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The multitasking role of monocytes in the bone marrow haematopoietic niche: from the drug-resistance to the maintenance of stem cells.

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To my family

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CHAPTER 1

GENERAL INTRODUTION

1. THE HAEMATOPOIETIC STEM CELL

Stem cells are uncommitted progenitor cells, defined by their ability to self-renew, propagate and generate progeny cells that can perform a more specialized function and an *in vivo* tissue reconstitution. Adult stem cells have been identified in many tissues and include epidermal stem cells, subventricular neural stem cells, mesenchymal stem cells, adipocytes, gastrointestinal stem cells and haematopoietic stem cells[1].
The haematopoietic stem cell (HSC) is functionally defined by its ability to provide reconstitution of all blood-cell lineages through a process called haematopoiesis. The self-renewal capacity of HSCs *in vivo* could be proved by serial bone marrow (BM) transplantation in mice, in which self-renewal depend on the ability of BM from recipients transplanted with donor cells, to reconstitute secondary recipients[2]. However, self-renewal capacity is correlated with a restricted number of transplants, after which this functionality decreases. On the base of these information, HSCs could be divided into Long-Term HSCs (LT-HSCs) and Short-Term repopulating cells (ST-HSCs). LT-HSCs are the most primitive progenitors that lie at the top of the haematopoietic cell hierarchy, being able to self-renew for the lifetime of the organism. ST-HSCs, instead, present a more limited self-renewal capacity and a shorter lifetime; they can give rise to multipotent progenitors (MPP) that can further differentiate to

unipotent committed progenitors of lymphoid and myeloid lineages, restricting their self-renewal capacity as the cells differentiate.

During the differentiation, HSCs go through an extensive cell surface alteration, essential for their immunophenotypic characterization, which is peculiar for mouse and human. For murine HSCs, Sca-1, c-kit, CD150 and CD48 are fundamental markers to isolate HSCs from murine BM[3]. $Lin^{-}Sca^{+}kit^{+}$ is a heterogeneous group also called LSK that contains LT-HSCs ($lin^{-}Sca^{+}kit^{+}CD150^{+}CD48^{-}$), ST-HSCs ($lin^{-}Sca^{+}kit^{+}CD150^{-}CD48^{-}$) and MPPs. For human HSCs identification two different superficial markers, CD34 and CD38, are utilized. CD34 positivity is observed in case of haematopoietic stem cell-enriched cell population. CD38 marker can be useful for a more accurate selection of HSCs in a $CD34^{+}$ population, since $CD34^{+}CD38^{-}$ cells show a prolonged repopulation capacity *in vivo*. In addition, CD45RA, Thy1.1 CD90 and CD49f can be utilized as supplementary markers which guarantee to distinguish a more immature HSCs population[4].

1.1. THE HAEMATOPOIETIC STEM CELL NICHE

A stem cells niche is a spatial structure that provides a specific microenvironment in which stem cells maintain undifferentiated state and cellular self-renewal, thanks to an intricate interaction of various cells, metabolic pathways, transcriptional and epigenetic regulators and humoral and/or neuronal signals. In adults, HSCs are primally located in BM niche. The haematopoietic stem cell niche was described for the first time by Scholfield in 1978. BM is a highly cellularized tissue that is located in the intra-trabecular spaces of

spongious bones and in the cavity of long bones where haematopoietic and non-haematopoietic cells are in contact through vascular and nervous networks[5]. Conventionally, two niches are distinguished into the BM: osteoblastic niche and vascular niche.

1.1.1. OSTEOBLASTIC NICHE

Inside the BM cavity, HSCs tend to normally home in proximity of the endosteum where they are maintained until their maturation, before the release inside the vascular system[6]. Within osteoblastic niche, HSCs are surrounded and regulated by osteoblasts. There is a straight reciprocal communication between haematopoietic cells and osteoblasts, underlined by the fact that osteolineage cells support haematopoietic progenitor cells expansion[5]. Moreover, *in vivo* osteoblastic depletion generates an altered haematopoiesis with a reduction of lymphoid, erythroid and myeloid progenitors[7]. The interaction between osteoblasts and HSCs is primary due to the presence of adhesion molecules (N-cadherin) and to the release of different haematopoietic growth factors and/or cytokines, such as Osteopontin (OPN), Thrombopoietin (THPO), Angiopoietin 1 (ANGPT1) and CXCL12[5]. However, heterogeneity of osteolineage cells frequently induces discrepant data. In fact, for example, mature osteoblasts do not have a direct role in the regulation of HSCs fate in contrast with the immature ones.

1.1.2 VASCULAR NICHE

Vascular niche in the BM is individuated near the fenestrated endothelium of BM sinusoids. BM endothelial cells (BMECs), similarly to osteoblasts, could sustain *in vitro* HSCs cell growth and differentiation[8]. Vascular niche is responsible for HSCs proliferation, differentiation and trafficking. Being in contact with blood vessels, it is a more nutrient-rich environment compared to osteoblast niche, suggesting that these two niches cooperate to a homeostatic HSCs regulation inside the BM. Alongside, HSCs and BMECs, that come from the same precursor haemangioblast, closely interact, payable to the fact that BMECs release cytokines (CXCL12) and adhesion molecules (E-selectin, VCAM1) that guarantee HSCs engraftment, mobilization and homing[6].

1.1.3. OTHER CELLULAR COMPONENTS

In the BM niche other cellular components involved in HSCs sustainment are detectable and mesenchymal stromal cells (MSCs) are an example. Haematopoietic progenitor cells maintenance is supported by MSCs, in particular by that expressing the filament protein Nestin ($Nestin^+$ MSCs). MSCs release different factors fundamental for HSCs maintenance, such as CXCL12, stem cell factor (SCF), ANGP-1, IL-7, vascular cell adhesion molecule 1 (VCAM1), and OPN[9]. Unlike MSCs and osteoblasts, adipocytes have an inhibitory effect on HSCs control due to the production of neuropilin-1, lipocalin-2, adiponectin, TNF-α and to the reduction of levels of G-CSF and GM-

CSF. In fact, marrow cavities with high percentage of adipose content present a low number of HSCs[4]. On the contrary, inhibition of adipogenesis is responsible for concomitantly increased formation of trabecular bone and osteoblastogenesis, supporting the idea of the balance between adipose and bone tissue in the marrow[4].

Along with non-haematopoietic cells, also the progeny of HSCs can regulate their fate. For example, megakaryocytes control HSCs quiescence by the secretion of different cytokines, such as CXCL4, TGFβ and TPO, promoting osteoblasts growth[10]. Differently, phagocytic cells, particularly $CD169^{+}$ macrophages, regulate HSCs migration to blood and contrast the oxidative stress by releasing prostaglandin E2[11]. Not only cells and molecules released, but also nervous network can regulate haematopoiesis inside the BM niche. In fact, the sympathetic and sensory nerves innervate the BM, favouring HSCs mobilisation out of the marrow through other organs and blood vessels. The system is also directly controlled by G-CSF, which by decreasing CXCL12 levels in the BM leads to a rapid haematopoietic progenitors cells (HSPC) egress from the BM[5].

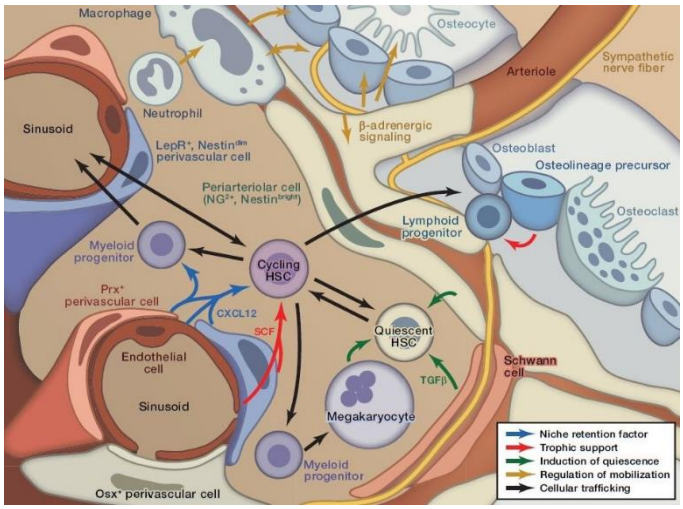

Fig. 1: Anatomical structure of the human haematopoietic niche[12]

1.2. HSC NICHE REGULATION

HSCs interact with other cells in the BM niche thanks to specific conserved signalling pathways that play a crucial role in the regulation of cell proliferation, death and differentiation. Indeed, in case of cellular stress, the haematopoiesis system reacts by activating or down-modulating different regulators involved in various pathways. The most studied signalling pathways are SDF-1, Wnt, BMP, Notch, and COX2. SDF-1 signaling pathway, for example, play a critical role in HSCs maintenance and attachment within the niche[13]. The chemokine SDF-1 is released especially by endothelial cells and interacts with the receptor CXCR4, highly expressed on HSCs surface, inducing their migration through the endothelial compartment[6]. HSCs proliferative and repopulating activities are, instead, controlled by the Wnt signaling. Administration of Wnt inhibitors in transplanted mice activate the cell cycling and reduce the

HSCs repopulating ability overall[14]. In addition, the involvement of BMP signalling, particularly BMP-4 that maintains HSCs proliferation and repopulating activities, is fundamental for controlling the number of HSCs in the adult BM niche. The *in vitro* supplement of BMP-4 in HSCs cultures increases their haematopoietic colony forming capacity, while HSCs repopulating activity is reduced by silencing BMP[15]. Despite the importance of these pathways, in the present work we focused our attention on two other fundamental signalling pathways, such as JAG/Notch and COX2/PGE2, described in detail in the next paragraphs.

1.2.1 NOTCH SIGNALLING PATHWAY

The Notch signalling pathway is one of these regulatory systems, in which the Notch transmembrane receptor interacts with an extracellular ligand on the surface of a contacting cell. In mammals, there are four different Notch receptors (Notch 1-4), composed by two domains: a big Notch extracellular domain (NECD) and a smaller Notch intracellular domain (NICD). After the ligand activation, the NECD is removed and destroyed by endocytosis into the ligand-expressing cells. At the same time, through a proteolytic cleavage of the receptor mediated by gamma-secretase, NICD is released in the cytoplasm of receptor-expressing cell and subsequentially translocates to the nucleus, where it interacts with recombinant signal binding protein (RBP-J) and initiates the transcription of Notch target genes[16]. The receptors can interact with five different Delta-Serrate-Lag (DSL) ligands (Jag1, Jag2, Dll1, Dll3, Dll4), creating different receptor-

ligand combinations, which could generate distinct responses. Between these, JAG1 is primarily involved in the highly conserved Notch signalling pathway[10].

The Notch pathway is one of the most important regulatory signals, fundamental not only for embryonic development, but also for HSCs maintenance within the niche. Indeed, administration of soluble Notch ligands in a HSCs culture, increases the number of stem cells that could successfully engraft in recipient mice. Moreover, it has been demonstrated in different *in vitro* studies that cells expressing Notch ligands could maintain or enhance HSCs self-renewal in culture[17]. Despite these *in vitro* results, the Notch pathway function in the mouse model is not completely clear. Indeed, Notch1 knockout in mice or combined silencing of Notch1 and its ligand Jagged1 does not reveal any effect on HSCs self-renewal[18]. Moreover, inhibition of all canonical Notch signals through an overexpression of a dominant negative form of Mastermind-like 1 (dnMALM1) does not influence HSCs repopulating activity[19]. Taken together, these results have suggested that HSCs are usually exposed to low level of Notch signalling, explaining why the inhibition of the Notch pathway does not result in an evident effect *in vivo*. Moreover, the BM niche is under the control of different stimuli, so the Notch signalling is only one of the systems through which HSCs maintenance is regulated. Differently, the Notch pathway acquires an indispensable role *in vitro* to compensate the absence of other regulatory pathways.

1.2.2. CYCLOOXYGENASE ACTIVITY

Cyclooxygenase (COX) is an enzyme that catalyse the conversion of arachnoid acid (AA) into the cyclic endoperoxidase PGG_2, that is then reduced into the corresponding alcohol PGH_2. PGH_2, depending on the specific cellular and tissue activity, could be converted by specific enzymes into various eicosanoids, such as PGE_2, PGD_2, $PGF_{2\alpha}$, and PGI_2[20]. Cyclooxygenase exists in two isoforms: COX-1 and COX-2. At protein level, the two isoforms are similar, because they are composed by three high mannose oligosaccharides and three different domains: epidermal growth factor domain, the membrane binding domain and the catalytic domain with the cyclooxygenase and peroxidase active sites. However, at gene level, they are expressed by two different gene: COX-1 (576 amino acids) by *PTGS-1* and COX-2 (581 amino acid) by *PTGS-2*. COX-1 is expressed ubiquitously in different tissues (brain, bone, kidney, etc.), while COX-2 is constitutive in kidney and brain and it is expressed in other tissues only after external stimuli or during pathophysiologic conditions, such as inflammation and cancer. Between eicosanoids, prostaglandins and their inhibition via cyclooxygenases activity are extremely important for controlling haematopoietic homeostasis[21]. Unfortunately, the information about this pathway in haematopoietic homeostasis control are controversial. Prostaglandin PGE_2, strongly produced by osteoblasts and monocytes/macrophages within the BM microenvironment, guarantees an evident activity in the regulation of progenitor and stem functions. In fact, it has been demonstrated in different works that PGE_2 is able to:

- increase a long-HSCs engraftment in mice transplanted with BM cells pre-treated *in vitro* with synthetic $PGE_{2;}$
- reduce apoptosis, favouring HSCs proliferation both in mice and human;
- induce HSCs entry into cell cycle, incrementing the typical self-renewal activity of stem cells[22].

Despite these evidences, some recent researches demonstrated that the COX-2 pathway has also a negative feedback control in haematopoiesis. Precisely, by administrating selective COX-2 inhibitors, neutrophils and white blood cells increase and haematopoietic regeneration is favoured. As a counterproof, *in vivo* experiments using COX-2 deficient mice have demonstrated that the knockdown animals have a better haematopoietic regeneration compared to wild-type mice.[21]

The COXs inhibitors are known as non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs are a group of molecules generally prescribed for the treatment of chronic pain and inflammation, but they seem to have an additional role in the treatment of certain solid tumors, reducing tumor cells proliferation. NSAIDs could be non-selective or selective cyclooxygenases COX-2 inhibitors. The first group includes drugs such as indomethacin, Diclofenac and Flurbiprofen, while selective cyclooxygenase-2 inhibitors are represented by NS-398 and Meloxicam. Unfortunately, the administration of NSAIDs is responsible for some undesirable effects, especially regarding the gastrointestinal tract, because they reduce the quantity of prostaglandins with a cytoprotective activity inside the gastrointestinal mucosa. To reduce this problem, the use of selective

COX2 inhibitors is desiderable, since they can reduce the incidence and the intensity of gastrointestinal effects, making the drug more permissive for the treatment of radiation-induced myelosuppression[21].

1.3. MODELS OF HAEMATOPOIETIC NICHE

The study of human haematopoiesis is extremely complicated due to high complexity of the haematopoietic system. For this reason, various groups of researchers have promoted different approaches to trying to reproduce this complicated environment through *in vitro* and *in vivo* models. 2D culture system is the first utilized model and consists in a co-culture system composed by HSCs and a stromal feeder, such as mesenchymal stromal cells, with or without addition of exogenous cytokines. It represents the simplest BM microenvironment model and permits to maintain HSCs *in vitro*. To recreate a more complex model, 2D culture can be modified in a 3D culture system, using bioreactors and platforms that reproduce the BM niche in major details, including vascularisation[23].

Recently, in literature, it has been reported a widespread interest in generating human BM niche into immunodeficient mice[24–26]. Ossicles are an example. They are formed starting from human MSCs *in vitro* cultured with different cytokines and growth factors and implanted, in presence or absence of scaffold, in subcutaneous tissues or kidney capsule of mouse host, where undergo to endochondral ossification differentiating in bone tissues. This heterotopic ossicle creates "niche-like units" in mice that sustain murine or human haematopoietic cells, permitting the study of cell-to-cell interactions inside the niche.

Ossicles partially resolve the problem of xenotransplant models, represented by the lacking cross-reactivity between mouse and human of factors involved in HSCs maintenance, by permitting a more rapid and successful engraftment[27]. Most importantly, heterotopic humanized ossicles provide support to human cancer cells, such as certain subtypes of AML, MPN as myelofibrosis, which normally are difficult to engraft in murine BM.

From the eighties, the most useful approach to study human normal and malignant haematopoiesis is represented by the use of human xenograft models, immunodeficient mice transplanted with human haematopoietic cells. Xenograft models have allowed to functional assessment of human HSCs[28]. However, human haematopoiesis is not completely reproducible in mice, due to problems with the maturation of T and B cells and with the paucity of erythrocytes, platelets and myeloid human cells developing in mice. Moreover, we must be taken into consideration different aspects that can influence human engraftment, such as haematopoietic cell sources, routes of injection, age, sex and conditioning regimen. Thus, the choice of mouse strains is important, because every strain has specific characteristics, and this could probably influence the *in vivo* experiment outcome.
One of the first xenotransplantation model, was obtained transplanting in the strain CB17-SCID (mice without mature T and B cells due to *scid* mutation), cells obtained from human foetal thymus, liver and/or lymph node. Various *in vivo* studies revealed that the SCID model is not so permissive for a good *in vivo* study of haematopoiesis, due to poor human engraftment resulting. For this reason, other mouse

strains are obtained with the aim of improving human engraftment efficiency. Among these, NOD/SCID mice are generated by backcrossing SCID/beige mice with nonobese diabetic (NOD)/severe ones for incrementing the human engraftment[28] harbouring innate immunity defects[29]. Indeed, the combination of SCID mutation to NOD background results in decrease in macrophages, DC, NK cells and deficiency in complement. Alongside, the NSG mice strain was obtained by combining NOD/SCID strains with mice characterised by interleukin 2 receptor gamma chain (IL2R-γ_c) *null* mutation. Due to the high capacity to engraft human cells, NSG mice are becoming extremely important for the study of pathologies, such as HIV, and for the *in vivo* study of HSCs self-renewal. Another kind of mouse model is represented by the transgenic mouse obtained through the Knockdown technology, in which specific genes could be silenced. This technology has permitted the study of specific genes correlated with primitive and definitive haematopoiesis. For instance, Gata1 knockdown induces a reduction of erythroid cells, influencing both primitive and definitive haematopoiesis, while Gata2 knockdown alters only the primitive haematopoiesis and Runx1 only the definitive one.

