Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

SCIENZE BIOMEDICHE e NEUROMOTORIE

Ciclo XXXII

Settore Concorsuale di afferenza: 06/D3

Settore Scientifico disciplinare: MED/15

THE INFLAMMATORY/IMMUNE SIDE OF

MYELOFIBROSIS:

<u>A BIOLOGICAL UPDATE</u>

<u>Presentata da: Martina Barone</u>

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Esame finale anno 2019

ABSTRACT

Myelofibrosis (MF) is a clonal disorders of hematopoietic stem cells. Mutations in 3 genes (*JAK2, CALR, MPL*) and chronic inflammation are the hallmark of MF. Triple negative patients (TN) are negative to all three mutations. In addition to molecular aberrations, MF is characterized by specific abnormalities in the development of megakaryocytes and platelet activation and immune dysfunction. In this scenario, infectious complications are the leading cause of morbidity and mortality. Therapy with Ruxolitinib (RUX), a JAK1/2 inhibitor, suppresses both clonal myeloproliferation and release of proinflammatory cytokines, reducing splenomegaly and constitutional symptoms in around 50% of patients (pts). RUX exerts also immunosuppressive activity, resulting in increased infectious risk in RUX-treated MF pts.

Inflammation plays a role in cancer and MF. However, the crosstalk between normal hemopoietic stem/progenitor cells (HSPC) and their inflammatory microenvironment is largely elusive. Circulating microvesicles (MVs; 0.1-1 μm), which are part of the inflammatory network, are small vesicles deriving from the cell plasma membrane with a role in intercellular communication. They are increased in inflammation and cancer, including MF. Most of circulating MVs are of platelet (PLT) and megakaryocyte (MK) origin. However, their pathogenetic role in the inflammatory microenvironment of MF is still elusive.

Furthermore, even though previous studies described immune dysfunction in pts with MF, it is still unknown whether the atypical infectious events are caused by specific deficit in the innate or adaptive immune response and whether RUX therapy may impact the monocyte (MO) "performance" or the circulating MV profile/composition.

Based on this background, the main aim of my PhD project was the functional characterization of the immune/inflammatory microenvironment of MF. Specific aims were: 1) to analyze the effects of inflammation on the functional behavior of normal HSPC; 2) to characterize the bio-molecular profile of circulating MVs in MF; 3) to functionally characterize the circulating immune microenvironment of MF and 4) to investigate the role of circulating MO in the inflammatory microenvironment of *JAK2^{V617F}* mutated MF pts and evaluate whether and to what extent RUX may influence their *in vitro/in vivo* behaviour.

Focusing on the functional effects of the inflammatory microenvironment on the HSPC compartment, we show that various combinations of inflammatory cytokines promote the in vitro survival of CD34+ cells from umbilical cord and increase proliferation/clonogenicity and in vitro migration of CD34+ cells from G-CSF-mobilized peripheral blood. We demonstrated that normal CD34+ cells from two different sources show distinctive response to inflammatory factors and that the balance between pro/anti-inflammatory signals play a very important role in the functional behaviour of normal CD34+ cells.

Focusing on the functional role of circulating MVs in MF, the results show that 1) the circulating MK/PLT-MVs profile is altered; 2) according to IPSS score, Intermediate 2/high risk pts show respectively reduced/increased MK/PLT-MVs proportion as compared with the intermediate1/low risk pts; 3) at baseline spleen-responders (SR) pts show a significant increased MK-MVs proportion as compared with the non-responder (NR) counterparts; importantly, a cut-off value below 19.95% of MK-MVs predicted the NR pts. Interestingly, RUX therapy restores the normal MK/PLT-MVs profile in SR pts only. On this basis, the circulating MK/PLT-MVs could have a diagnostic and prognostic role in MF.

Finally, focusing on the immune microenvironment of MF, the results of this thesis demonstrate that 1) there are phenotypic/functional alterations in key immune cell subsets such as reduced ability of monocyte to differentiate into dendritic cells, reduced plasticity of Th17 lymphocytes and reduced functional capacity of Innate Lymphoid Cells. Furthermore, selected immune defects were mainly associated with the presence of the *JAK2^{V617F}* or *CALR* mutation; 2) circulating MO show an altered activation/differentiation program and a reduced *in vitro* capacity to

produce/secrete inflammatory cytokines in response to an infectious stimulus. Importantly, at variance with previous studies on T cells, RUX improves intracellular pro-inflammatory cytokines production of MF-MO and promotes the release of inflammatory cytokines associated with MO-derived-MVs in response to an infectious stimulus. Overall, these immune system abnormalities could contribute to the development of an immunodeficiency state with the potential to promote immune evasion, cancer progression and increased susceptibility to infection. These findings further contribute to better understand immune biology in the setting of the MF and refines the biological effects of RUX, suggesting that RUX activity is cell type-dependent.

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Introduction

1.Myelofibrosis

1.1 Clinical features

The Myeloproliferative Neoplasms (MPN), including Essential Thrombocythemia (ET), Polycythemia Vera (PV) and Myelofibrosis (MF), are clonal disorders of the hematopoietic stem cells characterized by a myeloid proliferation driven by at least one somatically acquired driver mutation in *JAK2, MPL*, and *CALR* genes. Regardless of driver mutations, the JAK-STAT signalling pathway is hyperactivated in all MPNs (1). The World Health Organization (WHO) classification system for hematopoietic tumors was recently revised and the 2016 document recognizes several major categories of myeloid malignancies including acute myeloid leukemia (AML) and related neoplasms, myelodysplastic syndromes (MDS), MPN, MDS/MPN overlap, mastocytosis, eosinophilia-associated myeloid/lymphoid neoplasms with recurrent mutations involving *PDGFRA, PDGFRB, FGFR1*, and *PMC1-JAK2*, and myeloid neoplasms with germline predisposition. Within the WHO MPN category, PV, ET and primary (PMF) are grouped together as "the JAK2 MPNs". PMF is further sub-classified into "prefibrotic" and "overtly fibrotic" PMF. Furthermore, some patients with ET or PV develop a PMF-like phenotype over time, referred to as post-ET or post-PV MF, with similar treatment and outcome. Somatic mutations in MPN, including PMF, are classified into "driver" and "other" mutations; the former include mutations in *JAK2, CALR* and *MPL* genes and the latter mutations in genes mainly regulating methylation and splicing. It is generally believed that driver mutations are essential for the MPN phenotype whereas the "other" mutations might contribute to disease progression and leukemic transformation (2).

MF is a blood cancer with an incidence of about 0.58 new cases per 100.000 person-years, but with a much higher prevalence because of a chronic and disabling course leading always to death due to progression, disease-related or treatment-related complications. MF patients suffer from debilitating systemic symptoms, progressive splenomegaly and transfusion-dependent cytopenias. They also experience increased risk of thrombosis, second neoplasia, and evolution to acute leukemia. It is mainly characterized by a clonal myeloproliferation and medullary fibrosis, with consequent insufficiency and delocalization of medullary hemopoiesis at the spleen and liver level (splenomegaly and hepatomegaly). Bone marrow fibrosis is mainly caused by the expansion of monocytes, which secrete pro-angiogenetic factors (3) and megakaryocyte contribution (   ).. Inefficient hematopoiesis leads to a lower production of red blood cells (anemia), an impaired

megakaryocytopoiesis (thrombocytopenia or thrombocytosis), platelet activation, an increase of immature granulocytes and the appearance of myeloid precursors in the peripheral blood. Constitutional symptoms such as fatigue, dyspnoea, night sweats and fever are also observed (4). The diagnosis of MF is based on the observation of bone marrow morphology and on the search for the three "driver mutations" (*JAK2, MPL, CALR*). Based on histological analysis of the bone marrow, a pre-fibrotic state and a fibrotic state are also distinguished (5).
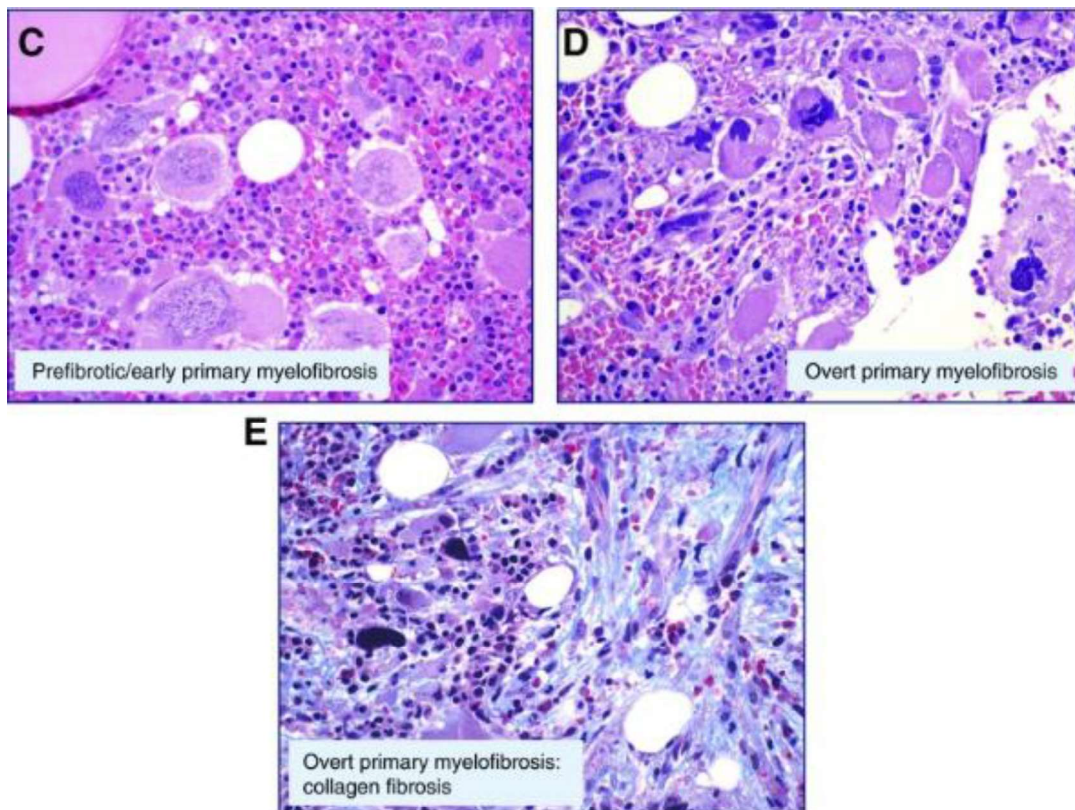

Fig 1: Examples of bone marrow sections of patients with MF (6).

The International Prognostic Scoring System (IPSS) risk score was designed in 2009 for use in the initial diagnosis and risk stratification of PMF patients according to the following prognostic factors: 1- age (above 65 years); 2- presence of constitutional symptoms; 3-hemoglobin levels (lower than 10 g/dL); 4- leukocyte count (higher than 25×10^9/L); 5- percentage of circulating blasts (higher or equal than to 1%).

Based on these factors, MF patients can be subdivided into 4 categories:

• low risk (0 adverse factors)

• intermediate risk 1 (1 adverse factors)

• intermediate risk 2 (2 adverse factors)

• high risk (≥3 adverse factors)

and the corresponding median survivals were reported at 11.3, 7.9, 4 and 2.3 years (7).
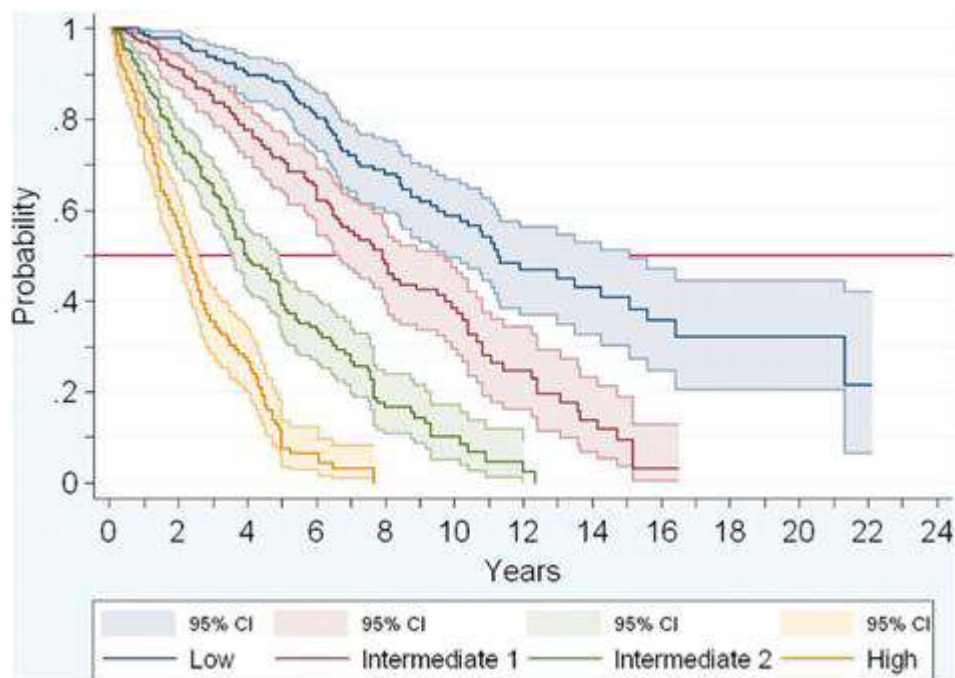
Fig 2: Stratification of patients according to the IPSS risk score (7).

The IWG-MRT subsequently developed a dynamic prognostic model (DIPSS) that utilizes the same prognostic variables used in IPSS but can be applied at any time during the disease course and used to predict the survival of the patients during the follow-up (8). The DIPSS prognostic score was then further extended to the DIPSS plus model, which also considers the patient's karyotype, platelet count and transfusion status (9).

Variable	IPSS	DIPSS	DIPSS-plus
Age >65 y	√	√	√
Constitutional symptoms	√	√	√
Hemoglobin <10 g/dL	√	√	√
Leukocyte count >25x10^9/L	√	√	√
Circulating blasts ≥ 1%	√	√	√
Platelet count <100x10^9/L			√
RBC transfusion need			√
Unfavorable karyotype +8,-7/7q-,i(17q),inv(3), -5/5q-,12p-, 11q23 rearr.			√

Fig 3: Prognostic models in MF (10).

In recent years two new prognostic scores have been developed: GIPSS (genetically-inspired prognostic scoring system) and MIPSS70 + version 2.0 (mutation- and karyotype-enhanced international prognostic scoring system) (5).

The GIPSS prognostic score estimates the risk based on the mutational status. The cytogenetic aspects, the presence of the three driver mutations (*JAK2, MPL, CALR*) and other additional mutations (*ASXL1, SRSF2, U2AF1Q157*) are also taken into consideration (11). Finally, the MIPSS 70+ prognostic score, used for patients with age eligible for hematopoietic stem cell transplant, includes the presence of various mutations (such as *ASXL1, SRFR2, EZH2, IDH1, IDH2, CALR*) and, at the same time, six clinical parameters (hemoglobin level, lymphocyte count, percentage circulating blasts, degree of medullary fibrosis, constitutional symptoms) (12). Based on the risk estimation, a different therapeutic approach is applied. A therapeutic option is the allogeneic stem cell transplant, which is recommended for high risk subjects. Patients with intermediate and low risk are treated with conventional therapy, including thalidomide, danazol, hydroxyurea and inhibitors of *JAK1/2* (5).
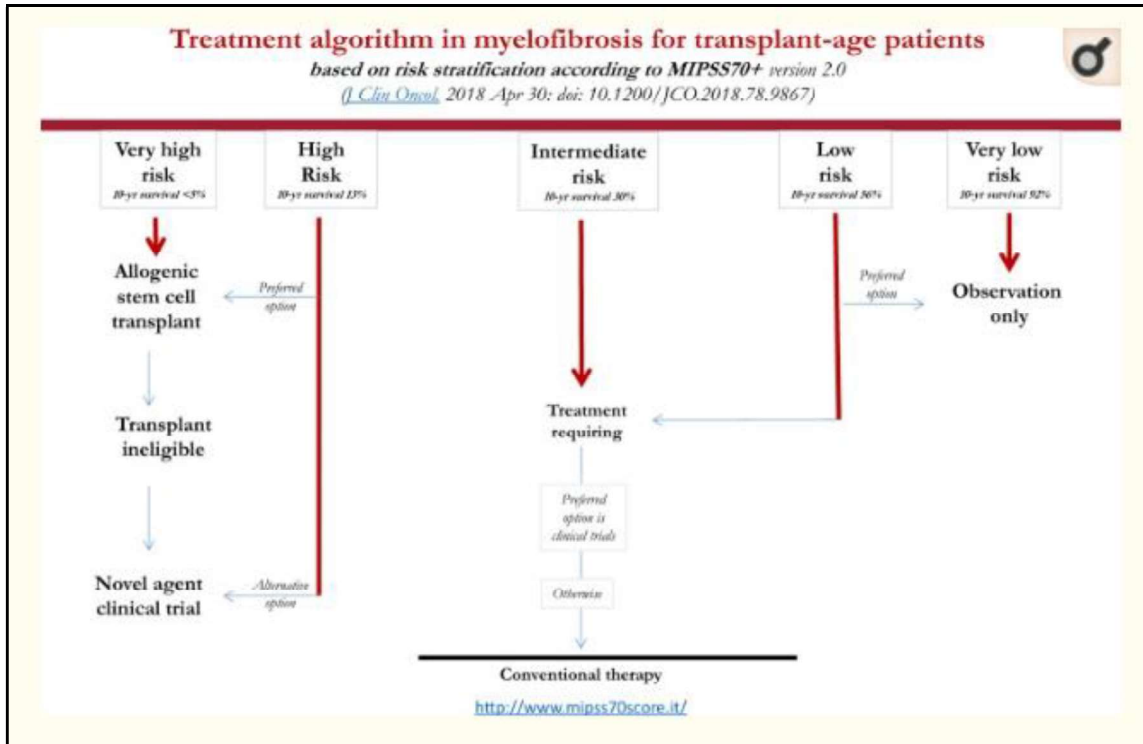
Fig 4: *The therapeutic approach to MF based on the risk assessment using the MIPSS70 + method (13).*
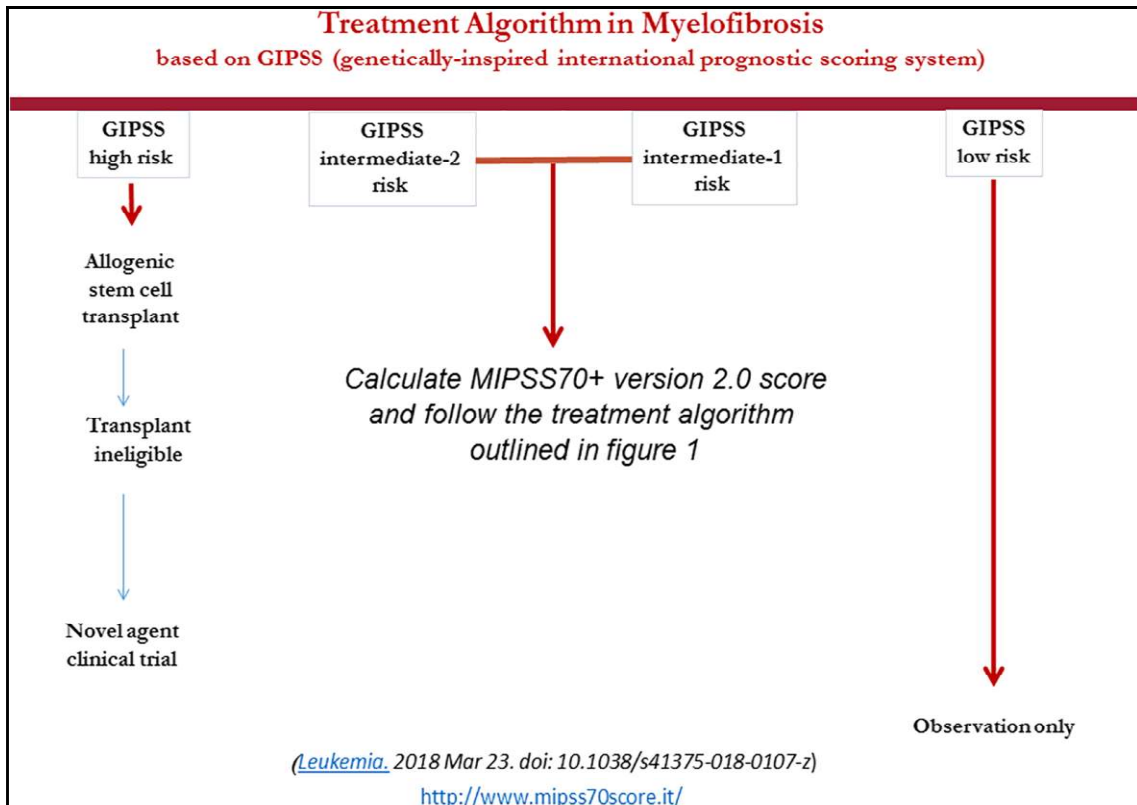

Fig 5: *GIPSS-based treatment algorithm of MF (11).*

1.2 Molecular pathogenesis

The molecular pathogenesis of MF relies on 3 driver mutations in *JAK2, MPL* and *CALR* genes. The first identified mutation (in 2005) was the *JAK2*^{V617F}, a gain-of-function mutation, caused by the replacement of a valine with a phenylalanine in position 617 (V617F) (14). JAK2 is a tyrosine kinase belonging to a large family of similar proteins that, in mammals, includes JAK1, JAK2, JAK3, JAK4 (15). The gene coding for *JAK2* is placed in position 9p24.1 26 and has 26 exons (16). Physiologically, JAK plays a fundamental role in signal transduction that occurs when a cytokine or a growth factor interacts with its receptor. When the JAK kinase is activated, it phosphorylates the STAT substrate by starting a fundamental signal pathway (JAK/STAT) involved into proliferation, survival and inhibition of apoptosis. JAK2 can also activate other downstream pathways, such as Ras and the PI3K/Akt pathways, which regulate these important cellular mechanisms (17).
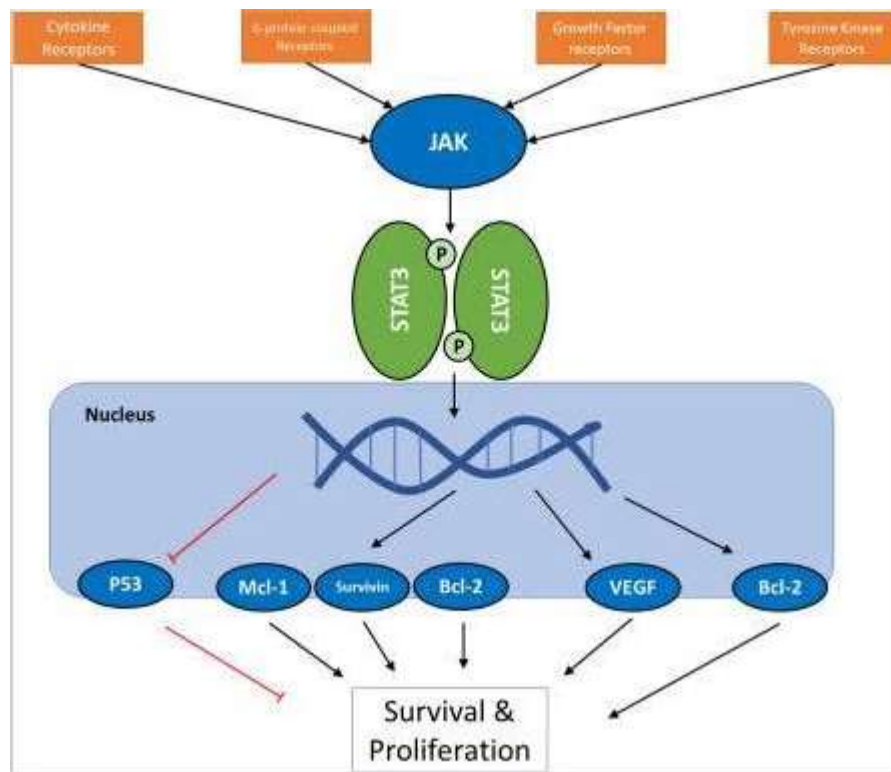

Fig 6: The JAK / STAT pathway (17)

To perform its crucial task, the kinase JAK2 is formed by two protein domains: a kinase domain (JH1 domain), which phosphorylates the downstream substrates allowing signal transduction and a pseudokinase domain (JH2 domain) with an inhibitory role against the kinase domain. According to some recent studies, the pseudokinase domain is able to phosphorylate two residues of the JAK2 protein (Ser523 and Tyr570), generating a conformational change that makes the kinase inactive. So, the JAK2 protein has an important feedbeck-negative regulation system (18).
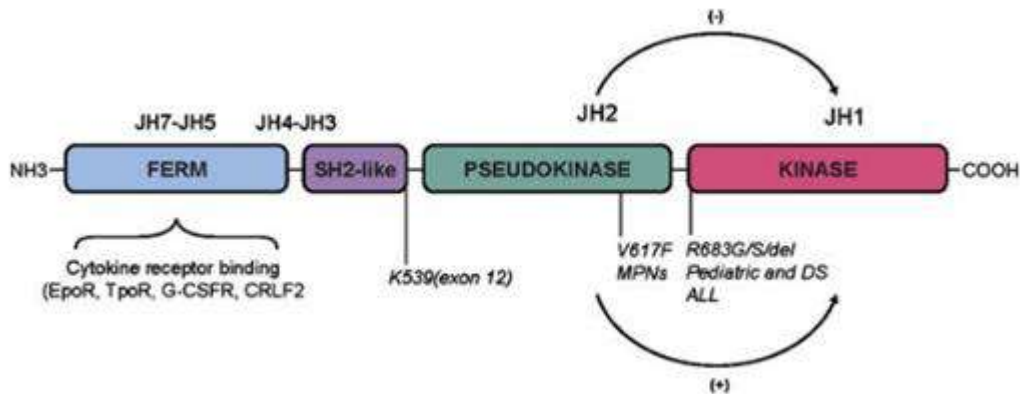
Fig 7: JAK2 protein domains (19)

The $JAK2^{V617F}$ mutation falls at the level of this regulatory domain (specifically in exon 14), thus causing a constitutive hyperactivation of the kinase. Therefore, the cell carrying this type of mutation is essentially independent of the stimulation of cytokines and growth factors, with consequent clonal proliferation (20). Other mutations have also been reported in other positions of the *JAK2* gene, such as a mutation in exon 12 (21). At the cellular level, the effect of these mutations is substantially the same as the $JAK2^{V617F}$ mutation.

The second driver mutation identified in MPN was detected in the *MPL* gene. This gene, placed in 1p34.2 position of the human genome, encodes the Thrombopoietin receptor, the most important regulatory factor of megakaryocytopoiesis and platelet formation. Once the ligand binds its receptor, there is an MPL receptor dimerization and the activation of the JAK/STAT pathway (22).
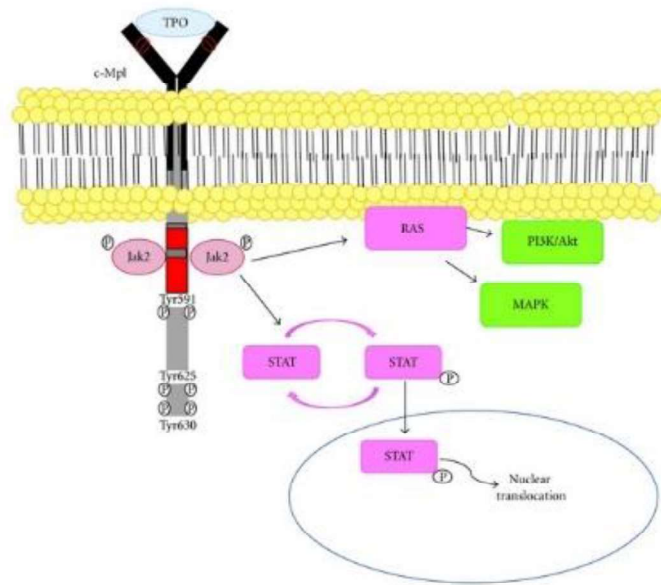
Fig 8: *The TPO/MPL pathway (23).*

The *MPL* mutation consists in the substitution of a tryptophan in 515 position with another aminoacid (W515L/K/A). In some cases, there is a deletion of 5 aminoacids (Δ5TpoR). In both cases, there is an abnormal constitutive activation of the MPL receptor (in the absence of the ligand) with consequent activation of the JAK/STAT pathway (24).

Finally, another mutation in the Calreticulin gene (*CALR*) was recently discovered. This gene, which is on chromosome 19p13.13, encodes a protein implicated in intracellular calcium homeostasis (transport of calcium from the endoplasmic reticulum) and in the correct "folding" of proteins; however, it can also play a role as a transcription factor (25). The Calreticulin protein is present at the intracellular, extracellular or transmembrane level. It is a very versatile protein, with roles in the regulation of proliferation, apoptosis and in the control of the immune response (26).
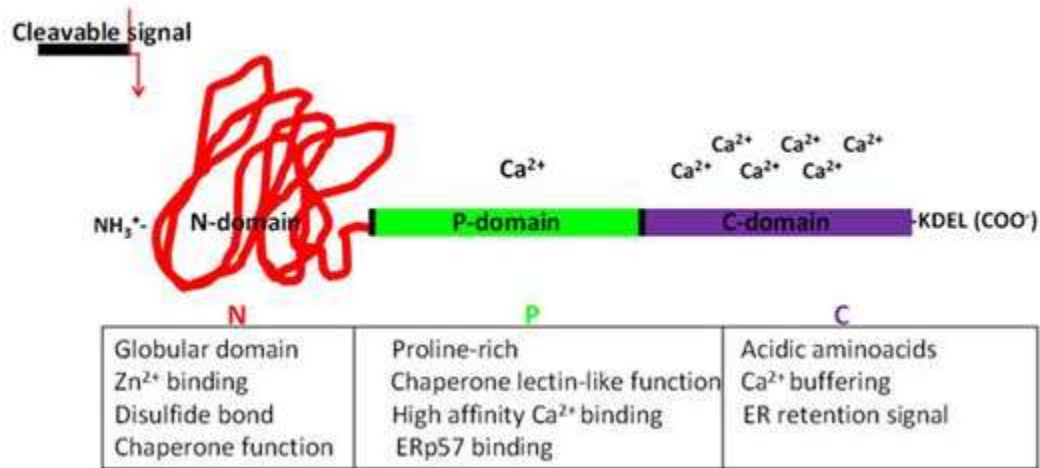
Fig 9: The structure of the Calreticulin protein (27)

Mutations in the *CALR* gene are generally deletions or insertions that usually fall into exon 9. There are two types of mutations of the CALR gene:

- Type 1: deletion of 52 base pairs
- Type 2: insertion of 5 base pairs

Both mutations affect the C-terminal domain of the protein (28).
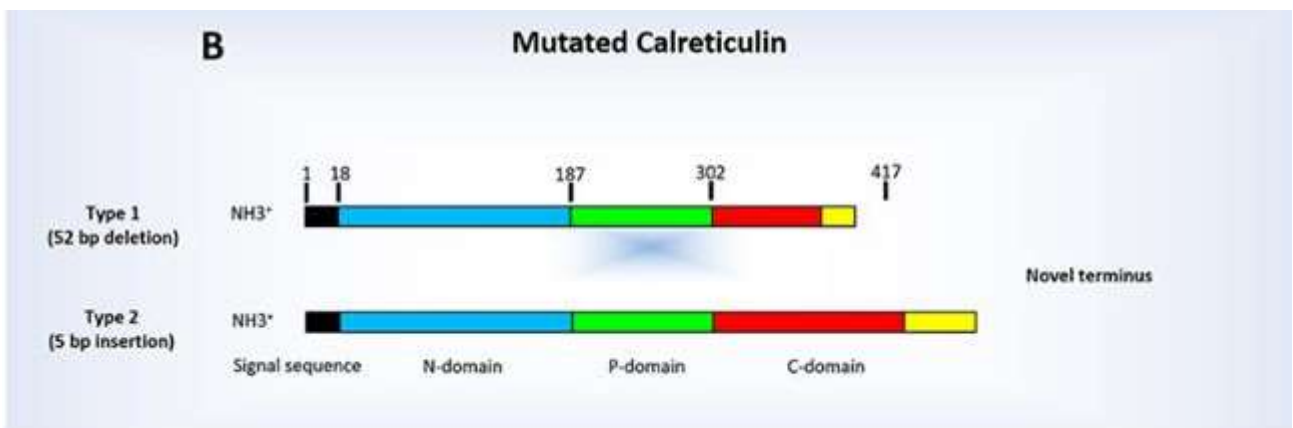
Fig 10: The mutated Calreticulin: type 1 and 2 (29)

According to Araki M. et al. and Elf S et al., the mutated Calreticulin undergoes a conformational change that allows it to interact with the MPL receptor, inducing a signal transduction via JAK/STAT in the absence of the ligand (Thrombopoietin). In particular, the mutated Calreticulin

would bind the extracellular part of the MPL receptor, determining its constitutive activation (30, 31).

Therefore, constitutive activation of the JAK/STAT signalling pathway is key to the development of the MF phenotype in all mutant backgrounds. $JAK2^{V617F}$ mutations can drive MF through activation of erythropoietin receptor (EPOR), thrombopoietin receptor (MPL) and granulocyte-colony stimulating factor receptor (G-CSFR) receptors present on different stages of a maturing myeloid cell. Clonal dominance of homozygosity or heterozygosity of $JAK2^{V617F}$, the presence and order of acquisition of co-operating mutations and additional factors such as iron deficiency and gender can impact on the resulting phenotype. *CALR* and *MPL* mutations result in a PMF phenotype through activation of the MPL receptor. All drivers mutations appear to be largely mutually exclusive although bi-clonal disease can occur. $JAK2^{V617F}$ and *CALR* mutations are detectable in the long term haematopoietic stem cell (LT-HSC) population and in all maturing stages of the haematopoietic hierarchy. Yet, these *JAK2* V617F LT-HSC population appear to exhibit reduced self-renewal and are skewed towards expansion of the progenitor pool instead (32).

In addition to the 3 driver mutations (*JAK2, MPL and CALR*), a range of genes are repeatedly found to be mutated in MF. These co-operating oncogenic mutations found alongside the driver mutations include genes involved in cell signalling pathways (*LNK*, *CBL*, *NRAS* and *NF1*), epigenetic regulation (*ASXL1*, *EZH2*, *TET2*, *DNMT3A*, *IDH1* and *IDH2*), transcriptional regulation (*TP53*, *RUNX1)* and mRNA processing (*SF3B1*, *SRSF2*, *U2AF1*, *ZRSR2*) (32). The most mutated genes are *TET2, ASXL1, DNMT3A, CBL, LNK, IDH1/2, IKF1, EZH2, TP53, SRSF2* (28). This creates a general genetic instability that characterizes and complicates the MF framework. As shown in **Fig. 10**, around 60% of MF patients carry the *JAK2V617F* mutation, 30% are *CALR* mutated and 8% are *MPL* mutated (33). However, there is a small group of patients (around 10%) in which none of the driver mutations has been observed; these patients are called "triple negative".

Distribution of JAK2, MPL, and CALR Mutations in Philadelphia Chromosome–Negative Myeloproliferative Neoplasms
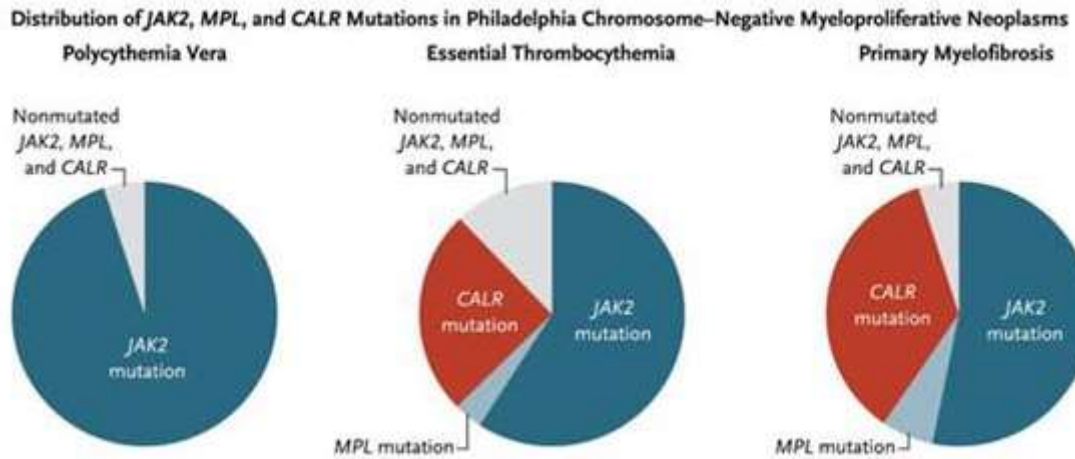
***Fig 11:** The distribution of the "driver" mutations in MPN (34).*

As demonstrated by Tefferi et al., the mutational spectrum of MF patients is correlated with survival. The "triple-negative" patients have the lowest survival (with an average of 2.3 years), while the *CALR* mutated patients have the highest (with an average of 15.9 years). Mutations in *JAK2* and *MPL* genes respectively confer an average life expectancy of 5.9 years and 9.9 years (35).
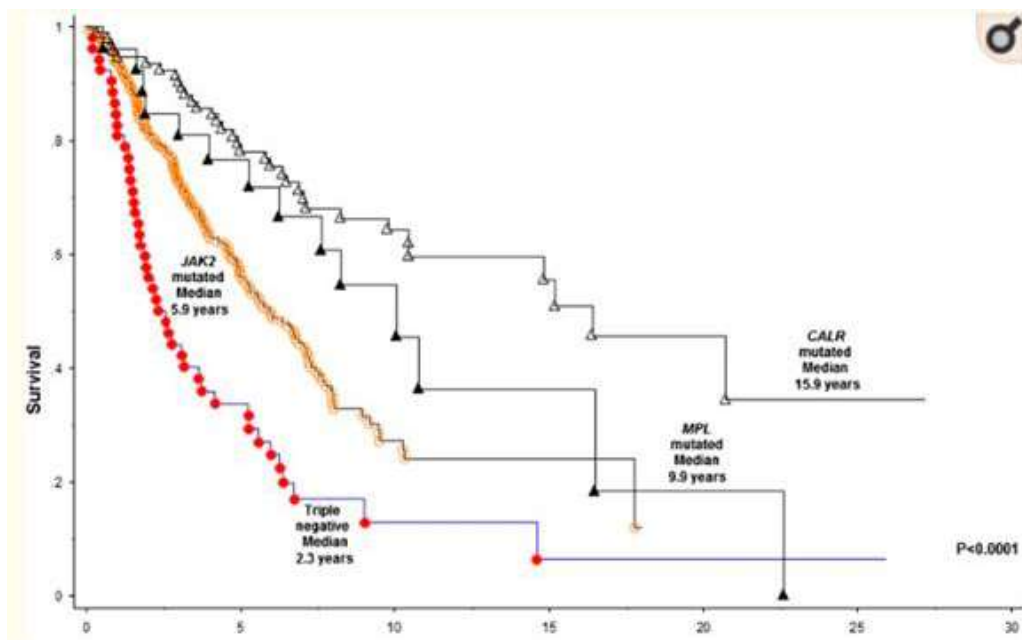
***Fig 12:** Survival of patients with MF according to mutational status (35).*

In addition, the allelic burden of the *JAK2* mutation (more than 56.7%) is associated with increased disease severity and increased risk of thrombosis in patients with MF (3, 36).

2. Inflammatory pathogenesis in MF

Chronic inflammation is the hallmark of MF. We know that inflammation has a protective role; however, in some cases, it can become harmful. Some authors describe "oncoinflammation", referring to the relationship between tumour cells and inflammatory microenvironment (37).

Cytokines are soluble proteins, commonly known for their immunomodulatory functions, that orchestrate both innate immunity and adaptive immunity. In addition to classically defined cytokines, such as interleukins and interferons, a variety of other soluble factors, including a range of growth factors, have been often classified as cytokines. Generally, cytokines are produced in response to cellular stresses including pathogen infections, inflammation, or injury. Their release exerts effects on different types of somatic cells by modulating different types of response. In case of infections or inflammations, monocytes, macrophages and neutrophils infiltrate and secrete numerous cytokines locally, including a variety of angiogenic factors, growth factors, and proteases. This results in a variety of cellular responses including increased angiogenesis, cell proliferation, migration of cells, and haematopoiesis (38) The immune response is mediated by the early reactions of innate immunity and other later ones of adaptive immunity. The innate immunity consists of cellular and biochemical defence mechanisms pre-existing to infection and ready to react quickly. In the context of innate immunity, the cytokines guarantee a rapid response from the leucocytes but also from the parenchymal cells, which are able to identify a pathogen by toll like receptor (TLR) expression. The main cytokines in this category are Interleukins (IL)-1, 6, 12, 18 (CXCL8), Tumor necrosis factor (TNF)-α, Granulocyte/Granulocyte-macrophage colony stimulating factor (G-CSF, GM-CSF). They activate a series of fundamental cells for innate immunity (monocytes, dendritic cells, T cells, NK etc.) as well as the processes aimed at the elimination of the pathogen (chemokine release, increase of adhesion molecules expression at the endothelial level, increased fluidity of blood etc.) (39).This category of cytokines is also called inflammatory cytokines because they induce an inflammatory state. Regarding adaptive immunity, one of the fundamental roles of cytokines is to guide the T-cell response, for example IL-12 induce a TH1-type response, resulting in the production of effector cytokines (interferon (IFN)-γ and TNF-α), while the Th2 response is induced by IL-4 production with consequent activation of the humoral response and IgE production (40).

Some cytokines have a negative regulatory role of immunity. They can be defined as anti-inflammatory cytokines. The suppression of the immune response is mainly driven by IL-10 and Transforming Growth Factor (TGF)-β.

They are produced by many cell type (monocytes, dendritic cells, T cells, regulatory T-cell (Treg) NK, etc) (41).

The released cytokines, both pro-inflammatory and anti-inflammatory, act to control cellular stress and minimize tissue damage. In general, after the resolution of the lesion or the inflammatory state, the cytokines return to the homeostatic levels. However, it is increasingly clear that chronic inflammation, resulting in abnormal production and dysregulation of cytokine levels, contributes to the pathogenesis of various diseases including cancer (42).

MF is typically characterized by a high level of proinflammatory cytokines both in the bone marrow and in the system. The constitutive activation of the JAK/STAT pathway leads to the excessive production of pro/anti-inflammatory cytokines. Therefore, a complex inflammatory microenvironment, supported by the activation of the JAK/STAT pathway, is created (43). These pro-inflammatory cytokines result from both mutant haematopoietic MPN clones and non–mutant haematopoietic cells as a direct result of JAK/STAT signalling (44).

Recent studies have examined the circulating levels of pro/ anti-inflammatory cytokines in patients with MF and have studied their prognostic significance. Various pro-inflammatory cytokines such as TNF-alpha, IL-6, IL-8, IL-1β are elevated in the circulation. It has also been shown that IL-8 and IL-2 receptor (IL-2R) are prognostic indicators of reduced survival and leukemic transformation (45). In particular, IL-8 can contribute to the development of the tumour microenvironment through its important role in angiogenesis and in the proliferation of endothelial cells (46).
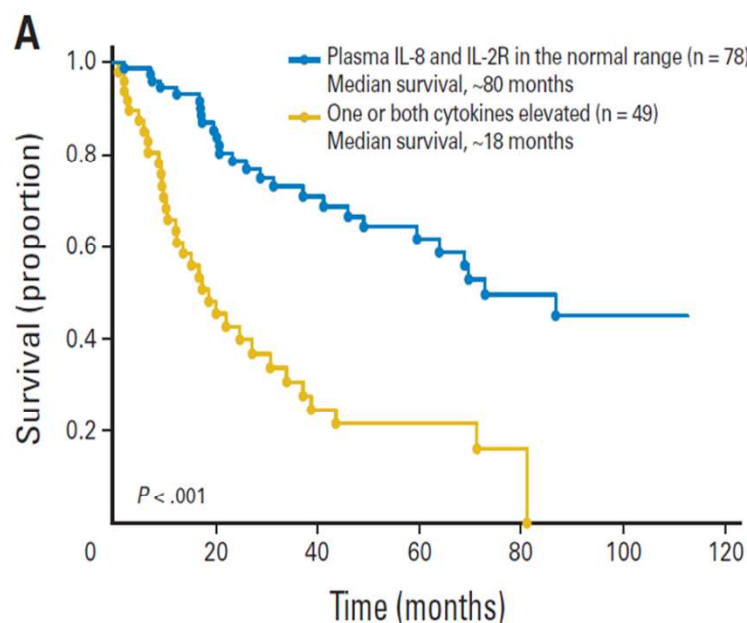

Fig 13: *Increased survival of patients with normal levels of IL-8 and IL-2R (blue) as compared to patients with one / both cytokines increased in plasma (yellow)(45).*

Skov et al., based on the study of the gene expression profile of whole blood in patients with MF, has shown upregulation of genes involved in inflammation and immunity, including Vascular Endothelial Growth Factor (*VEGF), Hepatocyte Growth Factor (HGF), G-CSF, monokine induced by IFN-γ (MIG)*. In particular, high levels of growth factor such as Platelet Derived Growth Fcator (PDGF), Fibroblast Growth Factor (FGFB) and VEGF have been implicated in fibrosis and angiogenesis. Furthermore, the expression of bone morphogenetic proteins (BMP), such as BMP1, BMP6, and BMP7, were higher in patients with advanced stages of MF (47). Increased BMP6 expression was also observed in the pre-fibrotic phases of MF. It has been suggested that progressive medullary fibrosis may be promoted by synergism between fibronectin and proinflammatory cytokines such as TGF-β1 and IL-1. Hoermann et al. has shown that the level of oncostatin M, a pleiotropic cytokine involved in a variety of physiological contexts including hematopoiesis, is elevated in $JAK2^{V617F}$-positive patients and this is mutation-linked. The authors concluded that overexpression of oncostatin M plays a role in bone marrow fibrosis and neo-angiogenesis of the bone marrow microenvironment and furthermore, amplifies cytokine production contributing to the cytokine storm observed in patients with MF (48).

