Department of Health Science
School of Medicine

Master degree in Medical Biotechnologies

Study of in vitro differentiation of macrophages from hemophilia A bone marrow cells

Tutor: **Candidate**

Dr Chiara BORSOTTI   Mohamad Baker Alhasan Alsheik TERKAWI

                                                         Matricula number: 20053340

Academic Year: 2023/2024

Contents

ABSTRACT

Hemophilia A (HA) is an X-linked bleeding disorder marked by a deficiency in coagulation factor VIII (FVIII), resulting in spontaneous bleeding episodes, predominantly within joints. These recurrent intra-articular bleedings lead to chronic hemarthrosis and ultimately osteoarthritis. The inflammatory response triggered by joint bleedings in HA patients significantly contributes to bone and joint deterioration and can affect macrophage function and polarization. Previous experiments done in our laboratory demonstrated defective murine osteoblast in vitro maturation and increased osteoclast differentiation capability in HA samples compared to WT, suggesting that FVIII deficiency may disrupt normal skeletal homeostasis. Given that osteoclasts are derived from macrophages and that bone marrow-derived macrophages (BMDM) play a significant role in bone health in this study we investigated whether the absence of FVIII impacts in vitro BMDM differentiation. To this aim we employed a preclinical mouse model of severe HA. We compared the in vitro differentiation of HA versus (vs) WT BMDM by stimulating them by different colony-stimulating factor (M-CSF) and assessed their morphology and phenotype at the end of the culture under the light microscope and by flow cytometry; TRAP staining was performed for confirming macrophage identity. Microscope observation revealed morphological changes of the attached cells that acquired by day 8 features of macrophage morphology for both HA and WT samples. Flow cytometry analysis confirmed the occurred differentiation since more than 80% of cells were positive for both CD11b and F480. Overall the results showed a trend toward a higher in vitro HA BMDM differentiation and their better survival rate upon prolongated exposure to M-CSF. These preliminary suggest that FVIII absence might facilitate higher macrophage production in the BM, in accordance to what previously observed for in vitro osteoclastogenesis. This could be favored by the inflammatory status generated in the bone proximity in HA patients. Further studies are needed to functionally assess in vivo and in vitro the impact of FVIII deficiency on macrophage maturation, polarization and behavior, for instance adding FVIII in the culture medium during the in vitro differentiation. Elucidation of new mechanisms of macrophages involvement in the pathophysiology of the HA disease might be instrumental to improve the current therapies.

INTRODUCTION

1. Hemophilia A

Hemophilia A (HA) is an X-linked bleeding disorder characterized by reduced or absent levels of coagulation factor (F) VIII, and according with the residual FVIII activity, HA can be distinguished in mild (5-40%), moderate (1-5%) and severe (<1%) (Bolton-Maggs & Pasi, 2003). The HA prevalence is approximately 1 in 5000-10000 male births. While initial indications of HA may manifest as easy bruising and excessive mucosal bleeding, the classical bleeding symptoms involve soft tissue bleeding and hemarthrosis (Zimmerman & Valentino, 2013). FVIII holds significant importance within the coagulation cascade, playing a crucial role in maintaining hemostasis, the natural process responsible for sealing vascular damage and preventing excessive blood loss following an injury (Smith et al., 2015). The cause of HA is the mutation of the FVIII gene (F8), which leads to the production of mutated or absent FVIII protein. In individuals with HA, mutations occur within the FVIII gene, leading to the production of abnormal or insufficient FVIII protein or, in some cases, no protein at all. These mutations can vary in type and location within the gene, resulting in different degrees of impairment in FVIII function. Some mutations may lead to a complete absence of functional FVIII, causing severe HA, while others may allow to produce partially functional FVIII, resulting in milder forms of the disorder. Understanding the specific genetic mutations involved in hemophilia A is crucial for diagnosis, genetic counseling, and the development of targeted treatments(Bolton-Maggs & Pasi, 2003).

Because of the reduced/absent FVIII activity, HA patients suffer from spontaneous bleedings, mostly in joints, and chronic damage to the soft tissues and skeletal muscles. Intra-articular hemorrhage, also known as hemarthrosis, primarily in the knee, elbow, and ankle joints, is the clinical hallmark of HA(Long et al., 2016). Repeated intra-articular bleeding is a critical concern due to its potential to cause cartilage damage and degenerative articular changes, ultimately resulting in severe osteoarthritis (Rodriguez-Merchan & Valentino, 2016).

The inheritance pattern of HA is X-linked recessive, meaning that the gene responsible for the disorder is located on the X chromosome. Because males have only one X chromosome (XY), a single copy of the mutated FVIII gene is sufficient to cause the disorder in males. Females (XX), on the other hand, have two X chromosomes, so they typically need to inherit two copies of the mutated gene to manifest the disorder. However, females who inherit only one copy of the mutated gene are carriers of HA and may exhibit mild bleeding symptoms or be asymptomatic carriers.

1.1 FVIII involvement in the coagulation cascade

The coagulation cascade involves a series of complex interactions between various clotting factors, ultimately leading to the formation of a blood clot. It comprises two primary pathways: tissue factor (TF) pathway and the contact pathway, formerly known as extrinsic pathway and intrinsic pathway, respectively (**Figure 1**).

The extrinsic pathway necessitates the introduction of an "extrinsic "element, specifically TF, for its activation. Upon vessel injury, FVII in the bloodstream enters in contact with TF to initiate the coagulation cascade. On the other hand, the intrinsic/contact pathway does not require TF for its activation, and it has a minor role in initiating the clot formation, while it plays an important role in clot stabilization and resolution, thus it seems more involved in inflammation and innate immunity (Peyvandi et al., 2016). In the intrinsic pathway, vascular injury leads to the activation of FXII which then activates FXI. Activated FXI subsequently activates FIX, forming a complex with calcium ions and FVIII. This complex, along with TF (also known as FIII) from the extrinsic pathway, activates FX to FXa, which then catalyzes the conversion of prothrombin to thrombin. Thrombin, in turn, converts fibrinogen to fibrin, which forms the meshwork of the blood clot (Chavin, 1984).

FVIII plays a crucial role as an amplifier in the coagulation cascade rather than being a starter. FVIII acts as a cofactor in the intrinsic pathway of the coagulation cascade: it enhances the activity of activated FIX by serving as a cofactor for the activation of FX by FIXa. This amplifies the generation of FXa, accelerating the conversion of prothrombin to thrombin and ultimately leading to the formation of a stable blood clot. Therefore, FVIII's role in the coagulation cascade is crucial for efficient clot formation and hemostasis. (Hoffman, M. 2003)
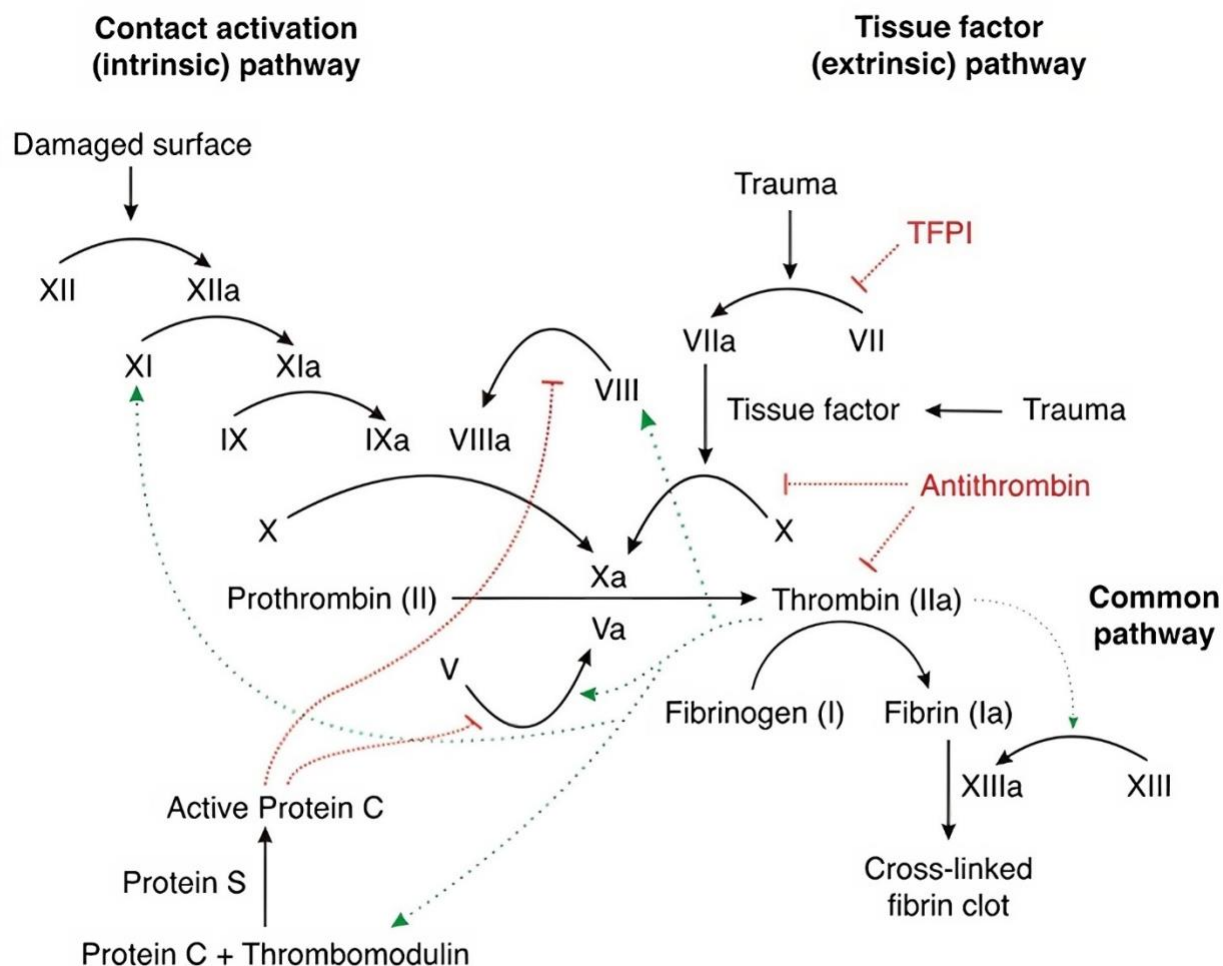
Figure 1. Schematic representation of the two pathways activating the coagulation cascade

1.2 Hemophilia A therapies

The bleeding incidents are treated/prevented with replacement therapy, consisting in prophylactic infusions of exogenous FVIII, either recombinant (r) or plasma-derived (pd) (Coppola et al., 2017). However, there are two significant downsides to this treatment: the high costs per patient and the short FVIII half-life (10–12 hours), which forces HA patients to have frequent FVIII infusions (every other day). Furthermore, approximately 30% of individuals afflicted with severe HA develop anti-FVIII antibodies, called inhibitors, able to inhibit the activity of infused FVIII (van Velzen et al., 2017).

The development of innovative therapeutic interventions aimed at improving HA patient quality of life (QoL) by reducing the number of required infusions and preventing inhibitor formation is increasing. In recent years, there has been notable progresses in the treatment of patients, with advancements such as the introduction of FVIII concentrates with longer half-lives, bispecific antibodies known as FVIII mimetics (Emicizumab), the targeting of natural anticoagulant pathways (Fitusiran), and gene therapy/editing (Weyand & Pipe, 2019). The extension of the half-life of FVIII recombinant proteins was achieved through the fusion of polyethylene glycol (PEG-FVIII) or IgG1-Fc (rFVIIIFc) (Mancuso & Santagostino, 2017). These modifications have provided modest benefits in HA with 14-19 hours half-life, due to the need of the FVIII interaction with von Willebrand factor (vWF). Indeed, is important to highlight the role of vWF in stabilizing FVIII in circulation, thus extending its half-life. vWF serves as a carrier protein for FVIII, protecting it from degradation and clearance from the bloodstream. This interaction between vWF and FVIII is crucial for maintaining hemostasis and preventing excessive bleeding episodes in individuals with HA. Consequently, therapies targeting vWF-FVIII interactions aim to enhance FVIII stability and prolong its presence in the bloodstream, thereby improving treatment outcomes for HA patients.

When injury occurs, vWF steps into action, anchoring itself to exposed collagen in the injured blood vessel walls. This action not only forms the initial scaffolding for platelet adhesion but also facilitates the release of FVIII. Additionally, vWF's engagement with platelet receptors promotes platelet adhesion and aggregation, fortifying the growing clot. This tight coordination between FVIII and VWF ensures efficient clot formation, maintaining vascular integrity and preventing excessive bleeding. However, disruptions in this partnership, as seen in conditions like HA or von Willebrand disease, can lead to bleeding disorders, underscoring the indispensable nature of their physiological interaction in hemostasis (Terraube et al., 2010).

The process of introducing a disulfide bond can result in the binding of heavy and light chains of rFVIII, leading to the formation of a novel product with an extended half-life, commonly referred to as single-chain rFVIII (Zollner et al., 2013). Modifying the method of drug delivery (e.g., oral, or subcutaneous), is an additional approach to minimize the treatment load. This is especially true for patients who encounter difficulties in obtaining venous access, such as neonates and young children (Weyand & Pipe, 2019). Although these FVIII products offer certain benefits, they may still result in the formation of inhibitors against FVIII. Therefore, the first commercially available non-factor replacement product, Emicizumab (Hemlibra®) was introduced(Blair, 2019). It is a subcutaneously

administered, recombinant, humanized, bispecific monoclonal antibody that has been designed to imitate the cofactor activity of activated FVIII. It has been granted approval in multiple countries for the regular prophylaxis of bleeding episodes in individuals diagnosed with HA, regardless of the presence of FVIII inhibitors.

Despite the progress made in this area, these non-factor replacement products are only capable of improving the bleeding phenotype, and patients will still require FVIII concentrate in cases of significant bleeding, severe trauma, or surgical interventions.

Researchers are currently focusing their attention on novel methodologies, including cell and gene therapy approaches, to ensure a consistent and persistent treatment of the hemorrhagic phenotype observed in patients.