The injection of human cell in a mouse model could induce the activation of murine innate immune elements, such as neutrophils, granulocytes, NK cells and macrophages. For this reason, the use of a conditioning regimen is extremely important in order to create an immunosuppressive condition for improving the engraftment. In mice, the conditioning regimen is usually obtained with sublethal irradiation, in the presence or absence of chemotherapeutic

agents/antibodies. For what concern irradiation, the source could affect the prognosis of xenotransplant. For example, X-ray is considered an ideal radiation, compared to Caesium-137, because it permits a strong penetration inside the tissue[30]. Irradiation is not sufficient for inducing a strong immune depletion in mice model. Thus, it is essential to couple with other strategies, such as antibodies, growth factors and chemotherapeutic agents. Antibodies can deplete specific subpopulations of immune system, improving HSCs engraftment. For example, anti-CD122 and anti-asialo GM1 are utilized respectively in SCID and NGS mice to induce a reduction of NK cells[31]. Differently, macrophages can be eliminated by administrating clodronate. Moreover, the administration of growth factors (EPO, MGF,PIXY321, TNF, etc.) may favour the human cells engraftment in mice, promoting a rapid haematopoietic cell reconstitution [32,33]. Finally, chemotherapeutic agents, such as bulsufan and cyclophosphamide, are used as an alternative conditioning regimen[34].

In refer to our work, we decided to use Fludarabine (9-β-D-arabinosyl-2-fluroadenine5'-monophosphate),a fluorinated nucleotide, analogous to the antiviral agent vidarabine, with the acquisition of antineoplastic activity. In clinics, Fludarabine is useful for the treatment of some kind of leukaemia and for the conditioning regimen prior to haematopoietic transplantation. Fludarabine is considered a prodrug, since it undergoes a process of dephosphorylation and rephosphorylation that, as a final result, induces DNA synthesis interruption, inhibition of tumor cell growth and, overall, apoptosis[35]. The incorporation into nucleic acids of the resulting active form of

Fludarabine determines the suppression of DNA and RNA synthesis and probably DNA repair by interfering with ribonucleotide reductase, DNA polymerases, DNA ligase I, DNA primase, and RNA polymerase II, resulting also in reduced protein synthesis. Moreover, it has been demonstrated that Fludarabine is a suppressor of signal transducer and activator of transcription 1 (STAT1). This effect can explain its immunosuppressive properties[36].

1.4. HSCs TRANSPLANTATION

Replacement of the haematopoietic system through the transplantation of haematopoietic stem cells (HSCT) is a procedure that was used for the first time in clinics 45 years ago[37]. Nowadays, HSCT is extremely useful for the treatment of different haematopoietic malignancies, such as leukaemia (particularly for acute myeloid leukaemia, 33% of incidence), Hodgkin and non-Hodgkin lymphomas (12%) and multiple myeloma (3%)[38]. Patients are subjected to high doses of chemotherapy for killing cancer cells, that, unfortunately, also destroy normal cells in the BM, including stem cells. Thus, after these pharmacological treatments, a supply of healthy stem cells by transplantation is necessary. After transplantation, donor-derived HSCs can restore normal BM function in the treated patients, mimicking the natural HSCs movement during ontogeny[39] into three different consecutive stages[40]. In the first one, the BM endothelium sinusoids express molecules, such as p-selectin, e-selectin and VCAM-1, that interact with different receptors (CD44, VLA-4) on the surface of circulating HSCs. These interactions permit the migration

of stem cells across the endothelium matrix barrier (step two) to lodge inside the specific BM compartment (step three). In the BM niche, transplanted HSCs undergo massive cell proliferation, initiating a new haematopoietic system able to recreate a normal BM situation.

On the basis of stem cells source, HSCT can be divided in autologous (HSCs from the same patient) or allogenic (HSCs from another healthy individual). Unfortunately, before allogenic HSCT, patients come under chemotherapy treatment, that causes drug toxicity and complications, such as infections. Moreover, the same transplant could cause graft versus host disease (GvHD), an immunological reaction occurring when the donor immune system rejects the recipient, that could be divided in acute (aGvHD, onset <100 days after transplantation) or chronic (cGvHD, onset >100 days after transplantation). aGvHD impairs principally skin, liver and intestinal tract and the administration of calcineurin inhibitors (cyclosporine or tacrolimus), before and after transplantation, can reduce the possibility of aGvHD occurrence. cGvHD presents similar symptoms, but with more aggressive traits.

Stem cells for haematopoietic transplantation are usually obtained from BM, peripheral blood (PB) after mobilisation, and umbilical cord blood (CB). BM stem cells are harvested by BM aspiration from posterior iliac crest from patients or from healthy donor under anaesthesia. However, this technique induces a moderate pain in the donor and a red blood cells transfusion is sometimes needed. For this reason, in the last years, in the case of allogenic HSCT, it is desirable to obtain HSCs from PB after mobilisation with G-CSF. Peripheral blood stem cells (PBSCs) guarantees high percentage of HSCs, with

shorter time of engraftment if compared to BM. However, lacking a suitably matched HLA donor can limit the chance of haematopoietic transplantation receiving. The use of CB could be a solution for this issue. In fact, the naïve nature of new-born's immune system allows transplantations with less restriction of the HLA system. Moreover, CB is easily available, their collection is safer, and its transplantation reduces the probability of viral infections and GVHD cases in patients. A setback of CB transplantation is the slow pace of engraftment. This fact is related to lower amount of cells contained compared to the other sources and increases the possibility of early post- transplantation mortality.

1.4.1. HOW TO IMPROVE CB-HSC SURVIVAL

Despite the advantages in using CB as a source of HSCs, the limited nucleated cell dose negatively affects the engraftment and the outcome of the transplantation, particularly in adult patients. For these reasons, different approaches have been proposed to overcome these issues. Firstly, the use of multidonor CBTs or/and a dual transplantation has been suggested, involving co-transplantation of CB samples with a component from haploidentical donor PB, with increased possibility of developing GvHD. As these methods failed to give satisfactory results, in the recent years, different research groups have studied different approaches to *ex vivo* expand $CD34^+$ prior to transplantation maintaining survival and stemness, using cytokines, stromal cells and/or a combination of both. For what concern

cytokines, the addition of specific molecules (G-CSF, SCF, EPO, IL-3, flt-3 ligand) to culture medium for about 10 days before infusion was suggested[41]. Another suggested approach was *in vitro* blocking of CB cells differentiation, by using various inhibitors or chemical substances, such as Copper chelator and Nicotinamide. Unfortunately, these systems are not enough to efficiently improve HSCs number and survival. Therefore, the use of stromal cells to support this activity has been proposed. As an example, MSCs have been used as a layer that adheres to the dish, where it is possible to cultivate the $CD34^+$ cells[42]. MSCs constitutively secret different cytokines, such as interleukins (IL-6, IL-7; IL-8, IL-11; IL-12, IL-14, and IL-15), thrombopoietin (TPO) and Fl3 ligand. Taken together, it seems that MSCs can improve HSCs expansion, inhibiting differentiation and decreasing apoptosis[43]. MSC-conditioned CB units, co-transplanted with unmanipulated units, accelerated haematopoietic recovery, probably through MSC-expanded monocytes, which contribute to HSCs retention in the BM[44]. Trento and colleagues have highlighted how the interaction between MSCs and myeloid cells is fundamental in the maintenance of haematopoietic homeostasis. In particular, monocytes are the first responders of inflammation, healing, vascular remodelling and tissue regeneration. Unfortunately, the characterisation and function of monocytes/macrophages in the BM haematopoietic niche is generally unknown. However, when HSCs are transplanted in combination with monocytes, there is a massive increment in human haemopoietic engraftment, supporting the role of monocytes in the haematopoiesis[45].

1.5. MONOCYTES

Monocytes are a kind of bone-marrow derived leukocytes, characterised by a nongranulated cytoplasm and a singular lobar nucleus. Monocytes phagocyte foreign bodies, but also release specific cytokines and present antigens, facilitating healing and repair[46,47]. Monocytes are originated in the BM from common myeloid progenitors (CMP), generated from the so-called ST-HSCs. How the release of monocytes from BM is controlled remains partially unclear. It seems that this activity is regulated though the interaction between various cytokines and their receptors. Once outside the BM, through PB, monocytes arrive at different tissues and organs, where they complete their differentiation into dendritic cells (DC) and macrophages. Macrophages are resident phagocytic cells, especially in lymphoid and non-lymphoid tissues, and phagocytosis of microbial invaders (bacteria, viruses, fungi and parasites) represents their fundamental role. Their function is carried out through the activation of different membrane receptors, which respond to host-derived and foreign ligands, but also by the activation of intracellular inflammasomes or by caspase processing. DCs are another distinct lineage, which monocytes could be differentiated. DCs, unlike macrophages, are highly migratory cells that reside in an immature state in non-lymphoid organs at the beginning. Only after an infection/inflammation, they leave the tissue, moving towards lymphoid tissues, in which they initiate an adaptive immune response. In fact, after the interaction with pathogens or foreign bodies, DCs can capture, process and present on the surface the antigens, and interact with $CD4^+$ and $CD8^+$ T-cells. Despite this main function, DCs also

perform two other lesser-known functions, such as immune tolerance and maintenance of immune memory[48].

During the past years, monocytes have been subdivided based on adhesion capacity and morphology, but, nowadays, it is preferred to divided monocytes in different groups according to the expression of specific markers. In mice, monocytes are generally subdivided into two different subpopulations on the base of LyC6 expression. $Ly6C^{hi}$ manifests proinflammatory antimicrobial functions and are characterised by high expression of C-C chemokine receptor 2 (CCR2) and low level of CX3CR chemokine receptor 1 (CX3CR1). On the contrary, $Ly6C^{lo}$ develops an early response to inflammation and tissue repair and has high CX3CR1 level and low CCR2 levels. Differently, CD14 and CD16 are fundamental markers in human monocytes. Regarding these markers, human monocytes are divided into three distinct populations: classical ($CD14^{+}CD16^{-}$), intermediate ($CD14^{+}CD16^{+}$) and non-classical ($CD14^{-}CD16^{+}$) monocytes. These three subsets have also different sites of residence. For example, classical monocytes (80-95% circulating monocytes) are recruited to the site of infection and injury, where they exhibit elevate plasticity[49]. On the other hand, non-classical ones (2-11% circulating monocytes) can patrol the vascular endothelium both in homeostatic and inflammatory conditions. Instead, the effective function and distribution of intermediate monocytes is still unknown because they have phenotype and roles overlapping partially with classical monocytes and partially with non-classical ones, underling the typically heterogeneity of this group. In more detail, classical monocytes leave the BM and arrive in the blood vessels where they

could be differentiated in intermediate monocytes or disappear by death or migration[49]. Intermediate monocytes share a similar fate, because they could give origin to non-classical monocytes or leave the circulation. As seen in this model, it is evident that classical monocytes have the shorter lifespan (on average 1 day) and, in turn, non-classical monocytes show the longest lifespan of about 4 days. These differences between subsets are also related to their roles. In fact, classical monocytes, usually involved in inflammation, tend to rapidly migrate to the site of injury, where they conduct a pro- or anti-inflammatory activity. Differently, non-classical monocytes present a more differentiated phenotype and tend to accumulate in the endothelium, although little is known about their role.

Besides their function in inflammation, monocytes are involved in healing, vascular remodelling and tissue regeneration. Moreover, they are involved in the HSCs maintenance within BM haematopoietic niche, even if their role in this context is largely unknown. Chow and colleagues have studied indirectly this activity through BM mononuclear phagocytes depletion by the use of Clodronate in the animal model[10]. They found that macrophages depletion reduces HSCs proliferation, as well as the pool of quiescent HSCs under both homeostatic and inflammation conditions and suppresses osteoblasts and different HSCs growth factors. These changes induce the destruction of the haematopoietic niche, with a massive HSCs mobilisation to the bloodstream and lymphoid organs due to BM CXCL12 levels reduction[10].

2. ACUTE MYELOID LEUKAEMIA

Leukaemia is a heterogenous group of malignant disease subdivided into myeloid or lymphoid (depending on the haematopoietic precursor type involved), and in acute or chronic (according to the maturation stage of the cells). Acute myeloid leukaemia (AML) is characterised by rapid growth and accumulation of immature precursors of myeloid white blood cells in the BM and in the peripheral blood[50], where they remain in an undifferentiated state. AML is rare and affects mainly adults (especially men), with an average age of 67 years and incidence that increases with age: there are 1.3 cases per 100,000 for those under 65 and approximately 12.2 cases per 100,000 over 65, as reported by American Cancer Society[51]. Relative survival for adults is 34% at 1 year from onset and 15% at 5 years, values that decrease markedly with age of patients.

2.1. CLINICAL PRESENTATION AND DIAGNOSIS

At diagnosis, patients mainly exhibit anaemia, neutropenia, peripheral blood leucocytosis and BM infiltration with an average of myeloblasts of 20-100%[50]. Due to BM failure, patients show fatigue, dizziness, anginal chest pain, palpitations seizures, fever, higher risk of infection, bleeding gums, multiple ecchymoses, anorexia, occasionally significant gastrointestinal, urinary or intracranial haemorrhage[50]. Infiltration is presented not only at BM level but also in spleen, liver, gums and skin. If untreated after diagnosis, secondary infections or bleeding can cause the death of the patient. When there is a suspected AML diagnosis, BM aspirate, PB and in some cases cerebrospinal fluid samples are collected to perform diagnostic studies. In the first case, morphological tests are carried out. At least more than 20% myeloid blasts should be present in the BM (3% blasts positive on Sudan-Black-B or Myeloperoxidase staining), with the exception of common chromosomal abnormalities t(15;17), t(8;21), inv(16), or t(16;16)[50]. If the results are controversial, immunophenotyping is necessary in order to detect specific myeloid antigens, such as cCD3, cMPO, TdT, CD34 and CD117, along with the analysis of monocytic, erythroid or megakaryocytic differentiations. Moreover, to identify chromosomal abnormalities and karyotyping changes, chromosome banding methods can be useful.

2.2. AML CLASSIFICATION

From the 1976, the French-American-British (FAB) classification system is used, according to which AML, based on morphological and cytochemical characteristics of the leukaemic cells[52], can be divided into eight different subtypes (M0-M7, Fig.2).

Subtype	Description	%
M0	Minimally differentiated acute myeloid leukemia	5
M1	Acute myeloid leukemia without maturation	15
M2	Acute myeloid leukemia with maturation	25
M3	Acute promyelocytic leukemia	10
M4	Acute myelomonocytic leukemia	20
M4 Eo	Myelomonocytic together with BM eosinophilia	5
M5(a,b)	Acute monoblastic leukemia (a), acute monocytic leukemia (b)	10
M6(a,b)	Acute erythroid leukemias, including erythroleukemia (a) and very rare pure erythroid leukemia (b)	5
M7	Acute megakaryoblastic leukemia	5

Fig 2 FAB classification

Among all AML subtypes, minimally differentiated acute myeloid leukaemia (M0) is one of the most difficult to diagnose because blasts are morphologically less differentiated and present lymphoblastic and myeloblastic properties. Cells from patients with FAB M1 differ from FAB M2 on myeloid maturation, with ≥20% myeloblasts, <10% promyelocytes and more mature neutrophils, monomorphic features and no particular cytogenetic abnormality associations. In AML with maturation (M2), ≥10% cells are at the post-myeloblast stage and promyelocytes, myelocytes and neutrophils can be found; it is

commonly associated with traslocation t(8;21) and a high response rate to chemotherapy. Hypergranular cells and leukopenia are typical of acute promyelocytic leukaemia (M3); it affects younger patients that in most cases present t(15;17) traslocation, hemorrhagic morbidity and a poorer prognosis. Myelomonocytic leukaemia has blasts with both myeloid and monocytic elements (M4), that can be identified morphologically by the partly differentiated monocytes present in the PB (≥20% leukaemic cells): no clinical or cytogenetic clustering can be associated with this variant because of a wide range of patients incorporated. The M4 subtype includes young patients characterised by dysplastic eosinophils at different maturation levels, with large basophilic granules, chromosome 16 abnormalities and an excellent prognosis. The M5 FAB represents 10% of all AML cases, seen in patients of all ages with higher blast counts and drug resistance; it is divided into acute monoblastic leukaemia (M5a), with a monoblast predominance, small cytoplasms, no morphological differentiation and common genetic alterations in chromosomes 9, 11, 19, and acute monocytic leukaemia (M5b) with mainly promonocytes characterised by abundant granulated cytoplasm. 5% of total AML patients belongs to the M6 subtype, in which morphologic abnormalities in erythropoiesis are prominent; it is described as a mixed proliferation of myeloid blasts and megaloblastic multinucleated erythroid precursors with increased mitoses number, resulting in resistance to therapy with no distinct karyotypic features associated. Acute myeloblastic leukaemia (M7) presents mostly morphologically undifferentiated and multinucleated blasts of

megakaryocytic lineage, abnormalities of chromosome 3 and poorer treatment response and survival.

Even though the FAB classification system is the most widely used for AML, the World Health Organization (WHO) established a more recent system in 2001[53], which defines six major disease types according to morphology, immunophenotype, clinical presentation and genetic information. Different chromosomal translocations delineate eleven subtypes among cases of AML with balanced cytogenetic abnormalities, while morphological evidence of dysplasia and the presence of cytogenetic abnormalities (monosomy 5 or 7 and deletion 5q or 7q) are incorporated in AML types with myelodysplasia-related changes, suggesting a poor prognosis. Other distinct categories in the classification remain the therapy-related myeloid neoplasms for patients who develop myeloid neoplasms following cytotoxic therapy, myeloid proliferations related to Down syndrome (usually megakaryoblastic proliferations), and not otherwise specified AML lacking prognostic significance. Myeloid sarcoma in any subtype of AML could occur *de novo*, as progression of a prior Myelodysplastic syndrome (MDS) or Myeloproliferative neoplasms (MPN), or as relapse of AML.

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 with minimal differentiation
	AML without maturation
	AML with maturation
	Acute myelomonocytic leukemia
	Acute monoblastic/monocytic leukemia
	Acute erythroid 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; WHO, World Health Organization.

Fig 3: WHO revised AML classification [53]

2.3 MOLECULAR SIGNATURE

Due to its heterogeneity, AML could be characterised based on its genetic and molecular peculiarity. From a cytogenetic point of view, AML patients could be classified into favourable, intermediate and unfavourable. In more details, favourable patients with t(8;21)(q22;q22) [RUNX1/RUNX1T1], inv(16)(p13q22) [CBFB/MYH11] and t(15;17)(q24;q21) [PML/RARA] have a good prognosis with a high possibility of complete remission, intermediate patients present t(9;11)(p22;q23) [MLLT3/MLL], while unfavourable ones with t(6;9)(p23;q34) [DEK/NUP214], inv(3)(q21q26) [RPN1/EVI1] and

t(1;22)(p13;q13) [RBM15/MKL1] show a poor prognosis due to the high aggressivity of this AML subtype[54]. Among the intermediate group, it is possible to detect patients with also some genetic modification, which represent more than 25% of total AML patients[55]. In these cases, genes involved in the mutation are various, but the most important ones are FLT3, NPM1 and CEBPα, associated with treatment response and progress of the disease[54]. Other involved genes are KIT, FLT3, NPM1, CEBPA, RAS, WT1, BAALC, ERG, MN1, DNMT, TET2, IDH, ASXL1, PTPN11 and CBL. Only recently, an involvement of the epigenetic changes (DNA methylation, histone modification and chromatin remodelling) has been also proposed, especially in pre-leukaemic cells[56].

2.4. AML TREATMENT

The standard treatment of AML patients is subdivided in an initial induction phase and a consolidation phase and it is different based on the risk group classification[57].