Splenomegaly in MF is associated with the migration of CD34+ stem/progenitor cells from the bone marrow to the spleen and it is supposed to be the result of the clonal expansion of neoplastic stem cells, associated with high levels of cytokines. Consistently, high number of CD34+ cells is observed in the peripheral blood of MF patients. The presence of the $JAK2^{V617F}$ mutation in both the bone marrow cells and the spleen confirms clonality. In addition, TNF-alpha, a cytokine known to be associated with clonal evolution and the selection of preleukemic stem cells in Fanconi anemia, is also associated with clonal expansion in MF and therefore splenomegaly. Many other cytokines, including HGF, MIG and IL-1RA have been associated with marked splenomegaly (49).

Frequent symptoms reported by patients with MF, such as night sweats and itching, are caused by high levels of cytokines, and more particularly, high circulating IL-8 levels have been associated with severe constitutional symptoms.

Based on *in vitro* studies and animal models, it has been hypothesized that chronic inflammation plays an important role in the initiation and progression of MF. It has been hypothesized that chronic inflammation can promote genetic instability and mutations (50) and may contribute to select the malignant clone and promote disease progression to leukemia (51).

More recently, it has been observed that, in addition to TNF-α, also IL1β and the Tissue Inhibitor of Metalloproteases (TIMP1-inhibitor of metalloproteases) are present at high levels in the blood of patients with MF (both *JAK2 and CALR* mutated). When combinations of these three factors are

added in culture *in vitro*, a significant increase in survival, migration and clonogenic capacity of circulating CD34+ cells of patients with MF is observed (52).

Interestingly, pre-existing inflammatory diseases, such as Crohn's disease or autoimmune diseases, can significantly increase the risk of developing MF. In addition, patients with MF have a high risk of developing secondary tumours, both haematological and non-haematological, and this risk is probably related to the $JAK2^{V617F}$ mutation (50).

2.1. Extracellular microvesicles

Among the possible mechanisms of inflammation development/propagation, it has been described that the contribution of extracellular microvesicles (EVs) is crucial. EVs, which are composed of microvesicles (MVs, 150-1000 nm) and exosomes (30-150 nm), are released by a wide variety of cells during homeostasis and cellular activation with pleiotropic effects on signalling between cells (53). EVs express antigens and contain constituents from the source cell including microRNAs. This mechanism supports cellular communication because, proteins, lipids and nucleic acids (DNA, miRNA, etc.) can be found within EVs with the potential to affect the short and long distance microenvironment. Therefore, they have the unique ability to transport membrane and cargo molecules between cells and quickly spread cellular information without the need for cell migration (53, 54).

The biogenesis of MVs occurs by extroflection of the cell membrane, which in turn, is made possible by the action of proteins and lipids that are able to modify the rigidity of the membrane itself. Subsequently, the detachment of the nascent vesicle from the plasma membrane takes place thanks to the actin-myosin contractile system and with ATP consumption. Their development is regulated by small GTPases from the ARF, Rab and Rho families. Contrary to MVs, the exosomes are released by the multivascular bodies of the cells (55).

Peripheral blood contains MVs resulting from platelets/megakaryocytes, red blood cells, leukocytes and endothelial cells; however, platelet/megakaryocytes-derived MVs are the most abundant (56). Specifically, Flaumenhaft et al identified the circulating MVs of megakaryocyte and platelet origin. According to this study, the megakaryocyte MVs are CD41+/CD62P- and express phosphatidylserine on the surface. Furthermore, these MVs are characterized by the presence of Filamin A. The platelet MVs are instead CD41, CD62P and LAMP-1 positive (57).

Numerous studies indicate that EVs, due to their cargo in lipids, inflammatory cytokines/proteins and nucleic acid, have pivotal role in the initiation, propagation and regulation of inflammatory diseases and might be used as biomarkers. They likely play a role in modulating inflammatory and autoimmune diseases, such as arthritis, diabetes and lupus. MVs enhance inflammation through secretion or surface expression of pro-inflammatory cytokines that promote an inflammatory microenvironment and drive immunomodulatory/immunosuppressive activities (58).

Fitzgerald et al. have systematically analyzed the association between 33 cytokines and EVs in eight in vitro, ex vivo and in vivo biological systems (cultured T cells, cultured monocytes, explants of tonsillar, cervical, placental villous, and amnion tissues, amniotic fluid, and blood plasma of healthy volunteers).

They found that a cytokine could be released predominantly either in soluble or in EV-associated form depending on the biological system. These two systems are not strictly separated, as many cytokines in vitro, ex vivo, and in vivo are released in EV-encapsulated forms and can elicit biological effects upon contact with sensitive cells. Moreover, upon stimulation, the pattern of encapsulation changes depending on the stimulus. This suggests that the encapsulation of cytokines in EVs is not simply the property of a particular cytokine, but rather a tight biological process that can be changed upon system activation. Such a targeting would require that EV-associated cytokines are biologically active, and they provided evidence of such activity. Their experiments demonstrated that the biological activity of the EV-encapsulated cytokines was the same whether they released the cytokines or provided them in EVs.

Multiple biologic meanings have been suggested to loading EVs with cytokines: (1) could be a mechanism to dispose of products when they are over-produced and simultaneously protecting the releasing cell from an autocrine effect; (2) EVs protect cytokines from environmental degradation. Indeed, EV-entrapped cytokines are protected from trypsin digestion; (3) may be a mechanism whereby the cytokine expressing cell could expand its sphere of influence to concentrate cytokines at the surface of other cells that might not otherwise be targeted by cytokines in solution (59).

These studies show that deciphering the regulatory mechanisms of EV encapsulation could lead to a better understanding of cell-cell communications in health and disease.

Based on the bidirectional transfer of molecules between tumour cells and the microenvironment, EVs are emerging players. Recent evidence suggests that EVs have crucial roles in cancer development, including pre-metastatic niche formation and metastasis, angiogenesis and suppression of the immune system. Cancer cells are now recognized to secrete more EVs than their non-malignant counterparts and EVs have strong potential as blood-based biomarkers for the diagnosis, prognostication and surveillance of cancer. Thus, EVs play a key role in the regulation of immunity/inflammation and cancer, which in turn can contribute to the further release of EV (60, 61).

Few studies have shown elevated circulating EVs levels in patients with MPN (62, 63). Trappenburg et al have shown that patients with ET have higher number of circulating microparticles with platelet and endothelial markers, suggesting an ongoing platelet and endothelial activation and a role of microparticles in thrombosis of ET (64). Furthermore, Timari et al found that EVs released by MSCs from patients with MPN were found to be selectively enriched in miR155, and they induced an increase in colony forming unit (CFU) ability of neoplastic CD34+ cells (65). However, the role of EVs in MPN, including MF, has yet to be addressed. A deepening

of their role in MPNs would be useful to better understand the mechanisms underlying the disease and to identify new therapeutic strategies.

3. Immune dysregulation in MF

The survey of US Surveillance, Epidemiology, and End Results, (SEER)-Medicare database (1,017 MPNs cases) has documented that autoimmune conditions, overall, are associated with an increased risk of MPN with respect to healthy controls. Autoimmune conditions can cause an immune-related and inflammation-driven tumorigenesis that may result in MPN. Furthermore, therapies given to patients with autoimmune disease (anti-inflammatory and immunosuppressive agents) can play a role in the risk of developing MPN. Moreover, there might be a shared common genetic and/or environmental susceptibility in autoimmune diseases and MPN (37).

Several studies provided evidence that T, B, and NK cell lineages could be involved by the MPN mutations, suggesting that the target cell in MPN is a myelo-lymphoid progenitor. Anyway, the consequences of the involvement of B, T, and NK cells by the somatic mutation that drives the clonal proliferation are still unclear, but they could underline some of the immunologic abnormalities of MPN patients.

Few studies analyzed lymphocyte subsets and their possible correlations with the immune/inflammatory features of MPNs. First of all, Cervantes et al have documented a reduced absolute circulating lymphocyte count in MF patients. Despite this, there was an increase of cytotoxic T cells (CD3+/ CD56+) in most of PMF patients and 10% of them showed an increased CD19+/CD5+ B cell subpopulation (66).

In another study, the presence of the *MPLW515K* gene mutation in CD4+ lymphocytes was observed, suggesting that the lymphoid compartment may also be affected by the mutation (67). Regulatory T lymphocytes (Treg), which are a subpopulation of T helper lymphocytes with the role of maintaining immune tolerance, show altered number. Zhao et al investigated the frequency/function of Tregs (CD4+CD25+ FOXP3+) in PV patients and healthy donors. Tregs were significantly increased in patients and the expression of FOXP3, the master regulator of the immunesuppressive activity of Tregs, was increased (37,68). Conversely, Keohane et al reported low levels of Tregs in 50 MPN patients as compared to healthy donors (69). It has also been observed that the mutational status can influence the proportion between the regulatory T lymphocyte subpopulations. If we consider the three regulatory T-subpopulations identified by Miyara et al, the "triple negative" MF patients show an increase in the circulating population I

(CD3+CCD4+ CCD45RA+ CD25+ CD127low); conversely, patients with the *JAK2V617F* mutation show an increase in circulating population III (CD3+CD4+CD45RA-CD25 + CD127-) (70).

Regarding other subset of immune cells, dysregulations have also been observed in the monocyte/macrophage population. Thiele et al. have revealed an expansion of the monocyte/macrophage cell population in the bone marrow of PMF patients, with a significant increase in the number of mature CD68+ macrophages. These macrophages are also morphologically altered (71). Anyway, monocytosis in MPNs is a rare event, restricted to PMF, and is associated with rapid disease progression. Furthermore, it has been described that the monocytes of patients with PMF are hyperactivated, because there is an increased production of proinflammatory cytokines and transforming growth factor-beta (TGF-β) through an autocrine mechanism (72).

Myeloid-derived suppressor cells (MDSCs) is a subpopulation of immune cells that plays an important role in the immune system and tumorigenesis. This population is part of a larger and more complex group of cells that are fundamental for the mechanisms of immunological tolerance towards the tumor: they guarantee an immunosuppression that allows the neoplastic clone to progress undisturbed (37). MDSCs are part of a generalized circuit of immunosuppression in which other tolerogenic cells are active participants. It is not surprising, therefore, that in MPN the MDSCs (CD11b+CD14-CD33+) are significantly increased, as demonstrated by Kundra et al. (73).

The network regulating the relationship between hematopoietic stem/progenitor cells and immunoregulatory cells has not been completely elucidated in MPN. There are many outstanding questions on the role of immunomodulation in the generation and progression of MPN that deserve to be addressed. For example, mesenchymal stem cells (MSCs) are key cells for immunoregulation and inflammation. MSCs are a key component of the hematopoietic niche, where they support the proliferation and differentiation of HSC. Furthermore, MSCs have a fundamental role in immune regulation, suppressing the proliferation of T cells and favoring the immunosuppressive function of regulatory T lymphocytes (Treg) (74). The bone marrow of MF patients has both cellular and extracellular changes due to inflammation/fibrosis that modifies the hematopoietic niche; for example, alterations of fibroblasts, osteoblasts, endothelial cells and even MSCs are found (75).

The MF inflammatory microenvironment shows a high production of FGF and VEGF, which stimulate neo-angiogenesis and marrow fibrosis, and of PDGF and TGF-β, which instead induce an increase in matrix proteins such as proteoglycans, fibronectin and collagen (76). Chronic inflammation therefore creates a microenvironment that facilitates the release of many molecules from the cells of the microenvironment and from the hemopoietic clone, that leads to an

"inflammatory storm" in the bone marrow (75). Due to the overproduction of these factors, the MSCs are stimulated to proliferate and to explicate their immunoregulatory and immunosuppressive role.
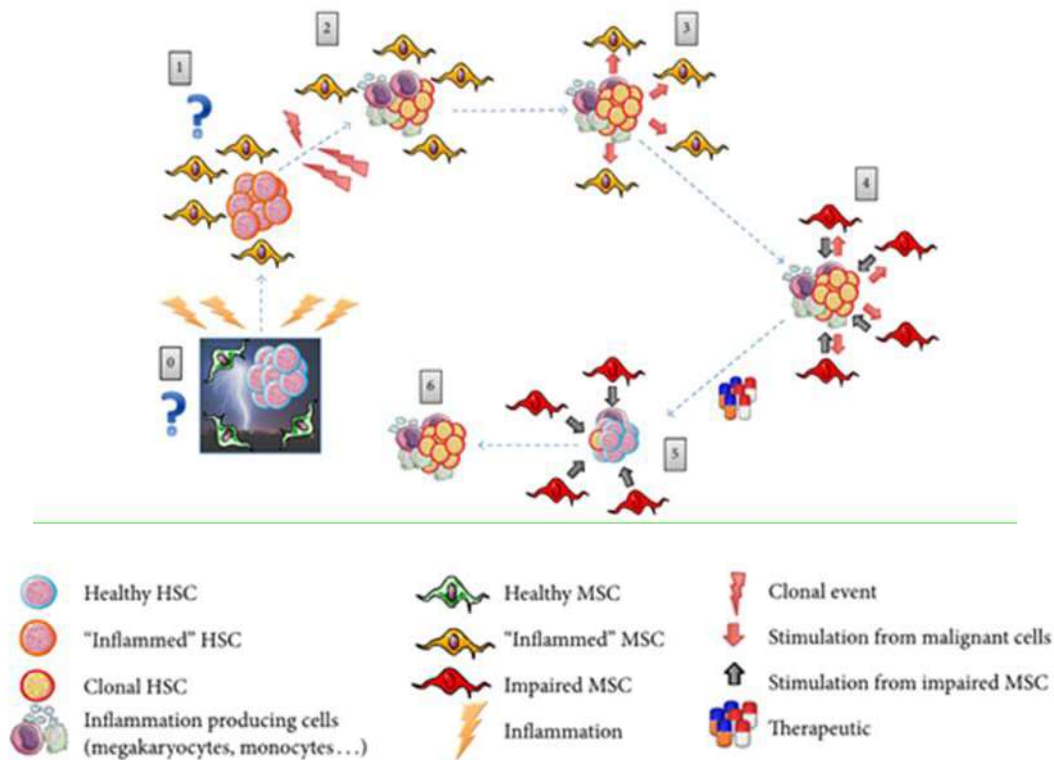
Fig 14: *The vicious circle that is created between cells and inflammatory microenvironment in the bone marrow of patients with MF. (75)*

The concept of "onco-inflammation" and immunoregulation in MPN offered further suggestions for therapeutic strategies. Approaches with anti-inflammatory/immunomodulatory drugs have been designed as promising drug therapies in MPN, including JAK1/2 inhibitors (such as Ruxolitinib), IFN, statins or specific anti-TGF beta agents or IL-8 antagonists (77).

3.1. Monocytes

3.1.1 Monocytes physiology

Monocytes are immune cells that are part of the reticuloendothelial system, which is a system playing a role of sentinel of the organism for protection from what is foreign. Monocytes are circulating cells that can be recruited from tissues and mature on macrophages. Alternatively, they can mature to dendritic cells (Monocyte derived DCs) (78). They derive from a common myeloid precursor and develop in the primary lymphoid organs (bone marrow in adults, liver in fetal life). Starting from this precursor, a factor called M-CSF (macrophage-colony stimulating factor) is essential for their terminal maturation. In the blood, mature monocytes account for about 5-10% of the circulating cells (79, 80).
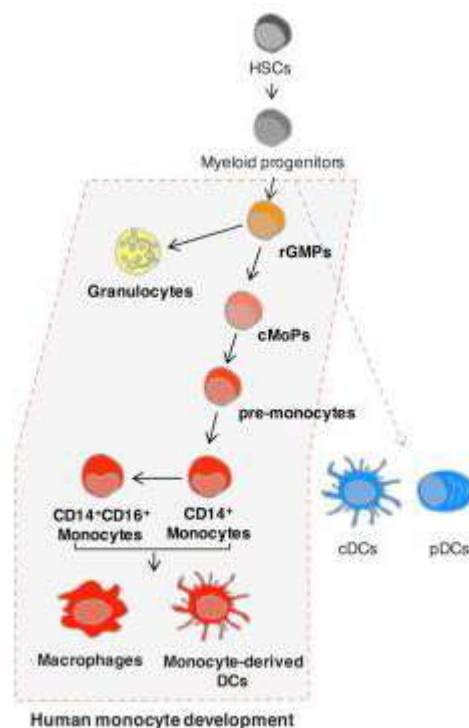

Fig 15: Development of the monocyte-macrophage line in humans (81)

Although monocytes are generally referred to as circulating precursors of macrophages, today their role in the immune system has been expanded. Thanks to the characterization of surface antigens, in fact, it is possible to identify the various phases of their development and to distinguish the different subsets that are present in humans.

In the murine model, circulating monocytes are LY6C + and lose this protein once activated and migrated into lymph nodes or tissues. In this second phase, there is also an increase in the transcriptional levels of IL-1β, TNF, MHC II, CD206, CD11c and CCR7. This indicates that the LY6C monocytes control the tissue environment and are able to present a possible antigen to the T lymphocytes, even without differentiating to macrophages (78).

In humans, LY6C + monocytes are defined as "classic" because they are the most represented population in the peripheral blood (80-90%). From the point of view of antigenic expression, classical monocytes are CD14 ++/CD16-. LY6C- monocytes, on the other hand, are defined as "non-classical" and are CD14+/CD16 ++. They can also be referred to as "patrolling subset" because their role is to patrol the vessels, checking the integrity of the endothelium (82). More recently, a third class of monocytes has been identified: these are the "intermediate" monocytes, with characteristic antigenic CD14 ++/CD16+ expression. Although numerically smaller than the other subset, they are interesting because they are able to respond to the lipopolysaccharide (LPS) stimulus by TNF-α secretion. In fact, CD14, strongly expressed by these monocytes, is the LPS receptor, whereas CD16 appears to be the receptor of FcγIII (83).

In addition to the expression of CD14 and CD16, the three subsets can also be distinguished by the different secretory capacities. Following stimulation with LPS, it appears that:

- **Classic monocytes** produce high levels and a wide variety of cytokines (G-CSF, IL-10, CCL2, IL-6 TNF-α, IL1β and IL-6)

- **Intermediate monocytes** produce high levels of pro-inflammatory cytokines such as TNF-α, IL1β and IL-6

- **Non-classical monocytes** produce the same cytokines as the other subset, but generally they mainly produce anti-inflammatory cytokines (such as IL10).

The same study also highlighted other differences regarding the phenotype of the three sub-sets:

- CCR2, CXCR1, CXCR2 and CD62L are highly expressed by classical monocytes.

- CD64, CCR1, CCR2, CX3CR1, CD11b, CD33 and CD115 are expressed at intermediate levels in intermediate monocytes; CD40, CD54 and HLA-DR are expressed instead in this subset at high levels.

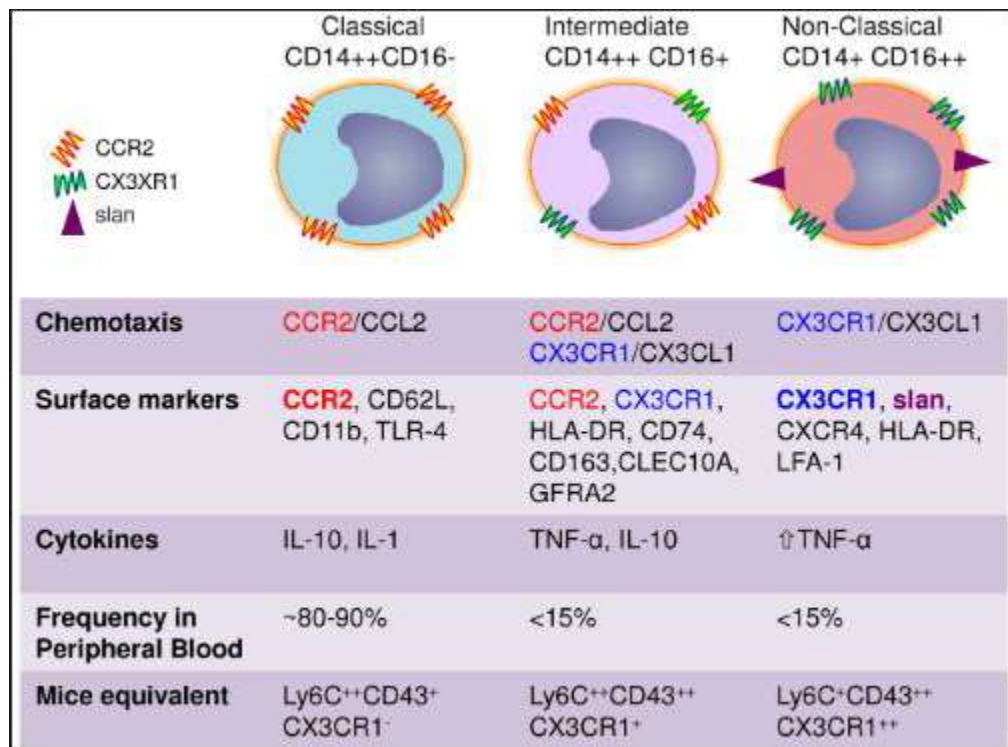- CX3CR1 and SLAN is expressed by non-classical monocytes (84)

	Classical CD14++CD16-	Intermediate CD14++ CD16+	Non-Classical CD14+ CD16++
Chemotaxis	CCR2/CCL2	CCR2/CCL2 CX3CR1/CX3CL1	CX3CR1/CX3CL1
Surface markers	CCR2, CD62L, CD11b, TLR-4	CCR2, CX3CR1, HLA-DR, CD74, CD163,CLEC10A, GFRA2	CX3CR1, slan, CXCR4, HLA-DR, LFA-1
Cytokines	IL-10, IL-1	TNF-α, IL-10	⇑TNF-α
Frequency in Peripheral Blood	~80-90%	<15%	<15%
Mice equivalent	Ly6C++CD43+ CX3CR1-	Ly6C++CD43++ CX3CR1+	Ly6C+CD43++ CX3CR1++

Fig 16: Phenotypic and biological characteristics of the three subsets of monocytes (85)

Based on this information, each of the three sub-sets can be assigned a specific role. Classic monocytes are the first to be recruited and come out from the bone marrow to go into the bloodstream. They have high phagocytic capacity, thanks to the production of peroxidase and produce high levels of Reactive oxygen species (ROS), IL10, IL1-β and low levels of TNF-α, when stimulated by LPS and they differentiate into intermediate and non-classical monocytes. They are involved in angiogenesis and coagulation.

Intermediate monocytes, on the other hand, are typically inflammatory and have a reduced phagocytic capacity and peroxidase activity but produce higher levels of TNF-α, IL-1β, IL-6 under inflammatory stimulus. From the bloodstream they reach the tissues and sites of inflammation by CX3CR1 and CCR5 receptors that mediate the accumulation of monocytes in inflammatory sites and that bind CCL3, expressed by macrophages in inflammatory sites, and CCL5 (RANTES), expressed and secreted by T cells to recruit leukocytes to the inflammatory sites. They are increased in diseases associated to chronic inflammation. They also express CD40 for the activation of T lymphocytes.

Non-classical monocytes patrol the vessels and from the bloodstream invade the tissues damaged by inflammation through the expression of CX3CR1, which is the receptor for chemokine CX3CL, and

contribute to angiogenesis and fibrosis, favouring the production of collagen (IL-10 and TGF- β-relate). They are in fact defined as "patrolling". They therefore have an anti-inflammatory function. They can produce IL1-β and TNF-α, but in response to nucleic acids (86).

3.1.2 Monocytes and MF

Previous studies have partially characterized the monocyte population in the course of MF. It has been shown that monocytes of patients with MPN are more functionally active and therefore produce large amounts of cytokines (TGF-β, IL-1 and substance P) (72);

In 2016, a possible role of monocytes was shown in the development of one of the major clinical features of MF: marrow fibrosis. In fact, high percentages of a particular monocyte-derived cell type, namely fibrocyte, has been identified in patients with MF at the fibrotic stage (87). The monocyte-fibrocyte transition is mediated by the Pentraxin-3 (PTX3), released by macrophages and endothelial cells, which binds the Fcγ receptor present on monocytes (88).

To carry out their task in the context of the immune system, monocytes are recalled at the site of inflammation thanks to some chemokines, including the most important Monocyte chemoattractant protein 1 (MCP-1). It has been recently described that a given polymorphism of MCP-1-2518A/G, may predispose to the development of MF. This polymorphism had already been associated with other pathological conditions (autoimmune disorders, atherosclerosis, chronic infections), which places monocytes at the centre of the etiopathogenesis of various immune defects (89).

To investigate their role in MPN, monocyte lines with stable *JAK2V617F* mutation were developed. They were then used as a study model and the levels of pro and anti-inflammatory factors were evaluated. The results show that the mutated cells produce a greater quantity of metalloproteases (and their inhibitors), growth factors and other crucial substances (such as PTX3) compared to "wild type" cells (90).

In addition to being predictive of disease development and as study model for the underlying etiopathogenetic mechanisms, it was tested whether monocytes could be a prognostic index for MF. In a recent study, patients were stratified based on absolute monocyte counts and it was shown that monocytosis is associated with a poorer prognosis (91).

It has also been shown that monocytes are able to express the receptor for angiopoietin 2, namely Tie-2, and therefore they can promote angiogenesis in an autocrine manner (92). In fact, patients with MF have an increased concentration of Tie-2+ monocytes in the peripheral blood. In this case we are dealing with monocytes of the "intermediate" subset (CD14 ++ CD16 +) (93).

However, monocytes expressing the same receptor for angiopoietin 2 have also been identified in the patient's spleen; in this case they are non-classical monocytes (CD14 + CD16 ++) (94). This last evidence is very relevant, as an increased neo-angiogenesis in the patient's spleen has been demonstrated (95). Monocytes therefore seem to be responsible for this phenomenon.

It has also been shown that the expression of IL-2Rα by monocytes could be associated with an increased risk of thrombosis in patients with MF who have the *JAK2V617F* mutation (96).

Based on these observations, monocytes may have a leading role in the development and maintenance of MF. Nevertheless, additional studies are needed to understand the mechanisms underlying their action within the inflammatory microenvironment of MF.

4.Ruxolitinib

MF is still a treatment-orphan disease that may be cured only by allogeneic stem cell transplant in younger selected patients. However, as above described, regardless of the type of mutation, patients with MF have hyperactivation of the JAK/STAT pathway. An effective therapeutic approach, therefore, would be to inhibit the action of the JAK kinase. The first drug developed and approved for the treatment of patients with MF was Ruxolitinib (or INCB018424): a JAK1 / 2 inhibitor (97). This drug was approved by the EMA in 2012 and is marketed under the trade name of JAKAVI (98).

The approval of the drug came thanks to two main clinical studies:

- COMFORT I: in which Ruxolitinib was compared with placebo (99)

- COMFORT II: in which Ruxolitinib was compared with the best available therapy (100)

Thanks to these two trials, Ruxolitinib has been approved for patients with intermediate (1-2) or high-risk MF who are not eligible for hematopoietic stem cell transplantation.

Ruxolitinib reduces inflammatory cytokine production (*JAK1*-driven) and exhibits myelosuppression (*JAK2*-driven).
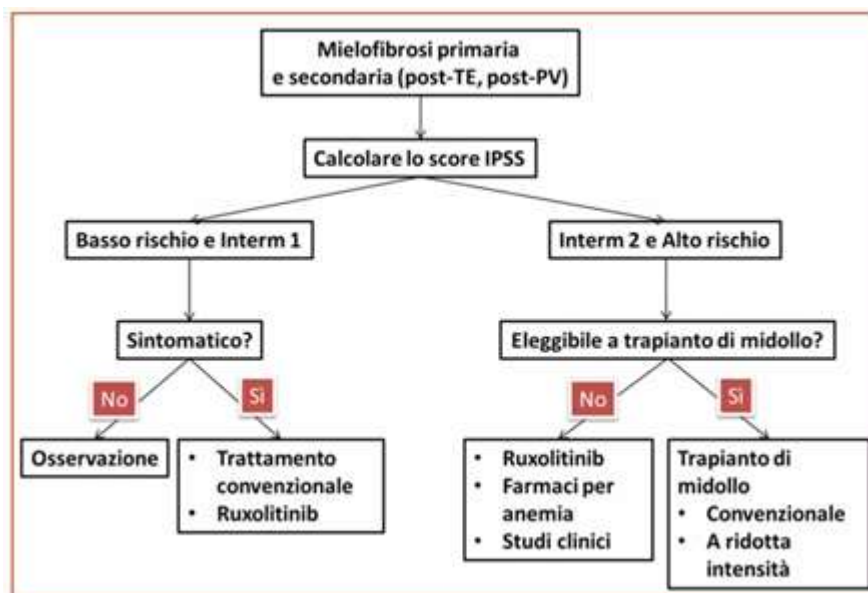

Fig 17: *The use of Ruxolitinib in the clinical practice of MF (101)*

Regarding the mechanism of action, Ruxolitinib is able to inhibit the kinase activity of JAK1/2 by binding to the ATP "binding domain" of the protein (the site where ATP normally resides). In particular, the Ruxolitinib molecule has a double ring system, through which it forms two hydrogen bonds with JAK, at the level of its kinase domain (102).
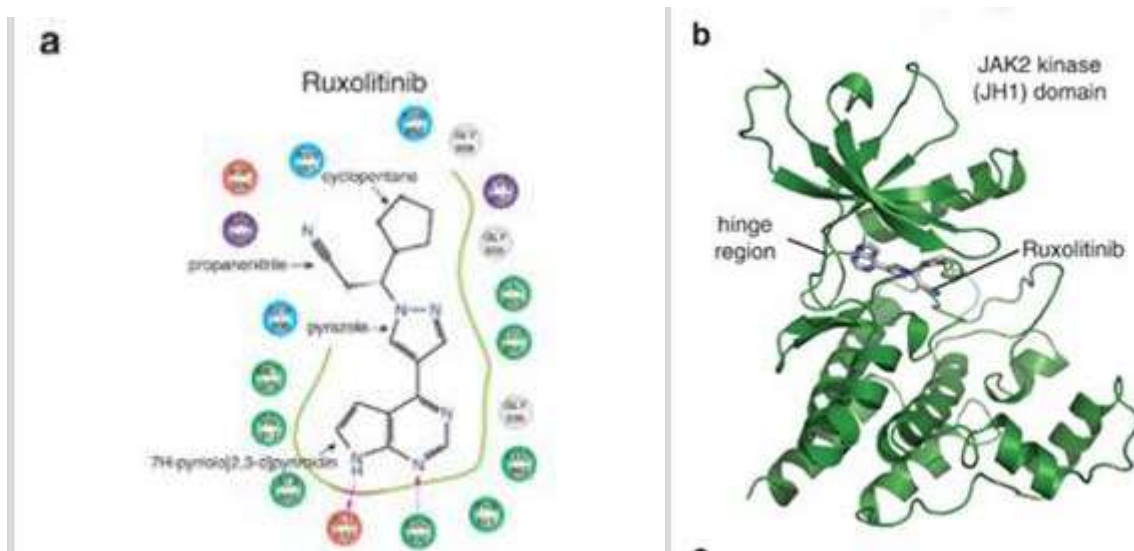

Fig 18: Molecular interaction between the JAK2 kinase and its inhibitor Ruxolitinib. (102)

The binding to the ATP "binding domain" turns out to be effective in inhibiting the activity of JAK1/2, since the ATP is not only used by the kinase to obtain phosphate groups, but it also seems to have a role in stabilizing the pseudokinase domain in the absence of stimulus. Ruxolitinib, therefore, would act as an ATP mimetic, stabilizing the inhibited form of JAK1/2 (103).

In these randomised controlled trials it has demonstrated efficacy in spleen volume reduction and symptom burden reduction with a moderate improvement in overall survival of PMF patients (104). Despite these benefits, there is limited impact to induce complete haematological remission with normalisation of blood counts, reduce the mutant allele burden or reverse bone marrow fibrosis. Clonal evolution has been observed on ruxolitinib therapy and transformation to acute leukaemia can still occur.

Spleen size reduction occurs in more than 50% of patients with MF and a significant reduction in constitutional symptoms is also observed. This can be related to the reduction of pro-inflammatory cytokines. Furthermore, this finding demonstrates how the inhibition of the JAK/STAT pathway has important anti-inflammatory implications (97).

Although important therapeutic effects have been demonstrated following treatment with Ruxolitinib, numerous side effects have however been reported. These are due to the fact that the drug is not selective for the mutated kinase, and therefore also acts on the wild type form. The clinical study COMFORT I showed that neutropenia, urinary tract infections and herpes zoster were observed in patients treated with Ruxolitinib (99). These evidences were confirmed following a 5-year follow-up in the context of the clinical study COMFORT II, with an increased risk of developing pneumonia, sepsis and tuberculosis (100). Interestingly, new drugs are being studied with increased inhibitory capacity against JAK and with greater selectivity towards the mutated protein (105).

Adverse Event	Ruxolitinib (N = 146)	Best Available Therapy (N = 73)
	number of patients (percent)	
Nonhematologic: all grades, grade 3 or 4		
Diarrhea	34 (23), 2 (1)	9 (12), 0
Peripheral edema	32 (22), 0	19 (26), 0
Asthenia	26 (18), 2 (1)	7 (10), 1 (1)
Dyspnea	23 (16), 1 (1)	13 (18), 3 (4)
Nasopharyngitis	23 (16), 0	10 (14), 0
Pyrexia	20 (14), 3 (2)	7 (10), 0
Cough	20 (14), 0	11 (15), 1 (1)
Nausea	19 (13), 1 (1)	5 (7), 0
Arthralgia	18 (12), 1 (1)	5 (7), 0
Fatigue	18 (12), 1 (1)	6 (8), 0
Pain in extremity	17 (12), 1 (1)	3 (4), 0
Abdominal pain	16 (11), 5 (3)	10 (14), 2 (3)
Headache	15 (10), 2 (1)	3 (4), 0
Back pain	14 (10), 3 (2)	8 (11), 0
Pruritus	7 (5), 0	9 (12), 0
Serious		
Anemia	7 (5)	3 (4)
Abdominal pain	3 (2)	1 (1)
Pyrexia	3 (2)	1 (1)
Esophageal varices	3 (2)	0
Dyspnea	2 (1)	3 (4)
Pneumonia	1 (1)	4 (5)
Actinic keratosis	0	2 (3)
Ascites	0	2 (3)
Peritoneal hemorrhage	0	2 (3)
Respiratory failure	0	2 (3)

Fig 19: Adverse effects observed in patients with MF treated with Ruxolitinib or with the best available therapy (100)

The risk of infections is a serious problem for individuals with MF, as it represents about 10% of the causes of death for these patients. This is due to the profound deregulation of the immune system in these subjects, which affects both the cellular component and mediators such as cytokines (106). The question then arose of verifying whether treatment with Ruxolitinib could exacerbate this situation of immunodepression in treated patients, since the JAK/STAT pathway plays a key role in many immune-related processes.

According to studies conducted both *in vivo* and *in vitro* on dendritic cells, Ruxolitinib is able to inhibit their differentiation capacity, the ability to produce IL-12, the migration and expression of activation markers (107). Dendritic cells are fundamental for the antigen presentation process to T cells and, moreover, are able to produce various cytokines (such as IL12, IL23), which in turn drive the Th1 and Th17 response. For this reason, reduced functionality of dendritic cells results in dysfunction of the immune system (108).

The effects of Ruxolitinib on natural killer cells were also studied. Natural killer cells are effector cells with a critical role in defence against viral infections and cancer cells. They are able to produce IFN-γ and TNF-α (108). In a recent study, an important reduction in the frequency of these cells were demonstrated in patients with MF treated with Ruxolitinib. This may probably be due to a defective maturation process. *In vitro*, a reduced capacity to produce cytokines and lytic activity typical of this cell type was then highlighted (109).

Tregs are reduced in MF. In a recent study, it was observed that this decrease is even more pronounced in patients treated with Ruxolitinib. These authors have in fact demonstrated a reduction of CD4+CD127low CD25high FOXP3 + cells in the peripheral blood of patients. They also demonstrated, *in vivo* and *in vitro*, a functional block of these cells (110).
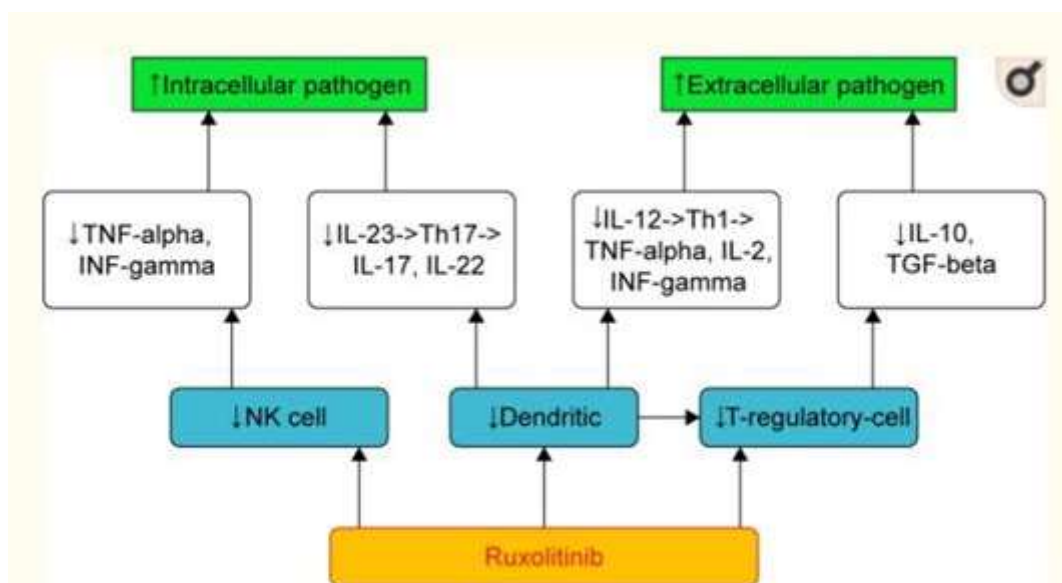

Fig 20: *Effects of Ruxolitinib on crucial cells of the immune system. (108).*

Of note, although monocytes are key players of the inflammatory microenvironment, in MF their pathogenetic role, both at baseline and following treatment with Ruxolitinib, is far from being defined.

Aims of the thesis

This thesis is based on four projects aiming to address the pathogenetic role of the immune/inflammatory microenvironment in MF.

Specific aims were:

1. **To analyze the role of inflammation on the functional behaviour of normal hemopoietic stem/progenitor CD34+ cells.** It has been hypothesized that the sustained inflammatory microenvironment of MF can alter crucial biological processes, leading to genomic instability and cancer progression. To mirror the *in vivo* inflammatory microenvironment, here we investigated the *in vitro* functional effects and role of combined crucial proinflammatory cytokines (IL-1β, TNF-α, IL-6, and tissue inhibitor of metalloproteases (TIMP-1)) on the functional behaviour of normal CD34+ cells from neonatal umbilical cord blood (CB) and adult normal G-CSF-mobilized peripheral blood (mPB) in the presence or absence of bone marrow MSCs. Specifically, we analysed the effects of these selected inflammatory mediators on the viability, proliferative activity, clonogenic potential and migration capability of CD34+ cells. **Results of these project have been published on** "**Mediators Inflammation",** 2018 Jul 4;2018:5974613. doi: 10.1155/2018/5974613. eCollection 2018".

2. **To characterize the bio-molecular profile of circulating MVs in MPN and particularly MF.** Circulating MVs, as biomarkers of disease/malignancy and as contributors of the inflammatory network in MPN, are an open question. Here we investigated: 1) the profile of MVs in MF and ET; 2) whether MVs proportions could be related to severity of disease; 3) the role of inflammation on MVs frequency in MF; 4) the effects of Ruxolitinib on MVs proportion in MF; 4) the microRNA (miR) cargo of circulating MVs from MF patients. **Results of these projects have been published on "British Journal of Haematology",** 2019 Jun;185(5):987-991. doi: 10.1111/bjh.15682. Epub 2018 Nov 18" **and presented to the following Congresses:** American Society of Hematology (ASH) 2017: Blood 2017 130:4220; European Hematology Association (EHA) 2017: abstract n. E1309; XV Congress of the Italian Society of Experimental Hematology (SIES) Rimini, Italy, 18-20 October 2018, Haematologica Abstract n° PO047; American Society of Hematology (ASH) 2018: Blood 2018 132:4334; doi: https://doi.org/10.1182/blood-2018-99-114507;

3. **To characterize the circulating immune microenvironment of MF.** Infectious complications are the leading cause of morbidity and mortality constituting more than 10% of all patient deaths. In order to understand whether the infectious events are caused by deficits in the innate or adaptive immune response, a comprehensive analysis of key immune cells is required. Based on this background and considering the essential role of the JAK/STAT pathways in shaping the immune response, we enumerated and functionally characterized key immune-cell subsets including (dendritic cells (DCs), T-helper (Th) 17 cells , regulatory T cells (Tregs) and innate lymphoid cells (ILC)) with the aim to investigate their putative role in immunosurveillance in MF. **Results of these project have been published on "Oncoimmunology",** 2017 Jul;6(10):e1345402. doi: 10.1080/2162402X.2017.1345402. eCollection 2017".

4. **To investigate the role of circulating monocytes in the inflammatory microenvironment of MF and to evaluate whether and to what extent Ruxolitinib may influence their in vitro / in vivo behavior.** Monocytes play a key role in the inflammatory microenvironment of MPN. Ruxolitinib improve the therapeutic scenario of MF by reducing splenomegaly and systemic symptoms in a significant fraction of patients. Nonetheless, Ruxolitinib is burdened by hematological and extra-hematological toxicity (i.e.: infections). The *in vitro* and *ex vivo* inhibitory effects of Ruxolitinib on number/function of dendritic cells and T-cells (including Tregs) have been previously described. However, to date, in MF the effects of Ruxolitinib on monocytes biology have never been investigated. Based on this evidence and considering the essential role of the JAK/STAT pathways in shaping the immune response, the main purpose was: 1) to phenotypically and functionally characterize circulating monocytes in MF before and after 6 months of *in vivo* treatment with Ruxolitinib; 2) to address monocyte interaction with the inflammatory microenvironment and 3) to investigate whether and at what extent Ruxolitinib affects the *in vitro/in vivo* behaviour of circulating monocytes from MF patients. The driving hypothesis of the present proposal is that the analysis of the *in vitro/in vivo* biological effects of Ruxolitinib on the monocytes compartment will contribute to clarify the role of JAK1/2 inhibition in the modulation of the immune landscape of MF. **Results of these project have been submitted for publication on "Frontiers in Immunology" and presented to the following Congress:** European Hematology Association (EHA) Congress 2018: abstract n. PS1345.

Results I

Published on

Mobilized Peripheral Blood versus Cord Blood: Insight into the Distinct Role of Proinflammatory Cytokines on Survival, Clonogenic Ability, and Migration of CD34+ Cells

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Supplementary materials: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6079419/bin/5974613.f1.pdf

ABSTRACT

Inflammation may play a role in cancer. However, the contribution of cytokine-mediated crosstalk between normal hemopoietic stem/progenitor cells (HSPCs) and their (inflammatory) microenvironment is largely elusive. Here we compared survival, phenotype, and function of neonatal (umbilical cord blood (CB)) and adult (normal G-CSF-mobilized peripheral blood (mPB)) CD34+ cells after in vitro exposure to combined crucial inflammatory factors such as interleukin-(IL-) 1β, IL-6, tumor necrosis factor- (TNF-) α, or tissue inhibitor of metalloproteinases-1 (TIMP-1). To mimic bone marrow (BM) niche, coculture experiments with normal BM stromal cells (BMSCs) were also performed. We found that combined inflammatory cytokines increased only the in vitro survival of CB-derived CD34+ cells by reducing apoptosis. Conversely, selected combinations of inflammatory cytokines (IL-1β + TNF-α, IL-6 + TNF-α, and IL-1β + TNF-α + TIMP-1) mainly enhanced the in vitro CXCR4- driven migration of mPB-derived CD34+ cells. TNF-α, alone or in combination, upregulated CD44 and CD13 expression in both sources. Finally, BMSCs alone increased survival/migration of CB- and mPB-derived CD34+ cells at the same extent of the combined inflammatory cytokines; importantly, their copresence did not show additive/synergistic effect. Taken together, these data indicate that combined proinflammatory stimuli promote distinct in vitro functional activation of neonatal or adult normal HSPCs.

INTRODUCTION

Hemopoietic stem/progenitor cell (HSPC) activation and retention are modulated by the bone marrow (BM) niche where they are located. In response to inflammation and/or BM injury, long-term quiescent hemopoietic stem cells (HSCs) are efficiently recruited into the cell cycle progression returning back to quiescence after reestablishment of homeostasis [1, 2]. Inflammation is a fundamental response that protects tissues from damage and preserves internal homeostasis. However, chronic inflammation may hinder functionality of different tissues and has been suggested to cover a key role in cancer [3].

Proinflammatory cytokines are emerging as key regulators of steady-state and infection-driven hemopoiesis. Recent findings contributed to highlight how HSPC fate could be dictated by inflammatory factors in the BM microenvironment as HSPCs may actively respond to danger signals and proinflammatory cytokines [4, 5]. However, excessive chronic signalling can have negative effects on HSPC regulation and function [6]. Moreover, abnormalities in the inflammatory signalling pathways have been discovered in both preleukemic and leukemic diseases [7]. BM mesenchymal stromal cells (BMSCs) are one of the most important components of the BM microenvironment. They respond to various microenvironment stimuli by changing their secretory capacity and displaying immune-suppressive activity through direct or indirect production of prostaglandin E-2, indoleamine 2,3-dioxygenase, interleukin- (IL-) 10 [8–10], and soluble receptors for IL-1 and tumor necrosis factor-α (TNF-α) [11]. However, a crosstalk between HSPCs and the stromal cells may also create a proinflammatory environment that promotes malignant transformation and disease progression [12]. In such process, several factors and pathways have been implicated but it is not clear how inflammation could affect or transform HSPCs. Understanding the direct cellular target(s) of proinflammatory cytokines is a critical step to better clarifying how HSCs/HSPCs are regulated in the BM niche.