2. Osteoporosis and osteopenia in HA patients

Over the last decades the widespread availability of replacement therapy has dramatically improved the QoL and expectancy of hemophilic patients. However, a large and growing body of literature has revealed an increased prevalence of skeletal fragility in adult and young patients (Blair, 2019) as early as 12 years old (IORIO et al., 2010). Indeed, metanalysis and cross-sectional studies reported that 70% of adult HA patients had low bone mass density (BMD), over 40% had osteopenia and 27% had osteoporosis (WALLNY et al., 2007)(Paschou et al., 2014)). Initially, the reduced BMD was attributed to decreased physical activity since the recurrent hemarthroses resulted in debilitating arthropathy while no other risk factors (e.g. bone mass index, hypovitaminosis D, hepatitis C or HIV) were found correlating with the low BMD (Kiper Unal, 2017) (Linari et al., 2013). One study reported that the percentage of FVIII residual activity correlated with the bone alteration since patients with severe HA had lower BMD score than patients with the mild and moderate form (Kempton et al., 2014). More recently, another clinical study found no correlation between BMD and either the severity of hemophilia or number of bleeding episodes within the 12 months prior to study enrollment, or even between patients receiving on-demand or prophylactic factor replacement (Sahin et al., 2019). Interestingly, HA patients receiving prophylactically Emicizumab did not display significative changes in plasma level of bone biomarkers before and after the beginning of the treatment (Kiialainen et al., 2022) or in comparison with patients in FVIII prophylaxis (Manco-Johnson, 2021). Moreover, clinical and experimental studies have begun to suggest a direct link between reduced BMD and FVIII

deficiency, raising the possibility that FVIII might play a role outside of the coagulation cascade and interact with the molecular pathways involved in bone biology (Gebetsberger et al,2022). While clinical observations regarding BMD decrease in hemophilia B (HB) patients are less prevalent and often associated with a less severe bleeding phenotype, similar trends have been noted, albeit to a lesser extent. Further research is warranted to elucidate the underlying mechanisms and potential therapeutic interventions for bone health management in both HA and HB patients. In adult life bone homeostasis depends on balanced bone formation by osteoblasts and bone resorption by osteoclasts (**Figure 2**) (Kim et al., 2020). Proper equilibrium is guaranteed by several coordinated signaling mechanisms (cell-cell contact, cytokines secretion and extracellular matrix interaction).
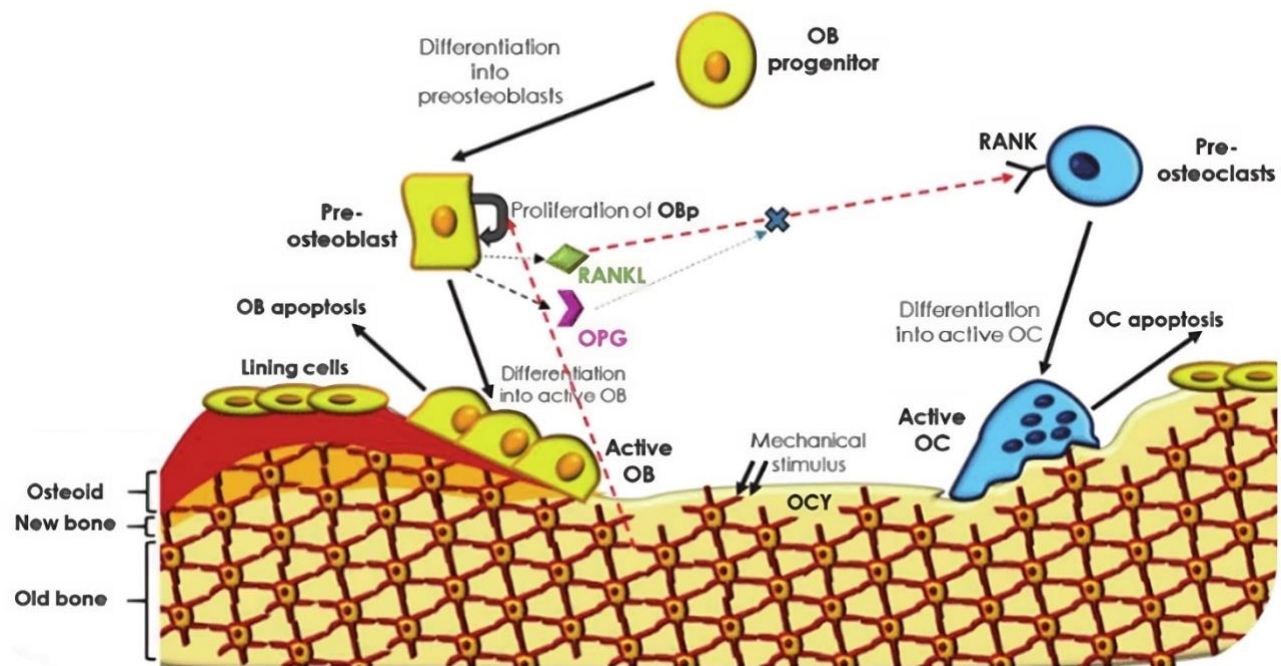

Figure 2. Schematic representation of bone cells main biochemical interactions during bone remodeling process (Ait Oumghar et al,2021).

Nonetheless, an imbalance between bone resorption and bone formation may occur under certain pathological conditions, which leads to abnormal bone remodeling and the development of bone disorders. Osteoblasts derive from committed mesenchymal precursors to the osteoprogenitor lineage and terminally differentiate in the osteocytes found in the bone lacunae (Harada & Rodan, 2003)(Harada & Rodan, 2003). They normally reside on the external surface of bones and secrete extracellular proteins including osteocalcin (OC), alkaline phosphatase (AP) and type I collagen

(COL1). Osteoclasts instead belong to the monocyte/macrophage lineage of bone marrow (BM) derived cells and therefore originate from hematopoietic stem cells (HSC) through progressive differentiation stages Unlike other tissue-resident macrophages, osteoclasts have a distinctive developmental pathway. While most tissue-resident macrophages arise from the proliferation and differentiation of precursor cells already present in tissues, osteoclasts undergo a unique process involving the fusion of multiple precursor cells. In the bone microenvironment, precursor cells of the monocyte/macrophage lineage are recruited to sites of bone remodeling. The precursor cells of the monocyte/macrophage lineage in the bone microenvironment are recruited to sites of bone remodeling. Under the influence of specific cytokines and signaling molecules such as macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL), these precursors differentiate into osteoclast precursors expressing the receptor activator of nuclear factor kappa-B (RANK). The binding of RANKL to RANK triggers a cascade of signaling events essential for osteoclast differentiation. These precursor cells then undergo fusion with one another to form multinucleated osteoclasts. This fusion process, regulated by molecules like dendritic cell-specific transmembrane protein (DC-STAMP) and osteoclast stimulatory transmembrane protein (OC-STAMP), is crucial for the development of osteoclasts with the specialized morphology and functional capacity required for bone resorption. The multinucleated osteoclasts mature, acquiring specialized features such as a ruffled border and a sealing zone necessary for effective bone resorption. This process of direct fusion, rather than differentiation into macrophages first, ensures the efficient formation of mature osteoclasts capable of maintaining bone homeostasis. (Horwood, N. J. 2016). The fusion of precursor cells to form multinucleated osteoclasts is regulated by various factors, including receptor activator of nuclear factor-kB (RANK) signaling and cell-cell interactions mediated by molecules such as dendritic cell-specific transmembrane protein (DC-STAMP) and osteoclast stimulatory transmembrane protein (OC-STAMP).

This unique mode of formation allows osteoclasts to efficiently carry out their bone resorption function by combining the cellular resources and enzymatic machinery of multiple precursor cells. This fusion-mediated process contributes to the generation of large, multinucleated osteoclasts that are essential for maintaining skeletal integrity and bone homeostasis (Boyle et al., 2003. They carry out their bone resorption activity by secreting acid and proteolytic enzymes (e.g. cathepsin K).

Many cytokines, hormones and signaling pathways are involved in bone metabolism/remodeling and act directly on the osteoclast and osteoblast differentiation. For instance, the receptor activator of nuclear factor-kB (RANK), normally expressed on the plasma membrane of osteoclasts and osteoclast precursors, promotes their differentiation and activation via interaction with its ligand, RANKL, which is expressed by osteoblasts, osteocytes, and activated T cells. On the other hand, one main RANKL decoy receptor, osteoprotegerin (OPG), is known to negatively regulate osteoclast differentiation and activation preventing RANK/RANKL interaction (Theoleyre et al., 2004). Thus, RANKL/OPG ratio is an important determinant of bone mass and skeletal integrity.

While BMD represents an indirect marker for bone health and structural integrity, several bone biomarkers such as serum AP, OC, RANKL and OPG could be used for clinical assessment of bone integrity and indirect evaluation of osteoclast and osteoblast *in vivo* activity (Eastell & Hannon, 2008). So far, the results of clinical studies conducted in HA patients for quantifying several bone biomarkers are not conclusive. Christoforidis et al (CHRISTOFORIDIS et al., 2011) reported increased level of circulating OC, RANKL, and decreased OPG in pediatric HA compared to healthy population, suggesting an increased osteoclast activity in the patients. Similar, several groups found increased osteoclastic activity by observing increased level of other bone resorption biomarkers (e.g. N- and C-terminal cross-linking telopeptide of collagen type I [NTX and CTX]) (Goldscheitter et al., 2021) (Katsarou et al., 2010) not matched by increased levels of bone formation biomarkers (RANKL or OPG), unless the samples were collected within 24 hours from intravenous (iv) FVIII injection (MELCHIORRE et al., 2012). OPG expression was found decreased in the synovium of hemophilic patients undergoing knee replacement while RANK and RANKL immunopositivity was strong in the arthropathy synovial tissues of the same patients (MELCHIORRE et al., 2012). In the same study they observed reduced serum levels of RANKL and OPG in HA patients compared to healthy controls.

One of the main limitations of the clinical assessments relates with the discrepancy in many parameters of tested patients, such as the timing of blood draw, the personal bleeding history, and the replacement therapy regimen. Therefore, many groups have been conducting their investigation on the available mouse model of hemophilia (Sabatino et al., 2012). In accordance with clinical data, FVIII knock out (KO) mice showed decreased BMD compared to wild type (WT) controls, despite the fact that they had a similar physical activity, no increased hemarthroses and were not affected by other comorbidities (Recht et al., 2013). Interestingly, this phenotype was shared by FVIII and FIX

KO but not by vWF KO mice (Lau et al., 2014) (Taves et al., 2019). In vitro studies have demonstrated that FVIII significantly influences both osteoclast and osteoblast activity in murine and human models. Regarding osteoclasts, FVIII has been observed to inhibit osteoclastogenesis in murine BM-derived macrophages by downregulating key osteoclastogenic factors such as RANKL and M-CSF (Cadé, M., et al 2022). This inhibition extends to the reduction of resorptive activity in mature osteoclasts, indicating that FVIII not only affects the differentiation of these cells but also their functional capacity in bone resorption. Similar findings have been reported in human cell cultures, where FVIII treatment resulted in decreased differentiation and activity of osteoclasts derived from human peripheral blood mononuclear cells (Gebetsberger, J., et al 2022).

For osteoblasts, in vitro studies have shown that FVIII positively influences their differentiation and activity (Cadé, M., et al 2022). In murine models, FVIII has been found to enhance the expression of osteoblastic markers and promote mineralization, suggesting a supportive role in bone formation (Taves et al., 2019). Human osteoblast cultures have also shown increased differentiation and function in the presence of FVIII, further highlighting its beneficial role in promoting bone health and formation. These observations collectively suggest a dual role of FVIII in inhibiting bone resorption by osteoclasts and promoting bone formation by osteoblasts, contributing to overall bone homeostasis. (Pagel et al., 2009)

3. Osteoclast and macrophage origin and functions

Various theories have been propounded to account for the origin of osteoclasts since they were discovered in 1873. However, Walker's pioneering experiments in the 1970s confirmed their hematopoietic origin, as transfusions of spleen and myeloid cells from WT mice reversed bone resorption in osteosclerotic and osteoporotic mice lacking osteoclasts (Walker, 1975). This demonstrated that hematopoietic organs could generate cells that cause hard tissues to be reabsorbed. In 1986, Scheven and coworkers (Scheven et al., 1986) were the first to show that osteoclasts can be produced from a subgroup of murine cells that are rich in hemopoietic progenitors. In 1990, the monocyte/macrophage origin of osteoclast was later certified by Udagawa et al (Udagawa et al., 1990) after they proved that HSCs differentiate into the monocyte-macrophages through the activation of M-CSF pathway. Subsequent studies further confirmed that osteoclasts can be formed from monocyte-macrophage precursor cells as well as from mature macrophages in tissues.

Furthermore, bones and BM contain unique macrophage populations, namely: osteoclasts and bone marrow macrophages (erythroid island macrophages), hematopoietic stem cell macrophages, and a recently discovered group of macrophages called osteal macrophages or "osteomacs". Cheng et al. (Chang et al., 2008) demonstrated that osteomacs, found in close association with osteoblasts on bone surfaces, play a supportive role in osteoblast differentiation by producing various cytokines and growth factors essential for creating a conducive microenvironment for bone formation. Additionally, osteomacs contribute to the maintenance of bone homeostasis by regulating the balance between bone formation and resorption processes. Their direct interaction with osteoblasts influences the latter's function and activity, thereby impacting new bone formation. Besides their role in osteoblast differentiation, osteomacs possess immune functions, participating in immune surveillance of bone tissue, protecting against infections, and aiding in the removal of debris, which can indirectly affect osteoblast health. Furthermore, osteomacs are integral to the bone remodeling process, assisting in the removal of apoptotic cells and matrix turnover, essential for continuous bone renewal and repair. It has been suggested that osteoclasts and macrophages may have a similar origin. Studies have shown that cells from the monocyte/macrophage system, which includes hematopoietic marrow cells, blood monocytes, and peritoneal macrophages, possess the ability to develop into bone-resorbing osteoclasts; thus, squarely classifying osteoclastic groups within these series of cells (Bar-Shavit, 2007).

