid mice. *J. Immunol.* **143**, 1087-93 (1989).

17. Mosier, D. E., Stell, K. L., Gulizia, R. J., Torbett, B. E. & Gilmore, G. L. Homozygous scid/scid;beige/beige mice have low levels of spontaneous or neonatal T cell-induced B cell generation. *J. Exp. Med.* **177**, 191-4 (1993).

18.   Carroll, A. M., & Bosma, M. J. Detection and characterization of functional T cells in mice with severe combined immune deficiency. *Eur. J. Immunol.* **18**, 1965-71 (1988).

Chapter 5

Summary, conclusions and future perspectives

The haematopoietic stem cell (HSC) niche is the physiological site in which HSC reside and in which haematopoiesis is strictly controlled.[1] Among the haematopoietic regulators in the bone marrow (BM) niche, mesenchymal stromal cells (MSC) play a critical role.[2]

In pathological conditions, MSC can be altered and support leukaemic cells and leukaemic stem cells (LSC) providing a chemoprotective milieu.[3,4]

Thus, the study of BM-MSC is crucial to better understand the pathophysiology and to ameliorate the current therapy of haematological disorders.

The investigation of the properties and functionality of these cells needs to integrate *in vitro* with *in vivo* assays to overcome *in vitro* limitations (heterogeneity of cell culture) and artificiality (influence of culture conditions on cell content and fate) to better approach reality and physiological states.[5,6]

In this PhD project, we studied the role of the BM microenvironment, especially of BM-MSC, in the pathogenesis and in the chemoresistance of two pathologies, aplastic anaemia (AA) and acute myeloid leukaemia (AML).

In AA, it is still unclear if BM-MSC alterations can be the cause or the consequence of the disease.[3,7] AA-MSC were characterised in few studies reporting contradictory results, probably due to the heterogeneity of patient cohorts, in terms of age and severity of the disease.[8]

Thus, deeper analyses are required and, especially, it is necessary to create an appropriate *in vivo* AA model to appreciate the role of the BM microenvironment in AA pathogenesis in itself. In fact, current *in vivo*

AA models develop the disease after chemical and drug exposure or following an immune-related approach,[9] and they do not allow this evaluation.

Considering AML, it is essential to improve its treatments as patient outcome is characterised by elevated rate of relapse and poor overall survival after therapy. Indeed, the lack of eradication of LSC, initiators and responsible for relapse, is a problem of current AML therapies.[10] BM-MSC/BM microenvironment protective effect,[4] in addition to LSC intrinsic features (e.g., quiescence and expression of adenosine triphosphate binding cassette [ABC] transporters),[10] is an aspect that must be considered in anti-AML drug studies. Indeed, it is important to examine the effects of promising novel therapeutic agents on leukaemia and primitive cell fractions in relation with other cellular components of the pathological BM niche.

Finally, the study of normal and malignant haematopoiesis, of potential drugs and of BM microenvironment can take advantage of humanised xenograft mouse models.

There are different strategies to boost the human haematopoietic engraftment in immunodeficient mice, from the choice of more permissive mouse strains to the conditioning regimen.

Indeed, in this PhD project, we investigated a new conditioning procedure to achieve better engraftment levels of human normal and, especially, malignant haematopoiesis in an already existing immunodeficient mouse strain. The main goal was to generate a novel AML xenograft model that, if implanted with chondroid pellets produced with human MSC, could be used to study the interaction of blasts with human BM microenvironment in AML.

In the first part of the project, we investigated the role of BM-MSC in the pathogenesis of AA in an innovative and comprehensive way, as shown in our recently published work.[11]

Here, for the first time, we integrated the *in vitro* characterisation of AA-MSC with the use of an *in vivo* BM niche model,[12] trying to overcome the limitations associated with *in vitro* experiments.