Granulocyte colony-stimulating factor- (G-CSF-) mobilized peripheral blood (mPB) and umbilical cord blood (CB) are two of the current sources of HSPCs for transplantation in hematological malignancies [13]; however, insights into the effects mediated by inflammation on neonatal and adult HSPCs are still elusive. In the last years, several phenotypic and functional differences between CB and mPB-derived HSPCs have been described [14–19]. However, so far, studies analysing the adaptations of HSPCs from these two sources to inflammatory cytokines were focused on a limited number of cytokines which were individually tested [20–24].

To mirror the in vivo inflammatory microenvironment, here we investigated the role of combined crucial proinflammatory cytokines (IL-1β, TNF-α, IL-6, and tissue inhibitor of

metalloproteases (TIMP-1)) on the in vitro functional behavior of CB- or mPB-derived CD34+ cells in the presence or absence of BMSCs.

RESULTS

1. Selected Combinations of Proinflammatory Cytokines Promote the In Vitro Survival of CB-Derived CD34+ Cells.

To test the role of proinflammatory factors on HSPCs, we firstly evaluated the in vitro survival of CB- and mPB derived CD34+ cells in the presence of IL-6, IL-1β, TNF-α, and TIMP-1, at concentrations previously shown by us to be effective in dose-response experiments [20].

Spontaneous survival rate of CB-derived CD34+ cells was higher as compared to mPB counterparts ($p \leq 0.05$; Figures 1(a) and 1(b)).

As shown in Figure S1, CB-derived CD34+ cell survival was further enhanced by TIMP-1, IL-1β, and IL-6 alone as compared with the mPB counterparts ($p \leq 0\ 05$; resp). Compared to untreated cells (control), TIMP-1, IL-1β, or IL-6 alone poorly promoted the survival of mPB- and CB derived CD34+ cells with the notable exception of TNF-α which significantly ($p \leq 0.05$) increased mPB-derived CD34+ cell survival.Therefore, based on these results and on data reported in literature [20, 25, 27], we hypothesized that combinations of cytokines can make CB- or mPB-derived CD34+ cells more responsive to inflammatory stimuli. As shown in Figure 1(a), when cytokines were two-by-two combined, we found that IL-1β + TNF-α ($p \leq 0.01$), IL-6 + IL-1β ($p \leq 0.05$), or TNF-α ($p \leq 0.01$) or TIMP-1 ($p \leq 0.01$) significantly increased the percentage of viable CB-derived CD34+ cells as compared with the untreated counterparts. In contrast, only IL-1β+TNF-α significantly increased the survival rate of mPB-derived CD34+ cells ($p \leq 0.001$).

Testing multiple cytokine combinations (Figure 1(b)), the survival of CB-derived and mPB-derived CD34+ cells was significantly increased in the presence of IL-1β + TNF-α + TIMP-1 ($p \leq 0.05$ and $p \leq 0.001$, resp.) as compared with untreated cells. When we compared CB and mPB (Figures 1(a) and 1(b)), the survival rate of CB-derived CD34+ cells was promoted in the presence of IL-1β + TIMP-1 and IL-6 + TNF-α ($p \leq 0.05$).

These data suggest that CD34+ cells from CB are more actively responsive to inflammatory cues than their mPB counterparts; however, multiple combinations are required to promote their survival.

Subsequently, we examined whether a combination of proinflammatory factors would trigger CD34+ progenitor cell differentiation. In selected experiments, freshly isolated CD34+ cells were cultured in RPMI medium supplemented with or without additional proinflammatory factors for 24 hours. The expression of selected myeloid-specific markers (CD11c, CD13, CD14, and CD45) along with HSPC markers (CD38, CD133) or specific marker for cell adhesion/proliferation (CD44) was analyzed by flow cytometry. The expression of CD11c, CD14, CD38, CD45, and

CD133 was not significantly affected by inflammatory factor treatment (data not shown). By contrast, after treatment with combined inflammatory cytokines, mPB- and CB-derived CD34+ cells upregulated the expression of CD13 and CD44 (Figures 1(c) and 1(d) and Supplementary Table 2). After treatment with IL-6 + IL-1β + TNF-α + TIMP-1, CB-derived CD34+ cells showed a 5-fold increase in geometric mean fluorescence intensity (gMFI) of CD13 as compared to untreated cells (gMFI, $p \leq 0.001$). Accordingly, a statistically significant difference was also found in the presence of IL-1β + TNF-α or IL-6 + TNF-α ± IL-1β (4.41- and 4.64-fold change; $p \leq 0.001$, resp.). A similar pattern was also found when mPB CD34+ cells were tested (Figure 1(c)).

Consistent with CD13 expression, the combination of IL-1β + TNF-α ($p \leq 0.01$) and IL-6 + IL-1β + TNF-α ($p \leq 0.05$) ± TIMP-1 ($p \leq 0.01$) induced a significantly higher CD44 expression in CB-derived CD34+ cells (>2-fold increase, respectively; Figure 1(d)). When we evaluated the mPB-derived CD34+ cells, CD44 expression markedly increased in the presence of IL-1β + TNF-α ($p \leq 0.001$), IL-6 + TNF-α ($p \leq 0.01$), and the combination of cytokines altogether ($p \leq 0.001$). Importantly, TNF-α alone increased the expression of CD13 and CD44 in CD34+ cells from both sources (Figure S2; $p \leq 0.001$).

Taken together, these results demonstrate that selected combinations of inflammatory cytokines, along with the promotion of the survival of CB-derived CD34+ cells, stimulate the expression of CD13, which is an early and late myeloid marker.
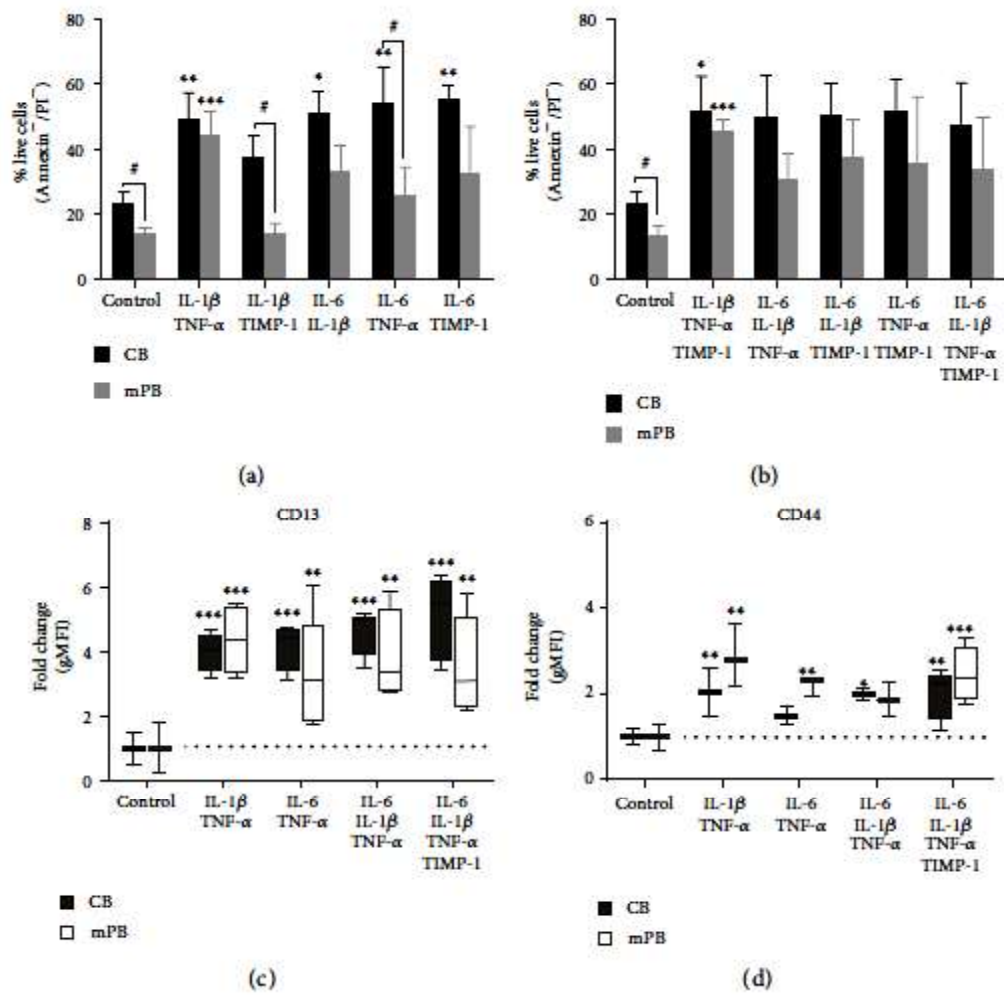
Figure 1: Survival and phenotype of CD34+ cells from CB or mPB in the presence of combined proinflammatory cytokines. *(a) Percentage of live CD34+ cells from CB (indicated as negative for Annexin V and PI (black columns, n=9) or mPB (grey columns, n=8) in vitro treated for 24 hours with a two-by-two-factor combination and assessed using Annexin V/PI staining, as described in Methods. (b) Percentage of live CD34+ cells in the presence of multiple combinations of proinflammatory cytokines. (c–d) Box-plot graphs with fold change of gMFI for CD13 and CD44 expression in CD34+ cells after treatment with different combinations of inflammatory cytokines. Dot lines were used to mark control samples without any treatment. All data are presented as mean ± SEM of n (as above described) experiments performed in duplicate (∗p ≤ 0 05, ∗∗p ≤ 0.01, and ∗∗∗p ≤ 0.001 versus untreated cells (control)). (#p ≤ 0.05 CB versus mPB).*

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2. Proinflammatory Cytokines Poorly Stimulate the Clonogenic Output of CD34+ Cells from CB and mPB.

To confirm the capacity of selected proinflammatory cytokines to drive the HSPCs toward a myeloid lineage, we performed clonogenic assay.

We therefore evaluated the CFU-C growth in the presence of proinflammatory factors in a two-by-two combination. As shown in Figure 2(a) and Supplementary Table 3, CFU-C growth from CB or mPB-derived CD34+ cells was not significantly affected by incubation with the proinflammatory cytokines as compared with untreated cells. When we used multiple combinations of cytokines (Figure 2(b)), we found an increased clonogenic output in the presence of IL-1β+TNF-α+TIMP-1 for CB-derived CD34+ cells and in the presence of IL-6 + IL-1β + TIMP-1 for the mPB counterparts (p ≤ 0.05, resp). Comparing the two sources, IL-1β+TNF-α+TIMP-1 led to an increase in the clonogenic output of CB-derived CD34+ cells (p ≤ 0.05).

Of note, when colony composition was analyzed, no significant difference was observed in the CFU-GM growth (Figures 2(c) and 2(d)) between treated and untreated cells with the notable exception of IL-6 + TIMP-1 for mPB-derived CD34+ cells (p ≤ 0.01). Moreover, comparing the two sources, the CFU-GM growth of the mPB-derived CD34+ cells was significantly enhanced by IL-6 + TNF-α (p ≤ 0.05). As regards the erythroid lineage (Figures 2(e) and 2(f)), only IL-1β + TNF-α + TIMP-1 significantly promoted the BFU-E growth of CB CD34+ cells as compared to untreated cells and the mPB counterparts (p≤ 0.01 and p ≤ 0.05, resp.). Interestingly, in the presence of IL-6 + TNF-α ± IL-1β, we found a statistically significant decrease in BFU-E growth of mPB CD34+ cells compared to untreated cells (p ≤ 0.05). Of note, IL-6 + TNF-α show opposite effects on the erythroid and granulomonocyte progenitors of mPB-derived CD34+ cells by enhancing CFU-GM (Figure 2(c)) and inhibiting BFU-E growth (Figure 2(e)).

To further investigate, we next examined the subtype compartment of CFU-GM and BFU-E as percentage of total CFU (Figure S3). As shown in Figure S3(A) and (B), we found that, after treatment with IL-6 + TNF-α ± IL-1β + TIMP-1, the CFU-GM growth of CB CD34+ cells was highly promoted (78.8 ± 8.4% and 74.5 ± 6.8% compared to 57% ± 3.2 of untreated cells; p ≤ 0.05, resp.), whereas it decreased in the presence of IL-1β + TNF-α + TIMP-1 (43.4 ± 3.7; p ≤ 0.05). No significant effects were observed when mPB CD34+ cells (Figure S3(C) and (D)) were analyzed.

These findings demonstrate that inflammatory cytokines slightly stimulate the hemopoietic functions of CB- and mPB-derived HSPCs.
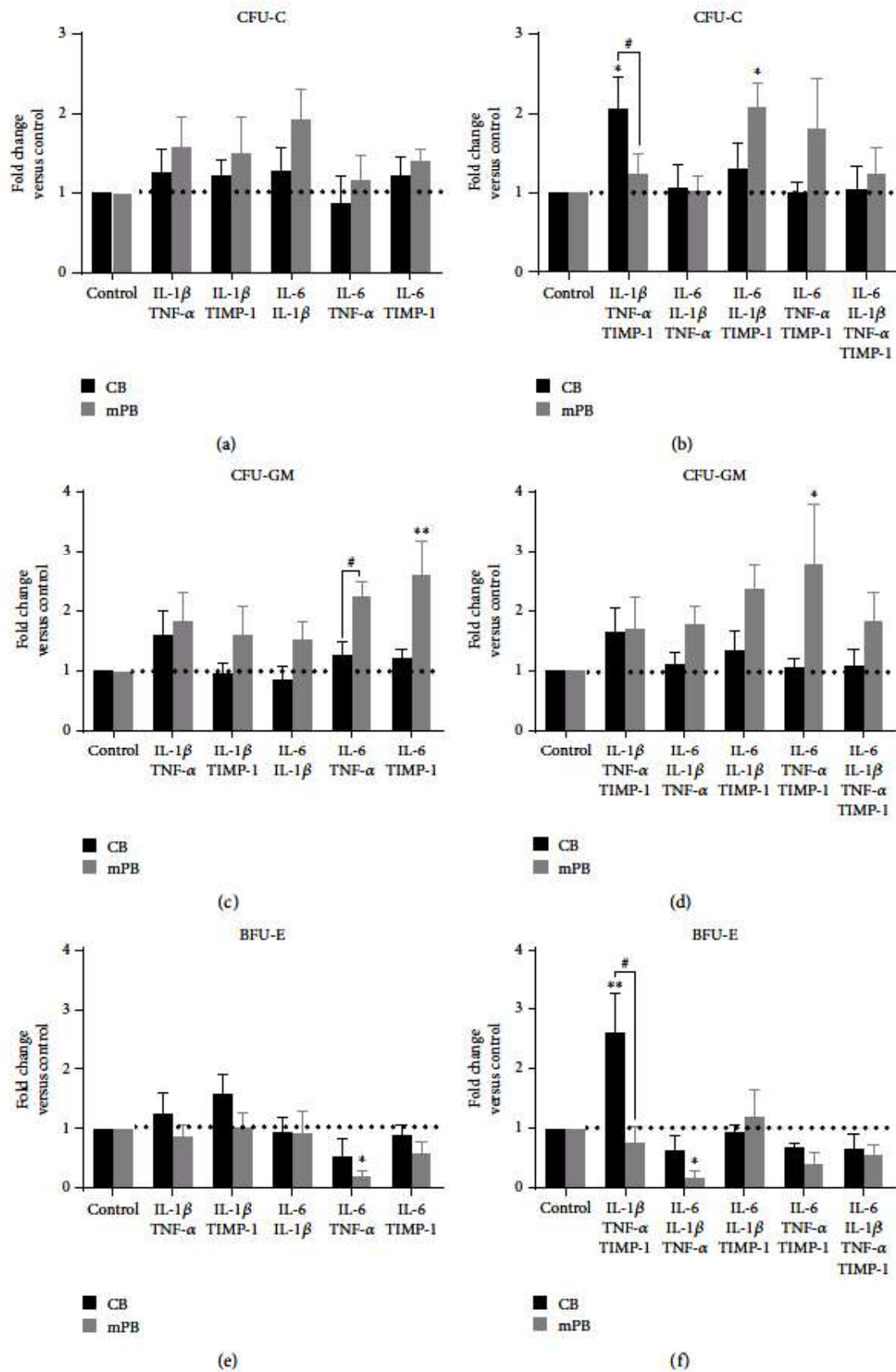
Figure 2: Clonogenic output of CB- and mPB-derived CD34+ cells after combined inflammatory stimuli treatment. *Comparison of CFU-C formation between CB- (n=9 independent experiments) and mPB-derived*

(n=10 independent experiments) CD34+ cells cultured for 14 days in methylcellulose-based medium is shown. The total CFU-C output was assessed in the presence of inflammatory cytokines with the two-by-two combination (a) or multiple combinations (b). The CFU-GM (c–d) and BFU-E (e–f) output was assessed. The results are expressed as growth fold change of inflammatory cytokine-treated versus untreated cells (control). Control samples were marked with a dot line. All data are presented as mean ± SEM (∗p ≤ 0.05, ∗∗p ≤ 0.01 versus untreated cells (control)). (#p ≤ 0.05 CB versus mPB).

3. Selected Combinations of Inflammatory Cytokines Mainly Enhance the In Vitro Migration of mPB-Derived CD34+ Cells.

It has been reported that CXCL12 is chemotactic for CD34+ cells and that the migratory behavior of CD34+ cells depends on their source of origin [28].

We firstly evaluated CXCR4 expression in CB- and mPBCD34+ cells. As shown in Figure 3(a), the absolute number of CD34+ cells co-expressing CXCR4 was significantly increased in mPB as compared with the CB counterparts (p ≤ 0.01). Accordingly, we observed a slight increase in the geometric mean value of CXCR4 in mPB CD34+ cells as compared to CB-derived CD34+ cells (Figures 3(b) and 3(c)).

Subsequently, we compared spontaneous versus CXCL12- driven migration and no significant differences were found in the migration rate when CXCL12 was added in culture (Figures 3(d) and 3(e)). This was probably due to the fact that, to highlight the effects of the inflammatory cytokines, low CXCL12 dose (120 ng/mL) was used.

To study the role of proinflammatory cytokines in the modulation of spontaneous or CXCL12-mediated migration, we set up the in vitro migration of CB- or mPB-derived CD34+ cells in the presence of CXCL12 alone or CXCL12 plus selected combinations of proinflammatory cytokines (Figures 3(d) and 3(e)). As compared to the spontaneous migration, CD34+ cells from mPB showed increased migration ability toward CXCL12 when IL-1β+TNF-α, IL-6 + TNF-α (p ≤ 0.05, resp.), and IL-1β + TNF-α + TIMP-1 or IL-6 + TNF-α + TIMP-1 (p ≤ 0.01, resp.) were added. IL-6 + TNF-α + TIMP-1 ± IL-1β significantly increased the migration of theCBCD34+ cells (p ≤ 0.01, resp.). As compared to migration toward CXCL12 alone, only IL-1β + TNF-α or IL-6 + TNF-α significantly enhanced the migration of mPB-derived CD34+ cells (p ≤ 0.05 and p ≤ 0.01, resp.). The migratory capacity toward CXCL12 of CB CD34+ cells was promoted only by IL-6+TNF-α+TIMP-1 +IL-1β (p ≤ 0.05). Comparing the two sources, the migration rate of CD34+ cells from mPB was significantly enhanced when CXCL12 + IL-1β + TNF-α ± TIMP-1 (p ≤ 0.05, resp.) and CXCL12 + IL-6 + TNF-α (p ≤ 0.05) were added to the lower transwell chamber. These results demonstrate the capacity of selected combinations of inflammatory cytokines to increase the

CXCR4-driven migration of CD34+ cells from mPB and CB. This effect was more prominent when mPB cells were assayed.
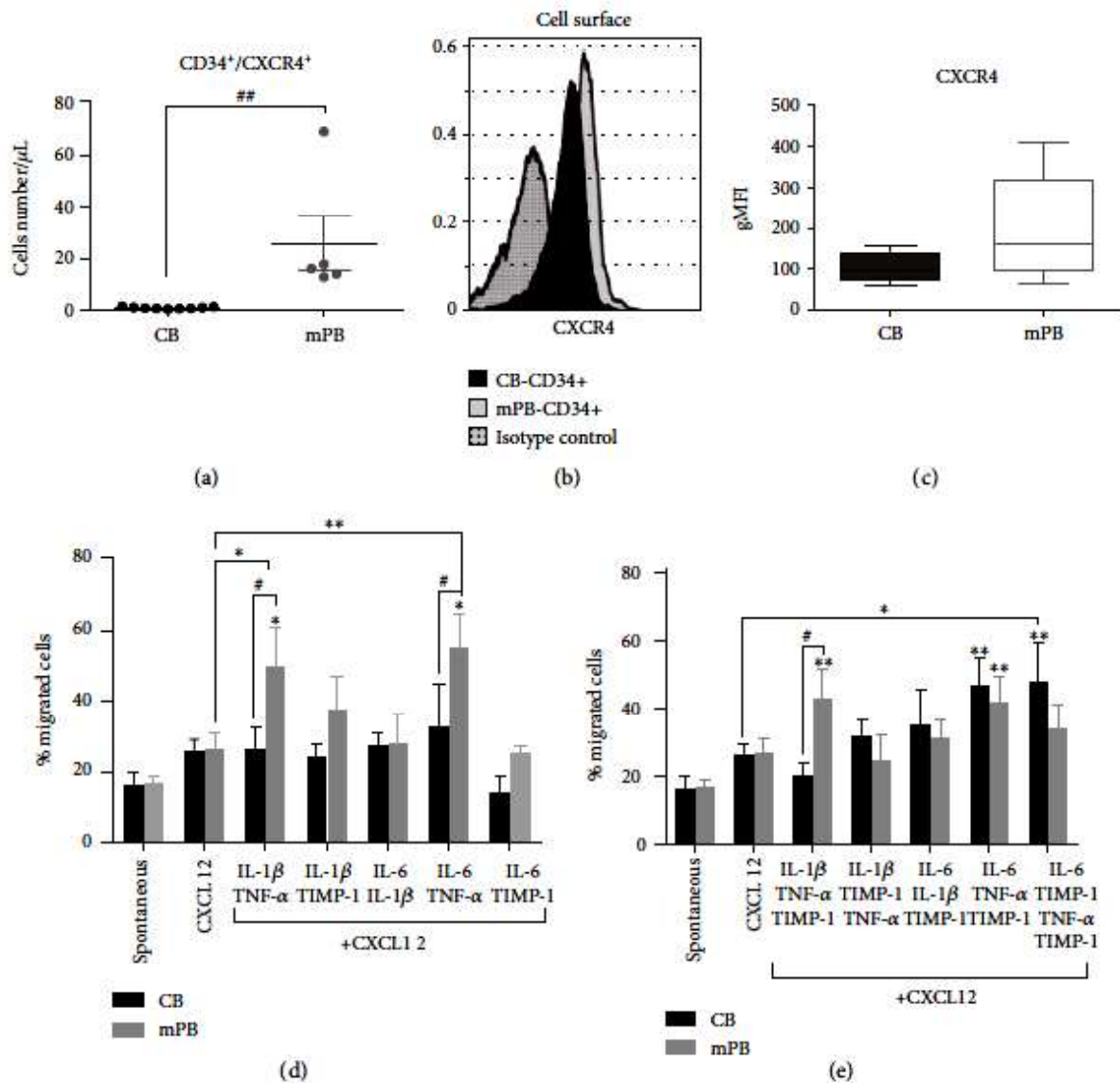

Figure 3: Migratory response of CB- and mPB-derived CD34+ cells in the presence of combined inflammatory stimuli. (a) The absolute number of circulating CD34+ cells from CB unit (n=9) or mPB (n=5) and coexpressing the CXCR4 receptor are shown. (b) Representative histogram of CXCR4 expression in CB- and mPB-derived CD34+ cells compared to isotype control. (c) Geometric mean value of CXCR4-positive cells on the CD34+ population after isolation from CB (n=5) and mPB (n=5) units. (d) Migration assay using transwell after o/n spontaneous migration (control) or exposure to CXCL12 (120 ng/mL) or to two-by-two inflammatory cytokines plus CXCL12 as chemoattractants. Percentages of migrated CD34+ cells from CB (black columns, n = 4) or mPB (grey columns, n = 4) are shown. (e) Migration assay using transwell after o/n spontaneous migration (control) or exposure to CXCL12 (120 ng/mL) or to CXCL12 plus multiple inflammatory cytokines as chemoattractants. Percentages of migrated CD34+ cells from CB (black columns, n = 4) or mPB (grey columns, n = 4) are shown. Data are presented as mean ± SEM of n (as above described) independent experiments ($*p \leq 0.05$ and $**p \leq 0.01$ versus untreated cells (control) or CXCL12 alone) ($\#p \leq 0.05$ and $\#\#p \leq 0.01$ CB versus mPB).

4. CD34+ Cells from mPB Show Increased Clonogenic Ability after In Vitro Migration toward Selected Combinations of Proinflammatory Cytokines.

Cell migration could be considered a selection of cells with different function and properties; for this reason, we tested the clonogenic potential of migrated CB and mPB CD34+ cells (Figure 2 and Supplementary Table 4). Of note, at variance with the results of freshly isolated cells (Figure 2) in terms of clonogenic output, CXCL12 + IL-6 + TNF-α ($p \leq 0.001$) ± IL1β ($p \leq 0.01$) ± TIMP-1 ($p \leq$

0.01) selected a subset of CD34+ cells from mPB with higher clonogenic potential as compared with CXCL12 alone (Figure 4(a). By contrast, no effects were found in the CB-derived counterparts (Figures 4(a) and 4(b)). Comparing the two sources, only the mPB-derived CD34+ cells migrated toward CXCL12 + IL-6 + TNF-α increased the CFU-C output ($p \leq 0.05$) (Figures 4(a) and 4(b)). We then analyzed separately CFU-GM (Figures 4(c) and 4(d)) and BFU-E (Figures 4(e) and 4(f)) growth, observing a significant promotion of the CFU-GM growth in mPB CD34+ cells after migration toward CXCL12 + IL-6 + TNF-α and CXCL12 + IL-6 + IL-1β + TNF-α ± TIMP-1 as compared to

CXCL12 alone ($p \leq 0.01$, $p \leq 0\ 05$ and $p \leq 0.01$, resp.). Comparing the two sources, CFU-GM growth was higher after migration of mPB CD34+ cells toward CXCL12 + IL-6 + TNF-α ($p \leq 0.01$) (Figure 4(c)). With regard the erythroid progenitors, no significant differences in BFU-E growth were observed between treated and untreated cells of either CB or mPB, with exception of the migrated mPB-derived CD34+ cells towards CXCL12 + all cytokines ($p \leq 0.05$; Figures 4(e) and 4(f)). Comparing the two sources, mPB CD34+ cells showed higher clonogenic ability ($p \leq 0.05$) after migration toward CXCL12 + IL-6 + TNF-α. When colony composition was analyzed (Figure S4), combined inflammatory factors do not significantly modify the CFU-GM/BFU-E proportion of migrated CD34+ cells of both sources. Overall, here we demonstrate that selected combinations of proinflammatory cytokines promoted the CXCR4-driven migration of mPB-derived CD34+ cells with higher clonogenic ability and granulomonocytic potential.
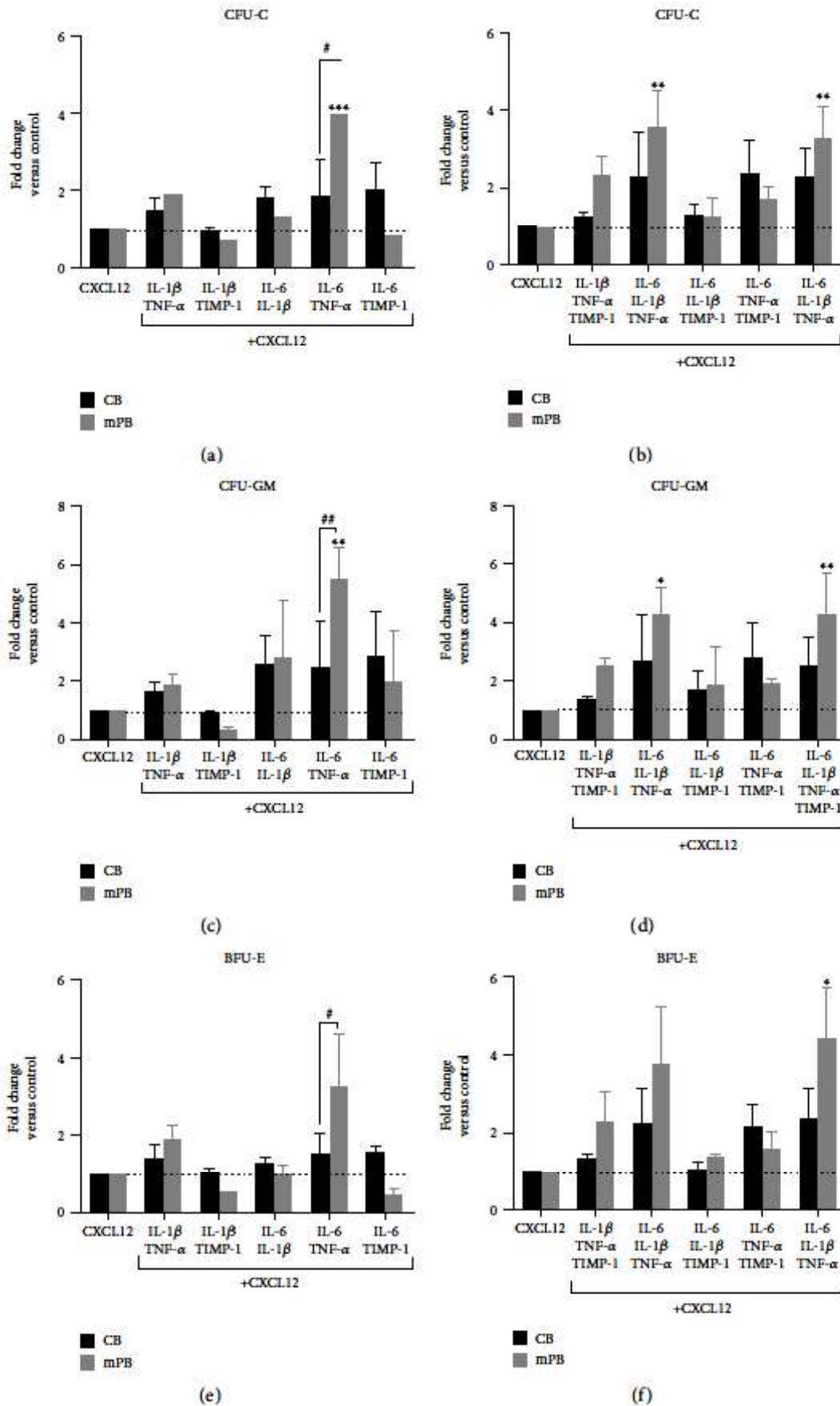
Figure 4: Clonogenic output of CB- or mPB-derived CD34+ cells after migration toward different combinations of inflammatory cytokines plus CXCL12. *Panels a and b show the fold change of clonogenic*

potential of CB-derived (n=4–8 independent experiments) and mPB-derived CD34+ cells (n=4–9 independent experiments) after migration toward CXCL12 with or without the two-by-two combination (a) or multiple (b) combinations of proinflammatory factors (postmigration CFU-C). (c–d) Fold change of CFU-GM (c–d) and BFU-E (e–f) growth after migration towards CXCL12 in the presence of various combinations of cytokines. Dot lines were used to mark control samples after migration towards CXCL12. Results are expressed as mean fold change of CFU − C ± SEM (∗p ≤ 0.05, ∗∗p ≤ 0.01, and ∗∗∗p ≤ 0.001; versus control cells (CXCL12)) (#p ≤ 0 05 and ##p ≤ 0 01 CB versus mPB).

5. The Copresence of BMSCs and Combined Inflammatory Cytokines Does Not Show Additive/Synergistic Effect in Terms of Hemopoietic Supportive Role.

To mimic the in vivo niche and to investigate the role of normal BMSCs in the inflammation-driven functional behaviour of normal HSPCs, we cocultured CB- or mPB-derived CD34+ cells with

BMSCs from HD in the presence or absence of combined proinflammatory cytokines.

As shown in Figure 5(a), the survival of CD34+ cells, either from CB or mPB, was significantly promoted by BMSCs (p ≤ 0.01 and p ≤ 0.05, resp.). Interestingly, cocultures with BMSCs decreased the percentage of apoptotic CB CD34+ cells compared with the monoculture counterparts, with 33.5 ± 8.8% of apoptosis in cocultures versus 76.71± 8.9% in monocultures (p ≤ 0.05, data not shown). Similar results were obtained when cocultures of mPB CD34+ cells were performed, with 48.63 ± 2.1% of apoptosis in cocultures versus 78.92 ± 6.05% in monocultures (p ≤ 0.05) (data not shown). However, in the presence of BMSCs (Figures 5(b) and 5(c)), the viability of cocultured CD34+ cells from CB or mPB was not significantly modified by the combined inflammatory factors as compared with the untreated counterparts. Comparing the two sources, only IL-6 + TIMP-1 (p ≤ 0.01) significantly increased the number of viable cocultured CB-derived CD34+ cells.

Altogether, these findings demonstrate that (1) the survival of normal CD34+ cells is highly promoted by normal BMSCs through a strong protection from apoptosis, (2) BMSCs alone or the combined proinflammatory cytokines stimulate the survival of normal HSPCs at the same extent, and (3) the copresence of BMSCs and the combined inflammatory cytokines does not show additive/synergistic effect in terms of hemopoietic supportive role.
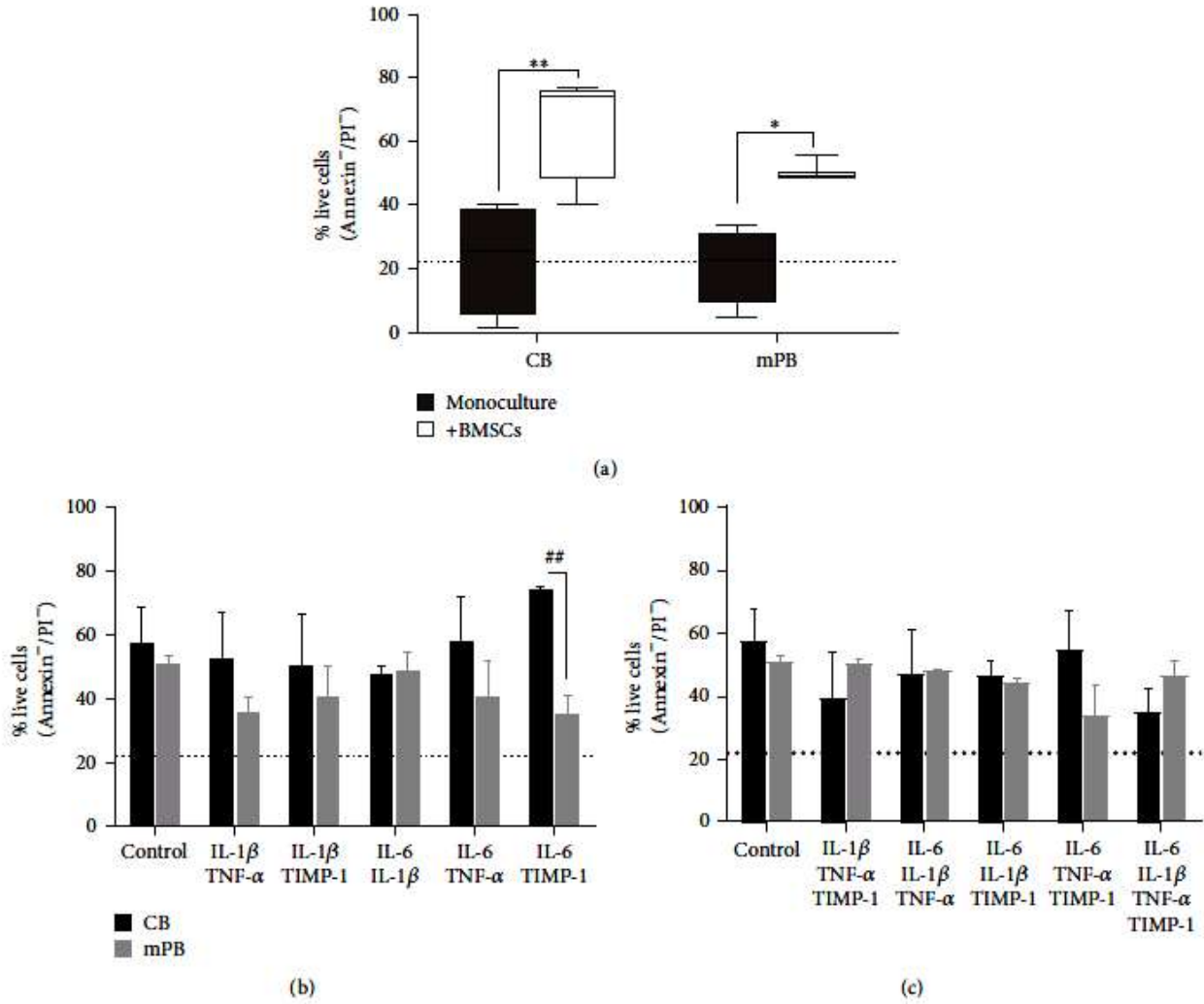
Figure 5: Survival of CB- and mPB-derived CD34+ cells after cocultures with HD-BMSCs and in the presence or the absence of combined inflammatory cytokines. *(a) Comparison between 24-hour monocultures and cocultures of CB and mPB-derived CD34+ cells (n=4 independent experiments, resp.) with BMSCs for in vitro survival (Annexin V/PI staining) is shown. Percentages of live CB- or mPB-CD34+ cells in the presence of BMSCs and/or proinflammatory cytokines with two by two combination (b) or multiple combinations (c) in comparison to CD34+ cells cocultured with BMSCs but without inflammatory stimuli are shown. For each graph, to highlight the comparison with cocultures, a dot line represented the mean percentage of live cells in all monocultures (CB and mPB CD34+ cells) (∗$p \leq 0.05$ and ∗∗$p \leq 0.01$ versus control cells) (##$p \leq 0.01$ CB vs mPB).*

6. Proinflammatory Cytokines Do Not Modify the In Vitro Migration of CD34+ Cells from CB and mPB toward BMSCs.

The BMSCs produce CXCL12 as mediator of migratory response of different cell types. CXCL12 is constitutively expressed by murine and human BM stromal cells [29]. To explore the effects of inflammation on the CXCR4-driven migratory ability of CD34+ cells in the presence or absence of BMSCs, we set up a migratory assay towards CXCL12 alone and BMSCs alone or in combination with various inflammatory cytokines. Of note, in order to mimic the in vivo pattern, along with inflammatory cytokines, a suboptimal concentration of CXCL12 (120 ng/mL) was also added.

As shown in Figure 6(a), we compared the spontaneous migration of CD34+ cells from CB and mPB with the migration towards BMSCs seeded on the bottom of the transwell system, as chemoattractant. We found that the migration of CD34+ cells from both sources was promoted by BMSCs, being significant ($p \leq 0.05$) with CB only. However, the migration rate of CB or mPB CD34+ cells was not increased by the presence of CXCL12 + BMSCs as compared with that of CXCL12 alone or BMSCs alone (Figures 6(b) and 6(c)). When we added various combinations of proinflammatory cytokines in the presence of CXCL12 and BMSCs, once again we did not find any significant difference in the migration rate of CD34+ cells between treated and untreated cells or between the two sources (Figures 6(b) and 6(c)).

These experiments demonstrate that the BMSCs exert a potent chemo attractive effect on normal HSPCs; moreover, in the presence of BMSCs, these combined proinflammatory factors are unable to significantly modify the CXCR-4-driven migratory behaviour of HSPCs from both sources.
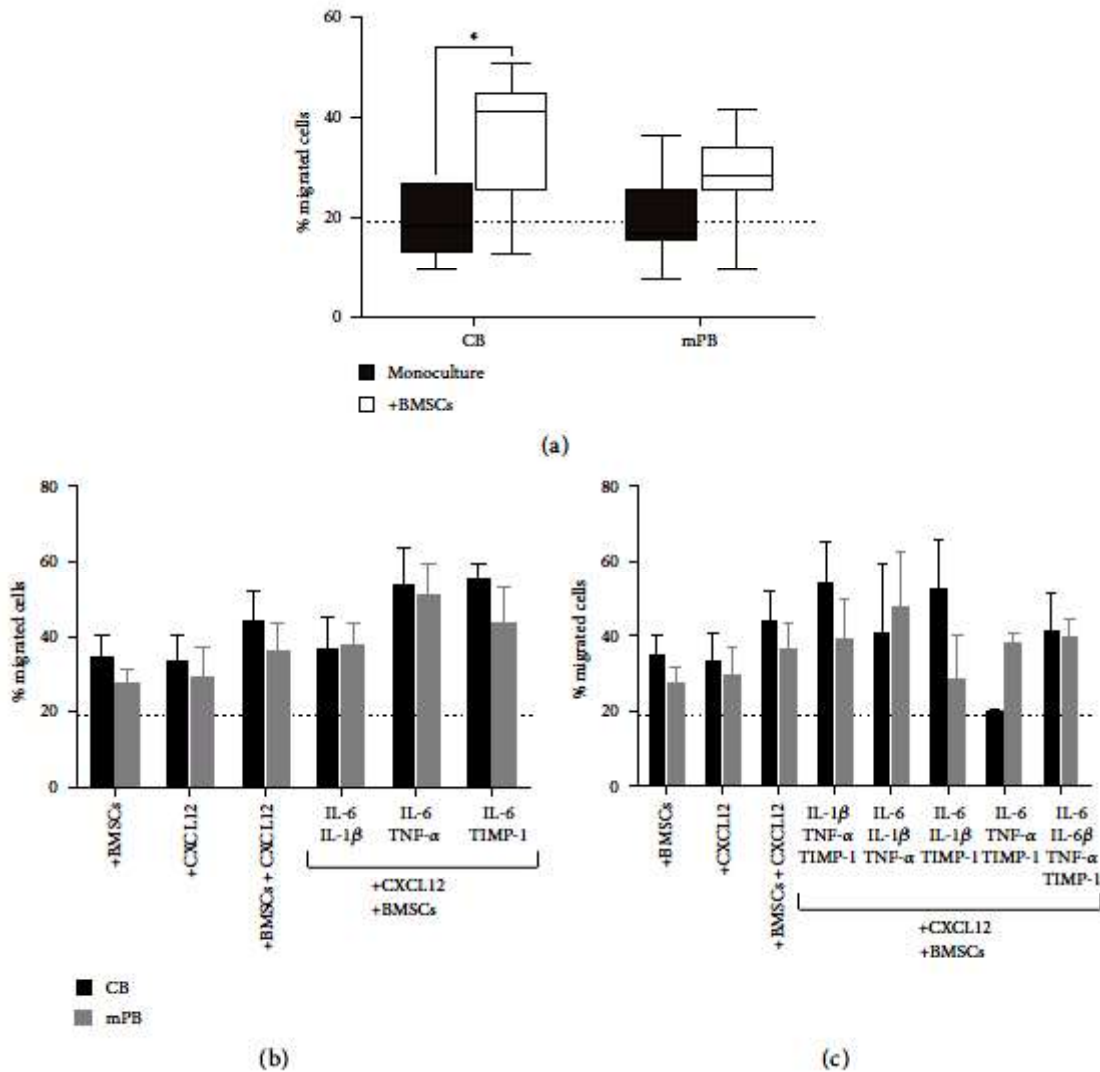
Figure 6: Migration of CB- and mPB-derived CD34+ cells towards CXCL12 and combined proinflammatory stimuli gradient and in the presence of normal BMSCs as further chemoattractant.

(a) Comparison between spontaneous migration and migration toward BMSCs precultured on the bottom for 24 hours before seeding CD34+ cells on the top of the transwell system (n=4 independent experiments, resp). Percentages of migrated CD34+ cells (seeded on the top of transwell) towards BMSCs (seeded 24 hours before on the bottom) plus CXCL12 and proinflammatory cytokines (two-by-two combination (b) or multiple combinations (c)) as chemoattractant are shown. For each graph, to highlight the comparison with cocultures, a dot line represented the mean percentage of migrated cells towards CXCL12 in all monocultures (CB and mPB CD34+ cells) ($*p \leq 0.05$ versus control cells).

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DISCUSSION

Several cytokine-based strategies enhancing hemopoiesis, homing, and subsequent engraftment of CB/mPB-derived HSCs have been previously described [30]. However, critical steps are involved in these processes and further insights are necessary to better understand HSPCs homing and engraftment [31]. Along with a role as activators of immune cell function, a growing evidence now demonstrates that proinflammatory cytokines strongly affect the size and lineage distribution of the blood cells via reprogramming of HSC/HSPC and the supporting BM niche [5, 32]. Along with the cytokine storm, the network created by danger associated molecular patterns (PAMPs/DAMPs) and alarmins could deviate HSCs fate, directly or indirectly via stromal cells [33]. Based on these evidences and due to the lack of informative data, it is of utmost importance to clarify the impact of proinflammatory cytokines on the biology of the normal HSPC and its BM microenvironment. A better understanding of the mechanisms driven by the inflammatory milieu in HSPCs may lead to better transplantation outcomes and knowledge of hematological defects or malignancies.