Induction phase is characterised by 7+3 regimen, meaning seven days of continuous cytarabine (100 mg/m^2/daily) and three days of anthracycline (60-90 mg/m^2 of daunorubicin or 10-12 mg/m^2 of idarubicin). Different dose combinations have been tested, especially for elders that are less likely to respond to chemotherapy and more susceptible to toxicities. Hypomethylating agents have shown some beneficial effects in older individuals, used in initial induction therapy and in relapsed setting[58]. Induction stage has the task of fostering a morphologic complete remission with the following values:

- <5% blasts in BM sample ≥200 nucleated cells;
- absolute neutrophil count (ANC)>1000/uL;
- platelets ≥100,000/uL[58]

Consolidation therapy can be useful before transplantation in order to eradicate minimal residual leukaemia, prolong initial remission and reduce relapse risk[30]. The consolidation phase is dependent on patients' characteristics. Patients with a favourable prognosis are generally treated with low dose cytarabine (1.5 g/m^2) or multi-agents setting. On the contrary, intermediate/high risk patients usually undergo allogeneic haematopoietic stem cell transplantation, following previous evaluation of transplant eligibility and comorbidity index, and prior conditioning if needed[59]. Unfortunately, relapse occurs within the first year in 50-80% of cases and represents the main reason of treatment failure.

Nowadays, thanks to the discover and approval of new efficient drugs and to increased understanding of AML pathogenesis, a more specific pharmacological treatment which selectively address biological processes deregulated in LSC and AML blasts and their interaction with BM niche can be proposed in order to improve therapeutic responses. The new agents are tested in preclinical and clinical phase studies, firstly for the treatment of relapsed or refractory AML or elderly patients, ineligible for current chemotherapy and comprehend novel chemotherapeutic agents, targeted molecular inhibitors, cell cycle regulators, pro-apoptotic agents, epigenetic modulators,

metabolic therapies, immunotherapies and therapies targeting the BM microenvironment. The Food and Drug Administration (FDA) has recently approved new specific therapies which range from targeted single agents (Enasidenib) and multi-kinase inhibitors (Midostaurin) to antibody-drugs (GO)[59]. Enasidenib and Midostaurin are specific inhibitors of two genes (respectively mIDH2 and FLT3) that often present epigenetic modifications in AML patients, while Gemtuzumab ozogamicin (GO) is a drug conjugated to the CD33 antibody, present in the 90% of AML blasts.

2.4.1. L-ASPARAGINASE

Although L-asparaginase (ASNase) has been principally used in the treatment of acute lymphoblastic leukaemia (ALL), recent *in vitro* and *in vivo* experiments have suggested its possible role in the treatment of AML patients[60]. ASNase is an enzyme that catalyses the deamination of asparagine or glutamine to respectively aspartic or glutamic acid, with ammonia release. It can be produced by various organisms, such as algal and yeast sources, actinomycetes, fungi and bacteria, especially Gram-negative species[61]**.** ASNase could be of type I or type II and, in particular, L-asparaginase of type II is clinically in use as chemotherapeutic for treating ALL. Nowadays, two native preparations are used in clinics: one from *Erwinia chrysanthemi* and the other one from *Escherichia coli. E. coli* ASNase is characterised by a molecular weight of 133-141kDa, an active site on each of the four subunits, a specific activity of 270 U/g and a low glutaminase activity. The *Erwinia* preparation, with similar molecular weight and

half-time (20 hours), possesses in contrast more specific activity (700 U/g) and complementary glutaminase properties up to 10%.[62] The short half-time and the high immunogenicity encouraged chemical modifications of *E. coli* ASNase in mid-1970s, consenting to obtain PEG-ASNase, covalently conjugated to polyethylene glycol (PEG). Usually ALL patients are treated with ASNase from *E. coli* but, unfortunately, more than 30% of patients develops allergic reaction to this kind of asparaginase due to the production of anti-ASNase antibodies. For these reason, patients are often switched to *Erwinia* asparaginase, guarantying a continuous treatment[62].

Leukaemic lymphoblasts depend upon exogenous aminoacid supply for survival and proliferation, because of their lack of L-asparagine synthetase (ASNS). Differently from healthy cells that have the ability to synthesize L-asparagine *de novo*, leukaemic cells die of starvation if factors essential for protein synthesis are depleted[63]. The decrease of L-asparagine and L-glutamine induces the activation of different metabolic pathways, resulting in global protein synthesis reduction and apoptosis[64].

In response to ASNase treatment, primary ALL cells exhibit higher ASNS expression, in particular it was found 3-fold increase in resistant blasts. As compared to ALL cells, AML blasts exhibited superior *ASNS* expression at mRNA level and present reduced susceptibility to ASNase. In case of ASNS suppression, AML cells lines resulted more responsive to the drug, but nonetheless ASNS mRNA did not proved to be an accurate predictive resistance determinant for ASNase susceptibility[65]. Several mechanisms of resistance to ASNase have been identified and could be mediated by

genetic, immunological, enzymatic, environmental and catabolic factors. Altered methylation of cytosine residues, causing modified regulation and de-repression of the asparagine synthetase gene in tumor cells, and changes in the expression of protein metabolism genes, such as ribosomal protein genes (e.g., RPL3, RPL4, RPL5, RPL6, and RPL11), in cytosketeleton and extracellular-matrix genes (e.g., TMSB10, PDLIM1 and DSC3) and apoptosis-regulatory genes (MCL1, BCL2L13, HRK, and TNF) have been associated with lower ASNase sensitivity and treatment response. Drug activity can be impacted by development of specific neutralizing antibodies, by lysosomal cysteine proteases, cathepsin B and asparaginyl endopeptidase (AEP), able to abrogate the enzymatic activity and accelerate their clearance *in vivo*[66].

2.5. CATHEPSIN B

Cathepsin is a lysosomal cysteine protease of the papain family that carries out its function usually in a slightly acidic environment. Cathepsin B is a bilobal protein with a central catalytic site, positioned between a left part containing cysteine and a right part with histidine. This protease has two distinct activities: endopeptidase (cleaving internal peptides bonds) and exopeptidase (carboxydipeptidase activity). The gene for the human Cathepsin B (*CTSB*) is located on chromosome 8p22 and it comprises 12 exons that synthesized a pre-pro-enzyme of 339 amino acids with a signal peptide of 17 amino acids. Pre-pro-enzyme is synthesized on the rough endoplasmatic reticulum (RER). In the lumen of RER, the signal peptide is removed and an inactivated form, procathepsin B, is created. Procathepsin B,

transported to the Golgi apparatus, is glycosylated and then is transported to lysosome trough transport vescicles. Only inside the acid environment of lysosome the protein is activated, modifying in Cathepsin B. Independently from the function, Cathepsin B is ubiquitously located into the lysosomal/endosomal compartment of various human tissues and is involved in the maintenance of homeostatic metabolic activity, creating a turnover of intracellular and/or extracellular proteins within the cell.

Despite this central role, Cathepsin B has other functions, such as pro-hormone and pro-enzyme activation, antigen processing, inflammatory response, tissue remodelling and apoptosis[67]. For this reason, alteration in the Cathepsin B expression can lead to the insurgence of different pathologies such as arthritis, pancreatitis and Alzheimer's disease. Moreover, the expression of Cathepsin B is also correlated to drug resistance. This role is partially associated with the typical acidic pH inside the lumen of the lysosomes where the drug is sequestrated, impeding the interaction with target cells. An example of drug-resistance by Cathepsin B has been shown by Van De Meer and colleagues in their recent work[68]. They have demonstrated that ASNase is accumulated especially in macrophage-rich tissues, such as liver, spleen and BM[68]. Inside these compartments there is a high accumulation of cathepsin B, that is required for a rapid degradation of this drug. This finding underlines the fact that this protease could have a role in ASNase resistance, creating a protective niche for the leukaemic cells. However, not all the patients have the same expression level of Cathepsin B, explaining why there are differences in prognosis[69]. In an ALL paediatric patient particularly responsive to

ASNase was detected a germ line mutation in *CTSB* gene which lowered the proteolytic activity of the enzyme extending ASNase half-life[70]. Moreover, an enhanced mRNA content and activity of CTSB was reported in PB mononuclear cells (PBMC) of AML paediatric patients and CTSB was proposed as a prognostic determinant[71].

2.6. THE LEUKEMIC STEM CELL AND THE AML NICHE

Biologically, AML cells could be represented as a hierarchy at the top of which there are leukaemic stem cells (LSCs)[72]. LSCs are cells with stemness properties, which are responsible for initiating and maintaining the disease giving rise to more differentiated blasts. Moreover, LSC refractoriness to conventional chemotherapies determines AML relapse. This is due to their peculiar characteristics (e.g., quiescence and expression of efflux pumps) and to the protection provided by the BM microenvironment. It has been observed indeed that leukemic cells hijack the normal HSCs-supportive microenvironment in favor of leukemogenesis and LSCs protection[73]. BM in AML patients at diagnosis is characterised by enhanced microvessel density, neuropathy, altered frequency of $CD146^+$ progenitors and osteoblasts number as compared to normal BM. 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. Evidences suggest that leukaemic cells reprogram MSCs at a transcriptional and epigenetic level, increasing the expression of pro-survival genes, reducing osteogenesis, reducing HSCs support, and

upregulating *VEGFA, VEGFB, CXCL12* and proinflammatory cytokine-related genes[74;75]. AML cells determine niche remodelling via direct cell contact and via soluble factors and exosome secretion[76]. 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 osteoblasts[77].

On the other way, several evidences demonstrate the involvement of the microenvironment in AML initiation. Mice carrying *Dicer1* deletion in immature cells committed to generate osteoblasts exhibited myelodysplastic and occasionally AML characteristics, even when transplanted with wild type BM cells[78]. However, when mutated haematopoietic cells were injected into wild type recipients no clinical manifestations were observed. Likewise, osteoblasts carrying activating mutation of β-catenin affected HSCs differentiation and generated AML *in vivo*[79].

3. SCOPE OF THE THESIS

The purpose of this PhD thesis was to study the role of monocytes/macrophages within the BM haematopoietic niche in normal condition and in haematological disorders such as Acute Myeloid Leukaemia.

In the first part of the project, we explored the possibility of improving the engraftment of human HSCs in SCID/beige mice, a poor permissive immunodeficient strain, by combining sub-lethal irradiation and Fludarabine, an immunosuppressive drug used in conditioning regimen in clinics. This conditioning regimens generate an effective xenograft model of human normal and malignant haematopoiesis.

The second part of the project aimed at evaluating the role of monocytes in *in vitro* maintenance of HSCs survival and self-renewal and their capability to boost human cells engraftment in xenograft models**,** in order to suggest new approaches to overcome the issues related to allogenic transplantation in clinics.

In the third and last part of the project, we investigated the role of monocytes/macrophages in malignant BM niche, particularly in AML. Precisely, we have focused our attention on the protective effect of BM microenvironment components, including mesenchymal stromal cells and monocytes/macrophages, to ASNase sensitivity of AML blasts.

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CHAPTER 2

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

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ABSTRACT

There is still an unmet need for xenotransplantation models that efficiently recapitulate normal and malignant human hematopoiesis. Indeed, there are a number of strategies to generate humanized mice and specific protocols, including techniques to optimize 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 hematopoiesis in immunodeficient mice. Quantification of human CD45^{+} cells in bone marrow revealed approximately a 102-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 hematopoiesis, 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 hematopoiesis in immunodeficient mice.

INTRODUCTION

In the 2000s, various immunodeficient models were developed by combining the IL-2rgnull 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, humanized 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 hematopoiesis 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 the transplant 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 7.4×10^6 in Irr+ Fluda, range from 2.25 to 13.6×10^6; 0.06×10^6 in Irr, range from 0.01 to 0.30×10^6; $p = 0.0015$) (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 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 hematopoiesis 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).

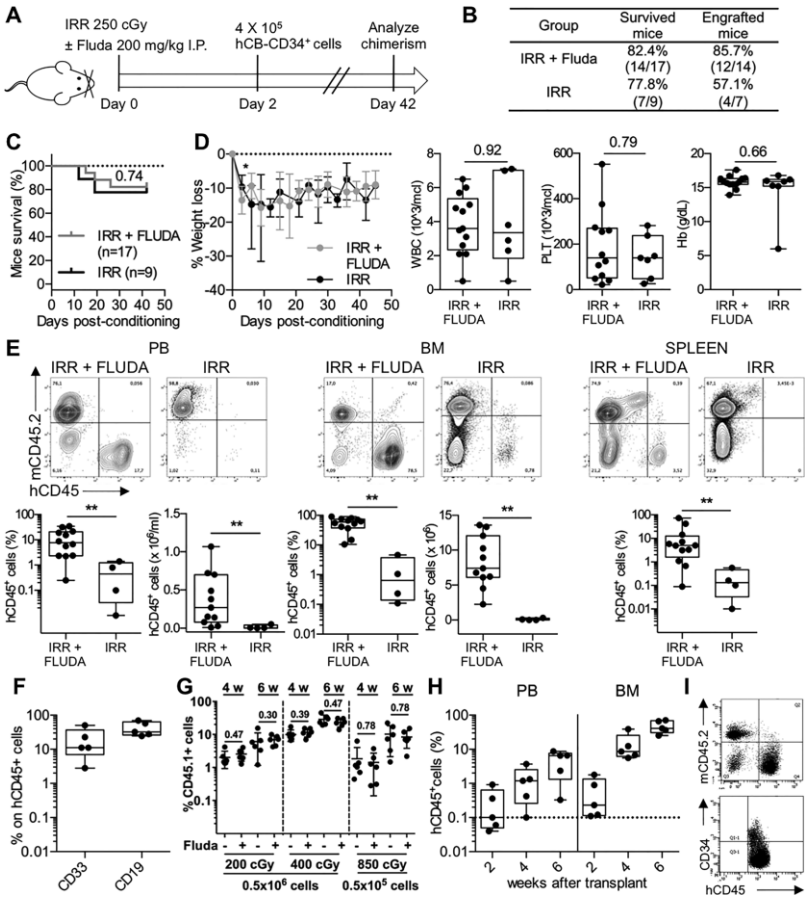

Figure 1. *Fludarabine enables efficient human cell reconstitution. (**A**) Representation of experimental outline: irradiation (250 cGy) and 200 mg/kg fludarabine treatment of SCID/beige mice followed by transplantation of hCD34⁺ cells. Where not otherwise specified, mice were euthanized 6 weeks (day 42) after treatment to analyze 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 (gray 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 hemoglobin (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 hematopoiesis 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 0.05 × 10^6 BM cells of congenic mice. 2 independent experiments (3 mice/group in each experiment);* ***(H)*** *Levels of human chimerism analyzed 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 2×10^6 KG-1/ mouse (Fig. 2A,B). Using the same conditioning strategy, it was possible to transplant lower numbers of cells (5×10^5 or 2×10^5/ mouse) obtaining dose-dependent sustained engraftment levels, with a longer overall survival (from 21 to 29 days) (Fig. 2C). Finally, we evaluated if this preconditioning strategy could sustain the engraftment of primary AML blasts derived from 8 patients with various genetic backgrounds, including NPM-1 mutated, Flt3 mutated, NPM-1/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).

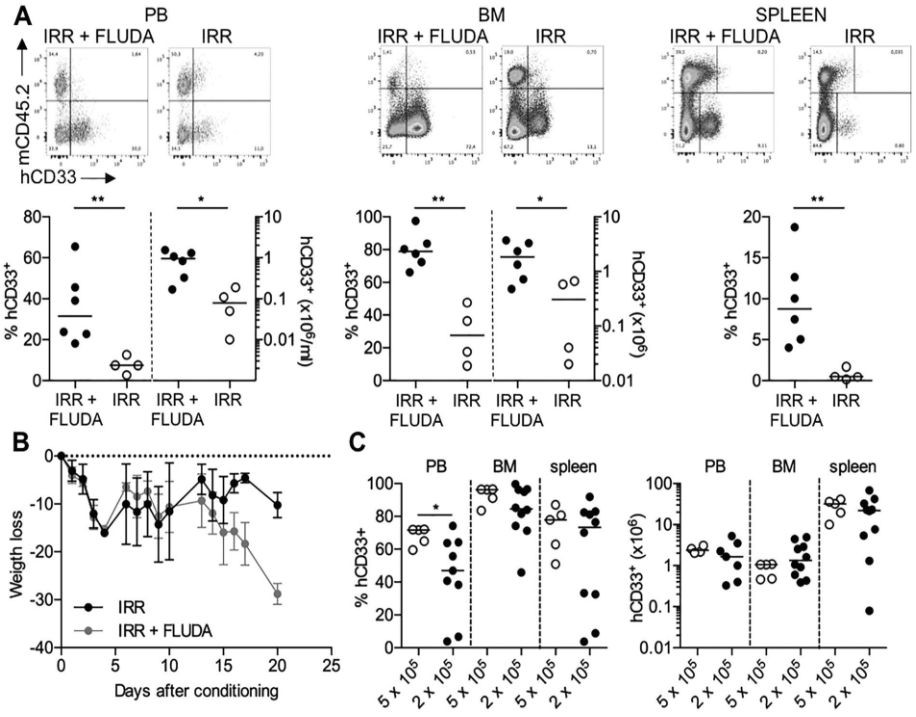

Figure 2. *Fludarabine promotes AML cell line engraftment. (**A**) SCID/beige mice were intravenously injected with 2 × 10^6 cells from the established human myeloid cell line KG-1 on day 2 post conditioning (irr+ fluda or irr). Mice were euthanized for the engraftment assessment in PB, BM and spleen 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 (gray 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 (5 × 10^5 or 2 × 10^5 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).