3.1 Pathway of macrophage differentiation

The monocyte/macrophage cell lineage derives from multipotent HSCs resident in the BM, and they originate from M-CFU in the yolk sac during embryonic development. Early expression of PU.1 and Mitf in M-CFU induces the emergence of M-CSFR (the receptor for M-CSF). Under the influence of M-CSF and RANKL, M-CFU can differentiate directly into osteoclasts. Separately, M-CSF can also direct M-CFU to differentiate into macrophage and dendritic cell progenitors (MDP). MDPs can then become dendritic cells or monocytes, which further differentiate into pro-monocytes under sustained M-CSF action. These pro-monocytes form LY6C$^+$ or LY6C$^-$ blood monocytes under stimuli like M-CSF and TNF. Ly-6C$^-$ monocytes develop into macrophages with M-CSF, while RANKL converts them towards osteoclast commitment. Early Ly-6C$^+$ monocytes are highly potential for osteoclast commitment in response to M-CSF and RANKL but can still become Ly6C$^-$ monocytes. Fully differentiated macrophages, induced by M-CSF and RANKL, can fuse to become osteoclasts. Pathologically, macrophages can form multinucleated giant cells (MGCs) with M-CSF, GM-CSF,

and interleukins (IL-4, IL-13), and these MGCs can differentiate into osteoclasts with common fusion mediators. Additionally, immature dendritic cells can differentiate into osteoclasts. (Sun, Y., Li, et, al 2021) (**Figure 3**). The classical view is that HSCs differentiate into lymphoid (CLP) and common myeloid progenitor (CMP) cells. After further differentiation, CLPs generate T cells, NK cells, and B cells, while the CMP7 differentiate into granulocyte-macrophage progenitors (GMPs). Under the influence of macrophage colony-stimulating factor (M-CSF), GMPs further develop into monocytes within the BM. Once formed, monocytes enter the bloodstream, where they circulate for a few days. These circulating monocytes can migrate into tissues in response to chemokines and cytokines, signaling molecules that guide their movement. Upon entering tissues, monocytes differentiate into macrophages. This differentiation process is influenced by the local microenvironment, including signals from damaged cells, pathogens, and other immune cells. These environmental signals help monocytes acquire the functional and phenotypic characteristics of tissue-resident macrophages. (Epelmans,.et al 2014)

With progress in single-cell analyses, this traditional and simplistic version of progenitor cell differentiation has been challenged. Many studies indicate that HSCs are heterogeneous and biased in their differentiation potential (Lunger, I., et al 2017). Studies also showed that HSC bias is regulated by the distinct niche they occupy in BM. Indeed, they become biased in their differentiation potential toward a specific cell lineage, such as osteoclast-biased (Os-Bi), myeloid-biased (My-Bi), platelet biased (Pl-Bi), and lymphoid-biased (Ly-Bi). Some HSCs have balanced differentiation potential and can develop into osteoclast, myeloid, platelet, and lymphoid lineage precursors. Os-Bi HSCs develop into preosteoclasts which, after fusion, create multinucleated osteoclasts. Osteoclasts can also derive from mature monocytes or macrophages. The My-Bi HSCs differentiate into neutrophils, eosinophils, basophils, erythrocytes, and monocytes forming dendritic cells and macrophages. Pl-Bi HSCs develop into megakaryocytes, which subsequently produce platelets. Megakaryocytes can also develop from My-Bi HSCs. Ly-Bi develops into T cells, NK cells, and B cells producing plasma cells (Bursuker,.et al 1983).
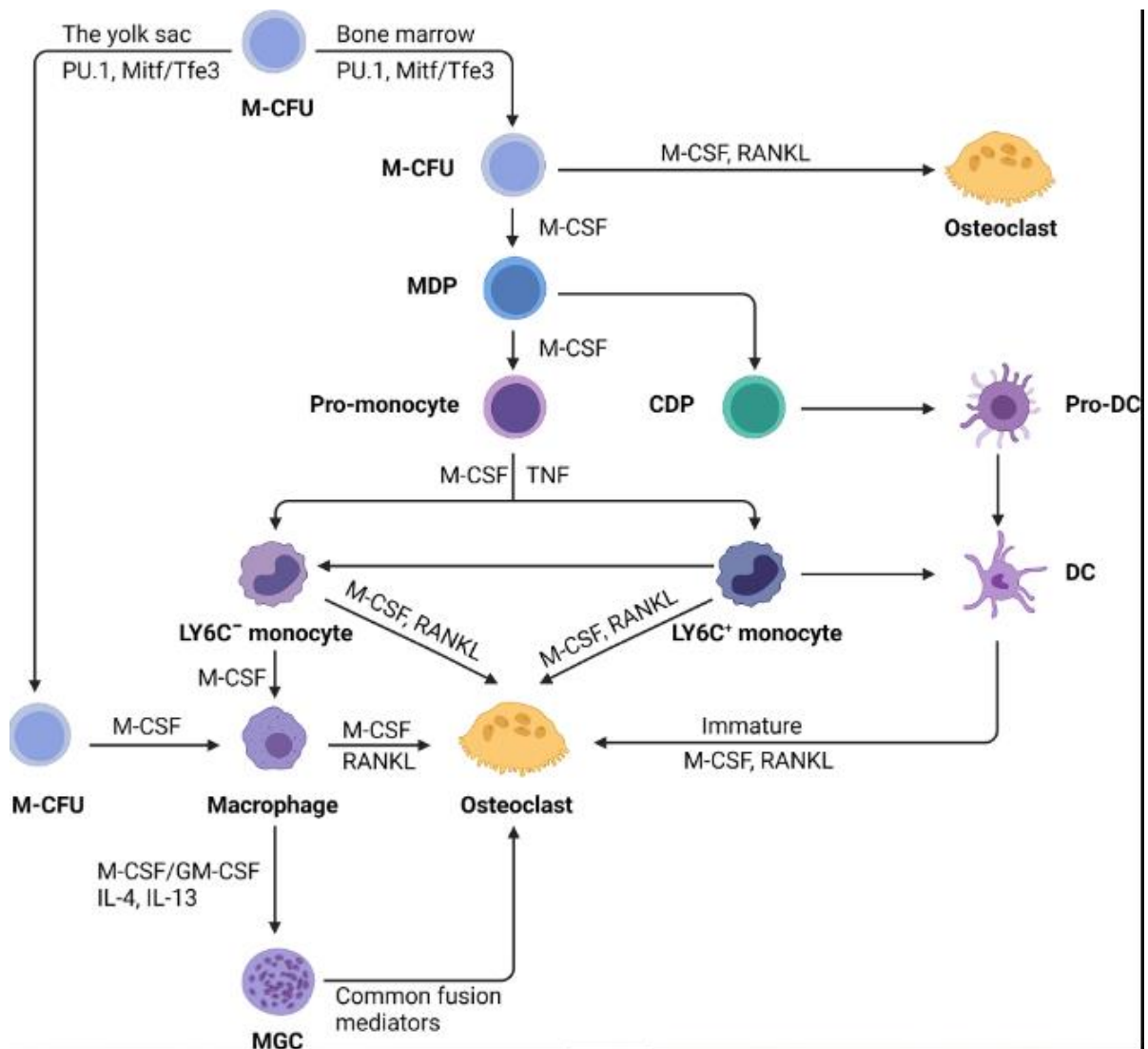
Figure 3. The monocyte/macrophage origin of osteoclasts. Hematopoietic stem cells myeloid colony-forming units (M-CFU) from bone marrow or yolk sac are the main site of myeloid cell production. Recent studies have shown that tissue-resident macrophages initially arise from myeloid stem cells (M-CFU) in the yolk sac of the developing embryo. In M-CFU, early expression of PU.1 and Mitf induces the emergence of M-CSFR (the receptor for M-CSF). Subsequently, in combination with macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL), M-CFU can differentiate directly into osteoclasts. But in the separate action of M-CSF, M-CFU can differentiate into M-CSF-dependent macrophage and dendritic cell progenitor (MDP). MDP can differentiate into both dendritic cells and monocytes, the latter of which can differentiate into pro-monocytes under the sustained action of M-CSF. Pro-monocytes can differentiate to form specifically marked monocytes, namely: $LY6C^+$ or $LY6C^-$ blood monocytes, under appropriate stimulation, such as M-CSF, TNF. Monocytes ($Ly\text{-}6C^-$) induced by M-CSF develop into macrophages, but the addition of RANKL converts monocytes into osteoblasts commitment. Early-stage $Ly\text{-}6C^+$ monocytes exhibit a high potential for osteoclast commitment in response to M-CSF and RANKL activation while still retaining the capacity to transform into $Ly6C^-$ monocytes. Completely differentiated macrophages induced by M-CSF and RANKL can fuse to become osteoclasts. Under pathological situations, macrophages can generate multinucleated giant cells (MGCs) when stimulated with M-CSF or stimulating factor (GM-CSF) and interleukins (IL-4, IL-13). MGCs continue to differentiate into osteoblasts in the presence of common fusion mediators. In addition, immature dendritic cells have the potential for osteolytic differentiation.

3.2 Macrophage functions

Macrophages are widely distributed across various tissues, where they often adopt specialized forms suited to their specific environments. Examples of tissue-resident macrophages include Kupffer cells (KCs) in the liver, alveolar macrophages in the lungs, microglia in the central nervous system, and osteoclasts in the bone. Each of these specialized macrophages plays crucial roles in maintaining tissue homeostasis and responding to local needs. For instance, KCs are involved in filtering the blood and removing pathogens, alveolar macrophages clear inhaled pathogens and particles, microglia participate in immune defense and maintenance of neural tissue, and osteoclasts are responsible for bone resorption and remodeling.

Some tissue-resident macrophages, such as microglia and KCs, originate from yolk sac progenitors and the fetal liver during embryogenesis. These macrophages have the ability to self-renew locally and maintain their population independently of monocyte recruitment in adults. In postnatal life, most tissue-resident macrophages are maintained through local proliferation rather than continuous recruitment from blood monocytes. This local self-renewal is essential for maintaining tissue homeostasis and responding to local needs.

Macrophages exhibit significant plasticity, allowing them to adapt to various functional roles depending on their microenvironment and pathological conditions. This plasticity is driven by local signals and involves changes in gene expression profiles, surface markers, and functional properties. The differentiation and function of macrophages are tightly regulated by various signaling pathways, transcription factors, and epigenetic modifications. Key transcription factors include PU.1, C/EBPα, and NF-κB, which play crucial roles in the development and activation of macrophages (Bonnardel, j .et al 2018).

Overall, macrophages undergo a series of differentiation steps, and adapt to various roles in different tissues. Their ability to respond to environmental cues and perform diverse functions makes them a central focus in immunology and biomedical research (Gordon, S., et al 2005). Understanding the origin and differentiation of macrophages is critical for developing therapeutic strategies for a wide range of diseases, including infections, chronic inflammatory conditions, and cancers. Modulating macrophage function and differentiation holds potential for treating diseases where macrophages play a key role in pathology. For example, in autoimmune diseases, macrophages can drive inflammation and tissue damage by presenting antigens to autoreactive T cells and producing pro-inflammatory cytokines. Conversely, they can promote tissue repair and resolution of inflammation by secreting anti-inflammatory cytokines and growth factors. (Yang et al., 2023) ·

Macrophages are pivotal players in the maintenance of bone health, particularly through their regulation of osteoclastogenesis. As highlighted, macrophages influence the differentiation and activity of osteoclasts, which are crucial for bone remodeling. In the context of HA, the role of macrophages extends beyond traditional immune responses. Chronic hemarthrosis (joints-bleeding) hemophilia A patients trigger inflammatory processes that macrophages are involved in resolving. This inflammatory response can exacerbate bone and joint deterioration. Thus, the anti-inflammatory and repair-promoting functions of macrophages are essential in mitigating these effects (Yang Sun et al,.202)

OBJECTIVE OF THE THESIS

In my thesis internship I investigated if and how the macrophage differentiation is altered in absence of FVIII. As model I employed cells derived from a model of severe HA available in the laboratory. One of the easiest ways to in vitro differentiate murine macrophages is to stimulate myeloid progenitors located in the BM. This study is a branch of a general project aimed to elucidate the mechanisms by which FVIII deficiency disrupts normal skeletal homeostasis and contributes to bone remodeling processes. Several experiments showed that both osteoblast maturation (**Figure 4A-B**) and calcium deposition (**Figure 4C**) were defective during in vitro differentiation of murine HA osteoblasts while in vitro osteoclastogenesis was increased in HA samples (**Figure 5A-C**). Since osteoclasts belong to the macrophage family, here I concentrated my attention to the BM-derived macrophages to understand if the observed alteration of osteoclastogenesis is in common with the formation of another macrophage population.

MATERIAL AND METHODS

Mice

Hemophilia A mice on a C57BL/6 background (Jackson laboratory U.S) were originally generated by targeted disruption of exon 16 (Bi et al., 1995) in FVIII gene. Males (brothers) of 8 weeks were used. All animals were kept under specific pathogen-free, and all the procedures were reviewed and approved by the Animal Care and Use Committee of Università del Piemonte Orientale, Novara, Italy.

Macrophage in vitro differentiation

Murine BM cells were isolated from 8 weeks old C57Bl6/J HA and WT mice: briefly, the limbs were harvested and cleaned by muscles, followed by flushing of the bones using a 1ml syringe and 25G needle with DMEM containing 10% FBS. The cell suspension was filtered through a 40 μm nylon mesh cell strainer and the obtained cellular suspension was centrifuged at 1500rpm for 5 minutes at 4°C and cells resuspended in DMEM+10% FBS at a volume of 2ml per mouse cells were counted in a Burker chamber using trypan blue for excluding dead cells. Cells were finally seeded at a density of 2×10^6 cells/mL in a total volume of 1 mL per well in a 12-well plate, with each well having a surface area of 3.5 cm in DMEM+10% FBS supplemented with M-CSF (1,3,5ng/ml), and incubated at 37°C and 5% CO_2 for 8-12 days, changing the differentiation medium every 3 day. At time of analysis, the adherent cells were detached by replacing the medium with cold PBS with 5 mM EDTA washed twice and centrifuged at 4℃ for 5 minutes at 1500rpm.

Flow Cytometry analysis

Samples were stained for 15 minutes with fluorochrome-conjugated monoclonal antibodies against mouse cells: F480, CD11b, CD115 from Thermofisher Scientific, The table below shows details about the antibodies and their dilutions. Samples were acquired on the Attune NxT Acoustic Focusing Cytometer (Thermofisher Scientific) and analysis was performed by FlowJo v10 (BD). Representative dot plots showing the progressive gating strategy for detecting myeloid cells and macrophages are shown in **Figure 6**.

Table 1. Antibodies Used for Flow Cytometry Analysis

Antibody	Clone	Fluorochrome	Working dilution
F480	BM8	PE	1:50
CD115	AFS98	APC	1:100
CD11b	M1/70	PE-Cyanine7	1:200

TRAP staining

Media was aspirated from the well and a wash with PBS was preformed; the samples were fixed in formalin for one minute and then rinsed in deionized water. Trap staining kit (SIGMA-ALDRICH) was used according to manufacturer's instruction.

Statistical analysis

Statistical analysis was performed with Graph-Pad Prism 9.0 (GraphPad Software). All data are expressed as average ± standard error median (SEM). Mann-Whitney t test was used for comparing the two experimental groups. A p-value of less than 0.05 was considered to be statistically significant.

RESULTS

1. Analysis of the myeloid BM cells in HA compared to WT mice

A single cell suspension of BM cells was obtained from one tibia and one femur of 8-week-old animals (**Figure 7A**). The total number of cells was not different between HA and WT samples (**Figure 7B**), indicating the absence of a global change in the marrow compartment.