In literature, there are conflicting results regarding AA-MSC ability to support haematopoiesis, which has been tested only through *in vitro* coculture.[7,13,14]

In our work, we mainly analysed this function taking advantage of the *in vivo* BM niche model that had been developed, in collaboration with Professor Mara Riminucci (Università Sapienza, Rome), starting from healthy donor (HD)-MSC. It consists in a scaffold-free system, which is based on the *in vitro* differentiation of human MSC into cartilaginous pellets subsequently implanted in SCID-beige mice. The harvested ossicles are perfectly similar to bone/marrow organ, with external cortical bone and inner marrow formed by murine haematopoiesis and sinusoids and human stroma.[12]

In the study of AA, we used this model for the first time to generate a pathological niche starting from patient MSC.

We analysed MSC derived from a homogeneous cohort of newly diagnosed paediatric patients affected by the acquired form of AA and, casually, mainly responsive to immunosuppressive therapy and to HSC transplantation. In our cohort, AA-MSC were unaltered in almost all the characteristics analysed *in vitro*, apart from the reduced clonogenic potential exhibited. The diminished clonogenicity observed could be a

consequence of the immune-mediated destruction, which decreases the number of progenitors,[15] rather than an intrinsic defect of AA-MSC.

The absence of alterations in AA-MSC ability to sustain haematopoiesis was highlighted *in vivo* as these cells could develop a functional (able to support murine haematopoiesis) and architecturally normal BM ossicle, excluding their role in the initiation of the disease.

In the second part of the project, we deeply investigated L-asparaginase (ASNase) effectiveness against AML cells derived from newly diagnosed patients.

ASNase, an enzyme with asparaginase and glutaminase activity, is widely used in the treatment of acute lymphoblastic leukaemia (ALL), but scarcely tested and employed in AML context.[16]

Firstly, we considered the anti-leukaemic effects of this drug on the bulk population and, most importantly, on AML progenitors (clonogenic cells and, especially, $CD34^+CD38^+$ $CD34^+CD38^-$ LSC-enriched fractions). Secondly, we evaluated ASNase toxicity against AML cells in relation with other BM niche cellular components that can regulate LSC sensitivity to chemotherapy.

In our work, ASNase was similarly effective on AML bulk population and on LSC-enriched compartments, while it showed negligible effects on healthy haematopoietic cells. The susceptibility of AML progenitors to the drug was confirmed by clonogenic assay and by experiments performed in LSC supportive culture conditions.[17]

However, in coculture experiments ASNase action on AML bulk and LSC-enriched populations was in part counteracted by both HD- and autologous AML-MSC. The protection exerted by MSC could be

related to their asparagine synthetase (*ASNS*) expression and, consequent, production of asparagine, as demonstrated in ALL.[18]

Other BM niche cell types, monocytes/macrophages, could play a role in the generation of a protective milieu because of their expression of cathepsin B (*CTSB*), a gene encoding for a lysosomal cysteine protease able to degrade ASNase,[19] accelerating drug turnover *in vivo*.[20]

This mechanism of resistance to ASNase could be even more important in AML-M5 subtype as blasts themselves expressed elevated levels of *CTSB* and, therefore, could contribute to ASNase inactivation and degradation.

In the third part of the project, we tested a new conditioning procedure in SCID-beige mice to generate an almost inexpensive but effective mouse model of human normal and, above all, malignant haematopoiesis, as reported in our recently published work.[21]

SCID-beige mice are less permissive for human haematopoiesis as compared to NSG mice and are used in human allograft rejection studies.[22] However, combining irradiation and fludarabine, an immunosuppressive drug used as conditioning regimen in the clinic,[23] we were able to augment the human engraftment level in SCID-beige mice as compared to irradiation only. This was probably due to fludarabine effect on murine leaky lymphocytes.[24]

As a result, we transformed poor permissive mice into more permissive ones for human normal haematopoiesis and AML blast engraftment.

SCID-beige was the mouse strain in which we developed the BM niche model recently described.[12]

For this reason, we would like to combine, in these mice, the implantation of cartilaginous pellets derived from AML-MSC and the transplantation of autologous blasts upon optimised conditioning in order to obtain a completely humanised AML BM microenvironment.

In conclusion, in this PhD thesis project:

- We took advantage of BM niche models (*in vitro* and *in vivo*) to generate and study a pathological BM niche.
  Indeed, we demonstrated the protective role of BM-MSC against ASNase both in AML bulk and in LSC-enriched fractions using *in vitro* coculture experiments.
  Moreover, we used the *in vivo* BM niche model generated by our group to show that AA-MSC were unaltered and able to form a complete BM niche.