A

Pat #	Age (y)	Source	% Blasts	FAB	Mutations	Transplanted cells per mouse (x 10^6)	% Human engraftment in BM
1	16	BM	90	M5	NPM1 Flt3-D835	8.3	NO
2	16	BM	90	M4	Flt3-D835	10.1	9.1
3	1	BM	98	M7	None	9.8	NO
4	13	PB	71	M2	None	27.3	33.7
5	14	BM	95	M4	None	6.8	NO
6	67	PB	99	M1	NA	11.6	71.5
7	58	BM	90	M5	NPM1	6	1.5
8	70	BM	50	M4	None	18.5	NO

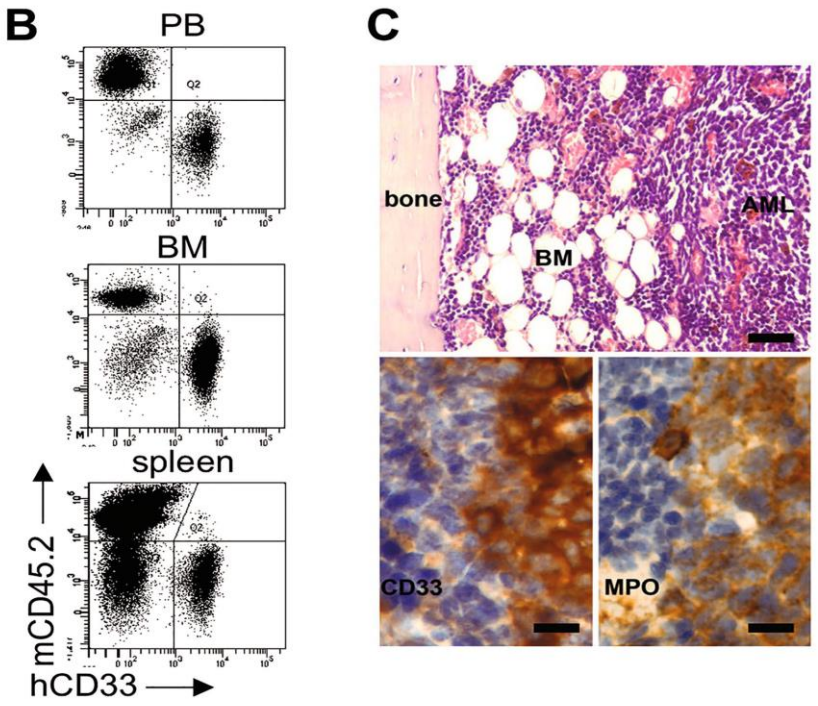

Figure 3. *Fludarabine permits engraftment of primary AML blasts. (**A**) Clinical characteristics of AML patients (age, source and percentage of blasts, Fab classification, NMP-1 and Flt3 mutational status) and engraftment details (transplanted cell dosage and % human engraftment detected in BM at final analysis) following transplantation in SCID/beige mice conditioned with irr+ fluda. NA, not analyzed. 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 Hematoxylin and Eosin (top panel) to show the 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 now 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 hematopoiesis 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 graft3. 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 signaling[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 graft16. 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 VDJ 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 hematopoiesis 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 provide 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 hematopoiesis 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 Gerando Hospital-Monza and carried out in accordance with the Declaration of Helsinki. All samples were only processed from patients who had consented 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 fetal 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 pediatric 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^{scid}Lystbg-$^{J/Crl}$) 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 h 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 cells 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 (analyzing 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, analyzed with hematology counter (Coulter AcT Diff, Beckman Coulter), and lysed with ACK (Ammonium-Chloride-Potassium) lysing buffer (Stem Cell 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 cells 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, eBiosciences) 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 Hematoxylin-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-week-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 0.5×10^6 donor cells/mouse and in the other one an irradiation of 850 cGy with 0.05×10^6 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, eBiosciences) and CD45.2 (clone 104, eBiosciences).

Immunosuppressive assay. 5×10^5 C57BL/6 murine splenocytes were labelled with PE-Cell Tracker (ThermoFisher Scientific), plated in the presence or in the absence of fludarabine (2.5 mcg/ml) for 24 hours, and then stimulated with ConA (3 mcg/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: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

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AUTHOR CONTRIBUTIONS

A.P. performed research, analyzed 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

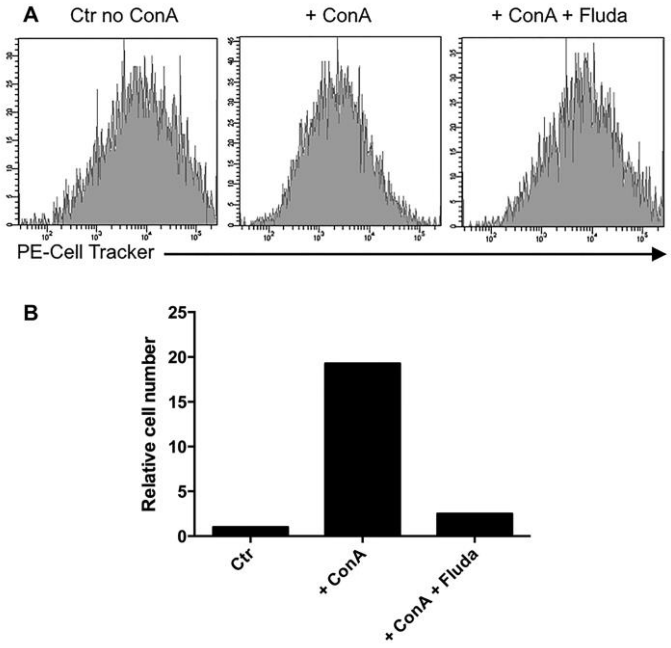

Figure S1. Treatment of C57BL/6 murine splenocytes with fludarabine leads to inhibition of mitogen-induced proliferation. *Splenocytes labelled with PE-Cell Tracker were treated or not with Fludarabine (2.5 mcg/ml) and cultured with or without Concanavalin A (ConA) for 72 hours. Proliferation and cells number were determined by flow-cytometry analyzing the Cell Tracker dilution and using the single-platform method.* ***A)*** *Representative hystogram plots were shown.* ***B)*** *Relative cell number was express in comparison to control (splenocytes not treated with Fludarabine and not stimulated with ConA).*

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CHAPTER 3

Human CD14^{+} cells accelerate engraftment and positively regulate haematopoietic stem and progenitor cells survival and differentiation.

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ABSTRACT

Allogeneic haematopoietic stem cell transplantation (HSCT) is the treatment of choice and a potentially curative approach for high-risk leukaemia. Sourcing incompletely matched donors would enable more patients to benefit of the procedure but also expose them to delayed haematopoietic and immune reconstitution and consequently to high morbidity and mortality. Therefore, there is a need to identify novel strategies to expedite haematopoietic recovery and thus improve the overall outcome of the procedure. Using a xenograft model, we have observed that when haematopoietic stem cell (HSCs) are transplanted in combination with $CD14^+$ monocytes, there is a massive increment in human haemopoietic engraftment. Moreover, monocytes, in particular $CD14^+CD16^-$ classical subset, support HSCs viability and prevent their differentiation *in vitro*. Both soluble factors and direct-cell contact interactions, such as JAG/NOTCH pathway, seems to be involved in HSCs-monocytes crosstalk. These results suggest that monocytes may be used to expedite the haemopoietic recovery after HSCT.

INTRODUCTION

Haematopoietic stem cell transplantation (HSCT) is a potentially curative therapeutic approach for a variety of haematopoietic malignancies. Preparative or conditioning regimens are administered as part of the procedure to reduce the tumour burden and provide sufficient immunoablation to prevent graft rejection. Although during the last decade reduced conditioning regimens have largely replaced conventional regimens, patients still incur in the risk of graft failure particularly when the HSC dose available is suboptimal. The prolonged haemopoietic ablation exposes patients to high-risk infections that represent a threat in the immediate post-transplant period[1] and impact on prolonged hospitalisation[2].

It has been shown that the administration of mega doses of $CD34^+$ cells can overcome graft failure in clinical situations in which the histo-incompatibility differences between donor and recipient are associated with increased risk of graft failure[3]. However, the doses required are often not available like in the case of cord blood (CB) transplantation[4]. Several approaches have been tested to expand HSCs *in vitro* using second-generation small molecules that inhibit HSCs differentiation and/or promote their self-renewal, including Notch ligands[5], nicotinamide[6] and StemRegenin-1[7]. Alternatively, mesenchymal stromal cells (MSC) have been used with the same purpose[8]. More clinical trials are warranted to understand whether approaches have been based on the use of T cells with 'veto' regulatory activity based on the evidence that the content of particular subset of T cells in the graft preparation was associated with better engraftment[9]. The immunological barrier between donor and recipient

could be overcome using donor-derived naïve CD8 T cells expanded against third-party alloantigens[10,11].

We have recently demonstrated the crucial role of myeloid cells in facilitating engraftment *in vivo* using animal models, whereby the administration of MSC-educated $CD11b^+$ cells accelerates haematopoietic reconstitution in bone marrow transplant recipients[12]. Although indirectly, these data are also supported by recent clinical trials in which MSC-conditioned CB units co-transplanted with unmanipulated units accelerated haematopoietic recovery, probably through MSC-expanded monocytes[8]. The innate immune system plays a critical role in tissue development, homeostasis, and repair of injured tissues. At the site of tissue injury, monocytes and macrophages are among the first responders and are required to orchestrate resolution of inflammation and healing[13]. There is plenty of evidence supporting their role in vascular remodeling, muscle and bone repair[14] and the possibility that they can be exploited therapeutically to favor tissue regeneration[15].

The characterization and function of monocytes/macrophages in the BM haematopoietic niche is largely unknown with evidence that they can regulate the mobilization of haematopoietic progenitors[16,17].

In the present study, we have demonstrated that human monocytes boost engraftment in a xenograft model and favour *in vitro* HCSs survival and maintenance of their undifferentiated phenotype.

MATERIALS AND METHODS

Cells isolation. $CD34^+$ progenitors from human CB units were obtained by Ficoll-Paque Plus (GE Healthcare Europe, Freiburg, Germany) separation of the mononuclear fraction followed by immunomagnetic selection using the CD34 MicroBeads kit (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's protocol with a median purity of 86.15% (range from 68% to 97.6%). $CD14^+$ monocytes and their subsets ($CD14^+CD16^-$ classical and $CD14^+CD16^+$ non-classical) were obtained from buffy coats using CD14 MicroBeads and Monocytes Isolation kit (Milteny Biotec) with a median purity of 96.25% for $CD14^+$, 63.33% for $CD14^+CD16^+$ and 89.6% for $CD14^+CD16^-$.
Purity has been determinate by flow cytometry using anti-CD34 PECy7 (clone 8G12; BD Biosciences Franklin Lakes, NJ, USA), CD14 FITC (clone M_φP9, BD Biosciences), and CD16 PE (clone B73.1, BD Biosciences).

Co-culture experiments. $CD34^+$ cells were cultured in StemSpan SFEN medium (Stem Cell Technologies; Grenoble, France) in 96 well-plate, alone or with monocytes ($CD14^+$, $CD14^+CD16^-$, or $CD14^+CD16^+$ subsets) at a ratio of 1:100. The co-cultures were performed both in direct contact and into transwells (0.4 um polyester membrane, Corning; NY; USA) with $CD34^+$ cells inside the transwell and monocytes in the well. After 48 hours cells were collected and analysed by flow cytometry for evaluating cell number, viability, and differentiation.

Flow cytometry. $CD34^+$ cells number was determined using single-platform method. Briefly, counting beads (CountBright™ absolute counting beads, Invitrogen™, Thermo Fisher Scientific, Waltham, Massachusetts, USA) were added before the acquisition to samples stained with CD34 PECy7 (clone 8G12, BD Biosciences) and the relative cell count was calculated following manufacturer's protocol. For analyses of $CD34^+$ cells apoptosis, cells were stained with CD34 PECy7 (clone 8G12, BD Biosciences) and AnnexinV/7-AAD (Apoptosis/Necrosis Detection Kit, Enzo Life Sciences, Farmingdale, NY, USA) following manufacturer's protocol. The percentage of $AnnexinV^+/7\text{-}AAD^{-/+}$ early and late apoptotic cells was evaluated by FACS.

For analyses of differentiation, cells were labelled with CD34 PECy7 (clone 8G12, BD Biosciences) and CD38 Alexa Fluor® 750 (clone LS198-4-3; Beckman Coulter Inc., Brea, CA, USA). The percentage of CD38 on $CD34^{+-}$ was determined compared to day 0.

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

Colony assay. Haematopoietic clonogenic assays were performed in 35mm low-adherent surface dishes (Thermo Fisher Scientific) using 1 ml/dish of MethoCult H4434 classic semisolid medium (StemCell™ Technologies) containing haematopoietic cytokines according to the manufacturer's instructions. Single-cell suspensions of $CD34^+$ cells cultured for 2 days alone or in presence of monocytes were plated in duplicate at $1x10^4$ cells per dish. Colonies were scored after 14 days of incubation based on the morphological criteria as erythroid

(BFU-E), granulocyte/erythrocyte/macrophage/megakaryocyte (CFU-GEMM) and granulocyte/macrophage (CFU-GM).

Gene expression. Total cellular RNA was isolated from $CD14^{+}CD16^{-}$ and $CD14^{+}CD16^{+}$ subsets of different donors using TRIzol™ reagent (Invitrogen, Thermo Fisher Scientific) according to manufacturer's protocol. One μg of total RNA was reversely transcribed using the SuperScript II Reverse Trascriptase (Invitrogen, Thermo Fisher Scientific).

Quantitative RT-PCR experiments were performed using Light Cycler 480II with Universal Probe Master system (Roche Diagnostics, Rotkreuz, Switzerland). Through the Software Probe Finder (Roche Diagnostics) the following primers for *JAG1* and *PTGS2* genes were designed: Left: 5'-GAATGGCAACAAAACTTGCAT-3' and Right: 5'-AGCCTTGTCGGCAAATAGC-3' for *JAG1* and Left: 5'-GCTTTTATGCTGAAGCCCR-3' and Right: 5'-TCCAACTC TGCAGACATT-3' for *PTGS2*.

ABL gene (left: 5'AGGAATCCAGTATCTCAGACGAA-3' and right:5'-GGAGGTCCTCGTCTTGGTG-3') was used as housekeeping gene. UPL probes number 42, 2 and 57 were used to detect genes. Gene expression relative to housekeeping gene was quantified by the ΔCt method, comparing classical with non-classical monocytes.

Inhibition of NOTCH and COX-2 pathways. 10.000 $CD34^{+}$ cells were cultured in StemSpan SFEN medium (StemCell™ Technologies) in a 96 well-plate, with $1x10^{6}$ $CD14^{+}CD16^{-}$ cells in the presence of 10 μM JAG1 inhibitor (DAPT, Sigma-Aldrich; Missouri, USA). DMSO

was used as control. The same set of experiments was also performed using a specific COX-2 inhibitor (NS-398, Cayman Chemical, Ann Arbor, Michigan; USA) at concentration of 100 μM. After 48 hours of culture, the percentage of CD38 positivity in CD34 cells was evaluated by flow cytometry as described before.

In vivo experiments. Procedures involving animal handling and care conformed to institutional guidelines (Italian Law D.L.vo 4 marzo 2014, n 26) and protocols approved by the local animal-health committee and authorized by the national authority. Animals were purchased from Charles River Laboratories (Calco, Italy).
For experiments of co-transplant, adult (10-12 weeks old) recipient CB17.Cg-Prkdc^{scid}Lyst^{bg} (SCID/beige) mice were irradiated (2.5 Gy) using a RADGIL X-Ray treatment unit (Gilardoni, Mandello del Lario, Italy) and treated with 200 mg/kg Fludarabine (Teva, Petha Tiqwa, Israel) by intraperitoneal administration, 48 h before transplantation[18]. 1 x 10^6 $CD34^+$ cells have been injected into the tail vein alone or with equal numbers of $CD14^+$. Human engraftment was evaluated in haematopoietic organs at 2 weeks after transplantation.
Differently, to evaluate the engraftment capacity of $CD34^+$ cells after *in vitro* co-culture, 1.5-2x10^5 $CD34^+$ cells, that have been cultured for 48 hours alone or with $CD14^+CD16^-$ monocytes, were transplanted into NOD/LtSz-scidIL2R$\gamma^{-/-}$ (NSG) mice previously irradiated (2.5 Gy). Mice were euthanized 4 weeks after transplantation to evaluate human chimerism.

Human engraftment evaluation. The engraftment of human cells was analysed at sacrifice on peripheral blood (PB) collected by tail

bleeding, on BM cells collected by flushing of femours and tibiae, and on splenocytes collected by smashing the spleens on a 70 µm cell strainer (Greiner Bio-One, Kremsmunster, Au). After lysis with ACK buffer (StemCell Technologies), the samples were stained with anti-mouse CD45.1 APC (clone A20, eBioscience, San Diego, CA, USA) and anti-human CD45 PE (clone HI30, BD Bioscience).

Statistical analyses. Data were analyzed using GraphPad Prism 7 (GraphPad Software, LA Jolla, CA, USA). Differences between groups were compared using a paired T test (for two groups comparison) and one-way ANOVA with the Bonferroni multiple comparison test (for more than two groups comparisons). All tests were two-sided alternative with a 5% significance level.

RESULTS

$CD14^+$ cells expedite engraftment of human $CD34^+$ in a xenograft model.

Starting from the evidence that murine $CD11b^+$ cells accelerate haematopoietic reconstitution in transplant recipients[12], we tested the role of human monocytes in a xenograft model. $CD34^+$ HSC cells have been injected alone (HSC only group) or with equal numbers of $CD14^+$ (HSC/CD14 group) into SCID/beige recipient mice pre-conditioned with sublethal irradiation and Fludarabine[18] (Fig 1A). The engraftment of human cells was monitored in the BM, PB and spleen at 2 weeks. The group given $CD14^+$ cells showed a higher proportion (17.7 ± 3.8% *vs.* 3.0 ± 1.4%, P=0.0007) and absolute number (702.7 ± 152.9 *vs* 105.1 ± 41.3 x 10^3, P=0.001) of $hCD45^+$ mononucleated cells in BM as compared to the control group receiving $CD34^+$ cells alone. Such an increment was also observed in PB (9.4 ± 3.2% *vs.* 1.2 ± 0.5%, and 97.8 ± 33.8 *vs* 14.0 ± 7.6 $hCD45^+$ mononucleated cells/μl, P=0.02) and spleen (1.8 ± 0.6% *vs.* 0.2 ± 0.1%, P=0.01) (Fig. 1B and C). Human chimerism analysis did not detect any $CD14^+$ cells engraftment in BM at 2 weeks, thus ruling out their direct contribution to the increased number of engrafted cells (data not shown).

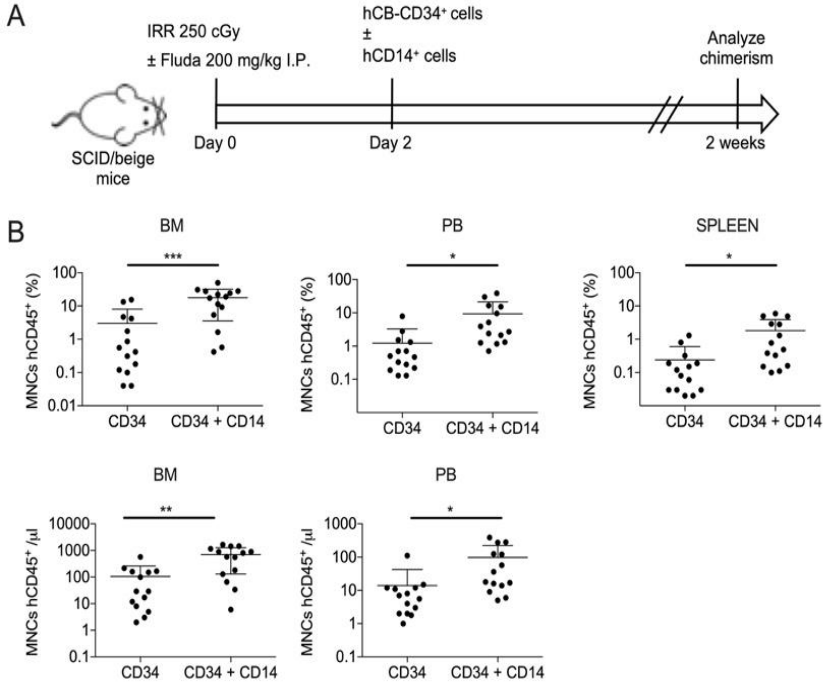


Figure 1. Human CD14$^+$ cells expedite engraftment of human haematopoiesis in a xenograft model. *SCID/beige recipients were conditioned by irradiation (250 cGy) and i.p. administration of Fludarabine (200 mg/kg) and injected 48 hours later with 1x10^6 human CD34$^+$ cells, either alone (HSC only group) or in combination with 1x10^6 CD14$^+$ cells (HSC/CD14 group). 14 days after the transplant, BM, PB, and spleen were analyzed by FACS.* ***(A)*** *Schematic representation of the experiment.* ***(B)*** *Frequency (on the top) and absolute number (on the bottom) of human haematopoietic cells (huCD45$^+$) in BM, PB, and spleen are represented. Each point represents a single mouse and bar represents the mean value of nine independent experiments ± SD, * p<0.05, **p<0.01, *** p<0.001, Paired t test.*

$CD14^+$ cells increase *in vitro* $CD34^+$ cell survival and retain $CD34^+CD38^-$ multipotent stem cells subpopulation.