Here we tested various combinations of proinflammatory cytokines such as IL-1β, IL-6, TNF-α, and TIMP-1 in order to investigate their functional role on the in vitro behavior of young (CB-derived) and adult (mPB-derived) CD34+ cells. To mirror the in vivo condition, tested cytokines were two to two or multiple combined. We selected these inflammatory cytokines for the following features: (i) IL-6 is a pleiotropic proinflammatory cytokine that acts on many cell types including hemopoietic cells. It has been implicated as a critical activator of myelopoiesis in response to pathogen infection and chronic inflammation [34]; (ii) IL-1β is a potent inflammatory cytokine that mediates leukocytosis and thrombocytosis under inflammatory conditions by inducing various cytokines (i.e., granulocyte colony-stimulating factor and IL-6) [7]. Moreover, Pietras et al. [35] recently demonstrated that while IL-1β is dispensable for steady-state hemopoiesis, acute exposure to IL-1β accelerates HSC proliferation and instructs HSC priming for a myeloid fate. Lastly, it is involved in the pathogenesis of solid tumors and hematological malignancies [36]; (iii) TNF-α negatively regulates the expansion and self-renewal of HSPCs [37]. However, other evidence suggests that TNF signalling may enhance HSC function [38, 39]; (iiii) TIMP-1, through receptor (CD63) binding, has a role in multiple biological processes, including inflammation and immune regulation. We recently demonstrated that it displays cytokine-like features in the normal and leukemic HSPC compartment [25, 26].

In addition, experimental evidence demonstrated that combined proinflammatory cytokines such as IL-6 and TNF-α are critical for both inflammation and cancer by activating STAT3 and the NF-κB complex [40]. Furthermore, other inflammatory cytokines and pathways (such as IL-6) are

induced by IL-1β and are involved in malignancy [36]. Thus, the combined action of these cytokines could constitute a central signalling pathway that promotes inflammation and tumor growth.

Comparing the two sources (neonatal versus adult) of HSPCs, here we demonstrated that the following:

(1) Various combinations of inflammatory cytokines mainly enhance the in vitro survival of CB-derived CD34+ cells.

(2) As compared to CB, mPB-derived CD34+ cells are susceptible to selected combinations of inflammatory factors in terms of proliferation and hemopoietic function.

(3) TNF-α, alone or in combination, promotes the expression of the CD13 myeloid marker and CD44, an adhesion/proliferation marker of normal and leukemic cells.

(4) Along with an increased number of circulating CXCR4+CD34+ cells, selected combinations of inflammatory cytokines mainly enhance the in vitro migration of mPB-derived CD34+ cells.

(5) BMSCs alone or combined inflammatory cytokines promote survival/migration of HSPCs from both sources at the same extent; moreover, their copresence does not show additive/synergistic effect in terms of hemopoietic supportive role.

Of note, despite that the use of frozen/thawed CD34+ cells in some cases might have influenced the variability of phenotype/clonogenic ability, our results clearly demonstrate that the selected network of proinflammatory factors has the potential to activate either neonatal or adult normal hemopoiesis and acts as regulator of HSPCs. Moreover, these results are consistent with previously described promoting effects of the inflammatory microenvironment on hemopoiesis [4,7,12,]. Taking into account that the functional consequences of inflammation-related molecules depend on the duration of exposure (acute versus chronic), our results may provide a starting point to investigate whether the inflammatory cues contribute to creating a favorable milieu for the development of hematological malignancies through hemopoietic activation.

Several groups evaluated the expression of hematopoietic markers, identifying various subpopulations of CD34+ cells in CB or mPB samples [41, 42]. Interestingly, we found that, when CB- or mPB-derived CD34+ cells were treated with combined proinflammatory cytokines including TNF-α, CD13 expression was highly promoted. The CD13/aminopeptidase N is expressed on various cell types, including myeloid hematopoietic cells, and regulates biological phenomena such as differentiation, proliferation, apoptosis, motility, and tumor cell invasion. Importantly, CD13

degrades the chemokine CXCL11 and modulates CXCL12-induced migration [43, 44]. It has also been reported that high levels of human CD13 correlate with leukemic cell resistance to apoptosis [45]. Moreover, CD13 is differentially expressed in discrete states of differentiation of neoplastic myeloid cells [46]. Along with CD13, here we found modulation of the homing associated cell adhesion molecule CD44. Recently, CD44, as a receptor for hyaluronan, emerges as mediator of cell-cell and cell-matrix interactions and as pivotal trigger in cancer stem cell communication with their microenvironment [47, 48]. Here we identified combined proinflammatory cytokines including TNF-α, which are able to upregulate CD44 expression on the surface of normal CD34+ cells. Although TNF-α-driven modulation of CD44 expression was already reportedin several cancers [49], this is the first time that a strong link has been found between combined inflammatory cytokines clearly indicate that the inflammation-driven CD13 and CD44 upregulation on neonatal or adult CD34+ cells has the potential of modulating key functional pathways (i.e., survival/differentiation) of the normal hemopoietic progenitor cells. Interestingly, these pathways may also play a role in myeloproliferation and leukemogenesis.

Several studies have shown that HSC can be expanded in cytokine-driven culture and by MSC feed layers [50, 51]. Consistently, our data clearly showed that normal BMSCs enhance survival and migration of CB- and mPB-derived CD34+ cells. Due to their capacity to modulate oxidative stress, it is likely that BMSCs are capable of inhibiting apoptosis; moreover, producing CXCL12 [52], they enhance cell migration. Interestingly, even though it is likely that different mechanisms are involved, the BMSC-driven promoting effect of the CB-derived CD34+ cell survival is similar to that induced by the combined inflammatory cytokines. A similar trend was observed in mPB. However, for the first time, we investigated the copresence of BMSCs and various combinations of selected proinflammatory cytokines. Surprisingly, the copresence of inflammatory stimuli with BMSCs did not significantly modify the survival-migration rate of normal HSPCs as compared with that observed after stimulation with BMSCs alone or combined inflammatory cytokines alone. Of note, the BMSCs are capable to sustain the survival of mPB-derived CD34+ cells in the presence of IL-1β + TIMP-1. These findings demonstrate that in our culture system an acute inflammatory stimulus does not impair the hemopoietic-supportive role of BMSCs. Interestingly, based on murine models, it has been previously demonstrated that MSCs can modulate inflammation by secreting soluble receptors for IL-1 and TNF, which bind to IL-1 and TNF-α and neutralize the activity of the cytokines [53–55]. We can therefore hypothesize that in our cocultures the promotion of normal HSPCs survival/migration is mainly due to BMSCs which show regulatory properties of the hemopoietic function of HSPCs because they are capable of balancing the proinflammatory signal-driven hemopoietic activation. These results suggest that

exploiting or modulating the thin balance between pro- and anti-inflammatory pathways may be a clinically relevant approach in hematological malignancies.

CONCLUSIONS

The goal of this study is the demonstration that an inflammatory microenvironment promotes distinct in vitro functional activation of neonatal and adult HSPCs and that an acute inflammatory stress does not impair the hemopoietic promoting effect of BMSCs. Moreover, this study may represent a starting point for future studies aiming at addressing the role of inflammation and the balance with anti-inflammatory signals in the functional behavior of normal HSPCs and their transformation to a leukemic phenotype.

MATERIALS and METHODS

Sample Collection

CB samples (n=14) from normal fullterm deliveries were provided by the Cord Blood Bank of the University Hospital of Bologna after written informed consent. mPB samples (n=14) were obtained from hemopoietic stem cell transplantation donors. This study was approved by the medical Ethical Committee of the University Hospital of Bologna and was conducted in accordance with the Declaration of Helsinki.

Cell Isolation

Mononuclear cells (MNCs) were separated from CB and mPB samples (maximum after 1 day from harvesting) by stratification on Lympholyte-H 1.077 g/cm3 gradient (Gibco-Invitrogen, Milan, Italy), followed by red blood cell lysis for 15 min at 4°C. MNCs were then processed on magnetic columns for CD34+ cell isolation (mean purity 94 ± 4%) (CD34 Isolation kit; Miltenyi Biotec, Bologna, Italy), as previously described [25], and treated with our combination of cytokines on the same day. In selected cases, CD34+ cells from CB or mPB were cryopreserved in liquid nitrogen and then thawed before testing with the combined inflammatory cytokines. Of note, to minimize the influence of freezing/thawing, only thawed CD34+ cells with a survival rate > 80% were used and the thawed CB/mPB cells were studied in the same experiment.

Phenotype of Circulating CD34+ Cells

The phenotype of circulating CD34+ cells was evaluated in CB and mPB samples by conventional flow cytometry, as previously described [20]. Antibodies used to characterize the CD34+ cells are listed in Supplementary Table 1. A minimum of 1×10^4 CD34+ cells were acquired by a BD Accuri C6 flow cytometer (Becton Dickinson, Milan, Italy). Analysis was performed excluding cellular debris in a SSC/FSC dot plot. The percentage of positive cells was calculated subtracting the value of the appropriate isotype controls. The absolute number of positive cells/μL was calculated as follows: percentage of positive cells × white blood cell count/100.

Apoptosis Assay

Freshly isolated CD34+ cells ($2–5 \times 10^5$) from CB units or mPB samples were maintained in RPMI 1640 with 10% fetal bovine serum (FBS), with or without IL-6 (10 ng/mL), IL-1β (1 ng/mL), TNF-α (10 ng/mL), and TIMP-1 (100 ng/mL), alone or in different combinations (all from Thermo Scientific, Rockford, IL, USA). After 24 hours, cells were stained for 15 min at RT with Annexin-VFLUOS Staining Kit (Roche, Penzberg, Germany). Samples were then immediately analyzed by a BD Accuri C6 flow cytometer. Results are expressed as percentage of live cells compared to the whole cells.

Erythroid and Granulocytic Progenitor Assays

CB/ mPB-derived CD34+ cells were cultured in vitro to achieve hematopoietic cell differentiation and the formation of colony-forming units (CFU-Cs), which is the sum of colony forming unit-granulocyte macrophage (CFU-GM) and erythroid burst-forming units (BFU-E). Specifically, CD34+ cells were seeded in methylcellulose-based medium (human Stem- MACS HSC-CFU lite w/ Epo, Miltenyi Biotech) at 5×10^2 cells/mL in 35mm Petri dishes in the presence or absence of the selected proinflammatory factors: IL-6 (10 ng/mL), IL-1β (1 ng/mL), TNF-α (10 ng/mL), and TIMP-1 (100 ng/ mL), alone or in combination. After 2 weeks of incubation at 37°C in 5% humidified CO_2 atmosphere, CFU-C growth was evaluated by standard morphologic criteria using an inverted microscope (Axiovert 40, Zeiss, Milan, Italy).

Migration Assay

Migration of CB/mPB-purified CD34+ cells was assayed in transwell chambers (diameter 6.5 mm, pore size 8 μm; Costar, Corning), as previously described [25]. In order to highlight the effects of the selected inflammatory cytokines, suboptimal CXCL12 gradient (120 ng/mL) was employed. Briefly, 50 μL of RPMI 1640 plus 10% FBS containing 0.5×10^5 cells was added to the upper chamber and 150 μL of medium with or without CXCL12 ± IL-6 (10 ng/mL), IL-1β (1 ng/mL), TNF-α (10 ng/mL), and TIMP-1 (100 ng/mL) (alone or in combination) was added to the bottom chamber. After overnight incubation at 37°C in 5% humidified CO_2 atmosphere, inserts (upper chambers) were removed and cells transmigrated into the lower chamber were recovered and counted by the Trypan Blue exclusion test. The amount of migrated cells was expressed as a percentage of the input, applying the following formula: (number of migrated cells recovered from

the lower compartment/total number of cells loaded in the upper compartment) × 100. In addition, migrated cells were assayed in methylcellulose-based medium for their ability to form hematopoietic colonies (as above described).

Isolation and Expansion of Mesenchymal Stromal Cells (BMSCs) from Healthy Donors (HD)

BMSCs were obtained from BM aspirates collected from HD (n = 3), as previously described [26]. BM-MNCs were separated by stratification on Lympholyte-H 1.077 g/cm^3 gradient (Gibco-Invitrogen) and then resuspended in culture medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% penicillin/ streptomycin, L-glutamine, and 10% FBS), plated, and maintained in a humidified incubator at 37°C and 5% CO_2. All nonadherent cells were removed after 24 hours. Medium was changed every 3–4 days until they reached 70–80% confluence. Cells were then trypsinized (Lonza, Verviers, Belgium), replated at a density of 3500 $cells/cm^2$, and used for experiments within passages 3-4 after flow cytometry analysis for immunophenotype.

BMSCs Coculture Assay

In selected experiments, CBand mPB-derived CD34+ cells were cocultured either without stromal support or directly seeded on a confluent layer of BMSCs in 96-well plates for 24 hours before use. CD34+ cells were then harvested and used to perform clonogenic and migration assay as above described; in addition, the selected cytokines were added to the bottom chamber. After overnight incubation at 37°C in 5% humidified CO_2 atmosphere, cells transmigrated into the lower chamber were recovered and counted, as previously described.

Statistical Analysis

Data are presented as mean ± SEM of at least three independent determinations. Statistical differences
between groups were determined by a two-tailed Student t-test and one- or two-way ANOVA, as appropriate. All analyses were performed using GraphPad Prism software (version 6.0; La Jolla, CA, USA); $p \leq 0.05$ was considered to indicate statistical significance.

Results IIa

Circulating megakaryocyte and platelet microvesicles correlate with response to Ruxolitinib and distinct disease severity in patients with Myelofibrosis

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Keywords: **Myelofibrosis, Essential Thrombocythaemia, Microvesicles, Megakaryocytopoiesis, Platelet activation, Ruxolitinib**

ABSTRACT

The role of circulating microvesicles (MVs) in Myelofibrosis (MF) and Essential Thrombocythemia (ET) is far to be defined. Here we found that 1) circulating megakaryocyte-MVs were reduced in MF and ET while platelet-MVs were increased; 2) the proportion of circulating megakaryocyte- and platelet-MVs was associated with disease severity in MF; 3) ruxolitinib normalized the profile of circulating megakaryocyte- and platelet-MVs in spleen responders MF patients only. Of note, a cut-off value of 19.95% of circulating megakaryocyte-MVs predicts ruxolitinib spleen response. In light of these findings, circulating megakaryocyte/platelet-MVs may have a tissue specific diagnostic and prognostic role in MF.

INTRODUCTION

Myelofibrosis (MF) and Essential Thrombocythemia (ET) are clonal disorders with abnormalities in megakaryocyte development and platelet activation, chronic inflammation and driver mutations (*JAK2, CALR, MPL*). The absence of the 3 "driver" mutations identifies triple negative (TN) patients. Ruxolitinib (*JAK1/2* inhibitor) reduces splenomegaly and constitutional symptoms in MF. However, over 50% of patients fail to achieve or lose the response over time (Tefferi *et al*, 2015; Vainchenker *et al*, 2018).

Extracellular microvesicles (MVs) are size-heterogeneous small vesicles (100-1000 nm) with pleiotropic effects on cell signalling including immunity and inflammation (Butler *et al*, 2018). Megakaryocyte- and platelet-MVs are the most abundant in peripheral blood (PB). However, while the MVs production by megakaryocytes is based on a constitutive mechanism, only activated platelets can produce CD62P+ MVs (Flaumenhaft *et al*, 2009). High serum levels of MVs have been detected in MF and ET (Caivano *et al*, 2015; Zhang *et al*, 2017).

Circulating MVs as biomarkers of disease/malignancy in MPNs is an open question. Here we investigated: 1) the profile of MVs in MF and ET; 2) whether MVs proportions could be related to severity of MF; 3) the role of inflammation on MVs frequency of MF; 4) the effects of ruxolitinib on MVs in MF.

RESULTS

Firstly, we characterized the circulating megakaryocyte- and platelet-MVs frequency. Comparing patients and healthy donors (HD; **Fig 1a, 1b**), megakaryocyte-MVs were significantly decreased in MF ($p<0.001$) and ET ($p<0.001$). By contrast, platelet-MVs were significantly increased in MF ($p<0.01$) and ET ($p<0.001$). Comparing patients groups, platelet-MVs were significantly increased in ET vs MF ($p<0.01$). No significant differences in megakaryocyte- and platelet-MVs distribution were observed between primary or post-PV/post-ET MF. According to mutation status (**Fig 1c, 1d**), the megakaryocyte-MVs of the $JAK2^{(V617F)}$-($p<0.001$)/$CALR$-($p<0.01$) mutated and TN ($p<0.01$) MF patients were significantly decreased as compared to HD. Conversely, the platelet-MVs were significantly increased in the $JAK2^{(V617F)}$-($p<0.001$)/$CALR$-($p<0.05$) mutated MF patients only. Comparing the molecular subtypes, the platelet-MVs of the $JAK2^{(V617F)}$-($p<0.05$)/$CALR$-($p<0.05$) mutated patients were significantly increased as compared with the TN counterparts.

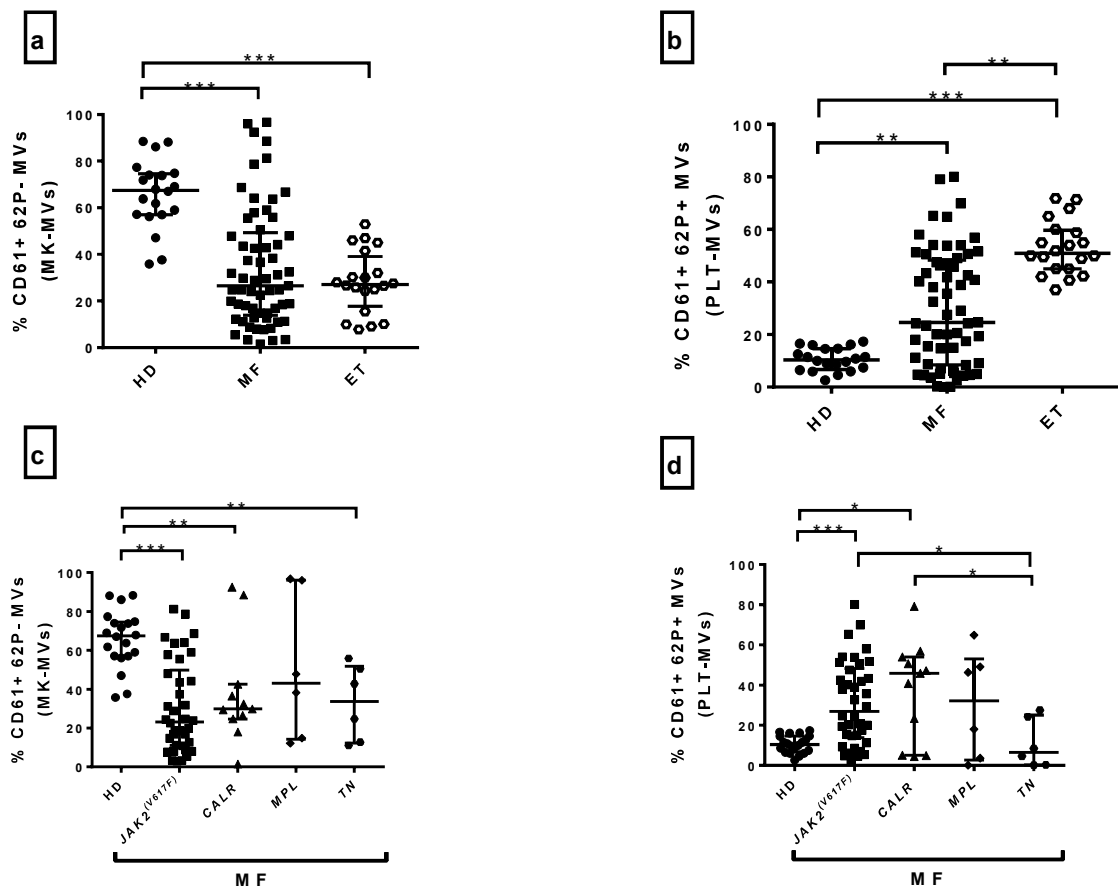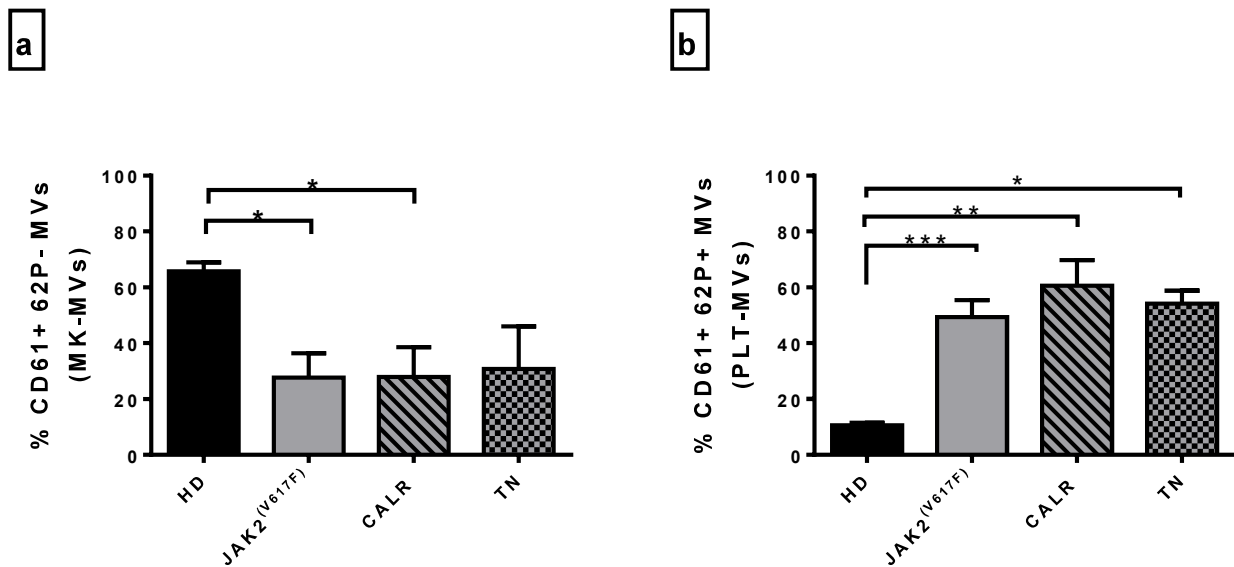***Fig 1**. Circulating megakaryocyte- and platelet-MVs frequency of MF and ET patients. Megakaryocyte-MVs (MK-MVs; CD61+CD62P-) and platelet-MVs (PLT-MVs; CD61+CD62P+) of MF (n=61), ET (n=20) patients and HD (n=20) are shown in panels **(a)** and **(b)**. Panels **(c)** and **(d)** show the frequency of MK- and PLT-MVs of MF patients according to mutation status (JAK2^(V617F) n=38; CALR n=11; MPL n=6 and TN*

In ET patients (**Supplementary Fig 2a, 2b**), only the megakaryocyte-MVs of the $JAK2^{(V617F)}$-(p<0.05)/$CALR$-(p<0.05) mutated patients were significantly decreased as compared to HD. By contrast, the platelet-MVs were significantly increased in $JAK2^{(V617F)}$-(p<0.001)/$CALR$-(p<0.01) mutated and TN patients (p<0.05). Comparing the three molecular subtypes, no significant differences were observed in megakaryocyte- and platelet-MVs of ET patients.

***Supplementary Fig 2**. **Circulating megakaryocyte- and platelet-MVs frequency in ET according to mutation status.** Megakaryocyte-MVs (MK-MVs; CD61+CD62P-) **(a)** and platelet-MVs (PLT-MVs; CD61+CD62P+) **(b)** frequency of ET patients (n=20) according to mutation status and HD (n=20) is shown. Results are reported as mean ± SEM. (Kruskal-Wallis test; *p<0.05; **p<0.01; ***p<0.001)*

Secondly, we explored the circulating megakaryocyte- and platelet-MVs of the MF patients according to the IPSS risk score. Intermediate-2/high IPSS risk patients showed a significant decrease in megakaryocyte-MVs along with a significant increase of platelets-MVs as compared to intermediate 1/low IPSS risk patients (p<0.05 and p<0.01, respectively) and HD (p<0.001) (**Fig 1e, 1f**). Comparing IPSS subgroups according to molecular subtypes and HD (**Fig 1g, 1h**), we observed that the megakaryocyte-MVs were significantly decreased in higher risk $JAK2^{(V617F)}$-/$CALR$-mutated patients (p<0.001, respectively). Concomitantly, the same group (higher risk $JAK2^{(V617F)}$-/CALR-mutated patients) presented a higher percentage of platelet-MVs (p<0.001, respectively), suggesting a disease-related specific pattern.
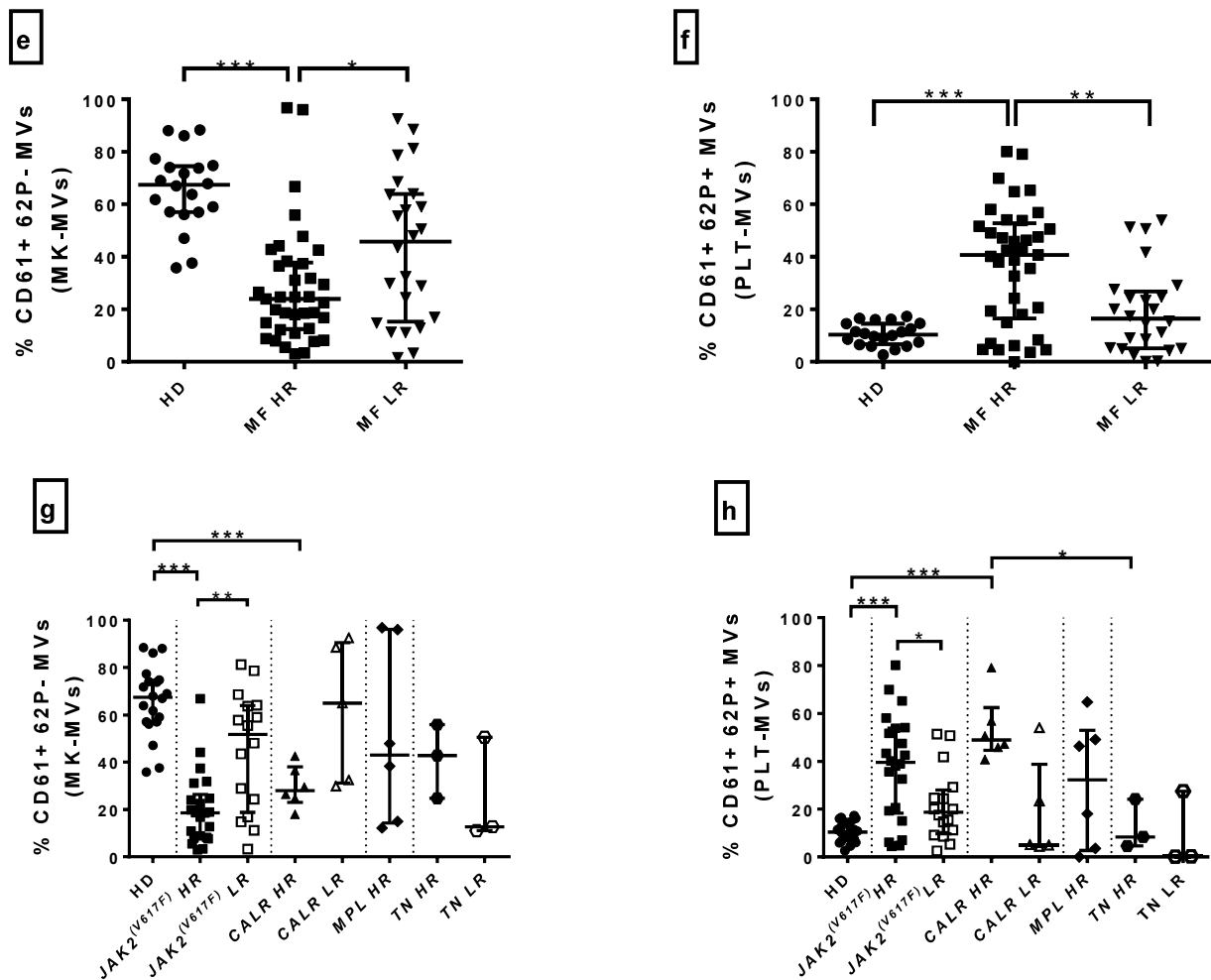
Fig 1. *Circulating megakaryocyte- and platelet-MVs frequency of MF patients.* Panels *(e)* and *(f)* depict MK- and PLT-MVs frequency of MF patients according to IPSS risk (HR= intermediate 2/high IPSS risk (n=37); LR=intermediate 1/low IPSS risk (n=24)). Frequency of MK- and PLT-MVs of MF patients according to mutation status and IPSS risk is shown in panels *(g)* and *(h)* (JAK2^(V617F)^HR n=22; JAK2^(V617F)^LR n=16; CALR HR n=6; CALR LR n=5; MPL HR n=6 and TN HR n=3; TN LR n=3). In addition to individual data, median values and interquartile ranges are shown. (Kruskal-Wallis test; *p<0.05; **p<0.01; ***p<0.001)

Surprisingly, we found a positive correlation between the megakaryocyte-MVs percentages of MF and platelets count (r=0.44; p<0.001; **Fig 2a**), suggesting a role of circulating megakaryocyte-MVs as biomarker of thrombopoiesis. In addition, the percentages of megakaryocyte-MVs of MF were inversely related to splenomegaly (r=-0.39; p<0.01; **Fig 2b**), confirming that a high disease severity is associated with reduced circulating megakaryocyte-MVs. Of note, no correlation was found between platelet-MVs and platelets count or splenomegaly.
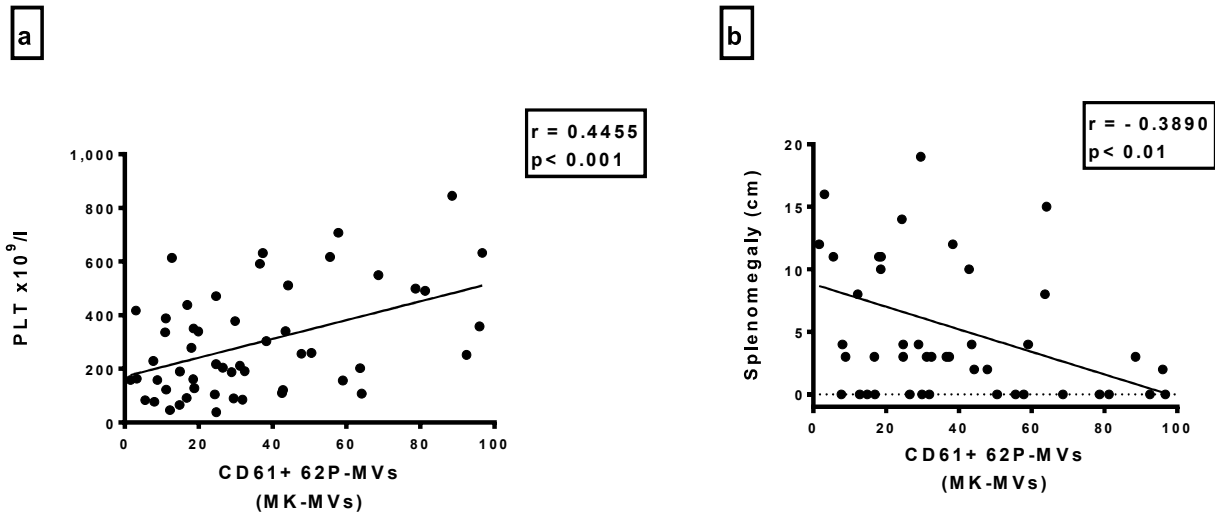
Fig 2. (a, b) Correlation between circulating megakaryocyte-MVs frequency and platelets count or splenomegaly in MF patients. *Megakaryocyte-MVs (MK-MVs; CD61+CD62P-) percentages **(a)** positively correlates with platelets count and **(b)** negatively with splenomegaly (Spearman's correlation test).*

Thirdly, despite crucial plasma pro-inflammatory cytokines and Thrombopoietin were increased in MF (**Supplementary Table 4**), only IL-6 plasma levels were inversely related with megakaryocyte-MVs percentages (r= -0.38; p<0.05; data not shown). We can therefore hypothesize that in MF IL-6 inhibits megakaryocyte-MVs production and/or increases their clearance. Conversely, the percentages of the platelet-MVs were positively correlated with the Thrombopoietin plasma levels (r=0.51; p<0.01; data not shown). Consistently, Thrombopoietin-driven platelets activation has been previously described (Kojima *et al*, 1995).

Finally, to investigate whether ruxolitinib therapy may affect circulating MVs, MF patients were studied before and after 6 months of therapy. After 6 months, 12 out of 27 (44%) patients were in spleen response. At baseline, the percentages of megakaryocyte-MVs were significantly decreased as compared with the HD counterparts (spleen responders/non-responders p<0.001, respectively), while platelet-MVs significantly increased (spleen responders/non-responders p<0.001, respectively) (**Fig 2c, 2d**). Importantly, non-responders showed a significantly lower median percentage of megakaryocyte-MVs as compared with the spleen responders counterparts (p<0.05) (**Fig 2c**). To further explore whether megakaryocyte-MVs proportion could be linked to ruxolitinib response, we performed a ROC analysis. A cut-off value of 19.95% of megakaryocyte-MVs was calculated with a specificity of 80%/sensitivity of 72% and discriminated the non-

responders (megakaryocyte-MVs < 19.95%). Ruxolitinib therapy, along with a significant decrease of platelet-MVs (p<0.01), promoted the release of megakaryocyte-MVs of spleen responders only (p<0.001) (**Fig 2c, 2d**), restoring the normal megakaryocyte- and platelet-MVs profile (**Fig 2e**).
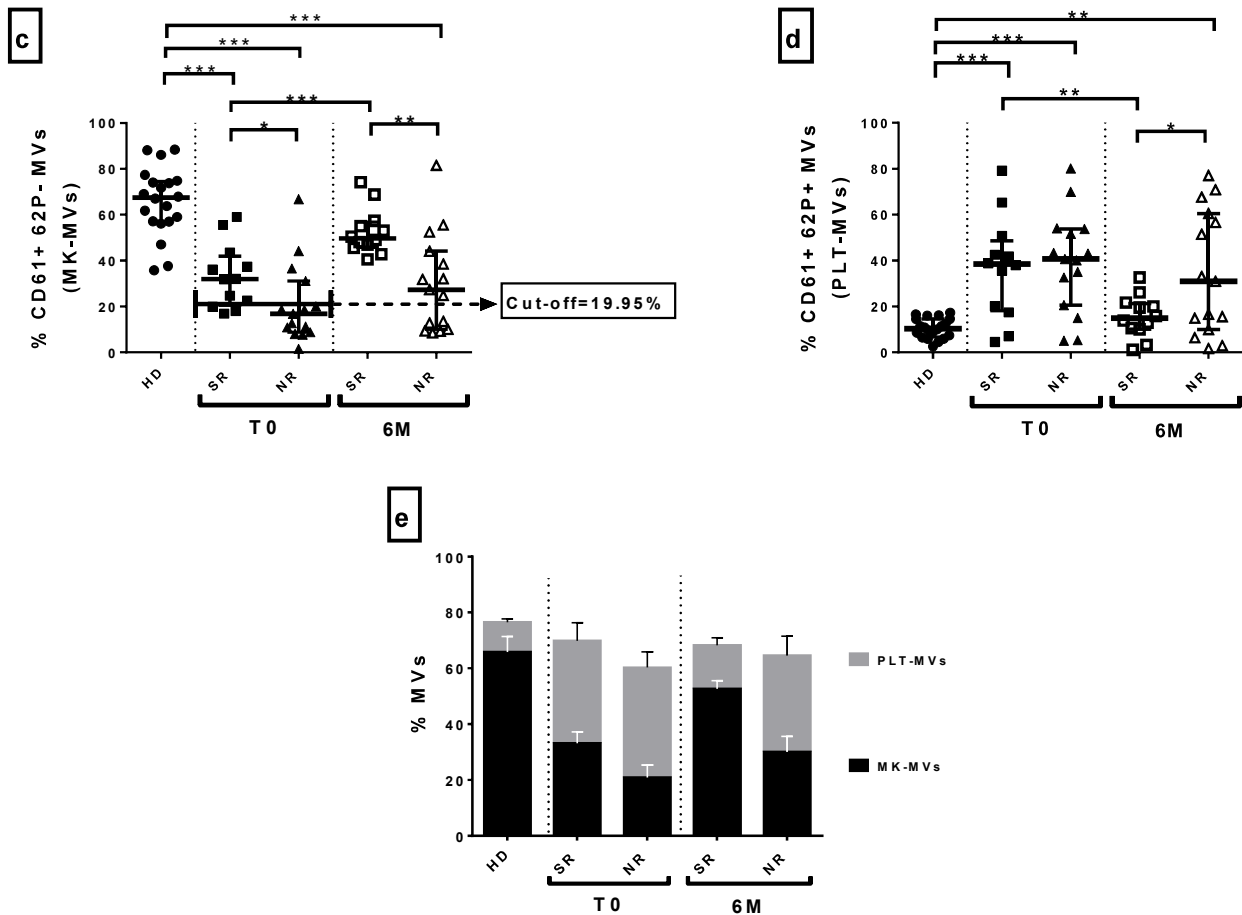
Fig. (c, d, e) Circulating megakaryocyte- and platelet-MVs frequency of MF patients according to ruxolitinib therapy response. (c) *and* ***(d)*** *show megakaryocyte-MVs (MK-MVs; CD61+CD62P-) and platelet-MVs (PLT-MVs; CD61+CD62P+) of HD (n=20), spleen responders (SR; n=12) and non-responder (NR; n=15) MF patients before (T0) and after 6 months ruxolitinib therapy (6M). In addition to individual data, median values and interquartile ranges are shown. (Kruskal-Wallis test; *p<0.05; **p<0.01; ***p<0.001).* ***(e)*** *the MK- and PLT-MVs combined profile of HD, spleen responders and non-responders before and after 6 months ruxolitinib therapy is shown (mean ± SEM).*

Interestingly, circulating monocyte- and endothelial-MVs (**Supplementary Fig 3a, 3b**) were significantly increased in MF patients (p<0.05 and p<0.01, respectively). At baseline, monocyte-

and endothelial-MVs were not significantly different between spleen responders and non-responders. Ruxolitinib therapy decreased the endothelial-MVs frequency in spleen responders only (p<0.05). A trend, albeit not statistically significant, toward a reduction of the monocyte-MVs was also observed in spleen responders.
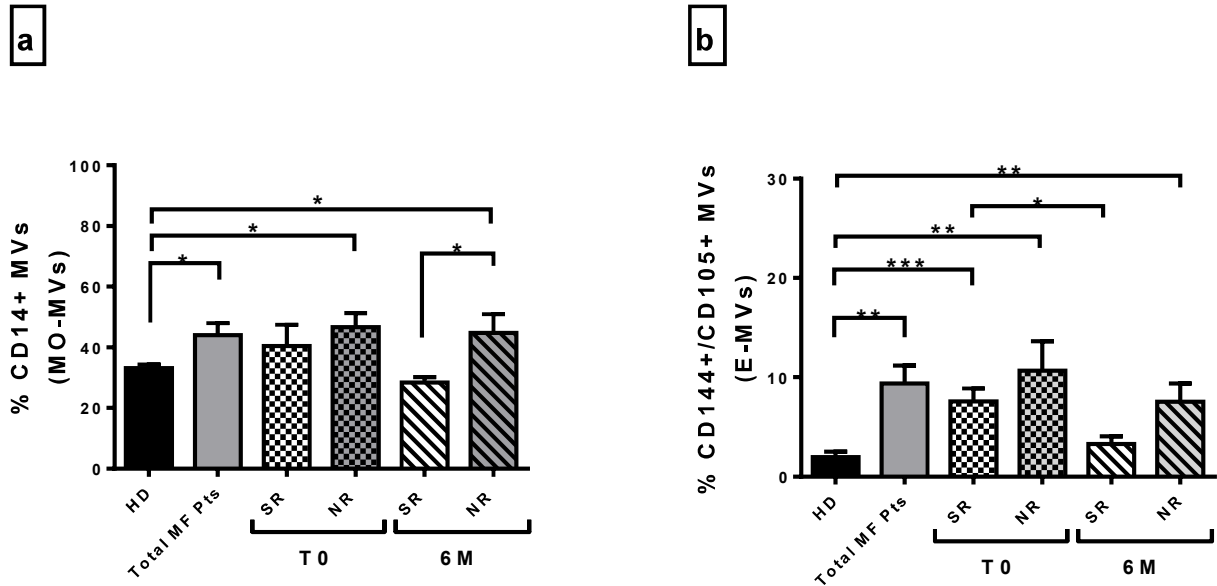
Supplementary Fig 3. ***Circulating monocyte- and endothelial-MVs frequency of MF patients according to ruxolitinib therapy response.*** *Monocyte-MVs (MO-MVs; CD14+) and endothelial-MVs (E-MVs; CD144+/CD105+) frequency of HD (n=20), spleen responders (SR; n=12) and non-responder (NR; n=15) MF patients before (T0) and after 6 months ruxolitinib therapy (6M) are shown in **(a)** and **(b)**, respectively. Results are reported as mean ± SEM. (Kruskal-Wallis test; *p<0.05; **p<0.01; ***p<0.001)*

CONCLUSIONS

Overall, these results demonstrate that distinct abnormalities of circulating megakaryocyte- and platelet-MVs profile are associated to MF and ET and suggest that: 1) platelets activation and abnormal/defective megakaryocytopoiesis may contribute to the increased/decreased proportion of circulating platelet- and megakaryocyte-MVs, respectively; 2) the activated JAK/STAT pathway plays a role in MVs biogenesis/clearance and, ultimately, in communication between megakaryocytes/platelets and the other cells. Additionally, circulating megakaryocyte-MVs may be considered a biomarker of thrombopoiesis in MF. Ruxolitinib therapy normalizes the profile of circulating MVs in spleen responders MF patients only by increasing the megakaryocyte-MVs and decreasing the platelet-MVs. Importantly, a cut-off value of 19.95% of megakaryocyte-MVs discriminates spleen responders and non-responders, demonstrating that circulating megakaryocyte-MVs, as a liquid biopsy assay, may be used as potential tool to predict response to ruxolitinib therapy. Therefore, despite the need to be confirmed in a larger casistic, circulating megakaryocyte/platelet-MVs may have a tissue-specific diagnostic and prognostic role in MF.

MATERIALS and METHODS

Casistic

This is a pilot study where patients were enrolled from May 2015 to May 2018. Sixty-one MF and 20 ET patients were included into the study. Patients were at diagnosis (n=31 MF; n=12 ET) or at least 3 months after cytoreductive therapy (n=30 MF; n=8 ET) **(Supplementary Table 1, 2).** Twenty-seven MF patients (24 *JAK2*$^{(V617F)}$- and 3 *CALR*-mutated) at intermediate-1 (n=7), intermediate-2 (n=12) or high (n=8) International Prognostic Scoring System (IPSS) risk (O'Sullivan *et al*, 2018) were studied before and after 6 months of ruxolitinib therapy. Spleen response in ruxolitinib-treated patients was evaluated according to the 2013 International Working Group-MPN Research and Treatment criteria (Tefferi *et al*, 2013). Twenty age/sex-matched healthy donors (HD) were also included.

Blood collection and Platelet Poor Plasma (PPP) preparation

EDTA-anticoagulated PB was collected from patients and HD. The first 2 ml of blood were discarded. PPP was obtained (within 2 hours from blood collection) after two consecutive centrifugations at 2500 g for 15 minutes at room temperature. PPP was then aliquoted and stored at -80°C until testing. The study was approved by the local Ethics Committee and was conducted accordingly to the Helsinki declaration (Informed consent was obtained from all subjects).

Flow cytometry MVs identification

Megakaryocyte-, platelet-, monocyte- and endothelial-MVs were analyzed in Platelet Poor Plasma (PPP; after thawing at 37°C) by flow cytometry (Cytoflex, Beckman Coulter, Milan Italy) *(Supplementary Fig 1 and Supplementary Table 3)*. The Violet Side Scatter laser (VSSC) is used as a trigger signal to discriminate the noise. To detect MVs the instrument was calibrated with MegaMix Beads (Stagò, Marseille, France). MVs identification was based on size (500-900 nm) and on the ability to bind lineage-specific monoclonal antibodies (**Supplementary Fig 1 and Supplementary Table 3**). Matched isotype controls were used to select the cut-off. Results are expressed as percentage of total MVs.

ELISA assay

Crucial plasma pro-inflammatory cytokines (*Interleukin (IL)1β, IL6, Interferon (IFN)-γ, Tumor Necrosis Factor (TNF)-α, Thrombopoietin (TPO))* of MF patients and HD were analyzed by ELISA (R&D Systems, Milan, Italy).

Genotype

Molecular genotyping was performed as previously described (Romano *et al*, 2017).