Flow cytometry was employed to analyze specific myeloid markers of BM cells: CD11b is a general marker for myeloid cells, such as granulocytes and monocytes, while F4/80 is a marker of mature murine macrophages. The frequency of CD11b$^+$ cells belonging to the total myelomonocytic lineage was similar in HA and WT BM (**Figure 7C**), as well as the number (**Figure 7D**) and percentage (**Figure 7E**) of mature CD11b$^+$F480$^+$ macrophages. Since the Macrophage Colony-Stimulating Factor Receptor (M-CSFR, CD115), plays a crucial role in the regulation of their growth, differentiation and survival, its expression was assessed on the CD11b$^+$F480$^+$ macrophages. Half of the mature BM macrophages displayed the M-CSFR on their surface (**Figure 7F**) with no disparity between HA and WT cells. Overall, these results indicate that there is no impairment in the in vivo BM macrophage differentiation in HA mice.

2. Microscopic analysis of in vitro maturation of BM derived macrophages

The progression of BM cell differentiation into macrophages under M-CSF exposure revealed intriguing insights into the cellular behaviors of HA and WT macrophages. On day 0 images taken at the light microscope showed the baseline characteristics of plated BM cells which attached to the surface: at this stage several round and bright cells were visible in suspension while no notable differences were observed between the two experimental groups (**Figure 8A-B**). By day 5, following exposure to M-CSF, most of the cells were growing in adherence and they began to display cytoplasmic elongations (**Figure 8C-D**). A similar morphology and behavior across the three concentrations of M-CSF (1 ng/mL, 3 ng/mL, 5 ng/mL) and between HA and WT samples was observed (data not shown). However, from day 8 onwards, HA cells demonstrated a slightly accelerated differentiation process (**Figure 8E-F**) and several macrophages with the classical "fried egg" morphology were visible (arrows in **Figure 8E-F**). On day 12 the HA cells exhibited a higher survival rate compared to WT cells (**Figure 8G-H**). Overall, the macrophage differentiation protocol

worked efficiently for both groups; however, HA samples seemed to have a slightly increase capability to in vitro differentiate in macrophages and to survive longer time at high M-CSF concentration when compared to WT BM cells.

3. Phenotypic characterization of in vitro differentiated BM macrophages

To further assess the in vitro BM differentiation efficiency, we conducted flow cytometry analysis on day 8 and 12. Specifically, as done on day 0, we employed antibodies detecting the myeloid CD11b, the macrophagic F4/80 and the CD115 marker. The number of cells was estimated by acquiring the same volume of each sample after having detached the cells by adding cold PBS plus 5mM EDTA. Already on day 8 most of the cells expressed both CD11b and F480 on their surface (**Figure 9A**) with the remaining displayed the CD11b only (**Figure 9B**), indicating that the majority of cells were mature macrophages. Overall, the three different M-CSF concentrations promoted the generation of the same percentage of CD11b$^+$F480$^+$ macrophages while their total number was enhanced by the higher cytokine concentration (**Figure 9C**). The number of CD11b$^+$F480$^-$ cells was very low (**Figure 9D**) in accordance with the low percentage detected. The latter cells were probably monocytes, based on their morphology observed at the light microscope (**Figure 8**). The recovery of CD11b$^+$F480$^+$ macrophages and of CD11b$^+$F480$^-$ cells was slightly incremented in HA compared to WT samples across all the M-CSF concentration, even if a statistical difference was not reached but in one case. Roughly 40-60% of CD11b$^+$F480$^+$ cells expressed still the M-CSFR as shown by the CD115 detection (**Figure 9E**). These results suggest that the in vitro differentiation of myeloid progenitors into macrophages and the subsequent proliferation of macrophages may be slightly higher in HA, in accordance with what visualized on the microscope

When the same analysis was performed on day 12, all the viable cells were CD11b$^+$F480$^+$ (**Figure 10A**) with a very little percentage expressing CD11b only (**Figure 10B**). The yield of cells (**Figure 10C**) slightly decreased compared to what obtained on day 8 (**Figure 9C**), suggesting a possible detrimental effect of continuous M-CSF stimulation on differentiated macrophages. Surprisingly, no difference was detected between HA and WT macrophages, neither in percentage nor in number. This is in contrast to what was observed on the light microscope on day 12, when the WT cells looked less than the HA ones.

CD115 percentage on CD11b$^+$F480$^+$ macrophages tended to decrease on day 12 in comparison to day 8 with no significative difference among HA and WT groups (**Figure 10E**), probably due to a down-regulation of the M-CSFR linked to overstimulation. In addition to the frequency, we investigated the mean fluorescent intensity (MFI) of CD115 since the latter value should better correlate with the number of molecules detected on the surface of each cell, rather than indicating the percentage of cells expressing it. Since the MFI can be greatly influenced by the flow cytometry parameters set for the daily acquisition, the results were plotted separately for each independent experiment (**Figure 11A-H**). Consequently, the limited number of samples per group makes it difficult to draw a definitive conclusion on the comparison of the expression levels of this marker between the two experimental groups.

4. In vitro macrophage visualization by TRAP staining

Tartrate-resistant acid phosphatase (TRAP) is a metalloprotease that catalyzes hydrolysis of phosphate esters, and it is highly expressed in osteoclasts and activated macrophages (Janckila, A. J., & Yam, L. T. 2009). TRAP staining allows the visualization under a microscope of cells expressing it which should show insoluble maroon deposits at sites of enzymatic activity. TRAP staining is particularly valuable in research focused on bone metabolism, immune responses, and various pathologies involving macrophage activity. Here it was used for confirming the presence of differentiated macrophages in the cultured BM-derived cells. On both day 8 (**Figure 12A-E**) and day12 (**Figure 13A-E**) the TRAP staining revealed a robust presence of TRAP-positive macrophages in both HA and WT groups, with no significant differences observed between the two groups across all M-CSF concentrations. Especially on day 8, several macrophages with the classical "fried egg" morphology were clearly visible (arrows in **Figure 12C-D**) while others looked more fibroblast-like elongated, which is reported for macrophages differentiated in vitro with M-CSF (Weiss, M., Blazek,et, al 2013). The density of cells appeared particularly high on day 8 at the 5ng/ml M-CSF concentration while on day 12 the cells were sparse and suffering. This indicates, at least for this single experiment, that the continuous stimulation with high dose of M-CSF brought to exhaustion of the cells.
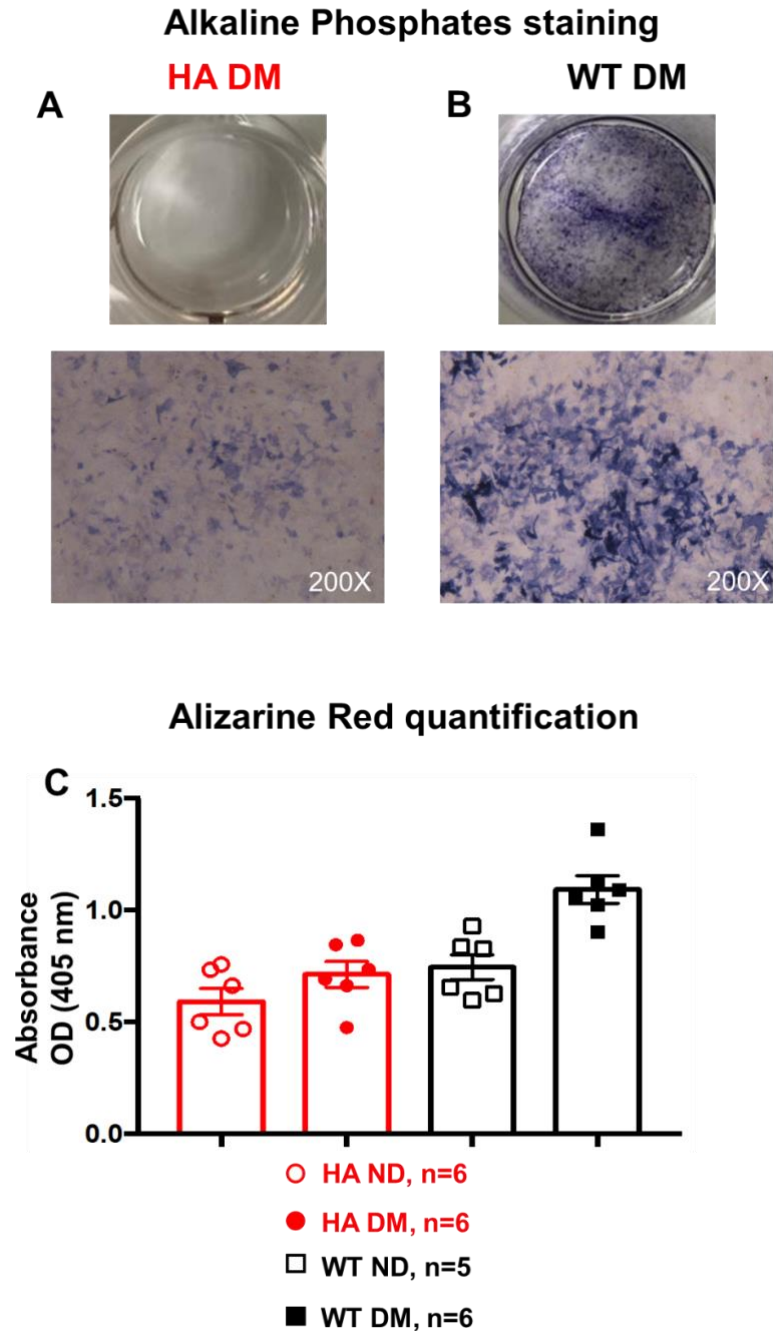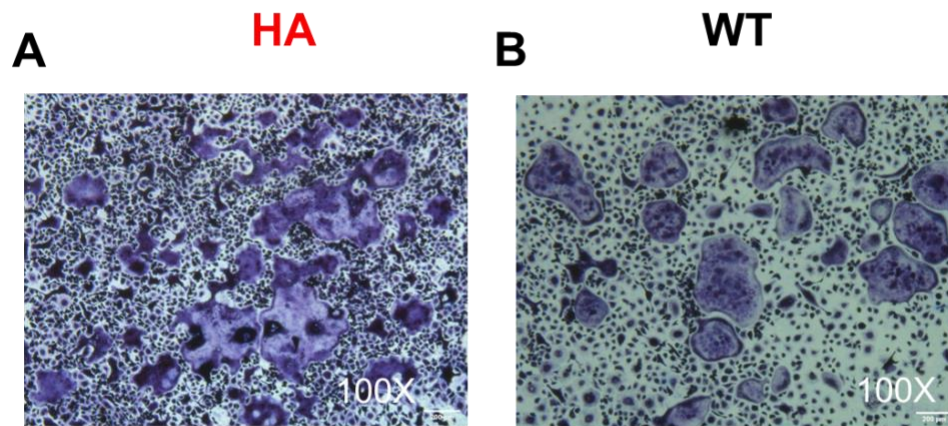
FIGURES

Figure 4

Figure 4. Murine osteoblast progenitors were isolated from HA or WT bones and cultured in vitro for 14 days in osteogenic medium (DM) supplemented with 50μg/ml ascorbic acid and 10mM β-glycerophosphate; cells cultured in non-differentiating medium (ND) were used as control. **A-B)** Representative images showing the alkaline phosphohatase positive cells. **C)** Histogram graph displaying the average ± SEM of the quantification of the alizarine red staining used for measuring the calcium deposition; one-way ANOVA test was applied - * $p < 0,05$
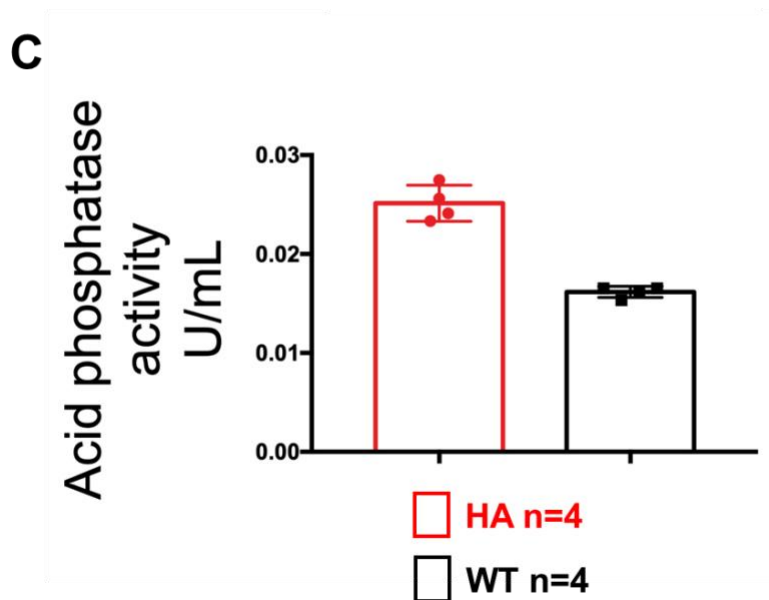
Figure 5