- We contributed to demonstrate the anti-neoplastic properties of ASNase in AML context, especially against LSC-enriched populations, one of the ultimate challenges of AML therapy.
  At the same time, we confirmed the mechanism of resistance mediated by *ASNS*-expressing MSC and we hypothesise the involvement of *CTSB*-expressing BM monocytes/macrophages and blasts themselves in ASNase degradation. These considerations must be taken into account by clinicians in the therapeutic use of ASNase in AML patients. For this reason, we suggest the administration of ASNase in combination with drugs able to downregulate *ASNS* expression, as cytarabine, or

with protease inhibitors, or to utilise functional ASNase variants resistant to proteases.

- We developed a robust AML xenograft model in SCID-beige mice, which, if implanted with chondroid pellets derived from human AML-MSC, could find its application in the study of anti-AML drug within AML BM microenvironment.

In the future, we would like to:

- Repeat the experiments of humanised BM niche generation using AA-MSC from patients not responsive to immunosuppression or HSC transplantation to evaluate, in these cases, the characteristics of the BM microenvironment in AA.
- Functionally evaluate the role of CTSB produced by monocytes/macrophages and AML blasts in ASNase degradation through the incubation of cellular lysates with the drug, in the presence or absence of the CTSB-specific inhibitor CA-074, and detection of ammonia release, using Nessler's reagent, or of ASNase cleavage, performing Western Blot analysis.
- Test ASNase in our *in vivo* model of AML (SCID-beige mice conditioned with irradiation and fludarabine). We have to optimise the dose and the timing of drug injections. Preliminary results were not encouraging as we did not appreciate the effect of ASNase on mice injected with the AML cell line KG-1, probably because we began to treat animals too late, when the tumour burden was too elevated. We would like to transplant

animals with primary AML blasts which are less aggressive than the cell line and in which it is much more probable to notice a drug effect.

- Use our *in vivo* BM niche generation protocol also in AML context, in the patient-derived xenograft model described, to recreate a completely humanised BM ossicle with human stromal and haematopoietic compartment.

The ossicles harvested will be analysed for the interactions between BM microenvironment and AML blasts in order to identify potential therapeutic targets.

Moreover, we would like to repeat these experiments in pharmacologically treated mice to evaluate the effect of the drug on AML cell distribution and connections within the niche.

References

1.   Wasnik, S., Tiwari, A., Kirkland, M. A. & Pande, G. Osteohematopoietic stem cell niches in bone marrow. *Int. Rev. Cell Mol. Biol.* **298,** 95–133 (2012).

2.   Dazzi, F., Ramasamy, R., Glennie, S., Jones, S. P. & Roberts, I. The role of mesenchymal stem cells in haemopoiesis. *Blood Rev.* **20,** 161–71 (2006).

3.   Kastrinaki, M. C. *et al.* Mesenchymal stem cells in immune-mediated bone marrow failure syndromes. *Clin. Dev. Immunol.* **2013,** 265608 (2013).

4.   Korn, C. & Méndez-Ferrer, S. Myeloid malignancies and the microenvironment. *Blood* **129,** 811–22 (2017).

5.   Bianco, P., Kuznetsov, S. A., Riminucci, M. & Gehron Robey, P. Postnatal skeletal stem cells. *Methods Enzymol.* **419,** 117–48 (2006).

6.   Bianco, P., Robey, P. G. & Simmons, P. J. Mesenchymal stem cells: revisiting history, concepts, and assays. *Cell Stem Cell* **2,** 313–9 (2008).

7.   Bacigalupo, A. *et al.* T-cell suppression mediated by mesenchymal stem cells is deficient in patients with severe aplastic anemia. *Exp. Hematol.* **33,** 819–27 (2005).

8.   Zeng, Y. & Katsanis, E. The complex pathophysiology of acquired aplastic anaemia. *Clin. Exp. Immunol.* **180,** 361–70 (2015).