Statistical analysis

Statistical analysis was performed with GraphPad Prism 6 using Kruskal-Wallis test, chi-square or Fisher exact test, as appropriate and the Spearman's correlation test. ROC analysis was performed with STATA Software 15. P values <0.05 were considered significant.
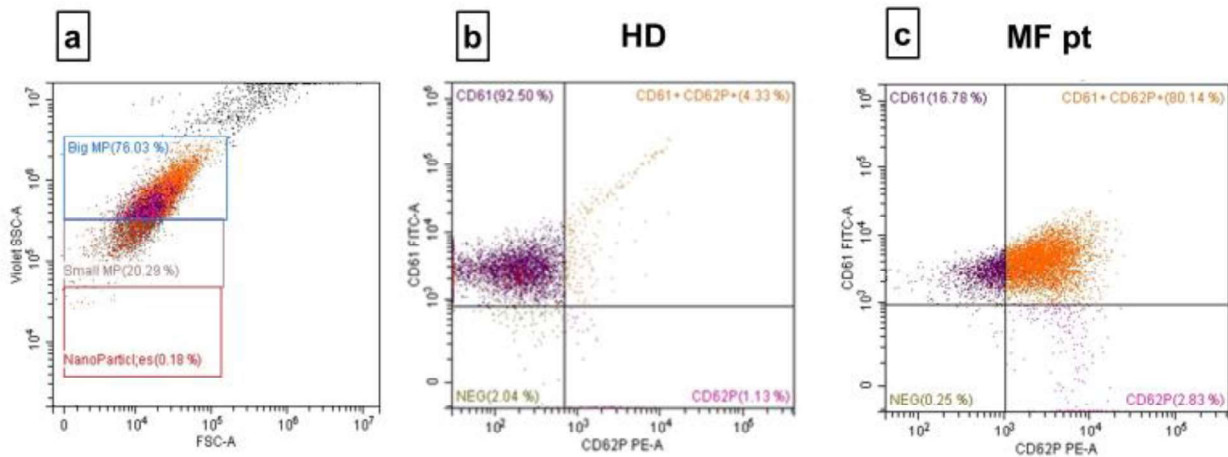
***Supplementary Fig 1. Gating strategy of circulating megakaryocyte- and platelet-MVs of 1 HD and 1 MF patient.** **(a)** Fluorescence gated polystyrene beads of different sizes were used to determine the gates identifying big (500-900 nm), small (200-300 nm) and nano (100-160 nm) MVs. Gating strategy to identify big MVs (500-900 nm) is shown. **(b)** and **(c)** show representative dot-plots of megakaryocyte- and platelet-MVs in plasma samples from 1 HD and 1 MF patient. Using the defined gate for big MVs, all events positive for surface markers staining (CD61+CD62P- megakaryocyte-MVs and CD61+CD62P+ platelet-MVs) were recorded.*

	Total MF (61 cases)	**JAK2$^{(V617F)}$** (38 cases)	**CALR** (11 cases)	**MPL** (6 cases)	**TN** (6 cases)
Median age, years (range)	70 (40-84)	65,5 (40-82)	72,5 (61-84)	65,5 (56-76)	77 (60-84)
Males, no. (%)	30 (49%)	19 (50%)	7 (64%)	1 (17%)	3 (50%)
Median Hemoglobin, g/dl; median (range)	11,5 (7,1-15,1)	12,4 (7,1-15,1)	10,0 (7,7-4,9)	9,1 (7,2-10,8)	9,7 (8,1-14,3)
Median Leukocytes, x 10^9/l; median (range)	9,4 (2,2-80,1)	9,4 (2,2-80,1)	7,5 (4,3-20,1)	15,9 (6,2-39,5)	10,8 (2,5-7)
Median Platelets, x 10^9/l; median (range)	229 (38-845)	229 (65-631)	217 (90-845)	280 (46-632)	121 (38-613)
Median Lymphocyte x 10^9/l; median (range)	1,7 (0,4-16,3)	1,4 (0,4-15,8)	1,85 (1,1-6)	2,4 (0,8 -16,3)	4,1 (0,4 -11,7)
Median Monocyte x 10^9/l; median (range)	0,6 (0,1-5,9)	0,6 (0,1-5,5)	0,6 (0,3-4,4)	1,3 (0,4-5,9)	0,7 (0,2-5,3)
BM fibrosis, no. of patients (%)					
Grade 1	30 (49%)	19 (50%)	5 (46%)	3 (50%)	3 (50%)
Grade 2	23 (38%)	14 (37%)	4 (36%)	2 (33%)	3 (50%)
Grade 3	8 (13%)	5 (13%)	2(18%)	1 (17%)	0
IPSS, Number of patients					
Low	4 (7%)	4 (10%)	0	0	0
Intermediate-1	20 (33%)	12 (32%)	5 (45,5%)	0	3 (50 %)
Intermediate-2	16 (26%)	11 (29%)	1 (9%)	3 (50%)	1 (17 %)
High	21 (34%)	11 (29%)	5 (45,5%)	3 (50%)	2 (33 %)
Previous treatment, no of patients (%)					
Hydroxyurea	30 (49%)	19 (50%)	3 (27%)	4 (67%)	4 (67%)
WHO Diagnosis					
PMF	39 (64%)	22 (58%)	7 (64%)	5 (83%)	5 (83%)
PPV-MF	14 (23%)	14 (37%)	0	0	0
PET-MF	8 (13%)	2 (5%)	4 (36%)	1 (17%)	1 (17%)

Supplementary Table 1. Clinical and laboratory features of MF patients according to mutational status
PMF: Primary Myelofibrosis; PPV-MF: Post Polycythemia Vera-Myelofibrosis; PET-MF: Post Essential Thrombocythemia-Myelofibrosis. The presence of 0, 1, 2 or 3 and >4 adverse factors defines low, intermediate-1, intermediate-2 and high-risk disease. IPSS, International Prognostic Scoring System. Comparisons of variables between groups of patients were carried out by Kruskal-Wallis test and by chi-square or Fisher exact test, as appropriate. No significant differences were observed among groups, except for Hb (JAK2$^{(V617F)}$ vs MPL, p<0.05) and PET-MF (JAK2$^{(V617F)}$ vs CALR, p<0.05).

	Total MF (20 cases)	**JAK2**[(V617F)] (13 cases)	**CALR** (5 cases)	**TN** (2 cases)
Median age, years (range)	65,5 (61-79)	66 (61-72)	65 (63-79)	69 (62-76)
Males, no. (%)	8(40%)	4 (30,8%)	3 (60%)	1 (50%)
Median Hemoglobin, g/dl; median (range)	13,7 (10-16,2)	13,7 (10-15,2)	13,5 (10,9-16,2)	12,6 (10,5-14,8)
Median Leukocytes, x 10^9/l; median (range)	7 (4,7-15,45)	8,7 (7-15,45)	6,5 (5,7-7,3)	5,3 (4,7-5,8)
Median Platelets, x 10^9/l; median (range)	647 (542-1069)	655 (542-1069)	639 (631-939)	665 (617-712)
Previous treatment, no of patients (%)				
Hydroxyurea	8 (40%)	4 (31%)	4 (80%)	0

Supplementary Table 2. Clinical and laboratory features of ET pts according to mutational status No significant differences were observed among groups. Comparisons of variables between groups of patients were carried out by Kruskal-Wallis test and by chi-square or Fisher exact test, as appropriate.

MVs type	**Identified as**	**List of monoclonal antibodies**
Megakaryocyte-MVs	CD61+/CD62P-	Anti-CD61 (Clone: SZ21; FITC-conjugated; Catalog number IM1758); Anti- CD62P (Clone: CLB-THROMB/6; PE-coniugated; Catalog number IM1759U). All antibodies from Beckman Coulter S.r.l.
Platelet-MVs	CD61+/CD62P+	Anti-CD61 (Clone: SZ21; FITC-conjugated; Catalog number IM1758); Anti- CD62P (Clone: CLB-THROMB/6; PE-coniugated; Catalog number IM1759U). All antibodies from Beckman Coulter S.r.l.
Monocyte-MVs	CD14+	Anti-CD14 (Clone: RM052; FITC-conjugated; Catalog number B36297) from Beckman Coulter S.r.l.;
Endothelial-MVs	CD144+/CD105+	Anti-CD144 (Clone: REA199; FITC-conjugated; Catalog number 130-100-742) from Miltenyi Biotec; Anti- CD105 (Clone; TEA3/17.1.1; PE-coniugated; Catalog number B92442) from Beckman Coulter S.r.l.

Supplementary Table 3. List of monoclonal according to MVs subtype

Cytokines (pg/mL)	HD (cases 20)	Total MF (cases 61)	*P*-value
IL-1β	0,2 (0-4,5)	1,5 (0,07-8,5)	<0.01
IL-6	5,4 (4,5-32,8)	24,8 (1,2-259)	<0.001
IFN-γ	0,2 (0,02-0,8)	1,3 (0-6,4)	<0.0001
TNF-α	0,4 (0-13,3)	6,8 (0,05-39,2)	<0.0001
TPO	22,2 (11,2-88)	124,4 (10,4-539,7)	<0.01

Supplementary Table 4. Plasma levels of crucial pro-inflammatory cytokines and thrombopoietin were increased in MF patients *Interleukin (IL)1β, IL6, Interferon (IFN)-γ, Tumor Necrosis Factor (TNF)-α, Thrombopoietin (TPO) plasma levels of HD (n=20), total MF patients (n=61) measured by ELISA. Results are expressed as median and range (Mann-Whitney test).*

Results IIb

Toward the identification of a microRNA-based signature of circulating microvesicles from Triple Negative and JAK2[(V617F)] mutated patients with Myelofibrosis

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INTRODUCTION

Myelofibrosis (MF) is characterized by clonal hemopoiesis, inflammatory microenvironment and mutations in *JAK2*, *MPL*, or *CALR* genes. Around 10% of patients (pts) do not carry the 3 mutations (Triple negative, TN) and show reduced survival (1). Microvesicles (MVs; 0.1 - 1 µm) are small vesicles deriving from the cell plasma membrane with a role in intercellular signalling (2). MVs can express antigens, and contain components (protein, mRNA, microRNA (miRs) from their cell of origin that determines their composition, characterization, and transfer of biologic information. Peripheral blood contains MVs deriving from megakaryocytes, platelets, leukocytes, red blood and endothelial cells. megakaryocytes- and platelets-MVs are the most abundant. Circulating MVs are increased in inflammation and cancer including MF. miRs contribute to MF pathogenesis (3). However, in MF the miRs profile of circulating MVs has never been investigated. Here we studied miR cargo of circulating MVs from JAK2$^{(V617F)}$ mutated and TN pts.

RESULTS

Card-based analysis demonstrated that many miRs were significantly upregulated in MVs from both JAK2$^{(V617F)}$ mutated and TN pts as compared with the HD counterparts (**Figure 1A, B**). Among them miR-21, known to be the most commonly upregulated miR in haematological tumours, is an anti-apoptotic factor with oncogenic potential and able to inhibit megakaryocyto-erithropoiesis (4,5). MiR-155, 222, 24 were upregulated in TN-MVs only. MiR-155 is upregulated in response to inflammation and, targeting p53 pathway, inhibits apoptosis. Its overexpression stimulates granulo-monocytopoiesis while impairing megakaryocytopoiesis (5). MiR-221/222, markers of poor prognosis in aggressive tumours and both increased in TN-MVs, show anti-apoptotic effect acting downstream of the oncogenic RAS-RAF-MEK pathway (5, 6). MiR-423-5p and miR-34a-5p, both pro-apoptic miRs (5, 8), show high expression in JAK2$^{(V617F)}$ and TN-MV, but are almost absent in HD counterparts. MiR-34a-5p is shown to be associated with MF (8), however, its expression is lower in TN-MV. Interestingly, many miRs regulating inflammation (miR-21, 146a, 223, 19a) and proliferation (miR-212-3p, -127-3p, -199a-3p, 21, 99b) were overexpressed in pts-MVs (5). Finally, miR-155, 146a, 19a and 194 overexpression affects the JAK-STAT pathway by blocking SOCS3, a negative regulator of the JAK-STAT signalling, and promoting cell survival. Comparing TN vs JAK2$^{(V617F)}$ MVs (**Figure 1C**), the expression of 6 miRs (miR-122, 27a, 744, 584c, 365, 483-5p) was increased, whereas 2 onco-suppressors miRs (let-7b and miR-361-5p) were less expressed. Of

note, 6 out of the above mentioned 8 miRs have SRSF1 gene as a common target which is both a protooncogene and a splicing regulator.
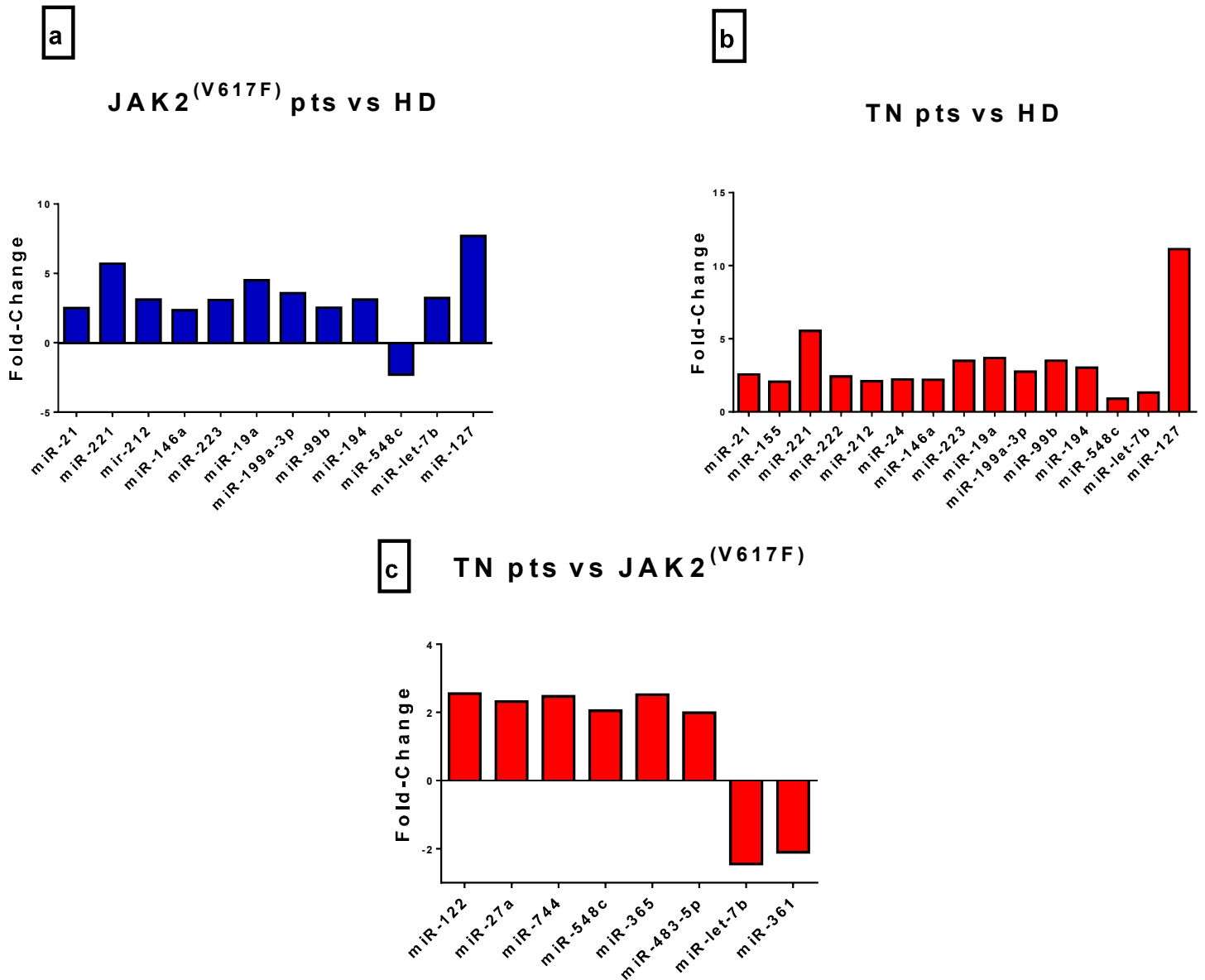

Figure 1. miRs profiling-Card analysis: *comparisons with fold change of significantly different miRs between A) JAK2 pts and HD; B) TN pts and HD; C) JAK2 and TN pts.*

Based on card analysis, we selected 21 miRs (-122-5p, -27a-3p, -365a-3p, -361-5p, let7b-5p, -744-5p, -548c-3p, -15b-5p, -221-3p, -202-3p, -212-3p, -409-3p, -24; -146a, 19a, -21, -155, -34a-5p, -127-3p, -222, -423-5p) to be validated with RT-PCR as potential circulating MV-associated signature. MiRs were selected according to cycle threshold (Ct<30) and amplification score (AmpScore >/= 1.24). Only miRs with significant fold changes ($\geq \pm 2$) between HD, JAK2$^{(V617F)}$ and TN-MVs were included in the study.

First, we investigated the difference between HD and total MF pts, and 4 miRs (-212-3p, -127-3p, -222, -34a-5p) were significantly increased in MF-MV (p<0.05 and p<0.001, respectively), as reported in **Figure 2**.
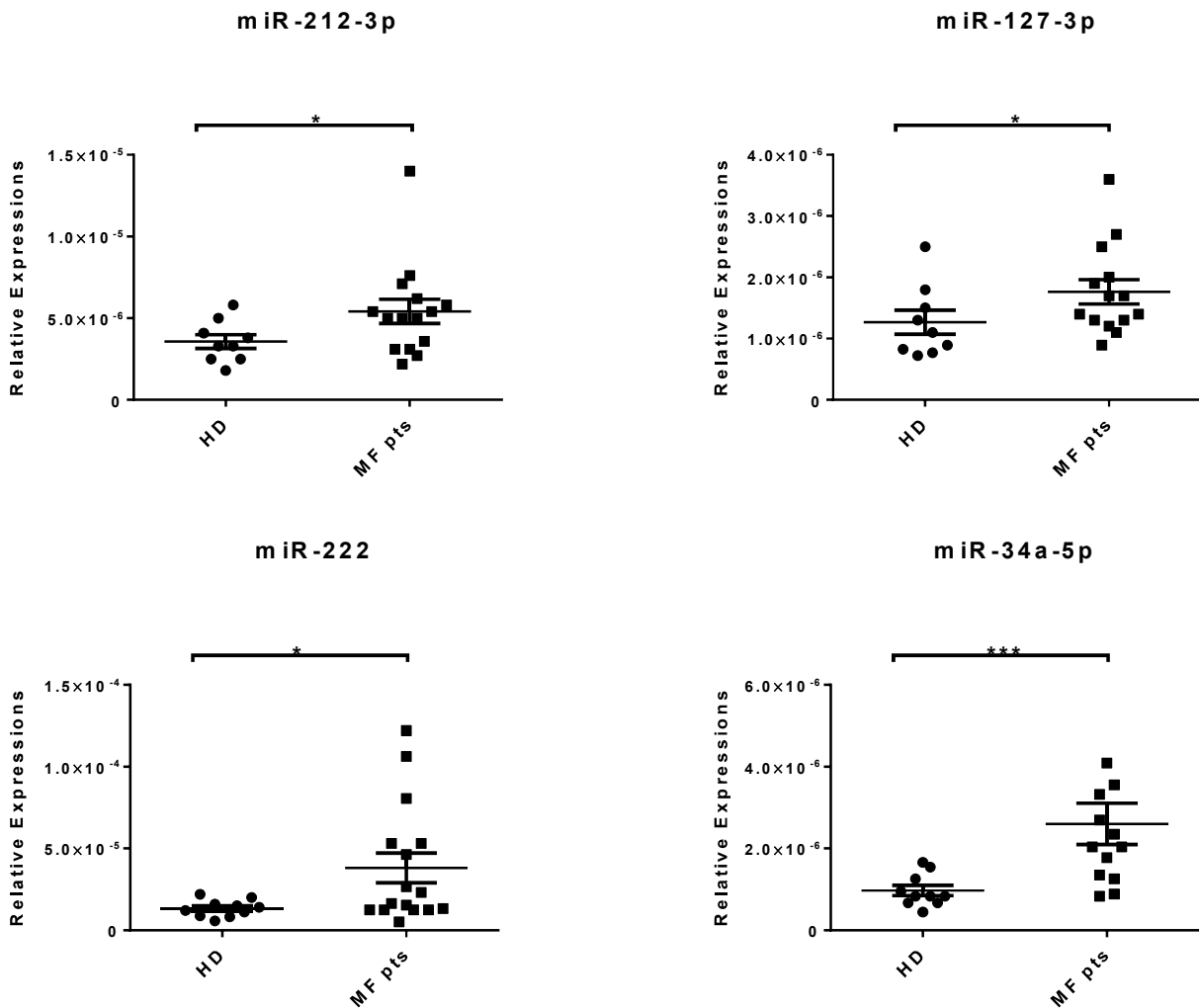

Figure 2. miRs profiling validation-RT-PCR analysis: comparison between MV from total MF patients and HD. The expression of miR-212-3p, -127-3p, -222 and -34a-5p were significantly increased in MF MVs. Data are reported as mean relative expression (normalized to cel-miR-39) ± SEM (* p<0.05, ***p<0.001).

Secondly, to identify whether miRs expression differs between JAK2$^{(V617F)}$/TN-MV and HD-MV, we analysed the miR cargo of MVs according to the mutational status. As reported in **Figure 3**, miRs -127-3p and -34a-5p resulted significantly upregulated in JAK2$^{(V617F)}$-MV as compared to HD (p<0.05 and p<0.01, respectively), while only miR-34a was overexpressed in TN-MV (p<0.05).
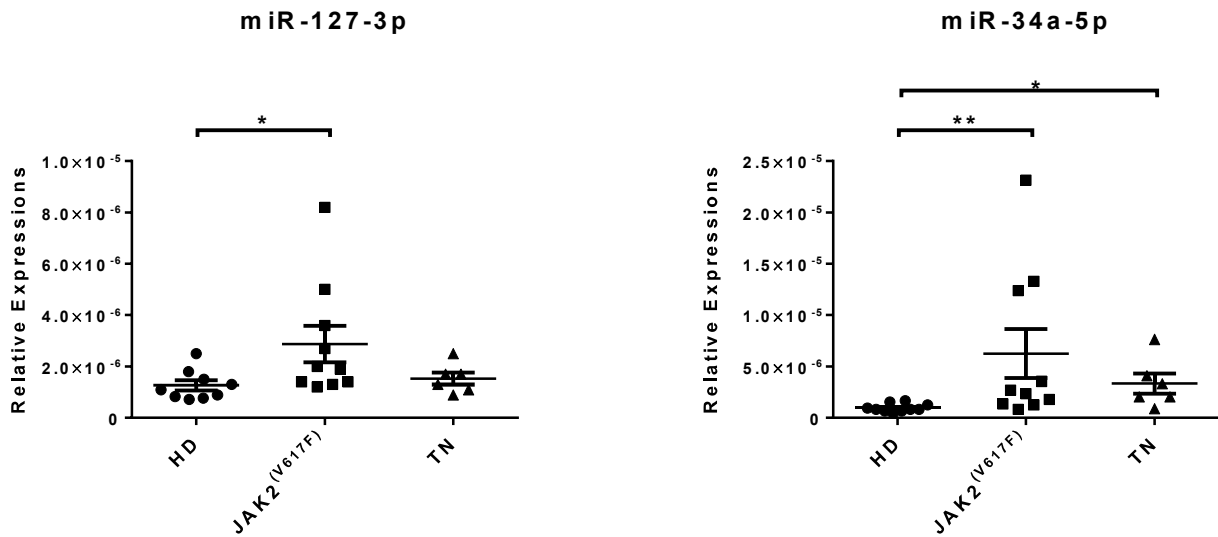
***Figure 3. miR profiling validations-RT-PCR analysis** comparison between TN/JAK2$^{(V617F)}$ and HD MV. miR-127-3p and miR-34a-5p were significantly increased in MVs from patients. Data are reported as mean relative expression, normalized to cel-miR-39 ± SEM (* p<0.05, **p<0.01).*

Finally, we compared the miR expression of JAK2$^{(V617F)}$ and TN-MV and only miR-361-5p was significantly increased in TN-MV as compared with the JAK2$^{(V617F)}$ counterparts (p<0.05; **Figure 4).**

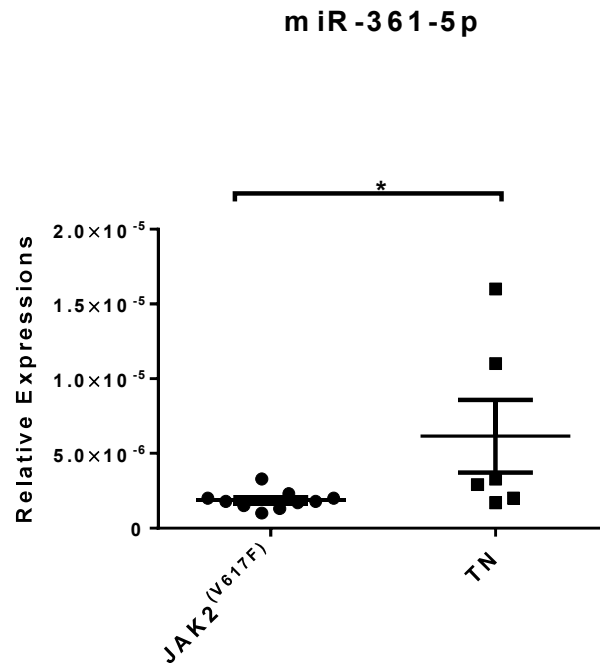**m iR -3 6 1 -5 p**

Figure 4. ***miRs profiling validation-RT-PCR analysis*** *comparison between TN and JAK2$^{(V617F)}$ MVs*. *MiR-361-5p expression was significantly increased in TN-MV as compared with the JAK2$^{(V617F)}$ counterparts. Data are reported as mean relative expression (normalized to cel-miR-39) ± SEM (* p<0.05).*

DISCUSSION

These results demonstrate that the miR profile of circulating MVs from MF patients is different from the normal counterparts and varies according to mutational status. Specifically, we found an upregulation of selected miRs, namely miR, -127-3p, -34a-5p -222, and -212-3p, in MF-MVs as compared to the normal counterpart. Analyzing patients according to the mutational status, only the MVs of JAK2$^{(V617F)}$ mutated patients show a significant upregulation of miR-127-3p compared to HD-MVs, while the miR-34a was overexpressed in both JAK2$^{(V617F)}$-/TN-MVs.

A previous study analysed differentially expressed genes and miRs in CD34+ cells from peripheral blood of patients with MF and found miR-127-3p to be upregulated. In particular, miR-127-3p was negatively associated with biological processes related to cell cycle and mitosis, suggesting that the high expression of miR-127-3p inhibits the proliferation of stem cells in peripheral blood (7). Therefore, miR-127-3p can function as a tumor suppressor and its high expression, also demonstrated in JAK2$^{(V617F)}$ -MVs, may influences the CD34+ cell cycle in peripheral blood, thus affecting the disease behaviour.

MiR-34a-5p is defined as tumor suppressor miR and its upregulation was found in the CD34+ hemopietic stem/progenitor cells (HPCs) of MF (8). Furthermore, they found that enforced expression of miR-34a-5p partially constrains proliferation and favours the megakaryocyte and monocyte/macrophage commitment of HPCs. The upregulation of miR-34a-5p in MF CD34+ cells and MF-MVs could be involved in the hyperplastic megakaryopoiesis and in some alterations of the bone marrow that are a hallmark of MF pathogenesis.

MiR-222 is associated with hematopoietic transformation of myeloid, erythroid and megakaryocytic progenitor cells during hematopoiesis (9). Mir-222 normally suppresses erythropoiesis by targeting c-KIT and is downregulated during the *in vitro* erythroid differentiation of CD34+ peripheral blood cells from healthy donors (10). Tombak A. et al., analyzing the miRs profile in the peripheral blood of patients with myeloproliferative neoplasms (MPN), reported that miR-222 expression was lower in Polycythemia vera (PV) patients and higher in Essential Thrombocythemia (ET) and MF patients as compared to control group (9). As suggested by Tombak A. et al, the detection of higher expressions of miR-222 in the peripheral blood from MF patients probably derives from an excessive cell proliferation. Interestingly, it has been hypothesized that, elevated mir-222 levels may contribute to anemia in MF by suppressing erythropoiesis. Therefore, this miR can be used as therapeutic targets for the treatment of anemia in MF (9).

Excluding the miR -127-3p, -34a-5p and -222, which have been previously described to be associated to MF, the other miRs (-212-3p and -361-5p), we found to be upregulated in MF-MVs, have never been described in MF.

MiR-212-3p promotes cell cycle progression, cell proliferation, migration, and invasion of non-small cell lung cancer and these effects are partially reversed by the miR-212-3p inhibitor or anti-miR-212-3p (11). These results suggest that miR-212 might have tumor-promoting properties. Potential targets of miR-212 is the tumor suppressor PTCH1, which may be responsible for the effect of miR-212-3p on cell proliferation (11). Furthermore, miR-212-3p was found to be downregulated in human Adult T-cell leukemia/lymphoma (ATL) cell lines as compared to normal T lymphocytes (12). Conversely, miR-212-3p restoration significantly inhibited ATL cell proliferation by repressing CCND3 expression (12). Therefore, the effect of miR-212 could be cell- or tumor- dependent and it would be interesting to study the effect of mir-212-3p in the context of MF and in particular, the effect of circulating MF-MVs, which show an upregulation of miR-212-3p, on different cell types. The upregulation of miR-212-3p in MF-MVs could be involved in hyperplastic hematopoiesis and in specific alterations of the immune system of MF.

Importantly, 4 miRs (-212-3p, -222, -127-3p and -34a-5p) overexpressed in MF-MVs have PABPC1 gene as a common target. This gene is involved in the processing and translation of mRNAs. The present study is still ongoing and validation of selected miR targets expression including PABPC1 is under investigation.

Interestingly, MiR-361-5p is significantly upregulated in TN-MVs as compared to JAK2-MVs. It was reported to suppress proliferation, migration and invasion of cancer cells in different tumors (13, 14, 15, 17). MiR-361-5p is downregulated in numerous human cancers, including cutaneous squamous cell carcinoma (14), gastric cancer (15), colorectal cancer (15) and prostate cancer (17). In these cancers, MiR-361-5p functions as a tumor-suppressor miRNA through directly binding to staphylococcal nuclease domain containing-1 (SND1), an endonuclease that regulates miRs involved in G1-to-S phase transition (13, 15, 16). For example, in colorectal cancer, miR-361-5p expression was negatively correlated with pulmonary metastasis and disease progression. Furthermore, the ectopic expression of miR-361-5p suppressed cell proliferation, migration and invasion by targeting SND1 (15). Liu et al showed that miR-361 inhibits prostate cancer growth and improves apoptosis (17). However, in cervical cancer, miR-361 has been reported to be upregulated, acting as an oncogene (18). The forced expression of miR-361 significantly increased the growth, migration and invasion of cervical cancer cells acting as a mediator of epithelial-mesenchymal transition (18). These conflicting results indicates that the expression and functions of miR-361-5p in tumors are tissue-specific.

Identification of miR-361-5p targets and functions would be important, especially to understand its role in circulating MVs and disease of TN patients.

Finally, the effects of miRs may be cell- or tumor- dependent, so it would be interesting to perform functional tests to investigate the effect of MF-MVs and of their upregulated miRs cargo, on different cell types, such as CD34+ cells and monocytes/T cells from HD and MF patients.

CONCLUSIONS

In conclusion, here we performed for the first time a characterization of miRs profile in circulating MF-MVs. Here we identified a miR signature of MF-MVs. Specifically, circulating TN-MVs show distinct miR signature as compared with the JAK2$^{(V617F)}$ mutated counterparts. This study has the potential to identify disease-related biomarker(s) and may provide a useful molecular target for MF diagnosis and treatment. We aim to increase our casistic in order to confirm these findings in MF-MVs, and to validate their role with functional studies.

MATERIALS and METHODS

Casistic

Peripheral blood was collected from MF pts (at diagnosis or out of cytotoxic treatment for at least 3 months) and age/sex-matched healthy donors (HD-n=10)). MF pts were *JAK2$^{(V617F)}$* mutated (n=10) and TN (n=6).

MVs isolation and count

After platelet poor plasma (PPP) collection, MVs were isolated from PPP by ultracentrifugation (45.000 rpm for 2 hours at 4°C) and quantified using the Nanosight technology.

MicroRNA expression assay

MiR expression of isolated MVs (10^9) was investigated after RNA extraction with the miRNeasy Mini Kit and by TaqMan™ Array Human MicroRNA A Cards (ThermoFisher), in 3 HD, 3 JAK2$^{(V617F)}$ and 3 TN pts. RT-qPCR validation assay was performed on 10 HD, 10 JAK2$^{(V617F)}$ and 6 TN, to discover significantly different expressed miRs. The validated miRs were selected as previously described.

Results III

Mutations in *JAK2* and *Calreticulin* genes are associated with specific alterations of the immune system in myelofibrosis

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(* or § equally contributed)

Supplementary materials: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5665081/bin/koni-06-10-1345402-s001.zip

ABSTRACT

Myelofibrosis (MF), a clonal neoplasia of the hemopoietic stem/progenitor cell, is associated with mutations in Janus Kinase 2 (*JAK2*) and calreticulin (*CALR*) genes. Chronic inflammation is the hallmark of MF. Infectious complications are the leading cause of morbidity and mortality. Based on this scenario, here we functionally evaluated key immune-cell subsets with the aim to investigate their putative role in immunosurveillance.

We found that MF patients (33 untreated patients - 20 *JAK2*$^{(V617F)}$ and 13 *CALR* mutated) were characterized by a reduced capacity of monocytes to differentiate into dendritic cells (DCs), reduced T-helper (Th) 17 plasticity and hypo-functional innate lymphoid cells (ILC). Furthermore, these patients showed reduced plasma levels of interleukin (IL)-4, -5 and Interferon-γ (IFN-γ) with concomitant increase of IL-1β, -6, -17, and Tumor Necrosis Factor-α (TNF-α). Additionally, we analysed the results according to the mutational status showing that patients carrying *JAK2*$^{(V617F)}$ mutation presented a reduction in Th17, myeloid-DCs and effector Tregs as well as an increase in ILC1. *CALR*-mutated patients revealed increased ILC3 levels, reduced Th1 and their monocytes had reduced capacity to mature *in vitro* into fully committed DCs. Their Tregs were also less effective in inhibiting the proliferation of autologous effector T-cells because of an increased proliferative status induced by *CALR* mutation.

Here, we have demonstrated that MF patients carry mutations that lead to phenotypic and functional alterations in key immune cell subsets. These alterations, contributing to the generation/maintenance of the inflammatory microenvironment, may have a potential role in susceptibility to infections and disease progression.

Background

Myelofibrosis (MF) refers to the Philadelphia chromosome-negative myeloproliferative neoplasms (MPNs) originating in the multipotent hematopoietic stem cells. It is clinically characterized by progressive anemia, splenomegaly, debilitating constitutional symptoms and by an increased risk to evolve in acute leukemia [1]. MF can develop *de novo* as primary MF (PMF) or secondary either from Polycythemia Vera (PPV-MF) or Essential Thrombocythaemia (PET-MF). Approximately 60% of MF patients carry a mutation in the Janus Kinase 2 (*JAK2*) [2] gene, and an additional 10% in the myeloproliferative leukemia protein gene (*MPL)*. Mutations in Calreticulin gene (*CALR*) have been reported in about 80% of *JAK2* and *MPL* unmutated patients [3]. Of note, regardless the driver mutations, the JAK-STAT signalling pathway is hyper-activated in all the MPNs [4].

Chronic inflammation, as result of aberrant cytokines production by mutated and unmutated cells, is considered the MF hallmark. In this scenario, infectious complications are the leading cause of morbidity and mortality constituting more than 10% of all patient deaths [5,6]. In order to understand whether the atypical infectious events are caused by deficits in the innate or adaptive immune response, a comprehensive analysis of key immune cells is required.

To date, it is well established that in PMF, the monocytes composition is different with a reduction in the classical (CD14^{bright}CD16$^-$) compartment [7]. Monocytes can differentiate, under inflammatory conditions, in dendritic cells (DCs); however, no data have been published so far about the ability of MF monocytes to differentiate into DCs. DCs are a heterogeneous group of professional antigen-presenting cells (APCs) including plasmacytoid (pDC), and myeloid (mDCs) DCs [8] . Thus far, no data have been reported on the frequency of circulating DC subsets in MF.

A recent report studied the T helper (Th)1, Th2 and Th17 compartments in MPNs patients under treatment. Of note, no differences between healthy donors and patients were found in Th cells polarization at baseline level [9]. Thymus derived regulatory T cells (Tregs) frequency has been already studied in MPNs, however conflicting results have been published [10–12].

MPNs, have reduced natural killer cell (NKs) compartment with impaired function [13,14]. NKs are part of the recently described family of innate lymphoid cells (ILCs), which play a role in autoimmunity, inflammation [15] and tumour immunosurveillance [16]. Beside conventional NKs, three distinct ILCs subsets have been described based on their transcriptional regulation and cytokine profiles mirroring those of Th cells [17]. We and others recently showed that acute myeloid leukaemia patients present an impaired ILC compartment [18,19] but no data are available in MF.

Based on this background and considering the essential role of the JAK/STAT pathways in shaping the immune response [20], we functionally evaluated key immune-cell subsets with the aim to investigate their putative role in immunosurveillance. We found that MF patients are characterized by a state of mutation-dependent immune alterations with key cellular components of the innate and adaptive immunity showing defective number and function.

RESULTS

Patients characteristics

33 MF patients were included in the study. Baseline features of the entire cohort are detailed in Table 1. Leukocytosis (leukocytes $\geq 25 \times 10^9$/L) was observed in 4 patients, while 6 patients had a low ($\leq 4 \times 10^9$/L) leukocyte count; lymphopenia (lymphocytes $\leq 10^9$/L) and monocytosis (monocytes $\geq 10^9$/L) were present in 14 and 9 patients, respectively. We studied 18 patients at the diagnosis while 15 patients received previous treatment for MF (Hydroxyurea/Ruxolitinib), as detailed in Table 1. In all cases, therapies had been discontinued for at least two months before sample collection. Only 2 patients presented an autoimmune clinical history.

Table 1. Clinical and laboratory features of MF patients according to mutational status

	Total MF (33 cases)	JAK2[(V617F)+] (20 cases)	CALR+ (13 cases)	P value
Median age, years (range)	70 (40-84)	67,5 (40-82)	73 (67-84)	**0.02**
Males, no. (%)	16 (48.4%)	9 (45%)	7 (54%)	0.72
Median Hemoglobin, g/dl; median (range)	11 (7.7-15.1)	12.6 (9.2-15.1)	9.3 (7.7-14)	**0.001**
Median Leukocytes, x 10^9/l; median (range)	7.3 (2.3-48.3)	9.3 (2.5-26.4)	6.4 (2.3-48.3)	0.6
Median Platelets, x 10^9/l; median (range)	216 (41-549)	248 (41-549)	175 (90-419)	0.26
Median Lymphocyte x 10^9/l; median (range)	1.5 (0.5-10.7)	1.45 (0.5-2.7)	1.6 (0.5-10.7)	0.16
Median Monocyte x 10^9/l; median (range)	0.6 (0.1-7.6)	0.58 (0.19-1.8)	0.6 (0.1-7.6)	0.23
BM fibrosis, no. of patients (%)				
Grade 1	11(33%)	11(55%)	0 (0%)	**0.0016**
Grade 2	15 (46%)	6 (30%)	9 (69%)	**0.03**
Grade 3	7 (21%)	3(15%)	4 (31%)	0.39
IPSS, Number of patients				
Low	3 (9%)	3 (15%)	0 (0%)	0.53
Intermediate-1	16 (49%)	11 (55%)	5 (38%)	0.48
Intermediate-2	4 (12%)	3 (15%)	1 (8%)	1
High	10 (30%)	3 (15%)	7 (54%)	**0.025**
Unfavorable Karyotype, no of patients (%)	3 (9%)	3 (15%)	0 (0%)	0.26
Previous treatment, no of patients (%)				
Hydroxyurea	11 (33%)	9 (45%)	2 (20%)	0.24
Ruxolitinib	4 (12%)	2 (10%)	2 (15%)	1
WHO Diagnosis				
PMF	22 (67%)	14 (70%)	8 (62%)	0.71
PPV-MF	4 (12%)	4 (20%)	0 (0%)	0.14
PET-MF	7 (21%)	2 (10%)	5 (38%)	0.08

PMF: Primary Myelofibrosis; PPV-MF: Post Polycythemia Vera-Myelofibrosis; PET-MF: Post Essential Thrombocythemia-Myelofibrosis. The presence of 0, 1, 2 or 3 and >4 adverse factors defines low, intermediate-1, intermediate-2 and high-risk disease. IPSS, International Prognostic Scoring System; unfavorable karyotype (presence of one or two abnormalities including þ8, 7/7q-, i(17q), inv(3), 5/5q-, 12p- or 11q23 rearrangement). P value between JAK2[(V617F)] and CALR mutated patients.

Dysregulated plasma levels of cytokines involved in differentiation/function of immune cells in MF patients

We firstly evaluated the plasma levels of cytokines involved in the differentiation and function of different immune cell types. In agreement with previous reports [23,24], we found reduced plasma levels of IL-4, -5 and IFN-γ with concomitant increased levels of IL-1β, -6, -10, -17, and TNF-α as compared to HD (Table S2; see Additional tables). Of note, IL-12 and -13 plasma levels from *CALR* mutated patients were not significantly different from the normal counterparts. No correlation between allele burden and cytokine plasma levels was observed, with the notable exception of TNF-α which highly correlated (R=0.63; p< 0.008) with *JAK2*$^{(V617F)}$ allele burden. Irrespective of mutational status, IL-12 and -13 plasma levels were negatively correlated with the IPSS score values (R=0.47; p<0.04; R=0.49; p<0.04, respectively). Conversely, we found a positive correlation between circulating IL-6 levels and splenomegaly/fibrosis (R=0.46; p=0.018 and R=0.49; p=0.003, respectively) (data not shown).

Reduced circulating mDCs in *JAK2*$^{(V617F)}$ mutated patients

Afterwards, we evaluated the number of circulating mDCs and pDCs in MF patients and controls (Figure 1A). As shown in Figure 1B, circulating mDCs were significantly reduced in MF compared to HD (7.8 ± 4.3 vs 12.7 ± 4 cells/μL, p ≤0.01). Interestingly, according to the mutational status, this reduction was significant only in *JAK2*$^{(V617F)}$ (12.7 ± 4 vs 6.2±2.7 cells/μL, p≤0.001), but not in *CALR* mutated patients. No significant differences were observed in the number of circulating pDCs (Figure 1C).
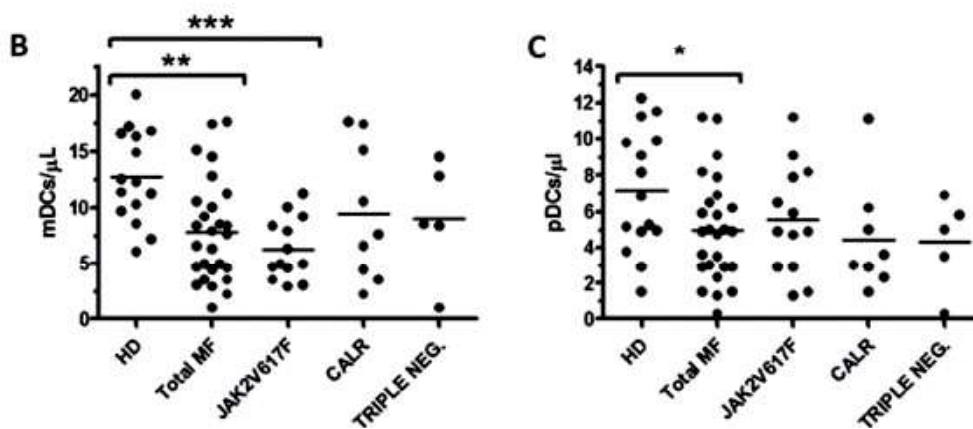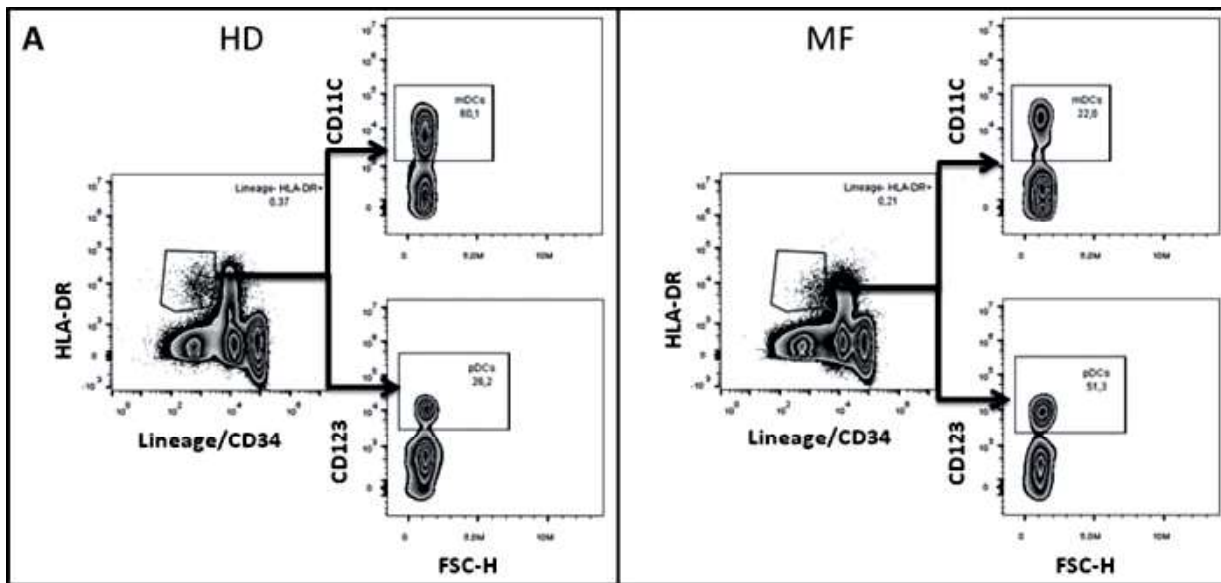
Figure 1. Reduced circulating mDCs in JAK2(V617F) mutated patients. *A) Representative example of the gating strategy used to determine, in HD (left panels) and MF patients (right panels), the percentages used to calculate the circulating level of mDCs (identified as Lin- HLA-DR+CD11c+ cells) and pDCs (identified as Lin-HLADR+ CD123+ cells). Circulating number of mDCs (B) and pDCs (C) in HD (n= 17), total MF (n D 27), JAK2(V617F) mutated (n=13), CALR mutated (n= 9) and triple negative (n=5) patients are shown; cell concentrations were calculated as follows: (percentage of positive cells) x (white blood cell count)/100. For all graphs one symbol represents one individual, and the height of the bar represents the mean (*p≤0.05, **p ≤ 0.01, ***p ≤0.001).*

Impaired DC differentiation capacity of monocytes from MF patients

Monocytes can differentiate into DCs *in vivo* mainly in infected or inflamed tissues, leading to the concept that monocytes are a precursor of inflammatory DCs. We thus studied the capacity of freshly isolated monocytes to differentiate into DCs *in vitro*. After 5 days culture, the phenotype of immature mo-DCs was evaluated by flow cytometry (Figure 2A). As shown in Figure 2A, monocytes from *CALR* but not those from $JAK2^{(V617F)}$ mutated patients were not able to differentiate into immature DCs, as indicated by the persistence of CD14 expression. In addition, irrespective of mutational status, immature mo-DCs failed to up-regulate CD1a ($85 \pm 4.6\%$ vs $69.7 \pm 19.6\%$, $p \leq 0.01$) and CD80 expression ($28.8 \pm 17.2\%$ vs $70.6 \pm 12.9\%$, $p \leq 0.01$) as compared with the normal counterparts. We then assessed the capacity of immature mo-DCs to mature in the presence of the previously described inflammatory cocktail. Surprisingly, immature mo-DCs were able to respond and mature as the healthy counterpart (Figure 2B). However, mature mo-DCs from *CALR* mutated patients continued to be defective in CD80 ($86.5 \pm 7.5\%$ vs $98.8 \pm 0.4\%$, $p \leq 0.01$) and CD40 expression ($71.1 \pm 7.6\%$ vs $93.7 \pm 3.7\%$, $p \leq 0.01$) as compared to the normal counterparts.