TRAP staining

Figure 5. Murine myeloid progenitors were isolated from HA or WT BM and cultured in vitro for 10 days in DMEM supplemented with 30ng/ml M-CSF and 100ng/ml RANKL. **(A-B)** Representative images showing the TRAP positive cells. **(C)** Histogram graph displaying the average ± SEM of the quantification of the TRAP activity; Mann-Whitney t-test test was applied - * $p < 0{,}05$
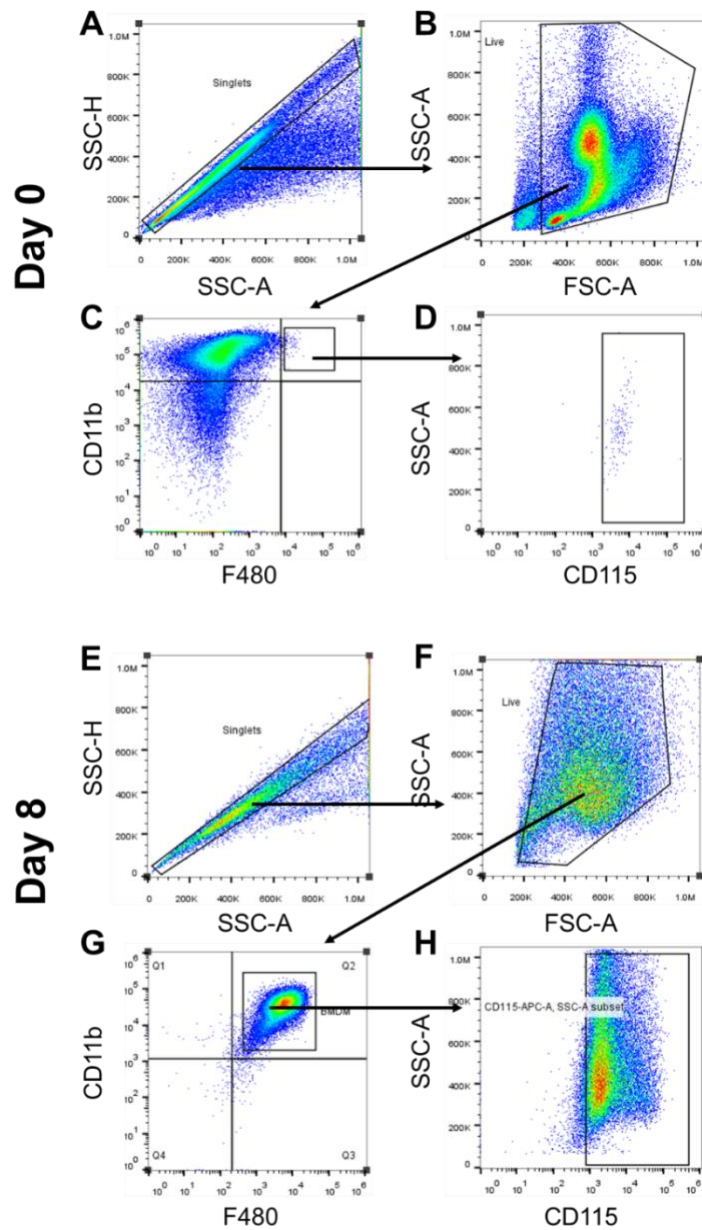
Figure 6

Figure 6. Representative dot plots showing the gating strategy used for the identification of CD11b$^+$F480$^+$ macrophages and the quantification of the CD115 (M-CSF receptor) expression on day 0 on the BM cells (**A-D**) and after 8 days of in vitro differentiation (**E-H**).
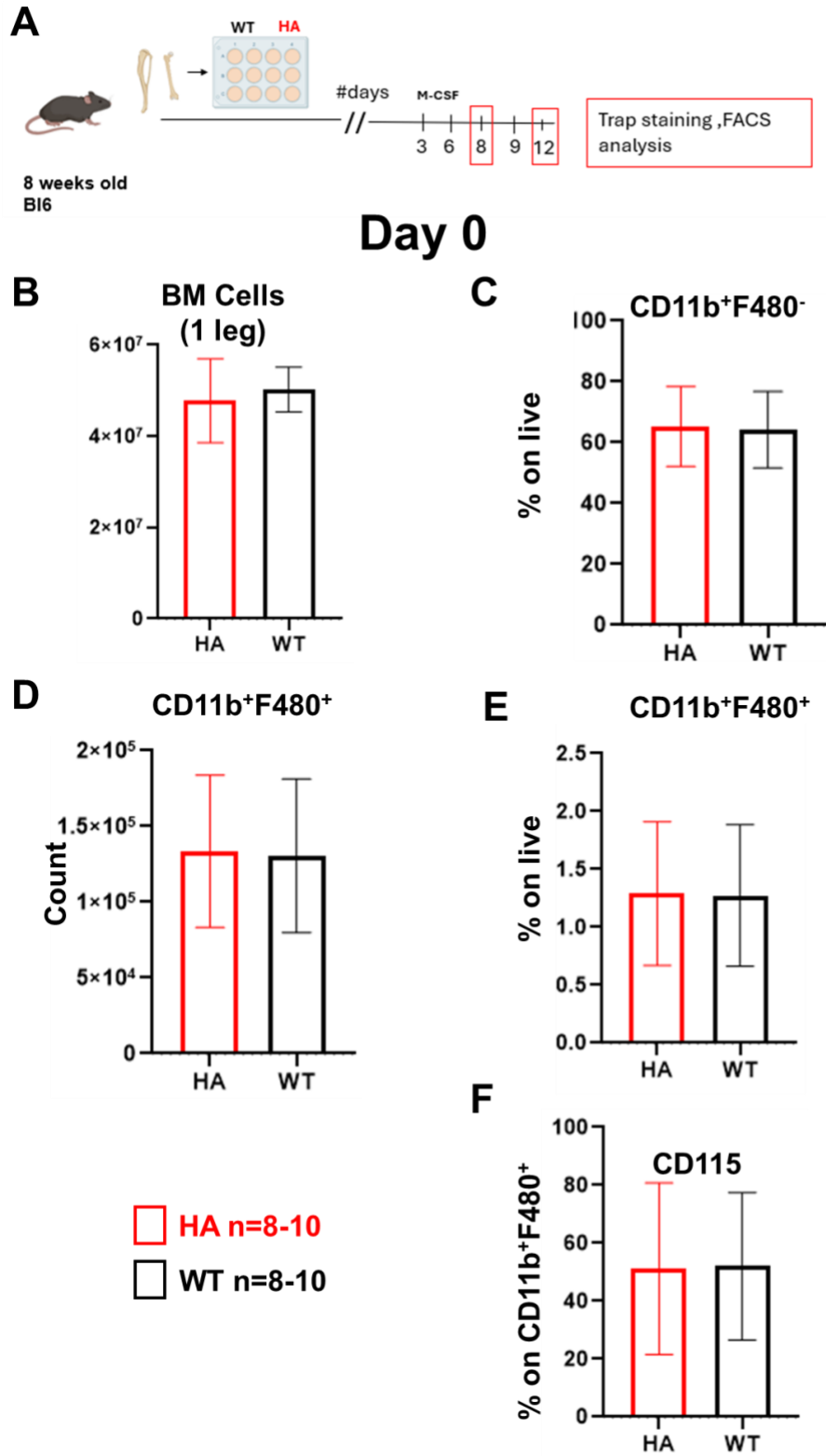
Figure 7

Figure 7. (**A**) Scheme representing the experimental outline. Histograms of total BM cells (1 leg) (**B**), percentage of CD11b$^+$F480$^-$ cells (**C**), count (**D**) and percentage (**E**) of CD11b$^+$F80$^+$ macrophage cells, percentage of CD115 positive cells (**F**). Bars represent the average while lines show the SEM (4 independent experiments).
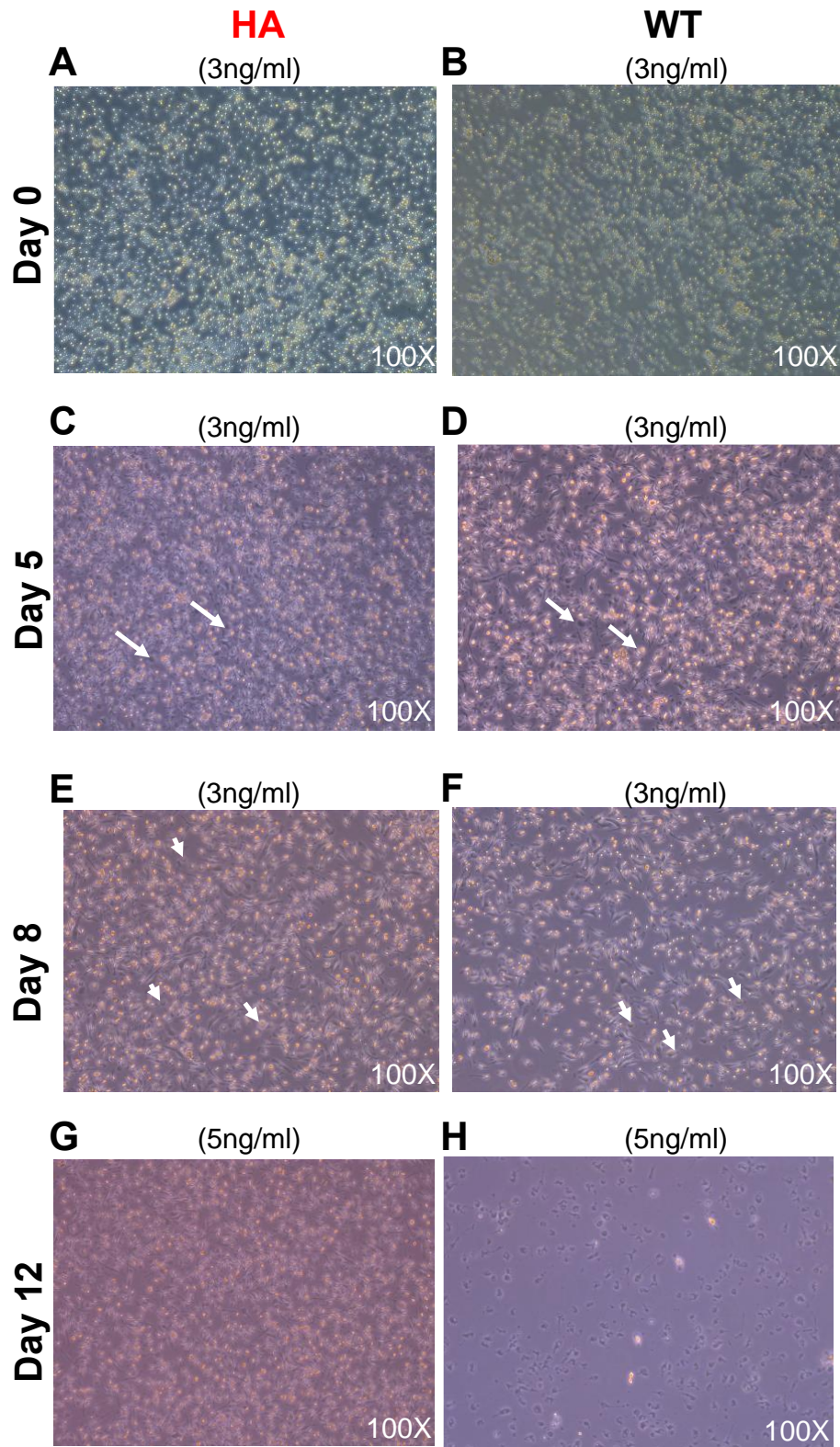
Figure 8

Figure 8. Representative pictures taken at the light microscope of the differentiating BM cells (3ng/ml) on day 0 (**A-B**), 5 (**C-D**), 8 (**E-F**) and 12 in (5ng/ml) (**G-H**) White arrows indicate macrophages with the classical "fried egg" morphology.
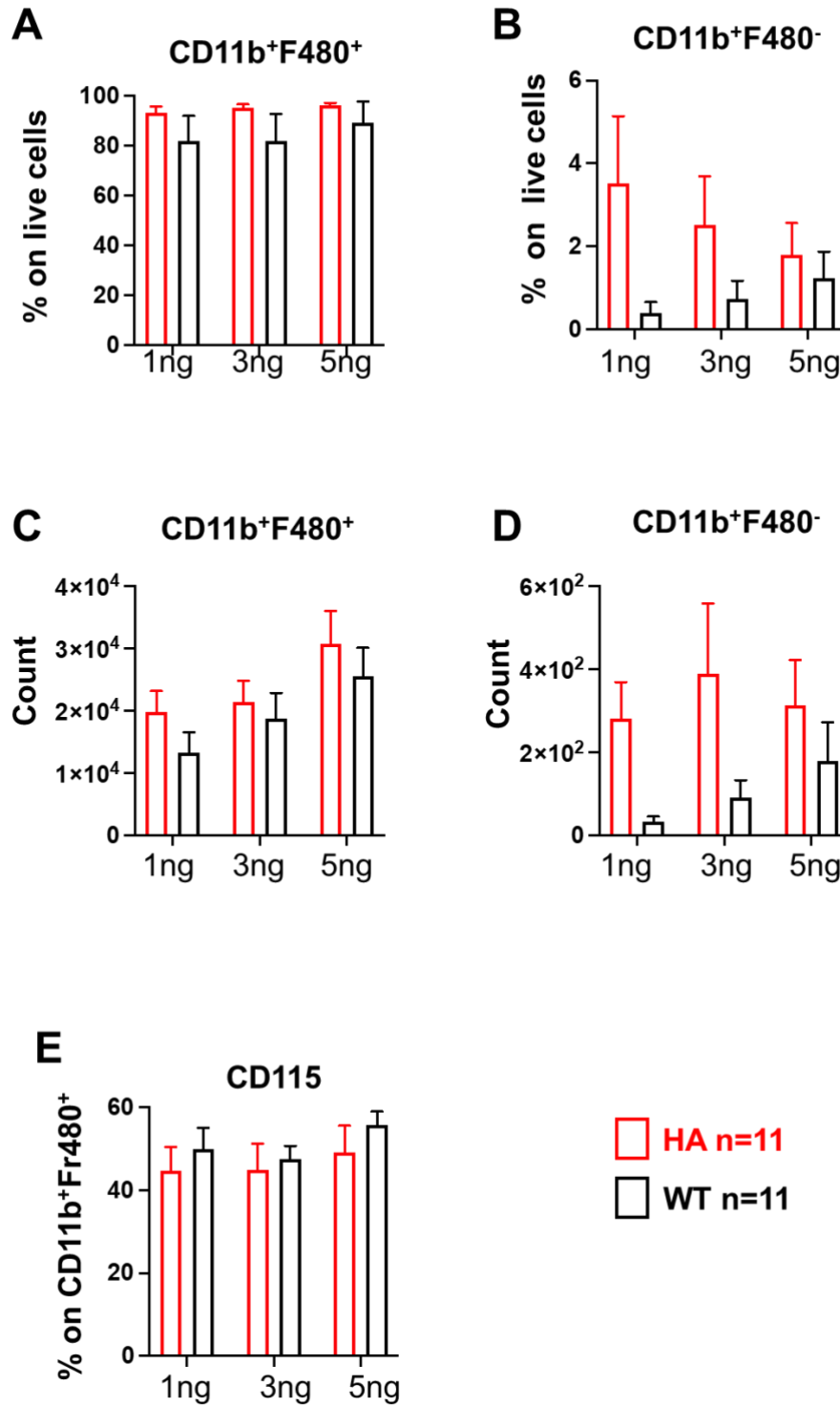
Figure 9