9.   Chen, J. Animal models for acquired bone marrow failure syndromes. *Clin. Med. Res.* **3,** 102–8 (2005).

10. Siveen, K. S., Uddin, S. & Mohammad, R. M. Targeting acute myeloid leukemia stem cell signaling by natural products. *Mol. Cancer* **16,** 13 (2017).

11. Michelozzi, I. M. *et al.* Human aplastic anaemia-derived mesenchymal stromal cells form functional haematopoietic stem cell niche in vivo. *Br. J. Haematol.* **179,** 669–73 (2017).

12. Serafini, M. *et al.* Establishment of bone marrow and hematopoietic niches in vivo by reversion of chondrocyte differentiation of human bone marrow stromal cells. *Stem Cell Res.* **12,** 659–72 (2014).

13. Bueno, C. *et al.* Bone marrow mesenchymal stem cells from patients with aplastic anemia maintain functional and immune properties and do not contribute to the pathogenesis of the disease. *Haematologica* **99,** 1168–75

(2014).

14. Hamzic, E., Whiting, K., Gordon Smith, E. & Pettengell, R. Characterization of bone marrow mesenchymal stromal cells in aplastic anaemia. *Br. J. Haematol.* **169,** 804–13 (2015).

15. Li, J. P., Zheng, C. L. & Han, Z. C. Abnormal immunity and stem/progenitor cells in acquired aplastic anemia. *Crit. Rev. Oncol. Hematol.* **75,** 79–93 (2010).

16. Emadi, A., Zokaee, H. & Sausville, E. A. Asparaginase in the treatment of non-ALL hematologic malignancies. *Cancer Chemother. Pharmacol.* **73,** 875–83 (2014).

17. Pabst, C. *et al.* Identification of small molecules that support human leukemia stem cell activity ex vivo. *Nat. Methods* **11,** 436–42 (2014).

18. Iwamoto, S., Mihara, K., Downing, J. R., Pui, C. H. & Campana, D. Mesenchymal cells regulate the response of acute lymphoblastic leukemia cells to asparaginase. *J. Clin. Invest.* **117,** 1049–57 (2007).

19. Patel, N. *et al.* A dyad of lymphoblastic lysosomal cysteine proteases degrades the antileukemic drug L-asparaginase. *J. Clin. Invest.* **119,** 1964–73 (2009).

20. van der Meer, L. T. *et al.* In vivo imaging of antileukemic drug asparaginase reveals a rapid macrophage-mediated clearance from the bone marrow. *J. Nucl. Med.* **58,** 214–20 (2017).

21. Pievani, A. *et al.* Fludarabine as a cost-effective adjuvant to enhance engraftment of human normal and malignant hematopoiesis in immunodeficient mice. *Sci. Rep.* **8,** 9125 (2018).

22. Kirkiles-Smith, N. C. *et al.* Development of a humanized mouse model to study the role of macrophages in allograft injury. *Transplantation* **87,** 189–97 (2009).

23. Montillo, M., Ricci, F. & Tedeschi, A. Role of fludarabine in hematological malignancies. *Expert Rev. Anticancer Ther.* **6,** 1141–61 (2006).

24. Mosier, D. E., Stell, K. L., Gulizia, R. J., Torbett, B. E. & Gilmore, G. L. Homozygous scid/scid;beige/beige mice have low levels of spontaneous or neonatal T cell-induced B cell generation. *J. Exp. Med.* **177**, 191–4 (1993).

Publications

Michelozzi, I. M.,[*] Pievani, A.,[*] Pagni, F., Antolini, L., Verna, M., Corti, P., Rovelli, A., Riminucci, M., Dazzi, F., Biondi, A., Serafini, M. Human aplastic anaemia-derived mesenchymal stromal cells form functional haematopoietic stem cell niche in vivo. *Br. J. Haematol.* **179,** 669–73 (2017). ([*]First co-authors)

Pievani, A., **Michelozzi, I. M.,** Rambaldi, B., Granata, V., Corsi, A., Dazzi, F., Biondi, A., Serafini, M. Fludarabine as a cost-effective adjuvant to enhance engraftment of human normal and malignant hematopoiesis in immunodeficient mice. *Sci. Rep.* **8,** 9125 (2018).