To examine the effects exerted by $CD14^+$ cells directly on HSCs, $CD34^+$ cells enriched from CB were cultured in StemSpan^{TM} SFEM medium for 48 hours in the presence or absence of $CD14^+$ isolated from the PB of healthy donors. As shown in Figure 2, a 1.6 ± 0.2-fold increase in $CD34^+$ cell number was found in co-cultures compared to $CD34^+$ cells cultured alone ($P<0.0001$). Co-cultures with PBMCs depleted from $CD14^+$ fraction did not impact on HSCs number ($P=0.834$, data not shown). A transwell assay suggest that cell contact may be important for $CD14^+$ cells to exert their effect on $CD34^+$ cells. In fact, in transwell culture the effect was impaired (fold change: 1.3 ± 0.3, $P=0.008$), thus indicating a primary but not exclusive role for direct cell contact (Fig 2A). The presence of $CD14^+$ cells increased survival of $CD34^+$ cells evaluated by AnnV/7AAD staining ($AnnV^-/7AAD^-$ live cells: 71.8 ± 9.3% *vs* 53.6 ± 3.3% in $CD34^+$ alone, $P=0.035$) (Fig 2B). Moreover, $CD14^+$ cells consent the retention of higher percentage of $CD34^+CD38^-$ multipotent haematopoietic stem cells compared to control (percentage of $CD38^-$ on $CD34^+$ cells: 17 ± 3.9% *vs.* 9.4 ± 2.6% in $CD34^+$ alone, $P=0.001$), maintaining a phenotype similar to that of freshly isolated $CD34^+$ (13.6 ± 1.7%, $P=0.191$; Fig. 2C).

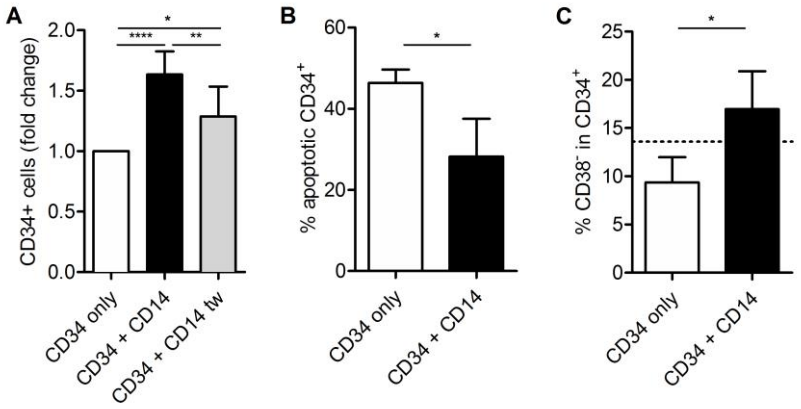


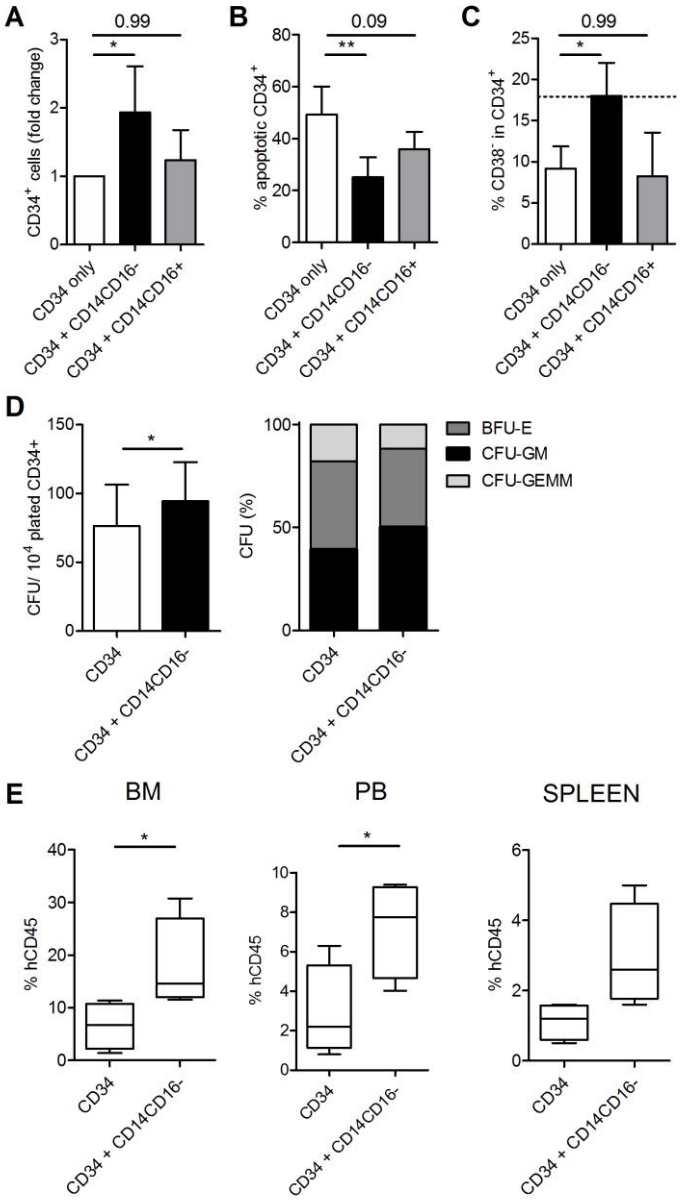
Figure 2. CD14$^+$ monocytes improve in vitro CD34$^+$ cells survival and stemness. *CD34$^+$ cells were cultured in the presence or absence of CD14$^+$ for 48 hours, in direct contact or in a transwell assay. (**A**) CD34$^+$ cell numbers were quantified by FACS using single-platform method. The data are depicted as fold changes normalized to CD34$^+$ cell numbers in CD34$^+$ only cultures. Mean of at least four independent experiments ± SD, * p<0.05, ** p<0.01, **** p<0.0001 One-way ANOVA with the Bonferroni multiple comparison test. (**B**) Percentage of CD34$^+$ apoptotic cells was measured by assessing AnnexinV/7AAD positivity after culture with or without CD14$^+$ cells. Mean of three independent experiments ± SD, * p<0.05, Paired t test. (**C**) Percentage of CD38$^-$ in CD34$^+$ cells was determined at day 0 and after 2 days in culture with or without CD14$^+$ cells. Mean of six independent experiments ± SD. The dashed line indicates the mean value for freshly isolated CD34$^+$. * p<0.05, One-way ANOVA with the Bonferroni multiple comparison test.*

$CD14^+CD16^-$ classical monocyte subset is primary responsible for increasing $CD34^+$ cell survival and preventing their differentiation.

$CD14^+$ cells from PBMCs can be subdivided based on CD14 and CD16 expression[19]. To individuate which monocyte subtype guarantees a better maintenance of $CD34^+$ cells we separated non-classical ($CD14^+CD16^+$) and classical ($CD14^+CD16^-$) subsets. Co-culture of $CD34^+$ cells with $CD14^+CD16^-$ cells resulted in 2 ± 0.7–fold increase in $CD34^+$ yield, compared to culture of $CD34^+$ cells alone (P=0.013). Co-culturing $CD34^+$ cells with $CD14^+CD16^+$ cells led to only a modest but not significant increase in $CD34^+$ (1.2 ± 0.4-fold change respect to $CD34^+$ alone, P>0.09; Fig. 3A). Survival of $CD34^+$ was significantly increased in the presence of classical monocytes (74.9 ± 7.7% *vs* 50.9 ± 10.9% in $CD34^+$ alone, P=0.002). On the contrast, $CD14^+CD16^+$ cells only partially protect $CD34^+$ from apoptosis, without any significant difference in viability respect to $CD34^+$ alone (64.1 ± 6.7%, P=0.094) (Fig. 3B). Moreover, the uncommitted $CD38^-CD34^+$ cells were maintained after culture in the presence of $CD14^+CD16^-$ cells (18.0 ± 4.0% *vs.* 17.9 ± 2.1% in $CD34^+$ at day 0, P>0.99) but not with $CD14^+CD16^+$ (8.2 ± 5.3%, P=0.013) (Fig. 3 C).

To further test the ability of $CD14^+CD16^-$ to preserve the haematopoietic progenitor cell function of $CD34^+$ cells we utilized an *in vitro* clonogenic colony-forming unit (CFU) assay. Equal numbers of $CD34^+$ cells that had been previously cultured for 48 hours with or without monocytes were plated in methylcellulose and haematopoietic colonies were counted after 14 days. Co-culture of $CD34^+$ cells with

$CD14^+CD16^-$ resulted in an increase in haematopoietic colonies formation compared to $CD34^+$ cells cultured alone (94.4 ± 28.4 vs. 76.3 ± 30.1, P=0.028). To examine an effect on lineage specification, the number of CFU-GM, CFU-GEMM, and BFU-E was determined. The overall distribution of CFU-GM, CFU-GEMM, and BFU-E after 2 days of co-culture with $CD14^+CD16^-$ cells was not biased, suggesting that monocytes do not push $CD34^+$ towards a specific lineage, but affect the overall yield of haematopoietic progenitors stem cells (HPSC) (Fig. 3 D). A xenotransplantation assay in NSG mice was then used to evaluate the *in vivo* repopulating function of $CD34^+$ cells previously co-cultured with $CD14^+CD16^-$, showing higher levels of engraftment in BM respect to $CD34^+$ cells cultured alone (17.9 ± 8.8% *vs.* 6.6 ± 4.5%, P=0.028) and higher capacity to colonize other haematopoietic tissues, in particular PB (7.2 ± 2.5% *vs.* 2.9 ± 2.4%, P=0.039) (Fig. 3E). Moreover, the percentage of $CD34^+$ within donor engraftment was almost double in the group of mice receiving $CD34^+$ cells pre-cultured with monocytes (11.7 ± 6.0% *vs.* 6.7 ± 2.8% in $CD34^+$ cells alone).


A
0.99
*
CD34+ cells (fold change)
CD34 only
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B
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% apoptotic CD34+
C
0.99
*
% CD38- in CD34+
D
*
CFU/ 10^4 plated CD34+
CFU (%)
BFU-E
CFU-GM
CFU-GEMM
CD34
CD34 + CD14CD16-
E
BM
PB
SPLEEN
% hCD45

Figure 3. Classical monocytes positively influence CD34$^+$ cell survival and stemness. A*) CD34$^+$ cells were cultured in the presence or absence of classical (CD14$^+$CD16$^-$) or non-classical (CD14$^+$CD16$^+$) monocytes for 2 days. The data are depicted as fold changes normalized to CD34$^+$ cell numbers of CD34$^+$ only cultures. Mean of at least six independent experiments ± SD, * p<0.05, One-way ANOVA with the Bonferroni multiple comparison test. (**B**) Percentage of CD34$^+$ apoptotic cells was measured after culture with or without CD14$^+$CD16$^-$ and CD14$^+$CD16$^+$. Mean of five independent experiments ± SD, ** p<0.01, One-way ANOVA with the Bonferroni multiple comparison test. (**C**) Percentage of CD38$^-$ in CD34$^+$ cells was determined in culture with or without monocytic subsets. Mean of five independent experiments ± SD. The dashed line indicates the mean value for freshly isolated CD34$^+$. * p<0.05, one-way ANOVA with the Bonferroni multiple comparison test. **(D)** Clonogenic potential of CD34$^+$ cells previously cultured alone or with CD14$^+$CD16$^-$ cells. Total number of colonies (on the left) and distribution of CFU-GM, CFU-GEMM, and BFU-E (on the centre) were reported. Mean of five independent experiments performed in duplicate ± SD, * p<0.05, Paired t test. **(E)** Analysis of multi-organ human chimerism in NSG mice transplanted 4 weeks before with CD34$^+$ cells previously co-cultured with or without CD14$^+$CD16$^-$ cells. Each point represents a single mouse and bar represents the mean value. Four independent experiments were performed, * p<0.05, Paired t test.*

JAG/NOTCH and COX-2/PGE2 pathways are involved in maintenance of HSCs stemness mediated by $CD14^+CD16^-$

In order to explore mechanisms responsible for the positive influence exerted by $CD14^+CD16^-$ on $CD34^+$ cells, we checked already published microarray analysis on human monocyte subsets[20,21]. We focused on two genes up-regulated in $CD14^+CD16^-$ population and known to be involved in HSCs maintenance, *JAG1* and *PTGS2*. We then validated by q-RT-PCR the expression of these genes on our cells. $CD14^+CD16^-$ express higher levels of *JAG1* compared to $CD14^+CD16^+$ subset (2^{-DCt}: 0.47 ± 0.44 *vs*. 0.14 ± 0.13, P=0.043) (Fig. 4A). *PTGS2* was also expressed at higher levels by $CD14^+CD16^-$ compared to $CD14^+CD16^+$ cells (2^{-DCt}: 4.75 ± 2.43 *vs*. 0.69 ± 0.37, P=0.025) (Fig. 4B).

Addition of DAPT, a specific inhibitor of Notch signaling, to the co-cultures, reduced the capacity of $CD14^+CD16^-$ cells to maintain the $CD34^+$ stemness (percentage of $CD38^-$ on $CD34^+$ cells: 9.36 ± 3.02% *vs.* 15.47 ± 5.65% in co-cultures without DAPT, P=0.016). However, the positive influence of classical monocytes was not completely abrogated by DAPT (percentage of $CD38^-$ on $CD34^+$ cells cultured alone: 4.94 ± 2.83%), suggesting that other mechanisms could be involved. Regarding the COX-2/PGE2 axis, the addition of COX-2 specific inhibitor NS398 to co-cultures of $CD34^+$ with $CD14^+CD16^-$ cells do not significantly alter the proportion of $CD34^+CD38^-$ (percentage of $CD38^-$ on $CD34^+$ cells: 18.96 ± 8.50% *vs.* 21.82 ± 6.74% in co-cultures without NS398, P>0.99).

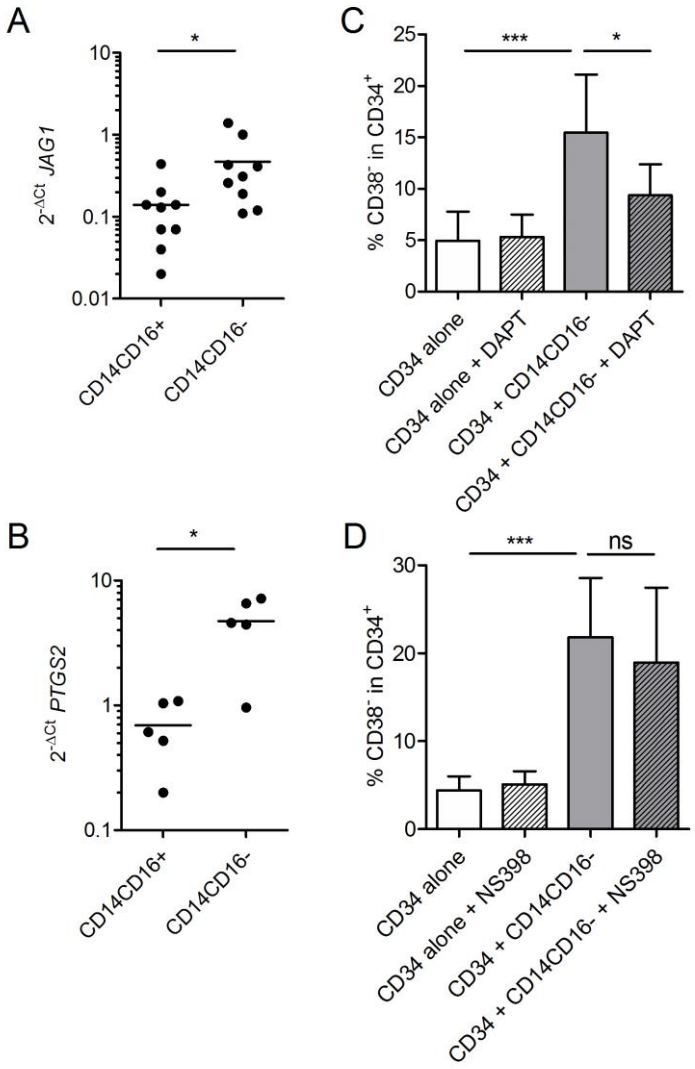

Figure 4. Role of JAG1/NOTCH and COX-2/PGE2 in CD14^{+}CD16^{-}/HSCs crosstalk ***(A)*** *qRT-PCR showing expression levels of JAG1 on CD14^{+}CD16^{+} and CD14^{+}CD16^{-} subsets. Each point represents a single sample from nine different donors analysed and bar represents the mean value. * p<0.05, Paired t test.* ***(B)*** *qRT-PCR showing expression levels of PTGS2 on CD14^{+}CD16^{+} and CD14^{+}CD16^{-} subsets from five donors. * p<0.05, Paired t test.* ***(C)*** *Percentage of*

*$CD38^-$ in $CD34^+$ cells was determined after 2 days in culture with or without $CD14^+CD16^-$ cells, in the presence or absence of 10 µM DAPT. Mean of four independent experiments ± SD. * $p<0.05$, *** $p<0.0001$, One-way ANOVA with the Bonferroni multiple comparison test. (D) Percentage of $CD38^-$ in $CD34^+$ cells was analyzed after 2 days in culture with or without $CD14^+CD16^-$ cells, in the presence or absence of 100 µM NS398. Mean of three independent experiments ± SD. *** $p<0.0001$, One-way ANOVA with the Bonferroni multiple comparison test.*

DISCUSSION

Survival of haematopoietic progenitor cells and retention of $CD34^+CD38^-$ multipotent stem cells subpopulation is crucial to avoid BM failure after BM transplantation and to consent the *ex vivo* expansion of haematopoietic stem and progenitor cells for cellular therapy.

At present, haematopoietic stem cell transplantation is usually performed with total mobilized peripheral blood mononuclear cells (PBMC) or BM rather than purified $CD34^+$ progenitor cells. Therefore, the effect of different haematopoietic effector cells on HSPCs engraftment is unclear. A great attention has been paid during years on identification of cells with facilitating potential to enhance HSC engraftment. One population is the $CD8^+/TCR^-$ facilitating cells that increase engraftment of purified allogeneic HSCs without causing GVHD[22]. Among these, a specific role of plasmacytoid precursor dendritic cells subpopulation has been reported[23].

Here, we shown the possible role of monocytes/macrophages in supporting the Short-Term HSCs survival *in vitro* and their engraftment *in vivo*.