Day 8

Figure 9. Histograms plotting the percentage (**A-B**) and count (**C-D**) of $CD11b^+F480^+$ and $CD11b^+F480^-$ cells after 8 days of in vitro exposure to 1, 3 or 4ng/ml M-CSF. CD115 percentage was evaluated on $CD11b^+F480^+$ macrophage cells (**E**). Bars represent the average while lines show the SEM (4 independent experiments). Mann-Whitney t-test test was run for comparing HA versus WT for each M-CSF concentration - * $p<0,05$
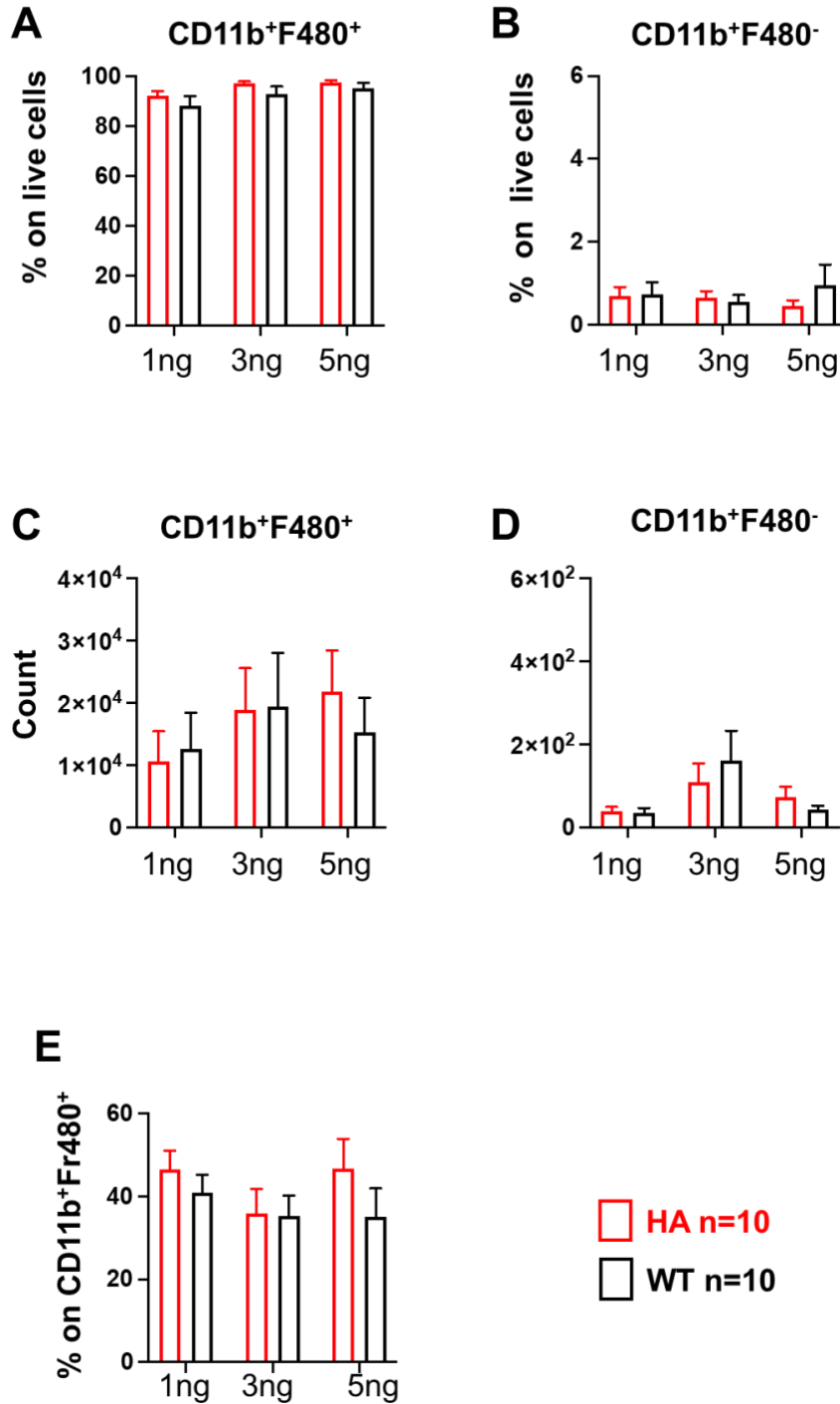
Figure 10

Day 12

Figure 10. Histograms plotting the percentage (**A-B**) and count (**C-D**) of CD11b$^+$F480$^+$ and CD11b$^+$F480$^-$ cells after 12 days of in vitro exposure to 1, 3 or 4ng/ml M-CSF. CD115 percentage was evaluated on CD11b$^+$F480$^+$ macrophage cells (**E**). Bars represent the average while lines show the SEM (4 independent experiments). Mann-Whitney t-test test was run for comparing HA versus WT for each M-CSF concentration.
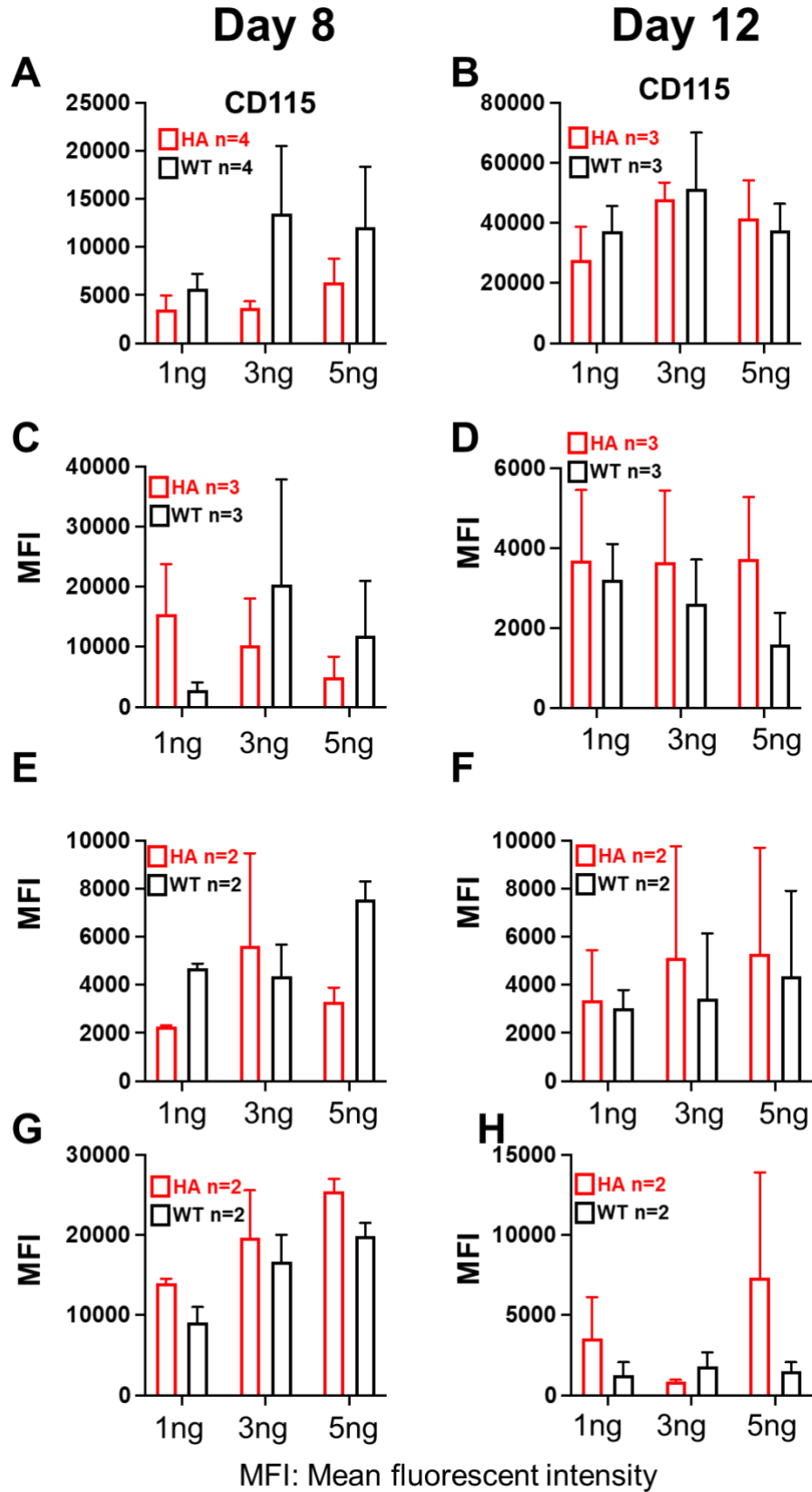
Figure 11

Figure 11. Histograms of single independent experiments (**A-H**) plotting the average + SEM of MFI (median fluorescent intensity) of CD115 calculated on CD11b$^+$F80$^+$ macrophages on day 8 and 12 of in vitro differentiation. Bars represent the average while lines show the SEM

Figure 12

Day 8

Figure 12. Representative pictures taken at the light microscope of the TRAP staining on day 8 of in vitro differentiation after exposure to 1 (**A-B**), 3 (**C-D**) and 5 (**E-F**) ng/ml M-CSF. White arrows indicate macrophages with the classical "fried egg" morphology.
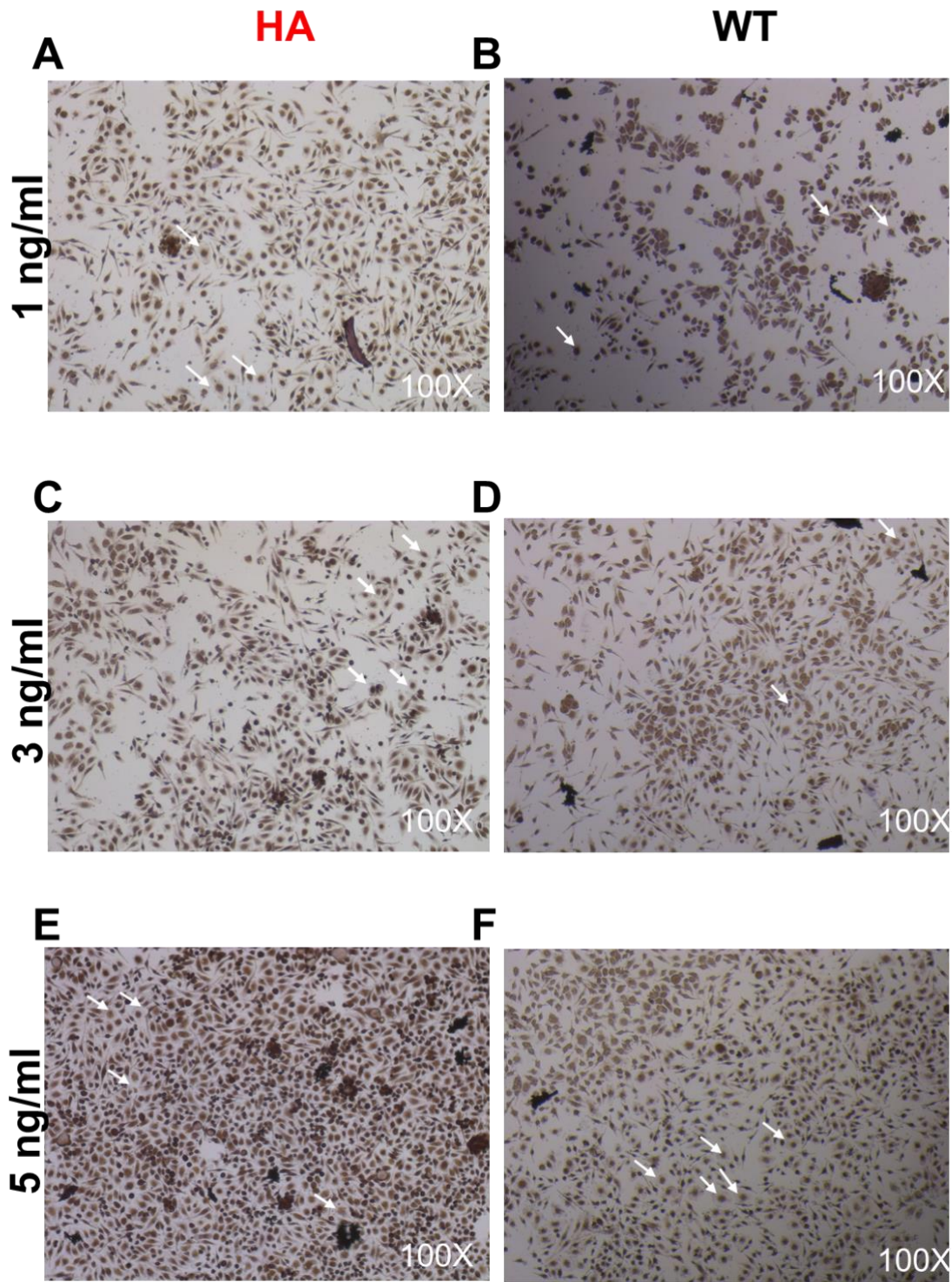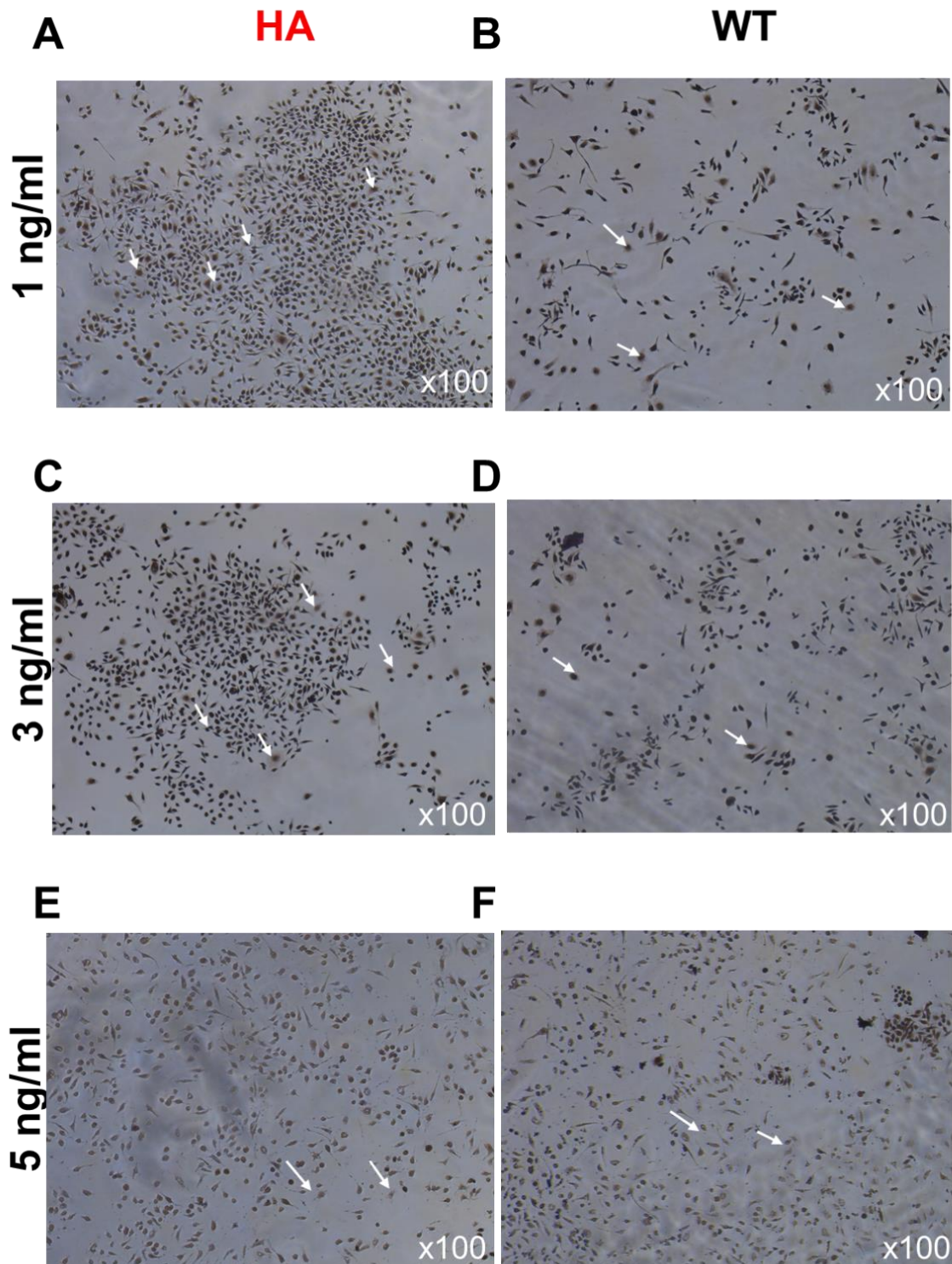
Figure 13

Day 12

Figure 13. Representative pictures taken at the light microscope of the TRAP staining on day 12 of in vitro differentiation after exposure to 1 (**A-B**), 3 (**C-D**) and 5 (**E-F**) ng/ml M-CSF. White arrows indicate macrophages with the classical "fried egg" morphology.