To investigate whether the impaired DCs phenotype was associated with altered function, we firstly assessed the ability of immature mo-DCs to prime allogeneic T-cell responses *in vitro*. Regardless of mutational status, patients' derived mo-DCs were unable to stimulate T cell proliferation to the same extent as the HD counterpart (Figure 2C). This data is supported by the defective CD25 up-regulation in T cells (Figure 2D). Migration towards the lymph node and the capacity to capture antigens are essential for DCs function. For this reason, we performed migration and endocytosis assays. No significant differences were found in the migratory capacity of MF-derived mature mo-DCs, both spontaneous or in the presence of CCL19, a chemokine essential for lymph node homing (Figure 2E). However, MF-derived immature mo-DCs were more efficient in capturing the antigen than the control counterparts (Figure 2F).

These results show an impaired MF-monocyte capacity to differentiate *in vitro* into mo-DCs associated with a defective priming ability.
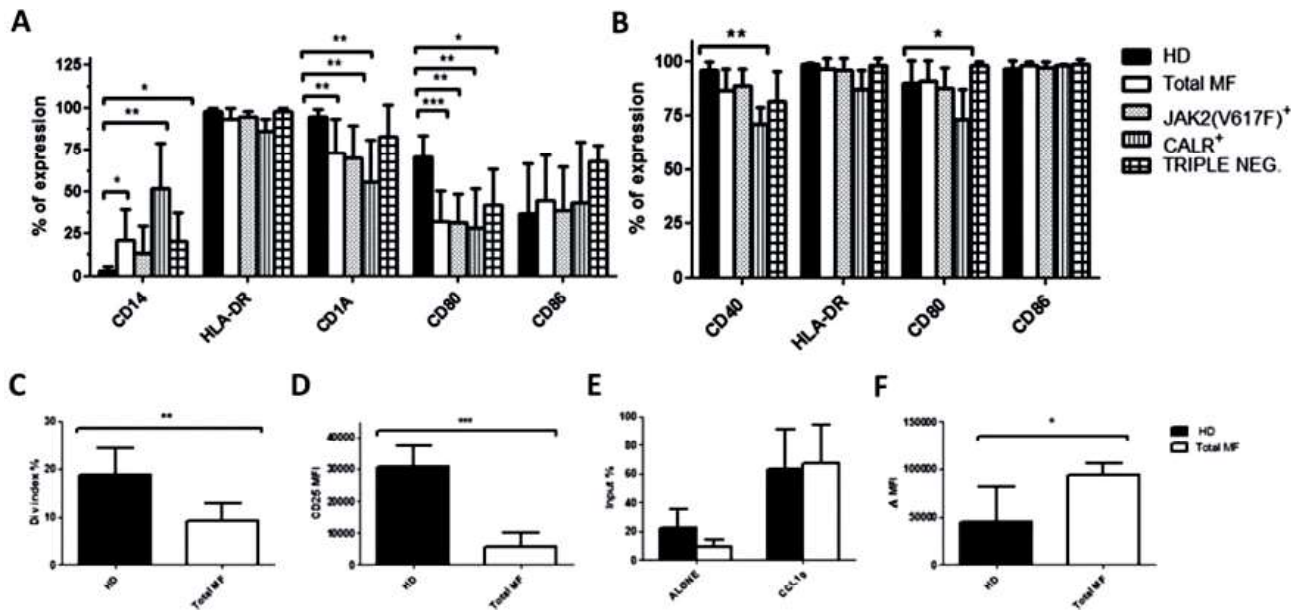
Figure 2. Impaired DCs differentiation capacity of monocytes from MF patients*. Immature (A) and mature (B) mo-DCs phenotype from HD (n=10) total MF (n=16), JAK2(V617F) mutated (n=7), CALR mutated (n=5) and triple negative (n=4) patients. The expression of HLA-DR, CD14, CD1a, CD40, CD80 and CD86 was evaluated by flow cytometry. Histograms represent the mean percentage of expression ± SD; C) ability of mo-DCs from HD (n= 8), total MF (n=8) to prime allogeneic T-cell responses in vitro. mo-DCs were cultured with allogeneic Tresp (mo-DCs/Tresp ratio 1:10) labeled with CFSE. The assays were performed over a period of 5 days and T cell proliferation was evaluated by division index. Histograms represent the mean § SD of the division index expressed as percentages; D) mo-DCs were cultured with allogeneic Tresp (mo-DCs/Tresp ratio 1:10) labeled with CFSE. The assays were performed over a period of 5 days and CD25 expression was evaluated by flow cytometry. Histograms represent the mean MFI ± SD of CD25 from HD (n=8) and total MF (n= 8) to prime allogeneic T-cell responses in vitro; E) evaluation of spontaneous and toward CCL19 (400 ng/mL) mature mo-DCs migratory capacity in HD (n= 6) and MF patients (n= 8). 1×10^5 cells were seeded in a transwell chamber (diameter 6.5 mm, pore size 8 mm) for 4 hours. The amount of migrated cells is expressed as a percentage of the input: (number of migrated cells in the lower compartment/loaded cells in the upper compartment) X 100. Histograms represent the mean ±SD of the input; F) Immature mo-DCs dextran uptake in HD (n=6) and MF patients (n=8). Cells were incubated for 30 min at 37°C or on ice (used as a background control). After washing, fluorescence was analyzed by flow cytometry. Uptake of FITC-dextran was expressed as delta (D) mean fluorescence intensity (MFI): MFI (uptake at 37°C) − MFI (uptake on ice). Histograms represent the mean ± SD of dextran uptake (*p≤0.05, **p ≤ 0.01, ***p ≤0.001).*

Reduced Th1 compartment in *CALR* mutated patients

Th cells play critical roles in the development and progression of infections, autoimmune diseases and tumours. Here, we firstly analysed the circulating quota of CD3$^+$CD4$^+$ cells and no significant differences between MF and HD were found (data not shown). These data were confirmed even when the percentages of total CD3, CD4 and CD8 positive cells have been evaluated in PBMCs (Figure 3A). However, when we evaluated the Th1 and Th2 balances [25], we found a reduction in the Th1 (24.2±3.6% vs 13.2±3.9%, p≤0.01) but not in the Th2 compartment (Figure 3B and C). Interestingly, only *CALR* mutated patients showed a significant reduction of Th1 cells (Figure 3B).
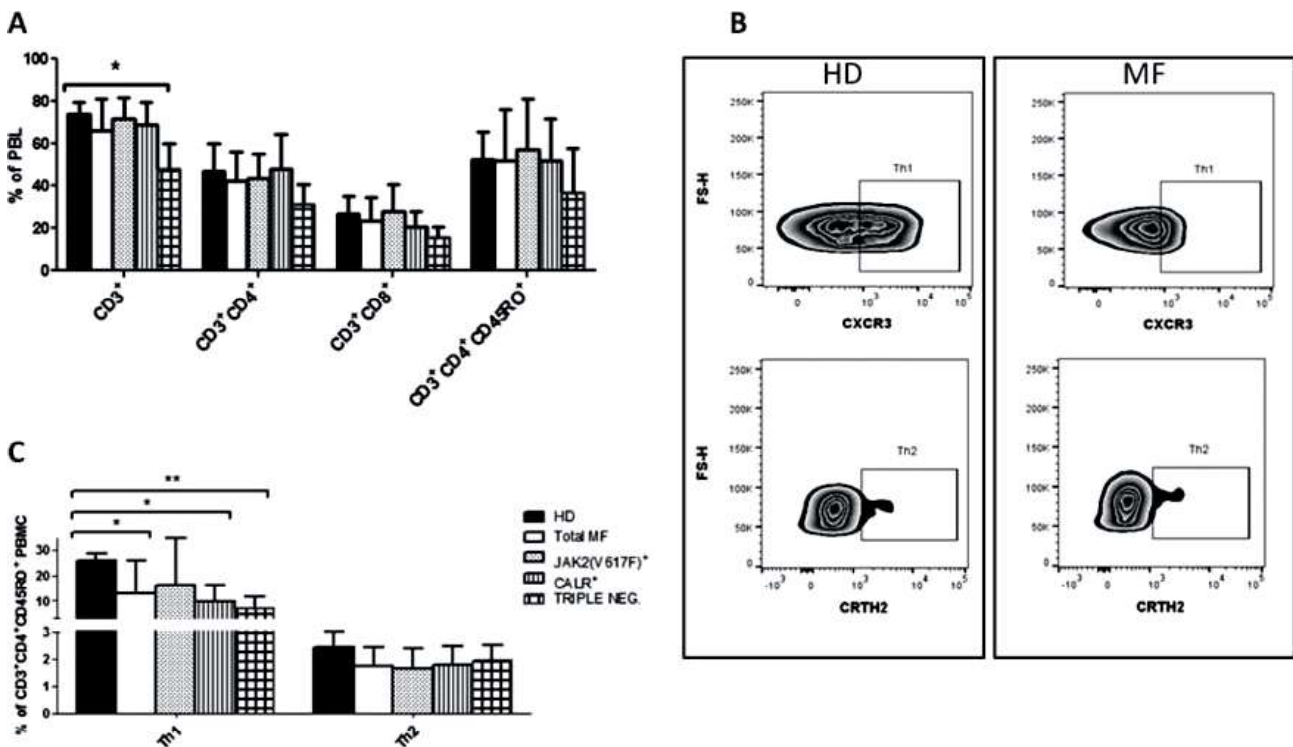

Figure 3. Reduced Th-1 compartment in CALR mutated patients *A) Percentages of CD3+, CD3+CD4+, CD3+CD8+, CD3+CD4+ CD45RO+ cells on PBMC from HD (n=14),total MF (n =16), JAK2(V617F) mutated (n=7), CALR mutated (n=5) and triple negative (n D 4) patients evaluated by flow cytometry; B) Representative example of the gating strategy used to determine by flow cytometry, in HD (left panels) and MF patients (right panels), the percentages used to identify Th-1 and Th-2 (identified as CD3+CD4+CD45RO+CXCR3+CRTH2-CCR6- and CD3+CD4+CD45RO+CXCR3-CRTH2+ cells,respectively); C) Percentages of Th1 and Th2 cells on the CD3+CD4+CD45RO+ population from HD (n=14), total MF (n=16), JAK2(V617F) mutated (n=7) and CALR mutated (n=5) and triple negative (n=4) patients. Histograms represent the mean percentages ± SD (*p≤0.05, **p ≤ 0.01, ***p ≤0.001).*

Reduced Th17 compartment in *JAK2*[(V617F)] mutated patients

Th17 can promote anticancer immunity; however, these cells exhibit also tumor-promoting properties. This dichotomy in cancer may be related to the versatile nature of these cells [26]. In MF the mean number of circulating Th17 was reduced as compared to HD (25.22 ± 17.8 vs 38 ± 16.8 cells/µL, $p\leq0.01$) (Figure 4A and B). However, when data were analysed according to the mutational status, only *JAK2*[(V617F)] mutated patients showed a statistically significant reduction (18.16 ± 9.3 vs 38 ± 16.8 cells/µL, $p<0.01$) (Figure 4B). Subsequently, we focused our attention on the intermediate Th populations recently described based on the expression of chemokine receptor and their capacity to secrete IL-17/IFN-γ and IL17/IL22, namely Th17/Th1 and Th17/Th22 respectively [27,28]. We found that MF patients showed a reduced percentage of circulating Th17/Th1 ($p\leq0.001$) and Th17/Th22 ($p\leq0.001$) cells (Figure 4C). These results suggest that in MF the reduced number of circulating Th17 is associated with a defective plasticity of this compartment.
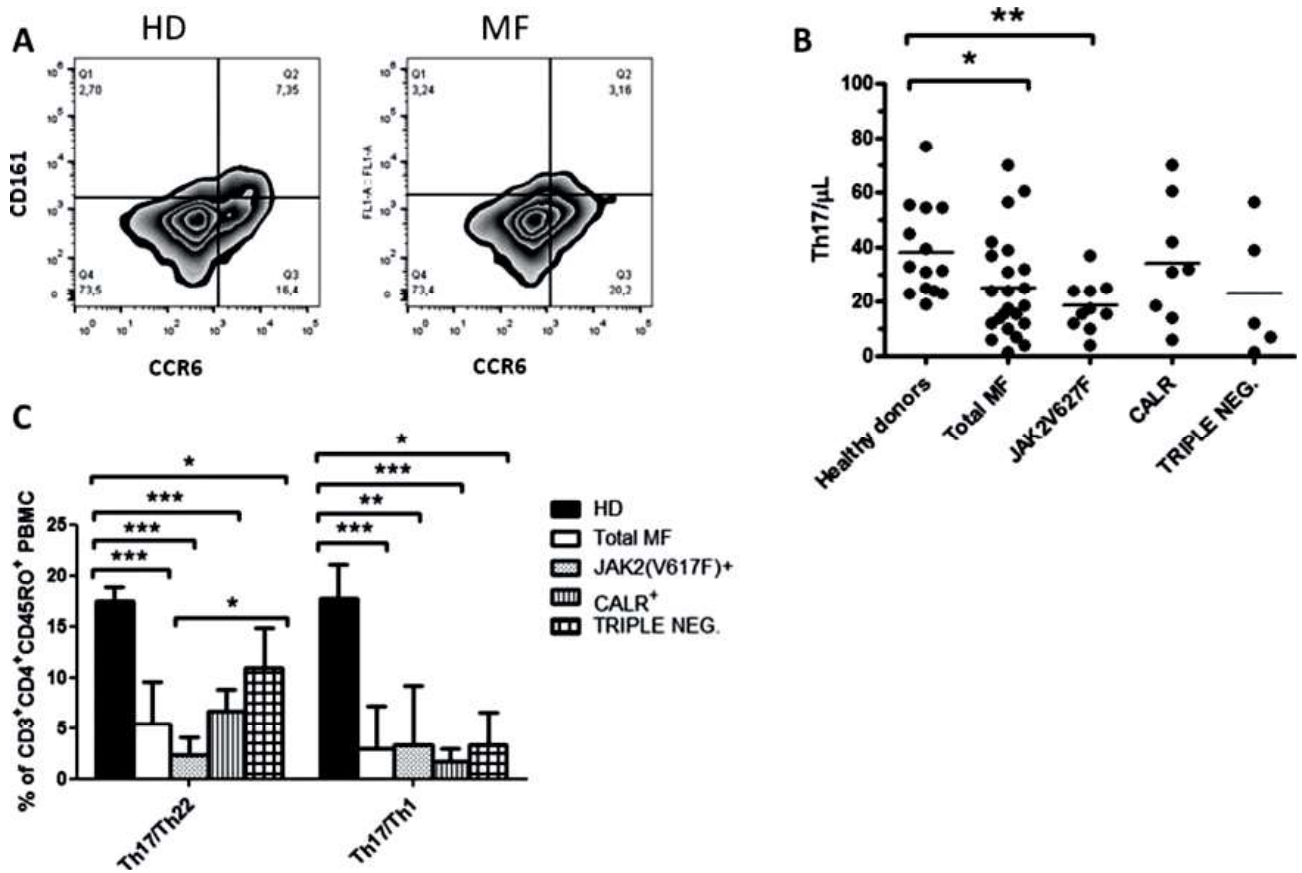
Figure 4. JAK2(V617F) mutated patients show a reduced Th17 compartment. *A) Representative example of the gating strategy used to determine by flow cytometry, in HD (left panels) and MF patients (right panels), the percentages used to calculate the circulating level of Th17 (identified as CD3+CD4+CCR6+CD161+ cells); B) PB circulating number of Th17 in HD (n=19), total MF (n =23), JAK2(V617F) mutated (n=10) CALR mutated (n=8) and triple negative (n=5) patients; cell concentrations were calculated as follows: (percentage of positive cells) x (Lymphocyte count)/100. Each symbol represents one individual and the height of the bar represents the mean; C) Percentages of Th17/Th1 and Th17/Th22 (identified as CD3+CD4+CD45RO+CXCR3+CRTH2-CCR6+ and CD3+CD4+CD45RO+CXCR3-CRTH2-CCR6+ cells, respectively) in HD (n=14), total MF (n=16), JAK2(V617F) mutated (n=7) CALR mutated (n=5) and triple negative (n=4) patients. Histograms represent mean percentage expression on the CD3+CD4+CD45RO+ population ±SD (*p≤0.05, **p ≤ 0.01, ***p ≤0.001).*

JAK2$^{(V617F)}$ mutated patients present an abnormal natural Treg heterogeneity in periphery

When we analysed the number of circulating Tregs we did not find any significant difference between patients and controls (Figure 5A). However, considering the three Treg sub-populations recently described by Miyara et al. [29] we found a reduction of the effector Tregs compartment identified as CD3$^+$CD4$^+$CD45RA$^-$CD25^{bright}CD127^{low} (Population II, p≤0.05) (Figure 5B and C). Nevertheless, the analysis according to the mutational status revealed that *JAK2$^{(V617F)}$* but not *CALR* mutated patients, showed this reduction (Figure 5C). In addition, we also found an inverse correlation between IL-12 plasma levels and the percentages of Population II Tregs (R=0.68; p<0.02).

To further understand the Treg role in MF we tested their suppressive ability *in vitro* and no significant differences were observed between patients and controls (data not shown). However, we found that Tregs from *CALR*, but not those from *JAK2$^{(V617F)}$* mutated patients, do not show inhibition of T cell proliferation as effectively as the normal counterparts (Figure 5D). In that regard, the effect of *CALR* mutation in T cell activation has been described [30]. Specifically, Tregs from *CALR* deficient mice are functional but effectors T cells are less sensitive to suppression by their ability to produce pro inflammatory cytokines like IL-2. In line with this hypothesis, we compared the proliferative ability of CD4$^+$CD25$^-$ T cells from *CALR$^+$* patients and HD. As predicted, CD4$^+$CD25$^-$ T cells from patients showed increased proliferation as compared to HD counterparts (p≤0.01) (Figure 5E). Interestingly, the CD4$^+$CD25$^-$ T cell population used in the assay carried the exon 9 *CALR* mutation (Figure 5F).

In conclusion, we show that *JAK2$^{(V617F)}$* mutated patients have an atypical composition in the Tregs compartment; moreover, the presence of CALR mutation in the effector T cells confer them a status of hyper-activation.
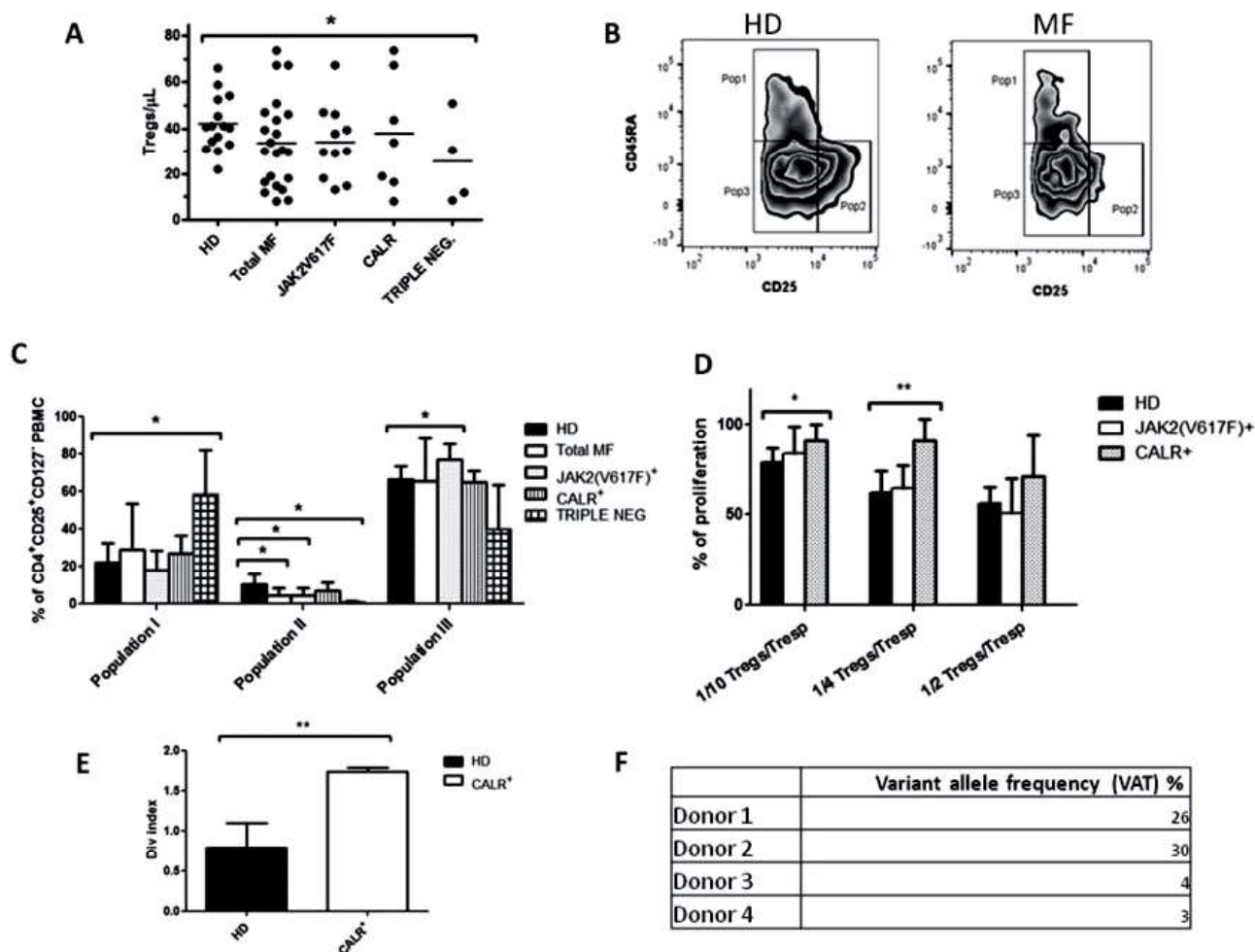
Figure 5. JAK2(V617F) mutated patients present a different Tregs heterogeneity in periphery. *A) PB circulating number of Tregs (identified as CD3+CD4+CD25+CD127low cells) in HD (n=17), total MF (n=22), JAK2(V617F) mutated (n=11), CALR mutated (n=7) and triple negative (n=4) patients; cell concentrations were calculated as follows: (percentage of positive cells) x (lymphocyte count)/100. Each symbol represents one individual and the height of the bar represents the mean; B) Representative example of the gating strategy used to determine by flow cytometry, in HD (left panels) and MF patients (right panels), the percentages of CD3+CD4+CD45RA+CD25+CD127low (population I), CD3+CD4+CD45RA-CD25brightCD127low (population II) and CD3+CD4+CD45RA-CD25+CD127- (population III) cells; C) Percentages of Population I, II and III in HD (n=14), total MF (n=16), JAK2(V617F) mutated (n=7) CALR mutated (n=5) and triple negative (n=4) patients.. Histograms represent mean percentage expression on the CD3+CD4+CD25+CD127- population ± SD; D) Co-colture (5 days) of autologous Treg from HD (n=9), JAK2(V617F) mutated (n=4) and CALR mutated (n=4) patients with autologous CD4+CD25- (Tresp) stimulated with anti-CD3 and anti-CD28 (5mg/mL) and labeled with CFSE. Percentage of proliferation was calculated as: (ratio between the" upper generation proliferation index" of Tresp cultured in the presence of increasing Treg ratios and the" upper generation proliferation index" of CTR culture, where no Treg were added)x100; histograms represent mean ± SD; E) Proliferation of CD4+CD25- from CALR mutated patients (n= 4) and HD (n= 4) stimulated with anti-CD3 and anti-CD28. Proliferation is calculated using the division index (average number of cell divisions that a cell in the original population has undergone); histograms represent mean ± SD; F) CALR exon 9 sequencing performed by Next Generation Sequencing to evaluate the variant allele frequency (VAT) expressed as percentages. Data indicate the percentages of mutated reads analyzed. (*p≤0.05, **p ≤ 0.01, ***p ≤0.001).*

Selected subsets of ILC with reduced functional capacity are expanded in MF patients

ILC function is tightly regulated by cytokines, and uncontrolled activation and proliferation can contribute to severe inflammation. Due to the aberrant cytokine compartment in MF, we evaluated ILC frequencies, phenotype and function.

The total ILC frequency was similar between MF patients and healthy controls (data not shown). We therefore analysed the relative frequency of the different ILC subsets as previously detailed (Figure 6A). We found that ILC1 and ILC3 NCR$^+$ fractions were significantly increased in MF patients as compared to the normal counterparts (ILC1: 38±4.4% vs 27.16±2.6%; ILC3 NCR$^+$: 23±3.58% vs 13.74±2.15%, p≤0.05) (Figure 6B). Of note, according to mutational status, ILC1 cells were significantly increased in *JAK2$^{(V617F)}$* patients (p≤0.05) while *CALR$^+$* patients showed an increased ILC3 NCR$^+$ compartment (p<0.05). Interestingly, total ILC3 percentages were significantly higher in patients with intermediate-2/high IPSS score compared to those with low/intermediate-1 IPSS score (p<0.05). Moreover, we found a positive correlation between the percentages of ILC3 NCR$^+$ and the circulating levels of IL-6 (R=0.51; p<0.04) (data not shown). Finally, we tested the functionality of ILCs by evaluating their cytokine producing capacity. Following short-term *ex vivo* activation*,* ILCs from MF patients showed dramatically impaired production of IFN-γ, IL-4, -5 and -13 (Figure 6C). In conclusion, we demonstrated that in MF the ILC compartment is functionally dysregulated.
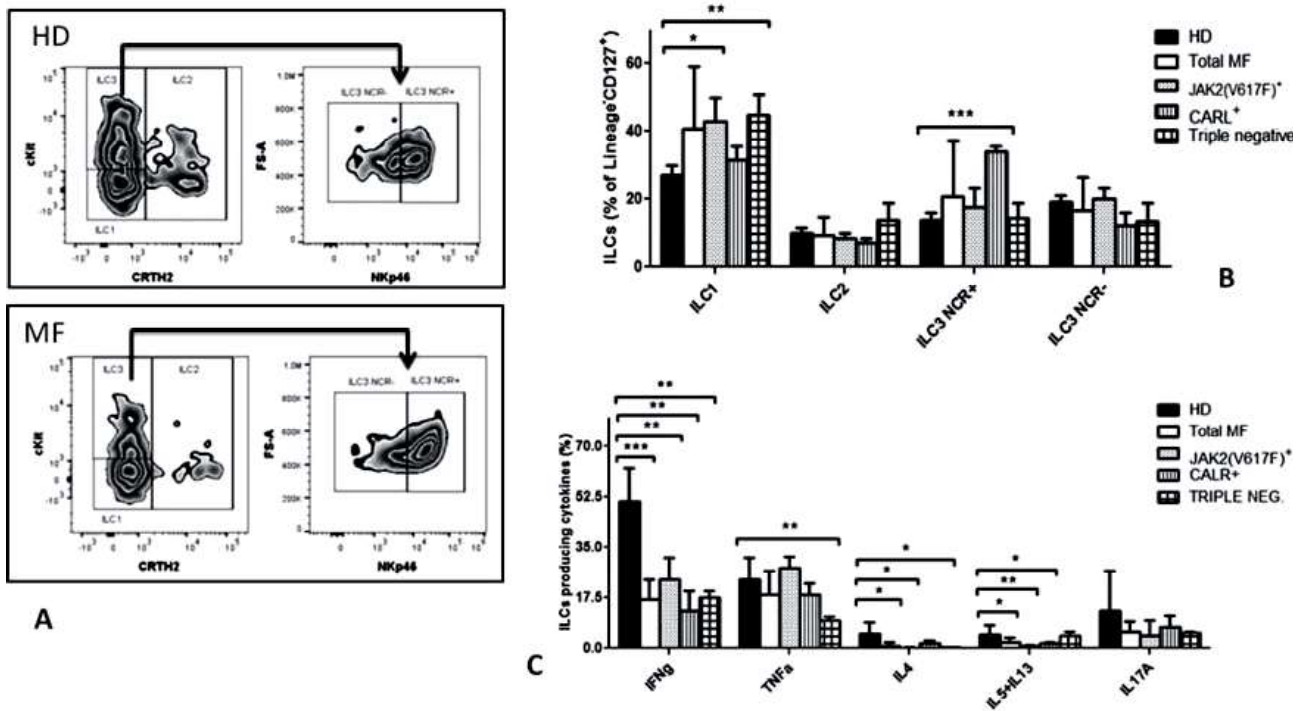
Figure 6. Selected subsets of ILCs with reduced functional capacity are expanded in MF patients. *A)*
Representative example of the gating strategy used to determine by flow cytometry, in HD (upper panels)
and MF patients (lower panels), the percentage of: ILC1 identified as Lin-CD127+CRTH2-cKit-CD56-
cells; ILC2 identified asLin¡CD127+ CRTH2+cKit+/- cells; ILC3 identified as Lin-CD127+CRTH2-
cKit+NKp46- cells that were further characterized by the expression of the natural cytotoxicity
receptor(NCR). B) Histograms represent the percentages ± SD of selected ILC subpopulation in HD (n=21),
total MF (n=23), JAK2(V617F) mutated (n=12) and CALR mutated (n=6) and triple negative (n=5)
patients. C) PBMCs were stimulated ex vivo for 3 hours, then an intracellular staining was performed.
Histograms represent the mean ± SD of the percentage of ILCs (Lin-CD127+cells) producing IFN-g, TNF-a,
IL4, IL5 plus IL13, IL17A in HD (n=21), total MF patients (n=21), JAK2(V617F)+ (n=12) CALR+ (n=6)
and triple negative patients (n=4). (*p≤0.05, **p ≤ 0.01, ***p ≤0.001).

DISCUSSION

MF is considered as "A Human Inflammation Model" [31] where the uncontrolled myeloproliferation and cytokine secretion creates a pro-inflammatory milieu influencing the immune system. Here we have demonstrated that several subsets of the adaptive and innate immune response show quantitative and/or qualitative abnormalities. Our data demonstrate how circulating mDCs, Th17, Th1, effectors Tregs, ILCs and cytokine plasma levels are dysregulated in our cohort of patients. Noteworthy, the presence of *CALR* or *JAK2*[(V617F)] mutation can affect this phenotype. Specifically, Th17, mDCs and Treg Population II reduction, associated with an increase in ILC1, was principally observed in the *JAK2*[(V617F)+] group. Patients carrying *CALR* mutation present a dysregulated IFN-γ axis. In particular, reduced circulating levels of INF-γ accompanied by Th1 reduction, hypofunctional ILC1 and mo-DCs. Notable, no association was found between allele burden and the number/phenotype/function of the studied cells. Furthermore, PET-MF and PV-MF did not show an immune pattern significantly different from PMF.

Along with a reduced amount of circulating mDCs, we found an impaired ability of MF-derived monocytes to differentiate *in vitro* toward DCs when cultured in the presence of IL-4 and GM-CSF. On monocytes, IL-4 receptor signals through JAK1/3 [32] while GM-CSF receptor through JAK2 and alternatively by IKK complex [33]. In MF, JAK2 constitutive activation confers to monocytes a high sensitivity to GM-CSF (commonly used *in vitro* for macrophages differentiation [34]) that results in a reduced capacity toward DCs differentiation and an increased macrophage-like phenotype. In line with these observations, 5 days cultured monocytes failed to fully down-regulate CD14 and presented a reduced expression of CD1a and CD80 compared to the normal counterpart. As a consequence, MF mo-DCs show a reduced capacity to stimulate T cell proliferation, and an enhanced endocytosis ability. In addition, mature mo-DCs from *CALR*[+] patients continued to be defective in CD80 and CD40 molecules providing an incomplete co-stimulatory signal essential for T cells activation and differentiation. These findings can explain, at least in part, the high infection rates seen in MF patients that are further exacerbated by ruxolitinib [35], a JAK1/2 inhibitor affecting DCs differentiation and function *in vitro* [36].

DCs have the unique capacity to direct T cell differentiation through the strength of TCR interaction and cytokines present in the microenvironment. In MF we found a Th1 reduction that was detectable in *CALR*[+] but not in *JAK2*[(V617F)+] patients. IL-12 is a key factor for Th1 differentiation; consistently, mature mo-DCs from *CALR* mutated patients show a reduced expression of CD40, a marker linked with DCs IL-12 production ability and Th1 differentiation in vivo [37,38].

The *JAK2*[(V617F)+] group is characterised by Th17 reduction with an impaired context-dependent plasticity since the percentage of Th17/Th1 and Th17/22 populations were reduced compared to controls. In addition, effector Tregs are reduced as well. This population show a potent suppressive function *in vivo*, and it dies quickly on activation. We can hypothesize that this deficiency may be the result of increased apoptosis or conversion in the context of chronic inflammation. In fact, we observed a negative correlation between effectors Tregs and IL-12 plasma levels, a cytokine increasing the outgrowth of non-Tregs in vivo [39]. Of note, Tregs from *CALR*[+] patients showed lower inhibition of autologous effector T cells proliferation than the normal counterpart. A more detailed analysis revealed that responder T cells, used in the assay, carried exon 9 *CALR* mutation conferring them an higher proliferative capacity *in vitro*. To our knowledge, a mutation in the T cell compartment in MF has been reported in MPN patients carrying *JAK2*[(V617F)+] [40–42] , with no data available on *CALR* mutated patients.

ILC rapidly respond to cytokines and microbial signals providing multiple pro-inflammatory and immuno-regulatory cytokines. Taking into account the aberrant cytokine production in MF we investigated the frequency of different ILC subsets. ILC1 and ILC3 NCR[+] were increased in *JAK2*[(V617F)] and *CALR* mutated patients, respectively. Nevertheless, irrespective of the mutational status, ILCs were hypofunctional. In *JAK2*[(V617F)+] patients, the increase of ILC1 can be explained considering the high IL-12 detected in circulation. This cytokine is essential for ILC1 differentiation and ILC2 conversion into ILC1 [43]. Equally, the ILC3 NCR[+] increase could be linked to the high circulating level of IL-1β and IL-23. Consistently, an ILC1 increase concomitantly with reduced functionality has been recently shown by us in patients with acute myeloid leukemia [18]. Noteworthy, total ILC3 percentages were significantly higher in patients with intermediate-2/high IPSS score indicating a possible role in Myelofibrosis progression. However, because of the current limited understanding in ILC biology, additional work needs to be performed to explain how the chronic inflammation status and the cytokine milieu influence this compartment.

<u>CONCLUSIONS</u>

Although this study is based on a limited number of patients, this limitation can be easily addressed in multicentric studies aiming to monitor MF patients before and under treatment. Of note, our data highlight the importance of investigating in larger cohorts of patients the role of the immune system in MF and other MPNs according to the mutational status.

Despite this limitation, this study gives an initial proof of concept that the immune landscape of MF varies among patients and that selected immune defects are principally associated with the presence of the *JAK2*$^{(V617F)}$ or *CALR* mutation. Overall, these abnormalities might contribute to the development of an immune defecting status with the potential to promote immune evasion, cancer progression and increased susceptibility to infections. In addition, a better understanding of the immune biology in the setting of MF would be important for designing novel therapies for MF.

MATERIALS and METHODS

Patients samples and cell isolation

EDTA-anticoagulated peripheral blood (PB) was obtained from 30 healthy age-matched volunteers and 33 patients with MF. Patients were at diagnosis (18 cases) or untreated for at least two months. The diagnosis of MF was made according to the WHO 2008 criteria (Table 1). No patients were previously treated with Interferon-α. This study was approved by the medical Ethical Committee of the University Hospital of Bologna and was conducted in accordance with the Declaration of Helsinki. Patients/controls provided written informed consent for the study. PB mononuclear cells (PBMCs) were separated by Lympholyte $1.077g/cm^3$ gradient (Cedarlane; CL5020) stratification. Subsequently, highly purified $CD4^+CD25^+$, $CD4^+CD25^-$ and $CD14^+$ cells were isolated using specific immunomagnetic cell isolation Kits (Miltenyi Biotech, 130-050-201) according to manufacturer's instructions.

Cell phenotype

The circulating immune cells were evaluated in PB from patients and controls by multiparametric flow cytometry. Th17, Tregs, mDCs and pDCs, were identified as listed in Table S1 (see Additional tables). A minimum of $1x10^5$ cells were acquired by flow cytometer BD Accuri C6 or FACSCanto (Becton Dickinson). Analysis was performed excluding cellular debris in a SSC/FSC dot plot. The percentage of positive cells was calculated subtracting the value of the appropriate isotype controls. Cell concentrations were calculated as follows: (percentage of positive cells) x (Lymphocyte count)/100 (Th17 and Tregs) or White Blood Cell counts/100 (mDCs and pDCs). PBMCs were used to assess the percentage of Th1, Th2, Th17/22, Th17/Th1, the three subpopulations of ILCs and Tregs. Gating strategy and antibodies used are listed in Table S1 (see Additional tables). The phenotype of circulating monocytes and monocyte-derived immature and mature DCs was also characterized (Table S1; see Additional tables). ILCs' cytokine production has been evaluated after PMA/Ionomycin stimulation by flow cytometry as described in Table S1 (see Additional tables).

Generation of monocyte-derived DCs

Monocyte-derived DCs (mo-DCs) were generated by a 5-day culture of CD14$^+$ cells in complete RPMI 1640 medium (Gibco-Invitrogen, BE12-167F) supplemented with 50 ng/mL Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) and 800 U/mL IL-4 (all from Endogen, 14-8339-62; 14-8049-80), at 37°C in 5% CO2, as previously described by us [21]. For maturation, day 5 mo-DCs were cultured for 48 hours in the presence of a pro-inflammatory cocktail: GM-CSF (50 ng/mL), IL4 (800 U/mL), IL6 (10 ng/mL; RIL6I), IL1β (10 ng/mL; RIL1BI), TNF-α (10 ng/mL; BMS301) and Prostaglandin (PGE)-2 (1 μg/mL; 14-8129-62) (all from Endogen).

Suppression assay

To assess the inhibitory capacity of freshly-isolated Tregs, we set-up a Mixed Leukocyte Reaction (MLR). Briefly, 10^5 CD4$^+$CD25$^-$ (Tresp) were labelled with Carboxyfluorescein succinimidyl ester (CFSE; Invitrogen; C34554), 5μM, according to the manufacturer's instructions. Tresp were co-cultured, for 5 days, alone or with autologous and irradiated (3000 cGy) Tregs at different Tresp/Treg ratios. MLR was set-up in 96-well plates pre-coated with anti-CD3 monoclonal antibody (mAb; clone UCHT1; BioLegend,; 317301) in presence of soluble anti-CD28 mAb (clone CD28; BioLegend; 302901). CFSE dilution has been exploited to assess cell division by flow cytometry (BD FACSCanto™). The capacity of Treg to modulate Tresp proliferation was analyzed using ModFit LT^{TM} 3.1 calculating the upper generation proliferation index.

Proliferation assay

Allogeneic purified CD4$^+$CD25$^-$ cells from healthy donors (HD) were labeled with CFSE and stimulated to proliferate by using immature mo-DCs (Tresp/DCs ratio 1:10) from HD and patients. The assays were carried out over a period of 5 days at 37°C and T cell proliferation was evaluated by flow-cytometry (BD FACSCanto™).

Endocytosis assay

Dextran uptake was measured by exposing $1x10^5$ immature mo-DCs to fluorescein isothiocyanate (FITC)-conjugated dextran (0.5 mg/mL; Sigma Aldrich; 74817). Cells were incubated for 30 minutes at 37°C or on ice (used as a background control). After washing, fluorescence was analyzed by flow cytometry (BD FACSCanto™). Uptake of FITC-dextran was

expressed as delta (Δ) mean fluorescence intensity (MFI): MFI (uptake at 37°C) − MFI (uptake on ice).

Migration assay

A total of 1×10^5 cells were seeded in a transwell chamber (diameter 6.5 mm, pore size 8 µm; Costar; Corning; CLS3464) in a 24-well plate and migration in response to CCL19 (400 ng/mL; Biolegend; 582104) was analyzed after 4 hours by Trypan Blue exclusion test. The amount of migrated cells was expressed as a percentage of the input: (number of migrated cells in the lower compartment/number of loaded cells in the upper compartment) x 100.

Plasma levels measurement of selected circulating cytokines

Selected cytokines plasma levels of patients/controls were measured by ELISA, according to the manufacturer's instructions. The IL-17 ELISA kit was provided by Boster Immunoleader (Boster Biological Technology Co.; EK0430). The Ciraplex™ immunoassay kit/Human 9-Plex Array (Aushon BioSystems, Cytokine 2 Array) was used for the measurement of various cytokines.

Mutation analysis

JAK2$^{(V617F)}$ allele-burden was assessed in granulocyte DNA with ipsogen *JAK2* MutaQuant Kit (Qiagen, Marseille, France) on 7900 HT Fast Real Time PCR System (Applied Biosystem, Monza, Italy). *CALR* exon 9 *s*equencing was performed by Next Generation Sequencing (NGS) approach with GS Junior (Roche-454 platform; Roche Diagnostics, Monza, Italy); analysis was carried out with AVA Software (GRCh38 as referenced). Rare *CALR* mutations identified by NGS were confirmed by Sanger sequencing. *MPL* mutations were investigated by ipsogen *MPLW515K/L* MutaScreen Kit (Qiagen) and by Sanger sequencing (for *MPLS505N* and other secondary exon 10 mutations).

Cytogenetic analysis

Chromosome banding analysis was performed on BM cells by standard banding techniques according to the International System for Human Cytogenetic Nomenclature. At least 20 metaphases were required. Unfavorable karyotype, defined according the Dynamic International

Prognostic Score System-plus (DIPSS) [22], included complex karyotype or single or two abnormalities including +8, -7/7q-, i(17q), -5%5q-, 12p-, inv(3) or 11q23 rearrangement.

Statistical analysis

Numerical variables have been summarized by their median and range, and categorical variables by count and relative frequency (%) of each category. All P values were considered significant when ≤ 0.05 (2-tailed). Statistical analyses were performed with Graphpad (Graphpad Software Inc., La Jolla, USA) using unpaired t test.

Results IV

Circulating Monocytes from Myelofibrosis show defective differentiation program and function. *in vivo/in vitro* JAK1/2 inhibition normalizes their phenotype and cell/microvesicles-linked inflammatory signaling

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Abstract

Myelofibrosis (MF) is a clonal disorder of the hemopoietic stem cell. Beyond mutations in 3 driver genes (*JAK2*, *CALR* and *MPL*), chronic inflammation is the hallmark of MF. Infections are one of the main causes of morbidity and mortality. Immune dysfunction, including T cells, natural killer and dendritic cells, which further aggravate after *JAK1/2* inhibition therapy, has been described. In this scenario, monocyte contribution to the inflammatory microenvironment needs to be clarified. To address the role of circulating monocytes within the inflammatory network of MF here we studied the *in vitro* and *ex vivo* functional behavior of monocytes from *JAK2V617F* mutated MF patients in the presence or absence of *in vitro/in vivo JAK1/2* inhibition.

We found that MF monocytes show defective differentiation program and activation capacity. Furthermore, their *in vitro* ability to produce and secrete free and microvesicles-linked inflammatory cytokines is severely impaired under lipopolysaccharides stimulation. This might be due, at least in part, to the inhibitory activity of MF isolated microvesicles on the inflammatory cytokines secretion of monocytes under lipopolysaccharides stimulation. Interestingly, *in vitro* and *in vivo* RUX therapy normalizes the monocyte chemokine expression and also their ability to produce intracellular and secrete microvesicles-bound inflammatory cytokines under lipopolysaccharides stimulation.

In conclusion, our data demonstrate that in MF circulating monocytes are functionally defective. Of note, upon infectious stimulus, JAK1/2 inhibition reactivate the monocyte-driven inflammatory cytokine signaling suggesting that the mutated pathway has an inhibitory role. These findings may have therapeutic implications because they contribute to better interpreting the off-target efficacy of JAK1/2 inhibition and to envisaging strategies aimed at facilitating antitumor immune response.

Introduction

Myelofibrosis (MF) is a clonal disorders of the hematopoietic stem/progenitor cell (HSPC) and may present as primary disease (PMF) or secondary to Essential Thrombocythemia (PET-MF) or Polycythemia Vera (PPV-MF). Typical clinical manifestations include debilitating systemic symptoms, progressive splenomegaly and transfusion-dependent cytopenia (1).

The molecular pathogenesis of MF relates on mutations in 3 "driver" genes (namely: *JAK2, CALR, MPL*) and more than 50% of patients carry a somatic mutation of JAK2[V617F] in hematopoietic cells. However, independently by the mutation status, a hyper-activation of the JAK-STAT pathway, that transduces most hematopoietic and inflammatory cytokines, is observed (2).