The presence of human $CD14^+$ monocytic cells isolated from PB induces a significant increment of CB-$CD34^+$ cells engraftment in the haematopoietic organs (BM, spleen, PB) of immunodeficient mice. We used a xenograft model that we have previously generated[18], consisting in SCID/beige mice pre-conditioned with sub-lethal irradiation and Fludarabine. This model has guaranteed an extensive and rapid engraftment of human haematopoiesis, already 2 weeks after

the transplantation. After 2 weeks, $CD14^+$ cells cannot be found in mouse BM, suggesting that the enhanced engraftment is completely ascribable to CB-$CD34^+$, thus highlighting that monocytic cells have only a supportive role for stem cell engraftment. These results are in agreement with recent demonstration that administration of MSC-educated murine $CD11b^+$ cells accelerates haematopoietic reconstitution in BM transplant recipients[12].

To further investigate the *in vivo* results, we have settled an *in vitro* co-culture system. *In vitro* experiments reveal that $CD14^+$ monocytic cells induces an increment in $CD34^+$ number preserving their viability, compared to $CD34^+$ cells cultured alone. Moreover, the presence of monocytes/macrophages prevent their differentiation, retaining a higher proportion of $CD34^+CD38^-$ multipotent stem cells.

Tissue-resident macrophage have been found in haematopoietic niche near to HSCs where they regulate maintenance of HSCs/progenitors in the niche during homeostasis[24]. Macrophages depletion in mice induce mobilization. Thus, besides being involved in HSC retention in the BM, monocytes/macrophages seem to be implicated also in supporting the survival of HSC. A similar role for $CD14^+$ cells in modulation of haematopoietic stem and progenitor cells survival has recently reported also by Eideveld E et al[25].

$CD14^+$ cells in PB are a mixed population of cells that include classical ($CD14^+CD16^-$), non-classical ($CD14^+CD16^+$), and intermediated subsets ($CD14^{++}CD16^+$)[26]. Among the two most represented subsets, it is the $CD14^+CD16^-$ subset of classical monocytes that is primary involved in positive regulation of HCSs survival and in maintenance of their undifferentiated phenotype. The

difference in positive influence on $CD34^+$ cells between classical and non-classical monocytes could be attributable to their different physiological functions[20]. Indeed, $CD14^+CD16^-$ intervene especially during inflammation with an anti-inflammatory activity, generating a more permissive environment[19,27].

The effect exerted by monocytes on support of cultured $CD34^+$ cells is due to different mechanisms, including both direct cell-to-cell contact pathways and soluble factors. Indeed, a greater increase is obtained when monocytes are in straight contact with $CD34^+$ cells compared to the transwell system, where the positive feedback of monocytes is less evident but still present. Moreover, the two described monocytic cells functions (increasing $CD34^+$ cells survival and retention of undifferentiated multipotent stem cells subpopulation) may be caused by independent factors. Among direct contact mechanisms, Notch pathway, already involved in the maintenance of HSC within the niche[28], could be implicated. Classical monocytes have a higher expression of the specific Notch ligand *JAG1* compared to non-classical monocytes. Other Notch ligands (*JAG2*, *DLL1* and *DLL4*) are not significantly expressed by neither of the monocytic subset (data not shown). The use of DAPT, a gamma-secretase inhibitor (GSI), induces a decrease in $CD38^-$ compartment within $CD34^+$ cells cultivated in the presence of $CD14^+CD16^-$, suggesting that this pathway is involved in monocytes-mediated HSCs maintenance *in vitro*.

Among the soluble factors we focused on the role of prostaglandin E2 (PGE2), released by cyclooxygenase COX-2 which is up-regulated on classical monocytes. *Ex vivo* treatment of murine BM cells with PGE2

promote HSC renewal and engraftment[29,30]. In our co-culture conditions, blocking of COX-2 expressed by $CD14^{+}CD16^{-}$ monocytes using the specific inhibitor NS398 did not alter the percentage of $CD38^{-}CD34^{+}$ cells. This result seems to be in contradiction with promoting effect of PGE2 on haematopoiesis. In reality, in co-cultures PGE2 can affect besides HSCs also monocytic cells, altering their capacity to support stem cells. Indeed, the production of regulatory cytokines by monocytes/macrophages could be modulated by treatment with COX-2 inhibitor[31,32]. It is known that *in vivo* administration of PGE2 preferentially expands short-term HSC/multipotent progenitors, through effects on other cells within BM niche, such as osteoblasts and osteoclasts[33]. These indirect effects of PGE2 on niche cells, such as monocytes/macrophages, may explain how our results differ from data on HSCs treatment *ex vivo*. Our data cannot at this stage determine whether direct and/or indirect (on monocytic cells) effects of PGE2 result in the HSCs effect we observed.

In conclusion, we have shown that $CD14^{+}$ cells provide support to HSPC cells leading to increased survival of $CD34^{+}$ cells and maintenance of their undifferentiated phenotype. These observations can explain the increased support to $CD34^{+}$ cells *in vivo* engraftment and unravel a novel role for $CD14^{+}$ cells in survival of haematopoietic progenitors.

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CHAPTER 4

Acute myeloid leukaemia niche regulates response to L-asparaginase.

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ABSTRACT

Eradicating the malignant stem cell is the ultimate challenge in the treatment of leukaemia. Leukaemic stem cells (LSC) hijack the normal haemopoietic niche, where they are mainly 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 explored the susceptibility of AML-LSC to ASNase as well as the role of the two major cell types that constitute the bone marrow (BM) microenvironment, i.e., mesenchymal stromal cells (MSC) and monocytes/macrophages. Whilst ASNase was effective on both $CD34^{+}CD38^{+}$ and $CD34^{+}CD38^{-}$ LSC fractions, MSC and monocytes/macrophages partially counteracted the effect of the drug. Indeed, the production of cathepsin B, a lysosomal cysteine protease, by BM monocytic cells and by AML cells classified as French-American-British M5 is related to the inactivation of ASNase. Our work demonstrates that, while MSC and monocytes/macrophages may provide a protective niche for AML cells, ASNase has a cytotoxic effect on AML blasts and, importantly, LSC subpopulations. Thus.

these features should be considered in the design of future clinical studies aimed at testing ASNase efficacy in AML patients.

INTRODUCTION

Acute myeloid leukaemia (AML), a heterogeneous blood cancer, represents the most frequently diagnosed leukaemia in adults (25%) and accounts for 15–20% cases in children (Siveen et al, 2017). Despite continuous progress in the comprehension of AML pathogenesis and in AML diagnosis and stratification, patients are still subject to a high rate of relapse and poor overall survival (Siveen et al, 2017).

Biologically, AML cells could be represented as a hierarchy, at the top of which are leukaemic stem cells (LSC) (Bonnet & Dick, 1997). 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 (Bonnet & Dick, 1997; Hope et al, 2004; Eppert et al, 2011). Moreover, LSC refractoriness to conventional chemotherapies determines AML relapse (Ishikawa et al, 2007; Pollyea & Jordan, 2017; Siveen et al, 2017). This is due to their peculiar characteristics (e.g., quiescence and expression of efflux

pumps) (Siveen et al, 2017) and to the protection provided by the bone marrow (BM) microenvironment (Ninomiya et al, 2007; Korn & Mendez-Ferrer, 2017). 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 the survival of AML blasts and LSC upon chemotherapy through several mechanisms, including release of factors (e.g., CXCL12/CXCR4 and VCAM1/VLA4 axis), modification of leukaemic metabolism and enhancement of MYC expression (Korn & Mendez-Ferrer, 2017). In addition, BM contains various mature immune cell types, such as T and B cells, dendritic cells and macrophages, that participate in a protective environment for leukaemic cells (Isidori et al, 2014; Riether et al, 2015). According to the importance of LSC in AML pathogenesis, therapeutic approaches aiming at targeting LSC are necessary to eradicate these cells, thus preventing their further evolution and consequent AML relapse (Pollyea & Jordan, 2017). L-Asparaginase (ASNase) is a deamidating enzyme that catalyses the hydrolysis of L-asparagine and L-glutamine, causing L-asparagine depletion in the blood and BM (Steiner et al, 2012; Tong et al, 2013), L-glutamine reduction (Steiner et al, 2012) and leukaemic cell death

under conditions deprived of these amino acids (Asselin et al, 1989; Willems et al, 2013). Although ASNase has been widely exploited in the treatment of acute lymphoblastic leukaemia (ALL) since the 1960s, (Egler et al, 2016) it has only been partly investigated in AML both *in vitro* and in clinical trials (Emadi et al, 2014). Despite the evidence of a higher efficacy of ASNase on ALL than AML blasts, (Zwaan et al, 2000; Okada et al, 2003) some specific subtypes and a subgroup of AML were reported to be more susceptible to ASNase as compared to others (Zwaan et al, 2000; Okada et al, 2003; Bertuccio et al, 2017). It has been recently demonstrated that AML cells are particularly addicted to glutamine for their energetic and biosynthetic metabolism (Willems et al, 2013; Jacque et al, 2015; Matre et al, 2016). Consequently, *Erwinia* ASNase, with 10-fold higher glutaminase activity than *E. coli* ASNase (Avramis, 2012), understandingly exhibits greater cytotoxicity on AML cells (Willems et al, 2013). Resistance to ASNase has been suggested to occur in ALL due to the L-asparagine and L-glutamine secreted by MSC and adipocytes surrounding blasts in BM (Iwamoto et al, 2007; Ehsanipour et al, 2013). A further mechanism proposed as a cause of therapy failure is the inactivation of ASNase mediated by cellular lysosomal cysteine proteases (Patel et al, 2009). Microenvironmental

cells, such as macrophages, can produce cathepsin B (CTSB) and contribute to ASNase turnover *in vivo* in mice (van der Meer et al, 2017). In this study, we aimed to investigate the effects of ASNase on AML blasts, focusing on the role of different players of the leukaemic microenvironment, e.g., LSC, MSC and monocytes/ macrophages, in the susceptibility to ASNase. Herein, we demonstrated that, while MSC and monocytes/macrophages contribute to a protective microenvironment for AML cells, ASNase exerts an effect on LSC subpopulations, as well as AML leukaemic blasts.

METHODS

Cell lines, patients and healthy donor samples. Peripheral blood or BM samples of 37 AML patients at diagnosis were collected after having obtained informed consent. Mononuclear cells were isolated using a Ficoll-PaqueTM Plus (GE Healthcare, Little Chalfont, Buckinghamshire, UK) density gradient separation and used either fresh or after cryopreservation for experiments. The study was approved by the Ethics Committee of San Gerardo Hospital-Monza (LMA ASNASE 2900). Clinical and biological patients' features are reported in Table SI. Details of cell lines and healthy donor samples are described in Data S1.

Reagents and compounds. We tested two formulations of L-asparaginase: *E. coli* ASNase (Kidrolase) and E. chrysanthemi ASNase (Erwinase) (Jazz Pharmaceuticals, Dublin, Ireland). StemRegenin1 (SR1) and UM729 (StemCell^{TM} Technologies, Vancouver, BC, CA) were used at a final concentration of 250 nmol/L and 1 µmol/L, respectively.

ASNase cytotoxicity. To determine the half maximal inhibitory concentration (IC50) of each ASNase formulation on AML cell lines,

$4x10^4$ cells/well 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 h of treatment, live cells were counted by flow cytometry and the IC50 was calculated using CompuSyn Software (www.combosyn.com). For primary AML samples, $2x10^5$ cells/well 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 h (Data S1). These experiments were also performed in LSC supportive culture conditions using complete medium supplemented with SR1 and UM729.

Real-time quantitative reverse transcription polymerase. chain reaction (RQ-PCR) RQ-PCR assays were used to determine asparagine synthetase (ASNS) expression in healthy donor (HD)- and AML-MSC, and cathepsin B (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 Data S1.

Degradation of ASNase. Washed cell pellets from primary healthy or AML BM samples were lysed by freeze-thawing in digestion buffer (50 mmol/L trisodium citrate buffer, pH 4.5, 5 mmol/L Dithiotreitol), clarified by centrifugation and stored at -80°C. ASNase (7.5 IU/mL) was incubated overnight at 37°C with 20 µg of whole-cell lysate. For inhibition of ASNase cleavage, lysates were incubated with protease inhibitor cocktail P8340 (PIC; Sigma-Aldrich, St. Louis, MO, USA) or CTSB-specific inhibitor Ca-074 (10 µmol/L; Sigma-Aldrich) before the addition of ASNase. After incubation, the residual ASNase activity was measured by spectrophotometric determination of the released ammonia after reaction with Nessler's reagent (Data S1).

Statistical analyses. Data were analyzed using GraphPad Prism 7 (GraphPad Software, LA Jolla, CA, USA). Differences between groups were compared with the Mann-Whitney test or Wilcoxon matched-pairs signed rank test in the case of matched values. All tests were two-sided with a 5% significance level.

RESULTS

Patient-derived AML cells are susceptible to ASNase

We tested the inhibitory effect on cell proliferation of two different formulations of ASNase (*E. coli* and *Erwinia* ASNase) on different AML cell lines (THP-1, KG-1 and HL-60) and on the 697 ALL cell line, used as a control. The IC50 of *Erwinia* ASNase was lower than that of *E. coli* ASNase for each cell line tested (Fig 1A), particularly for AML lines (*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). The superior efficacy of *Erwinia* ASNase was also observed in all AML cell lines tested in terms of induction of apoptosis. Indeed, *Erwinia* ASNase was able to induce apoptosis in THP-1, KG-1 and HL-60 at comparable levels to that of *E. coli* ASNase, used at doses that were 4-, 5- and 8-fold lower, respectively, than the latter (data not shown). The effects of *Erwinia* ASNase on primary AML samples representative of various leukaemia subtypes according to the French-American-British (FAB) classification (Table SI) were evaluated. Within specimens, we distinguished between blast and non-blast populations according to side scatter profile combined with CD45 intensity. Interestingly,

treatment with 1 IU/ml ASNase for 48 h caused a significant decrease in the number of live cells (median reduction treated versus untreated: 47.35%, P < 0.0001) within the AML blast population, along with an increase in the percentage of apoptotic cells (median apoptosis treated vs. untreated: 47.53% vs. 22.33%, P < 0.0001) (Fig 1B). Instead, the number of nonblast cells was only minimally reduced after exposure to ASNase (median reduction treated versus untreated: 5.62%, P = 0.0286) and the percentage of apoptosis after treatment was almost unaffected (Fig 1B). Comparing the 3 AML FAB subgroups that included more than 3 samples (FAB M1/M2, M4 and M5), there were no statistically significant differences with respect to the median reduction of live cell number and the median percentage of apoptosis of blasts after treatment (Figure S1).

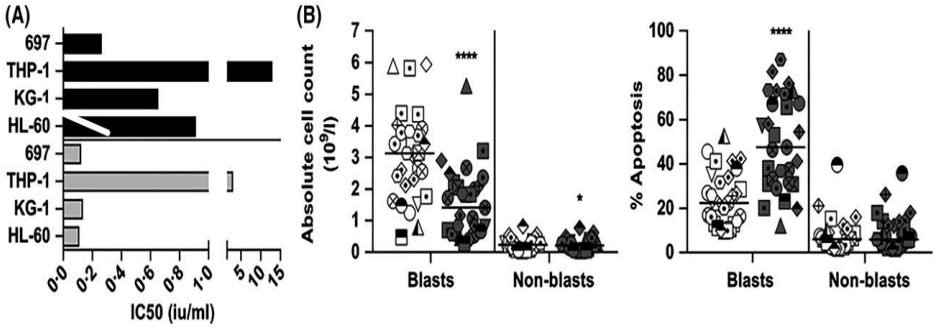

***Fig 1**. ASNase cytotoxicity on AML cell lines and on primary AML patient-derived cells. (**A**) Half maximal inhibitory concentration (IC50) values of E. coli (black) and Erwinia (grey) ASNase obtained for 697, THP-1, KG-1 and HL-60 cell lines, evaluating the reduction of cell number after 48 h of treatment. (**B**) Cell viability of primary acute myeloid leukaemia (AML) patient-derived cells after incubation for 48 h without (white) or with ASNase (1 IU/mL) (grey). We evaluated within blast and non-blast populations the absolute live cell count (x10^9/l) (on the left) and the percentage of apoptotic cells (on the right). Each symbol represents an individual AML patient (mean of technical triplicates). Bar indicates the median for each group. 29 independent experiments performed on 17 different patients are shown. *P < 0.05; ****P < 0.0001: Wilcoxon matched pairs signed rank test.*

ASNase affects leukaemic precursors within patient-derived AML cells

Next, in order to understand the ability of ASNase to target AML tumor-initiating cells, the cytotoxic effect of ASNase on leukaemic clonogenic cells within primary AML specimens was evaluated. 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 a low dose of ASNase (0.01 IU/mL) significantly reduced the clonogenic potential of AML cells as compared to untreated controls (median colony number treated vs. untreated: 2.75 vs. 28.75, P = 0.0001) (Fig 2A). Notably, colony formation was completely blocked by higher drug concentrations (0.1–1 IU/mL) (data not shown). Moreover, the effects of the drug on LSC subpopulations within AML samples, identified according to the expression of CD34 and CD38 markers, (Eppert et al, 2011) were investigated. Notably, the $CD34^+$ population was significantly susceptible to 48h treatment with 1 IU/mL ASNase ($P < 0.0001$ versus untreated control). In particular, a significant decrease in the number of live cells after treatment was observed in both $CD34^+CD38^+$ (median reduction treated versus untreated: 57.97%, $P < 0.0001$) and $CD34^+CD38^-$ subpopulations (median reduction treated versus untreated: 53.13%, $P < 0.0001$) (Fig 2B, top). Percentages of live cells after treatment in these subpopulations were comparable to those obtained on the bulk population of the same sample ($P = 0.4282$ calculated by Friedman test, Fig 2B, bottom). To deeply investigate the specific effect of ASNase on LSC, the culture conditions of primary AML specimens were modified by adding the SR1 and UM729 small molecules, which

have been previously described for their capability to better maintain the survival and stemness of AML-LSC *in vitro* (Pabst et al, 2014). As shown in Figure S2, the two compounds acted on the $CD34^+CD38^-$ fraction, significantly enhancing their viability as compared to a control population incubated without small molecules (median fold change of $CD34^+CD38^-$ in LSC supporting culture conditions versus control: 1.38, P = 0.0244). Of note, also in these LSC supportive culture conditions, $CD34^+CD38^+$ and $CD34^+CD38^-$ subpopulations displayed high sensitivity to ASNase (median $CD34^+CD38^+$ reduction treated versus untreated: 41.45%, P = 0.0015; median $CD34^+CD38^-$ reduction treated versus untreated: 46.71%, P = 0.0005). Similarly, the drug effect was maintained also on the bulk population (P = 0.0005 versus untreated) (Fig 2C). Furthermore, we observed a concomitant reduction of miR-126, a regulator involved in governing LSC self-renewal and quiescence (Lechman et al, 2016), for four out of five AML samples treated with ASNase in LSC-supporting culture conditions (Fig 2D).