DISCUSSION

This study aimed to investigate if the absence of FVIII has an impact on BM-derived macrophage differentiation. Preliminary experiments conducted in our laboratory have shown that in vitro osteoclastogenesis is increased in HA compared to WT samples suggesting a possible direct effect of FVIII on the macrophage formation. Here we focused on characterizing BM-derived macrophages due to the easy way to access the source for starting the differentiation process and for the strong link between osteoclasts and BM-resident macrophages (Chang et al., 2008). In future it would be interesting to investigate other tissue resident macrophages (e.g. Kupffer cells, microglia).

First, to ascertain that the difference was not already present in vivo in the BM region, we enumerated and calculated the frequency of total myeloid $CD11b^+$ cells and mature $CD11b^+F480^+$ macrophages in the BM single cell suspension prior to plating the cells in culture. The absence of significative difference between HA and WT mice indicates that FVIII does not affect those populations in vivo; this was not too surprising since previous experiments performed by other master students in the laboratory did not find changes in percentage or number of the main BM immune-hematological populations. Nevertheless, we cannot rule out that within the bone region there is a difference since our analysis was carried on cells flushed out from the BM while macrophages and osteoclasts are attached on the bone extracellular matrix. Additionally, the expression of the M-CSFR on mature macrophages was found consistent across both groups; however, to confirm if the differentiation process in vivo remains intact in HA mice, further studies are needed, examining M-CSFR expression on myeloid progenitors rather than on mature cells. This approach would provide more insights into the early stages of myeloid differentiation and help identify any potential disruptions in the developmental pathway.

In vitro macrophage differentiation under M-CSF stimulation was slightly more efficient in HA vs WT sample on day 8, a result that was shared also by $CD11b^+F80^-$ monocytes. This result agrees with the observed increase in vitro osteoclastogensis, even if here the statistical difference was not reached. It is intriguing that the M-CSF upward doses increased proportionally the number of recovered cells while maintaining the difference between the two experimental groups. On day 12 the difference disappeared, and the recovery of the cells was not enhanced but rather reduced, especially for the highest M-CSF concentration (5ng/ml). In this last case the decrease was particularly evident for the WT cells, as displayed by the pictures taken at the microscope and by the count obtained at the flow

cytometry instrument. This result may indicate a potential enhanced capability of HA cells to differentiate and survive under high M-CSF concentrations, although this observation requires further experiments to be confirmed. For instance, we could repeat the in vitro differentiation by using higher M-CSF concentration and/or testing the number of apoptotic cells or assess the survival of the macrophages (Eren,R.Fasel,N 2017).

TRAP staining confirmed the differentiation into macrophages, with robust TRAP-positive cells observed in both groups on days 8 and 12. Again, macrophage numbers peaked at day 8, with a decline by day 12. The lower TRAP staining intensity observed on day 12 confirms that prolonged exposure to M-CSF may lead to suffering and exhaustion of the cells. Indeed most of the protocol suggested for in vitro differentiation of murine BM-derived macrophages do not exceed 10 days (Mendoza,R 2022) (Toda et al ., 2021). For further characterization of macrophage activity, in future experiments the TRAP activity will be quantified by measuring the acid phosphatase activity at the spectrophotometer.

Overall, our preliminary findings suggest that FVIII absence might facilitate greater macrophage maturation in the BM. This could be favored by the inflammatory status generated in the bone proximity in HA patients, especially within the joints since hemarthrosis is one of the main comorbidities developed by HA patients. Macrophages are key players in both inflammation and blood clotting (Cadé et al., 2022). They respond to bleeding in joints and contribute to the immune response against FVIII. On the other hand, chronic low-grade inflammation established in HA patients may disrupt macrophage differentiation, potentially exacerbating disease progression. The inflammation/coagulation interaction involves complex processes like adjusting thrombin and FXa levels through Protease-Activated Receptors (PARs), which are influenced by the persistent inflammation seen in HA patients. In future experiment we could assess the expression levels of PARs on macrophages.

Moreover, in our in vitro experiments we did not check if FVIII could act directly on the differentiation process. Even if FVIII is well known as a clotting factor, it might have different functions. Indeed, our lab has shown that both murine monocytes and macrophages express FVIII (Zanolini et al., 2015) suggesting a possible autocrine signal. It has been shown that treatment of human healthy peripheral blood mononuclear cells (PBMCs) with FVIII, VWF, FVIII/VWF complex

and thrombin inhibits in vitro osteoclastogenesis (Lancellotti et al., 2023). They did not assess HA PBMC while for us, thanks to the availability of the mouse model of severe HA, it is possible to repeat the in vitro differentiation process toward macrophages or osteoclasts testing the different molecules, alone or in combination with each other. These experiments would help to clarify if those factors are able to act directly on the differentiation process.

Previously it has been shown that human macrophage polarization was deregulated in HA in response to M-CSF and that this alteration was mainly caused by the impaired clotting formation, which in turn increased inflammation and decreased the wound healing and tissue regeneration (Knowles et al., 2019). In our current study we concentrated on the assessment of macrophage differentiation without testing the M1 vs M2 polarization. To deepen our understanding of macrophage dysfunction, in future we will quantify gene expression levels of specific markers associated with the M1 (e.g. CD86, iNOS) or M2 (e.g. CD163, CD206) subtype.

Further in vitro and in vivo characterization of the formation, phenotype, polarization and functionality of HA macrophages would be important to investigate beyond the possible role played by the osteoclasts in the BMD loss. As discussed above, macrophages are highly involved in the pathophysiology of the HA disease and their activity could be manipulated in the patients for reducing the inflammation. Moreover, it is worthy to mention that macrophages, as part of the innate immune system, can be involved in the process of FVIII-antigen presentation that can lead to the formation of FVIII inhibitors (Lai et al., 2018)_, one of the main side effect of the replacement therapy. Thus, acquiring more knowledge on their alteration in HA patients can be instrumental in designing new strategies for eradicating and/or preventing FVIII-specific antibodies.

BIBLIOGRAPHY

Ait Oumghar, I., Barkaoui, A., & Chabrand, P. (2021). Mechanobiological Behavior of a Pathological Bone. In Biomechanics and Functional Tissue Engineering. IntechOpen.

Aronovich, A., Nur, Y., Shezen, E., Rosen, C., Zlotnikov Klionsky, Y., Milman, I., Yarimi, L., Hagin, D., Rechavi, G., Martinowitz, U., Nagasawa, T., Frenette, P. S., Tchorsh-Yutsis, D., & Reisner, Y. (2013). A novel role for factor VIII and thrombin/PAR1 in regulating hematopoiesis and its interplay with the bone structure. Blood, 122(15), 2562–2571.

Bar-Shavit, Z. (2007). The osteoclast: A multinucleated, hematopoietic-origin, bone-resorbing osteoimmune cell. Journal of Cellular Biochemistry, 102(5), 1130–1139.

Blair, H. A. (2019). Emicizumab: A Review in Haemophilia A. Drugs, 79(15), 1697–1707.

Bolton-Maggs, P. H., & Pasi, K. J. (2003). Haemophilias A and B. The Lancet, 361(9371), 1801–1809.

Bonnardel, J., & Guilliams, M. (2018). Developmental control of macrophage function. Current Opinion in Immunology, 50, 64–74.

Boyle, W. J., Simonet, W. S., & Lacey, D. L. (2003). Osteoclast differentiation and activation. Nature, 423(6937), 337–342.

Brinza, M., Uscatescu, V., Hemcinschi, M., Chiriac, E., Gherghe, G., & Coriu, D. (2024). Challenges in prophylactic therapy with Emicizumab in patients with hemophilia A: Focus on monitoring tests. Revista Romana de Medicina de Laborator, 32(1), 93–100.

Cadé, M., Muñoz-Garcia, J., Babuty, A., Fouassier, M., Heymann, M.-F., Monahan, P. E., & Heymann, D. (2022). FVIII at the crossroad of coagulation, bone and immune biology: Emerging evidence of biological activities beyond hemostasis. Drug Discovery Today, 27(1), 102–116.

Chang, M. K., Raggatt, L.-J., Alexander, K. A., Kuliwaba, J. S., Fazzalari, N. L., Schroder, K., Maylin, E. R., Ripoll, V. M., Hume, D. A., & Pettit, A. R. (2008). Osteal Tissue Macrophages Are Intercalated throughout Human and Mouse Bone Lining Tissues and Regulate Osteoblast Function In Vitro and In Vivo. The Journal of Immunology, 181(2), 1232–1244.

Chavin, S. I. (1984a). Factor VIII: Structure and function in blood clotting. American Journal of Hematology, 16(3), 297–306.

Chen, S., Saeed, A. F. U. H., Liu, Q., Jiang, Q., Xu, H., Xiao, G. G., Rao, L., & Duo, Y. (2023). Macrophages in immunoregulation and therapeutics. Signal Transduction and Targeted Therapy, 8(1), 207.

CHRISTOFORIDIS, A., ECONOMOU, M., PAPADOPOULOU, E., KAZANTZIDOU, E., FARMAKI, E., TZIMOULI, V., TSATRA, I., GOMPAKIS, N., & ATHANASSIOU-METAXA, M.

(2011). Comparative study of dual energy X-ray absorptiometry and quantitative ultrasonography with the use of biochemical markers of bone turnover in boys with haemophilia. Haemophilia, 17(1).

Chyu, K.-Y., Dimayuga, P. C., & Shah, P. K. (2017). Vaccine against arteriosclerosis: an update. Therapeutic Advances in Vaccines, 5(2), 39–47.

Coppola, A., D'Ausilio, A., Aiello, A., Amoresano, S., Toumi, M., Mathew, P., & Tagliaferri, A. (2017). Cost-effectiveness analysis of late prophylaxis vs. on-demand treatment for severe haemophilia A in Italy. Haemophilia, 23(3), 422–429.

Eastell, R., & Hannon, R. A. (2008). Biomarkers of bone health and osteoporosis risk. Proceedings of the Nutrition Society, 67(2), 157–162.

Epelman, S., Lavine, K. J., & Randolph, G. J. (2014). Origin and Functions of Tissue Macrophages. Immunity, 41(1), 21–35.

Eren, R. O., & Fasel, N. (2017a). Macrophage Survival Assay Using High Content Microscopy. BIO-PROTOCOL, 7(16).

Gebetsberger, J., Schirmer, M., Wurzer, W. J., & Streif, W. (2022a). Low Bone Mineral Density in Hemophiliacs. Frontiers in Medicine, 9. https://doi.org/10.3389/fmed.2022.794456

Goldscheitter, G., Recht, M., Sochacki, P., Manco-Johnson, M., & Taylor, J. A. (2021). Biomarkers of bone disease in persons with haemophilia. Haemophilia, 27(1), 149–155.

Gordon, S., & Taylor, P. R. (2005). Monocyte and macrophage heterogeneity. Nature Reviews Immunology, 5(12), 953–964.

Harada, S., & Rodan, G. A. (2003). Control of osteoblast function and regulation of bone mass. Nature, 423(6937), 349–355.

Hoffman, M., & Monroe, D. M. (2003). The action of high-dose factor VIIa (FVIIa) in a cell-based model of hemostasis. Disease-a-Month, 49(1), a29504b.

IORIO, A., HALIMEH, S., HOLZHAUER, S., GOLDENBERG, N., MARCHESINI, E., MARCUCCI, M., YOUNG, G., BIDLINGMAIER, C., BRANDAO, L. R., ETTINGSHAUSEN, C. E., GRINGERI, A., KENET, G., KNÖFLER, R., KREUZ, W., KURNIK, K., MANNER, D., SANTAGOSTINO, E., MANNUCCI, P. M., & NOWAK-GÖTTL, U. (2010). Rate of inhibitor development in previously untreated hemophilia A patients treated with plasma-derived or recombinant factor VIII concentrates: a systematic review. Journal of Thrombosis and Haemostasis, 8(6), 1256–1265.

Janckila, A. J., Lederer, E. D., Price, B. A., & Yam, L. T. (2009). Tartrate-resistant acid phosphatase isoform 5a as an inflammation marker in end-stage renal disease. Clinical Nephrology, 71(04), 387–396.

Katsarou, O., Terpos, E., Chatzismalis, P., Provelengios, S., Adraktas, T., Hadjidakis, D., Kouramba, A., & Karafoulidou, A. (2010). Increased bone resorption is implicated in the pathogenesis of bone loss in hemophiliacs: correlations with hemophilic arthropathy and HIV infection. Annals of Hematology, 89(1), 67–74.

Kempton, C. L., Antun, A., Antoniucci, D. M., Carpenter, W., Ribeiro, M., Stein, S., Slovensky, L., & Elon, L. (2014). Bone density in haemophilia: a single institutional cross-sectional study. Haemophilia, 20(1), 121–128.