Beyond "driver" mutations, chronic inflammation with abnormal release of pro-inflammatory cytokines by activated leukocytes and megakaryocytes/platelets is considered the MF hallmark and it has been indicated as main contributor in MF initiation/clonal evolution. An abnormal expression and activity of several pro-inflammatory cytokines are associated with MF phenotype and prognosis (3-7). More recently, it has been demonstrated that cytokine overproduction in MF myeloid cells is driven by multiple signaling pathways (NF-KB and MAPK) beyond JAK-STAT (8). Consistently, NF-KB signaling is hyperactivated in MF and contributes to myeloproliferation and inflammation (8, 9).

Infections are one of the main causes of morbidity and mortality in MF, representing the cause of death in around 10% of the cases (10-13). The increased risk of infections is thought to arise from deregulation of key mediators of the immune system (14-21). To further aggravate the clinical landscape, prior studies have demonstrated significant inhibitory effects within the T cells, natural killer cells and dendritic cells after exposure to JAK inhibitors (22-27). Of note, due to the involvement of multiple signaling pathways beyond JAK/STAT, MF cytokine overproduction is reduced but not abrogated by JAK1/2 inhibition with Ruxolitinib (RUX) (8).

Despite a key role in regulating inflammation and immune response, the role of monocytes in the pathogenesis of MF still needs to be fully addressed. Moreover, the impact of JAK1/2 inhibition on monocyte behavior has never been investigated. Monocytosis may occur in patients with MF (around 15%) and is associated with poor outcome (28). Additionally, there is evidence that monocytes are over-activated (29, 30), show inflammatory features (31) and represents the principal cellular source for most inflammatory cytokines (8).

Extracellular vesicles (EVs), which are composed of microvesicles (MVs; 200-1000 nm) and exosomes (30-150 nm), are released from a broad variety of cells during homeostasis and cell

activation with pleiotropic effects on signalling among cells. MVs affect normal and malignant hemopoiesis (32) and are critical players in the regulation of inflammation/immunity (33, 34). Prior study demonstrated that exosomes associated-cytokines, either surface-bound or encapsulated, can be detected in body fluids including plasma (35). High serum levels of MVs have been detected in haematological malignancies including MPN (36, 37) and we recently described that circulating monocyte-derived MVs are increased in MF (38).

However, their role in the immune microenvironment of MF, especially on the function of specific subsets of immune cells, such as monocytes, has not yet been defined.

To increase knowledge in the pathogenesis of the inflammatory/immune microenvironment of MF and to address the role of monocytes within this inflammatory network and in recurrent infections in MF, here we studied the in vitro and ex vivo functional behavior of monocytes from JAK2^{V617F} mutated MF patients and their ability to respond to an infectious stimulus, in the presence or absence of JAK1/2 inhibition. We also evaluated whether circulating MVs from patients may influence the immune response to infections of monocytes.

Results

Circulating monocyte subsets of MF are dysregulated and their profile is not affected by in vivo RUX therapy

Based on the expression of CD14/CD16, monocytes can be classified into Classical-CD14++/CD16-, Intermediate-CD14+/CD16+ and Non-Classical-CD14-/CD16++ monocytes fractions. There is a gradual acquisition of the surface marker CD16 during maturation in intermediate monocytes, playing an inflammatory role, and Non-Classical monocytes, having an anti-inflammatory activity (39, 40).

We therefore analyzed in MF the ex vivo profile of the 3 different subsets before and after RUX therapy. As shown in **Figure 1**, at baseline only the mean percentages of Intermediate/Non-Classical monocytes (**B and C**) were significantly increased/reduced as compared with the normal counterparts (p<0.05, respectively). In vivo RUX therapy did not significantly modify the monocyte subsets profile.
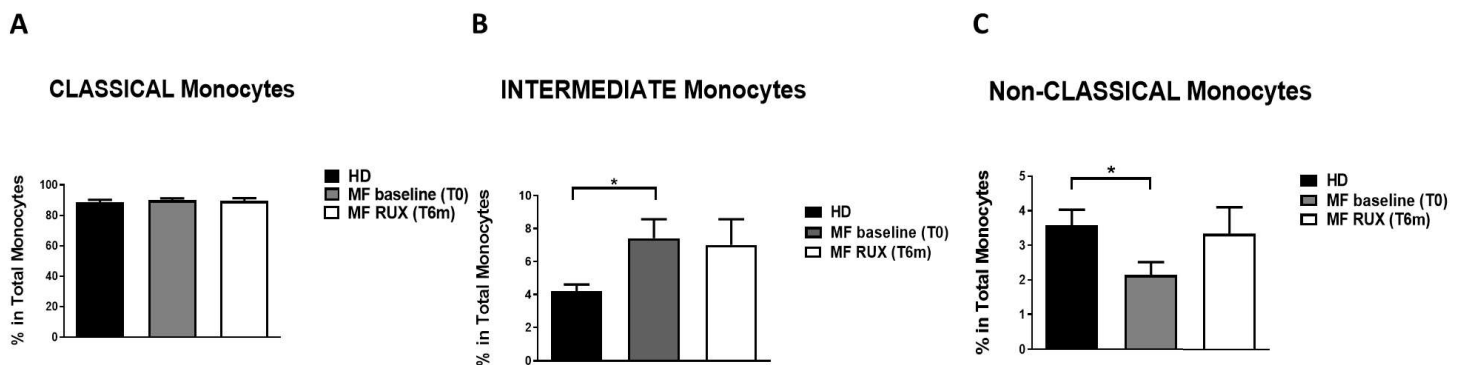

Figure 1. Frequency of circulating monocyte subsets (Classical, Intermediate and Non-Classical, according to CD14/CD16 expression) in PBMCs of HD (n=30) and MF pts at baseline (MF baseline T0; n=30) and after 6 months of in vivo RUX therapy (MF RUX (T6m); n=20). Histograms represent the mean percentage of monocyte subsets in total CD14+ monocytes ± S.E.M. (*p < 0.05).

Chemokine receptors expression of MF monocytes is dysregulated and in vivo RUX therapy normalizes the chemokine receptor repertoire

Chemokine-driven migration toward inflammatory/damaged tissues is a crucial monocyte-related feature. We thus studied the expression of crucial chemokine receptors on total circulating monocytes and subsets from MF patients and HD.

CCR2, which play a role in the mobilization of monocytes from bone marrow to peripheral blood, is primarily expressed by Classical monocytes. During differentiation, CCR2 is progressively downregulated in the Intermediate and Non-Classical monocytes. Conversely CX3CR, which in turn plays a role in the migration of circulating monocytes from peripheral blood toward inflamed/damaged tissues, is upregulated in Intermediate and Non-Classical monocytes. CCR5, which is also involved in the migration to inflamed tissues, is expressed by all monocyte fractions.

In MF patients, at baseline, only CCR2 (percentage of positive cells, **Figure 2A**; MIF, **Supplementary Figure 1A**) and CCR5 expression (MIF, **Supplementary Figure 1C**) of total monocytes was significantly increased/decreased as compared with the normal counterparts ($p < 0.05$, respectively). Analyzing the monocytes subsets, CCR2 expression was significantly higher in intermediate and Non-Classical monocytes as compared with the normal counterparts (percentage of positive cells, $p < 0.01$ and $p < 0.001$, **Figure 2A**; MIF, **Supplementary Figure 1A,** $p < 0.05$, respectively). Conversely, CX3CR1 expression of Intermediate and Non-Classical monocytes was significantly reduced (percentage of positive cells, **Figure 2B,** $p < 0.01$, respectively; MIF, **Supplementary Figure 1B** $p < 0.05$ and $p < 0.01$, respectively,). CCR5 expression was always significantly reduced as compared with the normal counterparts (MIF, **Supplementary Figure 1C,** $p < 0.05$).

Interestingly, *in vivo* Ruxolitinib therapy normalizes the CX3XR1 expression of Intermediate and Non-Classical monocytes (percentage of positive cells, **Figure 2B,** $p < 0.05$, respectively; MIF, **Supplementary Figure 1B**, $p < 0.05$, respectively) and CCR5 expression of all monocytes subsets ($p < 0.05$, respectively). Of note, there was the normalization of CCR2 expression also in Intermediate and Non-Classical monocytes (percentage of positive cells, **Figure 2A,** $p < 0.05$ and $p < 0.01$).

Collectively, these data demonstrated that in MF circulating monocytes show a dysregulated activation/differentiation program. Interestingly, *in vivo* RUX therapy normalizes the expression of crucial chemokine receptors.
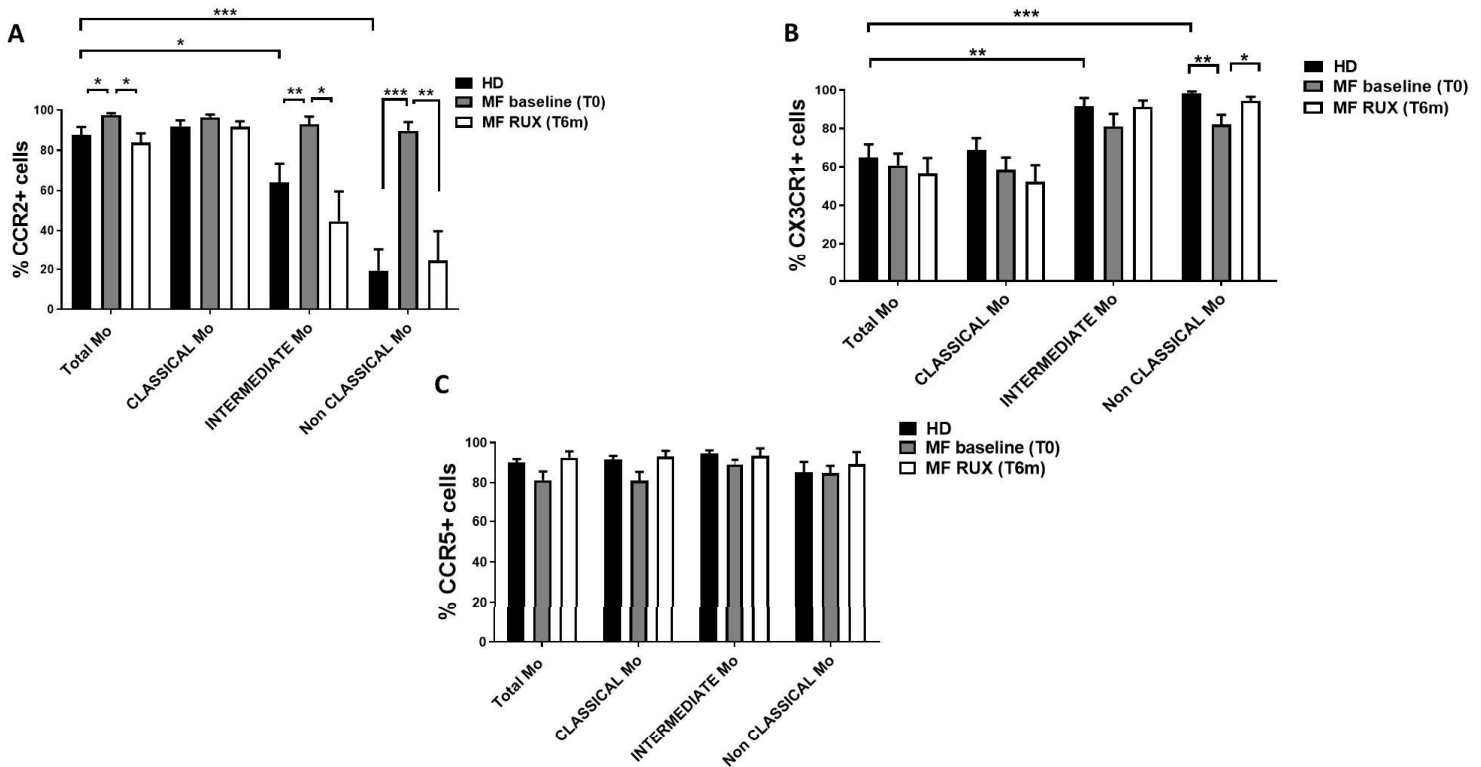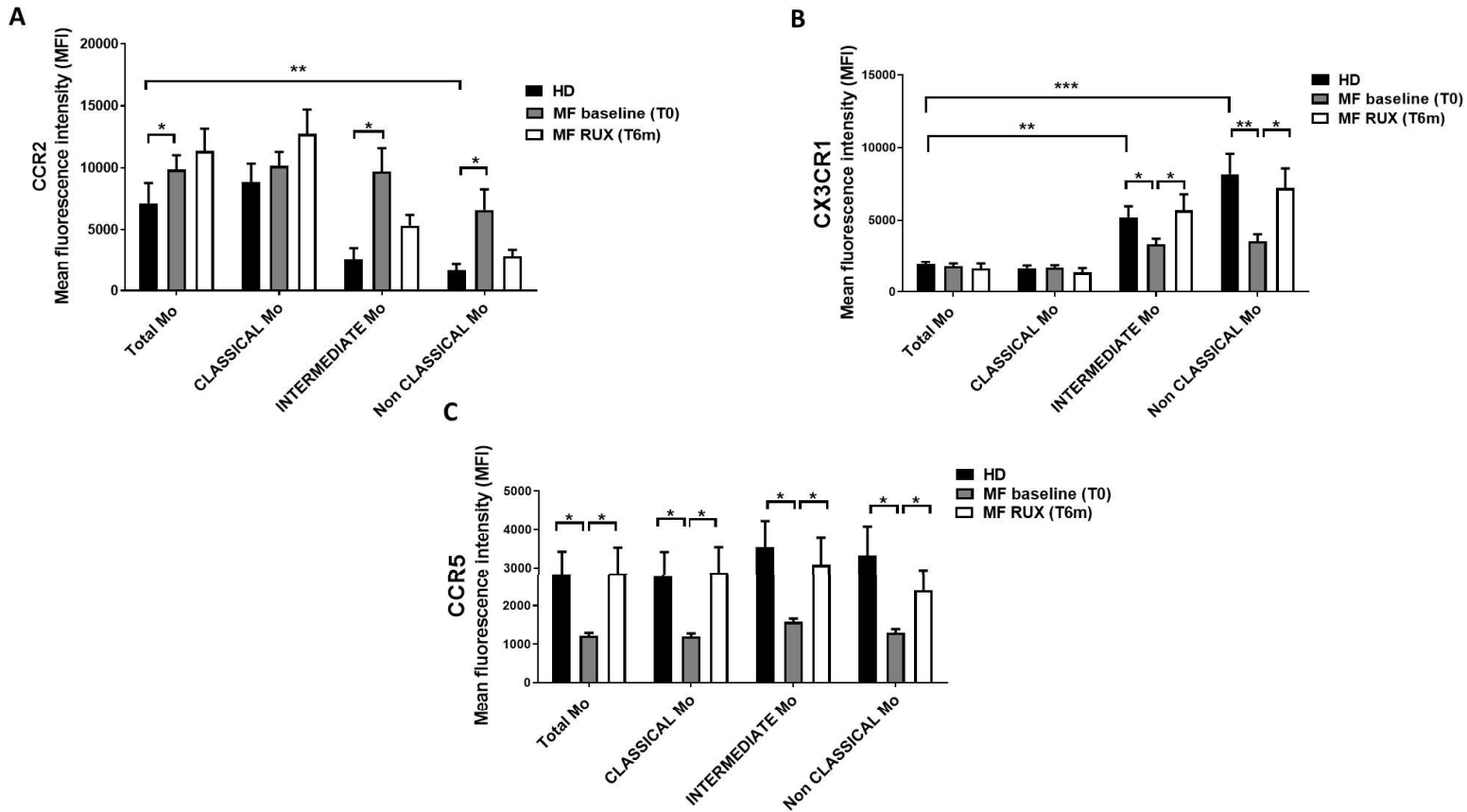

Figure 2. *Crucial chemokine receptors (CCR2, CX3CR1, CCR5) expression of total monocytes and of the three subsets (Classical, Intermediate and Non-Classical) from PBMCs of HD (n=30) and MF pts were analysed at baseline (MF baseline T0; n=30) and after 6 months of in vivo RUX therapy (MF RUX (T6m); n=20). Histograms represent the mean percentage of chemokine receptors positive monocytes ± S.E.M. (*p < 0.05; **p<0.01; ***p<0.001; Mo=monocytes).*

Supplementary Figure1. *Crucial chemokine receptors (CCR2, CX3CR1, CCR5) expression (MFI) of total CD14+ monocytes and of the three subsets (Classical, Intermediate and Non-Classical) from PBMCs of HD (n=30) and MF patients were analyzed at baseline (MF baseline T0; n=30) and after 6 months of in vivo RUX therapy (MF RUX (T6m); n=20). Histograms represent the mean MIF of CCR2, CX3CR1, CCR5 positive cells ± S.E.M. (*p < 0.05; **p<0.01, ***p<0.001).*

MF monocytes subsets show a dysregulated inflammatory cytokine receptors expression and in vivo RUX therapy partially modifies the cytokine receptors repertoire

To evaluate how MF circulating monocytes sense the inflammatory microenvironment, we tested the expression of crucial pro/anti-inflammatory cytokine receptors on monocytes from pts/HD.

Figure 3 shows a decreased percentage of TNF-R1 positive (**A**; $p<0.01$) and an increased percentage of TNF-R2 and IL10-R positive total MF monocytes (**B, C**: $p<0.01$, respectively) as compared with the normal counterparts. Comparing subsets, the percentages of TNF-R1 positive cells from MF pts were significantly reduced in all subsets (Classical and Non-Classical monocytes, $p<0.01$ and Intermediate monocytes, $p<0.001$). No differences were observed for TNF-R2 expression. Conversely, the percentages of IL-10R positive cells were significantly increased in all subsets ($p<0.01$ (Classical monocytes), $p<0.05$ (Intermediate and Non-Classical monocytes)). *In vivo* RUX therapy normalized the percentages of IL10R positive cells in intermediate and non-classical monocytes only.

Of note, the percentages of IL-1βR and IL-6R positive total monocytes and monocyte subsets of MF pts were not significantly different from the normal counterparts (data not shown). However, as shown in **Supplementary Figure 2**, total monocytes of MF pts showed significant increased IL-1βR and IL-6R expression (MIF; $p<0.05$) as compared with the normal counterparts. When monocytes subsets were analyzed, the expression of IL1β-R (MIF; Intermediate and Non-Classical monocytes; $p<0.05$ and $p<0.001$) and IL6-R (MIF; Classical and Non-Classical monocytes; $p<0.05$, respectively) were significantly increased as compared with the normal counterparts and *in vivo* RUX therapy does not affect their expression.

These results demonstrate that JAK2[V617F] mutation can alter the expression of inflammatory cytokine receptors and that MF monocytes may abnormally sense the pro/anti-inflammatory microenvironment. *in vivo* RUX therapy partially normalize their cytokine receptors profile.
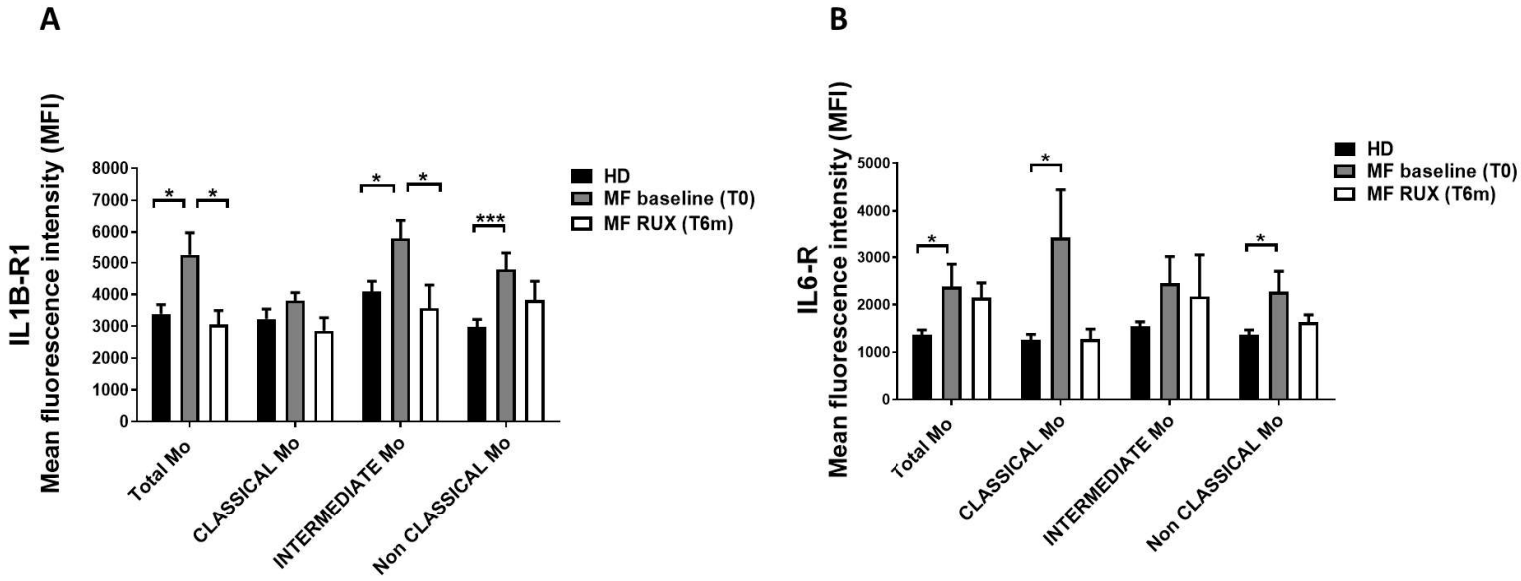
Supplementary Figure 2. *Crucial pro-inflammatory cytokines receptors (IL-1β-R1, IL-6-R1) expression (MFI) of total monocytes (identified by CD14 expression) and of the three subsets (Classical, Intermediate and Non-Classical) from PBMCs of HD (n=30) and MF patients at baseline (MF baseline T0; n=30) and after 6 months of in vivo RUX therapy (MF RUX (T6m); n=20). Data are shown as mean MIF of IL-1β-R1, and IL-6-R ± S.E.M. (*p < 0.05; ***p<0.001).*
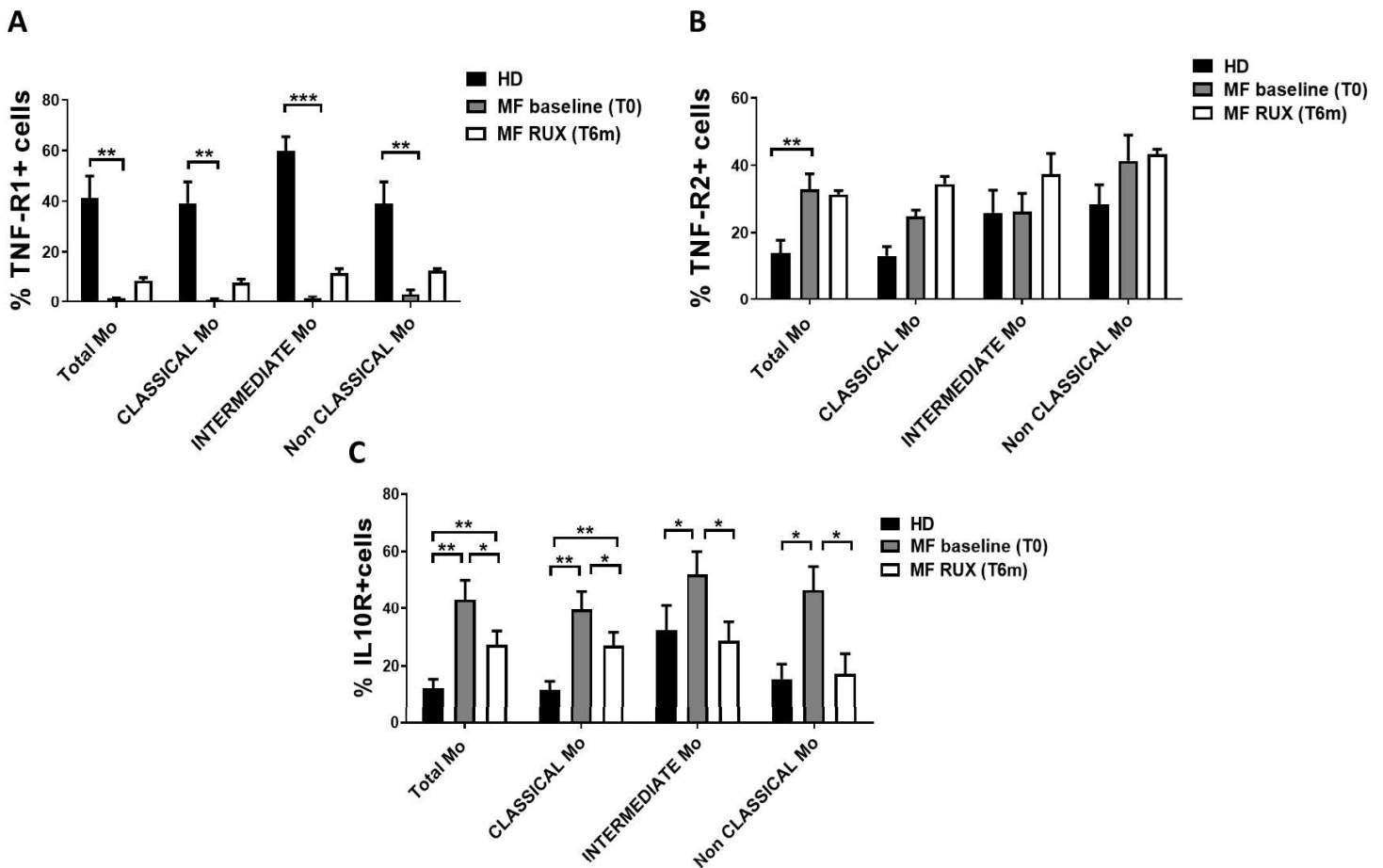
Selected activation markers are dysregulated in MF monocytes and in vitro and in vivo RUX treatment does not significantly modify their profile

To evaluate whether MF monocytes are hyperactivated and whether RUX might influence monocyte activation, we analyzed *ex vivo* and *in vitro* the expression of crucial activation markers in immunomagnetically isolated monocytes from MF pts/HD in the presence/absence of LPS stimulation and of *in vitro* RUX treatment.

As shown in **Figure 4**, at baseline only the mean percentages of IL-10R (**D**) and CD163 (**E**) positive MF monocytes were significantly increased/decreased (p<0.05, respectively), as compared with the normal counterparts.

Upon LPS stimulation, no significant differences in HLA-DR, CD86, CD40 and IL-10R (**Figure 4 A, B, C, D**; both percentages and MIF (MIF data not shown)) were observed between HD and MF monocytes. Interestingly, LPS stimulation significantly decreased the mean percentages of CD163 positive HD monocytes, but not MF monocytes, as compared with unstimulated cells (**E**; p<0.05). Thus, the percentages of CD163+ cells from MF pts was significantly increased as compared with the HD counterpart (p<0.05) (**Figure 4).**

in vitro RUX treatment did not significantly modify CD86, HLA-DR, CD40, IL10-R and CD163 expression (both percentages and MIF) of normal and MF total monocytes in the absence of LPS stimulation (data not shown). Accordingly, in vivo RUX treatment did not significantly modify the percentages of HLA-DR, CD86, CD40 and CD163 positive total MF monocytes as compared with baseline (data not shown); only the percentage of IL-10R positive cells was significantly reduced but not normalized (**Figure 3C;** p<0.05).

When monocytes from HD and MF pts were stimulated with LPS (**Figure 4**), *in vitro* RUX treatment did not significantly modify the mean percentages of HLA-DR positive MF /HD monocytes (**A**). Conversely, the mean percentages of CD86 (**B**; 1 μM RUX), CD40 (**C**;10 μM

RUX) and IL-10R (**D**; 0.2, 0.5, 1.5 µM RUX) positive monocytes from HD were significantly reduced as compared with RUX untreated cells (p<0.05, respectively). Of note, *in vitro* RUX treatment significantly reduced the percentage of CD163 positive MF monocytes (**Figure 4E;** 0.2, 1, 5, 10 µM; p<0.05, respectively) only ().

These data demonstrate that the activation marker profile of MF monocytes is partially altered and that, with the exception of CD163, in vitro/in vivo RUX treatment does not significantly modify this repertoire.

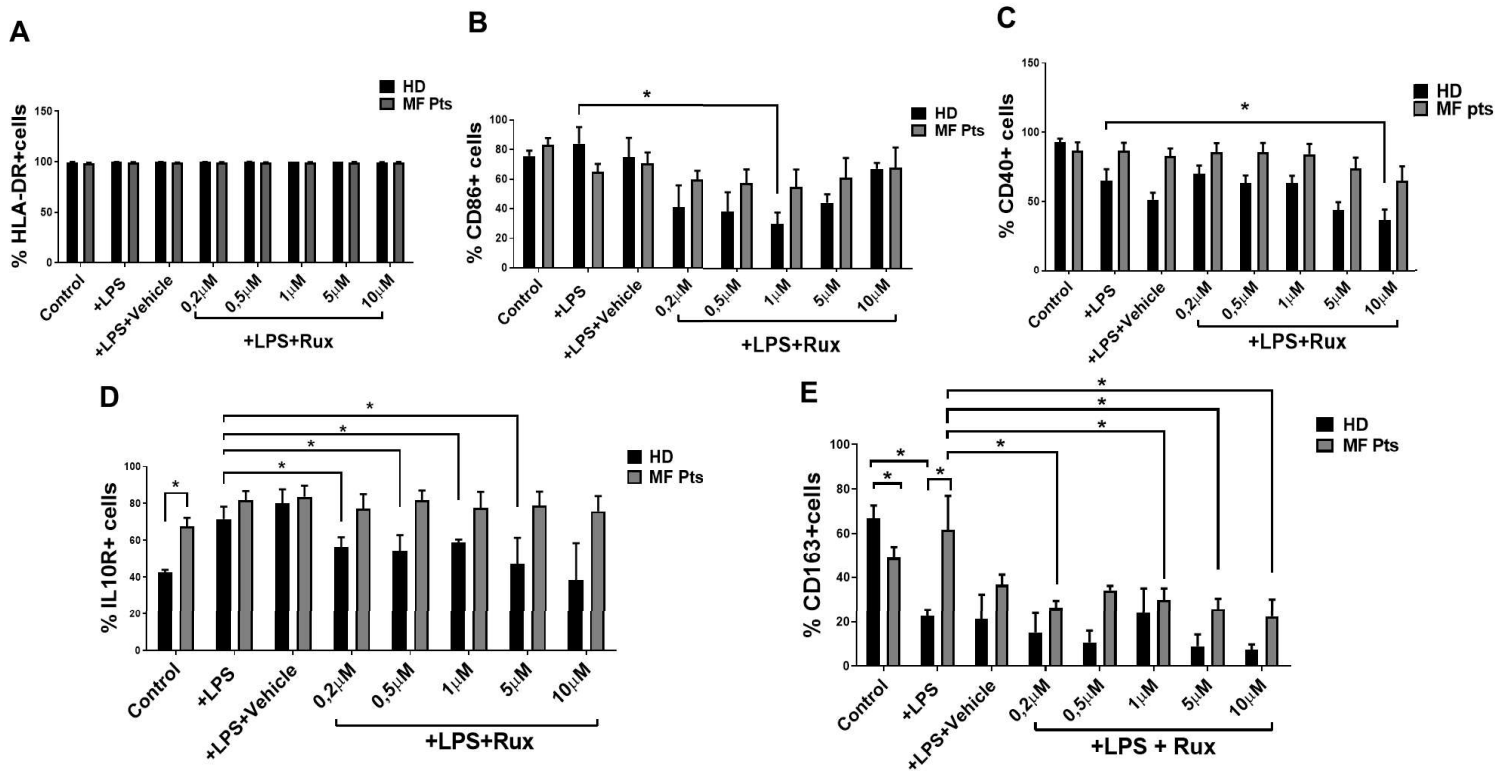
Figure 4. *The expression of crucial markers of activation (HLA-DR, CD86, CD40, IL-10R, CD163) on immunomagnetically isolated monocytes from HD (n=20) and MF pts (n=20) in the presence or the absence of LPS stimulation (24 hours) and titrating doses of in vitro RUX treatment is shown. Histograms represent the mean percentage of activation markers-positive monocytes ± S.E.M. (*p < 0.05).*

The ability of MF monocytes to produce pro-inflammatory cytokines under LPS stimulation is reduced and in vivo RUX therapy normalizes this pattern

Cytokine production represent the main mechanisms used by monocytes to respond to the external stimuli. We evaluated ex vivo the percentages of IL1-β, TNF-α, IL-10 and IL-6 producing monocytes with/without LPS stimulation (4 hours) in HD and patients before and after 6 months of in vivo RUX therapy. No IL-10 positive cells were detected with/without LPS stimulation (4 hours; data not shown).

As shown in **Figure 5**, in the absence of LPS stimulation the mean percentages of IL1-β (**A**), TNF-α (**B**) and IL-6 (**C**) positive cells were very low and no differences were observed between HD and MF pts.

LPS stimulation of MF monocytes failed to increase the percentages of IL1-β, TNF-α and IL-6 positive monocytes, which were therefore significantly reduced as compared with the normal counterparts (**Figure 5;** $p<0.001$, respectively). Interestingly, *in vivo* 6 months-RUX therapy restored and normalized the mean percentages of IL1-β, TNF-α and IL-6 positive monocytes after LPS stimulation.

These data demonstrate an impaired ability of MF monocytes to produce inflammatory cytokines in response to an infectious stimulus and show that *in vivo* inhibition of JAK1/2 restores normal cytokine production ability.
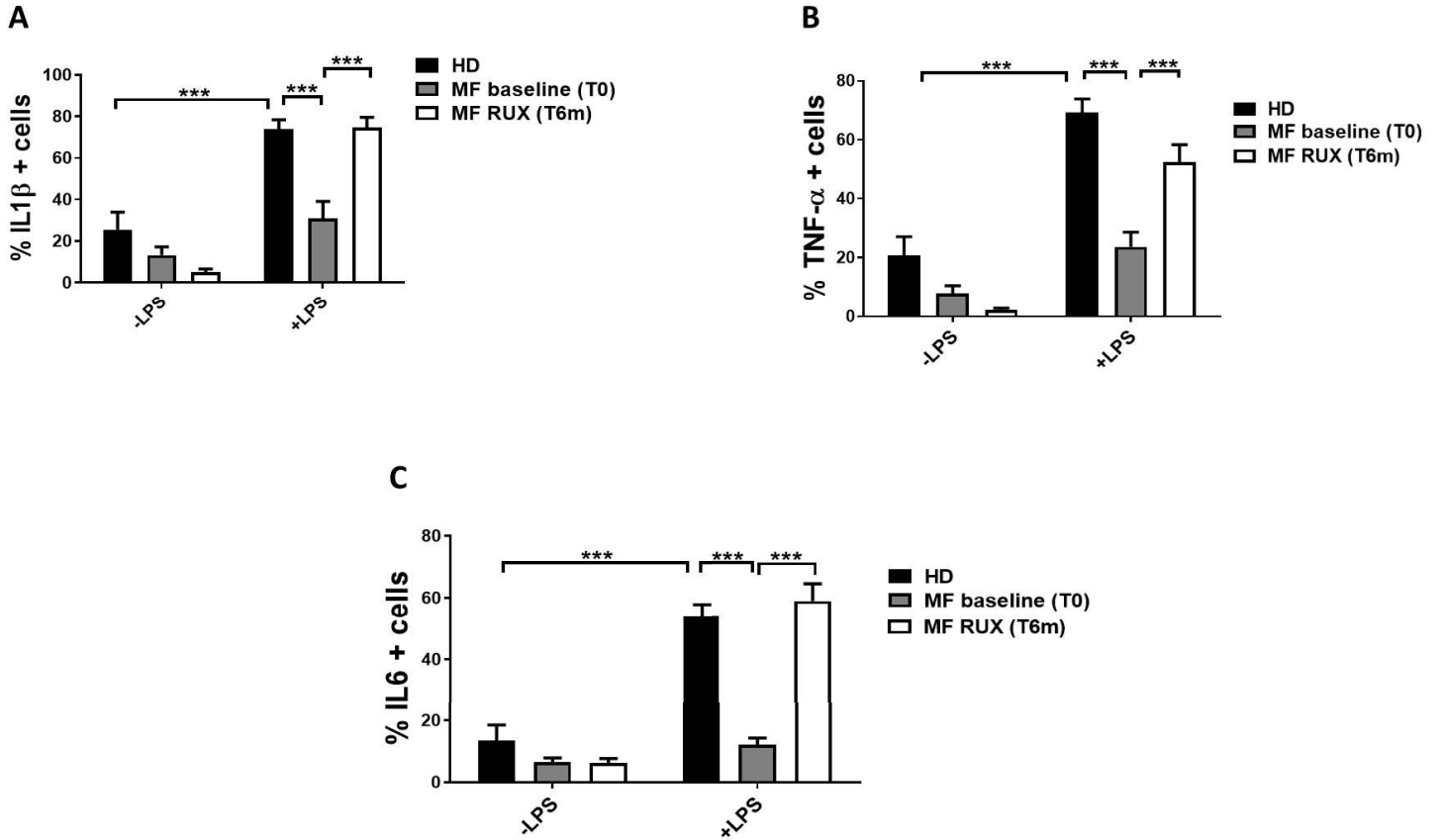
Figure 5. *Intracellular expression of pro-inflammatory cytokines in total monocytes from HD (n=30) and MF (n=30) PBMCs (at baseline (MF baseline T0) and after 6 months of in vivo RUX therapy (MF RUX (T6m); n=20) with or without stimulation with LPS (4 hours) is shown. Histograms represent the mean percentage of monocytes producing IL-1β, TNF-α and IL-6 ± S.E.M. (***p < 0.001).*

Monocytes from MF patients show reduced ability to secrete selected pro/anti-inflammatory cytokines and in vitro, but not in vivo, RUX treatment modify this profile

In parallel experiments we tested the concentrations of selected pro/anti-inflammatory cytokines in the supernatants of MF and HD monocytes upon LPS stimulation and before and after 6 months of *in vivo* RUX therapy. After LPS stimulation, normal monocyte activation involves the production of cytokines at different times points. As shown in **Figure 6**, the concentrations of IL-1β (A; 4 or 24 hours; $p<0.001$)   TNF-α (B; 4 hours; $p<0.001$), IL-6 (C; 4 or 24 hours; $p<0.01/p<0.001$, respectively) and IL-10 (D; 24 hours; $p<0.001$) in the supernatants of HD monocytes, were significantly increased after stimulation with LPS. Conversely, upon LPS stimulation, no significant differences were observed between stimulated and unstimulated MF monocytes. As a consequence, at baseline the concentrations of IL-1β (A; 4 or 24 hours; $p<0.001$), TNF-α (B; 4 hours; $p<0.01$), IL-6 (C; 4 or 24 hours; $p<0.01$) and IL-10 (D; 24 hours; $p<0.001$) were significantly reduced in the supernatants of MF monocytes as compared with the normal counterparts. Of note, 6 months of *in vivo* RUX therapy did not significantly modify the concentrations of the 4 selected cytokines in the supernatants of MF monocytes (**Figure 6**).

We then functionally analyzed the effects of *in vitro* titrating doses of RUX on the ability of isolated MF or HD monocytes to release crucial pro-anti-inflammatory cytokines in the supernatants of cultures upon LPS stimulation. As shown in **Figure 7**, the IL-6 and IL-1β concentrations in the supernatants from HD or MF monocytes were not significantly modified by increasing doses of RUX (**Figure 7** A, B, E, F) as compared with untreated cells. By contrast, RUX dose-dependently reduced the IL-10 concentration in the supernatants from MF (0.2, 0.5, 1, 5, 10 µM; $p<0.05/p<0.01$) or HD (1, 5, 10 µM; $p<0.05/p<0.01$; **Figure 7 G** and **H**) monocytes. Interestingly, low dose RUX treatment significantly increased the concentration of TNF-α in the supernatants from HD (0.2, 0.5, 1 µM) and MF (0.5, 1 µM) monocytes (**Figure 7** C and D) ($p<0.05$, respectively). Conversely, high dose RUX (10 µM) significantly decreased the concentration of TNF-α in the supernatants from HD and MF monocytes ($p<0.05$, respectively).

Collectively, these results indicate a reduced ability of MF monocytes to secrete inflammatory-related signals and demonstrate that in MF, *in vitro*, but not *in vivo*, RUX treatment distinctly modify the cytokine secretion ability of monocytes.

Figure 6. *Concentrations of crucial pro (IL-1β, TNF-α, IL-6)/anti (IL-10)-inflammatory cytokines in the supernatants of immunomagnetically isolated monocytes from HD (n=20) and MF pts (n=20) (at baseline (MF baseline T0) and after 6 months of in vivo RUX therapy (MF RUX (T6m); n=20) in vitro cultured for 4/24 hours in the presence or absence of LPS stimulation. Histograms represent the mean concentration of IL-1β, TNF-α, IL-6 and IL-10 ± S.E.M. (*p<0.05, **p<0.01, ***p < 0.001).*
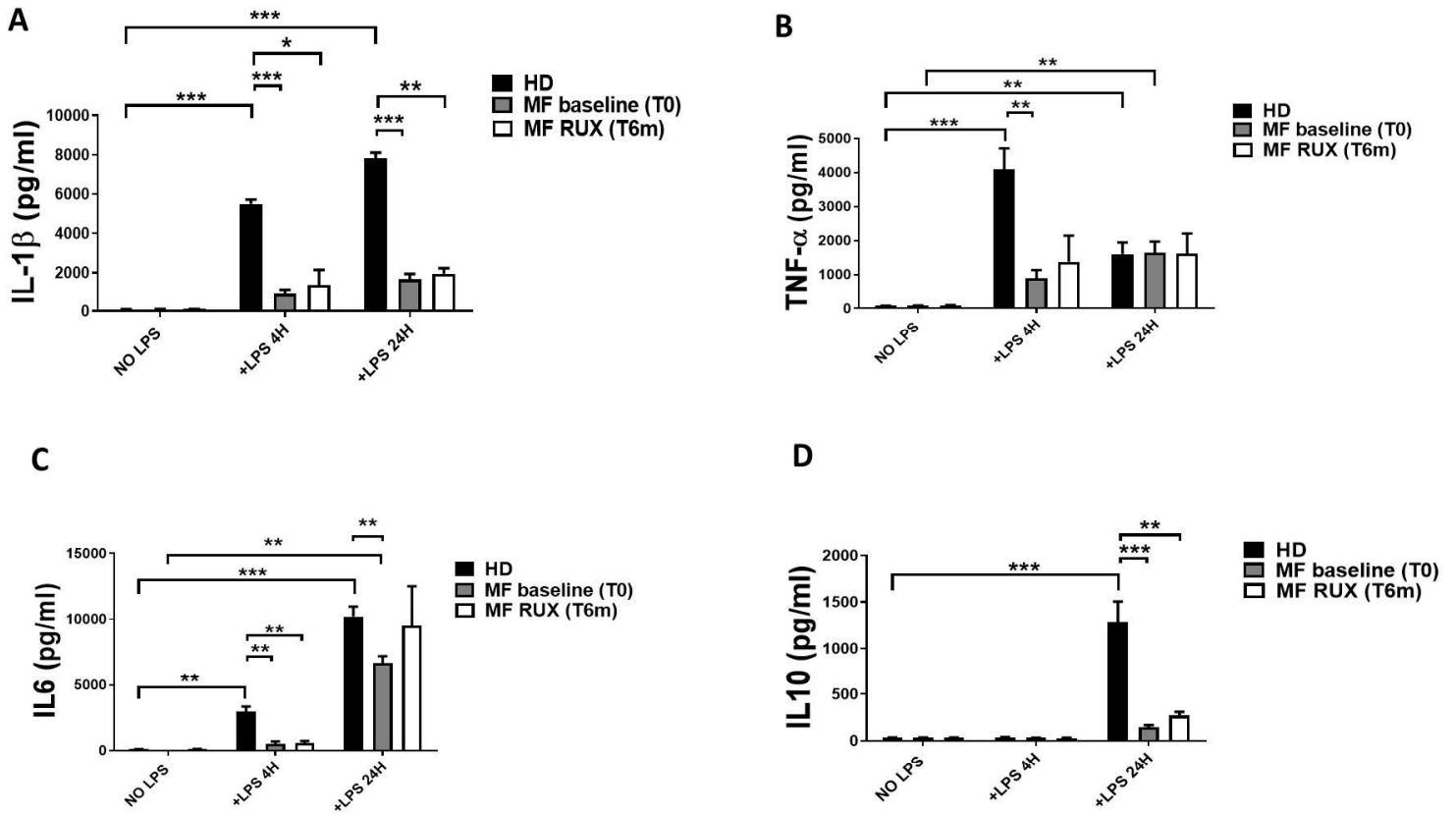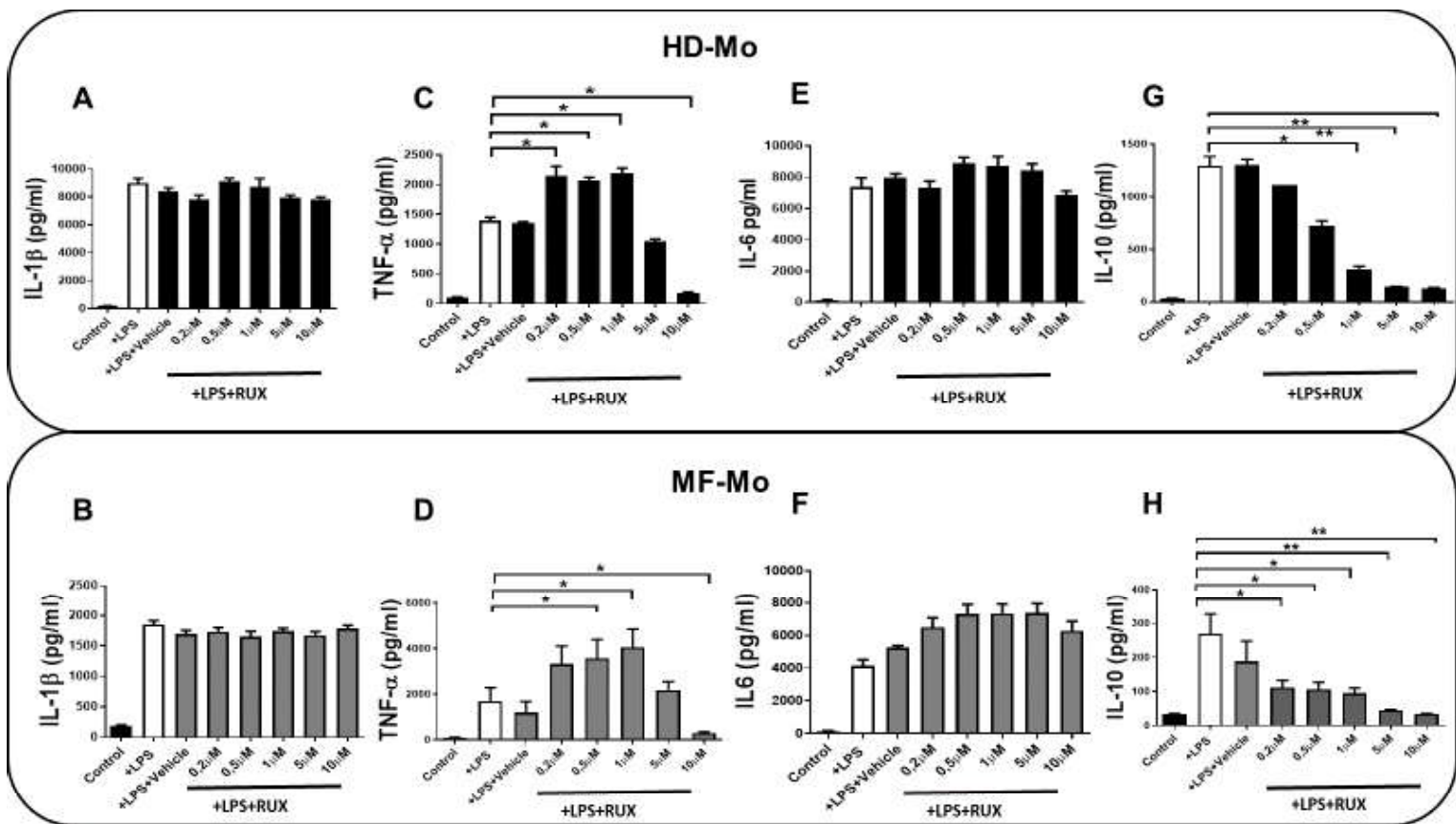
Figure 7. *Concentrations of crucial pro (IL-1β, TNF-α, IL-6)/anti(IL-10)-inflammatory cytokines in the supernatants of immunomagnetically isolated monocytes from HD (n=20;panels A, C, E, G) and MF pts (n=20; panels B, D, F, H) in vitro cultured for 24 hours in the presence or absence of LPS stimulation and titrating doses of RUX (0.2-10 μM). Histograms represent the mean concentration of IL-1β, TNF-α, IL-6 and IL-10 ± S.E.M (*p<0.05, **p<0.01).*

The expression of pro-inflammatory cytokines on the surface of monocyte-derived MVs from MF patients is reduced and in vivo/in vitro RUX treatment normalizes their profile

To investigate the surface associated-cytokines of monocyte-derived MVs and whether RUX treatment may affect their profile, we analyzed the presence of selected pro/anti-inflammatory cytokines on the surface of MVs produced by HD and MF monocytes (at baseline and after *in vivo/in vitro* RUX treatment). As shown in **Figure 8,** in the absence of LPS stimulation, no significant differences were observed between pts and HD or before and after *in vivo* RUX therapy. When monocytes were stimulated with LPS, the percentages of IL-6, IL1β, TNF-α, IL-10-positive MVs of MF pts were significantly decreased as compared with the HD counterparts (**Figure 8 A, B,**

C, D; p<0.001, respectively). Interestingly, there was a trend, albeit non statistically significant, toward increased percentages of cytokines-positive MVs after *in vivo* RUX treatment. Consistently, after LPS stimulation, *in vitro* RUX treatment significantly increased the percentages of IL1β, TNF-α, IL-6 and IL-10-positive MVs in the supernatants of isolated monocytes from MF pts at baseline (**Figure 9 A, B, C, D**). Interestingly, no effects were observed after LPS stimulation alone.