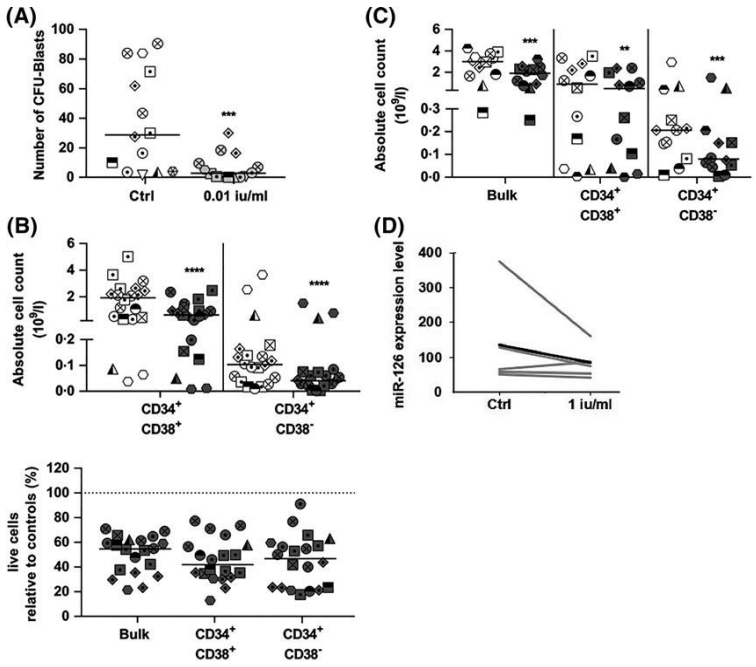

Fig 2. *ASNase treatment affects primitive populations within primary AML patient-derived samples. (**A**) The effect of the drug on the clonogenic potential of 9 primary acute myeloid leukaemia (AML) patient-derived samples was assayed after 14 days of culture on methylcellulose without (white) or with 0.01 IU/mL of ASNase (grey). Numbers of colony-forming unit (CFU)-Blasts (mean of technical duplicates) counted in 14 independent experiments are shown. (**B**) The effect of the drug on cell viability was nalysed also in primitive $CD34^{+}CD38^{+}$ and $CD34^{+}CD38^{-}$ fractions of 9 primary AML patient-derived samples. We evaluated the absolute live cell count (9109/l) (top) and the proportion of live cells in treated samples relative to the*

*vehicle control (bottom). 20 independent experiments are shown. The dotted horizontal line (100%) represents the control. P = 0.4282: Friedman test. (**C**) Primary AML patient-derived cells were incubated without (white) or with (1 IU/mL) ASNase (grey) in the presence of SR1 and UM729. The absolute live cell count (x10^9/l) 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. (**D**) miR-126 expression levels evaluated by quantitative droplet digital polymerase chain reaction in primary AML patient-derived cells after being incubated for 48 h without (Ctrl) or with 1 IU/mL ASNase in the presence of SR1 and UM729. 5 independent experiments performed on 5 different patients (grey lines) and the mean (black line) are shown. Each symbol represents an individual AML patient. **P < 0.01; ***P < 0.001; ****P < 0.0001: Wilcoxon matched-pairs signed rank test (Panels A, B top, and C).*

MSC show a protective role on AML cells against ASNase cytotoxicity by asparagine synthetase expression

In order to elucidate whether the BM microenvironment could exert an effect against the action of ASNase on AML blasts, primary AML samples were maintained in culture in the presence either of a normal

or of a patient-derived MSC layer and treated with ASNase. The effect due to the coculture with MSC was determined, comparing both 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 the respective untreated control. MSC derived from both healthy and AML BM were poorly sensitive to ASNase. Indeed, neither cell growth nor apoptosis were affected after exposure for 48 h at the higher ASNase concentration tested (3 IU/mL) (Figure S3). We showed that MSC derived from healthy donors (HDMSC) were able to counteract ASNase cytotoxicity on AML blasts, significantly increasing the number of live cells (median increase of live cells number with HD-MSC versus without HD-MSC: 35.43%, P = 0.001) and decreasing the percentage of apoptotic cells (median decrease of apoptosis with HD-MSC versus without HD-MSC: 33-73%, P = 0.0005) induced by treatment (Fig 3A). Similarly, the presence of HD-MSC significantly enhanced the viability of both $CD34^{+}CD38^{+}$ (median percentage of remaining live cells with HD-MSC vs. without HD-MSC: 62.67% vs. 37.3%, P = 0.0078) and $CD34^{+}CD38^{-}$ fractions (median percentage of remaining live cells with HD-MSC vs. without HD-MSC: 72.29% vs. 23.68%, P = 0.0039) upon ASNase treatment (Fig 3B), demonstrating that MSC also protect these primitive

populations from drug cytotoxicity. Considering that several microenvironmental features could be modified by the disease, additional experiments were performed 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 (median increase of live cells number with AML-MSC vs. without AML-MSC: 47.37%, P = 0.0078) and reduced the percentage of leukaemic apoptotic cells (median decrease of apoptosis with AML-MSC vs. without AML-MSC: 39.23%, P = 0.0391) in treated AML samples (Fig 3C). In a similar fashion, the presence of AML-MSC significantly decreased ASNase cytotoxicity against the $CD34^{+}CD38^{+}$ subpopulation (median percentage of remaining live cells with HD-MSC vs. without HD-MSC: 59.96% vs. 38.02%, P = 0.0078) and showed an effect on the $CD34^{+}CD38^{-}$ cells, where a positive trend was found in the majority of the performed experiments, almost approaching significance (P = 0.0547, Fig 3D). Furthermore, considering that the protective capacity of MSC may be dependent on the release of asparagine within the microenvironment, we evaluated the expression of ASNS in patient-derived MSC. The mean 2^{-DDCt} value of ASNS mRNA in AML-MSC was comparable to HD-MSC (respectively: 4.86 vs. 3, P = 0.0952) (Fig 3E).

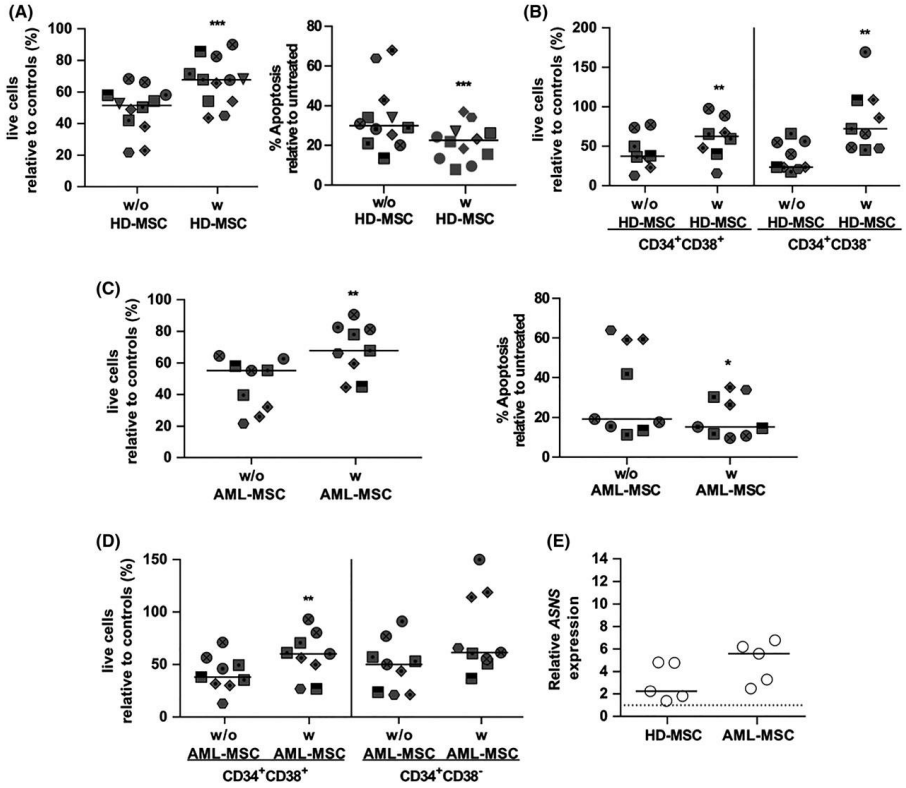

***Fig 3**. Protective role of MSC against ASNase cytotoxicity. (**A**) Primary acute myeloid leukaemia (AML) patient-derived cells were cultured without or with healthy donor mesenchymal stromal cells (HD-MSC) in the presence of ASNase (1 IU/mL) for 48 h. The proportion of live cells (left) and the percentage of apoptosis (right) normalised to untreated control in the presence (w) or in the absence (w/o) of HD-MSC are represented. Each symbol represents an individual AML patient (mean of technical triplicates). Bar indicates the median for each group. 12 independent experiments performed on*

*9 different AML patient-derived cells and 2 HD-MSC lines are shown. ***P < 0.001: Wilcoxon matched-pairs signed rank test. (**B**) Analysis of ASNase effect in the presence or in the absence of HD-MSC on the proportion of live cells (relative to control) in primitive $CD34^+CD38^+$ and $CD34^+CD38^-$ AML fractions. 9 independent experiments performed on samples derived from 6 different AML patients are shown. **P < 0.01: Wilcoxon matched-pairs signed rank test. (**C**) Primary AML patient-derived cells were cultured without or with AMLMSC in the presence of ASNase (1 IU/mL) for 48 h. The proportion 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 cocultures of blasts and MSC, both derived from 6 different AML patients are shown. *P < 0.05; **P < 0.01: Wilcoxon matched-pairs signed rank test. (**D**) Analysis of ASNase effect in the presence or in the absence of AML-MSC on number of live cells (relative to control) in primitive $CD34^+CD38^+$ and $CD34^+CD38^-$ AML fractions. 9 independent experiments performed on samples derived from 6 different AML patients are shown. Each symbol represents an individual AML patient. **P < 0.01: Wilcoxon matched-pairs signed rank test. (**E**) Expression of ASNS in HD- versus*

AML-MSC. The expression is showed as fold change, calculated as $2^{-\Delta\Delta Ct}$ *using the 697cell line as the reference (*$2^{-\Delta\Delta Ct} = 1$*, dotted horizontal line). 10 different MSC donors (5 HD-MSC and 5 AML-MSC) were analyzed. P = 0.0952: Mann Whitney test*

CTSB over-expressed in BM monocytes/macrophages and in AML FAB M5 blasts degrades ASNase

Another mechanism of ASNase resistance could be the drug clearance mediated by lysosomal cysteine proteases, such as CTSB expressed by cells of the BM microenvironment or by blasts themselves. In this respect, we found that in normal BM, CTSB was expressed primarily by $CD14^+$ monocytes/macrophages. Indeed, the median 2^{-DDCt} value of CTSB mRNA of $CD14^+$ samples (26.08) was observed to be higher as compared to the median 2^{-DDCt} value of CTSB mRNA in $CD14^-$ samples (5.96; P = 0.0005; Fig 4A). To determine whether proteases present in BM $CD14^+$ monocytes/macrophages, in particular CTSB, degrade ASNase, cell lysates were incubated with the drug. The mean residual ASNase activity after incubation with $CD14^+$ samples was reduced to 43% and was only slightly affected (83% of activity) after incubation with $CD14^-$ depleted BM cell lysates (P = 0.03; Fig 3B). Moreover, the degradation mediated by $CD14^+$ samples was prevented

by the addition of a protease inhibitor cocktail (residual ASNase activity: 81%, P = 0.03 versus control) and also by the CTSB-specific inhibitor Ca-074 (residual ASNase activity: 73%, P = 0.03 versus control), confirming the potential role exerted on ASNase cleavage by CTSB present in $CD14^+$ BM monocytes/macrophages (Fig 4B). AML BM exhibited an average of 3.4-fold higher CTSB mRNA as compared to normal BM. When examining relative expression levels among the 28 AML patients, CTSB was upregulated by 4- to 12-fold in 10 out of 28 (35.7%) patients in comparison to controls. Among these CTSB overexpressing patients, 9 (90%) belong to the FAB M5 subtype. The relative expression of CTSB mRNA was elevated in 9 out of 12 (75%) FAB M5 AML samples, with a median $2^{-\mathbf{DDCt}}$ value of 30.55. Instead, CTSB was constitutively expressed at lower levels by FAB M0/M1 (median $2^{-\mathbf{DDCt}}$ value of 4.82; P vs. AML-M5 = 0.0037), FAB M2 (median 2^{-DDCt} value of 4.86; P vs. AML-M5 = 0.0044) and FAB M4 (median $2^{-\mathbf{DDCt}}$ value of 5.8; P vs. AML-M5 = 0.0039) AML samples (Fig 4C). As for healthy BM monocytic cells, also in the case of leukaemic blast cells we found that cell lysates degraded ASNase through proteases such as CTSB. ASNase activity was reduced when incubated with whole cell lysates from blasts characterised by high CTSB expression,but was only slightly

decreased in the case of low CTSB expressing blasts (Fig 4D). Also, in this case, PIC and the CTSB-specific inhibitor, in particular, reduced degradation (Fig 4D).

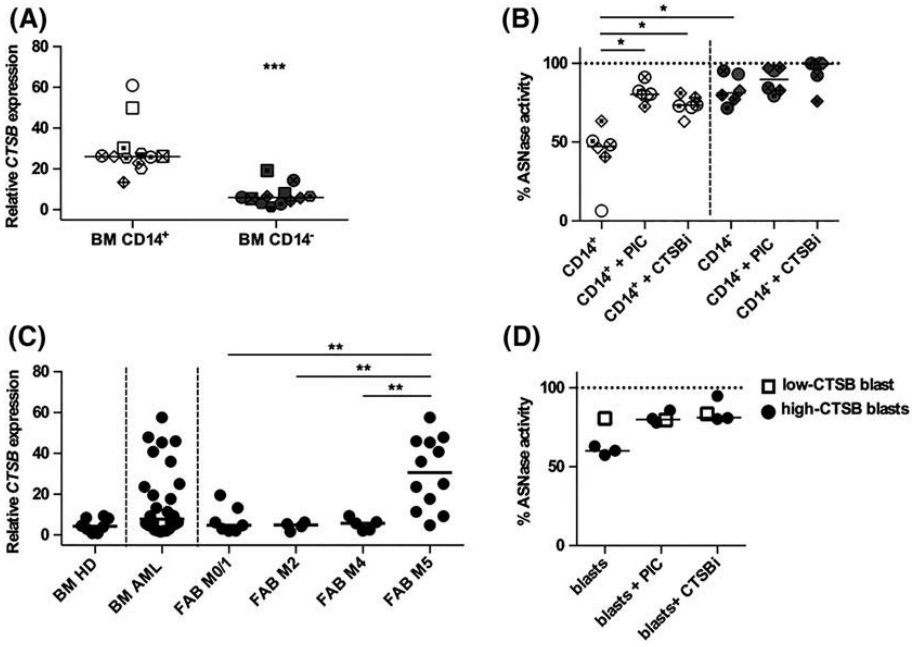

Fig 4. *CTSB expressed by BM CD14$^+$ monocytes/macrophages and by FAB M5 subset of AML degrades ASNase. (**A**) CTSB expression by CD14$^+$ and CD14$^-$ cells isolated from healthy donor bone marrow (BM). The expression was analyzed by RQ-PCR and is showed as fold change, calculated as 2^{-DDCt} using the REH cell line as the reference (2^{-DDCt} = 1). CD14$^+$ samples (average purity 86%) and CD14$^-$ samples (average purity 85%) from 12 different donors were*

*analyzed. ***P < 0.001: Wilcoxon matched-pairs signed rank test. (**B**) Residual ASNase activity following incubation of the drug with whole-cell lysates from BM CD14$^+$ and CD14$^-$ cells was quantified by Nessler assay. To prevent ASNase cleavage, lysates were incubated with protease inhibitor cocktail (PIC) or the CTSB-specific inhibitor (CTSBi), Ca-074, prior to incubation with the drug. CD14$^+$ and CD14$^-$ samples from 6 different BM donors were analyzed. *P < 0,05: Wilcoxon matched-pairs signed rank test. (**C**) CTSB expression by primary acute myeloid leukaemia (AML) cells. AML (n = 28, with >70% of blast cell content) and HD BM samples (n = 8) were analyzed by RQ-PCR. Within AML patients, 7 were FAB M0/M1, 4 were FAB M2, 5 were FAB M4 and 12 were FAB M5. **P < 0.01: Mann-Whitney test. (**D**) Residual ASNase activity following incubation with whole-cell lysates obtained from 3 different primary AML blasts with high CTSB expression (black circles) and from one AML sample with low CTSB expression (white squares) in the presence of PIC and Ca-074. Each symbol represents an individual AML patient.*

DISCUSSION

In the last few years it has been clarified that, in order for any AML therapy to be curative, it needs to be effective against the cells that propagate and sustain the disease, the so called LSC which reside in BM microenvironment. As a consequence, to assess the potential of new compounds, it is necessary 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 monocyte/macrophages in counteracting the effect of the drug. Even though the anti-leukaemic effect of ASNase has been extensively demonstrated in ALL therapy (Pession et al, 2005; Faderl et al, 2011), it has been used only occasionally, with different modalities and with alternate effects, to treat other haematological malignancies like AML (Gibson et al, 2011; Buaboonnam et al, 2013; Emadi et al, 2014, 2018; Ahmed et al, 2015) and solid tumors, these latter with scarce results (Taylor et al, 2001). ASNase acts firstly by inhibiting the proliferation of leukaemic cells and, subsequently, by inducing their apoptosis (Ueno et al, 1997). For this reason, we

analyzed the drug effect in terms of both absolute number of live cells and percentage of apoptotic cells. ASNase, used at clinically attainable dose, reduced the number and induced apoptosis of AML blasts, while minimally affecting non-blast cells. The susceptibility of FAB M5 subgroup to ASNase appeared to be higher compared to other FAB subtypes but this was not statistically significant. A further analysis on a larger number of samples is warranted to address the question of selectivity amongst different FAB/molecular subtypes. Effects of ASNase on AML primitive cell fractions have not been examined so far. In this work, we analyzed 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 $CD34^{+}CD38^{-}$ (LSC-enriched) and $CD34^{+}CD38^{+}$ fractions because LSC are not characterised by an unique phenotype and both compartments were reported to contain most of the LSC (Eppert et al, 2011). Notably, we showed that ASNase could reduce the number of live cells within the $CD34^{+}CD38^{+}$ and $CD34^{+}CD38^{-}$ fractions in a proportion similar to the 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* and retaining their engraftment capacity (Pabst et al, 2014). Also in these culture conditions favourable for LSC maintenance, we were able to further support the cytotoxic 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, (Lechman et al, 2016) offered 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 tumors and was related to its glutaminase activity (Liao et al, 2017). Indeed, in the absence of glutamine, the levels of reactive oxygen species augmented through attenuation of glutathione synthesis, leading to the downregulation of the b-catenin pathway and, subsequently, to the reduction of cancer stem cells (Liao et al, 2017). As LSC show susceptibility to oxidative stress and to alterations in the b-catenin pathway, (Wang et al, 2010) we can speculate that the cytotoxic effect of ASNase on $CD34^{+}CD38^{+}$ and $CD34^{+}CD38^{-}$ compartments observed in our work could be linked to the glutaminase activity of the drug. Concerning ASNase activity within the microenvironment,