Kiialainen, A., Niggli, M., Kempton, C. L., Castaman, G., Chang, T., Paz-Priel, I., Adamkewicz, J. I., & Levy, G. G. (2022). Effect of emicizumab prophylaxis on bone and joint health markers in people with haemophilia A without factor VIII inhibitors in the HAVEN 3 study. Haemophilia, 28(6), 1033–1043.

Kim, J.-M., Lin, C., Stavre, Z., Greenblatt, M. B., & Shim, J.-H. (2020). Osteoblast-Osteoclast Communication and Bone Homeostasis. Cells, 9(9), 2073.

Kiper Unal, H. D. et al. (2017). Evaluation of bone mineral density and related parameters in patients with haemophilia: a single center cross-sectional study.

Lai, J. D., Cartier, D., Hartholt, R. B., Swystun, L. L., van Velzen, A. S., den Haan, J. M. M., Hough, C., Voorberg, J., & Lillicrap, D. (2018). Early cellular interactions and immune transcriptome profiles in human factor VIII-exposed hemophilia A mice. Journal of Thrombosis and Haemostasis, 16(3), 533–545.

Lancellotti, S., Battafarano, G., Sacco, M., Tardugno, M., di Gennaro, L., Luciani, M., de Cristofaro, R., & del Fattore, A. (2023). In Vitro Analysis of Bone Remodeling Alterations in Hemophilia: Influence of Coagulation Factors on Bone Cells. Blood, 142(Supplement 1), 2605–2605.

Larson, E. A., Larson, H. J., Taylor, J. A., & Klein, R. F. (2021). Deletion of Coagulation Factor IX Compromises Bone Mass and Strength: Murine Model of Hemophilia B (Christmas Disease). Calcified Tissue International, 109(5), 577–585.

Lau, A. G., Sun, J., Hannah, W. B., Livingston, E. W., Heymann, D., Bateman, T. A., & Monahan, P. E. (2014). Joint bleeding in factor VIII deficient mice causes an acute loss of trabecular bone and calcification of joint soft tissues which is prevented with aggressive factor replacement. Haemophilia, 20(5), 716–722.

Lee, J.-H., Parthiban, P., Jin, G.-Z., Knowles, J. C., & Kim, H.-W. (2021). Materials roles for promoting angiogenesis in tissue regeneration. Progress in Materials Science, 117, 100732.

Li, M., Yang, Y., Xiong, L., Jiang, P., Wang, J., & Li, C. (2023). Metabolism, metabolites, and macrophages in cancer. Journal of Hematology & Oncology, 16(1), 80.

Linari, S., Montorzi, G., Bartolozzi, D., Borderi, M., Melchiorre, D., Benelli, M., & Morfini, M. (2013). Hypovitaminosis D and osteopenia/osteoporosis in a haemophilia population: a study in HCV / HIV or HCV infected patients. Haemophilia, 19(1), 126–133.

Long, A. T., Kenne, E., Jung, R., Fuchs, T. A., & Renné, T. (2016). Contact system revisited: an interface between inflammation, coagulation, and innate immunity. Journal of Thrombosis and Haemostasis, 14(3), 427–437.

Lunger, I., Fawaz, M., & Rieger, M. A. (2017). Single-cell analyses to reveal hematopoietic stem cell fate decisions. FEBS Letters, 591(15), 2195–2212.

M. Manco-Johnson. (2021). Emicizumab: Will it Suffice for Bone Metabolism.

Mackie, E. J., Pagel, C. N., Smith, R., de Niese, M. R., Song, S. - J., & Pike, R. N. (2002). Protease-Activated Receptors: A Means of Converting Extracellular Proteolysis into Intracellular Signals. IUBMB Life, 53(6), 277–281.

MANCUSO, M. E., MANNUCCI, P. M., ROCINO, A., GARAGIOLA, I., TAGLIAFERRI, A., & SANTAGOSTINO, E. (2012). Source and purity of factor VIII products as risk factors for inhibitor development in patients with hemophilia A. Journal of Thrombosis and Haemostasis, 10(5), 781–790.

Mancuso, M., & Santagostino, E. (2017). Outcome of Clinical Trials with New Extended Half-Life FVIII/IX Concentrates. Journal of Clinical Medicine, 6(4), 39.

MELCHIORRE, D., MILIA, A. F., LINARI, S., ROMANO, E., BENELLI, G., MANETTI, M., GUIDUCCI, S., CECCARELLI, C., INNOCENTI, M., CARULLI, C., CIVININI, R., MORFINI, M., MATUCCI-CERINIC, M., & IBBA-MANNESCHI, L. (2012). RANK-RANKL-OPG in Hemophilic Arthropathy: From Clinical and Imaging Diagnosis to Histopathology. The Journal of Rheumatology, 39(8), 1678–1686.

Mendoza, R., Banerjee, I., Manna, D., Reghupaty, S. C., Yetirajam, R., & Sarkar, D. (2022). Mouse Bone Marrow Cell Isolation and Macrophage Differentiation (pp. 85–91).

Miyamoto, T., Ohneda, O., Arai, F., Iwamoto, K., Okada, S., Takagi, K., Anderson, D. M., & Suda, T. (2001). Bifurcation of osteoclasts and dendritic cells from common progenitors. Blood, 98(8), 2544–2554.

Nathwani, A. C. (2022). Gene therapy for hemophilia. Hematology, 2022(1), 569–578.

Oldenburg, J., Mahlangu, J. N., Kim, B., Schmitt, C., Callaghan, M. U., Young, G., Santagostino, E., Kruse-Jarres, R., Negrier, C., Kessler, C., Valente, N., Asikanius, E., Levy, G. G., Windyga, J., & Shima, M. (2017). Emicizumab Prophylaxis in Hemophilia A with Inhibitors. New England Journal of Medicine, 377(9), 809–818.

Pagel, C. N., Song, S.-J., Loh, L. H., Tudor, E. M., Murray-Rust, T. A., Pike, R. N., & Mackie, E. J. (2009). Thrombin-stimulated growth factor and cytokine expression in osteoblasts is mediated by protease-activated receptor-1 and prostanoids. Bone, 44(5), 813–821.

Paschou, S. A., Anagnostis, P., Karras, S., Annweiler, C., Vakalopoulou, S., Garipidou, V., & Goulis, D. G. (2014). Bone mineral density in men and children with haemophilia A and B: a systematic review and meta-analysis. Osteoporosis International, 25(10), 2399–2407.

Peyvandi, F., Mannucci, P. M., Garagiola, I., El-Beshlawy, A., Elalfy, M., Ramanan, V., Eshghi, P., Hanagavadi, S., Varadarajan, R., Karimi, M., Manglani, M. V., Ross, C., Young, G., Seth, T., Apte, S., Nayak, D. M., Santagostino, E., Mancuso, M. E., Sandoval Gonzalez, A. C., … Rosendaal, F. R. (2016). A Randomized Trial of Factor VIII and Neutralizing Antibodies in Hemophilia A. New England Journal of Medicine, 374(21), 2054–2064.

Recht, M., Liel, M. S., Turner, R. T., Klein, R. F., & Taylor, J. A. (2013). The bone disease associated with factor VIII deficiency in mice is secondary to increased bone resorption. Haemophilia, 19(6), 908–912.

Rodriguez-Merchan, E. C., & Valentino, L. A. (2016). Orthopedic disorders of the knee in hemophilia: A current concept review. World Journal of Orthopedics, 7(6), 370.

ROODMAN, G. D. (2006). Regulation of Osteoclast Differentiation. Annals of the New York Academy of Sciences, 1068(1), 100–109.

Sabatino, D. E., Nichols, T. C., Merricks, E., Bellinger, D. A., Herzog, R. W., & Monahan, P. E. (2012). Animal Models of Hemophilia (pp. 151–209).

Sahin, S., Sadri, S., Baslar, Z., & Ar, M. C. (2019). Osteoporosis in Patients With Hemophilia: Single-Center Results From a Middle-Income Country. Clinical and Applied Thrombosis/Hemostasis, 25, 1076 02961986168.

Scheven, B. A. A., Visser, J. W. M., & Nijweide, P. J. (1986). In vitro osteoclast generation from different bone marrow fractions, including a highly enriched haematopoietic stem cell population. Nature, 321(6065), 79–81.

Sivagurunathan, S., Pagel, C. N., Loh, L. H., Wijeyewickrema, L. C., Pike, R. N., & Mackie, E. J. (2013). Thrombin inhibits osteoclast differentiation through a non-proteolytic mechanism. Journal of Molecular Endocrinology, 50(3), 347–359.

Smith, S. A., Travers, R. J., & Morrissey, J. H. (2015). How it all starts: Initiation of the clotting cascade. Critical Reviews in Biochemistry and Molecular Biology, 50(4), 326–336.

Sun, Y., Li, J., Xie, X., Gu, F., Sui, Z., Zhang, K., & Yu, T. (2021). Macrophage-Osteoclast Associations: Origin, Polarization, and Subgroups. Frontiers in Immunology, 12.

Taves, S., Sun, J., Livingston, E. W., Chen, X., Amiaud, J., Brion, R., Hannah, W. B., Bateman, T. A., Heymann, D., & Monahan, P. E. (2019). Hemophilia A and B mice, but not VWF−/−mice, display bone defects in congenital development and remodeling after injury. Scientific Reports, 9(1), 14428.

Theill, L. E., Boyle, W. J., & Penninger, J. M. (2002). RANK-L and RANK: T Cells, Bone Loss, and Mammalian Evolution. Annual Review of Immunology, 20(1), 795–823.

Theoleyre, S., Wittrant, Y., Tat, S. K., Fortun, Y., Redini, F., & Heymann, D. (2004). The molecular triad OPG/RANK/RANKL: involvement in the orchestration of pathophysiological bone remodeling. Cytokine & Growth Factor Reviews, 15(6), 457–475.

Toda, G., Yamauchi, T., Kadowaki, T., & Ueki, K. (2021). Preparation and culture of bone marrow-derived macrophages from mice for functional analysis. STAR Protocols, 2(1), 100246.

Tudpor, K., van der Eerden, B. C. J., Jongwattanapisan, P., Roelofs, J. J. T. H., van Leeuwen, J. P. T. M., Bindels, R. J. M., & Hoenderop, J. G. J. (2015). Thrombin receptor deficiency leads to a high bone mass phenotype by decreasing the RANKL/OPG ratio. Bone, 72, 14–22.

Udagawa, N., Takahashi, N., Akatsu, T., Tanaka, H., Sasaki, T., Nishihara, T., Koga, T., Martin, T. J., & Suda, T. (1990). Origin of osteoclasts: mature monocytes and macrophages are capable of differentiating into osteoclasts under a suitable microenvironment prepared by bone marrow-derived stromal cells. Proceedings of the National Academy of Sciences, 87(18), 7260–7264.

van Velzen, A. S., Eckhardt, C. L., Peters, M., Leebeek, F. W. G., Escuriola-Ettingshausen, C., Hermans, C., Keenan, R., Astermark, J., Male, C., Peerlinck, K., le Cessie, S., van der Bom, J. G., & Fijnvandraat, K. (2017). Intensity of factor VIII treatment and the development of inhibitors in non-severe hemophilia A patients: results of the INSIGHT case–control study. Journal of Thrombosis and Haemostasis, 15(7), 1422–1429.

Walker, D. G. (1975). Bone Resorption Restored in Osteopetrotic Mice by Transplants of Normal Bone Marrow and Spleen Cells. Science, 190(4216), 784–785.

WALLNY, T. A., SCHOLZ, D. T., OLDENBURG, J., NICOLAY, C., EZZIDDIN, S., PENNEKAMP, P. H., STOFFEL-WAGNER, B., & KRAFT, C. N. (2007). Osteoporosis in haemophilia – an underestimated comorbidity? Haemophilia, 13(1), 79–84.

Weiss, M., Blazek, K., Byrne, A. J., Perocheau, D. P., & Udalova, I. A. (2013). IRF5 Is a Specific Marker of Inflammatory Macrophages In Vivo. Mediators of Inflammation, 2013, 1–9.

Weitzmann, M. N., Roser-Page, S., Vikulina, T., Weiss, D., Hao, L., Baldwin, W. H., Yu, K., del Mazo Arbona, N., McGee-Lawrence, M. E., Meeks, S. L., & Kempton, C. L. (2019). Reduced bone formation in males and increased bone resorption in females drive bone loss in hemophilia A mice. Blood Advances, 3(3), 288–300.

Weyand, A. C., & Pipe, S. W. (2019). New therapies for hemophilia. Blood, 133(5), 389–398.

Wiktor-Jedrzejczak, W., Bartocci, A., Ferrante, A. W., Ahmed-Ansari, A., Sell, K. W., Pollard, J. W., & Stanley, E. R. (1990). Total absence of colony-stimulating factor 1 in the macrophage-deficient osteopetrotic (op/op) mouse. Proceedings of the National Academy of Sciences, 87(12), 4828–4832.

Xiao, Y., Palomero, J., Grabowska, J., Wang, L., de Rink, I., van Helvert, L., & Borst, J. (2017). Macrophages and osteoclasts stem from a bipotent progenitor downstream of a macrophage/osteoclast/dendritic cell progenitor. Blood Advances, 1(23), 1993–2006.

Yang, D., & Wan, Y. (2019). Molecular determinants for the polarization of macrophage and osteoclast. Seminars in Immunopathology, 41(5), 551–563.

Yao, Y., Cai, X., Ren, F., Ye, Y., Wang, F., Zheng, C., Qian, Y., & Zhang, M. (2021). The Macrophage-Osteoclast Axis in Osteoimmunity and Osteo-Related Diseases. Frontiers in Immunology, 12.

Zanolini, D., Merlin, S., Feola, M., Ranaldo, G., Amoruso, A., Gaidano, G., Zaffaroni, M., Ferrero, A., Brunelleschi, S., Valente, G., Gupta, S., Prat, M., & Follenzi, A. (2015). Extrahepatic sources of factor VIII potentially contribute to the coagulation cascade correcting the bleeding phenotype of mice with hemophilia A. Haematologica, 100(7), 881–892.

Zimmerman, B., & Valentino, L. A. (2013). Hemophilia: In Review. Pediatrics in Review, 34(7), 289–295.

Zollner, S. B., Raquet, E., Müller-Cohrs, J., Metzner, H. J., Weimer, T., Pragst, I., Dickneite, G., & Schulte, S. (2013). Preclinical efficacy and safety of rVIII-SingleChain (CSL627), a novel recombinant single-chain factor VIII. Thrombosis Research, 132(2), 280–287.