Collectively, these results demonstrate that in MF the MVs-related pro/anti-inflammatory signals of monocyte are impaired and that *in vivo* and *in vitro* RUX treatment normalizes the cytokine-bound MVs repertoire.

Figure 8. *The expression of surface-bound IL-1β, TNF-α, IL-6 and IL-10 positive MVs in the supernatants of immunomagnetically isolated monocytes from HD (n=20) and MF pts (n=20; at baseline (MF baseline T0) and after 6 months of in vivo RUX therapy (MF RUX (T6m); n=20), in the presence/absence of LPS stimulation is shown. Histograms represent the mean percentage of monocyte-derived cytokine-positive MVs ± S.E.M. (*p < 0.05).*

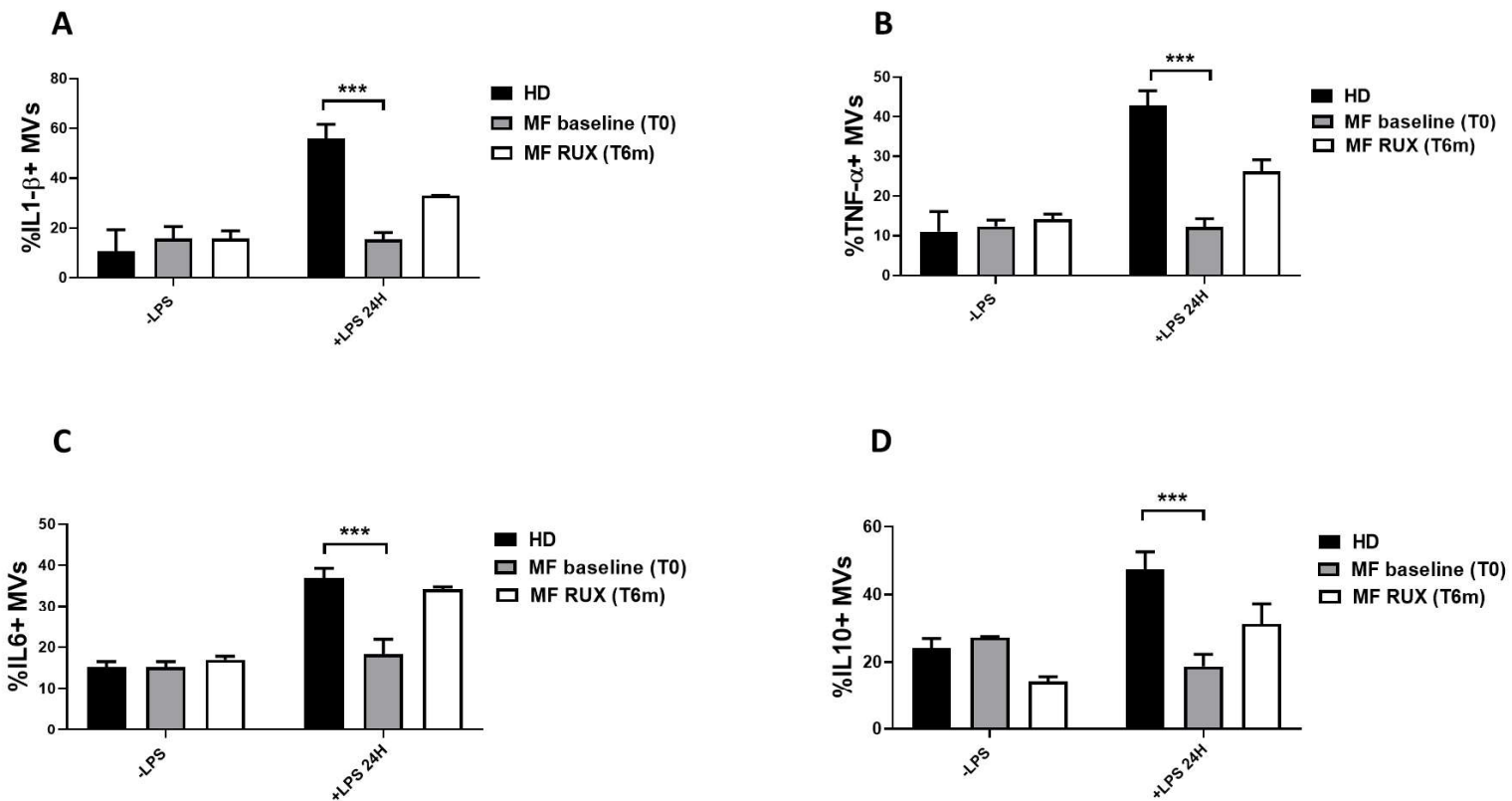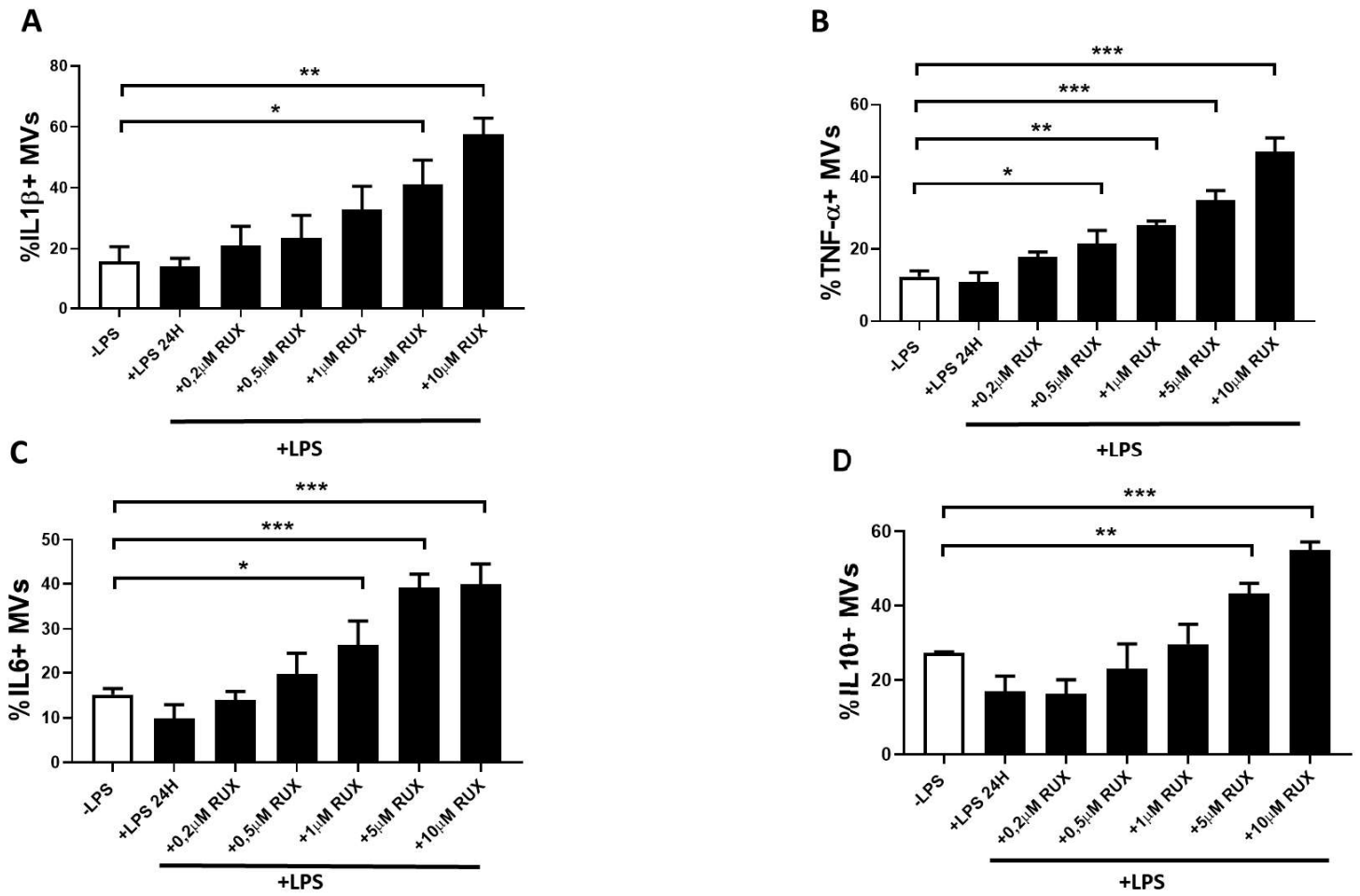**Figure 9**. *Surface-bound IL-1β, TNF-α, IL-6 and IL-10-positive MVs in the supernatants of immunomagnetically isolated monocytes from MF pts (n=20) in the presence or the absence of LPS stimulation (24 hours) and titrating doses of in vitro RUX treatment is shown. Histograms represent the mean percentage of monocyte-derived cytokine-positive MVs ± S.E.M. (*p < 0.05).*

in vitro RUX treatment inhibits the ability of monocytes from MF patients or HD to promote T cells proliferation/activation

To investigate whether RUX may affect the ability of monocytes from pts/HD to activate CD4+ T cells, we co-cultured monocytes and autologous CD4+ T cells in the presence of anti-CD3 stimulation and we analyzed the percentage of proliferating T cells. As shown in **Figure 10A**, RUX (0.2, 0.5, 1, 5, 10 µM) significantly reduced the ability of HD and MF monocytes to promote the proliferation of T cells (p<0.05, respectively). This reduction was associated with a significant decrease of the expression of early (CD69; **10B**) and late (CD25; **10C**) markers of activation in T cells from HD (CD69 and CD25) and MF patients (CD25 only).

These results demonstrate that MF monocytes are capable to fully activates autologous CD4+ T cells; however, *in vitro* RUX treatment inhibits this ability.
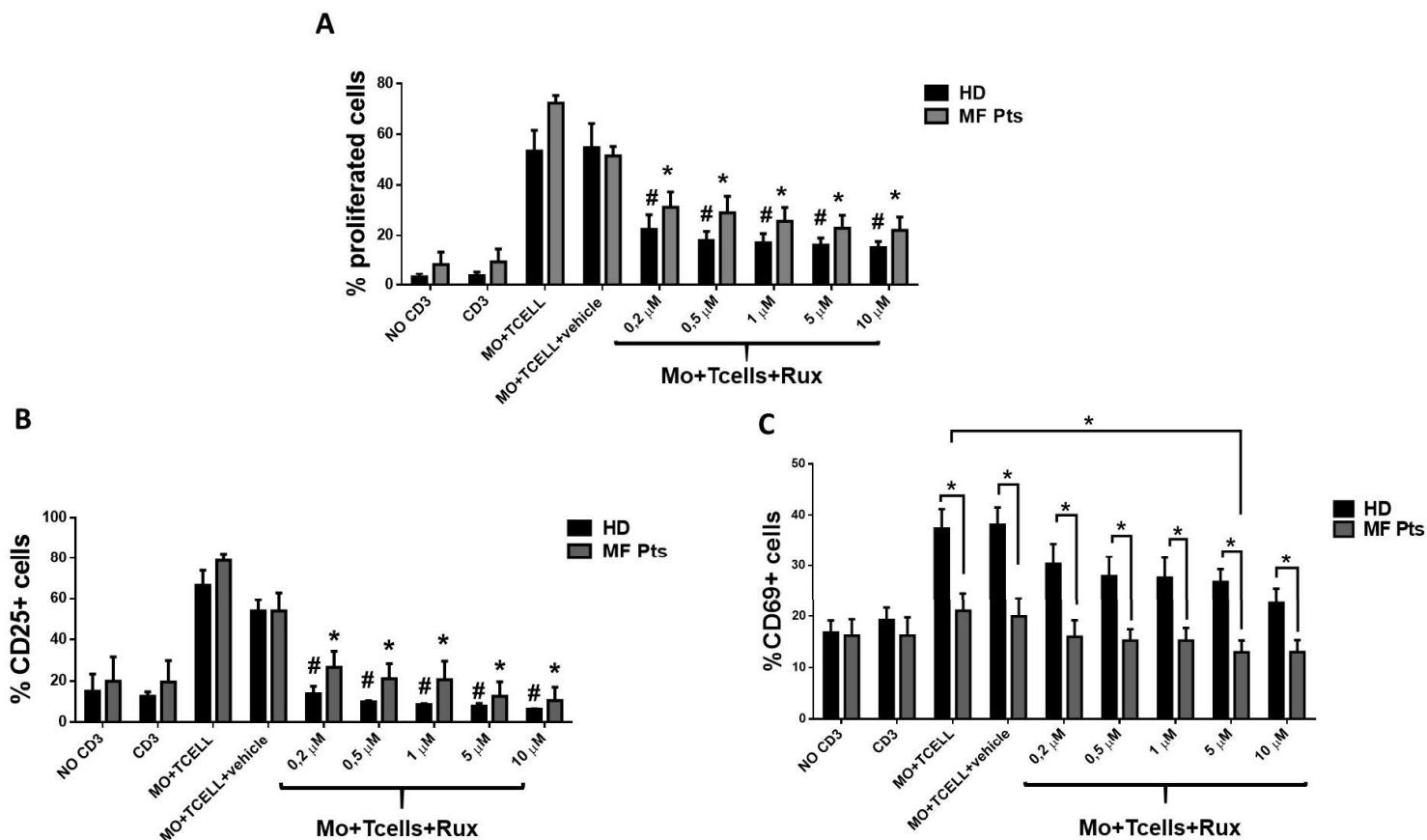
Figure 10. *Ability of immunomagnetically isolated monocytes from HD (n=10) or MF pts at baseline (n=10) to stimulate T cells proliferation in the presence or the absence of in vitro RUX treatment. After LPS pre-stimulation of monocytes, we performed co-cultures of immunomagnetically isolated monocytes and autologous CFSE-labeled CD4+ T cells (monocytes-CD4+ T cell ratio 1:1) in the presence of CD3 stimulation and titrating doses of RUX for 5 days. The proliferation of CD4+ T cells was evaluated by flow cytometry (A). The expression of selected activation markers of CD4+ T cells such as CD69 (B; 24h) and CD25 (C; 5 days) were also analysed. Histograms represent the mean percentage of proliferating and*

Isolated MVs from PPP of MF patients decrease the in vitro secretion of pro/anti-inflammatory cytokines from HD monocytes upon LPS stimulation

To evaluate whether circulating MVs may affect the ability of normal monocytes to secrete pro/anti-inflammatory cytokines we performed *in vitro* co-cultures of immunomagnetically isolated HD monocytes with isolated MVs from PPP of HD or MF pts, in the presence or the absence of LPS stimulation. As shown in **Figure 11**, in the absence of LPS stimulation, MVs from HD or MF pts did not affect the secretion of pro/anti-inflammatory cytokines from HD monocytes in the supernatants. Interestingly, after LPS stimulation, only MVs from MF patients significantly reduced the concentrations of pro (IL-6; p<0.01, TNF-α; p<0.05)/anti (IL-10; p<0.05)-inflammatory cytokines in the supernatants. No differences were observed in the IL-1β production (data not shown).

These data demonstrate that circulating MVs from MF patients do not affect the pro/anti-inflammatory cytokines secretion of normal monocytes; however, in the presence of an infectious stimulus, only MF MVs inhibit the cytokines secretion of normal monocytes.

A

TNF-α (pg/ml)

- MVS
+HD-MVs
+MF-MVs

-LPS   +LPS

*

B

IL6 (pg/ml)

- MVS
+HD-MVs
+MF-MVs

-LPS   +LPS

**

C

IL10 (pg/ml)

- MVS
+HD-MVs
+MF-MVs

-LPS   +LPS

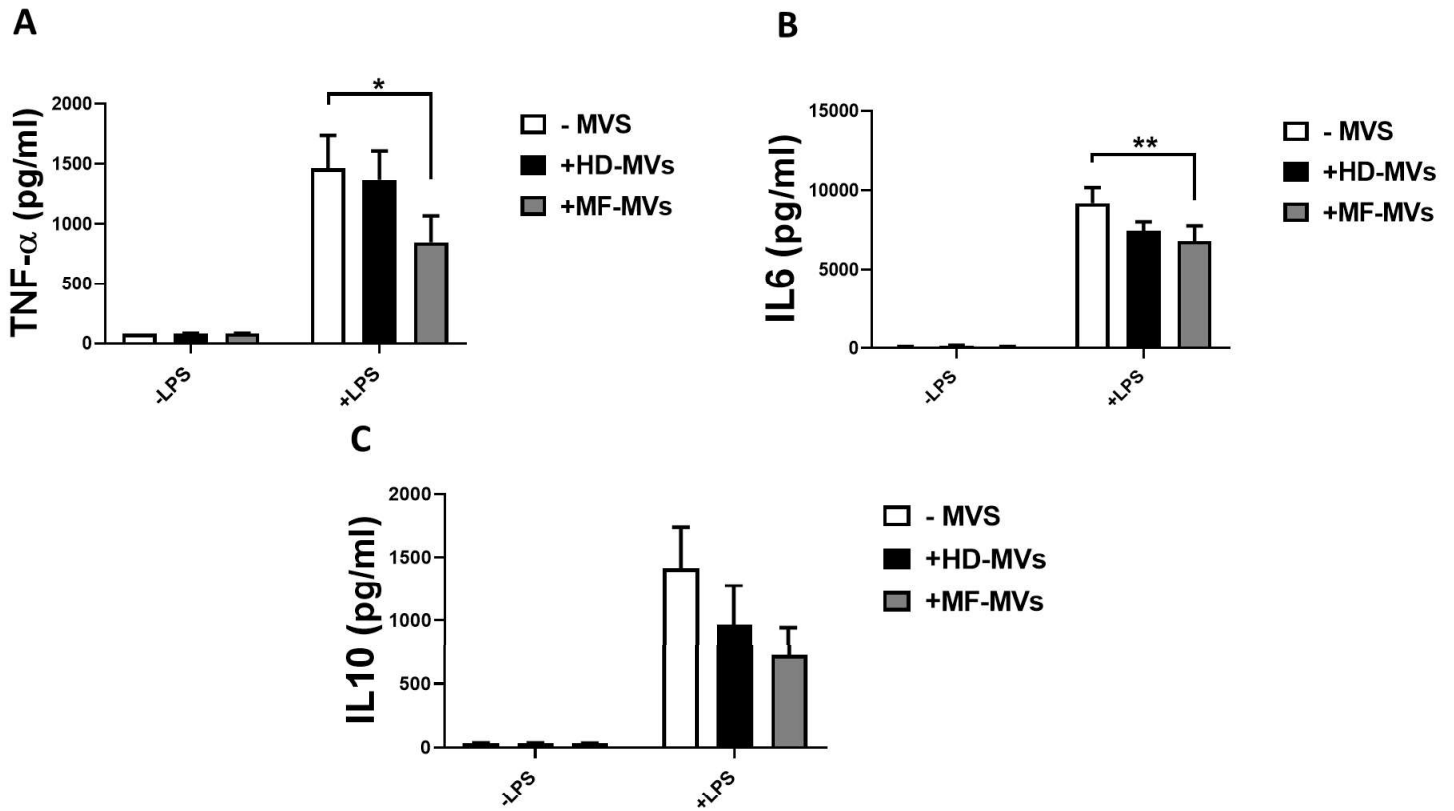*Figure 11.* *Concentrations of pro/anti-inflammatory cytokines (TNF-α, IL-6 and IL-10) in the supernatants of immunomagnetically isolated HD monocytes (n=15) in vitro cultured with or without LPS stimulation (24 hours) and allogeneic isolated MVs (1/100 monocyte/MVs ratio) from HD and MF pts at baseline. Histograms represent the mean concentration of TNF-α, IL-6 and IL-10 ± S.E.M (*p<0.05, **p<0.01).*

Discussion

Immune dysregulation is a common feature of patients with MF. JAK1/2 inhibitors therapy further aggravates this pattern. This results in impaired immunosurveillance which is associated to the occurrence of infectious complications with unusual site and type of disease (10-27).

Here we demonstrated that in *JAK2V617F* mutated MF patients the monocyte differentiation program is dysregulated as referred to subsets population profile and chemokine/cytokine receptors repertoire. Of note, *in vivo* RUX therapy distinctly normalize this behaviour, suggesting that the RUX therapy, reducing plasma inflammatory cytokines, may indirectly support monocyte homing to inflamed/damaged tissue through CX3CR1 and CCR5 upregulation. Conversely, the downregulation of CCR2 may promote monocyte mobilization from bone marrow to peripheral blood.

We have also found that *in vitro* the intra-cellular pro-inflammatory cytokine production of unstimulated MF monocytes is low and superimposable to the normal counterparts. Thus, our data suggest that the contribution of steady-state monocytes to the inflammatory microenvironment of *JAK2V617F* mutated MF is low. This finding is in contrast with Fisher DAC et al (8) showing that 14/15 cytokines measured by mass cytometry were found to be constitutively overproduced in MF, with the principal cellular source for most cytokines being monocytes. Whether this is due to the fact that different techniques have been used (mass cytometry vs traditional flow cytometry analysis) or to the fact that their analysis focused on cytokine produced by CD14 high monocytes only remains a matter of discussion.

Upon LPS stimulus, the *in vitro* intracellular production and secretion of pro/anti-inflammatory cytokines by monocytes of JAK2V617F mutated MF pts was severely impaired. Of note, recent study has shown that the binding of TNF-α leads to a sustained upregulation of proinflammatory cytokines via TNF-R1 and the binding through TNF-R2 upregulates the anti-inflammatory cytokine, IL-10, in the absence of any upregulation of proinflammatory cytokines (41). This study supports our hypothesis that the reduced ability of MF monocytes to produce inflammatory cytokines in response to infections stimuli might be due, at least in part, to the reduced frequency of circulating TNF-R1 positive monocytes. Furthermore, we can not also rule out the hypothesis that, in MF, monocytes might be exhausted by the continuous stimulus of the inflammatory microenvironment.

Importantly, in the absence of LPS stimulation, allogenic isolated circulating MVs from *JAK2V617F* mutated MF pts and HD failed to *in vitro* activate normal monocytes. When LPS

stimulation occurred, isolated circulating MVs of MF pts, but not those of HD, inhibited the *in vitro* pro-inflammatory cytokines secretion of normal monocytes. This finding suggests that the decreased ability of monocytes from *JAK2V617F* mutated MF pts to secrete inflammatory cytokines might be due, at least in part, to a MVs-driven mechanism of inhibition.

Interestingly, *in vivo* RUX therapy promoted the *in vitro* LPS-driven intracellular cytokines production, but not secretion, of monocytes from JAK2V617F mutated MF patients. Moreover, we provided also evidence that, upon *in vitro* LPS stimulus, the frequency of monocyte-derived MVs expressing surface pro/anti-inflammatory cytokines is strongly reduced. Importantly, *in vitro* and *in vivo* RUX treatment increases their proportion. Thus, JAK1/2 inhibition restores cytokine production and MVs-driven inflammatory signaling of MF monocytes. LPS-driven-cytokines production of monocytes is activated by MyD88 transduction signaling pathway (42); therefore, these data support the hypothesis that, upon LPS stimulus, the cytokines production by monocytes is JAK1/2 signaling independent. We can also hypothesize that the decrease of plasma inflammatory cytokines after JAK1/2 inhibition therapy may favour MF monocyte regeneration and activation.

Our findings are consistent with a prior study demonstrating that isolated circulating monocytes from MF fail to respond to LPS and the levels of pro-inflammatory cytokines such as IL23, IL12 and TNF-α remain very low or fail to increase in the case of IL-8 (31).

On the other hand, we are in contrast to a recent report (43) showing that monocytes from patients with MPN have defective negative regulation of Toll-like receptor (TLR) signalling leading to unrestrained production of TNF-α after TLR activation. Specifically, they demonstrated that monocytes of patients with MPN are insensitive to IL-10 which negatively regulates TLR-induced TNF-α production. Whether this discrepancy is due to the fact that Lai YH et al. studied, in addition to MF, also ET and PV monocytes remains a matter of discussion. Nevertheless, according to the present study, they also demonstrated that, upon LPS stimulation, the percentage of TNF-α+ monocytes were significantly decreased in MPN.

In conclusion here we demonstrated that circulating monocytes from *JAK2V617F* mutated MF patients are dysregulated and show a reduced *in vitro* ability to produce/secrete inflammatory cytokines in response to an infectious stimulus. These findings suggest that monocytes are not the principal source of inflammation in *JAK2V617F* mutated MF patients and that this monocyte dysfunction may result in altered immune surveillance against infectious complications or cancer. Importantly, *in vivo/in vitro* JAK1/2 inhibition ameliorates their cytokines production and promotes the MVs-based inflammatory cytokine signaling. Therefore, we can not draw the conclusion that in

MF infections occurring following exposure to JAK1/2 inhibition are due to monocyte compartment dysregulation.

Finally, our findings may have therapeutic implications because they contribute to better interpreting the off-target efficacy of JAK1/2 inhibition in MF and to envisaging strategies aimed at facilitating antitumor immune response rather than to promoting a direct effect on tumor cells.

MATERIALS and METHODS

Patients samples and cell isolation

EDTA-anticoagulated peripheral blood (PB) was collected from *JAK2V617F* mutated MF patients (pts) before (n=30) and after 6 months of Ruxolitinib (RUX) therapy (n=20) and from age/sex-matched healthy donors (HD; n=30). Pt characteristics are shown in Table I. Pts were at diagnosis or at least after 3 months from cytotoxic therapy. This study was approved by the local Ethical Committee and was performed according to the declaration of Helsinki. Pts/controls signed informed consent.

After discarding the first 2 ml of blood, Platelet Poor Plasma (PPP) was obtained (within 2 hours from blood collection) after centrifugation at 2500g for 15 minutes at room temperature and then stored at -80°C with 1% of dimethyl sulfoxide (DMSO). PB mononuclear cells (PBMCs) were separated by Lympholyte 1.077g/cm3 gradient (Cedarlane; CL5020) stratification, cryopreserved in liquid nitrogen and then thawed at 37°C before testing. Of note, to minimize the influence of freezing/thawing, only thawed PBMCs with a survival rate > 80% were used.

CD14+ monocytes and CD4+ T cells Isolation

Circulating CD14+ cells were immunomagnetically isolated from thawed PBMCs of MF pts (at baseline and after 6 months of RUX therapy) and HD using a commercially available kit (CD14 Isolation kit, human; Miltenyi Biotec, Bologna, Italy) and purity (95 ± 3%) was routinely checked by flow cytometry. After monocytes isolation, circulating CD4+ T lymphocytes cells (CD4+ T cells) were immunomagnetically isolated from CD14- PBMCs using a commercially available kit (CD4+ T Cell Isolation Kit, human; Miltenyi Biotec, Bologna, Italy) and purity (93 ± 4%) was routinely checked by flow cytometry.

Phenotype of Monocytes

Total monocytes and subsets (Classical- CD14++/CD16-, Intermediate- CD14+/CD16+, Non-Classical-Mo CD14-/CD16++) were phenotypically characterized by flow cytometry (chemokine receptors: C-C chemokine receptor type 2 (CCR2), C-X3-C motif chemokine receptor 1 (CX3CR1), C-C chemokine receptor type 5 (CCR5); cytokine receptors: Tumor Necrosis Factor (TNF)-αR1, TNF-αR2, Interleukin (IL)-10R, IL-1βR, IL-6R) in PBMCs from MF pts at baseline and after 6 months of RUX therapy and HD (*chemokine receptors/cytokine receptors panels are shown in Table 2*). All flow cytometry analysis were performed on a FACSs Canto II (BD Biosciences) and analysed using FlowJo (FlowJo, LLC) (**Gating Strategy 1 A, B, D, E, F** is shown in Supplementary materials).

Functional assay of monocytes in the presence or the absence of *in vivo* RUX therapy

PBMCs from MF pts at baseline and after 6 months of RUX therapy and HD were stimulated with lipopolysaccharides (LPS, 100 ng/ml) in the presence of 5 ng/ml Brefeldin A (Sigma-Aldrich®) for 4h. After *in vitro* stimulation, PBMCs were incubated with anti-CD14 and anti-CD16 monoclonal antibodies for 15 minutes at room temperature. Cells were then fixed/permeabilized according to standard procedures (IntraPrep Permeabilizaton kit, Beckman Coulter® Life Sciences) and IL-1β, IL-6, and TNF-α producing monocytes were measured by intracellular flow cytometry analysis (**Gating Strategy 1A, 2** is shown in Supplementary materials; *Intracellular Cytokine Production Panel is shown in Table 2*). In parallel experiments, immunomagnetically isolated monocytes from pts/HD were stimulated with LPS (100 ng/ml), on the same day. After 4/24h, culture supernatants were obtained after centrifugation at 400g, collected and stored with 1% DMSO at -80°C for further analysis.

Functional Assay of Monocytes in the presence or absence of *in vitro* RUX treatment

To investigate the in vitro biological/functional effects of RUX, circulating monocytes from MF pts and HD have been immunomagnetically isolated. We analysed by flow cytometry the effect of titrating doses (0.2-10 µM) of RUX (1) on the expression of crucial monocytes activation markers (CD86, HLA-DR, CD40, CD163, IL10R; *Activation markers panel 1/2 is shown in Table 2*) after 24 hours of incubation in the presence or absence of LPS (100 ng/mL); (2) on the monocyte-derived pro-anti/inflammatory cytokines production (TNF-α, IL-6, IL-10 and IL-1β); (3) on the expression of surface-bound inflammatory cytokines expression of monocyte-derived MVs in the supernatants of cultures (24 hours); (4) the effect of titrating doses of RUX (0.2-10 µM) on the capacity of MF and HD monocytes to activate autologous CD4+ T cells. Specifically, after LPS pre-stimulation of monocytes (4hours), co-cultures of immunomagnetically isolated monocytes and autologous CD4+ T cells (Mo:CD4+ ratio 1:1) in the presence of CD3 stimulation (5ng/ml; CD3 Monoclonal Antibody (OKT3), Functional Grade, eBioscience™) and titrating doses (0.2-10 µM) of RUX for 5 days have been performed. CD4+ T cells were previously labelled with Carboxyfluorescein succinimidyl ester (CFSE (5µM); Invitrogen; C34554), according to the manufacturer's instructions. The proliferation (CFSE assay) and the expression of selected activation markers such as CD69 (after 24h of co-cultures) and CD25 (after 5 day of co-cultures) of CD4+ T cells were analysed by flow cytometry (*Tcell Activation markers panel is shown in Table 2*).

Isolation of circulating MVs

Circulating MVs were isolated from cryopreserved pts/HD-PPP. Briefly, after thawing at 37°C, the PPP was centrifuged at 1000g for 15 minutes to eliminate further cellular or fibrin contamination. The PPP was then diluted with a double filtered saline solution (0.22 µm) up to a final volume of 20 ml and placed in ultracentrifuge tubes. The ultracentrifugation was performed by Optima L-90K (Beckman Coulter) at 45,000 rpm for 2 hours at 4°C. After ultracentrifugation, the supernatant was eliminated, and the MVs pellet was resuspended in 300µl of double filtered RPMI (0.22 µm filter). The isolated MVs were stored at -80°C after addition of 1% DMSO. The quantification of MVs was carried out by Nanosight instrument (NS300, Malvern Instruments Ltd, UK) to obtain the number of MVs/ml.

MVs and monocytes co-cultures

To evaluate the functional effect of circulating MVs on the production of cytokines by monocytes, co-cultures of immunomagnetically isolated HD-Mo and isolated MVs from MF pts and allogeneic HD have been performed, in the absence/presence of LPS. Specifically, monocytes were seeded (10^6/ml) in 12 wells plates with twice filtered RPMI (filter 0.22 μm) in the presence/absence of MVs and with/without LPS stimulation (100 ng/ml). The MVs were added at different monocytes/MVs ratio (1/100; 1/1000; 1/10000). After 4/24 hours of incubation at 37°C and 5% CO2, supernatants of monocytes were obtained after centrifugation at 400g for 5 minutes and stored at -80 ° C. The supernatants were then analyzed by flow cytometer to evaluate the concentration of IL-1β, IL-6, TNF-α and IL-10.

Cytokine concentration of the monocyte cultures supernatants

Supernatants from monocytes cultures, with/without RUX (*in vivo/in vitro*), and HD-Mo/MVs co-cultures (in the presence or absence of LPS stimulation (4/24h)) were harvested (centrifugation at 400g) and frozen at -80°C with 1% of DMSO until assays were performed. Cytokine concentration was determined by commercially available MACSPlex Cytokine 12 Kit (Miltenyi Biotec, Bologna, Italy) for human IL-6, TNF-α and IL-10 according to the manufacturer's instruction. IL-1β was determined by Human IL-1 beta/IL-1F2 Quantikine ELISA Kit; R&D Systems.

Flow cytometric analysis of the inflammatory cytokine's expression on the surface of MVs from monocyte culture supernatants

Cytofluorimetric analysis and staining method was developed to study the expression of surface attached pro/anti-inflammatory cytokines in MVs from monocyte culture supernatants in the presence/absence of LPS stimulation and with/without RUX (*in vivo/in vitro*). Supernatants (50μl) were incubated with anti-CD14 (5μl), IL-1β (5μl), IL-6 (5μl), TNF-α (5μl) and IL-10 (5μl) monoclonal antibody for 30 minutes at room temperature and then MVs were analyzed by flow cytometry (Navios, Beckman Coulter, Milan, Italy) (*Cytokine-bound MVs Panel is shown in Table 2*). To detect MVs the instrument was calibrated with MegaMix Beads (Stagò, Marseille, France). Fluorescence gated polystyrene beads of different sizes were used to determine the gates identifying big (500-900 nm), small (200-300 nm) and nano (100-160 nm) MVs. The Violet Side Scatter laser (VSSC) is used as a trigger signal to discriminate the noise. Big MVs identification was based on size (500-900 nm) and on the ability to bind specific monoclonal antibodies. Gating strategy to

identify big MVs (500-900 nm) is shown (**Gating Strategy 3**, in Supplementary materials). Matched isotype controls were used to select the cut-off. Using the defined gate for big MVs, all events positive for markers staining were recorded. The expression of the pro/anti-inflammatory cytokines was expressed as percentage of positive MVs.

Statistical analysis

Statistical analyses were performed with GraphPad (GraphPad Software Inc., La Jolla, USA). Data are expressed as mean ± SEM. P values were considered significant when ≤ 0.05 (2-tailed). The differences between the groups were analysed with Mann Whitney, Kruskal Wallis, one-way and two-way ANOVA tests as appropriate.

Conclusions

Inflammation plays a very important role in cancer, including blood cancer. MF is considered "A Human Inflammation Model" where the uncontrolled myeloproliferation and cytokine overproduction creates a pro-inflammatory microenvironment influencing the HSC/HSPC compartment and the immune system. Along with a role as activators of immune cell function, a growing evidence now demonstrates that proinflammatory cytokines strongly affect the size and lineage distribution of the blood cells via reprogramming of HSC/HSPC and supporting the bone marrow niche. Based on this evidence, it is very important to clarify the impact of proinflammatory cytokines on the biology of the normal HSPC and its BM microenvironment. A better understanding of the role and effects of inflammatory cytokines in the signals between HSPC and their inflammatory microenvironment would help to understand the mechanisms underlying tumorigenesis, particularly in hematological diseases and MF.

In addition to the inflammatory cytokines, other components of the inflammatory network may play a role in MF pathogenesis. Among the possible mechanisms of development/propagation of inflammation and tumorigenesis, it has been described that the contribution of MV is crucial. The MV, expressing antigens and containing constituents from the cell of origin, are part of a mechanism that supports cellular communication, with the potential to influence the short and long-distance microenvironment. Focusing on the inflammatory microenvironment that characterizes MF, the study of circulating MV would allow to deepen and understand the inflammatory pathways activated in the MF and to identify possible therapeutic targets.

The importance of immune cells should not be underestimated. They are the main regulators of the inflammatory microenvironment, producing pro/anti-inflammatory cytokines in response to infectious/inflammatory stimuli and are also the cells mainly and functionally influenced by the inflammatory regulatory effects. A better understanding of the immune biology in the setting of MF would be important to understand if atypical infectious events in MF are caused by deficits in the innate or adaptive immune response and for the design of new therapies.

Among the targets of the JAK1/2 inhibitor Ruxolitinib, we hypothesized that circulating MV, being involved in inflammation, and monocytes, given the importance of the JAK-STAT pathway in their differentiation/function, could be strongly influenced by Ruxolitinib.

Based on this background, the present thesis aimed: 1) to study the unexplored mechanisms that regulate the interaction between healthy HSPCs and their microenvironment; 2) focusing on MF, to investigate the immune/inflammatory microenvironment and characterize the pathogenetic role of crucial components of this inflammatory network, such as circulating MV, and specific subset of key immune cells.

Specifically:

1) we found that various combinations of inflammatory cytokines (IL-1β, TNF-α, IL-6, TIMP-1) promote *in vitro* survival of CB-derived CD34+ cells and increase proliferation, clonogenic ability and *in vitro* migration of mPB-derived CD34+ cells. Inflammatory cytokines combined with bone marrow MSC promote the survival/migration of CD34+ cells from both sources. These results suggest that the inflammation and signal balance between HSPC and their microenvironment play a very important role in the functional behavior of normal HSPCs; suggesting that the modulation of this balance might be a clinically relevant approach in hematological malignancies. However, critical steps are involved in these processes and further insights are necessary to better understand HSPCs homing and engraftment.

2) we found that in MF the profile of circulating MK/PLT derived MV is altered. Specifically, MK-MV and PLT-MV were respectively decreased/increased, in MF patients as compared to the normal counterparts. This finding suggests that the altered MK/PLT-MV profile might be due to the impaired megakaryocytopoiesis and platelets activation, which have been previously described in MF. Furthermore, according to IPSS score, Intermediate 2/high risk pts showed a respectively reduced/increased MK-/PLT-MV proportions, as compared with the intermediate1/low risk group. We also found that Ruxolitinib (JAK1/2 inhibitor) therapy restores the normal MK/PLT-MV profile of spleen-responders patients only, suggesting that constitutive activation of JAK/STAT pathway plays a role in the biogenesis/clearance of MV. Importantly, a cut-off value of 19.95% of MK-MVs discriminated the non-responders patients. Therefore, even though to be confirmed in a larger casistic, the MK/PLT-MV profile in MF can be used to predict the response to Ruxolitinib therapy and could have a diagnostic and prognostic role in MF. Further characterization demonstrated that the miR cargo of circulating MF-MV shows upregulation of 4 miRs (-212/-127/-222/-34a) which are involved in apoptosis and that regulate inflammation and proliferation. Furthermore, we also identified a 1-miR signature with the potential to differentiate TN- and JAK2-MV. Therefore, this study has the potential to identify disease-related biomarker(s) and may provide a useful molecular target for MF diagnosis and treatment.

3) we found that MF patients are characterized by a state of mutation-dependent immune alterations with key cellular components of the innate and adaptive immunity showing defective number and function. MF patients were characterized by a reduced ability of monocytes to differentiate into dendritic cells, reduced plasticity of Th17 lymphocytes and reduced functional capacity of ILCs. Analyzing the results by mutational status, we demonstrate that the MF immune microenvironment varies between patients and selected immune defects are mainly associated with the presence of the

JAK2 or CALR mutation. Overall, these abnormalities could contribute to the development of an immunodeficiency state with the potential to promote immune evasion, cancer progression and increased susceptibility to infection. Although this study is based on a limited number of patients, our data highlight the importance of investigating the role of the immune system in MF and other MPNs according to mutational status in larger cohorts of patients. Furthermore, a better understanding of immune biology in the setting of MF would be important for the design of new MF therapies.

4) we found that MF monocytes show defective differentiation program and activation capacity. Furthermore, their in vitro ability to produce/secrete free and microvesicles-linked inflammatory cytokines in response to an infectious stimulus is severely impaired. This might be due, at least in part, to the inhibitory activity of circulating MF isolated-MV on the inflammatory cytokines secretion of monocytes under lipopolysaccharides stimulation. Our data demonstrate that in MF, circulating monocytes are functionally defective. Furthermore, these findings suggest that monocytes are not the principal source of inflammation in JAK2-MF pts and support the hypothesis that the cytokines production by MF monocytes is JAK1/2 signalling independent. Interestingly, *in vitro/in vivo* Ruxolitinib therapy normalizes the monocyte chemokine expression and improves their cytokine production in response to an infectious stimulus, promoting the release of inflammatory cytokines associated with monocyte-MV. Upon infectious stimulus, JAK1/2 inhibition reactivates the monocyte-driven inflammatory cytokine signalling, suggesting that: 1) the mutated pathway may have an inhibitory role; 2) the decrease of plasma inflammatory cytokines after JAK1/2 inhibition therapy may favour monocyte regeneration and activation. Therefore, we can not draw the conclusion that in MF infections, occurring following exposure to RUX, are due to monocyte dysregulation. This finding further refines the effects of Ruxolitinib on the MF immune system and suggests that Ruxolitinib activity is cell type-dependent. These findings may have therapeutic implications because they contribute to better interpreting the off-target efficacy of JAK1/2 inhibition and to envisaging strategies aimed at facilitating antitumor immune response.

Taken together, the results of this thesis:

1) *Further characterize the promoting effects of combined inflammatory factors on the HSPC functional behaviour*

2) *Increase knowledge on the profile and cargo of circulating MV of MF and identify the circulating MV as diagnostic and prognostic biomarkers in MF*

3) *Increase knowledge on the immune dysfunction of MF*

4) *Discover promoting effects of the JAK1/2 inhibitor Ruxolitinib on monocyte function*

In conclusion, this thesis provides scientific advances in understanding the functional role of the immune/inflammatory microenvironment in the pathogenesis of MF. Therefore, in MF, both the pathogenetic hypothesis and the therapeutic attempts should take into consideration not only the malignant clone but also the microenvironment, including soluble proinflammatory factors, signals mediated by immune cells and circulating MV. Understanding the mechanisms altering the interaction between the malignant clone and its microenvironment has the potential to promote the design of therapeutic strategies based on the manipulation of key components within the tumor microenvironment.

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Introduction

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