Iwamoto et al (2007) proposed that 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 levels. Therefore, ASNS silencing decreases the protection, whereas enforced expression gives enhanced protection (Iwamoto et al, 2007). Laranjeira et al (2012) showed that insulin-like growth factor-binding protein 7 (IGFBP7) released by leukaemic cells boosts asparagine synthesis by stromal cells. 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. Bulk AML cells, particularly the $CD34^{+}CD38^{+}$ and $CD34^{+}CD38^{-}$ fractions, containing 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, particularly LSC, relatively insensitive to ASNase therapy and be 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 supporting leukaemogenesis and chemoresistance in their transcriptome, due to the leukaemia-induced remodelling of the BM microenvironment (Kim et al, 2015). Similarly to HD-MSC, also AML-MSC significantly increased the resistance to ASNase of $CD34^{+}CD38^{+}$ cells; a similar trend was found in the majority of experiments performed (7/9) in the $CD34^{+}CD38^{-}$ compartment that approached, but did not reach, statistical significance. This result could be explained by the limited number of samples analyzed 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 (2003) found that the interaction between VLA4 on AML cells and fibronectin on MSC was essential for the persistence of cytarabine-resistant disease and that VLA4 expression is an adverse prognostic factor in patients with AML. Moreover, Konopleva et al (2002) observed that MSC increased the expression of anti-apoptotic proteins and augmented the resistance to cytarabine in AML cells. 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 cells. The development of appropriate techniques to reduce the expression of ASNS by the AML-MSC could improve the effect of ASNase therapy. Cytarabine, a first-line AML chemotherapeutic agent, has been reported to induce downregulation of ASNS transcription (Takagaki et al, 2003). Thus, the combination of ASNase with conventional chemotherapy may provide a potentially synergistic effect. Although the UK Medical Research Council AML12 trial did not show any outcome benefit in patients randomized to receive an additional single consolidation course including high-dose cytarabine and a very limited amount of ASNase (Gibson et al, 2011), a more appropriate use of ASNase (in terms of dosing and timing) could potentially provide a clinical benefit. Indeed, in ALL a more intensive and prolonged exposition to ASNase has been very often associated with a better survival, with the extended asparagine depletion the mainstay of its therapeutic efficacy (Silverman et al, 2001). 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 lysosomal CTSB and asparaginyl endopeptidase (AEP) hydrolyse ASNase, resulting in

inactivation and exposure of immune epitopes (Patel et al, 2009). AEP and CTSB are expressed by lymphoblasts, particularly Philadelphia positive (Ph+) and iAMP21 leukaemia cells, two high-risk cytogenetic subtypes (Strefford et al, 2006; Patel et al, 2009). Moreover, a germ line mutation in the CTSB gene has been linked with a strongly prolonged ASNase turnover in a patient (van der Meer et al, 2014). Increased CTSB activity has been described in solid tumors, and it derives not only from the tumor mass but also from the cells surrounding the tumor, with a role in cancer progression and metastasis (Rakashanda et al, 2012). In particular, tumor-associated macrophages have been identified as the primary source of high levels of cathepsin activity in pancreatic islet cancers, mammary tumors and lung metastases (Gocheva et al, 2010). Phase I and phase I-II clinical trials using ASNase were conducted in patients with solid tumors showing that a large proportion of patients was not responsive to the treatment, mainly because the active dose of the drug quickly decreased after administrations, probably due to the proteolytic inactivation (Taylor et al, 2001). In healthy human BM samples, the expression of CTSB was found to be attributable to monocytic $CD14^+$ cells. Moreover, lysates from BM $CD14^+$ specifically degraded ASNase. This is consistent with findings of *in vivo* ASNase

distribution, showing that the drug is rapidly cleared from the serum by murine BM-resident phagocytic cells (van der Meer et al, 2017). Therefore, BM-resident monocytes/macrophages may collaborate to establish a protective niche for leukaemic cells by effectively removing ASNase from the BM through the release of CTSB. It is known that macrophages in the tumor microenvironment can protect tumor cells from cell death induced by a range of additional chemotherapeutic drugs (e.g. taxol, etoposide and doxorubicin), via a cathepsin-dependent mechanism (Shree et al, 2011). Moreover, myeloid blasts themselves can produce CTSB. Increased expression of cathepsin L and CTSB in AML patients seems to be associated with reduced overall survival (Jain et al, 2010). Notably, we found that in primary AML, the majority of FAB M5 samples specifically overexpressed CTSB, showing a 5.1-fold increase in mRNA levels compared to the other subtypes. Although the above-mentioned data had been assayed in unsorted AML BM samples, we included in our analysis only specimens with >70% blast cell content, assuming that the results will remain roughly the same, even in purified blast cells. We showed that lysates from overexpressing CTSB FAB M5 blasts specifically degrade ASNase. 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* (Okada et al, 2003). Zwaan et al (2000) reported that FAB M5 is equally sensitive *in vitro* to ASNase as ALL and this can be explained by the low level of ASNS in FAB M5 (Dubbers et al, 2000). 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 expression levels of proteases, e.g., CTSB, are likely to significantly modulate the therapeutic response to ASNase. Thus, the elimination of ASNase from the systemic circulation can be subject to the presence of enzymes produced by leukaemic cells. This suggests the possibility that patients with a high leukaemic burden, who positively respond to anti-leukaemic drugs before ASNase administration, may release more of these proteases in serum, which would then reduce the stability of ASNase. 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 (Olson & Joyce, 2015). 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 (Offman et al, 2011; Maggi et al,

2017). In conclusion, we demonstrated that, whilst ASNase was effective on AML bulk blasts and LSC fractions, MSC have a protective effect, nurturing blast survival. Moreover, monocytes/ macrophages and FAB M5 blasts themselves can partially counteract the effect of the drug via a CTSB-dependent mechanism. 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. Based on the well-known ASNase mechanism of action, pharmacokinetic and pharmacodynamic characteristics, clinical advantages routinely reported in front-line and relapsed ALL and sparsely in relapsed AML, and the new insights of its mechanism of action in BM niche here described, it is possible that a more extensive use of ASNase might be beneficial in the treatment of patients with AML when combined with conventional AML-directed chemotherapy and could, hopefully, ensure the desired favourable effects without increasing haematological toxicity.

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AUTHOR CONTRIBUTIONS

I.M.M. and V.G. performed research and analyzed the data; G.D., G.A. and C.T. performed research; L.A. supervised the statistical analyses; C.G.P. provided patient samples and edited the manuscript; T.C. and C.R. designed research, provided patient samples and contributed to the writing of the paper; 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.

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SUPPLEMENTARY INFORMATION

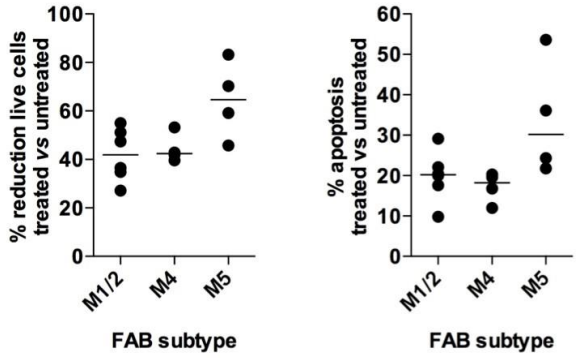

***Figure S1*. Comparison of ASNase susceptibility between samples from different FAB subtypes.** *Distribution and median values of reductions of live cell number (on the left) and percentage of apoptosis (on the right) of blasts after treatment in 3 AML FAB subtypes (FAB M1/M2, FAB M4, and FAB M5) were shown. Each dot represents an individual AML patient. Horizontal bar in the graph indicates the median for each subtype. Mann-Whitney test was used for comparison between pairs of groups.*

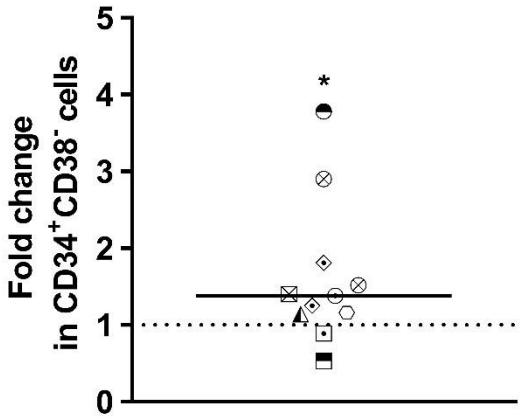

Figure S2. Effect of SR1 and UM729 on maintenance of primary CD34$^+$CD38$^-$ AML cells in culture. *Primary AML patient-derived cells were cultured with or without SR1 and UM729 for 48 hours. The effect of these two reagents on CD34$^+$CD38$^-$ subpopulation was expressed as fold change relative to the control cultured for 48 hours without the two compounds. Each symbol represents an individual AML patient (mean of technical triplicates). 11 independent experiments performed on 9 different patients are shown. Bar indicates the median and the dotted horizontal line represents the control. *P<0.05: Wilcoxon signed rank test.*

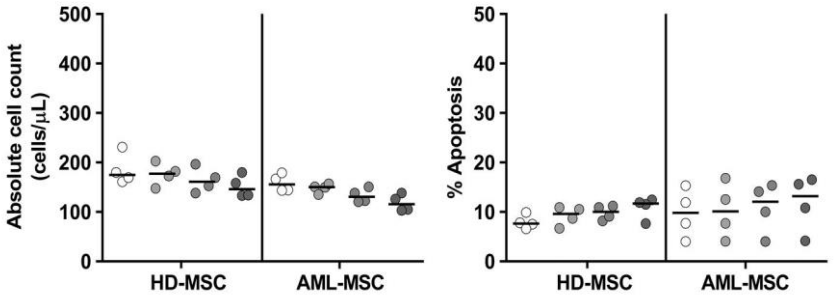

Figure S3. ASNase cytotoxicity on HD- and AML-MSC. *HD- and AML-MSC were incubated for 48 hours in the absence (white) or in the presence of 0.1-1-3 IU/ml of ASNase (represented by darker shades of grey as the dose increase). The absolute cell count (10^9/l) (on the left) and the percentage of apoptotic 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 technical triplicates and bar indicates the median for each group.*

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.[1] 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.[2] To evaluate the number of viable cells, counting beads (CountBright™ absolute counting beads, Invitrogen™, Thermo Fisher Scientific) were added to samples before the acquisition and the absolute cell count (10^9/l) was calculated following manufacturer's protocol. Primary patient-derived AML cells were labelled with pacific orange-anti CD45 (clone HI30; Invitrogen, Thermo Fisher Scientific), phycoerythrin-cyanine™ 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 non-blast cells within the sample, the bulk blast population and the leukaemic $CD34^+CD38^-$ and $CD34^+CD38^+$ subpopulations.[3]Experiments were performed on a FACSCanto™ II (BD Biosciences) and analyzed with FACSDiva™ software v.6.1.3 (BD Biosciences).

Coculture experiments. The susceptibility of MSC to ASNase was previously determined seeding HD- and AML-MSC (passage 5 to 6) at 1.7-2x10^4 cells/well in 96-well plates in complete RPMI 1640 medium and, when confluent (typically in 1-2 days), treating 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. Coculture experiments were performed seeding HD-MSC (passage 4 to 7) at 1.7-2x10^4 cells/well in 96-well plates. When confluent, primary AML patient-derived cells (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 analyzed for viability by flow cytometry. We executed the same experiment coculturing primary AML cells with the autologous AML-MSC (passage 3 to 6). All experiments were performed in triplicate.

Clonogenic assay. 1x10^4 of primary AML cells were resuspended with 1 mL of MethoCult™ H4434 classic (StemCell™ 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™ Nunc™, 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. Primary AML cells after 48 hours of incubation in the presence of SR1 and UM729 with or without ASNase, as previously described, were resuspended in TRIzol™ reagent (Invitrogen™, 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™ 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 analyzed using QX200 Droplet Reader (Bio-Rad) and QuantaSoft™. miR-126-3p levels were normalised by the geometric

mean of let-7a-5p, miR-16-5p, SNORD24 and SNORD48.

RQ-PCR. Total cellular RNA was isolated using TRIzol™ reagent according to manufacturer's protocol. One μg of total RNA was reversely transcribed using the SuperScript II Reverse Trascriptase (Invitrogen™, Thermo Fisher Scientific). Quantitative RT-PCR 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'-CA GCCACCCAGATGTAAGC-3' and h*CTSB*upl-right 5'-GCCGGATC CTAGATCCACTA-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. RQ-PCR data were calculated with the ΔΔCt method using as a reference the 697 and REH cell lines.

Nessler assay. The colorimetric Nessler method consists of the reaction between Nessler's reagent (potassium tetraiodomercurate(II), Sigma-Aldrich) and the ammonia released during the conversion of L-Asn into L-Asp, providing a characteristic yellow reaction mixture that can be quantified by spectrophotometry. Briefly, 60 µl of 44 mM L-asparagine (Sigma-Aldrich) dissolved in 15 mM Tris-HCl buffer, pH 7.3, supplemented with 0.015% w/v BSA was added to 15 µl of sample and incubated at 37°C for 30 minutes. The reaction was stopped by the addition of 50 µl trichloroacetic acid (24.5% w/v, Sigma-Aldrich). After centrifugation, 15 µl of the supernatant was plated and 120 µl Nessler's solution diluted with ddH_2O (1:8) was added. The adsorbance of the reaction product was read at 450 nm using the Tecan GENios microplate reader fluorometer (Tecan, Mannedorf, Switzerland).

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CHAPTER 5

CONCLUSIONS AND FUTURE PERSPECTIVES

The haematopoietic stem cells niche is the anatomical structure where HSCs reside, and where the haematopoiesis is controlled through interactions between HSCs and different cell types. Among these, monocytes play in the BM context a critical role which is still partially unknow. Monocytic cells are involved in tissue development, homeostasis, and repair of injured tissues. They are among the first responders in wound and are required to orchestrate resolution of inflammation and healing[1]. Thus, they can be exploited therapeutically to favour tissue regeneration[2]. Recent evidences suggest that monocytes/macrophages are capable of boosting haemopoietic regeneration in mouse model[3] and can regulate the mobilization of haematopoietic progenitors in bloodstream[4,5].

The counterbalance of these beneficial effects, is that monocytes/macrophages are also crucial component of the malignant niche whereby they are converted by the tumour into active suppressors of the immune system and provide an indicator of poor clinical outcome[6]. The information available about this activity in leukaemia is limited but recent data indicate that macrophages support the leukaemic process in JAK2V617F-driven polycythemia vera[7].

Moreover, BM resident monocyte/macrophages may provide a protective environment for leukemic cells by effectively removing chemotherapeutic drugs from the BM niche[8].

In this PhD thesis, we have focused our attention on the capability of monocytes to boost human engraftment in xenotransplantation models and on their role in the maintenance of HSC *in vitro*. Moreover, we evaluated the protective effect of the BM microenvironment components, including MSC and monocytes/macrophages, to L-asparaginase sensitivity of acute myeloid leukemia blasts.

First of all, we have tested conditioning regimens in SCID/beige mice to generate an effective xenograft model of human normal and malignant haematopoiesis[9]. SCID/beige mice are less permissive for human haematopoiesis as compared to other strains[10,11]. However, combining sub-lethal irradiation and Fludarabine, an immunosuppressive drug used in conditioning regimen in clinic[12], we were able to increase the human engraftment level in SCID/beige mice as compared to irradiation only. This was probably due to Fludarabine effect on murine leaky lymphocytes[13].

Taking advantage of this mouse xenograft model, we have highlighted the capacity of human monocytes to boost the engraftment of human $CD34^+$ cells isolated from CB. This confirm the hypothesis of Trento and colleagues according to which the administration of MSC-educated murine $CD11b^+$ cells accelerates haematopoietic reconstitution in BM transplant recipients[3]. These data are also supported by clinical trials in which MSC-conditioned CB units co-

transplanted with unmanipulated units accelerated haematopoietic recovery, probably through MSC-expanded monocytes[14]. Moreover, the *in vitro* co-culture of $CD34^+$ cells with monocytes, in particular with the classical $CD14^+CD16^-$ ones, reveals a significant increment of HSCs survival, and prevention of their differentiation, compared to $CD34^+$ cells cultivated alone. However, the explanation of this positive influence exerted by $CD14^+CD16^-$ on $CD34^+$ cells remains largely unknown. Both direct cells-to-cells contact mediated signals, such as JAG/NOTCH pathway, then soluble factors seem to be involved.

Other cell types, such as MSCs have already been used with the purpose to expand HSCs *in vitro*[14]. Alternatively, several approaches have been tested with the same purpose, using small molecules that inhibit HSCs differentiation and/or promote their self-renewal, including Notch ligands[15], nicotinamide[16] and StemRegenin-1[17].

Monocytes could have a central role also in tumour microenvironment. In fact, we demonstrated that bulk blasts and leukemic stem cell (LSCs) fractions, both the $CD34^+CD38^-$ and the $CD34^+CD38^-$, are intrinsically sensitive to L-asparaginase. However, cells in the BM microenvironment seems to partially counteract the effect of the drug. These cells included MSCs and monocytes/macrophages[18]. L-asparaginase may be inactivated by the lysosomal cysteine protease cathepsin B (CTSB) and by asparaginyl endopeptidase[19]. Monocytic cells express CTSB and may also produce other factors as well, potentially interfering with the effect of L-

asparaginase. Of interest, AML cells themselves, in particular the M5 subtype, may also produce CTSB. All these factors contribute to increased resistance to L-asparaginase. Thus, our work highlights crucial aspects, which should be considered in the design of future clinical studies aimed at testing L-asparaginase efficacy in AML patients. L-asparaginase should be tested in combination with other drugs that overcome the protective effect of the BM microenvironment. Obvious candidates are inhibitors of CTSB and/or novel modified versions of L-asparaginase resistant to proteolytic cleavage without impairment of their enzymatic function[20].

In the future, we would like to:

- better characterise the engraftment produced by HSCs co-transplanted with $CD14^+$ monocytes;
- use monocytes to expedite engraftment of genetically modified $CD34^+$ cells in a xenograft model;
- test whether the number of monocytes contained in the donor HSCs preparation impacts on the kinetics of haemopoietic engraftment in patients who have undergone allogeneic HSCT for any haematological malignancy;
- further study the potential beneficial effect of L-asparaginase in AML, and how to optimize that benefit. L-asparaginase should be tested in combination with other drugs that counteract any factor resulting in its decreased efficacy. Obvious candidates are inhibitors of CTSB and/or ASNS.

Moreover, considering that AML is a heterogeneous disease, sensitivity to L-asparaginase of different subgroups, as defined by cytogenetic features, have to be studied. Indeed, AML with 7q- or monosomy 7 might be more sensitive, because these AML cells seem to produce less ASNS[21]. Moreover, AML with IDH1 or IDH2 mutations may be particularly sensitive to glutamine depletion[22].

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Pievani A, Michelozzi IM, Rambaldi B, **Granata V**, Corsi A, Dazzi F, Biondi A, Serafini M. Fludrabine as a cost-effective adjuvant to enhance engraftment of human normal and malignant hematopoiesis in immunodeficient mice. Sci.Rep. 8, 9125 (2018)

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